

## Isolation and Characterization of a Molybdenum-reducing and Coumaphos-degrading *Bacillus* sp. strain Neni-12 in soils from West Sumatera, Indonesia

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### HISTORY

Received: 25<sup>th</sup> August 2019  
Received in revised form: 14<sup>th</sup> of October 2019  
Accepted: 18<sup>th</sup> of November 2019

### KEYWORDS

molybdenum  
molybdenum-reducing  
*Bacillus* sp.  
Molybdenum blue  
coumaphos

### ABSTRACT

The inappropriate removal, manufacturing and prospecting actions and unnecessary use of agricultural chemical compounds have triggered an international issue. Eliminating these kinds of contaminants by means of bioremediation is a less expensive approach in the long term notably at low concentrations, in which various other approaches for instance physical or chemical approaches is probably not useful. In this work we screen the ability of a molybdenum-reducing bacterium isolated from polluted soil to make use of pesticides as electron donor sources for assisting reduction and as carbon sources for growth. The bacterium was not able to use pesticides as electron donors, nonetheless, the bacterium can grow on coumaphos separate from molybdenum reduction. Optimum conditions for Mo-blue production were at pH 6.3 and between 25 and 37 °C, glucose as electron donor, phosphate at 5.0 mM and sodium molybdate between 15 and 20 mM. Reduction was inhibited by Hg, Ag and Cr at 2 ppm by 91.9, 82.7 and 17.4 %, respectively. Biochemical analysis tentatively and partially identified the bacterium as *Bacillus* sp. strain Neni-12. This is a novel Mo-reducing bacterium with coumaphos degrading capability.

### INTRODUCTION

Industrial and mining activities, improper disposal, and excessive use of agricultural chemicals have resulted in the pollution of the environment. Removing these toxic pollutants through bioremediation in the long run is a less costly approach. This is especially so at low concentrations of pollutants where other strategies for example physical or chemical methods may not be effective. Organophosphorus (OP) compounds are parts of pesticides that have garner global attention due their applications as pesticides and chemical weapons making them extremely hazardous in properties and becoming significant pollutants. These kinds of pesticides are necessary for the majority of agriculture-developing plans.

The comprehensive utilization or mistreatment of OP compounds in numerous ways has ended in quite a few unwelcome consequences which include toxicity to mankind and polluting the environment. It is estimated by the World Health Organization that exposure to OPs resulting in roughly 220,000 deaths and three million cases of severe poisoning globally [1].

Coumaphos [O-(3-chloro-4-methyl-2-oxo-2H-1-benzopyran-7-yl) O,O-diethylphosphorothioate] has a broad spectrum of application for crops and animal protection, but its main uses is to control a number of arthropod pest in animals.

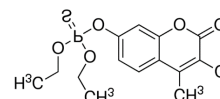


Fig. 1. Chemical structure of coumaphos.

Molybdenum is probably the crucial heavy metals that are essential at trace amount and is also dangerous to a selection of organisms at enhanced quantities. It has quite a few uses in industrial sectors which include as engine anti-freeze component, alloying agent, corrosion resistant steel and as molybdenum disulphide-based lubricant. The vast use of molybdenum in industry has ended in a number of water pollution instances all across the globe which include the Tyrol in Austria, Tokyo Bay and the Black Sea [2]. In organisms such as catfish and mice, molybdenum inhibits reproductive process including inhibiting

spermatogenesis and arresting embryogenesis [3,4]. Classically, molybdenum is very toxic to ruminants especially cows at low levels of concentration [5,6].

At low concentrations bioremediations of heavy metals can be an economical approach compared to physicochemical approaches [7]. The biotransformation of heavy metals coupled with the degradation of xenobiotics an important emerging bioremediation tools and has garnered numerous interests globally [8–21]. Here, a novel molybdenum-reducing bacterium has been isolated from an agricultural soil with the capacity to grow on the pesticide coumaphos.

