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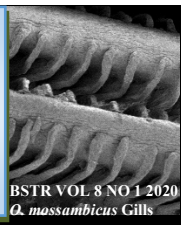

Characterization of a Molybdenum-reducing and Carbamate-degrading *Bacillus amyloliquefaciens* strain Neni-9 in soils from West Sumatera, Indonesia

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ABSTRACT

The isolation of a molybdenum-reducing bacterium with the capacity to grow on the insecticides carbaryl and carbofuran is reported. Characterization of the bacterium was carried out using resting cells in a microtiter plate. The bacterium reduced molybdenum to molybdenum blue optimally between pH 6.3 and 6.5, between 30 and 37 °C, between 5.0 and 7.5 mM phosphate, and between 20 and 30 mM sodium molybdate. Molybdenum reduction was inhibited by silver, copper, mercury and chromium at 2 ppm by 67.6, 58.4, 57.3 and 17.4%, respectively. The bacterium was identified as *Bacillus amyloliquefaciens* strain Neni-9. Growth on carbaryl and carbofuran modelled according to the modified Gompertz model gave maximum specific growth rates of 1.194 h^{-1} and 0.913 h^{-1}, for carbaryl and carbofuran, respectively, while the lag periods of 1.67 h and 2.63 h were observed for carbaryl and carbofuran, respectively. This is a novel molybdenum reducing bacterium showing pesticide-degrading capacity.

INTRODUCTION

Contamination of the soil and water bodies is caused by the production of heavy metals and xenobiotic chemicals from crop, manufacturing, and mining operations. Their elimination is being intensively investigated worldwide through numerous processes, such as biological physical and chemical. Pollution from molybdenum comes from the mining and manufacturing operations. Industrial source of molybdenum emissions also comes from the steel sector because almost 60 per cent of global molybdenum supply reaches this sector. For some locations related to the steel industry for Russia, the Black Sea, China and Austria, fumes and aqueous effluents from these plants have poisoned water sources and soils [1].

Mining operations are the primary use of emissions from molybdenum. The New Mexico Molycorp molybdenum mine held tailings and mines in many areas. Seepage of metal including molybdneum from these areas into the Red River has triggered river habitat destruction [2]. Mining operations to remove metals from the copper-gold-molybdenum porphyry formation in Batu Hijau, Sumbawa, Indonesia have slowly poisoned the adjacent coastal areas. The mine annually dumped more than two million tons of waste tailings into the surrounding sea resulting in water contamination and a loss in fish stocks between 2006 and 2010 [3,4]. Recent research on molybdenum toxicity revealed that ruminants are the most prone to molybdenum. Molybdenum toxicity at levels between 5 and 10 ppm caused hypocuprosis or a deficiency in copper.

Molybdenum is related to the cause of this disease. Ruminants ingesting high amounts of molybdenum would make it easier to turn this metal into thiomolybdate compounds; the latter are important copper chelators. When copper is deficient, the loss in copper enzymes leads to scouring and in some cases to death [5]. A coherent body of information has revealed that molybdenum is toxic to spermatogenesis. Early works in the fruit fly drosophila have shown that supplementation of molybdenum severely affected spermatogenesis with the most severe genetic abnormality occurring at the initial phases of spermatogenesis [6]. Experiments in rats [7] and the Japanese eel (*Anguilla japonica*) [8] also conclusively shown that molybdenum affected spermatogenesis. The concentrations of molybdenum utilized in these studies range from 50 to 150 mg/Kg in the diet. Molybdenum's contamination and toxicity means their elimination is of considerable significance. One of the best

examples of a large-scale removal of molybdenum contamination from soils is in Tyrol, Austria, where bioremediation using plants and sewage microbes was introduced to remedy contaminated agricultural soils [9]. Bioremediation via reduction of molybdenum to the colloidal molybdenum blue [10–12] is an emerging new candidate. Several Mo-reducing bacteria have been reported such *E. coli* K12 [13], *Acinetobacter calcoaceticus* [14], *Klebsiella* spp. [15–17], *Bacillus* spp. [18,19], *Pseudomonas* spp. [20,21], *Enterobacter* spp. [22,23], and *Serratia* spp. [24–27] with the last genera is the dominant species reported.

