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Isolation and Characterisation of a Molybdenum-reducing and Metanil Yellow Dye-decolourising *Bacillus* sp. strain Neni-10 in Soils from West Sumatera, Indonesia

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Abstrak: Satu bakteria penurun molibdenum dengan keupayaan baru untuk menyahwarna pewarna azo Kuning Metanil dilaporkan. Kondisi optimum untuk penurunan molibdenum adalah pada pH 6.3 dan pada suhu 34°C. Glukosa adalah penderma elektron yang terbaik. Keperluan-keperluan lain termasuklah kepekatan fosfat yang sempit di antara 2.5 dan 7.5 mM. Profil masa pengeluaran Mo-biru menunjukkan tempoh sela masa kira-kira 12 jam, jumlah maksimum Mo-biru dihasilkan pada kepekatan molibdat 20 mM dan pengeluaran maksimum pada masa 52 jam inkubasi. Logam-logam berat seperti merkuri, perak, tembaga dan kromium merencat penurunan sebanyak 91.9, 82.7, 45.5 dan 17.4%, masing-masing. Penyahwarnaan pewarna Kuning Metanil pada kepekatan 100 dan 150 mg/L berlaku pada hari ketiga dan hari enam inkubasi, masing-masing. Kepekatan yang lebih tinggi menghasilkan degradasi separa, dengan penyahwarnaan warna sebanyak 20% berlaku pada kepekatan 400 mg/L. Bakteria ini dikenal pasti secara separa berdasarkan analisis biokimia sebagai *Bacillus* sp. strain Neni-10. Spektrum penyerapan Mo-biru mencadangkan bahawa kompaun terhasil adalah fosfomolibdat terturun. Pengasingan bakteria ini, yang menunjukkan penurunan logam berat dan keupayaan penyahwarnaan pewarna, adalah diperlukan, terutamanya untuk bioremediasi.

Kata kunci: Penurun Molibdenum, Molibdenum Biru, *Bacillus* sp., Pewarna Azo, Kuning Metanil

Abstract: A molybdenum reducing bacterium with the novel ability to decolorise the azo dye Metanil Yellow is reported. Optimal conditions for molybdenum reduction were pH 6.3 and at 34°C. Glucose was the best electron donor. Another requirement includes a narrow phosphate concentration between 2.5 and 7.5 mM. A time profile of Mo-blue production shows a lag period of approximately 12 hours, a maximum amount of Mo-blue produced at a molybdate concentration of 20 mM, and a peak production at 52 h of incubation. The heavy metals mercury, silver, copper and chromium inhibited reduction by 91.9, 82.7, 45.5 and 17.4%, respectively. A complete decolourisation of the dye Metanil Yellow at 100 and

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150 mg/L occurred at day three and day six of incubations, respectively. Higher concentrations show partial degradation, with an approximately 20% decolourisation observed at 400 mg/L. The bacterium is partially identified based on biochemical analysis as *Bacillus* sp. strain Neni-10. The absorption spectrum of the Mo-blue suggested the compound is a reduced phosphomolybdate. The isolation of this bacterium, which shows heavy metal reduction and dye-decolorising ability, is sought after, particularly for bioremediation.

Keywords: Molybdenum-reducing, Molybdenum Blue, *Bacillus* sp., Azo Dye, Metanil Yellow

INTRODUCTION

Molybdenum has a variety of uses in many industries, including alloys, lubricants, pigments and electronics. These industries are a major contributor to the molybdenum pollution. For example, incandescent light bulb producers in China have polluted surrounding soils with molybdenum levels reaching 252 mg/kg (Geng *et al.* 2014). When compared to the industrial contribution to molybdenum pollution, mining activities present a far greater hazard. For example, waste tailings from a uranium-molybdenum mine produced an exceptionally high concentration of molybdenum, reaching a concentration of 6,550 mg/kg in soil (Stone & Stetler 2008). In Batu Hijau, Sumbawa, Indonesia, the copper-gold-molybdenum porphyry deposit has resulted in reduced fish populations and water pollution in the ocean (Angel *et al.* 2013). Earlier research of the toxicity of molybdenum demonstrated that ruminants are the most sensitive to molybdenum. Exposure to molybdenum at levels of between 5 to 10 ppm causes hypocuprosis or copper shortage (Kessler *et al.* 2012). More recent findings have indicated that molybdenum is toxic to spermatogenesis. For example, a testicular organ culture of Japanese eels exposed to heavy metals, including molybdenum, at concentrations between 0.1 to 10 mg/L shows a synergistic interaction amongst these metal ions in inhibiting spermatogenesis (Yamaguchi *et al.* 2007). Furthermore, adult male Wistar rats exposed to ammonium molybdate exhibited abnormalities in the testes, which is supported by histopathological and histomorphometric analyses (Pandey & Jain 2015).

Molybdenum reduction into molybdenum disulfide, an insoluble compound (Tucker *et al.* 1997), and reduction to molybdenum blue, a colloidal blue precipitable compound (Ghani *et al.* 1993), has been proposed as feasible methods for molybdenum bioremediation. The first process is catalysed by bacterial genera, such as *Desulfovibrio* and *Desulfotomaculum*, which required complete anaerobicity. On the other hand, the second process is catalysed by numerous heterotrophic bacteria from various genera (Shukor *et al.* 2007; Shukor *et al.* 2008a; Rahman *et al.* 2009; Shukor *et al.* 2009a; Yunus *et al.* 2009; Shukor *et al.* 2009b; Shukor *et al.* 2010a; Shukor *et al.* 2010b; Lim *et al.* 2012; Ahmad *et al.* 2013a; Halmi *et al.* 2013; Ibrahim *et al.* 2015; Masdor *et al.* 2015; Nuraini *et al.* 2015). The second process is a more feasible tool as the conversion occurs under facultative anaerobic conditions, and no toxic gas is produced (Ghani *et al.* 1993).

