Assessing Resistance and Bioremediation Ability of *Enterobacter sp.* Strain Saw-1 on Molybdenum in Various Heavy Metals and Pesticides

Mohd Khalizan Sabullah[1,2], Mohd Fadhil Rahman[2], Siti Aqlima Ahmad[2], Mohd Rosni Sulaiman[3], Mohd Shukri Shukor[4], Nor Aripin Shamaan[5] & Mohd Yunus Shukor[1]

[1]Faculty of Science and Natural Resources, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia
[2]Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, UPM 43400 Serdang, Selangor, Malaysia
[3]Faculty of Food Science & Nutrition, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia
[4]Snoc International Sdn Bhd, Lot 343, Jalan 7/16 Kawasan Perindustrian Nilai 7, Inland Port, 71800, Negeri Sembilan, Malaysia
[5]Faculty of Medicine and Health Sciences, Islamic Science University of Malaysia, 55100 Kuala Lumpur, Malaysia
E-mail: ejanz_sart2@yahoo.com

Abstract. One of the most economical approaches for removal of toxic compounds is bioremediation. In the long term, bioremediation is economic and feasible compared to other methods, such as physical or chemical methods. A bacterium that can efficiently reduce molybdenum blue was isolated from polluted soil. Biochemical analysis revealed the identity of the bacterium as *Enterobacter* sp. strain Saw-1. The growth parameters for optimal reduction of molybdenum to Mo-blue or molybdenum blue, a less toxic product, were determined around pH 6.0 to 6.5 and in the range of 30 to 37 °C, respectively. Glucose was selected as preferred carbon source, followed by sucrose, maltose, l-rhamnose, cellobiose, melibiose, raffinose, d-mannose, lactose, glycerol, d-adonitol, d-mannitol, l-arabinose and mucate. Phosphate and molybdate were critically required at 5.0 mM and 10 mM, respectively. The scanning absorption spectrum acquired to detect the development of complex Mo-blue showed similarity to previously isolated Mo-reducing bacteria. In addition, the spectrum closely resembled the molybdenum blue from the phosphate determination method. Heavy metals, including mercury, copper (II) and silver (I), inhibited reduction. Moreover, the bacterium also showed capability of exploiting the pesticide coumaphos as an alternative carbon source for growth. As the bacterium proved its ability to detoxify organic and inorganic xenobiotics, the usefulness of this microorganism for bioremediation is highlighted.

Keywords: *bioremediation; coumaphos; Enterobacter sp. strain Saw-1; heavy metals; molybdenum.*

Received 11th September 2015, Revised June 4th, 2017, Accepted for publication June 14th, 2017.
 ISSN: 2337-5760, DOI: 10.5614/j.math.fund.sci.2017.49.2.8

1 Introduction

At low concentrations of toxicants, their elimination by means of bioremediation is perhaps the most cost effective approach in the long term. In addition, physical or chemical methods are usually not cost effective to treat diluted toxicants. Organophosphorus (OP) compounds have garnered global attention due to their application in chemical weapons and, chiefly, in pesticides, giving them extremely hazardous properties and making them significant pollutants. Pesticides containing OP compounds are necessary for the majority of agriculture-developing applications. The extensive use or abuse of OP compounds has triggered a number of unwanted outcomes, such as toxicity to humans and environmental pollution problems. It is estimated by the World Health Organization (WHO) that roughly 220,000 deaths and three million cases of severe poisoning globally are caused by exposure to OPs [1]. Coumaphos [O-(3-chloro-4-methyl-2-oxo-2H-1-benzopyran-7-yl) O,O-diethylphosphorothioate] (Figure 1) features a wide range of applications for crop and animal safety, but its main use is to control a number of arthropod pests in animals.

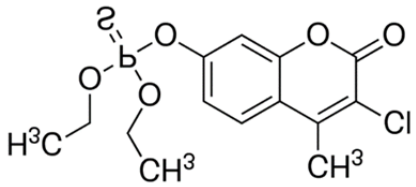

Figure 1 Chemical structure of coumaphos.

Numerous enzymes, including xanthine oxidoreductase, sulphite oxidase and aldehyde oxidase, require molybdenum as a cofactor. Its uses in industry are numerous and the element is very important as anti-freeze component of the automobile engine, preventing corrosion of steel alloys. Molybdenum polluting water bodies and soils worldwide chiefly originates from these industries. In the waters of Tokyo Bay in Japan, molybdenum concentrations have been reported to be in the hundreds of ppm. Similar concentrations have been reported in the Black Sea [2]. In agricultural regions in Tyrol, Austria, air pollution containing molybdenum released from industrial gases has contaminated grazing areas of cows, leading to deaths and serious illnesses [3]. In the worst case of molybdenum pollution reported to date, the soil from a molybdenum mine in New Mexico was reported to contain nearly 2,000 parts per million of molybdenum [4]. Like many metal ions they are toxic at concentrations slightly above trace. For instance, the spermatogenesis of fish from the Mekong delta have been demonstrated to be affected by low concentrations of heavy metals, including molybdenum [5]. In addition, at low concentrations molybdenum has

been reported to affect the morphology and function of mouse ovarian tissue [5,6]. Furthermore, ruminants including cows and goats exposed to several parts per million of molybdenum in their diet showed evidence of hypo cuprosis [7].

It has been demonstrated that in numerous cases, bioremediation of heavy metals and organic pollutants offers an economical approach in the long run, especially at low concentrations [8]. In the case of heavy metal bioremediation through bioreduction, the microorganisms involved could also use other organic xenobiotics as electron donor or growth source, independent of bioreduction [9,10]. Previously, a molybdenum-reducing bacterium has been shown to grow on a detergent, i.e. SDS, as an optimal carbon source, even though SDS cannot assist molybdenum reduction [11]. This method of microbial-bioremediation that is resistant towards toxicant exposure should be developed in addition to facilitating the agencies and departments related to environmental care and preservation to overcome excessive levels of molybdenum in any medium. More data are needed to identify any molybdenum-reducing bacterium that has tolerance towards a broad type of contaminants, such detergents, nerve agents, drugs and toxic metals.

In this study, the isolation of a coumaphos-degrading molybdenum-reducing bacterium originating from a polluted agricultural soil was determined. The bacterium is expected to be well suited for bioremediation of polluted sites containing molybdenum and the pesticide coumaphos.