Besides heavy metals, the pollution caused by pesticides is also a problem in Asian countries. Carbamate pesticides such as carbofuran and carbaryl are still utilized in Asia even though in some countries they are banned [28]. Carbamates comprise of the chemical compound carbamate with ester functional group. They include compounds such as carbofuran, fenoxycarb, aldicarb, carbaryl, and fenobucarb. They inhibit the activity of the acetylcholine esterase enzyme leading to the accumulation of acetylcholine at the synapses [29].

Among the carbamates, carbofuran is one of the most toxic insecticides. This is evident with the LD_{50} of carbofuran compared to carbaryl in mice with values of 2 mg/kg and from 100 mg/kg to 650 mg/kg, respectively. Carbamates mainly inhibit the activity of acetyl cholinesterase although other targets such as protein synthesis, detoxification function, metabolisms of carbohydrate have been reported [29]. As carbamates are very mobile in soil, their removal, especially through microbiological action is being intensively sought [30].

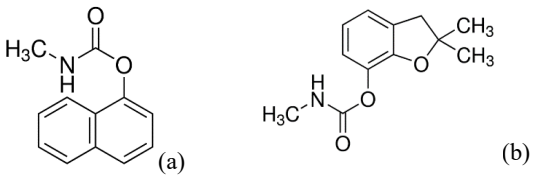

Fig. 1. Chemical structure of carbaryl (a) and carbofuran (b).

A molybdenum-reducing and carbamate-degrading bacterium isolated from agricultural soil is reported. This is the first report on a bacterium having heavy metal and pesticide detoxification property. The bacterium will be a good candidate for bioremediation of polluted sites containing these toxicants.

MATERIALS AND METHODS

Mo-reducing bacterium isolation and characterization

An agricultural soil with previous history of intensive pesticide application was chosen as the site of sampling. The location of sampling was in Bukittingi, West Sumatera, Indonesia, in January 2009. A minimal salts media with a low phosphate (5 mM) content supplemented with sodium molybdate was utilized for screening of Mo-reducing bacteria. A soil bacterial suspension was prepared by adding one gram of soil to 10 ml of distilled water. An aliquot of the soil suspension of about 0.1 mL of was spread onto a solid low phosphate agar (pH 6.5) and incubated at room temperature for 48 hours.

The composition (w/v) of the media was as follows: $MgSO_4.7H_2O$ (0.05%), yeast extract (0.5%), $Na_2MoO_4.2H_2O$ (0.242 % or 10 mM), glucose (1%), NaCl (0.5%), $(NH_4)_2.SO_4$ (0.3%), agar (1.5%), and Na_2HPO_4 (0.071% or 5 mM) [27].

Several white and blue colonies appeared, and the bacterial colony showing strong blue intensity was then restreaked on the LPM agar several times in order to get pure culture. Mo-blue production was also monitored in liquid culture. The blue supernatant from the liquid culture was centrifuged at 10,000 x g for 10 minutes at room temperature, and then was scanned from 400 to 900 nm (UV-spectrophotometer, Shimadzu 1201) with low phosphate media minus bacterium was the baseline correction. The bacterium was identified via biochemical and phenotypical methods in accordance to the Bergey's Manual of Determinative Bacteriology [31]. The analysis was computed into the ABIS online system [32].

Bacterial resting cells preparation

The characterization works on molybdenum reduction, which include concentrations of phosphate, molybdate, carbon sources, effect of heavy metals, pH and temperature from this bacterium utilized resting cells in a microtiter as before [33]. The total volume of the culture media in each well of the microtiter plate was 200 µL. Readings at 750 nm was periodically taken using a BioRad Microtiter Plate reader (Model No. 680, Richmond, CA). The specific extinction coefficient of 11.69 $mM.^{-1}.cm^{-1}$ at 750 nm was utilized to quantify Mo-blue production. The effect of several heavy metals was studied utilizing Atomic Absorption Spectrometry calibration standard solutions from MERCK.

Pesticides as carbon sources for bacterial growth

Pesticides tested include imidacloprid, carbaryl, carbofuran, coumaphos, endosulfan, flucythrinate, atrazine, glyphosate, diazinon, metolachlor, paraquat, diuron, parathion dicamba, and simazine. Preliminary works showed that none of the pesticides was able to support molybdenum reduction. Hence, these pesticides were tested for their ability to support growth using the microplate format above.

