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AIsolation and Characterization of a Heavy Metal-reducing Enterobacteriaceae Bacterium Strain DRY 7 with the Ability to Assimilate Phenol and Diesel

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Abstract

Background/Objectives: Molybdenum, phenol and diesel are toxic to organism, and are part of global pollution. Their removal using microorganisms with multiple detoxification ability is being intensely sought as a cleaner and economic approach. **Methods/Statistical analysis:** A soil suspension was spread plated on a minimal salts media supplemented with molybdenum. Blue colonies, indicating molybdenum reduction was then screened for phenol and diesel degradation capabilities. **Findings:** A molybdenum-reducing bacterium locally isolated showed the ability to grow on phenol and diesel. The bacterium required pHs of between 5.8 and 6.3 and temperatures of between 30 and 40°C for optimal reduction. Among the carbon sources tested for supporting reduction, glucose was the best. A critical concentration of phosphate at just 5 mM was required, while molybdenum (sodium molybdate) was required between 15 and 25 mM. The absorption spectrum of the Mo-blue produced showed a characteristic maximum peak at 865 nm. The reduction of molybdenum was inhibited by the ions mercury, copper, chromium, lead and silver by 78.9, 78.4, 77.4, 53.5 and 36.8%, respectively. Analysis using phylogenetic analysis identifies the bacterium as Enterobacteriaceae bacterium strain DRY7. Growth on phenol and diesel as carbon sources showed that the optimal concentrations supporting growth was between 300 and 400 mg/L and between 300 and 500 mg/L, respectively. **Application/Improvements:** The capacity of this bacterium to detoxify a number of toxicants is an important property or bioremediation of soils contaminated with multiple toxicants.

Keywords: Bioremediation, Detoxification, Diesel, Enterobacteriaceae, Molybdenum, Phenol

1. Introduction

Heavy metals including molybdenum, and organic pollutants such as phenolics, amides and detergentsare ubiquitously present globally[1,2]. Molybdenum pollution in the waters of the Black Sea, Japan Bay and soils in Tyrol, Austria is due to industrialization activities[3,4]. Metals mining areas are additional major caused of molybdenum contamination. In southern Colorado, molybdenum concentration as high as 6,500 mg/Kg in soils and dissolved

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molybdenum as high 900 mg/L have been reported[5]. In western Liaoning, China, molybdenum level exceeding the regulatory levels by several hundred times has been reported coming from molybdenum mining activities, presenting a high risk to the environment and humans's health[6]. In Malaysia, molybdenum is produced in a small scale level from a copper-gold-molybdenum mine in Ranau, Sabah. Pollution from this site poses heavy metal toxicity risksto the people consuming plants and animals from this region[7]. Molybdenum, found in the muscles of the Mekong fishPangasianodonhypophthalmus, pose a spermatogenesis inhibitory risksat levels between 0.1 and 10 mg/L[8]. Spermatogenesis inhibition was also observed in drosophila[9]and male rats[10]. Historically, molybdenum shows extreme toxicity effects to ruminants including cattle and sheep,with a dietary intake of between 5 and 10 ppm molybdate leads to scouring and in certain cases deaths due to hypocuprosis[11].

Microbiological conversion of soluble molybdenum (molybdate ions) into insoluble products is a potential candidate of bioremediation. Under low oxygen tension, bacteria reduce the soluble molybdate anion into the colloidal molybdenum blue, which can be entrappedin dialysis tubing[12]. Numerous Mo-reducing bacteria have been described, and include Gram negative and positive bacteria[13-25]. The purification of the enzyme responsible for this phenomenon has only been carried out recently[26].

Hydrocarbons in the forms of grease, phenol and oil are ranked as the primary scheduled wastes produced by industries in Malaysia[27]. The Department of Environment of Malaysia estimates that the yearly wastes generated is more than 1000 metric tons[28]. Hydrocarbon and phenol are generally toxic to all organisms, and their global pollution is well known[29]. Certain microorganisms could degrade a variety of xenobiotics. The resourcefulness of these microorganismswill be beneficial for the removal of pollutants where the presence of multiple contaminants isfound[2]. Based on this premise, we have isolated a novel molybdenum-reducing bacterium which could grow on diesel and phenol. The multiple pollutants detoxificationability can be utilized as a future potential tool for bioremediation.

