

RESEARCH ARTICLE

A PEG 4000-degrading and hexavalent molybdenum-reducing *Pseudomonas putida* strain Egypt-15

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Abstract: The objectives of this work were to isolate and characterise a heavy metal-reducing bacterium with the capability to degrade another xenobiotic; an organic pollutant. Six molybdenum-reducing bacteria from soil that can reduce sodium molybdate into the colloidal molybdenum blue (Mo-blue) were isolated. One of these isolates identified as *Pseudomonas putida* strain Egypt-15 was capable of growing on PEG 4000. The optimal conditions for Mo-blue production were 34 °C, pH 6.5, 20 mM molybdate, and glucose as the electron donor. The optimum concentration supporting the growth on PEG 4000 was between 600 and 800 mgL⁻¹. PEG degradation showed a lag period of about two days and 75 % degradation of PEG 4000 was achieved on day six at 800 mgL⁻¹. Growth on PEG 4000 at 800 mgL⁻¹ modelled according to the modified Gompertz model gave a maximum specific growth rate of 2.216 d⁻¹ and a lag period of 1.45 days. Growth on PEG was optimum at 30 °C and pH 7.5. The dual ability of this bacterium to detoxify molybdenum and degrade PEG is novel and will be very useful for bioremediation.

Keywords: Modified Gompertz, molybdenum blue, molybdenum-reducing bacteria, polyethylene glycol biodegradation, *Pseudomonas putida* strain Egypt-15.

INTRODUCTION

Industrial and mining activities of molybdenum are two major sources of molybdenum pollution worldwide. The principal molybdenum usage is for the alloy and

steel industries. Areas near these industries have been reported to be contaminated with molybdenum. The Black Sea and the Japan Bay and soils from a grazing area of cattle in Austria (Neunhäuserer *et al.*, 2001) are reported to be affected with molybdenum pollution. Aside from industrial activity, a more serious pollution of molybdenum is reported from mining activities. The highest concentration of molybdenum in the environment to date is about 6,500 mgKg⁻¹ (approximately 68 mM) from an abandoned uranium mine (Stone & Stetler, 2008). In Malaysia, molybdenum is produced in small quantities from copper mining. Although the mine has been abandoned for several years, its mine tailings and holding dams still contain elevated concentrations of toxic metal ions, polluting the surrounding ecosystems (Ali *et al.*, 2006).

Molybdenum transformations into either insoluble molybdenum disulphide or to colloidal molybdenum blue (Mo-blue) are potential mechanisms for molybdenum bioremediation (Ghani *et al.*, 1993). As the production of molybdenum disulphide results in the production of toxic hydrogen sulphide gas, bacterial transformation of molybdenum to Mo-blue is a better mechanism for the bioremediation of molybdenum as reduction occurs under near neutral pH and facultative conditions (Ghani *et al.*, 1993). Numerous Mo-reducing bacteria have been isolated with Mo-reducing properties from the bacterial

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genera *Klebsiella* (Lim *et al.*, 2012; Halmi *et al.*, 2013; Masdor *et al.*, 2015; Sabullah *et al.*, 2016), *Bacillus* (Abo-Shakeer *et al.*, 2013; Othman *et al.*, 2013), *Pseudomonas* (Shukor *et al.*, 2010a; Ahmad *et al.*, 2013), *Enterobacter* (Shukor *et al.*, 2009a), *Escherichia* (Capaldi & Proskauer, 1896; Campbell *et al.*, 1985), *Acinetobacter* (Shukor *et al.*, 2010b) and *Serratia* (Shukor *et al.*, 2008b; Rahman *et al.*, 2009; Shukor *et al.*, 2009b; Yunus *et al.*, 2009), and have been characterised.

Industries such as pharmaceutical, lubricant, cosmetic, and antifreeze are the main consumers of polyethylene glycol (PEG) and non-ionic surfactant-based PEGs. Several millions of tons of this compound are consumed and produced annually. PEGs are water-soluble polymers and have the common structural formula of $\text{HO}(\text{CH}_2\text{CH}_2\text{O})_n\text{CH}_2\text{CH}_2\text{OH}$, which differ in their chain lengths and molecular weights. PEG with $n = 9$ possesses an average molecular weight of about 400 daltons, and the PEG is labelled as PEG 400 (Traverso-Soto *et al.*, 2016). The toxicity of PEG to mammals has been documented. PEG is a parasymphomimetic-like chemical. Wounded rabbits that are exposed topically to PEG-based antimicrobial cream showed that PEG absorption leads to symptoms of nephrotoxicity with several deaths after one week of exposure (Traverso-Soto, *et al.*, 2016). Absorption of PEG into the bloodstream causes a fatal toxic syndrome through the formation of mono- and dicarboxylated forms (Traverso-Soto *et al.*, 2016). Effluents contaminated with PEGs usually reach conventional sewage treatment systems making them a significant pollutant (Watanabe & Kawai, 2010). PEG is a recalcitrant xenobiotic, and contamination of aquatic sediments in seas and rivers have been reported, and PEG degradation in these sediments are even slower. A study has shown that 5 mgL^{-1} of various PEGs of up to PEG 400 require about 170 days to completely degrade a small concentration of PEG under anaerobic conditions (Traverso-Soto *et al.*, 2016). In general, PEGs with longer carbon chains degrade more slowly than shorter chains. Chains longer than PEG 10000 are either slowly degraded anaerobically with incubation time in hundreds of days or in the presence of bacterial co-culture aerobically. The existence of higher chain-PEG-degrading monoculture (PEG 20000) bacteria are rare and a few exists (Watanabe & Kawai, 2010). The principal PEG-degrading enzymes under aerobic conditions are alcohol dehydrogenase, aldehyde dehydrogenase and an ether-bond splitting enzyme that progressively oxidises PEG into carboxylic acid groups, eventually producing glyoxylate (GOA), which can be integrated into generic anabolic pathways starting from the glyoxylate shunt (Marchal *et al.*, 2008; Watanabe & Kawai, 2010).