In Indonesia, aside from heavy metals, the organic pollutant azo dyes are often present as co-pollutants in water and soils and in wastewater treatment plants (Meitiniarti *et al.* 2007). Basic and diazo direct dyes are being produced on the order of nearly one million tons yearly. Unfortunately, they have the highest rates of toxicity (LD_{50} or median lethal dose), as reported by The Ecological and Toxicological Association of the Dyestuff Manufacturing Industry (ETAD) (Shore, 1996). Being brightly coloured and water soluble, these reactive and acidic dyes tend to pass through conventional water treatment systems unscathed (Lazim *et al.* 2015). Due to this, it is well known that the textile finishing industries are one of the largest contributors to 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), colour, pH and the presence of toxic metal ions make effluent dyes a significant threat to ecology (Johari *et al.* 2014). Toxicology research shows that the administration of Metanil Yellow (Figure 1) to animals through various routes, such as oral, intraperitoneal and intratesticular, resulted in testicular lesions, probably due to damage to the seminiferous tubules. The end result is a decreased rate of spermatogenesis (Anjaneya *et al.* 2011). Oral consumption in humans causes cyanosis and toxic methaemoglobinaemia. Skin contact with the dye results in allergic dermatitis. It has been shown that Metanil Yellow also has tumour-producing properties in the human body (Anjaneya *et al.* 2011).

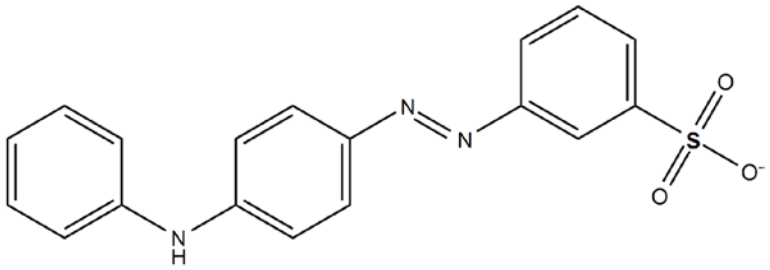

Figure 1: The structure of Metanil Yellow (Anjaneya *et al.* 2011).

Polluted sites often contain a mixture of heavy metals and toxic xenobiotics. The search for multiple detoxification microorganisms is on the rise globally (Ahmad *et al.* 2013b; Agrawal *et al.* 2014; Ahmad *et al.* 2014; Muhamad *et al.* 2015). Examples include the heavy metal chromate reduction coupled to azo dye decolourization (Chaudhari *et al.* 2013) and the isolation of Mo-reducing bacteria showing sodium dodecyl sulfate (SDS)-degrading (Halmi *et al.* 2013; Masdor *et al.* 2015), phenol-degrading (Ibrahim *et al.* 2015) and glyphosate-degrading (Sabullah *et al.* 2016) properties.

A novel molybdenum-reducing bacterium that has the capacity to decolorize the azo dye Metanil Yellow is reported here. This is the first report of the isolation of a dye decolorising Mo-reducing bacterium and will be beneficial in the bioremediation of sites contaminated with these pollutants.

MATERIALS AND METHODS

Soil Sampling

A clay-type soil from an industrial site producing textiles (0°56'47.8"S 100°22'09.1"E) was chosen as the sampling site. The location of sampling was in Padang, West Sumatera, Indonesia. A sterile spatula was utilised to take soil samples (ten grams) and placed in a sterile polycarbonate container. A soil sample was taken in January 2009.

Isolation, Growth and Maintenance of Bacterium

Preparation of a soil bacterial suspension was carried out by adding one gram of soil to 10 mL of deionised water. An aliquot of the soil suspension (0.1 mL) was then spread onto an agar of the following media (pH 6.5) and incubated at room temperature. The composition (w/v) of the media is as follows: yeast extract (0.5%), $MgSO_4 \bullet 7H_2O$ (0.05%), $Na_2MoO_4 \bullet 2H_2O$ (0.242% or 10 mM), glucose (1%), $(NH_4)_2 \bullet SO_4$ (0.3%), NaCl (0.5%), agar (1.5%), and Na_2HPO_4 (0.071% or 5 mM) (Masdor *et al.* 2015). After 48 hours of incubation, several white and five blue colonies appeared. All five isolates were then re-streaked on the Low Phosphate Medium (LPM) agar several times to obtain a pure culture.

Quantification of Mo-blue Production and Scanning Absorption Spectrum

Mo-blue production from these bacteria was monitored in liquid culture to obtain the best isolate. Mo-blue production from these bacteria was quantified in 100 mL of liquid culture (LPM) to select the best isolate. Mo-blue production was determined at 865 nm utilizing the extinction coefficient of 16.3 $mM^{-1}cm^{-1}$ (Shukor *et al.* 2007). Characterisation of the molybdenum blue produced was carried out by scanning the absorption spectrum of the blue supernatant from the liquid culture from 400 to 900 nm (UV-spectrophotometer, Shimadzu 1201, Shimadzu Corporation, Kyoto, Japan), with low phosphate media minus bacterium as the baseline correction. Culture supernatant from a 48 h incubation was centrifuged at 10,000 x g for 10 minutes at room temperature (Shukor *et al.* 2003).