2. Materials and Methods

2.1 Molybdenum-Reducing Bacterium Isolation and Maintenance

The sampling was carried out in January 2005, in Ipoh, a city in the state of Perak, Malaysia. A low phosphate molybdate medium or LPM was utilized to isolate and maintain molybdenum-reducing (molybdenum-blue producing) bacteria. The followings are the composition of the LPM, adjusted to pH 7.0:MgSO4•7H2O (0.05%), NaCl (0.5%), (NH4)2•SO4 (0.3%), Na2MoO4•2H2O (0.242 % or 10 mM), glucose (1%), yeast extract (0.5%), and Na2HPO4 (0.071% or 5 mM)[17]. The process of bacterial isolation started with the preparation of a soil suspension. This was carried out by mixing 1 gram of soil with 10 ml of sterile distilled water. A 0.1 mlaliquot of the soil suspension was spread on solid LPM medium (adding 1.5% agar). The plate was then incubated for 2 days at room temperature in an incubation oven (Memmert, GmbH, Germany). Blue colony with the highest intensity was purified by restreaking several times on fresh LPM agar. Growth of the purified molybdenum-reducing bacterium in liquid LPM medium (100 mL) turned the culture medium blue after 2 days of incubation at room temperature. A centrifuged (10,000g, 5 min) of the culture supernatant was placed in a plastic cuvette and scanned from 400 to 900 nm (Shimadzu 1201) to obtain the absorption spectrum.

2.2 16sr DNA Gene Sequencing

Extraction of bacterial genomic DNA was carried out utilizing the alkaline lysis method. PCR amplification of the 16s rDNA on a thermal cycler (Biometra, Gottingen, Germany) utilize the 16s rDNA forward primer (First Base Sdn Bhd., Malaysia) (5'-AGAGTTTGATCCTGGCTCAG-3') and a reverse primer (5'-AAGGAGGTGATCCAGCCGCA-3') (Devereux et al., 1995). The final volume of the composition was 50 µL, and the composition of the reaction mixture was as follows: 1 x reaction buffer, 0.5 pM of each primer, 200 µM of each deoxynucleotide triphosphate, and 2.5 U of Taq DNA polymerase (Promega). PCR of the 16s rDNA gene was carried out with an initial denaturation for 3 minutes at 94 °C; 25 cycles of 94 °C for 1 minute, 50 °C for 1 minute, 72 °C for 2 minutes, and a 10-minute final extension at 72 °C. The PCR product was sequenced via a cycle sequencing operation (Perkin-Elmer Applied Biosystems Big Dye terminator kit). Sequence data was recorded and edited on the CHROMAS software (Version 1.45). The resultant bases were blasted through the GenBank database utilizing the Blast server (http://www.ncbi.nlm.nih.gov/BLAST/). The 16s rDNA gene sequence was deposited in the Gen Bank databasewith the accession number of DQ226208.

2.3 Identification of Bacterium

Biochemical identification was carried out using the Biolog GN microplate (Biolog, Hayward, CA, USA). In addition,a molecular phylogenetic analysis was carried out by first aligning multiple 16S rRNA gene sequences based obtained from a BLAST exercise utilizing the program clustal_W(Thompson et al., 1994). The phylogenetic tree was developed by using the PHYLIP suits of program version 3.573.). A distance-based method was utilized to calculate the phylogenetic relationship.

