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Isolation and Characterization of a Molybdenum-reducing and Carbamate-degrading *Serratia* sp. strain Amr-4 in soils from Egypt

AbdEl-Mongy, M. Abd.[1], Rahman, M.F.[1] and Shukor, M.Y.*[2]

[1]Microbial Biotechnology Department, Genetic Engineering and Biotechnology Institute, Sadat City University, Egypt.
[2]Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, UPM 43400 Serdang, Selangor, Malaysia.

*Corresponding author:
Prof Dr. Yunus Shukor,
Department of Biochemistry,
Faculty of Biotechnology and Biomolecular Sciences,
Universiti Putra Malaysia,
UPM 43400 Serdang,
Selangor,
Malaysia.
Email: yunus.upm@gmail.com / mohdyunus@upm.edu.my

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ABSTRACT

Physical or chemical procedures could efficiently remove contaminants including pesticides such as carbamates from high concentrations of toxicants. Bioremediation, on the other hand, is frequently a less expensive option in the long term when used at low concentrations. Isolation of multiple toxicants removing microorganisms is the goal of bioremediation. In this paper we report on the molybdenum reduction of the bacterium and its ability to grow on the carbamates carbofuran and carbaryl as carbon sources. Both the carbamates carbofuran and carbaryl cannot support molybdenum reduction when used as the sole carbon sources. Between pH 6.0 and 6.8 and between 30 and 34 °C, the bacterium is most efficient in converting molybdate to Mo-blue. For molybdate reduction, glucose was shown to be the strongest electron donor, with maltose and sucrose coming in second and third, respectively, and d-mannitol and d-adonitol coming in last. Phosphate concentrations of 2.5 to 7.5 mM and molybdate concentrations of 20 to 30 mM are also needed. Identical to that of a decreased phosphomolybdate, the Mo-blue produced by the new Mo-reducing bacteria has an absorption spectrum similar to prior Mo-reducing bacteria. Inhibition of molybdenum reduction was 73.3, 50.1, 50.1 and 20.7 percent, respectively, by mercury, copper, silver and lead at 2 ppm. The bacterium was tentatively identified as *Serratia* sp. strain Amr-4 after biochemical investigation. This bacterium's ability to detoxify a variety of toxicants is highly sought after, making it a significant bioremediation agent.

INTRODUCTION

Pesticides based on carbamate have a fascinating history of research and discovery. Calabar bean paste, which is rich in carbamate alkaloids, was employed in West Africa to determine whether or not a person was guilty or innocent of witchcraft accusations. After being forced to consume calabar bean paste, the suspected 'witches' either died or were deemed innocent. In the calabar bean, physostigmine was shown to be the active carbamate carbamate, according to research. Indeed, the West African term for calabar bean, esere, is eserine's counterpart. A series of carbamate insecticides was originally created in the mid- to late 1940s in an attempt to create new insect repellents, but their insecticidal qualities were immediately discovered and recognized [1–6]. In agriculture, the carbamate chemicals are esters of carbamic acid. N-methylcarbamates are the name given to these substances. Weed killers often make use of carbamic acid and its derivatives thiocarbamic acid and dithiocarbamic acid. To the advantage of society, carbamate insecticides safeguard and boost agricultural productivity and protect human and animal health from insect-vector-mediated illnesses when applied appropriately. Nevertheless, excessive exposure to these chemicals may lead to pesticide poisoning in people and animals. The acetylcholinesterase enzyme is inhibited by N-methylcarbamate pesticides. Hypercholinergic activity, as a consequence, is a poisonous indication. N-methyl-d-aspartate receptors are likewise hyperactivated by triggering a series excitotoxicity. Carbamate poisoning is normally treated within six to 24 h for both humans and animals. Atropine sulphate is the mainstay of treatment [7–12].