2 Materials and Methods

2.1 Isolation of Molybdenum-Reducing Bacterium

Screening and isolation of Mo-reducing bacteria from soil is straightforward, as the formation of blue colonies on solid media supplemented with molybdenum indicates molybdate reduction. A contaminated agricultural land in Kuching, Sarawak, Malaysia was chosen for the isolation of potential pesticide-degrading and Mo-reducing bacteria. One gram of soil sample, taken in January 2010 at a depth of about 5 cm from the topsoil, was suspended in clean tap water. Then 100 μL was streaked onto low-phosphate molybdate media (LPM, pH 7.0) agar. The LPM mixture was as follows: 3% of $(NH_4)_2.SO_4$, 1% of glucose, $MgSO_4.7H_2O$ (0.05%) and Na_2HPO_4 (0.071% or 5 mM) [12]. Plate incubation was performed for 48 hours at ambient temperature. The presence of molybdenum-reducing bacteria was indicated by the appearance of blue colonies. Subsequently, the Mo-blue that had formed diffused into the agar. In order to obtain a pure culture, only the microbial colony with the strongest visible blue intensity was selected to isolate and then restreaked onto the same agar. Growth and reduction in liquid molybdate media was carried out in 100

mL of LPM media (at pH 7.0) by the increase of the phosphate concentration to 100 mM [13]. A single blue colony was added into the high phosphate LPM media and incubated at room temperature. Incubation was carried out for 48 hours via continuous orbital shaking at a speed of 120 rpm. The high phosphate concentrations prevented the production of blue cellular aggregates that complicate cell harvesting, but the Mo-reducing activity was retained [13-15].

2.2 Identification of the Mo-Reducing Bacterium

Standard biochemical and phenotypical identification and determination of the purely isolated bacterium were carried out based on Bergey's Manual [16], while the ABIS online system was utilized to interpret the results [17]. In brief, the standard methods include observation of colony shape and size on the nutrient agar plate, Gram staining and motility via the hanging drop method. Oxidase and catalase tests were carried out after 24 h. Other tests included presence of ornithine decarboxylase, detection of arginine dihydrolase activity, lysine decarboxylase detection, β-galactosidase activity detection using the ortho-Nitrophenyl-β-galactoside colorimetric method, positive test for hydrolysis of esculin, nitrate (NO_3) to nitrite (NO_2) reduction, presence of methyl red, production of indole, acetoin production via the Voges-Proskauer test, tartrate utilization ability of Jordans agar, the release of hydrogen sulfide (H_2S) through triple sugar iron (TSI) agar, acetate utilization as a carbon source, malonate utilization as a carbon source, citrate utilization as a carbon source, test for the presence of gelatinase via gelatin hydrolysis test for the presence of DNAse or deoxyribonuclease on DNAse agar with methyl green indicator, lipase production using corn oil as a substrate, urease test for media containing 2% urea with phenol red as the pH indicator, detection of deamination of phenylalanine via the phenylalanine deaminase activity, and the final product generated from carbohydrate metabolism such gas production and acids.

2.3 Resting Cells Preparation

The cell was initially grown in a 1-L shake flask culture containing high phosphate media (HPM) with a continuous shake at 150 rpm for 48 hours at ambient temperature. The cells containing media were then centrifuged at 10,000 x g for 15 minutes and the pellets were collected and then washed with 20 mL of LPM for several times minus glucose to remove residual phosphate and then resuspended in the same volume of buffer. Then, 180 µL was added into each well of a clean microplate, followed by the addition of 20 µL sterile glucose to a final concentration of 1% (W/V) to induce Mo-blue formation. The microtiter plate was then sealed with a sterile and clean sealing tape that permits air exchange (Corning® microplate sealing tape) and then incubated at room temperature. The specific extinction coefficient (EC: 11.69 $mM^{-1}.cm^{-1}$) was

utilized to monitor Mo-blue production at a wavelength of 750 nm in a Microtiter Plate reader (BioRad, Richmond, 153 CA, Model No. 680). Determination of heavy metal effects on molybdenum reduction was carried out in the microplate format as well. The heavy metals tested were copper ion (2+), silver ion (1+), chromium ion (6+) mercury ion (2+), lead ion (2+), arsenic ion (5+), and cadmium ion (2+).

2.4 Effect of Pesticides Supplementation as Carbon Sources for Growth

The pesticides utilized in this study were coumaphos, atrazine, imidacloprid, carbofuran, metolachlor, carbaryl, glyphosate, paraquat, dicamba, diuron, diazinon, endosulfan, parathion, simazine and flucythrinate, and were sourced from Pestanal, Sigma Aldrich. Atrazine, diazinon, diuron, endosulfan, parathion, coumaphos, flucythrinate and simazine need to be dissolved in the carrier solvent methanol. Glucose was replaced with the pesticides in the LPM at a final concentration of 200 mg/L. The LPM was supplemented with 1 mL of trace element solution (mg/L) as follows: $CaCl_2$ (40), $ZnSO_4 \cdot 7H_2O$ (20), $CoCl_2 \cdot 6H_2O$ (5), $MnSO_4 \cdot 4H_2O$ (40), $CuSO_4 \cdot 5H_2O$ (5), $FeSO_4 \cdot 7H_2O$ (40) and $Na_2MoO_4 \cdot 2H_2O$ (5). An indication of bacterial growth at room temperature after an incubation period of three days was obtained by measuring absorbance at 600 nm.

2.5 Statistical Analysis

Values are means ± standard error of three replicates. $P < 0.05$ was regarded as statistically significant. Graphpad Prism version 3.0 available from www.graphpad.com was utilized for the data analyses.

3 Results and Discussions

Molybdenum possesses multiple functions in the biological system, especially as a cofactor for several enzymes that are involved in the anabolism and catabolism of biomolecules. Continuous accumulation of this metal exceeding the permissible limit in the body may cause overproduction of free radicals associated with the induction of programmed cell death, which may lead to organ damage. Molybdenum in the form of molybdate is highly toxic unless it is reduced to form heteropolymolybdate and isopolymolybdate complexes, also known as molybdenum blue, which are less toxic and insoluble. This study shows the optimal growth conditions for *Enterobacter sp.* strain Saw-1 as well as the function of anionic phosphate concentration in the formation of molybdenum blue and the tolerance of this bacteria towards various heavy metals and pesticides.