2.4 Preparation of Resting Cells

The preparation and use of resting cells in a microtiter format was carried out as before[25,26]. The bacterium was grown in 1 L of the LPM medium for 2 days at room temperature, with the phosphate concentration increased to 100 mM to prevent molybdenum blue reduction that complicates cell harvesting. Cells were centrifuged (10,000 g, 10 min) and the resultant cellular pellets were resuspended in 20 mL of LPM medium. Bacterial cell suspension (180 µL) was pipetted into the wells of a sterile microplate. Sterile glucose (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). Mo-blue production was monitored at 750 nm using a microtiter plate reader (BioRad Model No. 680, Richmond, CA). The effect of heavy metals on Mo-blue production was studiedutilizing calibration solutions for Atomic Absorption Spectrometry (AAS) (Merck Chemical Co., Germany). The substances phenol, acetamide, acrylamide, nicotinamide, iodoacetamide, propionamide, Sodium Dodecyl Sulfate (SDS) and diesel to support molybdenum reductionwas tested at the final concentration of 200 mg/L (Arif et al., 2013). Glucose was omitted. If these xenobiotics can be utilized as electron donors, Mo-blue production will be observed. Simultaneously, bacterium was tested on its ability to grow on these compounds separate from molybdenum-reduction was carried out in H[illegible] Phosphate Medium (HPM) medium without molybdenum and glucose. A similar concentration of the xenobiotics was utilized. Bacterial growth after 72 hours of incubation was monitored at room temperature was assessed at 600 nm and visually observed through the increase in turbidity.

2.5 G C Analysis of Diesel Degradation

Confirmation of degradation was monitored via gas chromatographic analysis as carried out before[30] with slight modifications on a gas chromatography (Varian 3900 model, USA) equipped with a 25 m X 0.32 mm SE-54 capillary column and a Flame Ionization Detector (FID). The programming of the oven was as follows: 40 oC (4 min), which was then increased to 325 oC for 5 min at a rate of 8 oC per min. The carrier gas was helium at a flow rate of 30 ml/min. The detector and injector temperatures were 325 oCand 275 oC, respectively.

2.6 Analysis of Statistics

Data were analysed using Graphpad Prism version 5.0 available from www.graphpad.com.

3. Results

Moderate bootstrap value (58.7%) was seen when strain DRY7 was associated to Enterobacteriaceae bacterium Strain PH31 suggesting that the phylogenetic linkage to this bacterium was moderately strong. A strong linkage was suggested if the bootstrap value exceeds 75% [31]. Strain DRY7 is also linked to several sister groups having Enterobacteriaceae species from the genus Pseudomonas, Enterobacterium, XenoharbdusandSerratia indicating that it is quite difficult to assign Strain DRY7 to any of the genus (Figure 1). The results from the biochemical identification usingBiolog GN also gave inconclusive identification to any species. Due to this the assignment to the species level for Strain DRY7 cannot done and the bacterium is assigned tentatively as Enterobacteriaceae bacterium Strain DRY7. The bacterium exhibits optimum pH for reduction of molybdenum at pH of between 5.8 and 6.3. The optimum temperature was between 30 and 40 °C (data not shown). Inhibition of molybdenum reduction using several metals at 1 ppm showed that molybdenum reduction was inhibited by mercury, copper, chromium, lead and silver by 78.9, 78.4, 77.4, 53.5 and 36.8%, respectively (data not shown). Most of the Mo-reducing bacteria isolated to date are inhibited by similar toxic heavy metals (Table 1). Mercury and copper are strong inhibitors to bacterial chromate reduction from the toxic 6+ state to the 3+ state. This inhibition is seen inBacillus sp.[32] and Enterobacter cloacae strain H01[33].