## MATERIALS AND METHODS

### Molybdenum-reducing bacterium isolation and maintenance

Soil samples were taken in Bukittinggi, the province of West Sumatera, Indonesia in January 2009. Soils were taken about 5 cm deep from the topsoil. Screening and isolation works were carried out on a solid low phosphate agar medium (pH 6.5), at room temperature and incubated for 48 h. The composition (w/v) of the media is as follows: yeast extract (0.5%),  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  (0.242 % or 10 mM), glucose (1%), NaCl (0.5%),  $(\text{NH}_4)_2\text{SO}_4$  (0.3%),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.05%), and  $\text{Na}_2\text{HPO}_4$  (0.071% or 5 mM). Solid medium was prepared by adding agar (1.5%) [22]. This is low phosphate molybdenum or LPM media. Blue colonies indicate molybdenum has been reduced to molybdenum blue. The blue colonies were repeatedly streaked on agar to purify the Mo-reducing bacterium. LPM liquid culture was utilized to characterize the Mo-reducer. To obtain the scanned spectrum of the resultant Mo-blue, 1 mL of the blue supernatant was centrifuged at  $10,000 \times g$  for 10 min and scanned from 400 to 900 nm (UV-spectrophotometer, Shimadzu 1201). The Bergey's Manual of Determinative Bacteriology [23], Identification of the bacterium was carried out utilizing biochemical and phenotypical methods and the results plugged into the ABIS online system software [24] as before [25].

### Bacterial resting cells preparation

Characterization of molybdenum reduction such as molybdate, carbon sources, phosphate, effect of pH, heavy metals and temperature was carried out using resting cells preparation as before [25,26]. Briefly, 1 L of cells were first grown in high phosphate molybdate media or HPM at room temperature at 120 rpm on an orbital shaker (Yihder, Taiwan) for 48 h. Cells were centrifuged ( $15,000 \times g$ ) at  $4^\circ\text{C}$  for 10 minutes. The pellets were first rinsed with deionized water and resuspended in 20 mL of LPM with glucose not included. 180  $\mu\text{L}$  aliquot of the resting cells was sterically transferred to a sterile microplate. Glucose or other carbon sources (20  $\mu\text{L}$ ) were then added to the final concentration of 1.0 % (w/v). Readings at 750 nm was periodically after the microplates were sealed and incubated for 48 h at room temperature. The effects of several heavy metals were also studied using metal ion solutions from Atomic Absorption Spectrometry standards.

### Pesticides as carbon sources for bacterial growth

As the soil sample came from an agricultural land with a history of pesticides application, the Mo-reducing bacterium was tested for its ability to grow on the pesticide's atrazine, paraquat, diuron, imidacloprid, carbaryl, glyphosate, coumaphos, endosulfan, flucythrinate, diazinon, metolachlor, carbofuran, parathion, dicamba, and simazine. These pesticides were tested for their ability to support growth as preliminary works showed that none of the pesticides was able to support molybdenum reduction. Glucose was replaced with the pesticides at the final concentration of 200 mg/L. Atrazine, diazinon, endosulfan,

flucythrinate, diuron, coumaphos, parathion and simazine were dissolved in the carrier solvent methanol in a minimal volume and added to the HPM media. The growth media omitted molybdenum, as this element might have a detrimental effect to growth on xenobiotics. The microplate was incubated for ten days at room temperature. Bacterial growth was monitored at 600 nm. Growth of the bacterium on the pesticide was modelled according to the modified Gompertz model [27].