3.1 Molybdenum Reducing Bacterium Identification

Gram staining showed that the bacterium was Gram-negative. It is motile and rod-shaped. The ABIS online software [17] identification results (Table 1) suggested two possible bacterial identities. The first was *Enterobacter aerogenes* and the second was *Enterobacter cloacae*, both showing 100% accuracy with 90% homology and 91% homology, respectively. Based on this, however, species level assignment could not be carried out. A molecular technique to identify the bacterium via comparison of the 16srRNA gene is needed in the future. However, at this juncture, based on the biochemical tests alone, the bacterium can be tentatively identified as *Enterobacter* sp. strain Saw-1.

Table 1 Biochemical Tests for the Mo-reducing Bacterium *Enterobacter sp.* strain Saw-1.

Motility	+	Acid production from:	
Pigment	-	Alpha-Methyl-D-Glucoside	+
Catalase production (24 h)	+	D-Adonitol	+
Oxidase (24 h)	-	L-Arabinose	+
ONPG (beta-galactosidase)	+	Cellobiose	+
Arginine dihydrolase (ADH)	+	Dulcitol	d
Lysine decarboxylase (LDC)	-	Glycerol	+
Ortnithine reduction	+	D-Glucose	+
Nitrates reduction	+	Myo-Inotisol	d
Methyl red	-	Lactose	+
Voges-Proskauer (VP)	+	Maltose	+
Indole production	-	D-Mannitol	+
Hydrogen sulfide (H_2S)	-	D-Mannose	+
Acetate utilization	+	Melibiose	+
Malonate utilization	+	Mucate	+
Citrate utilization (Simmons)	+	Raffinose	+
Tartrate (Jordans)	+	L-Rhamonse	+
Esculin hydrolysis	+	Salicin	+
Gelatin hydrolysis	-	D-Sorbitol	+
Urea hydrolysis	+	Sucrose (Saccharose)	+
Deoxyribonuclease	-	Trehalose	+
Lipase (com oil)	-	D-Xylose	+
Phenlyalanin deaminase	-		

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

Bacteria from this genus have been demonstrated to be capable of reducing molybdenum. Both of these bacteria, i.e. *Enterobacter cloacae* strain 48 or EC 48 [13] and *Enterobacter* sp. strain Dr.Y13 [18], have been isolated from contaminated soils. Although bacterial growth in a shake flask allows for rapid growth, the use of the microplate method has the advantage that it is simple, is a high-throughput method, and uses less media [19,20]. Resting cells have been used to characterise molybdenum reduction in EC 48 with satisfactory results [13]. Resting cells have also been utilized in several bacterial heavy metal reduction researches, for example on selenate reduction [21] and on degradation of SDS [22].

3.2 Molybdenum Absorbance Spectrum

The production of Mo-blue shows a distinctive absorption spectrum with a shoulder at a wavelength of approximately 700 nm and a maximum peak between 860 and 870 nm, near the infra-red region (Figure 2). Even though the optimal absorption wavelength for Mo-blue production is 865 nm, Mo-blue measurement in this work was carried out at 750 nm. At this wavelength, the absorbance value was 30% lower than at 865 nm, however, as cellular absorption at 600-620 nm shows a negligible effect on the measurement at 750 nm, the use of the latter as the default wavelength in the microplate format was deemed adequate [19]. Several studies have been conducted by monitoring Mo-blue production at a different wavelength, such as 820 nm [23] and 710 nm [13]. The spectrum was extraordinarily similar to the spectrum of the phosphate determination method. The Mo-blue produced from the latter method exhibited optimal absorption reached around 880 to 890 nm with another peak around 700 to 720 nm [24,25]. We have previously utilized this similarity to propose a hypothesis that an intermediate species, phosphomolybdate, is formed in the entire bacterial reduction phenomenon of molybdenum to Mo-blue. In fact, all of the Mo-blue spectra from numerous other bacteria are very similar, showing the unique spectra mentioned above. The existence of an intermediate species is a common phenomenon in Mo-blue production. For instance, bacterial reduction of chromate from the 6^+ to 3^+ in both *Pseudomonas ambigua* [26] and *Shewanella oneidensis* (previously known as *S. putrefaciens*) [27] occurred through the presence of the intermediate species Cr^{5+}, recognized through spectroscopic analysis and paramagnetic resonance work.

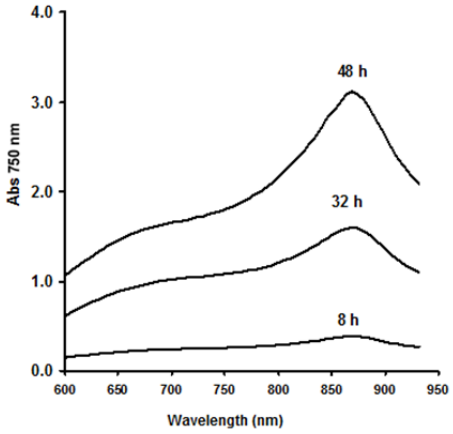

Figure 2 Mo-blue scanning absorption spectrum from the bacterium *Enterobacter sp.* strain Saw-1.

3.3 Optimum Temperature and pH on Molybdenum Reduction

Incubation of *Enterobacter sp.* strain Saw-1 at various initial pHs ranging from 5.5 to 8.0 showed that pH between 6.0 and 6.5 was optimal (Figure 3). Analysis of the effect of temperature on molybdenum reduction exhibited that temperatures between 30 and 37 °C (Figure 4) were optimal, with no significant difference compared to the other values measured ($p > 0.05$).

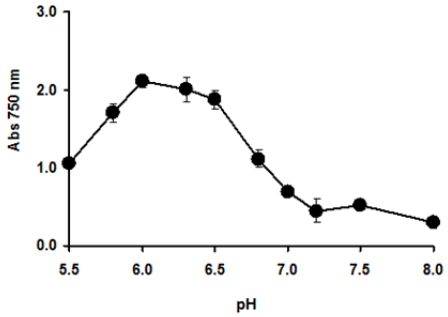

Figure 3 Molybdenum reduction by *Enterobacter sp.* strain Saw-1 at various pHs. The error bars represent mean ± standard deviation (n = 3).

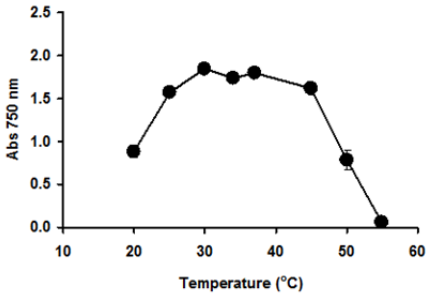

Figure 4 Molybdenum reduction by *Enterobacter sp.* strain Saw-1 at various temperatures. The error bars represent mean ± standard deviation (n = 3).