The carbon source glucose was replaced from the low phosphate medium, and replaced with the pesticides at the final concentration of 200 mg/L. Atrazine, diazinon, coumaphos, endosulfan, flucythrinate, diuron, parathion and simazine were dissolved in minimal volume of methanol as the carrier solvent 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 these pesticides was modelled according to the modified Gompertz model [34].

RESULTS

Identification of molybdenum reducing bacterium

The bacterium was a rod-shaped, motile, Gram-positive bacterium. Various biochemical test results (Table 1) computed into the ABIS online software gave three suggestions for the bacterial identity with the highest homology (86%) and accuracy at 88% as *Bacillus amyloliquefaciens*. Currently, the 16s rRNA gene from this bacterium is being sequenced. This is important for further identification of the bacterium through molecular phylogenetic analysis. At this juncture, the bacterium is tentatively identified as *Bacillus amyloliquefaciens* strain Neni-9 in honor of the late Dr. Neni Gusmanizar. The optimum pH for reduction was between 6.3 and 6.5 while the optimum temperature was from 30 to 37 °C. The Mo-blue spectrum from this bacterium displayed a maximum peak at 865 nm and a shoulder at 700 nm. This unique profile was conserved as Mo-blue production increases (**Fig. 2**).

Table 1. Physiological and Biochemical tests for *Bacillus amyloliquefaciens* strain Neni-9.

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

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

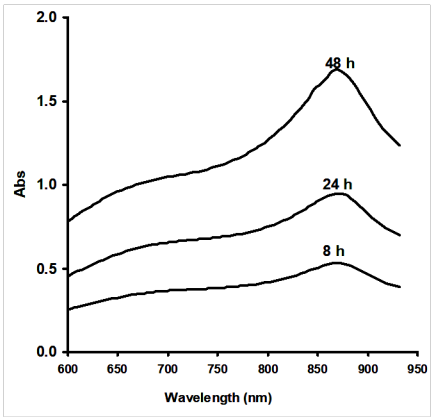

Fig. 2. Scanning absorption spectrum of Mo-blue from *Bacillus amyloliquefaciens* strain Neni-9 at different time intervals.

Effect of electron donor on molybdate reduction

Glucose was the most optimal electron donor followed in descending order by sucrose, fructose, maltose, lactose, l-arabinose, cellobiose, glycerol, meso-inositol, d-mannose, mannitol, melibiose and sorbitol (**Fig.** 3). The optimal concentration was 1% (w/v) (data not shown).

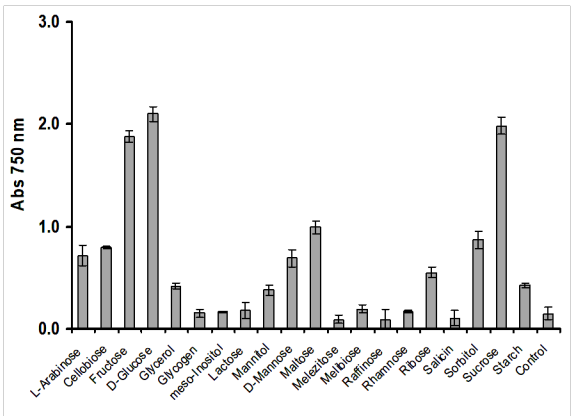

Fig. 3. Molybdenum reduction utilizing various electron donor sources. The error bars indicate mean ± standard deviation of three replicates.

Mo-blue production at various phosphate and molybdate concentrations

Concentrations of phosphate of between 5.0 and 7.5 mM were required for optimal reduction, while concentrations higher than 20 mM strongly inhibited reduction (**Fig. 4**). Optimal reduction was observed at concentrations of sodium molybdate between 20 and 30 mM. Higher concentrations especially more than 50 mM strongly inhibited reduction (**Fig. 5**). A time profile of Mo-blue production at various molybdate concentrations showed a lag period of about 10 hours. Maximum amount of Mo-blue produced was seen at concentrations of molybdate of between 20 and 25 mM, and at 48 hours of incubation approximately (**Fig. 6**).