These pesticides contain carbamate insecticides, which are made up of carbamate and an ester group. Carbonyl, aldicarb and fenoxycarb are only few of the chemicals that make up this group. Anti-acetylcholine esterase enzyme inhibitors cause the buildup

of acetylcholine at synapses [13]. Among the carbamates, carbofuran is one of the most toxic insecticides registered in some parts of the world. Carbofuran is displayed high mammalian toxicity with an LD_{50} of 2 mg/kg in mice, while carbaryl is a broad-spectrum insecticide often used in the agricultural sector.

Acetyl cholinesterase activity is permanently inhibited by both pesticides. Mammalian liver metabolic activities such as protein synthesis, glucose metabolism, and detoxification have been shown to be affected by nontarget toxicity [13]. This medicine is also known as a mild inducer of hepatic microsomal drug metabolism (WHO) activity. Both carbamates are very mobile in soil, and excessive usage of them might have detrimental effects on non-targeted creatures and human health. Numerous researches have been conducted on how to remove them from the environment as a result [14–21].

Molybdenum is one of the necessary heavy metals that is required in small levels but is hazardous to a wide range of species when in large quantities. It is one of the necessary heavy metals that is required in small amounts but is harmful to a wide range of species when in large quantities. Molybdenum disulphide is used as an alloying agent, as an anti-freeze component in automotive engines, and as a lubricant in the form of molybdenum disulphide, among many other industrial applications. As a result of the widespread use of molybdenum in industry, there have been a number of instances of water contamination. On the ground, however, it has been demonstrated to be a significant contaminant in sewage discharge that has been polluted [22–26].

Molybdenum has been proven to disrupt spermatogenesis and to delay embryogenesis in species such as catfish and mice at concentrations as low as a few parts per million of the elements. In addition, molybdenum is extremely toxic to ruminants, particularly cows, even at low concentrations of a few parts per million or less [27–31]. Even if physical or chemical treatments may be effective at larger concentrations (e.g., eliminating heavy metals), bioremediation is more cost-effective in the long run, particularly at lower concentrations of contaminants. In the past, it has been demonstrated that the degradation of xenobiotics and heavy metals operate in concert. However, despite the fact that sodium dodecyl sulphate (SDS) does not allow for the reduction of molybdenum, a molybdenum-reducing bacteria was found to be able to thrive when SDS was used as the only carbon source. The use of non-native microorganisms in bioremediation is discouraged since it has the potential to cause ecological issues. As a result, it is advised that local bacteria be screened in advance of cleanup activities [32,33].

It is our goal in this study to determine if a unique molybdenum-reducing bacteria isolated from polluted soils can reduce molybdenum on its own, either even without the assistance of a pesticide, by using pesticides as electron donors for reduction or as carbon sources for growth.

MATERIALS AND METHODS

Chemicals
All chemicals were of analytical grade unless stated otherwise.

Isolation of molybdenum-reducing bacterium
A polluted land site in Sadat City, Egypt, was sampled (5 cm deep from the topsoil) in 2014. A single gramme of dirt was dissolved in sterile tap water for the analysis. At room temperature for 48 h, the soil in tap water suspension was pipetted and distributed on agar with a medium of low phosphate (pH 7.0). As a result, the low phosphate media (LPM) was composed of glucose (1 percent), $(NH_4)_2.SO4$ (0.3 percent), $MgSO_4.7H_2O$ (0.05 percent) and yeast extract (0.5 percent), as well as sodium chloride (0.5 percent), NaCl (0.5 percent) and Na_2HPO_4 (0.071 percent or 5 mM) [34]. Molybdate reduction by molybdenum-reducing bacteria is shown by the presence of blue colonies. It was isolated and restreaked on LPM to get a pure culture of the organism (low phosphate media). It took 48 hours at room temperature on an orbital shaker set at 120 rpm for the same medium to decrease molybdenum in liquid media using the same procedure (pH 7.0). 1.0 mL of Mo-blue was obtained by centrifuging the liquid culture at 10,000 x g for 10 minutes at room temperature, yielding 1.0 mL of Mo-blue for analysis. UV-spectrophotometer 400-900 nm scan of supernatant (Shimadzu 1201). We utilised low phosphate medium for baseline correction.