Since rivers and soils receive effluents from many industries, contamination of the environment with heavy metals and toxic xenobiotics including molybdenum and PEG is anticipated. This presents a challenge for efficient bioremediation. The use of several microorganisms each displaying multiple detoxification attributes can reduce the cost of bioremediation. Hence, the focus of this research was to identify a microorganism with both heavy metal and xenobiotic detoxification property, with a distinctive focus on molybdenum as the model metal and PEG as the model xenobiotic. In this study, a molybdenum-reducing bacterium that can grow on PEG 4000 is first reported. In addition, the modified Gompertz model will be utilised to model the growth on PEGs to obtain important growth parameters. The multiple detoxification potential of this bacterium will be an important component of a battery of microbial degraders needed to cleanup multiple toxicant-containing polluted sites.

METHODOLOGY

Site of soil sampling

Ten grams of soil was taken from about 5 cm below the topsoil using a sterile large steel spatula and placed in a sterile polycarbonate container. The soil sample was taken from a riverbank land receiving agricultural run offs and industrial effluents in Sadat City, Egypt, in 2014.

Molybdenum reducing low phosphate molybdate media

The isolation of Mo-reducing bacterial candidates was carried out on a low phosphate minimal salts medium (MSM), which is also called low phosphate molybdate (LPM) medium. Another growth medium is the high phosphate molybdate medium or HPM, which is similar to the LPM but the phosphate concentration is increased to 100 mM to prevent cell aggregation with the colloidal Mo-blue. Mo-reducing enzyme activity was similar either in LPM or HPM medium (Ghani *et al.*, 1993; Shukor *et al.*, 2008a; 2014). A soil suspension was initially prepared by mixing 1 g of soil with 10 mL of deionised water. Then, 0.1 mL of the soil suspension was spread onto a Petri dish containing LPM medium (w/v) at pH 6.5 with the following composition: $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.242 % or 10 mM), NaCl (0.5 %), agar (1.5 %), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.05 %), glucose (1 %), yeast extract (0.5 %), $(\text{NH}_4)_2\text{SO}_4$ (0.3 %), and Na_2HPO_4 (0.071 % or 5 mM) (Masdor *et al.*, 2015). After 48 h of incubation at room temperature, 6 blue colonies appeared on the plate.

The isolates were repeatedly transferred onto fresh LPM agar plates to purify the isolate for further screening on growth on various PEGs.

Assay for Mo-blue production and scanning absorption spectrum

Mo-blue production was quantified in a 100 mL liquid culture of LPM medium. This was carried out to choose the most efficient isolate. The culture supernatant was first centrifuged at 10,000 g for 5 min and the absorbance of the supernatant was read at 750 nm. An extinction coefficient of 11.69 mM⁻¹cm⁻¹ at 750 nm was utilised to choose the best isolate (Masdor *et al.*, 2015). The centrifuged culture supernatant was also scanned between 400 and 900 nm utilising LPM medium as the baseline correction.

Identification of Mo-reducing bacterium

The bacterium was identified based on phenotypical and biochemical methods as outlined in the Bergey's Manual of Determinative Bacteriology and computed into the ABIS online system as before (Masdor *et al.*, 2015).

Preparation of resting cells for Mo-blue production, characterisation and PEG screening

Preparation of resting cells was carried out according to a previous method (Shukor *et al.*, 2014). The bacterium was grown in 1 L of HPM on an orbital shaker (120 rpm) at room temperature (28 °C) for 48 h. Cells were centrifuged for 10 min at 10,000 g, and the cellular pellets were then washed twice with deionised water, followed by resuspension in 20 mL of LPM medium (glucose omitted initially) giving an optical density reading of about 1.0 at 600 nm. The cell suspension prepared (180 µL) was transferred to each well of a sterile microplate. Sterile glucose or other sugars as carbon sources (20 µL) from a 10 % (w/v) stock solution was mixed with the cell suspension to initiate Mo-blue production. The microplate was covered with a sterile sealing tape to allow gas exchange (Corning® microplate). The microplate was incubated at room temperature and Mo-blue production was determined at 750 nm in a microtiter plate reader model no. 680 (BioRad, Richmond, CA). The specific extinction coefficient of 11.69 mM⁻¹cm⁻¹ was utilised to determine Mo-blue concentration (Shukor *et al.*, 2003).