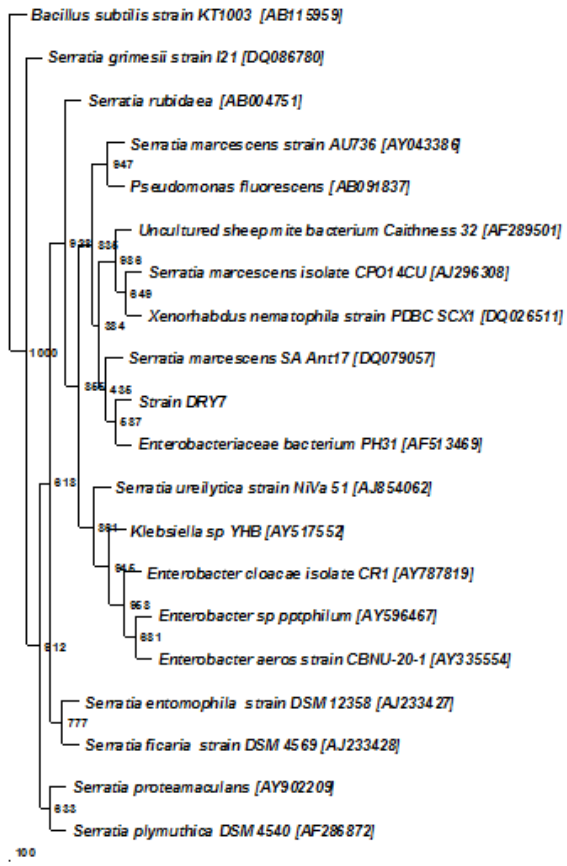

Figure 1. Compressive test of cube.

3.1 Molybdenum-Blue Absorption Spectrum

The Mo-blue produced from bacterial reduction was scanned between 400 and 900 nm. A unique property of the spectrum was observed with a shoulder at 700 nm and a maximum peak observed at 865 nm. It was also noted that this unique profile was conserved at several incubation periods (Figure 2).

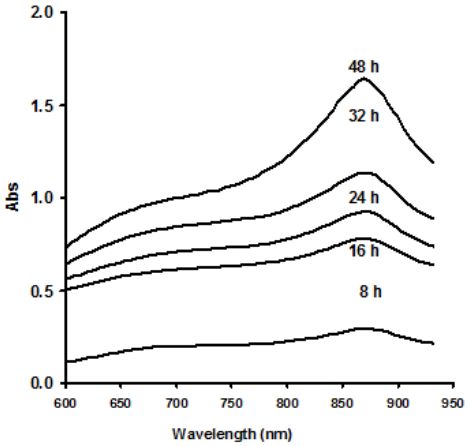

Figure 2. Mo-blue scanning absorption spectrum at different time intervals.

3.2 Effect of Various Carbon Sources as Electron Donors for Molybdate Reduction

Among the carbon sources tested as electron donor, glucose was found to be the best. This wasfollowed by sucrose and trehalosein descending order. Other carbon sources failed to support reduction (Figure 3).

3.3 Effect of Phosphate and Molybdate Anions on Molybdenum Reduction

Mo-blue production required a low concentration of phosphate at 5 mM as the optimal concentration. Higher concentrations inhibited reduction with concentrations higher than 40 mM showed no Mo-blue production (Figure 4). Despite this, reduction to Mo-blue was observed at the high concentration of 60 mM but with a much-reduced efficiency. Optimal reduction occurred from 15 to 25 mM (Figure 5).

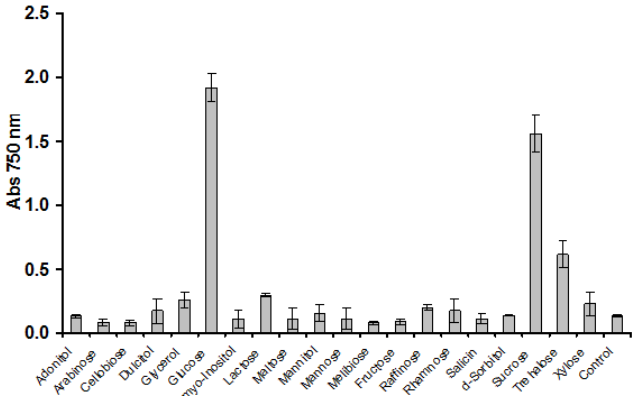

Figure 3. Effect of different electron donor sourceson molybdenum reduction. Error bars are mean ± standard deviation of three replicates.