Partial identification of the Mo-reducing bacterium
In order to identify the bacterium, standard methods such as size and colour on nutrient-agar plates, Gram staining, motility and other biochemical tests were used [35]. The ABIS online system was utilized to interpret the results [36].

Preparation of resting cells for molybdenum reduction characterization
It was done in the same manner as previously, using a microplate or microtiter format, to undertake a range of molybdenum reduction to Mo-blue characterisation tests. These included the effects of temperature, phosphate, pH, and molybdate concentrations, among other things. The specific extinction coefficient was used to quantify the amount of Mo-blue that was produced by the medium in a microplate configuration where the value of 11.69 $mM.^{-1}.cm^{-1}$ at 750 was used [37]. Heavy metals from commercial salts or from Atomic Absorption Spectrometry standard solutions from MERCK were utilized in this study. The capability of several pesticides to provide for molybdenum reduction as source of electron was analyzed using the microplate format with substituting the glucose from the LPM with these xenobiotics at 200 mg/L final concentration. Non-water soluble pesticides were dissolved in methanol as the carrier solvent [38].

Statistical analysis
Means ± standard deviation of tripilates are used in the calculation of values. Comparing groups was done using a Student's t-test or a one-way analysis of variance followed by a Tukey's post hoc analysis. Statistical significance was defined as a $P<0.05$.

RESULTS AND DISCUSSIONS

Identification of Mo-reducing bacterium
Based on standard biochemical and physiological methods, the microorganism was a Gram-negative, facultative anaerobe, rod-shaped, motile microorganism. Approximately 1 to 3 mm in diameter, the colonies had a glossy cream-white appearance, and were smooth and round in shape. Comparing the results of numerous biochemical and morphological tests helped identify the bacterium (**Table 1**) to the Bergey's Manual of Determinative Bacteriology [35] and using the ABIS online software [36]. T*Serratia* plymuthica was the most accurate and homologous of the four possible bacterial identities suggested by the programme. The assignment to the species level is not possible at this time. In order to identify this species further, additional research, including a comparison of the 16srRNA gene by molecular identification, is required. As of right now, the bacterium is temporarily identified as *Serratia* sp. strain Amr-4.

Table 1. Biochemical tests for *Serratia* sp. strain Amr-4.

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

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

Molybdenum reduction is dominated by the *Serratia* genus [39]. The species of this genus have also been reported to be able to degrade pesticides such as nicosulfuron [40] and diazinon [41].

Methods to speed up characterisation and collect more data were employed in this study using a simple, high-throughput method employing microplates [38,42]. Resting cells have been utilized in exploring the biological reduction of heavy metals including vanadate [43], selenate [44], chromate [45], reductions and xenobiotics biodegradation such as dyes [46,47], diesel [48], SDS [49], phenol [50], amides [51] and pentachlorophenol [52].

Molybdenum absorbance spectra

Absorption spectra of Mo-blue generated by the Serratia sp. strain Amr-4 has a maximum peak at 860-870nm with a median value of 865nm, with a shoulder at roughly 700nm (**Fig. 1**). The Mo-blue is difficult to identify because of its complicated structure and numerous species [53]. Mo-blue can be either the molybdenum complexes- isopolymolybdate or heteropolymolybdate in reduced forms. In the reduction of molybdenum by *E. coli* K12, it has been suggested that the Mo-blue detected is decreased phosphomolybdate, however the authors did not provide a mechanism [54]. No biological reducing agent can produce isopoly Mo-blue from molybdate because the conversion requires powerful reducing agents and acidic circumstances. Using ascorbic acid as a phosphate determination method, the likelihood of heteropoly Mo-blue formation by biologically-based reducing agents such ascorbic acids or enzymatic reduction increases [55]. Molybdenum reduction in phosphate and molybdate-rich media is likely to occur via a phosphomolybdate intermediate, according to our hypothesis. This intermediate is formed when the pH of the phosphate and molybdate-rich medium is reduced during bacterial growth. This bacterium's Mo-blue should have an absorption spectrum that closely resembles the phosphate determination method if it proceeds through this mechanism.