## RESULTS AND DISCUSSIONS

Molybdenum reduction to molybdenum blue by microorganisms is first reported more than a century ago in 1896 in *E. coli* [28]. The soluble molybdenum in the form of sodium molybdate is reduced to the colloidal molybdenum blue, which is relatively insoluble and forms an opportunity for the reduced product to be removed from aqueous solution. Since then, plentiful of Mo-reducing bacteria have been reported (Table 1), including an Antarctic psychrophilic Mo-reducing bacterium. Resting cells utilization in a microtiter plate format allow more characterization to be made [25,26,29]. Although the resting cells or whole cells has been utilized in optimizing molybdate reduction in the bacterium *Enterobacter cloacae* strain 48 [30]. Several bacterial characterizations work such as in selenate [31], chromate [32] and vanadate [33] reductions also utilize resting cells. Furthermore, biodegradation of xenobiotics for example SDS [34] and diesel [35] also takes advantage of resting cells. The use of resting cells bypasses the initial stage of the growth process that is normally affected by toxic xenobiotics.

Table 1. Characterization of Mo-reducing bacteria isolated to date.

Bacteria	Optimal C source	Optimal Molybdate (mM)	Optimal Phosphate (mM)	Heavy metals inhibition	Author
<i>Klebsiella oxytoca</i> strain Aft-7	glucose	5-20	5-7.5	$\text{Cu}^{2+}$ , $\text{Ag}^+$ , $\text{Hg}^{2+}$	[25]
<i>Bacillus pumilus</i> strain lbna	glucose	40	2.5-5	$\text{As}^{3+}$ , $\text{Pb}^{2+}$ , $\text{Zn}^{2+}$ , $\text{Cd}^{2+}$ , $\text{Cr}^{6+}$ , $\text{Hg}^{2+}$ , $\text{Cu}^{2+}$	[36]
<i>Bacillus</i> sp. strain A.rzi	glucose	50	4	$\text{Cd}^{2+}$ , $\text{Cr}^{6+}$ , $\text{Cu}^{2+}$ , $\text{Ag}^+$ , $\text{Pb}^{2+}$ , $\text{Hg}^{2+}$ , $\text{Co}^{2+}$ , $\text{Zn}^{2+}$	[37]
<i>Serratia</i> sp. strain Dr.Y8	sucrose	50	5	Cr, Cu, Ag, Hg	[38]
<i>S. marcescens</i> strain Dr.Y9	sucrose	20	5	$\text{Cr}^{6+}$ , $\text{Cu}^{2+}$ , $\text{Ag}^+$ , $\text{Hg}^{2+}$	[22]
<i>Serratia</i> sp. strain Dr.Y5	glucose	30	5	n.a.	[39]
<i>Pseudomonas</i> sp. strain DRY2	glucose	15-20	5	$\text{Cr}^{6+}$ , $\text{Cu}^{2+}$ , $\text{Pb}^{2+}$ , $\text{Hg}^{2+}$	[40]
<i>Pseudomonas</i> sp. strain DRY1	glucose	30-50	5	$\text{Cd}^{2+}$ , $\text{Cr}^{6+}$ , $\text{Cu}^{2+}$ , $\text{Ag}^+$ , $\text{Pb}^{2+}$ , $\text{Hg}^{2+}$	[41]
<i>Enterobacter</i> sp. strain Dr.Y13	glucose	25-50	5	$\text{Cr}^{6+}$ , $\text{Cd}^{2+}$ , $\text{Cu}^{2+}$ , $\text{Ag}^+$ , $\text{Hg}^{2+}$	[42]
<i>Acinetobacter calcoaceticus</i> strain Dr.Y12	glucose	20	5	$\text{Cd}^{2+}$ , $\text{Cr}^{6+}$ , $\text{Cu}^{2+}$ , $\text{Pb}^{2+}$ , $\text{Hg}^{2+}$	[43]
<i>Serratia marcescens</i> strain DRY6	sucrose	15-25	5	$\text{Cr}^{6+}$ , $\text{Cu}^{2+}$ , $\text{Hg}^{2+}$	[44]
<i>Enterobacter cloacae</i> strain 48	sucrose	20	2.9	$\text{Cr}^{6+}$ , $\text{Cu}^{2+}$	[30]
<i>Escherichia coli</i> K12	glucose	80	5	$\text{Cr}^{6+}$	[45]
<i>Klebsiella oxytoca</i> strain hkeem	fructose	80	4.5	$\text{Cu}^{2+}$ , $\text{Ag}^+$ , $\text{Hg}^{2+}$	[46]
<i>Morganella</i> sp.	glucose	40	3.5	n.a.	[47]
<i>Enterobacter cloacae</i>	glucose	80-100	5-7.5	n.a.	[48]
<i>Pantoea</i> sp. strain HMY-P4	glucose	20-40	5		[49]
<i>Pseudomonas</i> sp.	glucose	40-60	3.5		[50]
<i>Serratia</i> sp. strain HMY1	sucrose/cyanide	55	3.95	n.a.	[51]
<i>Raoultella ornithinolytica</i> strain Mo1 and <i>Raoultella planticola</i> strain Mol	glucose	20	3.9	n.a.	[52]