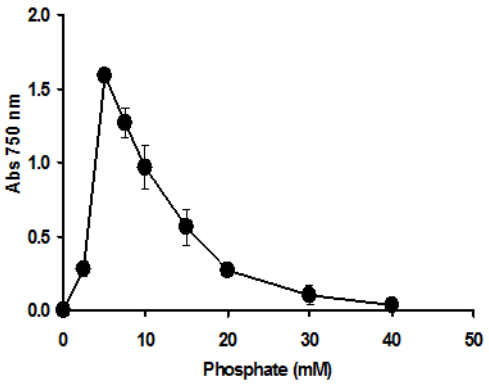

Figure 4. Molybdenum reduction in the presence of various phosphate by Enterobacteriaceae bacterium strain DRY7. The error bar represents standard deviation of three replicates.

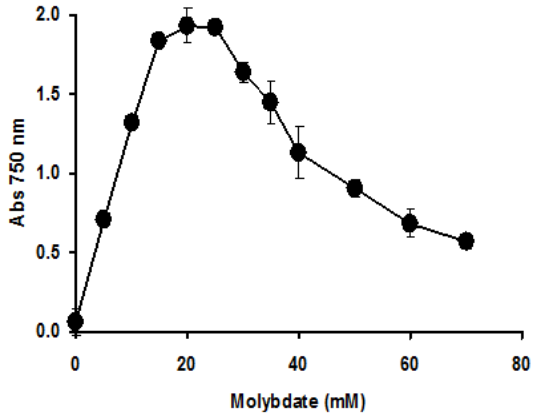

Figure 5. Molybdenum reduction in the presence of various molybdate concentrations by Enterobacteriaceae bacterium strain DRY7. The error bar represents standard deviation of three replicates.

3.4 Xenobiotics as Source of Electron Donors or Growth

Not all of the xenobiotics tested could support molybdenum reduction. Previously isolated Mo-reducing bacteria with the capability to degrade the xenobiotic Sodium Dodecyl Sulphate(SDS)[25,34] could also not use SDS as an electron donor source for molybdenum reduction. However, the bacterium shows the novel ability to grow on diesel and phenol as carbon sources. However, the utilization of these substrates appears to be at a muchlower efficiency than glucose (Figure 6).When phenol was utilized as a carbon source, the nitrogen source was ammonium sulphate. The results showed that the optimal phenol concentrations supporting growth was between 300 and 400 mg/L (Figure 7A). Growth on diesel under similar conditions showed an optimal growth between 300 and 500 mg/L (Figure 7B). GC analysis proved that diesel is degraded based on the depletion of the aliphatic carbons signals seen from minute 14 onwards (Figure 8).

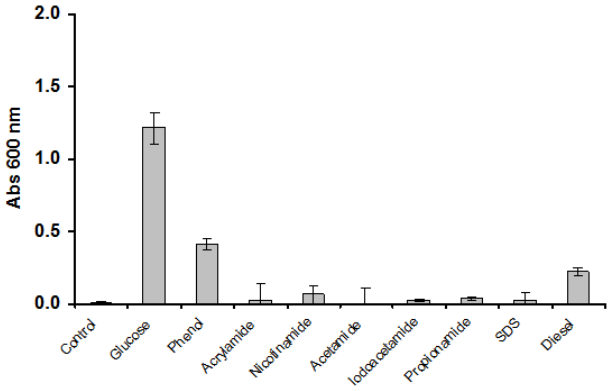

Figure 6. Growth of Enterobacteriaceae bacterium strain DRY7 on xenobiotics. The positive control was glucose. The error bar represents standard deviation of three replicates.