In general, both temperature and pH affect enzyme activity, including molybdenum reduction. Deviation from the optimal conditions will affect protein folding and enzyme activity, leading to molybdenum reduction inhibition. The broad optimum conditions exhibited by the bacterium fit exceptionally well to a tropical country like Malaysia having 25 to 35°C as the yearly average temperature [28]. The majority of molybdenum reducers are isolated from tropical soils and display optimal reduction between 25 and 37 ºC [15,29-33] with the exception of a reducer that was isolated from Antartica. The

Antarctic bacterium exhibited molybdenum reduction between 15 and 20ºC [34].

The optimal pH for reduction was observed to be slightly acidic. This is similar to all isolated Mo-reducing bacteria that have been reported, exhibiting optimal conditions from the lowest pH at 5.0 to the highest pH at 7.0 [15,29-34]. As the stability and formation of phosphomolybdate are optimal under acidic conditions, determination of optimal pH plays a significant role. The choice of optimal pH needs to take into account both enzyme activity and the stability of the substrate [25].

3.4 Effect of Carbohydrate on Molybdate Reduction

Simple carbohydrates were screened for their electron donor capability. Glucose exhibited the best carbon sources for complementary molybdate reduction, followed by sucrose, maltose, l-rhamnose, cellobiose, melibiose, raffinose, d-mannose, lactose, glycerol, d-adonitol, d-mannitol, l-arabinose and mucate. (Figure 5).

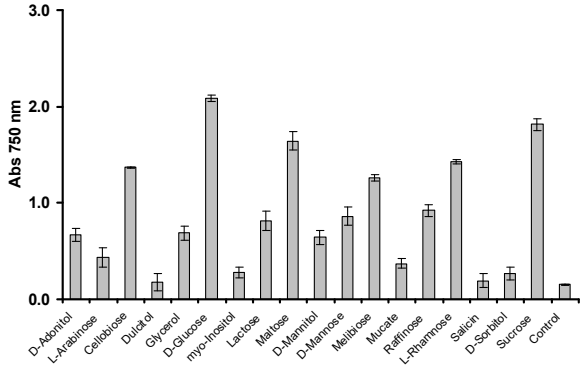

Figure 5 Molybdenum reduction utilizing various electron donor sources (1% w/v) in LPM contained with 10 mM of molybdate. Error bars represent mean ± standard deviation (n = 3).

The rest did not support molybdenum reduction. These include *Enterobacter* sp. strain Dr.Y13, *Escherichia coli* K12, *Bacillus pumilus* strain lbna, *Bacillus* sp. strain A.rzi, *Pseudomonas* sp. strain DRY1, *Serratia* sp. strain Dr.Y5, *Acinetobacter calcoaceticus* strain Dr.Y12 and *Pseudomonas* sp. strain DRY2 [18, 23, 31, 32, 34, 35, 37, 38]. Other Mo-reducing bacteria prefer sucrose, such as *S. marcescens* strain Dr.Y9, *Enterobacter cloacae* strain 48, *Serratia marcescens* strain DRY6 and *Serratia* sp. strain Dr.Y5 [12, 13, 28, 35]. The only exception is *Klebsiella oxytoca* strain hkeem, which has fructose as the best electron donor [30]. Glucose and sucrose are easily assimilable carbon

sources. They produce the reducing equivalents NADH as well as NADPH (efficient electron donors, especially for the Mo-reducing enzyme) far easier than other carbon sources [15,38] through metabolic pathways. This includes glycolysis, the citric acid cycle and the electron transport system.

3.5 Effect of Phosphate and Molybdate Concentration on Mo-Reduction

Phosphate anions have been demonstrate to be critical to Mo-blue production. Based on previous studies, the phosphate concentration required is around 5 mM and lower for optimal Mo-reduction [13,15,18, 23, 28, 30-37,39]. The result of the present study showed that maximum concentration of phosphate also occurred at 5.0 mM. Concentrations higher than this, especially more than 20 mM, were strongly inhibitory to reduction (Figure 6). Glenn and Crane [40] and Shukor, *et al*. [29] have proposed that high phosphate concentrations inhibit Mo-blue production through affecting phosphomolybdate stability. This is because the complex requires acidic conditions for stability. The high concentration of phosphate anions possibly destabilizes the complex.

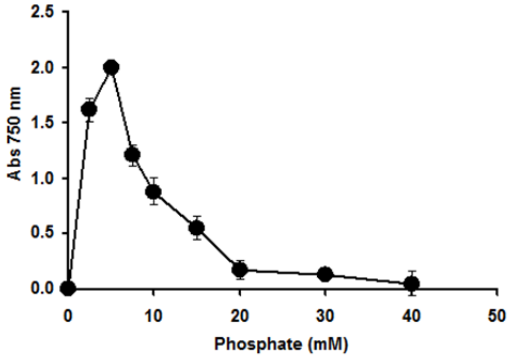

Figure 6 Molybdenum reduction by *Enterobacter sp.* strain Saw-1 utilizing resting cells incubated under various phosphate concentrations. The error bars represent mean ± standard deviation (n = 3).

If the concentration level of molybdenum in the environment exceeds 20 mM it is considered pollutant [4]. Mo-reducing bacteria need to cope with and handle such a high toxic concentration during bioremediation of the metal. The newly isolated bacterium showed optimal reduction at an initial molybdenum concentration of 10 mM, with reduction seen at molybdenum concentrations up to 60 mM, but with a significant reduction of Mo-blue production beyond 10 Mm of molybdate (Figure 7). It is anticipated that reduction at this high level of molybdenum into an insoluble form enables the strain to remediate molybdenum pollution, as stated previously. This is among the lowest optimal

concentrations of molybdenum required for optimal reduction. Several Mo-reducing bacteria show optimal reduction at 15 mM, such as *Pseudomonas sp* strain Dr.Y2 [36]. Other Mo-reducing bacteria produce optimal Mo-blue at concentrations between 20 and 55 mM, such as EC48 [13], *S. marcescens*. Dr.Y9 [12], *Pseudomonas sp*. strain Dr.Y2 [36], *S. marcescens* strain Dr.Y6 [29], *Serratia sp*. strain Dr.Y5 [37], *Enterobacter sp*. strain Dr.Y13 [18], *Acinetobacter calcoaceticus* [37], *Bacillus sp*. strain A.rzi [32], *Bacillus pumilus* strain lbna [31] and *Serratia sp*. strain Dr.Y8 [41]. On the other hand, the highest molybdenum concentration for optimal reduction was 80 mM in *Klebsiella oxytoca* strain hkeem [30] and *E. coli* K12 [23].