Screening of PEGs as carbon sources for growth

Preliminary studies show that none of the PEGs utilised in this work such as PEG 4000, PEG 5000, PEG 8000, PEG 10000, PEG 14000 and PEG 20000 (Sigma Aldrich,

USA) can support molybdenum reduction to Mo-blue. Thus, the various PEGs were tested for their ability to support growth instead. The study was carried out using the microplate format above in the growth medium below with PEGs utilised at a final concentration of 1000 mgL⁻¹ in a final volume of 250 µL. A 20 µL cell suspension from the above resting cell preparation was added as an initial inoculum. The ingredients of the growth medium (pH 7.0) were as follows: NaNO₃ (0.2 %), (NH₄)₂SO₄ (0.3 %), yeast extract (0.01 %), MgSO₄·7H₂O (0.05 %), Na₂HPO₄ (0.705 % or 50 mM), NaCl (0.5 %), and 1 mL of trace elements solution. The trace elements solution composition (mgL⁻¹) was as follows: FeSO₄·7H₂O (40), CaCl₂ (40), MnSO₄·4H₂O (40), CuSO₄·5H₂O (5), ZnSO₄·7H₂O (20), Na₂MoO₄·2H₂O (5) CoCl₂·6H₂O (5). Bacterial growth was determined optically at 600 nm after five days of incubation at room temperature (28 °C) using the microplate reader (Bio-Rad 680).

Assay for PEG

PEG was determined through a colourimetric method that is sensitive, simple, and applicable to all PEG derivatives (Sims & Snape, 1980). Stock solutions of various PEGs were prepared as 10 mgL⁻¹. The total volume of the mixture was 2 mL and consisted of stably diluted stock solutions of various PEGs to make the final concentration between 1 to 7.5 mgL⁻¹. Samples from the resting cell preparation were suitably diluted before being added to the assay reagents. Samples were taken at initial incubation and after an extended incubation period of 10 ds at room temperature. The colour developing reagents consist of a 200 µL solution of 5 % (w/v) BaCl₂·H₂O in 1 M HCl mixed with 200 µL of a solution of 1.27 g iodine dissolved in 100 mL of 2 % (w/v) potassium iodide. Colour that developed after 10 min of incubation at room temperature (28 °C) was read at 535 nm against a reagent blank using water. The concentration of PEG was determined based on the formation of an intensity of brownish orange colour by the complex solution (Watanabe & Kawai, 2010).

The percentage of PEG degradation was calculated as:

$$\% \text{ PEG degradation} = [1 - ([\text{PEG}]_{t_f} / [\text{PEG}]_{t_i})] \times 100\% \quad \dots(1)$$

where [PEG]_{t_f} indicates the concentration of PEG at the time of final incubation, and [PEG]_{t_i} indicates the concentration of PEG at the time of initial incubation.

Mathematical modelling of growth on PEG

The modified Gompertz model (equation 2) (Gibson *et al.*, 1987) is a robust model that can describe the growth

of a bacterium on a multitude of substrates. The model was utilised to describe the growth of the bacterium on PEG 4000. The absorbance value at 600 nm was first converted to natural logarithm. This study was carried out in a scale-up version aerobically. The bacterium was grown aerobically on an orbital shaker (120 rpm) at room temperature in 50 mL of culture media for this purpose. The initial bacterial inoculum was 1 % (v/v).

$$y = A \exp \left\{ - \exp \left[\frac{\mu_m e}{A} (\lambda - t) + 1 \right] \right\} \quad \dots(2)$$

where A = bacterial growth at lower asymptote; μ_m = maximum specific bacterial growth rate, λ = lag time, e = exponent (2.718281828) and t = sampling time.

The growth parameters μ_m and λ were obtained using a nonlinear regression analysis employing the Levenberg-Marquardt algorithm (LMA) through the CurveExpert Professional software (version 1.6). The algorithm minimises the sum of the squares of the differences between the measured and predicted values (Zwietering *et al.*, 1990). In non-linear regression, a modified form of the coefficient of determination, the adjusted coefficient of determination ($adjR^2$) was utilised instead of the often used R^2 , as the former takes into account the number of parameters of a model. The modified Gompertz model has 3 parameters to be solved. The formulae are as follows (equations 3 and 4), where RMS is the residual mean square and S_y^2 is the total variance of the y variable (Zwietering *et al.*, 1990).

$$Adjusted(R^2) = 1 - \frac{RMS}{S_y^2} \quad \dots(3)$$

$$Adjusted(R^2) = 1 - \frac{(1 - R^2)(n - 1)}{(n - p - 1)} \quad \dots(4)$$

Identification of the bacterium through 16S rDNA gene sequencing

Bacterial genomic DNA was obtained through alkaline lysis. The 16S rDNA from the genomic DNA was amplified *via* PCR (Biometra, Montreal Biotech Inc., Kirkland, QC) using the following primers; 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-AAGGAGGTGATCCAGCCGCA-3' corresponding to the forward and reverse primers of 16S rDNA, respectively (Devereux & Willis, 1995). The 16S rRNA gene sequence of this bacterium (1447 bp) has been submitted to the DNA Data Bank of Japan under

the accession number LC150887. Several 16S rRNA gene sequences of other bacterial isolates that closely matched this isolate were retrieved from GenBank and then aligned using the programme Clustal Omega. A bootstrapped phylogenetic tree was constructed utilising the suits of programmes in PHYLIP, version 3.573.