**Identification of molybdenum reducing bacterium**

The bacterium was motile, a rod-shaped Gram-positive bacterium. Based on the Bergey’s Manual of Determinative Bacteriology (Table 2) and the ABIS online software the bacterium was tentatively identified as *Bacillus* sp. strain Neni-12 in honor of the late Dr. Neni Gusmanizar.

**Table 2.** Biochemical tests for *Bacillus* sp. strain Neni-12.

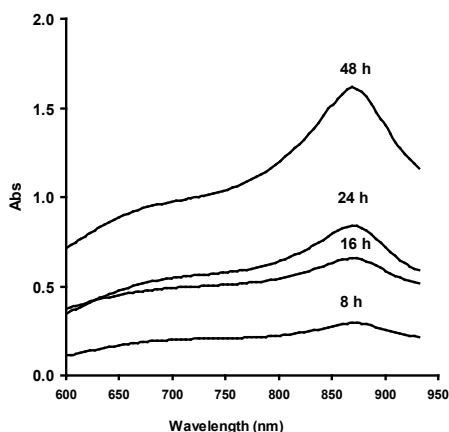
Gram positive staining	+	Acid production from:	
Motility	+	N-Acetyl-D-Glucosamine	+
Growth on usual media *	+	L-Arabinose	+
Growth at 45 °C	+	Cellobiose	+
Growth at 65 °C	-	Fructose	+
Growth at pH 5.7	+	D-Glucose	+
Growth on 7% NaCl media	+	Glycerol	+
Anaerobic growth	-	Glycogen	-
Casein hydrolysis	+	meso-Inositol	-
Esculin hydrolysis	d	Lactose	-
Gelatin hydrolysis	+	Mannitol	+
Starch hydrolysis	-	D-Mannose	+
Tyrosine degradation	d	Maltose	-
Beta-galactosidase (ONPG)	+	Melezitose	-
Catalase	+	Melibiose	-
Oxidase	+	Raffinose	+
Urease	-	Rhamnose	-
Arginine dehydrolyase (ADH)	-	Ribose	+
Lysine decarboxylase (LDC)	-	Salicin	+
Ornithine decarboxylase (ODC)	-	Sorbitol	-
Indole production	-	Sucrose	+
Citrate utilization	+	Starch	-
Egg-yolk reaction	-	Trehalose	+
Nitrates reduction	-		

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

Several Mo-reducing bacteria from the *Bacillus* genus have been isolated and include bacteria such as *Bacillus* sp. strain A.rzi [37] and *Bacillus pumilus* strain lbna [36].