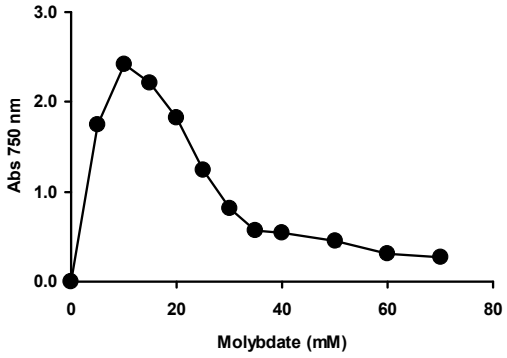

Figure 7 Molybdenum reduction by *Enterobacter sp.* strain Saw-1 utilizing resting cells incubated under various molybdenum (sodium molybdate) concentrations. The error bars represent mean ± standard deviation (n = 3).

3.6 Effect of Heavy Metals

The binding of heavy metals to Mo-reducing bacteria can reduce their metal-reducing ability. In this bacterium, mercury, copper (ii) and silver (I) at two ppm inhibited molybdenum reduction by 47.1, 28.4 and 24.4%, respectively (Figure 8). Toxic metal ions such as Hg^{2+}, Cd^{2+}, Ag^{2+} and Cu^{2+} are known enzyme inhibitors. They usually target sulfhydryl groups of enzymes [42]. The heavy metal mercury targets sulfhydryl groups and also carboxyl, amide, phosphoryl and amine groups of enzymes. This makes mercury one of the most toxic metal ions [43]. Like mercury, silver binds to thiol groups (-SH) in enzymes. Silver also binds to DNA and RNA, causing indirect inhibition to enzyme production, leading to cessation of metabolism in general [44]. Copper binds with high affinity to cysteine, histidine and methionine residues of enzymes, inactivating them as a result [45]. All the metal ions that have been mentioned in this study are also able to inhibit numerous of the molybdenum-reducing bacteria isolated to date (Table 2).

Several works have demonstrated that mercury and copper inhibit the metal-reducing enzyme chromate reductase by binding at the sulfhydryl group of the enzyme in the bacteria *Bacillus sp.* [46] and *Enterobacter cloacae* strain H01 [47]. The presence of these ions reduces the detoxification of chromium in the environment. Several works [48,49] have proposed that sites that harbor these toxic metal ions could be remedied by reducing the bioavailability and mobility of these ions. This can be carried out by inclusion of chemical additives such as magnesium hydroxide, phosphate, thiosulphate, manganese oxide, sulphur and calcium carbonate.

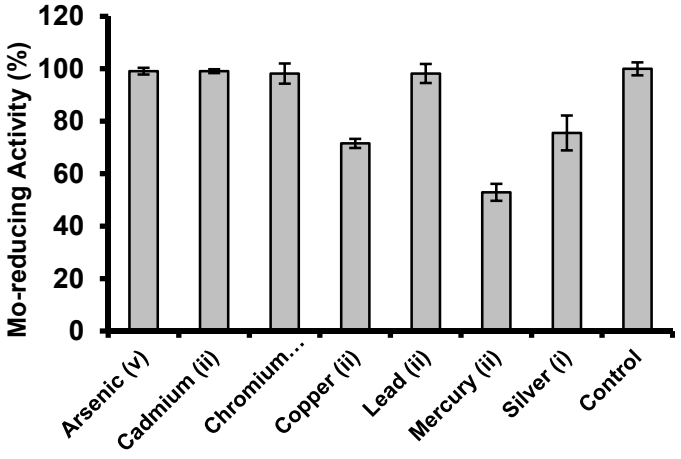

Figure 8 Mo-blue production by *Enterobacter sp.* strain Saw-1 in the presence of various metal ions utilizing resting cells optimized conditions for 48 hours in a microtiter plate. The error bars represent mean ± standard deviation (n = 3).

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

Heavy metals that inhibit reduction	Bacteria	Author
As^{3+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Cr^{6+}, Hg^{2+}, Cu^{2+}	*Bacillus pumilus* strain lbna	[31]
Ag^{+}, Cd^{2+}, Co^{2+}, Cr^{6+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, Zn^{2+}	*Bacillus* sp. strain A.rzi	[32]
Cr^{6+}, Cu^{2+}, Hg^{2+}, Pb^{2+}	*Serratia* sp. strain Dr.Y8	[41]
Cr^{6+}, Cu^{2+}, Pb^{2+}, Hg^{2+}	*Pseudomonas* sp. strain DRY2	[36]
Ag^{2+}, Cd^{2+}, Cr^{6+}, Cu^{2+}, Hg^{2+}, Pb^{2+}	*Pseudomonas* sp. strain DRY1	[34]
Ag^{2+}, Cr^{6+}, Cu^{2+}, Hg^{2+}	*S. marcescens strain* Dr.Y9	[12]
n.a.	*Serratia* sp. strain Dr.Y5	[35]
Cr^{6+}, Cu^{2+}, Hg^{2+}	*Serratia marcescens* strain DRY6	[28]
Cd^{2+}, Cr^{6+}, Cu^{2+}, Pb^{2+}, Hg^{2+}	*Acinetobacter calcoaceticus* strain Dr.Y12	[37]
Cr^{6+}, Cd^{2+}, Cu^{2+}, Ag^{+}, Hg^{2+}	*Enterobacter* sp. strain Dr.Y13	[18]
Cr^{6+}, Cu^{2+}	*Enterobacter cloacae* strain 48	[13]
Cu^{2+}, Ag^{+}, Hg^{2+}	*Klebsiella oxytoca* strain hkeem	[30]
Cr^{6+}	*Escherichia coli* K12	[23]

3.7 Growth of Enterobacter sp. Strain Saw-1 on Pesticides

In a previous work, a Mo-reducing bacterium showed the capability to detoxify a detergent, i.e. SDS, another toxic xenobiotic. As the bacterium was isolated from a contaminated agricultural soil sample, we monitored the capacity of the microorganism to use pesticides as an electron donor for molybdenum reduction or to degrade pesticides independent of molybdenum reduction. None of the pesticides can assist molybdenum reduction; nevertheless, the bacterium can grow on the pesticide coumaphos (Figure 9). As coumaphos is a toxic pesticide, this bacterium can be a good candidate for pesticide bioremediation, especially coumaphos. However, degradation needs to be confirmed using HPLC analysis. There are still few studies on coumaphos-degrading bacteria. These include *Flavobacterium sp.* [50], *Nocardiodes simplex* [51], *Pseudomonas monteilli* [52] and a microbial consortia [53]. There is still limited reporting on bacteria with dual action that are able to detoxify both xenobiotics and heavy metals at the same time. Thus, the ability of *Enterobacter sp.* strain Saw-1 in this study could be used as an alternative bioremediation tool for the polluted area.