Partial Identification of Mo-reducing Bacterium

The bacterium was identified via biochemical and phenotypical methods (Masdor *et al.* 2015) in accordance to Bergey's Manual of Determinative Bacteriology (Holt *et al.* 1994) with the analysis computed into the Advanced Bacterial Identification Software (ABIS) online system (Costin & Ionut 2015).

Preparation of Resting Bacteria

The characterization of molybdenum reduction by this bacterium, including carbon sources, effect of heavy metals, concentrations of phosphate, molybdate, pH and temperature, utilized resting cells in a microtiter plate as before with slight

modifications (Shukor & Shukor 2014). Briefly, bacterial cells were grown aerobically with shaking at 120 rpm [orbital shaker (Yihder Technology Co. Ltd., Taipei, Taiwan)] in a volume of 1 L and distributed in several 250 mL shake flasks. Incubation was carried out at room temperature. The media utilised is a modified low phosphate media (HPM or high phosphate medium) with the phosphate concentration increased to 100 mM to prevent the formation of Mo-blue because the low phosphate media leads to cell harvesting complications. Cells were centrifuged at 15,000 x g at 4°C for 10 minutes. The bacterial pellets were then rinsed twice with deionised water. The pellets were then resuspended in 20 mL of LPM with glucose omitted. Appropriate alterations to the LPM were performed as a way to meet the needs of the modification of the carbon sources, phosphate, molybdate and pH conditions during the characterisation studies. Approximately 180 µL of the appropriately modified LPM was sterilely transferred into the wells of a sterile microplate. This was followed by the addition of 20 µL of sterile glucose or other carbon sources from a stock solution to make a final concentration of 1.0 % (w/v). The total volume of the culture media in each well of the microplate was 200 µL. The microplates were then sealed (Corning® microplate, Corning Inc., Corning, NY, USA) and incubated at room temperature. Readings at 750 nm were periodically taken using a BioRad Microtiter Plate reader (Model No. 680, BioRad, Richmond, CA, USA). This wavelength is the maximum filter available for the microplate unit (Shukor & Shukor 2014). Quantification of the Mo-blue produced was carried out utilising the extinction coefficient of 11.69 $mM^{-1}cm^{-1}$ at 750 nm. The effect of several heavy metals was studied utilising Atomic Absorption Spectrometry calibration standard solutions from MERCK (Merck KGaA, Darmstadt, Germany).

Screening for Bacterial Dye Decolorisation

As the soil was sampled near a textile-producing company, the Mo-reducing bacterial isolates were also screened for their ability to decolorise dyes. Simultaneous Mo-reduction and dye decolourisation was not studied as the overlapped spectra would prevent the quantification of the decolourisation activity. The ability of the bacterium to decolourise dyes (final concentration of 100 mg/L) was carried out using the microplate format above. Dyes (Sigma-Aldrich (St. Louis, U.S.A.) with maximum wavelength in parentheses were as follows: Cresol Red (Colour Index [C.I.]. 1733-12-6) (570 nm), Crocein Orange G (C.I. 15970) (482 nm), Congo Red (C.I. 22120) (498 nm), Evans Blue (C.I. 23860) (594 nm), Fuchsin Basic (C.I. 42510) (625 nm), Crystal Violet (C.I. 42555) (590 nm), Fast Green FCF (C.I. 42053) (620 nm), Methyl Orange (C.I. 13025) (505 nm), Methyl Red (C.I. 13020) (493 nm), Metanil Yellow (C.I. 13065) (414 nm), Methyl Green (C.I. 42590) (635 nm), Orange G (C.I. 16230) (476 nm), Orange II sodium salt (C.I. 15510) (483 nm), Methylene Blue (C.I. 52015) (590 nm), Naphthol Blue Black (C.I. 20470) (618 nm), Nigrosin (C.I. 50415) (570 nm), Remazol Black B (C.I. 20505) (597 nm), Rhodamine B (C.I. 45170) (554 nm), Ponceau 2R (C.I. 16150) (388 nm), Sudan Black B (C.I. 26150) (600 nm), Tartrazine (C.I. 19140) (427 nm), Toluidine Blue (C.I. 52040) (626 nm), Ponceau

S (C.I. 27195) (352 nm), Safranin O (C.I. 50240) (530 nm), Direct Blue 71 (C.I. 34140) (586 nm), and Trypan Blue (C.I. 23850) (607 nm).

The ingredients of the growth media (% w/v) were modified from the LPM above to cater for dye-decolorization requirement, which include sodium lactate and sodium nitrate (Jain *et al.* 2012; Ng *et al.* 2014), and 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%), and Na_2HPO_4 (0.705% or 50 mM). The media was adjusted to pH 7.0.

Monitoring of Dye Decolorisation

Some of the dyes change in colour as the pH changes, and the phosphate concentration was increased to 50 mM at pH 7.0 to prevent colour change. Decolourization was monitored utilizing three standard wavelengths, which were 405, 490 and 595 nm, to cover the maximum absorption values for specific dyes (Solís *et al.* 2012), and these wavelengths were read using a BioRad 680 microplate reader. Additionally, the use of these pre-set wavelengths considers that the maximum absorption spectrum of water soluble dyes were typically shallow, and a difference of ±20 nm from the maximum absorption wavelength does not give dramatic reduction of absorbance values. The difference of the absorbance values from the initial measurements were subtracted from the final measurements after an incubation period of 48 h, and a percentage of decolourisation was calculated.