The absorption peak was found to be between 860 and 870 nm, with a shoulder at around 700 nm. An analysis of the Mo-blue spectrum from the phosphate determination method typically indicated a peak around 880–890 nm and a shoulder between 700 and 720 nanometers [56].

Other bacteria's Mo-blue spectra also follow this rule, as we have shown [53]. In this study, the absorption spectra clearly indicate a comparable spectrum, which supports the idea. The complicated structure of the phosphomolybdate compound necessitates the use of n.m.r. and e.s.r. for precise identification.

Heteropolymolybdate species can be characterised using spectrophotmetric methods such as scanning spectroscopic profiles. [57]. Even though Mo-maximal blue's absorption wavelength was 865 nm, routine monitoring of its synthesis at 750 nm was sufficient due to the intensity achieved being significantly higher than 600-620 nm, which is the range cellular maximum absorption [38]. Earlier determination of Mo-blue production utilizes a number of wavelengths such as 820 nm [54] and 710 nm [58].

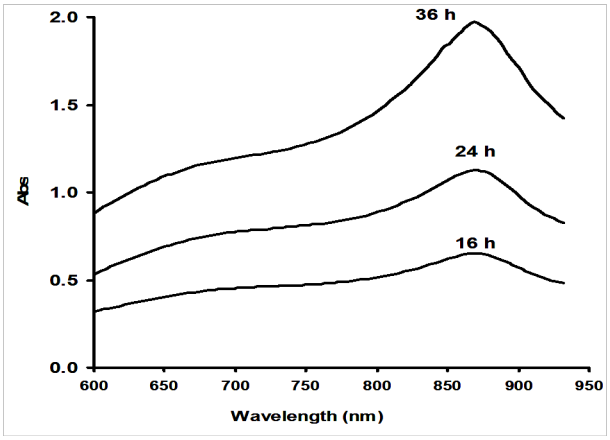

Fig. 1. Absorption spectra of Mo-blue scanned from 500 to 950 nm from *Serratia* sp. strain Amr-4 at various growth stages.

Effect of pH and temperature on molybdate reduction

Varied buffers were used to incubate *Serratia* sp. strain Amr-4 at different pH levels, ranging from 5.5 to 8.0. (20 mM). An ANOVA study found that the ideal pH range for reducing was between 6.0 and 6.8." (**Fig. 2**). An optimal temperature ranged from 30 to 34 °C with no significant difference ($p>0.05$) between values recorded as analysed using ANOVA (**Fig. 3**) spanning the range of 20 to 60 °C. Serratia sp. strain Amr-4 was unable to produce Mo-blue at temperatures over 34 ^{O}C.

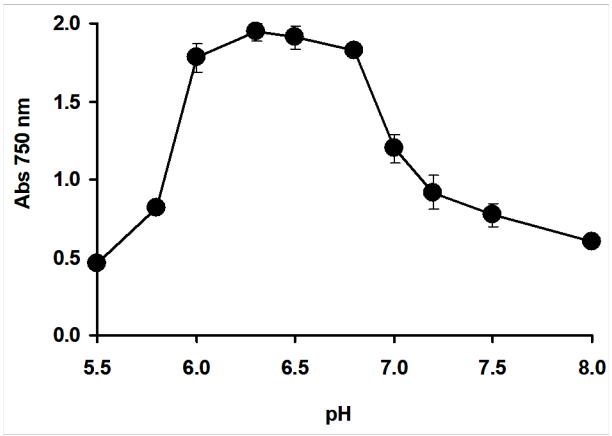

Fig. 2. Effect of pH on Mo-blue production by *Serratia* sp. strain Amr-4. Error bars are ± standard deviation (n=3).