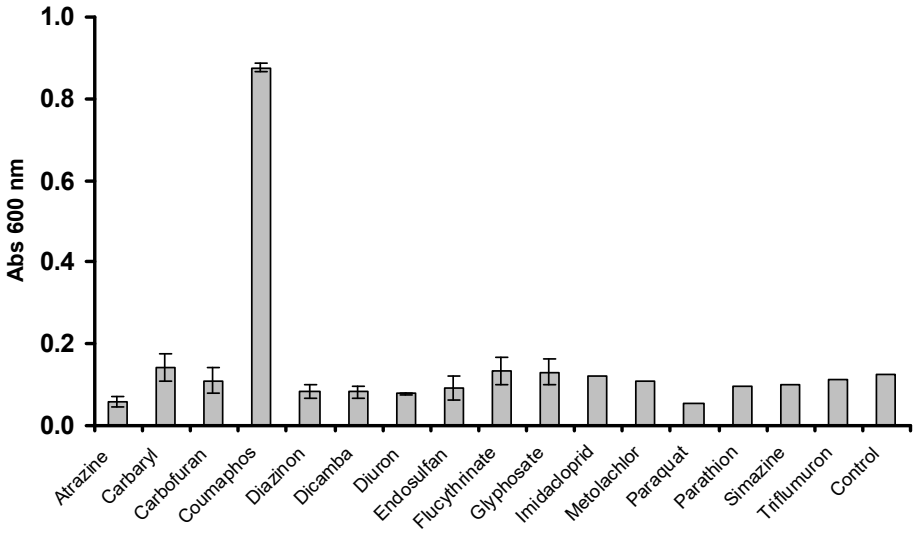

Figure 9 Growth ability of *Enterobacter* sp. strain Saw-1 on various pesticides using resting cells in a microtiter plate. The error bars represent mean ± standard deviation (n = 3).

4 Conclusion

A bacterium capable of reducing molybdenum and detoxifying coumaphos was successfully isolated. A slightly acidic condition was needed for optimal

reduction. In addition, the bacterium was able to reduce molybdenum in a broad range of temperatures. The best carbon source or electron donor for supporting molybdate reduction was glucose. The most critical requirement was a phosphate concentration of 5.0 mM. The Mo-blue absorption spectrum from this bacterium was similar to that of previously isolated Mo-reducing bacteria, resembling a reduced phosphomolybdate. The bacterium was identified as *Enterobacter sp.* strain Saw-1. The heavy metals mercury, copper and silver, inhibited molybdenum reduction. The capacity of this microbe to remove a number of contaminants is a desired property, making the microbe an essential instrument for xenobiotic detoxification and environmental rehabilitation. At present, efforts are done to purify the Mo-reducing enzyme, to determine the pesticides biodegradation parameter and to confirm degradation via HPLC analysis.

Acknowledgment

Snoc International Sdn Bhd. financially supported this project with partially funding by SGPUMS (SLB1032-2017).

References

[1] Mansee, A.H., Chen, W. & Mulchandani, A., *Detoxification of the Organophosphate Nerve Agent Coumaphos using Organophosphorus Hydrolase Immobilized on Cellulose Materials*, Journal of Industrial Microbiology and Biotechnology, **32**(11-12), pp. 554-560, 2005.

[2] Davis, GK., *Molybdenum*, In: Merian E, ed., Metals and their Compounds in the Environment, Occurrence, Analysis and Biological Relevance, pp. 1089-100, VCH Weinheim, New York, 1991.

[3] Neunhäuserer, C., Berreck, M. & Insam, H., *Remediation of Soils Contaminated with Molybdenum using Soil Sediments and Phytoremediation,* Water, Air and Soil Pollution, **128**(1-2), pp. 85-96, 2001.

[4] Runnells, D.D., Kaback, D.S., & Thurman, E.M., *Geochemistry and Sampling of Molybdenum in Sediments, Soils, and Plants in Colorado*, In: Chappel, W.R. & Peterson, K.K., eds., Molybdenum in the Environment.. Marcel and Dekker, Inc., New York, 1976.

[5] Yamaguchi, S., Miura, C., Ito, A., Agusa, T., Iwata, H., Tanabe, S., Tuyen, B.C. & Miura, T., *Effects of Lead, Molybdenum, Rubidium, Arsenic and Organochlorines on Spermatogenesis in Fish: Monitoring at Mekong Delta Area and in-vitro Experiment*, Aquatic Toxicology, **83**(1), pp. 43-51, 2007.

[6] Zhang, Y.L., Liu, F.J., Chen, X.L., Zhang, Z.Q., Shu, R.Z., Yu, X.L., Zhai, X.W., Jin L.J., Ma, X.G., Qi, Q. & Liu, Z.J., *Dual Effects of*

Molybdenum on Mouse Oocyte Quality and Ovarian Oxidative Stress, Systems Biology in Reproductive Medicine, **59**(6), pp. 312-318, 2013.

[7] Ward G.M., *Molybdenum Toxicity and Hypo Cuprosis in Ruminants A Review*, Journal of Animal Sciences, **46**(4), pp. 1078-85, 1978.

[8] Lim, K.T. Shukor, M.Y., & Wasoh, H., *Physical, Chemical, and Biological Methods for the Removal of Arsenic Compounds*, BioMed Research International, 2014. DOI:10.1155/2014/503784

[9] Chirwa, E.N. & Wang, Y.T., *Simultaneous Chromium(VI) Reduction and Phenol Degradation in an Anaerobic Consortium of Bacteria*, Water Research, **34**(8), pp. 2376-2384, 2000.

[10] Chung, J., Rittmann, B.E., Wright, W.F. & Bowman, R.H., *Simultaneous Bio-Reduction of Nitrate, Perchlorate, Selenate, Chromate, Arsenate, and Dibromochloropropane Using A Hydrogen-Based Membrane Biofilm Reactor*, Biodegradation, **18**, pp. 199-209, 2007.