RESULTS AND DISCUSSION

The phenomenon of molybdenum reduction to Mo-blue is observed predominantly in heterotrophic bacteria from the Enterobacteriaceae family using minimal salts or complex media under low phosphate (< 10 mM) and elevated molybdate (> 10 mM) conditions (Campbell *et al.*, 1985). Bacterial reduction of molybdate to Mo-blue was first mentioned more than a century ago in the year 1896 (Capaldi & Proskauer, 1896). Intermittent or sporadic reports on this phenomenon since then reflects the rarity of this phenomenon (Campbell *et al.*, 1985; Ghani *et al.*, 1993). Of these reports, comprehensive studies were carried out by Campbell *et al.* (1985) on the bacterium *Escherichia coli* K12 and by Ghani *et al.* (1993) on the bacterium *Enterobacter cloacae* strain 48 (EC 48) with the latter quickly recognising the potential application of this phenomenon for molybdenum bioremediation. Table 1 shows the characteristics of Mo-reducing bacteria isolated to date.

Selection of Mo-reducing bacterium exhibiting PEG degradation

Six Mo-reducing bacterial candidates were isolated and then screened for growth on PEG. None of the PEGs tested could be used as electron donor for supporting molybdenum reduction (Table 2). The best candidate giving the highest Mo-blue was isolate O with 12.04 nmole Mo-blue produced after five days of incubation at room temperature. However, isolate N can reduce molybdenum and grow on PEG 4000 (Table 2), and it was chosen for further studies.

Microscopic analysis showed that the isolate was a Gram-negative, rod-shaped and motile bacterium. The results from the biochemical tests (Table 3) were then analysed using the ABIS online software. The two suggestions for the bacterial identity were *Pseudomonas putida* and *P. monteilii* with a similar homology score of 80 % and an accuracy score of 85 %. A molecular identification technique through phylogenetic analysis of the 16S rRNA gene was also carried out. The phylogenetic tree in Figure 1 shows the relationship between strain Egypt-15 and other closely related species. Strain Egypt-15 was closely related to *P. putida* with a high bootstrap value.

Table 1: Characterisation of Mo-reducing bacteria isolated to date

Bacteria	Optimal C source	Optimal molybdate (mM)	Heavy metals inhibition	Author
<i>Klebsiella oxytoca</i> strain Saw-5	glucose	20 – 30	Cu ²⁺ , Ag ⁺ , Hg ²⁺ , Cd ²⁺	(Sabullah <i>et al.</i> , 2016)
<i>Pseudomonas aeruginosa</i> strain Amr-11	glucose	20 – 30	Cd ²⁺ , Cr ⁶⁺ , Cu ²⁺ , Ag ⁺ , Hg ²⁺	(Ibrahim <i>et al.</i> , 2015)
<i>Klebsiella oxytoca</i> strain Afi-7	glucose	5 – 20	Cu ²⁺ , Ag ⁺ , Hg ²⁺	(Masdor <i>et al.</i> , 2015)
<i>Bacillus</i> sp. strain A.rzi	glucose	50	Cd ²⁺ , Cr ⁶⁺ , Cu ²⁺ , Ag ⁺ , Pb ²⁺ , Hg ²⁺ , Co ²⁺ , Zn ²⁺	(Othman <i>et al.</i> , 2013)
<i>Bacillus pumilus</i> strain lbna	glucose	40	As ³⁺ , Pb ²⁺ , Zn ²⁺ , Cd ²⁺ , Cr ⁶⁺ , Hg ²⁺ , Cu ²⁺	(Abo-Shakeer <i>et al.</i> , 2013)
<i>Pseudomonas</i> sp. strain DRY1	glucose	30 – 50	Cd ²⁺ , Cr ⁶⁺ , Cu ²⁺ , Ag ⁺ , Pb ²⁺ , Hg ²⁺	(Ahmad <i>et al.</i> , 2013)
<i>Klebsiella oxytoca</i> strain hkeem	fructose	80	Cu ²⁺ , Ag ⁺ , Hg ²⁺	(Lim <i>et al.</i> , 2012)
<i>Pseudomonas</i> sp. strain DRY2	glucose	15 – 20	Cr ⁶⁺ , Cu ²⁺ , Pb ²⁺ , Hg ²⁺	(Shukor <i>et al.</i> , 2010a)
<i>Acinetobacter calcoaceticus</i> strain Dr.Y12	glucose	20	Cd ²⁺ , Cr ⁶⁺ , Cu ²⁺ , Pb ²⁺ , Hg ²⁺	(Shukor <i>et al.</i> , 2010b)
<i>Serratia marcescens</i> strain Dr.Y9	sucrose	20	Cr ⁶⁺ , Cu ²⁺ , Ag ⁺ , Hg ²⁺	(Yunus <i>et al.</i> , 2009)
<i>Serratia</i> sp. strain Dr.Y8	sucrose	50	Cr, Cu, Ag, Hg	(Shukor <i>et al.</i> , 2009b)
<i>Enterobacter</i> sp. strain Dr.Y13	glucose	25 – 50	Cr ⁶⁺ , Cd ²⁺ , Cu ²⁺ , Ag ⁺ , Hg ²⁺	(Shukor <i>et al.</i> , 2009a)
<i>Serratia</i> sp. strain Dr.Y5	glucose	30	n.a.	(Rahman <i>et al.</i> , 2009)
<i>Serratia marcescens</i> strain DRY6	sucrose	15 – 25	Cr ⁶⁺ , Cu ²⁺ , Hg ²⁺	(Shukor <i>et al.</i> , 2008b)
<i>Enterobacter cloacae</i> strain 48	sucrose	20	Cr ⁶⁺ , Cu ²⁺	(Ghani <i>et al.</i> , 1993)
<i>Escherichia coli</i> K12	glucose	80	Cr ⁶⁺	(Campbell <i>et al.</i> , 1985)