RESULTS AND DISCUSSION

The bacterial reduction of molybdenum to molybdenum blue was initially reported more than one century ago in 1896 (Capaldi & Proskauer 1896). In the last century, isolation of Mo-reducing bacteria were reported as early as 1939 (Jan 1939). After a long absence, it was reported again in 1985 (Campbell *et al.* 1985) and in 1993 (Ghani *et al.* 1993). Ghani *et al.* (1993) were the first to quickly recognize the potential of molybdenum-reducing bacterium for the bioremediation of molybdenum. The search for newly isolated Mo-reducing bacterium with the ability to detoxify other xenobiotics is indeed valuable.

Identification of Molybdenum Reducing Bacterium

Five Mo-reducing bacterial isolates were quantified for their capacity to produce Mo-blue by monitoring production at 865 nm. Out of the five isolates (Table 1), isolate 5 gave the highest absorbance value and was chosen for further studies.

Table 1: Mo-blue production by bacterial isolates.

Isolate	Abs 865 nm (±std. deviation, *n*=3)
1	0.23
	1±0.
	013
2	1.873±0.073
3	1.032±0.021
4	0.45
	2±0.
	011
5	2.198±0.034

The best Mo-reducing bacterium was a Gram-positive, rod-shaped structure as observed under a microscope, and it was motile as determined by the hanging drop method. The results for the various biochemical tests (Table 2) were input into the ABIS online software. The software suggested that the identity of the bacterium was *Bacillus circulans*, with a similarity score of 92%, and an accuracy score of 84%. The molecular identification method of 16s rRNA gene sequencing is being carried out to identify this species further. At this juncture, the bacterium is tentatively identified as *Bacillus* sp. strain Neni-10. The optimum pH for reduction of molybdenum was 6.3, and the optimum temperature was 34°C (data not shown). The pH and temperature optima are within the range reported for most of the Mo-reducing bacteria isolated to date, which range from 6.0 to 7.0 and from 25 to 37°C (Shukor *et al.* 2007; Shukor *et al.* 2008a; Rahman *et al.* 2009; Shukor *et al.* 2009a; Yunus *et al.* 2009; Shukor *et al.* 2009b; Shukor *et al.* 2010a; Shukor *et al.* 2010b; Lim *et al.* 2012; Halmi *et al.* 2013; Ibrahim *et al.* 2015; Masdor *et al.* 2015; Sabullah *et al.* 2016). The only reported psychrotolerant Mo-reducing bacterium was isolated from Antarctic soil and showed an optimum temperature supporting molybdenum reduction between 15 and 20°C (Ahmad *et al.* 2013a). The effective use of resting cells in analysing molybdenum reduction is undoubtedly an advantage, as all of the Mo-reducing bacteria isolated thus far reduce molybdenum under static or low oxygen tension (Shukor & Syed 2010; Masdor *et al.* 2015). The utilization of the microtiter plate allows a high throughput characterization of the molybdenum reduction process (Shukor & Shukor 2014). The utilisation of resting cells in the bacterial characterization of xenobiotic degradation and metal reduction in bacteria is observed in studies of selenate reduction by *Enterobacter cloacae* (Losi & Frankenberger Jr. 1997) and SDS biodegradation by several bacteria (Chaturvedi & Kumar 2011).

Table 2: Biochemical tests for *Bacillus* sp. strain Neni-10.

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

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

Molybdenum Absorbance Spectrum

The bacterium exhibited a distinctive Mo-blue spectrum having a maximum peak at 865 nm and a shoulder at 700 nm. This particular profile was preserved as the fermentation period increases (Figure 2). The production of molybdenum blue by microorganisms was initially suggested to require an enzymatic reduction from the Mo^{6+} to Mo^{5+} accompanied by the inclusion of phosphate ions, creating Mo-blue (Ghani *et al.* 1993). However, this mechanism is not consistent with molybdate chemistry. The Mo-blue spectra obtained from this work and nearly all of the Mo-reducing bacteria isolated to date (Shukor *et al.* 2007; Shukor *et al.* 2008a; Rahman *et al.* 2009; Shukor *et al.* 2009a; Yunus *et al.* 2009; Shukor *et al.* 2009b; Shukor *et al.* 2010a; Shukor *et al.* 2010b; Lim *et al.* 2012; Ahmad *et al.* 2013a; Halmi *et al.* 2013; Ibrahim *et al.* 2015; Masdor *et al.* 2015; Sabullah *et al.* 2016) are very similar to the Mo-blue spectrum determined by the phosphate determination method (PDM), which uses a reduced phosphomolybdate, having