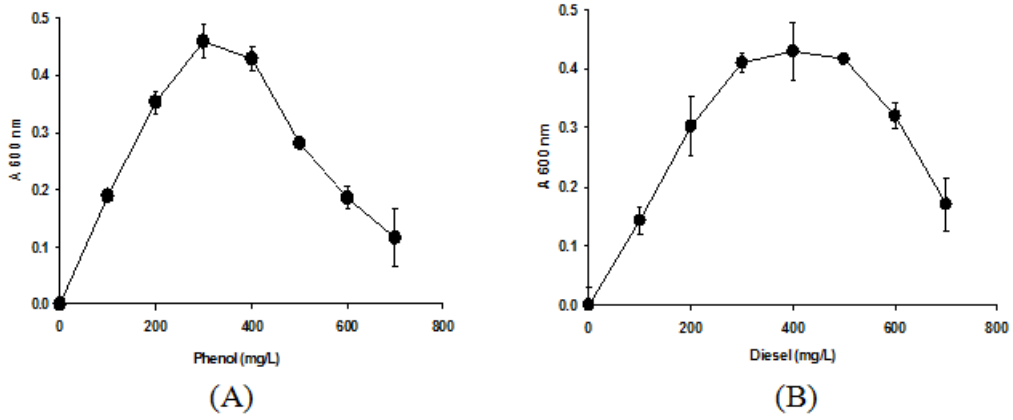

Figure 7. Growth of Enterobacteriaceae bacterium strain DRY7 on various concentrations of phenol (A) and diesel (B). Growth was carried out in shake flask culture (100 ml) on high phosphate media (pH 7.0) incubated at 30oC on an orbital shaker (120 rpm).The error bar represents standard deviation of three replicates.

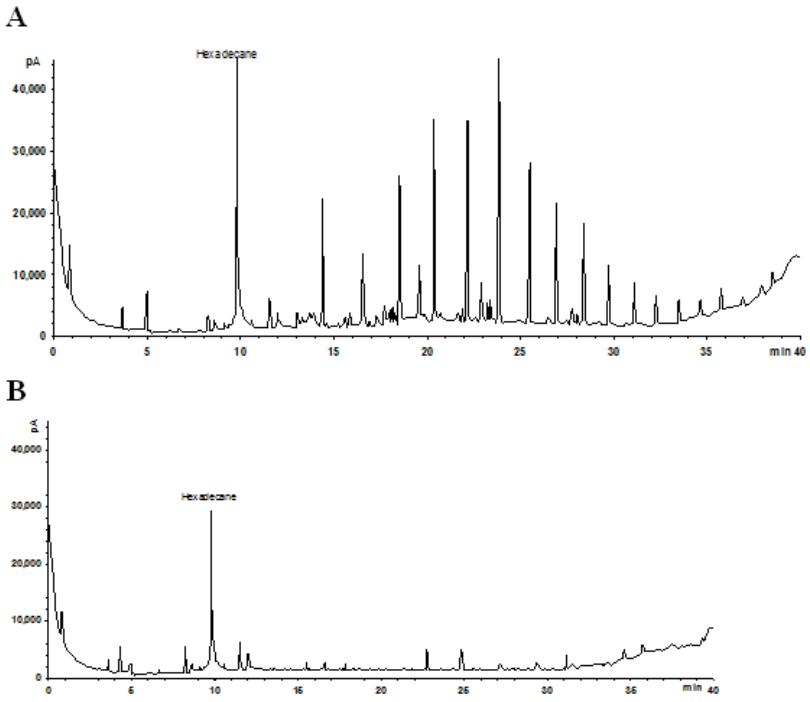

Figure 8. A GC chromatogram of diesel biodegradation by Enterobacteriaceae bacterium strain DRY7 at the start of incubation (A), and after 10 days of incubation at room temperature (B). Hexadecane is the internal standard.