**Molybdenum absorbance spectrum**

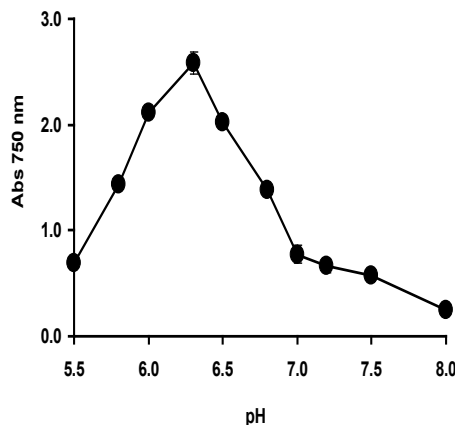
When scanned from 400 to 900 nm, the absorption spectrum of the Mo-blue exhibited a shoulder at approximately 700 nm and a maximum peak at 865 nm (Fig. 2) which is similar to many other Mo-reducing bacteria isolated to date, and generally indicating that a phosphomolybdate is involved as an intermediate.



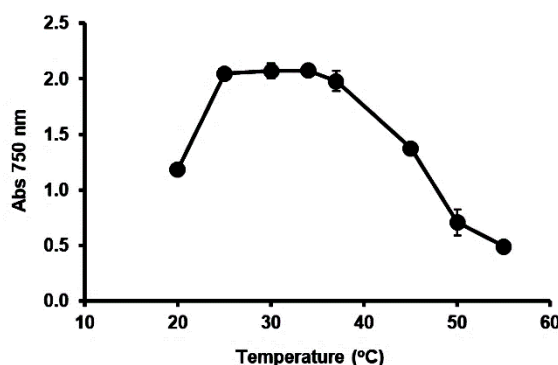
**Fig. 2.** Scanning absorption spectrum of Mo-blue from *Bacillus* sp. strain Neni-12 at different time intervals.

**Effect of pH and temperature on molybdate reduction**

*Bacillus* sp. strain Neni-12 was incubated at different pHs and temperatures and analysis by ANOVA showed that the optimum pH for reduction was at 6.3 (Fig. 3) and from 25 °C to 37 °C (Fig. 4).



**Fig. 3.** Optimization of pH by *Bacillus* sp. strain Neni-12. Error bars represent mean ± standard deviation (n=3).



**Fig. 4.** Optimization of temperature by *Bacillus* sp. strain Neni-12. Error bars represent mean ± standard deviation (n=3).

The majority of molybdenum reducers shows an optimal temperature of between 25 and 37 °C (Table 1). The only psychrotolerant Mo-reducing bacterium came from Antarctic soil with temperature optimum between 15 and 20 °C [41]. An important observation is the optimal pH reduction is slightly acidic (Table 1) since reduction needs an acidic pH as the important intermediate; phosphomolybdate’s formation and its stability require and an acidic condition [53].

**Effect of electron donor on molybdate reduction**

Glucose at the optimal concentration of 1% (w/v) was the best electron donor followed by sucrose, fructose, mannitol, raffinose, d-mannose, trehalose, d-xylose and glycerol in descending order (Fig. 5). Other carbon sources did not support molybdenum reduction. The electron donating substrates; NADH and NADPH are the products of the basic metabolic pathways and these substrates are the substrates for the molybdenum reducing-enzyme [54,55].

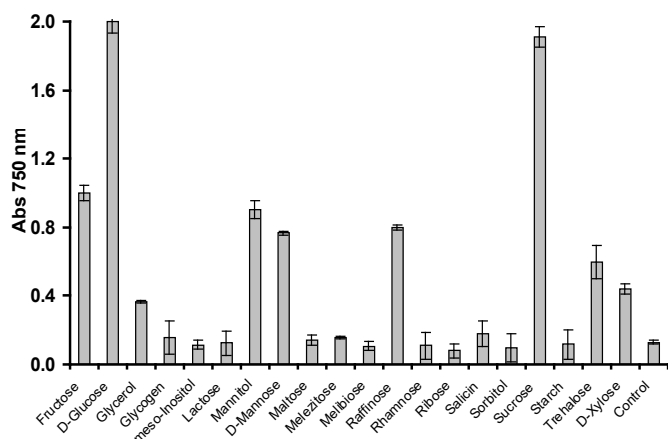


Fig. 5. Optimization of carbon sources by *Bacillus* sp. strain Neni-12. Error bars represent mean  $\pm$  standard deviation (n=3).