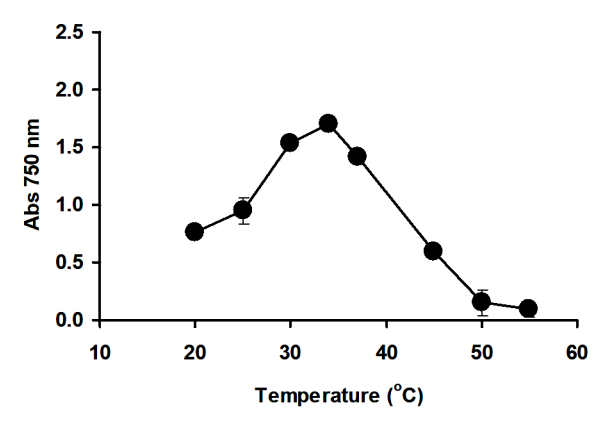

Fig. 3. Effect of temperature on Mo-blue production by *Serratia* sp. strain Amr-4. Error bars are ± standard deviation (n=3).

Temperature and pH alter enzyme activity and protein folding, resulting in the suppression of molybdenum reduction, which is an enzyme-mediated process. *Serratia* sp. strain Amr-4 may be a promising choice for soil bioremediation of molybdenum in both the local and international contexts. The bulk of the reducers operate well at temperatures ranging between 25 and 37 degrees Celsius [39,56,59–68] with a psychrotolerant reducer showing an optimal temperature sbetween 15 and 20 ºCas it was isolated from Antarctica [69].

When it comes to supporting molybdenum reduction, Serratia sp. strain Amr-4 has an ideal pH range that corresponds to the bacterium's ability to function as a neutrophile. The ability of neutrophils to develop between pH 5.5 and 8.0 is one of their distinguishing properties. The best pH for molybdenum reduction in bacteria is somewhat acidic, with optimal pHs ranging from pH 5.0 to pH 7.0. This is a significant fact about molybdenum reduction in bacteria [54,56,58–70]. The creation and stability of phosphomolybdate before it is reduced to Mo-blue have been postulated in the past, and it has been suggested that an acidic pH is necessary in this process. As a result, the optimal reduction is achieved by a delicate balance between enzyme activity and substrate stability [71].

Effect of carbon sources as electron donor for Mo reduction

Earlier works of Shukor et al. indicated that the majority of the Mo-reducing bacteria prefers glucose, followed by sucrose and fructose [63]. These simple sugars produces NADH and NADPH much easier than complex carbon sources such as acrylamide or acetamide [68,72]. Glucose was the most efficient electron donor for promoting molybdate reduction, followed by maltose, sucrose, d-mannose, myo-inositol, d-sorbitol, trehalose, glycerol, salicin, d-adonitol, and d-mannitol, in that order, and then maltose and sucrose (**Fig. 4**). Other carbon sources were found to be insufficient for molybdenum reduction.

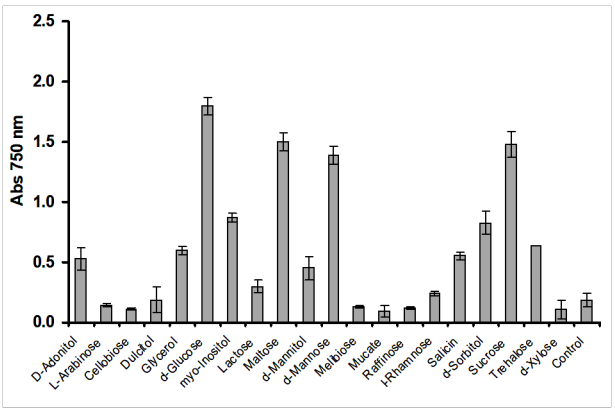

Fig. 4. The effect of different electron donor sources (1 percent weighted average) on Mo-blue production by *Serratia* sp. strain Amr-4. Error bars are ± standard deviation (n = 3).