4 Discussions

Microbiological conversion of soluble molybdenum (molybdate ions) into the colloidal molybdenum blue is an essential instrument for the process of bioremediation. The Bacterial reduction of molybdate to molybdenum blue was first reported more than one hundred years ago in E. coli[35], and further studied in E. coli K12[36] in greater detail. This was followed by a detailed study in another bacterium, Enterobacter cloacae strain 48[37], but without citing earlier works of Mo-blue reduction, possibly due to the long absence of any reported works in this area since the publication in E. coli K12. As the oxygen concentration and pH level dropped during incubation, molybdenum was converted to phosphomolybdate, a heteropolymolybdate, and the reduction product was Mo-blue[31]. By using this method with a modification, the reduction of molybdate to Mo-blue in Enterobacter cloacae strain 48 and other Mo-reducing heterotrophic bacteria was demonstrated to be catalyzed by enzyme and not by abiotic processes[38]. The purification of the enzyme responsible for this phenomenon has only been carried out recently[26]. The purification of this enzyme helps to solve a more than a century phenomenon first reported in 1896[35]. To date, numerous Mo-reducing bacteria have been isolated and characterized (Table 1). The use of the microplate format speeds up characterization works[39,40], while the use of resting cells in studying molybdate reduction was first initiated by Ghani[37]. Other studies on heavy metals reduction such as in selenate[41], chromate[42] and vanadate[43] reductions have also benefitted from the use of resting cells. As molybdenum blue production increases, the profile was preserved[44]. The spectra look very similar to the ascorbic acid-reduced Mo-blue or reduced phosphomolybdate from the method to determine phosphate using ascorbic acid as the reducing agent[45]. The latter exhibits a peak maximum at 880 nm and a shoulder at 700 nm[46]. Previously, we showed that numerous Mo-reducing bacteria have similar spectra and we proposed that phosphomolybdate is an intermediate based on the similarity to the phosphate determination method. We utilized phosphomolybdate as the electron accepting substrate for the Mo-reducing enzyme and found the activity was increased almost 200 times. The original substrate is molybdate. We manage to purify this enzyme using phoshomolybdate as a substrate.

Several researchers have shown that the oxidation state of Mo-blue or reduced phosphomolybdate is between 5+ and 6+. Phoshomolybdate has the general formula of [XM12O40]n? and is a Keggin structure. The presence of an intermediate was also observed in the reduction of chromate from 6+ to 3+ in the bacterium Pseudomonas ambigua[47]. This indicates that the presence of intermediate species is not unique to molybdenum reduction.All of the previously isolated Mo-reducing bacteria utilize either glucose or sucrose as carbon sources (Table 1). These carbon sources are easily converted to the reducing equivalents NADH and NADPH, both sources of electron donating substrates for the Mo-reducing enzyme[13, 26]. The phosphomolybdate complex is known for its instability in solutions containing high phosphate[44,48,49], this could explain the instability of the complex. Previously isolated molybdenum-reducing bacterium exhibited requirement for phosphate concentration around 5 mM for optimal reduction (Table 1).

The optimal concentration of molybdate supporting Mo-blue production was within the range reported in other Mo-reducing bacteria with optimal reduction at concentrations in between 10 to 80 mM (950 to 7,600 mg/L) (Table 1). The highest level of molybdenum was discovered in an abandoned uranium mine in Colorado where a molybdenum concentration as high as 6,550 mg/Kg in the soils have been reported (Stone, 2008). Thus, the bacterium isolated so far including this bacterium will be a suitable candidate as all of them could tolerate very high concentrations of molybdenum.

At high concentrations,diesel exhibits toxic effects to bacterium as it acts as a solvent that strips of bacterial phospholipid cell membrane[27].To date, numerous bacteria have been reported with diesel-degrading property. The main bacterial genuswith diesel degradation capability includeAcinetobacter, Staphylococcus, Pseudomonas, Bacillus, Proteus, Aeromonas, Micrococcus, Klebsiella, SerratiaandFlavobacterium[30,50, 51].The concentrations of diesel supporting optimal growth seen in this work is slightly lower than the optimal concentrations reported in the above diesel-degraders with diesel concentrations ranging from 500 to 1,000 mg/L are reported to be optimal. Another hydrocarbon, phenol, is much more toxic than diesel. Despite this toxicity, numerous phenol-degrading bacteria have been isolated and include Bacillus brevis[52], Alcaligenes sp.[53], Ochrobactrum sp.[54], Pseudomonas species[55-57], Acinetobacter sp.[58](Ahmad et al., 2012) and Rhodococcus species[59] with optimal phenol concentrations supporting growth ranging from 200 to 1750 mg/L. The range of optimal phenol concentration reported in this work is within this range. There are also microorganisms that could grow on both phenol and diesel[59,60]. Microorganismsexhibiting multiple xenobiotic-degrading capacityincluding metal detoxifiers are very useful in bioremediation. However, feware reported[61,62]. As an example, in chromate reduction, phenol can be utilized asan electron donor for chromatereduction[63].