a characteristic shoulder between 700 and 720 nm and a peak maximum between 870 and 890 nm (Kazansky & Fedotov 1980; Clesceri *et al.* 1989). Based on this similarity, we put forward the hypothesis that a phosphomolybdate intermediate is involved in molybdate reduction by bacteria (Shukor *et al.* 2007). The presence of an intermediate species in molybdate reduction is not unique to Mo-reducing bacteria. In the reduction of another similar heavy metal, chromate, the reduction from Cr^{6+} to Cr^{3+} goes through an unstable intermediate species, Cr^{5+}, as observed in the bacteria *Pseudomonas ambigua* (Suzuki *et al.* 1992) and *Shewanella putrefaciens* (Myers *et al.* 2000), where electron paramagnetic and UV-spectroscopic studies have confirmed the formation of the Cr^{5+} intermediate substrate. This occurrence is probably the result of the near likeness in the chemistry of molybdate and chromate anions (Lee 1977). Additional support for the formation of a phosphomolybdate intermediate during the bacterial reduction of molybdenum is provided by the observation that the enzymatic reduction of phosphomolybdate to Mo-blue by several enzymes, such as aldehyde and xanthine oxidases (Glenn & Crane 1956), including the Mo-reducing enzyme (Shukor *et al.* 2014), cannot utilize molybdate as a substrate (Glenn & Crane 1956; Shukor *et al.* 2008b; Shukor *et al.* 2014). Even though analyses utilizing nuclear magnetic resonance (NMR) and electron spin resonance (ESR) are needed for the detailed identification of the many lacunary species of phosphomolybdate, the spectroscopic technique is regarded as suitable for distinguishing heteropolymolybdates, such as silicomolybdate, phosphomolybdate, and sulfomolybdate (Sims 1961; Kazansky & Fedotov 1980).

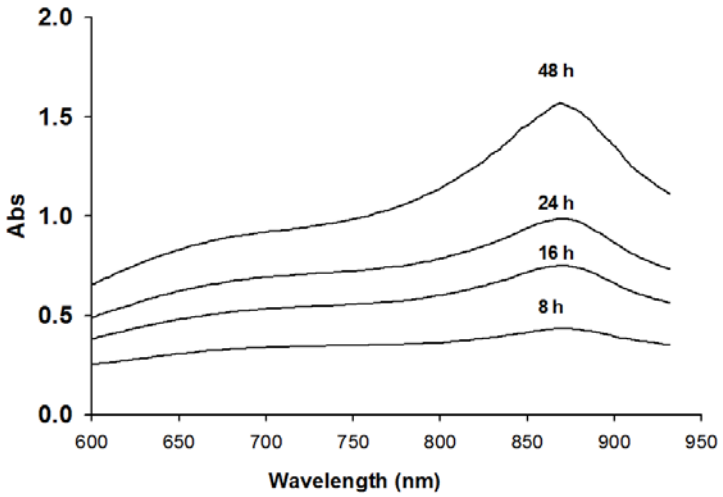

Figure 2: Mo-blue scanning absorption spectrum at different time intervals.

Effect of Various Carbon Sources as Electron Donors for Molybdate Reduction

Among the electron donors tested for aiding molybdenum reduction, glucose was the best electron donor followed by sucrose, lactose, maltose, raffinose, meso-inositol, melibiose, mannitol, d-mannose, melezitose, glycogen, glycerol and trehalose (Figure 3). The optimal concentration for glucose was 1% (w/v). Concentrations of glucose higher than 1% show a steady inhibition (data not shown), where the inhibition is probably caused by an increase in osmolarity (Shukor *et al.* 2009a). Molybdenum reduction in bacteria generally is best supported by easily assimilable sugars, such as glucose and sucrose. Mo-reducing bacteria *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) require sucrose as the ideal carbon source to aid reduction. 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.* 2013a), *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), *Bacillus* sp. strain A.rzi (Othman *et al.* 2013), *Klebsiella oxytoca* strain Aft-7 (Masdor *et al.* 2015), *Pseudomonas aeruginosa* strain Amr-11 (Ibrahim *et al.* 2015) and *Klebsiella oxytoca* strain Saw-5 (Sabullah *et al.* 2016) prefer glucose as the carbon source. The only notable exception is *Klebsiella oxytoca* strain hkeem, which prefers fructose as its carbon source, although both glucose and sucrose are also utilised (Lim *et al.* 2012). These carbon sources produce the reducing equivalents NADH (or reduced nicotinamide adenine dinucleotide) and NADPH (or reduced nicotinamide adenine dinucleotide phosphate) easily through generic metabolic pathways, and both reducing equivalents are known substrates for Mo-reducing enzymes (Shukor *et al.* 2008b; Shukor *et al.* 2014). A less expensive carbon source, for instance, molasses, may be used rather than sucrose and glucose in the foreseeable future, especially for bioremediation studies, considering that molasses can be purchased in large quantities as agricultural waste products of sugar cane in Indonesia (Nuraini *et al.* 2015). Molasses is utilized as a cheap carbon source in the bacterial reduction of hexavalent chromate (Smith *et al.* 2002; Sugiyama *et al.* 2012) and selenate (Zhang *et al.* 2008). The impact of molasses as an alternative carbon source is presently being studied.

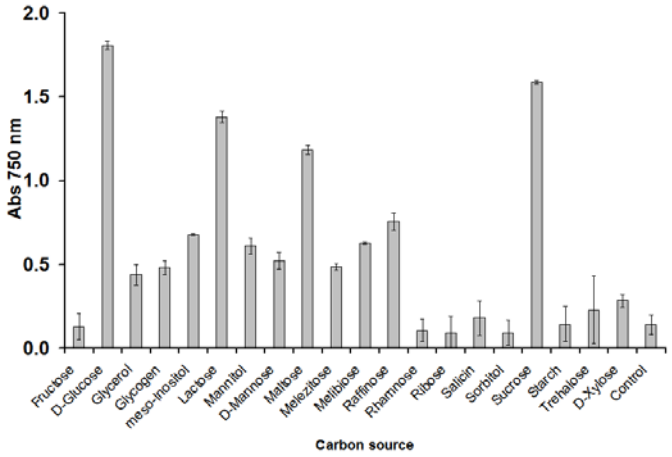

Figure 3: The effect of various carbon sources as electron donors for molybdenum reduction to molybdenum blue by *Bacillus* sp. strain Neni-10. The error bars indicate mean ± standard deviation of three replicates.