[11] Halmi, M.I.E., Zuhainis, S.W., Yusof, M.T., Shaharuddin, N.A., Helmi, W., Shukor, Y., Syed M.A. & Ahmad, S.A., *Hexavalent Molybdenum Reduction to Mo-blue by a Sodium-dodecyl-sulfate- Degrading Klebsiella Oxytoca Strain Dry14*, BioMed Research International, **2013**, ID 384541, 2013.

[12] Yunus, S.M., Hamim, H.M., Anas, O.M., Aripin, S.N. & Arif, S.M., *Mo (VI) Reduction to Molybdenum Blue by Serratia marcescens Strain Dr. Y9*, Polish Journal of Microbiology, **58**(2), pp. 141-147, 2009.

[13] Ghani, B., Takai, M., Hisham, N.Z., Kishimoto, N., Ismail, A.K.M., Tano, T. & Sugio, T., *Isolation and Characterization of a Mo^{6+}-Reducing Bacterium*, Appl. Environ. Microbiol., **59**(4), pp. 1176-80, 1993.

[14] Shukor, A., Yunus, M., Lee, C.H., Omar, I., Karim M.I.A., Syed M.A. & Shamaan, N.A., *Isolation and Characterization of a Molybdenum-reducing Enzyme in Enterobacter cloacae strain 48*, Pertanika Journal Science and Technology, **11**(2), pp. 261-272, 2003.

[15] Shukor, M.Y., Halmi, M.I.E., Rahman, M.F.A., Shamaan, N.A. & Syed, M.A., *Molybdenum Reduction to Molybdenum Blue in Serratia sp. strain DRY5 is Catalyzed by a Novel Molybdenum-reducing Enzyme*, BioMed Research International, **2014**, ID 853084, 2014.

[16] Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T., & Williams, S.T., *Bergey's Manual of Determinative Bacteriology*, 1994.

[17] Costin, S., & Ionut, S., *Bacterial Identification Software, Database Version*: *Bacillus 022012-2.10*, ABIS Online, http://www.tgw1916.net/bacteria_logare.html, (15 March 2015).

[18] Shukor, M.Y., Rahman, M.F., Shamaan, N.A. & Syed, M.S., *Reduction of Molybdate to Molybdenum Blue by Enterobacter sp. strain Dr.Y13,* Journal of Basic Microbiology, **49**(SUPPL. 1), pp. S43-S54, 2009.

[19] Shukor, M.S. & Shukor, M.Y., *A Microplate Format for Characterizing the Growth of Molybdenum-reducing Bacteria*, Journal of Environmental Microbiology and Toxicology, **2**(2), pp. 42-44, 2014.

[20] Iyamu, E.W., Asakura, T. & Woods, G.M., *A Colorimetric Microplate Assay Method for High-Throughput Analysis of Arginase Activity in Vitro*, Analytical Biochemistry, **383**(2), pp. 332-334, 2008.

[21] Losi, M.E. & Frankenberger, W.T., *Reduction of Selenium Oxyanions by Enterobacter Cloacae Strain SLD1a-1: Reduction of Selenate to Selenite,* Environmental Toxicology and Chemistry, **16**(9), pp. 1851-1858, 1997.

[22] Chaturvedi, V. & Kumar, A., *Diversity of Culturable Sodium Dodecyl Sulfate (SDS) Degrading Bacteria Isolated from Detergent Contaminated Ponds Situated in Varanasi City*, India, International Biodeterioration and Biodegradation, **65**(7), pp. 961-971, 2011.

[23] Campbell, A.M., Campillo-Campbell, A.D. & Villaret, D.B., *Molybdate Reduction by Escherichia coli K-12 and Its Chl Mutants*, Proc Natl Acad Sci, **82**(1), pp. 227-231, 1985.

[24] Kazansky L.P. & Fedotov M.A, *Phosphorus-31 and Oxygen-17 N.M.R. Evidence of Trapped Electrons in Reduced 18-molybdodiphosphate(V),* $P_2Mo_{18}O_{62}^{8-}$, J. Chem. Soc., Chem. Commun., (14), pp. 644-6, 1980.

[25] Shukor, Y., Adam, H., Ithnin, K., Yunus, I., Shamaan, N.A., & Syed, A., *Molybdate Reduction to Molybdenum Blue in Microbe Proceeds via a Phosphomolybdate Intermediate*, Journal of Biological Sciences, **7**(8), pp. 1448-1452, 2007.

[26] Suzuki, T., Miyata, N., Horitsu, H., Kawai, K., Takamizawa, K., Tai, Y. & Okazaki, M., *NAD(P)H-dependent Chromium(VI) Reductase of* Pseudomonas ambigua *G-1: A Cr(V) Intermediate is Formed during the Reduction of Cr(VI) to Cr(III)*, Journal of Bacteriology, **174**(16), pp. 5340-5345, 1992.

[27] Myers. C.R., Carstens, B.P., Antholine, W.E. & Myers, J.M. *Chromium(VI) Reductase Activity is Associated with the Cytoplasmic Membrane of Anaerobically Grown Shewanella Putrefaciens MR-1*, Journal of Applied Microbiology, **88**(1), pp. 98-106, 2000.

[28] Shukor, M.Y., Habib, S.H.M., Rahman, M.F.A., Jirangon, H., Abdullah, M.P.A., Shamaan, N.A. & Syed, M.A., *Hexavalent Molybdenum Reduction to Molybdenum Blue by* S. *Marcescens Strain Dr. Y6*, Applied Biochemistry and Biotechnology, **149**(1), pp. 33-43, 2008.

[29] Shukor, M.Y., Syed, M.A., Lee, C.H., Karim, M.I.A., & Shamaan, N.A., *A Method to Distinguish between Chemical and Enzymatic Reduction of Molybdenum in Enterobacter cloacae strain 48*, Malaysian Journal of Biochemistry, **7**, pp. 71-72, 2002.

[30] Lim, H.K., Syed, M.A. & Shukor, M.Y., *Reduction of Molybdate to Molybdenum Blue by Klebsiella sp. strain hkeem*, Journal of Basic Microbiology, **52**(3), pp. 296-305, 2012.

[31] Abo-Shakeer, L.K.A., Ahmad, S.A., Shukor, M.Y., Shamaan, N.A., & Syed, M.A., *Isolation and Characterization of a Molybdenum-reducing Bacillus pumilus strain lbna*, Journal of Environmental Microbiology and Toxicology, **1**(1), pp. 9-14, 2013.