Table 1. Characteristics of various Mo-reducing bacteria isolated to date.

Bacteria	Optimal Molybdate (mM)	Optimal Phosphate (mM)	Optimal C source	Heavy metals inhibition	Author
Klebsiellaoxytocastrain Aft-7	5-20	5-7.5	glucose	Cu^{2+}, Ag^{+}, Hg^{2+}	25
Bacillus sp. strain A.rzi	50	4	glucose	Cd^{2+}, Cr^{6+}, Cu^{2+},Ag^{+}, Pb^{2+}, Hg^{2+}, Co^{2+},Zn^{2+}	23
Bacillus pumilus strain lbna	40	2.5-5	glucose	As^{3+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Cr^{6+}, Hg^{2+}, Cu^{2+}	21
Pseudomonas sp. strain DRY1	30-50	5	glucose	Cd^{2+}, Cr^{6+}, Cu^{2+},Ag^{+}, Pb^{2+}, Hg^{2+}	22
Klebsiellaoxytocastrainhkeem	80	4.5	fructose	Cu^{2+}, Ag^{+}, Hg^{2+}	20
Acinetobactercalcoaceticusstrain Dr.Y12	20	5	glucose	Cd^{2+}, Cr^{6+}, Cu^{2+}, Pb^{2+}, Hg^{2+}	19
Pseudomonas sp.strainDRY2	15-20	5	glucose	Cr^{6+}, Cu^{2+}, Pb^{2+}, Hg^{2+}	18
Enterobactersp. strain Dr.Y13	25-50	5	glucose	Cr^{6+}, Cd^{2+}, Cu^{2+}, Ag^{+}, Hg^{2+}	15
S. marcescens strain Dr.Y9	20	5	sucrose	Cr^{6+}, Cu^{2+}, Ag^{+}, Hg^{2+}	17
Serratiasp. strain Dr.Y5	30	5	glucose	n.a.	14
Serratiamarcescens strain DRY6	15-25	5	sucrose	Cr^{6+}, Cu^{2+}, Hg^{2+}	13
Enterobacter cloacae strain 48	20	2.9	sucrose	Cr^{6+}, Cu^{2+}	37
Escherichia coli K12	80	5	glucose	Cr^{6+}	36

5. Conclusion

A local isolate of bacterium is reported to show multiple xenobiotics detoxification capability including molybdenum reduction, phenol and diesel degradation. Diesel degradation was confirmed via GC analysis. The bacterium reduced molybdate to molybdenum blue optimally at a broad temperatures range. Glucose was the most optimal electron donor for aiding molybdenum reduction. The most critical requirementisa phosphate concentration at 5.0 mM. The bacterial molybdenum reduction product; molybdenum blue exhibits an absorption spectrum that suggests phosphomolybdate is an intermediate species. The heavy metalsmercury, copper, chromium, lead and silver inhibited molybdate reduction. Analysis using phylogenetic approach partially identifies the bacterium as Enterobacteriaceae bacterium strain DRY7. The bacterium ability to detoxify multiple toxicants including heavy metal will be an important tool for bioremediation of sites contaminated with numerous toxicants. Presently, studies are being carried outto purify the Mo-reducing enzyme and to fully characterize the phenol and diesel-degrading ability of this bacterium.

6. Acknowledgment

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