Mo-Blue Production at Various Phosphate and Molybdate Concentrations

The optimum concentration of phosphate occurred between 5.0 and 7.5 mM, with higher concentrations strongly inhibiting reduction (Figure 4). When the phosphate concentration was fixed at 5 mM, the bacteria required 20 mM sodium molybdate for optimal reduction (Figure 5). A time profile of Mo-blue production shows a lag period of approximately 12 hours. The lag period observed is probably due to the conversion of molybdate to the intermediate phosphomolybdate, which needs to reach a critical value before reduction can take place as discussed previously (Shukor & Syed 2010). The maximum amount of Mo-blue produced was observed at molybdate concentrations of 20 mM and after an incubation period of approximately 52 hours (Figure 6). Mo–blue production from bacteria is generally restricted by high concentrations of phosphate (Campbell *et al.* 1985; Ghani *et al.* 1993; Shukor *et al.* 2007; Shukor *et al.* 2008a; Rahman *et al.* 2009; Shukor *et al.* 2009a; Yunus *et al.* 2009; Shukor *et al.* 2009b; Shukor *et al.* 2010a; Shukor *et al.* 2010b; Lim *et al.* 2012; Ahmad *et al.* 2013a; Halmi *et al.* 2013; Ibrahim *et al.* 2015; Masdor *et al.* 2015; Nuraini *et al.* 2015). The main mechanism of restriction is by affecting the stability of the phosphomolybdate complex, as it requires acidic conditions for stability (Lee 1977; Shukor *et al.* 2007). Phosphate at high concentrations, and especially at a neutral pH, greatly affects the formation and stability of phosphomolybdate (Glenn & Crane 1956). Another plausible mechanism is through the phosphate anion destabilizing the complex itself, irrespective of the pH. In a study, a 100 mM phosphate concentration set at pH 5.0 destabilizes Mo-blue formed from an ascorbate-reduced phosphomolybdate, indicating that the mechanism of destabilization also includes the effect of phosphate (Shukor *et al.* 2002).

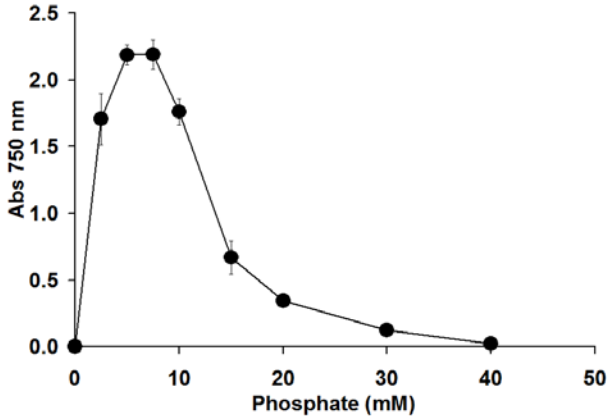

Figure 4: The effect of phosphate concentrations on molybdenum blue production. The error bars indicate mean ± standard deviation of three replicates.

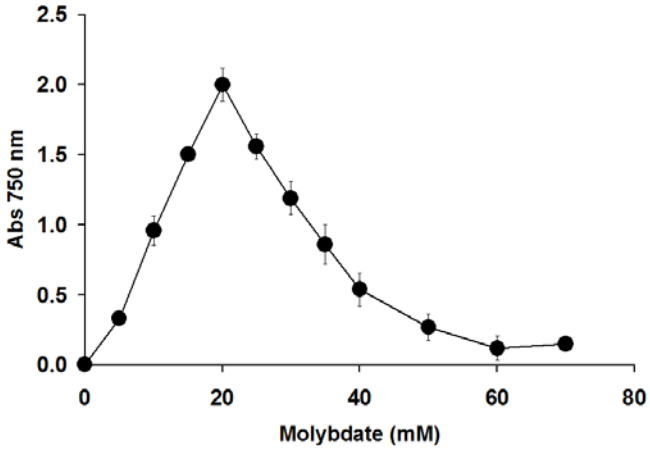

Figure 5: The effect of sodium molybdate concentrations on molybdenum blue production. The error bars indicate mean ± standard deviation of three replicates.

The range of molybdate required for optimal Mo-blue production by Mo-reducing bacteria is wide and ranges between 5 and 80 mM (Campbell *et al.* 1985; Ghani *et al.* 1993; Shukor *et al.* 2007; Shukor *et al.* 2008a; Rahman *et al.* 2009; Shukor *et al.* 2009a; Yunus *et al.* 2009; Shukor *et al.* 2009b; Shukor *et al.* 2010a; Shukor *et al.* 2010b; Lim *et al.* 2012; Ahmad *et al.* 2013a; Halmi *et al.* 2013; Ibrahim *et al.* 2015; Masdor *et al.* 2015; Nuraini *et al.* 2015). The tolerance and reduction of molybdenum at high concentrations are advantageous for the bioremediation of the molybdenum, as the highest molybdenum concentration found in the environment is 900 mg/L (approximately 9.4 mM) in water and 6,500 mg/kg in soils (approximately 68 mM) (Stone & Stetler 2008). Most of the Mo-reducing bacteria isolated so far are excellent candidates to detoxify molybdenum in bioremediation sites.