Hence, based on biochemical and molecular phylogeny results, the bacterium was assigned as *Pseudomonas putida* strain Egypt-15. Two previous Mo-reducing bacteria from this genus *Pseudomonas* sp. strain DRY2 (Shukor *et al.*, 2010a) and the Antarctic bacterium *Pseudomonas* sp. strain DRY1 (Ahmad *et al.*, 2013) have been reported. *Pseudomonas* spp. are well known PEG degraders (Pan & Gu, 2007; Watanabe & Kawai, 2010). Mo-blue production was optimum at 34 °C and pH 6.5 (data not shown). The use of a microplate format expedite and simplify PEG-screening processes as well as simplifying Mo-reduction characterisation (Shukor & Shukor, 2014). The use of washed, whole or resting cells in studying xenobiotic detoxifications has been carried out in the characterisation of molybdenum reduction (Ghani *et al.*, 1993) and also in PEG degradation studies (Kawai & Yamanaka, 1989).

Molybdenum absorbance spectrum

The absorption spectrum of Mo-blue produced by *P. putida* strain Egypt-15 exhibited a maximum peak near the infrared region at 865 nm and a shoulder at 710 nm (Figure 2). The Mo-blue spectrum from this bacterium is very similar to the Mo-blue spectrum seen in the phosphate determination method (PDM) using ascorbic acid as a reducing agent. The PDM's Mo-blue spectrum has a maximum absorption between 880 and 890 nm and a shoulder between 700 and 720 nm (Clesceri *et al.*, 1989). Incidentally, the entire Mo-blue spectra from other Mo-reducing bacteria are very similar (Shukor *et al.*, 2007; Shukor & Syed, 2010; Masdor *et al.*, 2015; Sabullah *et al.*, 2016). As the Mo-blue from the PDM method is a reduced phosphomolybdate, it is proposed that the identity of the Mo-blue from bacterial reduction of molybdate is also a reduced phosphomolybdate.

Table 2: Growth on various PEGs measured as optical density increase at 600 nm and Mo-blue production after 5 days of incubation at room temperature. Absorbance increase of less than 0.075 absorbance unit at 600 nm was considered as no growth (-)

Isolate	nmole Mo-blue (± standard deviation, n = 3) on glucose	nmole Mo-blue (± standard deviation, n = 3) on PEG	PEG 4000	PEG 5000	PEG 8000	PEG 10000	PEG 14000	PEG 20000
K	8.13 ± 0.02	-	-	-	-	-	-	-
L	9.43 ± 0.03	-	-	-	-	-	-	-
M	10.24 ± 0.04	-	-	-	-	-	-	-
N	11.76 ± 0.08	-	0.42 ± 0.26	-	-	-	-	-
O	12.04 ± 0.05	-	-	-	-	-	-	-
P	5.35 ± 0.03	-	-	-	-	-	-	-

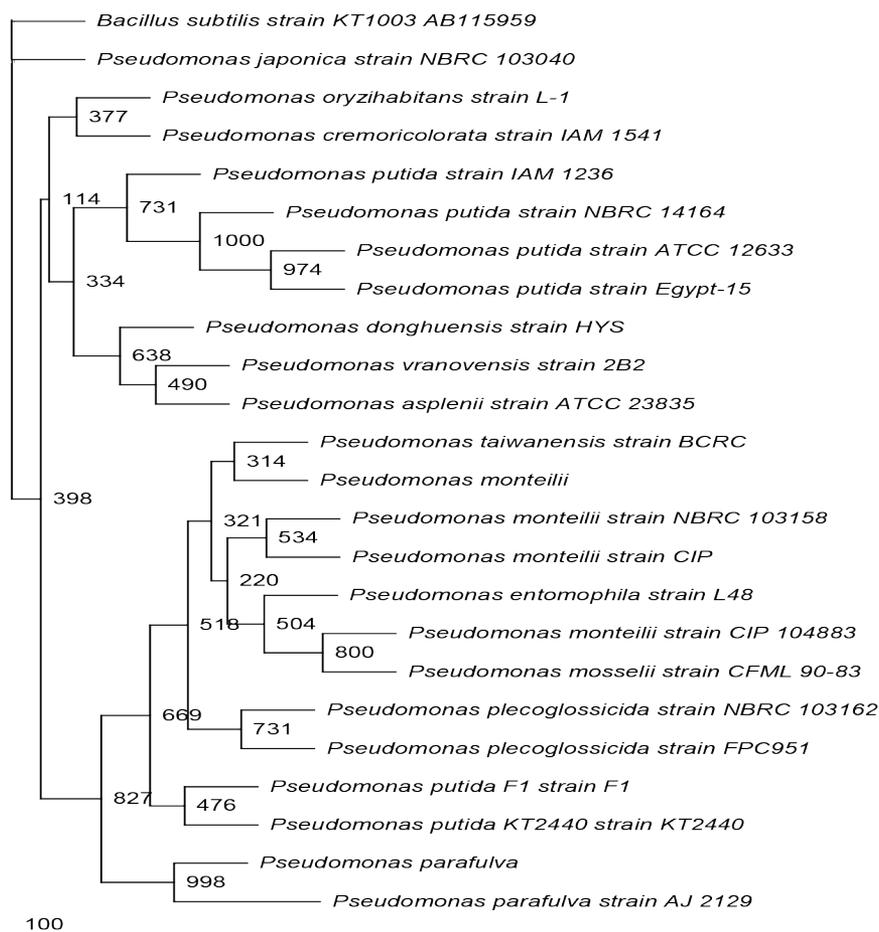
**Figure 1:** A neighbour-joining method phylogram based on the 16S rRNA gene sequence analysis showing the linkage between strain *P. putida* Egypt-15 and other reference microorganisms. *Bacillus subtilis* is the outgroup. The bootstrap values, which are based on 1000 re-samplings is shown at the branching points. Scale bar indicates 100 nucleotide substitutions.