Role of phosphate and molybdate in molybdate reduction

A balance amount of molybdenum and phosphate is critical as unbalanced level impede Mo-blue synthesis in bacteria, making this research extremely essential [39,56,59,61–63,66,68–70]. Phosphate was required from 2.5 to 7.5 mM with higher concentrations were strongly inhibitory to reduction (**Fig. 5**). In order for the phosphomolybdate complex to be stable, it is believed that a high phosphate concentration would hinder the complex's stability.

However, this has not been shown yet. The buffering strength of the phosphate buffer, on the other hand, rises with increasing phosphate content. It is also observed that the phosphomolybdate complex is unstable when exposed to phosphate [73–75]. So far, all known bacteria that reduce molybdenum require phosphate concentrations no greater than 5 mM to function properly [54,56,58–70]. When the influence of molybdenum concentration on molybdenum reduction was investigated, it was shown that the newly isolated bacteria was capable of reducing molybdenum at concentrations as high as 60 mM, albeit at the expense of Mo-blue synthesis. The best reduction range was found to be between 20 and 30 mM (**Fig. 6**).

Molybdenum contamination can be reduced if the strain is reduced to an insoluble form at this high concentration. Molybdenum's optimum concentrations for supporting bacterial reduction range from 15 to 80 mM [54,56,58–70]. As a contaminant, molybdenum has a maximum concentration of roughly 20 mM in the environment [76].

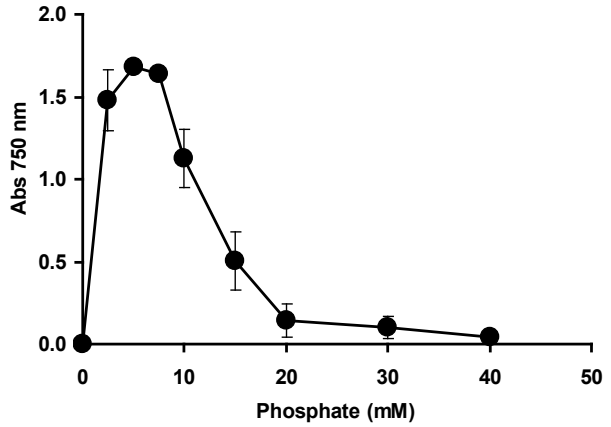

Fig. 6. *Serratia* sp. strain Amr-4 molybdenum reduction at various phosphate content. Error bars are ± standard deviation (n = 3).

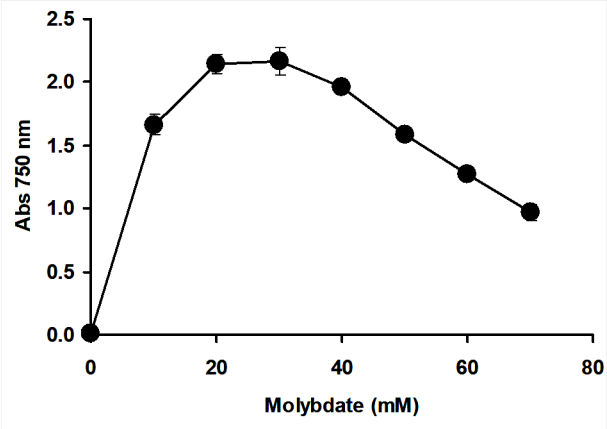

Fig. 6. The effect of molybdate concentration on molybdenum reduction by *Serratia* sp. strain Amr-4. Error bars are ± standard deviation (n = 3).