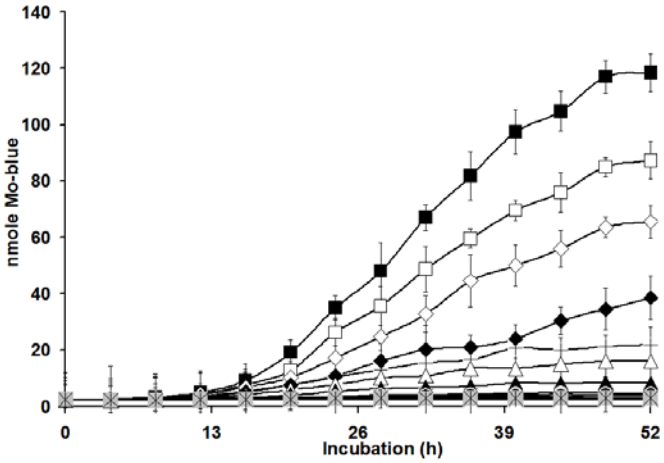

Figure 6: A time profile of molybdenum blue production at sodium molybdate concentrations of 0 (∗), 5 (×), 10 (+), 15 (□), 20 (■), 25 (◇), 30 (◆), 35 (△), 40 (▲), 50 (○), 60 (●) and 70 mM (∗). The absorbance values measured at 750 nm were converted to Mo-blue concentration measured as nmole Mo-blue. The error bars indicate mean ± standard deviation of three replicates.

Effect of Heavy Metals

Molybdenum reduction was inhibited by 91.9, 82.7, 45.5 and 17.4% by mercury, silver, copper and chromium, respectively, at 2 ppm (Figure 7). Similar heavy metals also inhibit molybdenum reduction in many of the Mo-reducing bacteria isolates to date, with mercury, silver and copper forming the majority of the inhibitory metal ions (Campbell *et al.* 1985; Ghani *et al.* 1993; Shukor *et al.* 2007; Shukor *et al.* 2008a; Rahman *et al.* 2009; Shukor *et al.* 2009a; Yunus *et al.* 2009; Shukor *et al.* 2009b; Shukor *et al.* 2010a; Shukor *et al.* 2010b; Lim *et al.* 2012; Ahmad *et al.* 2013a; Halmi *et al.* 2013; Ibrahim *et al.* 2015; Masdor *et al.* 2015; Nuraini *et al.* 2015). Copper and mercury also inhibit chromate reduction in *Bacillus* sp. (Elangovan *et al.* 2006) and *Enterobacter cloacae* strain H01 (Rege *et al.* 1997). The target site of inhibition by these two heavy metals is suggested to be the sulfhydryl group (Rege *et al.* 1997; Elangovan *et al.* 2006) and is probably similar to the Mo-reducing enzyme, as the sulfhydryl group is known to be inhibited by these metals ions (Shukor *et al.* 2006). Since most of the toxic heavy metals above are cations, the addition of cationic metal-sequestering compounds, such as manganese oxide, phosphate, calcium carbonate, and magnesium hydroxide, have already been used to remediate cationic heavy metals toxicity (Hettiarachchi *et al.* 2000). Another alternative is to immobilize the Mo-reducing bacterium in dialysis tubing to reduce the inhibitory effects of toxic cations (Halmi *et al.* 2014; Shukor & Shukor 2015).

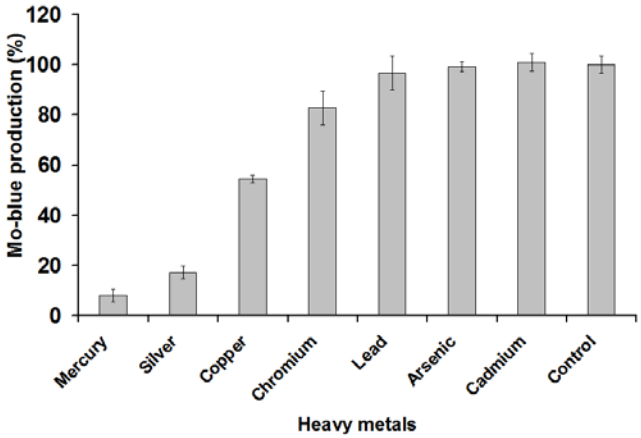

Figure 7: The effect of heavy metals on Mo-blue production by *Bacillus* sp. strain Neni-10. The error bars indicate mean ± standard deviation of three replicates.

Azo Dye-Decolorising Ability of the Molybdenum-Reducing Bacterium

Screening for decolorising ability by various dyes has shown that the best Mo-reducing bacteria, which was identified as *Bacillus* sp. strain Neni-10, can efficiently decolorise the azo dye Metanil Yellow. Other Mo-reducing bacterial isolates were not able to decolorise any of the other dyes tested. Other azo dyes, such as Methylene Blue, Crocein Orange G and Direct Blue 71, are poorly decolorised by the bacterium, whereas other azo dyes, such as Congo red, Orange II, Evans Blue, Ponceau S, Remazol Black B or 5, Methyl Orange, Tartrazine, Methyl Red, Naphthol Blue Black and others, failed to be decolorised when compared to the control (Figure 8). Azo dyes are strongly resistant to biodegradation under normal conditions, but the azo bond (–N=N–) is vulnerable to reductive cleavage (Syed *et al.* 2009). Bacterial species that have been reported to degrade this dye are *Pseudomonas* sp., *Alteromonas* sp., *Enterococcus* sp., *Serratia* sp. and *Enterobacter* sp. (Kamble & More 2013), *Vibrio harveyi* (Ozdemir *et al.* 2008), *Bacillus* sp. strain AK1 and *Lysinibacillus* sp. (Anjaneya *et al.* 2011), *Oenococcus oeni* (El Ahwany 2008) and an unknown bacterial species (Johari 2014).