Table 3: Biochemical tests for *P. putida* strain Egypt-15

Test	Result
Motility	+
Haemolysis	+
Growth at 4 °C	-
Growth at 41 °C	+
Growth on MacConkey agar	-
Arginine dihydrolase (ADH)	+
Alkaline phosphatase (PAL)	+
Indole production	d
Nitrates reduction	-
Lecithinase	-
Lysine decarboxylase (LDC)	-
ONPG (beta-galactosidase)	d
Esculin hydrolysis	-
Gelatin hydrolysis	d
Starch hydrolysis	-
Urea hydrolysis	+
Oxidase reaction	+
Utilisation of: L-Arabinose	+
Citrate	+
Fructose	+
Glucose	+
Meso-inositol	d
Mannose	+
Mannitol	-
Sorbitol	-
Sucrose	+
Lysine sucrose	+
Trehalose	-
Xylose	-

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

It is also proposed that phosphomolybdate is an intermediate substrate and have replaced molybdate with phosphomolybdate as a substrate for the Mo-reducing enzyme (Shukor *et al.*, 2008a) resulting in the first purification of the enzyme (Shukor *et al.*, 2014) since it was first reported in 1896 (Capaldi & Proskauer, 1896).

Carbon sources as potential electron donors to Mo-blue production

Glucose at 1 % (w/v) was found to be the best electron donor for supporting Mo-blue production, followed by sucrose, d-mannose, lactose, melibiose, maltose,

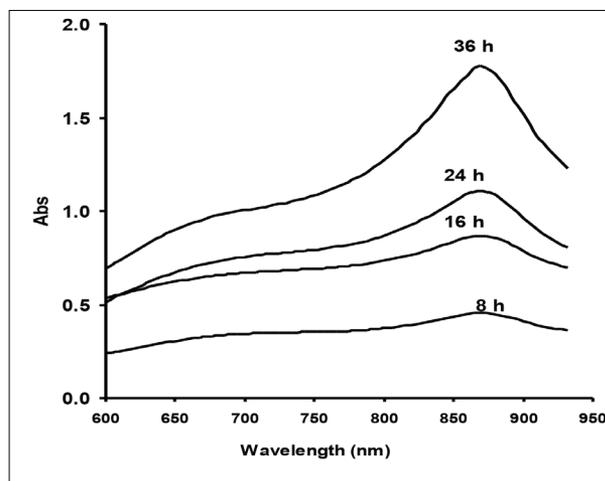


Figure 2: Scanning absorption spectrum of Mo-blue from *P. putida* strain Egypt-15 at different time intervals

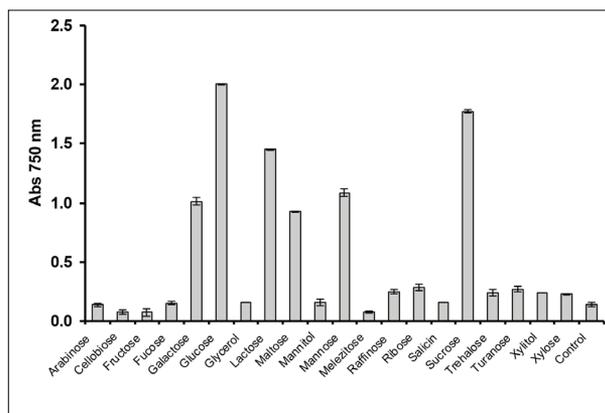


Figure 3: The effect of various carbon sources as potential electron donor sources (1 % w/v) for Mo-blue production. The error bars indicate standard deviation of the average of triplicates

trehalose, l-rhamnose, l-arabinose and myo-inositol in descending order (Figure 3). Glucose was optimal at this concentration, while higher concentrations inhibit Mo-blue production (data not shown) possibly due to osmotic stress (Shukor *et al.*, 2009b; Masdor *et al.*, 2015). The simple glucose formate or lactate are among the most optimal electron donors for supporting metal reductions in bacteria such as in vanadate reduction (Antipov *et al.*, 2000). In nearly all the Mo-reducing bacteria studied, either glucose or sucrose is the best electron donor for molybdenum reduction (Table 1). These carbon sources are efficiently converted to the reducing equivalents NADH or NADPH, with the latter compounds being substrates for the Mo-reducing enzyme

(Ghani *et al.*, 1993; Shukor *et al.*, 2008a; Shukor *et al.*, 2014). As some metal reduction studies have turned to the more economic substrate molasses (Zhang *et al.*, 2008; Ahmad *et al.*, 2013), the current work include the use of molasses as a replacement for glucose.