[32] Othman, A.R., Bakar, N.A., Halmi, M.I.E., Johari, W.L.W., Ahmad, S.A., Jirangon, H., Syed M.A. & Shukor, M.Y., *Kinetics of Molybdenum Reduction to Molybdenum Blue by* Bacillus sp. *strain A.rzi*, BioMed Research International, **2013**, ID 371058, 2013. DOI:10.1155/2013/371058

[33] Khan, A., Halmi, M.I.E. & Shukor, M.Y., *Isolation of Mo-reducing Bacterium in Soils from Pakistan*, Journal of Environmental Microbiology and Toxicology, **2**(1), pp. 38-41, 2014.

[34] Ahmad, S.A., Shukor, M.Y., Shamaan, N.A., Mac Cormack, W.P. & Syed, M.A., *Molybdate Reduction to Molybdenum Blue by an Antarctic Bacterium*, BioMed Research International, **2013**, ID 871941, 2013. DOI:10.1155/2013/871941

[35] Rahman, M.F.A., Shukor, M.Y., Suhaili, Z., Mustafa, S., Shamaan, N.A. & Syed, M.A., *Reduction of Mo(VI) by The Bacterium Serratia sp. Strain DRY5*, Journal of Environmental Biology, **30**(1), pp. 65-72, 2009.

[36] Shukor, M.Y., Ahmad, S.A., Nadzir, M.M.M., Abdullah, M.P., Shamaan, N. A., & Syed, M.A., *Molybdate Reduction by Pseudomonas sp. strain DRY2*, Journal of Applied Microbiology, **108**(6), pp. 2050-2058, 2010.

[37] Shukor, M.Y., Rahman, M.F., Suhaili, Z., Shamaan, N.A. & Syed, M.A., *Hexavalent Molybdenum Reduction to Mo-blue by Acinetobacter Calcoaceticus*, Folia Microbiologica, **55**(2), pp. 137-143, 2010.

[38] Shukor, M.Y., Rahman, M.F.A., Shamaan, N.A., Lee, C.H., Karim, M.I.A. & Syed M.A., *An Improved Enzyme Assay for Molybdenum-reducing Activity in Bacteria*, Applied Biochemistry and Biotechnology, **144**(3), pp. 293-300, 2008.

[39] Mansur, R., Gusmanizar, N., Roslan, M.A.H., Ahmad, S.A. & Shukor, M.Y., Isolation and *Characterisation of a Molybdenum-reducing and Metanil Yellow Dye-decolourising* Bacillus sp. *strain Neni-10 in Soils from West Sumatera, Indonesia*, Tropical Life Sciences Research, **28**(1), pp. 69-90, 2017.

[40] Glenn, J.L. & Crane, F.L., *Studies on Metalloflavoproteins. V. The Action of Silicomolybdate in the Reduction of Cytochrome c by Aldehyde oxidase*, Biochimica Et Biophysica Acta, **22**(1), pp. 111-115, 1956.

[41] Shukor, M.Y., Rahman, M.F., Suhaili, Z., Shamaan, N.A., & Syed, M.A., *Bacterial Reduction of Hexavalent Molybdenum to Molybdenum Blue*, World Journal of Microbiology and Biotechnology, **25**(7), pp. 1225-1234, 2009.

[42] Sugiura, Y. & Hirayama, Y., *Structural and Electronic Effects on Complex Formation of Copper(II) and Nickel(II) with Sulfhydryl-containing Peptides*, Inorganic Chemistry, **15**(3), pp. 679-682, 1976.

[43] Sabullah, M.K., Ahmad, S.A., Shukor, M.Y., Gansau, A.J., Syed, M.A. Sulaiman, M.R. & Shamaan, N.A., *Heavy Metal Biomarker: Fish Behaviror, Cellular Alteration, Enzymatic Reaction and Proteomic Approaches*, International Food Research Journal, **22**(2), pp. 435-454, 2015.

[44] Arakawa, H., Neault J.F. & Tajmir-Riahi, H.A., *Silver(I) Complexes with DNA and RNA Studied by Fourier Transform Infrared Spectroscopy and Capillary Electrophoresis*, Biophysical Journal, **81**(3), pp. 1580-7, 2001.

[45] Camakaris, J., Voskoboinik, I. & Mercer, J.F., *Molecular Mechanisms of Copper Homeostasis*, Biochemical Biophysical Research Communications, **261**(2), pp. 225-232, 1999.

[46] Elangovan, R., Abhipsa, S., Rohit, B., Ligy, P. & Chandraraj, K., *Reduction of Cr(VI) by a Bacillus sp.,* Biotechnology Letter, **28**(4), pp. 247-252, 2006.

[47] Rege, M.A., Petersen, JN, Johnstone, D.L., Turick, C.E., Yonge, D.R. & Apel, W.A., *Bacterial Reduction of Hexavalent Chromium by Enterobacter cloacae strain H01 Grown on Sucrose,* Biotechnology Letter, **19**(7), pp. 691-694, 1997.

[48] Hettiarachchi, G.M., Pierzynski, G.M. & Ransom, M.D., *In-situ Stabilization of Soil Lead using Phosphorus and Manganese Oxide*, Environmental Sciences and Technology, **34**(21), pp. 4614-4619, 2000.

[49] Deeb, B.E. & Altalhi, A.D., *Degradative Plasmid and Heavy Metal Resistance Plasmid Naturally Coexist in Phenol and Cyanide Assimilating Bacteria*, American Journal of Biochemistry and Biotechnology, **5**(2), pp. 84-93, 2009.

[50] Sethunathan, N. & Yoshida, T., *A Flavobacterium sp. that Degrades Diazinon and Parathion*, Canadian Journal of Microbiology. **19**(7), pp. 873-875, 1973.

[51] Mulbry, W., *Characterization of a Novel Organophosphorus Hydrolase from Nocardiodes Simplex NRRL B-24074*, Microbiology Research, **154**(4), pp. 285-288, 2000.

[52] Horne, I., Harcourt, R.L., Sutherland, T.D., Russell, R.J. & Oakeshott, J.G., *Isolation of a Pseudomonas monteilli strain with a Novel Phosphotriesterase*, FEMS Microbiology Letter, **206**(1), pp. 51-55, 2002.

[53] Ha, J., Engler, C.R. & Wild, J.R., *Biodegradation of Coumaphos, Chlorferon and Diethylthiophosphate using Bacteria Immobilized in Ca-Alginate Gel Beads*, Bioresource Technology, **100**(3), pp. 1138-1142, 2009.