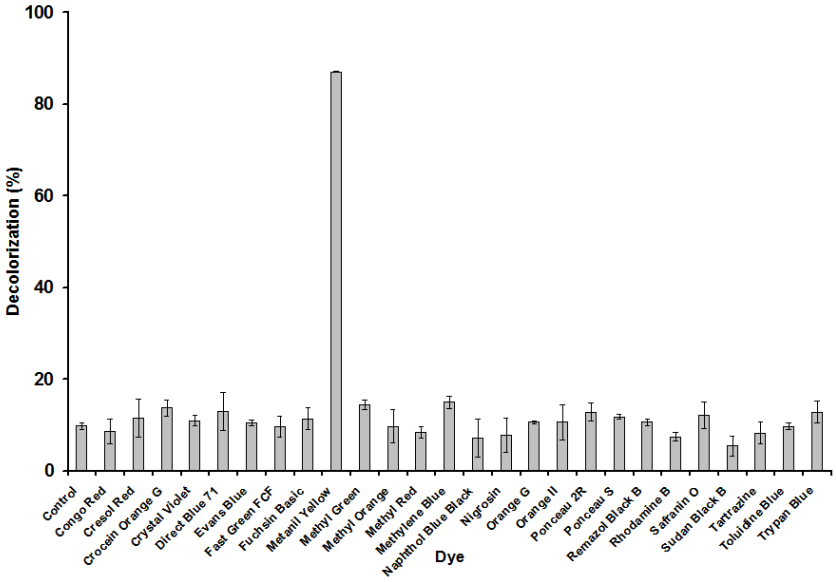

Figure 8: Decolourisation of various dyes by *Bacillus* sp. strain Neni-10. The error bars indicate mean ± standard deviation of three replicates.

The bacterium was then tested with various concentrations of dye, utilising the microplate format as before. The results show that the complete decolourisation of the dye at 100 and 150 mg/L occurred at day three and day six of incubation, respectively, whereas higher concentrations show partial degradation, with a 20% decolourisation observed at the highest dye concentration tested, 400 mg/L (Figure 9). A similar work carried out in the bacteria *Bacillus* sp. strain AK1 and *Lysinibacillus* sp., utilizing this dye under static conditions, indicated a better decolourization capability with complete decolourization at 1000 mg/L after 78 and 84 hours of incubation with *Bacillus* sp. strain AK1 and *Lysinibacillus* sp., respectively (Anjaneya *et al.* 2011). The researchers suggested that the inhibition of decolourisation at high concentrations is probably due to the inhibitory effect of the dye to the decolorising activity. A proposed route of Metanil Yellow degradation by these bacteria is suggested through the action of azoreductase producing the metabolites metanillic acid and p-aminodiphenylamine based on High-Performance Liquid Chromatography (HPLC) and Gas Chromatography/Mass Spectrometry (GC/MS) analysis (Anjaneya *et al.* 2011).

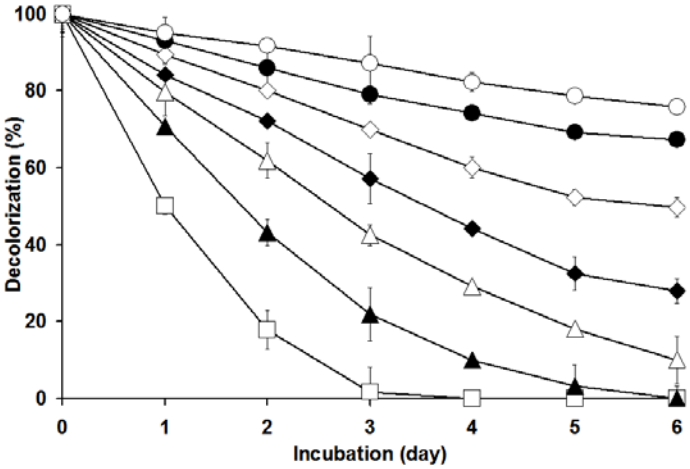

Figure 9: Decolourisation of various concentrations of Metanil Yellow at 100 (□), 150 (▲), 200 (△), 250 (◆), 300 (◇), 350 (●) and 400 (○) mg/L by *Bacillus* sp. strain Neni-10. The error bars indicate mean ± standard deviation of three replicates.

CONCLUSION

The screening of soil samples yielded five molybdenum-reducing isolates. The best isolate shows the novel ability to decolorise the azo dye Metanil Yellow, whereas other azo dyes poorly decolorise. Characterisation was carried out in a microtiter or microplate format using a preparation of resting cells. This is the first report of a molybdenum reducing bacterium with the ability to decolorise dye. Glucose was the best electron donor, whereas the most important requirement is phosphate, where a narrow concentration of between 2.5 and 7.5 mM was optimal. The absorption spectrum of the Mo-blue suggested the compound is a reduced phosphomolybdate. Molybdenum reduction was inhibited by heavy metals. The isolation of this bacterium, which displays heavy metal reduction and dye-decolorising ability, is sought after, particularly for bioremediation. Currently, work is being carried out to characterise dye decolourisation in greater detail, specifically to identify the bacterium via molecular techniques and purify the enzyme response for reducing molybdenum.

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