Inhibitory effect of heavy metals

Mmercury, copper, silver and lead at 2 ppm inhibited reduction by 73.3, 50.1, 50.1 and 20.7%, respectively (**Fig. 7**). Because of their inhibitory effects on bioremediation, other metal ions and heavy metals have a significant influence on the process. As a result, screening and isolation of metal-resistant bacteria are critical steps in the process. As explained earlier by Shukor et.al. [77], mercury is a true physiological inhibitor to molybdate reduction as it did not inhibit chemical reduction of molybdenum to molybdenum blue while other metal ions either inhibit or act as a promoter in producing Mo-blue from phosphomolybdate, which is a common problem in many metal reduction works involving microorganisms where careful experiments are needed to distinguish between abiotic and biological reduction [77]. Almost all Mo-reducing bacteria to date are inhibited by heavy metals (**Table 2**). In general, heavy metals such as silver, cadmium, mercury and copper tend to target enzymes in their sulfhydryl group [78]. Chromate can inhibit enzymes such as glucose oxidase [79]. The enzymes responsible for reducing heavy metals were rendered ineffective by heavy metal binding.

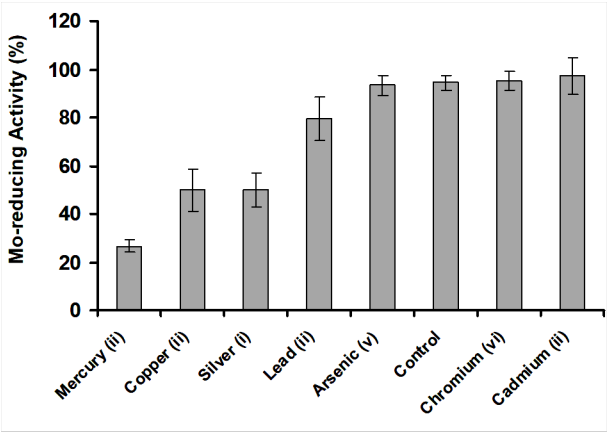

Fig. 7. The influence of metals on *Serratia* sp. strain Amr-4's Mo-blue synthesis. Resting bacteria cells were cultured for 48 hours under optimal conditions on a microtiter plate.. Error bars are ± standard deviation (n = 3).

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

Bacteria	Heavy Metals that inhibit reduction	Author
Bacillus pumilus strain lbna	As^{3+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Cr^{6+}, Hg^{2+}, Cu^{2+}	[64]
Bacillus sp. strain A.rzi	Cd^{2+}, Cr^{6+}, Cu^{2+},Ag^{+}, Pb^{2+}, Hg^{2+}, Co^{2+},Zn^{2+}	[66]
Serratia sp. strain Dr.Y8	Cr, Cu, Ag, Hg	[61]
S. marcescens strain Dr.Y9	Cr^{6+}, Cu^{2+}, Ag^{+}, Hg^{2+}	[39]
Serratia sp. strain Dr.Y5	n.a.	[60]
Pseudomonas sp. strain DRY2	Cr^{6+}, Cu^{2+}, Pb^{2+}, Hg^{2+}	[56]
Pseudomonas sp. strain DRY1	Cd^{2+}, Cr^{6+}, Cu^{2+},Ag^{+}, Pb^{2+}, Hg^{2+}	[69]
Enterobacter sp. strain Dr.Y13	Cr^{6+}, Cd^{2+}, Cu^{2+}, Ag^{+}, Hg^{2+}	[70]
Acinetobacter calcoaceticus strain Dr.Y12	Cd^{2+}, Cr^{6+}, Cu^{2+}, Pb^{2+}, Hg^{2+}	[62]
Serratia marcescens strain DRY6	Cr^{6+}, Cu^{2+}, Hg^{2+}*	[59]
Enterobacter cloacae strain 48	Cr^{6+}, Cu^{2+}	[58]
Escherichia coli K12	Cr^{6+}	[54]
Klebsiella oxytoca strain	Cu^{2+}, Ag^{+}, Hg^{2+}	[63]