**Effect of phosphate and molybdate concentrations to molybdate reduction**

The optimum concentration of phosphate supporting the reduction of sodium molybdate occurred at 5 mM with higher concentrations were strongly inhibitory to reduction (Fig. 6). High phosphate concentration destabilizes the phosphomolybdate complex and this is likely the reason for the diminishing reduction at high phosphate concentrations [56–58]. Studies The bacterium was able to reduce molybdenum as high as 60 mM at a cost of diminishing Mo-blue production.

The optimal concentration was between 15 and 20 mM (Fig. 7). Numerous metal anion reduction can tolerate high concentrations of anionic metal evident in the reduction of selenate [31] and vanadate [59] of which concentrations as high as 60 mM can be tolerated due to the comparatively nontoxic characteristics of nonionic metal ions compared to cationic metal ions like Hg, Ag or Pb. The only exception is chromate where this metal anion is very toxic compared to other anions mentioned above.

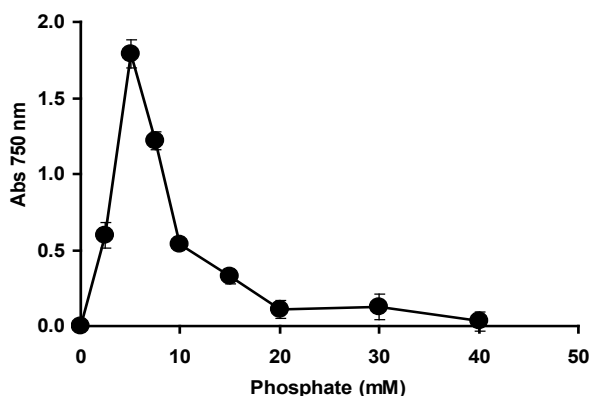


Fig. 6. Optimization of phosphate concentration by *Bacillus* sp. strain Neni-12. Error bars represent mean  $\pm$  standard deviation (n=3).

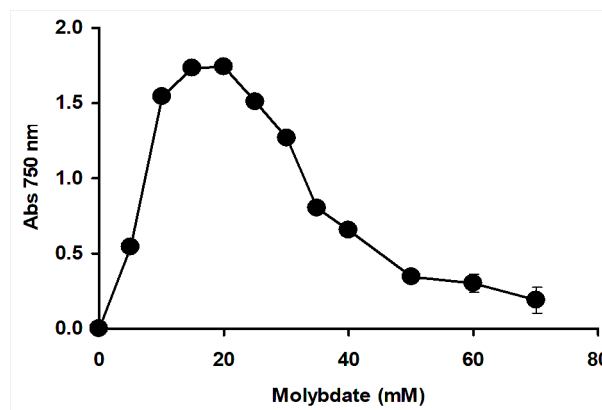


Fig. 7. Optimization of molybdate concentration by *Bacillus* sp. strain Neni-12. Error bars represent mean  $\pm$  standard deviation (n=3).

**Effect of heavy metals**

Molybdenum reduction was inhibited by Hg, Ag and Cr at 2 ppm by 91.9, 82.7 and 17.4 %, respectively (Fig. 8). Cationic metal ions inhibition by anionic metal ions is a major issue in the bioremediation of anionic metal ions through bioreduction. Almost all of the molybdenum reducers are inhibited by cationic heavy metals especially mercury (Table 1). Cationic heavy metals target sulphydryl group of enzymes [60]. Even the anionic chromate, if present together with molybdenum, will form a problem since chromate is a very toxic anionic metal ions and is known to inhibit glucose oxidase [61].