The effect of sodium molybdate concentrations to Mo-blue production

Both molybdate and phosphate are important for efficient Mo-blue production. Of the two, phosphate concentration is a more critical factor. The concentration supporting optimal Mo-blue production is very narrow from 2.5 to 7.5 mM, but the most reported is at 5 mM (Table 1). On the other hand, optimal molybdate concentration varies in a large range between 5 to 80 mM. In this bacterium, the optimal reduction value for molybdate which supports optimal Mo-blue production was at 20 mM. However, the bacterium can tolerate concentrations as high as 70 mM but the reduction capability is severely affected (Figure 4). Compared to anionic heavy metals such as mercury and cadmium, anionic heavy metals including molybdenum are tolerable by bacteria. For example, tolerance and reduction at high concentrations of vanadate, at 50 mM has been reported (Antipov *et al.*, 2003). The highest concentration of molybdenum in the environment is about 6,500 mgkg⁻¹ (approximately 68 mM) (Stone & Stetler, 2008). At this concentration, majority of the Mo-reducing bacteria including *P. putida* strain Egypt-15 are capable of converting toxic molybdenum into a less soluble and less toxic colloidal Mo-blue.

PEGs as carbon sources for growth

The optimum concentrations supporting growth on PEG 4000 for the bacterium was studied by utilising PEG concentrations between 100 and 1200 mgL⁻¹, where growth was best supported at concentrations between 600 and 800 mgL⁻¹. PEG 4000 at higher concentrations was slightly inhibitory to growth (Figure 5). Confirmation of degradation was then monitored colourimetrically. Degradation started slowly with a detectable lag period of about two days. A 75 % degradation of PEG 4000 was observed on day six (Figure 6). The growth of the bacterium on PEG 4000 at 800 mgL⁻¹ was modelled according to the modified Gompertz model (Figure 7). The growth parameters obtained were, maximum specific growth rate of 2.216 ± 0.118 d⁻¹ and a lag period of 1.45 ± 0.04 day. The model showed an adjusted coefficient of determination ($adjR^2$) of 0.995, indicating a good agreement between predicted and observed values. The presence of a lag period in PEG degradation was also

observed in other bacterial PEG degradation studies, and generally is shorter (about one to two days) under aerobic conditions and longer (> 10 days) under anaerobic conditions (Huang *et al.*, 2005; Traverso-Soto *et al.*, 2016). Growth on PEG was optimum at 30 °C and pH 7.5 (data not shown). The principal PEG-degrading enzymes under aerobic conditions are alcohol dehydrogenase, aldehyde dehydrogenase and an ether-bond splitting enzyme that progressively oxidises PEG into carboxylic acid groups, eventually producing glyoxylate (GOA),

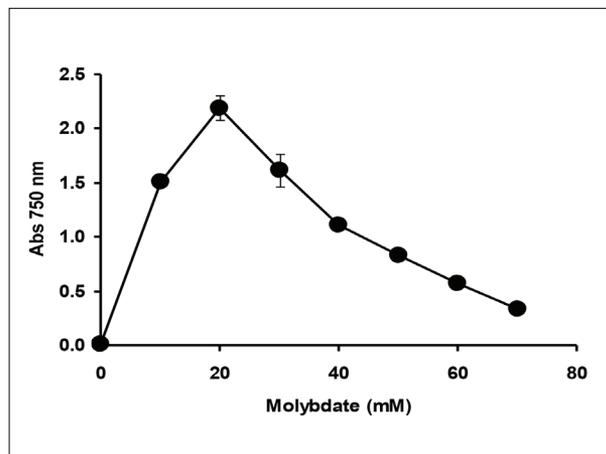


Figure 4: Mo-blue production at different sodium molybdate concentrations. The error bars indicate standard deviation of the average of triplicates

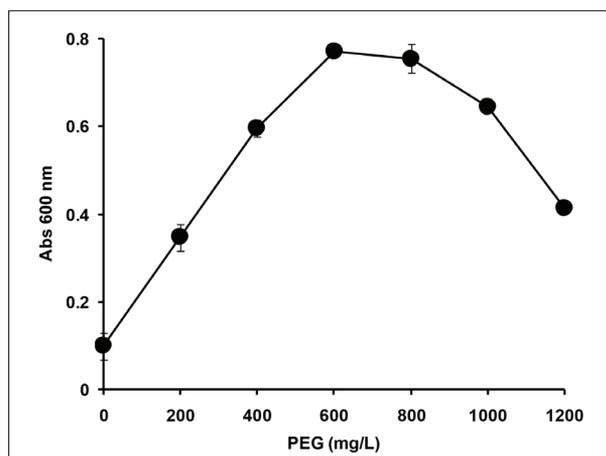


Figure 5: Growth of *P. putida* strain Egypt-15 on PEG 4000. Growth on PEGs was carried out aerobically in a 50 mL growth media at room temperature and shaken at 120 rpm. The error bars indicate standard deviation of the average of triplicates.

which can be integrated into generic anabolic pathways starting from the glyoxylate shunt (Marchal *et al.*, 2008; Watanabe & Kawai, 2010).