Potential of pesticides as electron donors for Mo reduction or to support growth

Pesticides were tested to see whether they could help reduce molybdenum levels. Although molybdenum reduction was not supported by any of the insecticides, carbofuran and carbaryl grew the bacterium (**Fig. 8**). Carbohydrate-degrading bacteria from a variety of taxa have been identified including *Rhodococcus, Sphingomonas, Sphingobium, Bosea,* and *Microbacterium* [80] and bacterial species such as *Burkholderia cepacia* [81], Novosphingobium sp. [82], Sphingomonas sp. [83].

Carbofuran-degrading bacteria isolated from soils were subjected to a complete molecular investigation, which found numerous known bacterial taxa as well as new ones *Aminobacter* spp., *Arthrobacter* spp., *Bacillus* spp., *Chelatobacter* spp., *Ochrobactrum* spp., *Pseudomonas* spp., *Ralstonia* spp., *Rhodanobacter* spp., *Rhodococcus* spp. [84]. Carbofuran-degrading microbes include *Micrococcus arborescens, Pseudomonas aeruginosa, Brachybacterium* sp. and *Salsuginibacillus kocurii* [85,86][86], *Rhizobium* sp. [87], *Micrococcus* species [88], *Arthrobacter* sp. [89] and *Rhodococcus* sp. [90].

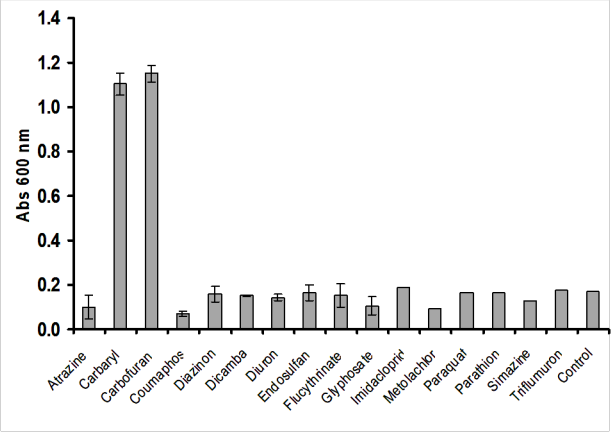

Fig. 8. Growth of *Serratia* sp. strain Amr-4 on various pesticides. Error bars are ± standard deviation (n = 3).

CONCLUSION

The carbamate herbicides carbofuran and carbaryl can be used as carbon sources for growth by a local Mo-reducing bacterium. *Serratia* sp. strain Amr-4 has been provisionally identified as the bacterium that caused the outbreak. It is the first time that a molybdenum-reducing bacterium capable of thriving on carbofuran and carbaryl has been discovered in nature. The bacterium is most effective in converting molybdate to Mo-blue when the pH is between 6.0 and 6.8 and the temperature is between 30 and 34 degrees Celsius. When it comes to molybdate reduction, glucose was shown to be the most effective electron donor, followed by maltose and sucrose, which came in second and third, respectively, and d-mannitol and d-adonitol, which came in last and last, respectively. A phosphate concentration of 2.5-7.5 mM as well as molybdate concentrations of 20-30 mM are also required in this experiment. The Mo-blue generated by the novel Mo-reducing bacteria has an absorption spectrum that is identical to that of a reduced phosphomolybdate, and its absorption spectra is comparable to that of previous Mo-reducing bacteria. Mercury, copper, silver, and lead all decreased molybdenum reduction by 73.3, 50.1, 50.1, and 20.7 percent, respectively, when used at 2 parts per million (ppm) mercury, copper, silver. The capacity of this bacterium to detoxify a wide range of toxicants is greatly sought after, making it a valuable bioremediation agent. We are now working on the purification and characterization of the molybdenum-reducing enzyme isolated from this bacterium.

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