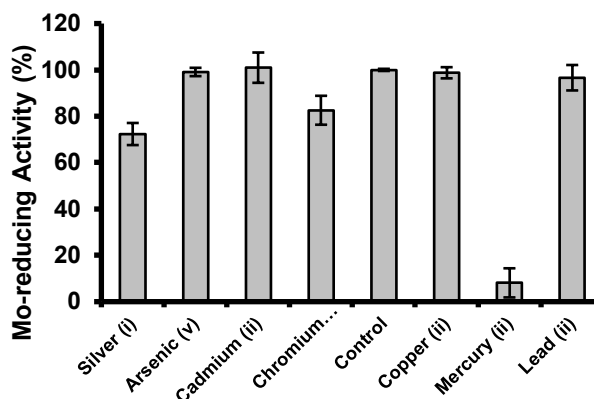


Fig. 8. Effect of metal ions to *Bacillus* sp. strain Neni-12 reduction of molybdate. Error bars represent mean  $\pm$  standard deviation (n=3).

**Pesticides as electron donors for molybdenum reduction and independent growth**

The ability of the pesticides to support growth showed that the bacterium can grow on coumaphos (Fig. 9). Bacteria that have been reported to degrade coumaphos are few, and include *Flavobacterium* sp. [62], a microbial consortium [63], *Pseudomonas monteilli* [64] and *Nocardiodes simplex* [65].

The inclusion of great number of microorganisms with pesticide-degrading potential tends to make bioremediation the greater suitable solution to pesticide degradation. On the other hand, hardly any bacteria have already been stated to be capable of breaking down xenobiotics and detoxify heavy metals at the same time, and the potential of this bacterium to accomplish the two suggest that this bacterium can be very beneficial as a bioremediation agent in contaminated sites co-contaminated with xenobiotics and cationic heavy metals.

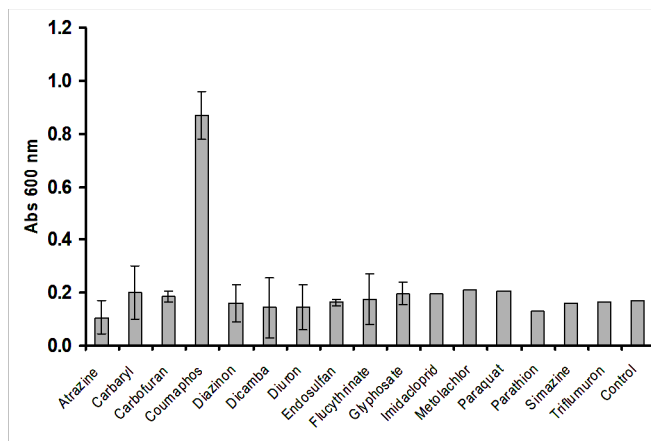


Fig. 9. Effect of various pesticides as growth substrate to *Bacillus* sp. strain Neni-12. Error bars represent mean  $\pm$  standard deviation (n=3).

## CONCLUSION

A novel Mo-reducing bacterium tentatively identified as *Bacillus* sp. strain Neni-12 that can grow on the pesticide coumaphos as a source of carbon is reported. Molybdenum reduction to molybdenum blue by the bacterium is optimally supported at pH 6.3, at temperatures between 25 and 37 °C, 1% (w/v) glucose, phosphate concentration of 5.0 mM and sodium molybdate concentrations between 15 and 20 mM. The absorption spectrum resembles a reduced phosphomolybdate. Reduction was inhibited by the cationic metal ions; Hg, Ag and the anionic metal Cr at 2 ppm by 91.9, 82.7 and 17.4 %, respectively. As hardly any bacteria are capable of breaking down xenobiotics and detoxify heavy metals at the same time, the potential of this bacterium to accomplish the two suggest that this bacterium can be very beneficial as a bioremediation agent in contaminated sites co-contaminated with xenobiotics and cationic heavy metals. Currently, work is underway to characterize the pesticide-degrading ability and to partially and characterize the bacterial's molybdenum-reducing enzyme.

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