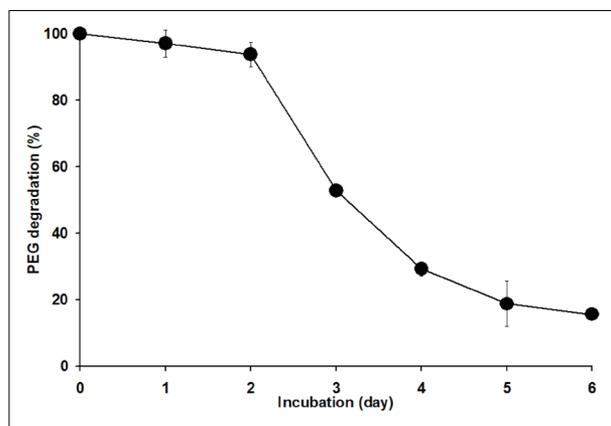


Figure 6: Percentage degradation of PEG 4000 at a starting concentration of 800 mgL⁻¹ by *P. putida* strain Egypt-15. Growth on PEGs was carried out aerobically in a 50 mL growth medium at room temperature and shaken at 120 rpm. PEG degradation was monitored colourimetrically. The error bars indicate standard deviation of the average of triplicates

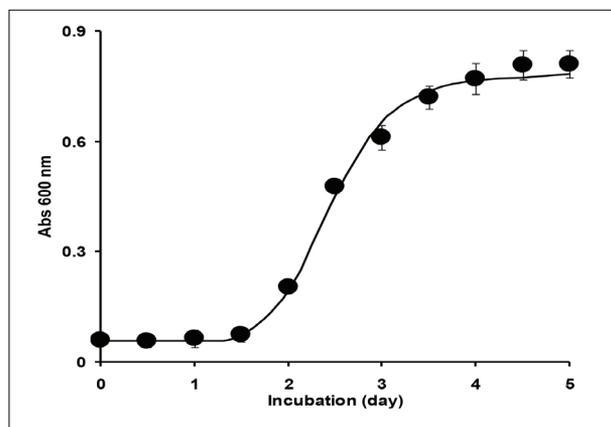


Figure 7: *P. putida* strain Egypt-15 growth on 800 mgL⁻¹ of PEG 4000 as modelled using the modified Gompertz model (solid lines). Growth on PEGs was carried out aerobically in a 50 mL growth medium at room temperature and shaken at 120 rpm. The error bars indicate standard deviation of the average of triplicates

About 80 % degradation of PEG 4000 observed in this work within six days is considered achievable as the PEG chain length is relatively short and the concentration used

is not high. Even higher chain lengths of PEGs and higher concentrations are reported in literature, but those require longer incubation periods. For instance, Haines and Alexander (1975) used soil bacteria to show that 10,000 mgL⁻¹ of various PEGs from 400 to 20000 were degraded completely by 20 days of incubation. Lag periods of between two and five days were observed in correlation with an increasing PEG size. *Pseudomonas aeruginosa* isolated from soil can grow on PEG 20000 as a carbon source at 10,000 mgL⁻¹, but the extent of degradation was not mentioned. Degradation was carried out using resting or whole cells, and ethylene glycol was detected as a metabolite of PEG degradation. The ethylene glycol produced may be metabolised through the glyoxylate shunt (Watanabe & Kawai, 2010).

Anaerobic degradation of PEGs was studied using a methanogenic bacterial consortium from sewage sludge at a final concentration of 5 mM of PEG 400, 2 mM of PEG 1000 and 0.1 mM of PEG 20000, which was equal to the final concentration of approximately 2,000 mgL⁻¹. After 12 days of incubation under anaerobic conditions, PEG 400, PEG 1000 and PEG 20000 were degraded to about 100, 82 and 82 %, respectively. The rate of PEG degradation was approximately inversely related to the ethylene glycol number of units in the PEG molecules (Watanabe & Kawai, 2010). In another study, *Pelobacter venetianus* sp. nov degraded 1,000 mgL⁻¹ of PEG 200, PEG 6000, and PEG 20000 completely, forming acetate and methane under anaerobic conditions after five weeks of incubation. Acetaldehyde was among the first metabolite detected and the glyoxylate route of PEG metabolism has been suggested (Marchal *et al.*, 2008). Huang *et al.* (2005) reported that 1,000 mgL⁻¹ of PEG 600, PEG 6000 and PEG 20000 were aerobically degraded to about 85 % after 5 days, 88 % after 4 days, and 77 % after 5 days of incubation, respectively using sludge microbes. More recently, an *Acinetobacter* strain degraded 83 % of 1,650 mgL⁻¹ of PEG 400 within 5 days of aerobic growth at 30 °C, at pH between 7.2 and 8.0 (Jiang *et al.*, 2013). Bacteria that could detoxify heavy metals and degrade xenobiotics are rarely reported. For example, in chromate reduction, a xenobiotic such as phenol can be used as an electron donor for reduction (Anu *et al.*, 2010). Hence, the ability of this bacterium to degrade PEG and reduce molybdenum is considered novel and useful for bioremediation.

CONCLUSION

The aim of this work was two-fold; to isolate Mo-reducing bacteria with PEG-degrading characteristics from soil. A Mo-reducing bacterium with the ability to use PEG

200, 300 and 600 as sources of carbon for growth is reported. This is the first report of a PEG-degrading and molybdenum-reducing bacterium. Characterisation of Mo-blue production from this bacterium shows that the results conform to previously published results. Growth of the bacterium on PEG was then successfully modelled using the modified Gompertz model, showing the applicability of this model to obtain growth parameters on PEG. The important growth parameters obtained indicate that PEG 200 was the best substrate while predictably, the longer chain PEG 1000 was the least preferred substrate. The ability of this bacterium to degrade PEG and reduce molybdenum is a novel and useful process for the future bioremediation of PEG as well as molybdenum.

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