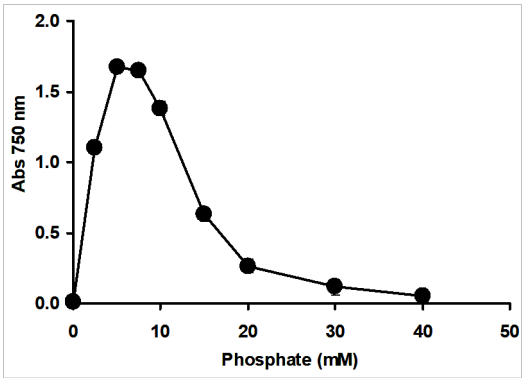

Fig. 4. Molybdenum reduction at various phosphate concentrations. The error bars indicate mean ± standard deviation of three replicates.

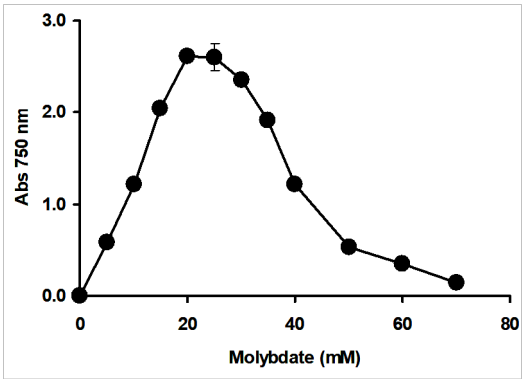

Fig. 5. Molybdenum reduction at various sodium molybdate concentrations. The error bars indicate mean ± standard deviation of three replicates.

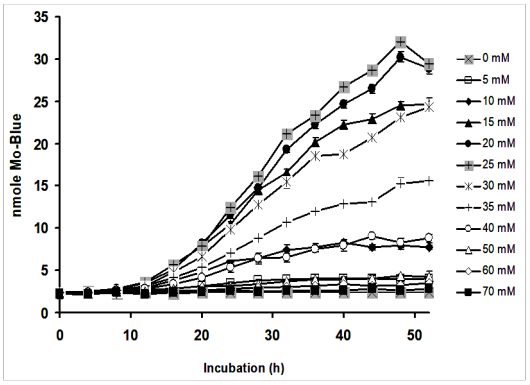

Fig. 6. A time profile of Mo-blue production at various sodium molybdate concentrations. The error bars indicate mean ± standard deviation of three replicates.

Effect of heavy metals

The effect of heavy metals on molybdate reduction to molybdenum blue shows that molybdenum reduction was inhibited by silver, copper, mercury and chromium at 2 ppm by 67.6, 58.4, 57.3 and 17.4%, respectively. Lead, arsenic and cadmium did not inhibit reduction (**Fig. 7**).

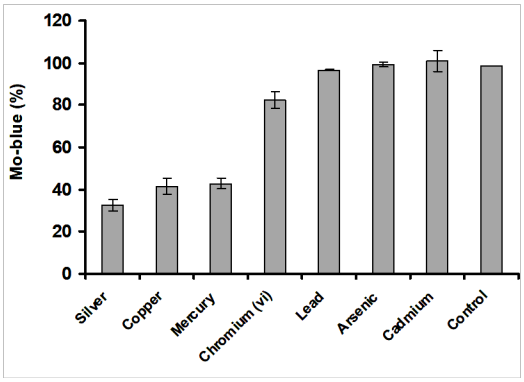

Fig. 7. Mo-blue production under several heavy metals. The error bars indicate mean ± standard deviation of three replicates.

Pesticides as carbon sources for growth

Even though the pesticides tested cannot support molybdenum reduction, two of the pesticides; carbofuran and carbaryl can support bacterial growth (**Fig. 8**). The growth of this bacterium on carbaryl and carbofuran as carbon sources was modelled according to the modified Gompertz model (**Fig. 9**). The absorbance values at 600 nm was converted to natural logarithm before modelling was carried out.

The correlation coefficients for the model were good at 0.97 and 0.98 for carbaryl and carbofuran, respectively. The growth parameters obtained were maximum specific growth rates of 1.194 h^{-1} and 0.913 h^{-1}, for carbaryl and carbofuran, respectively, while the lag periods of 1.67 h and 2.63 h were observed for carbaryl and carbofuran, respectively. The presence of lag periods indicates that the bacterial cells spend energy to tolerate and activate metabolic pathways needed for pesticide assimilation.

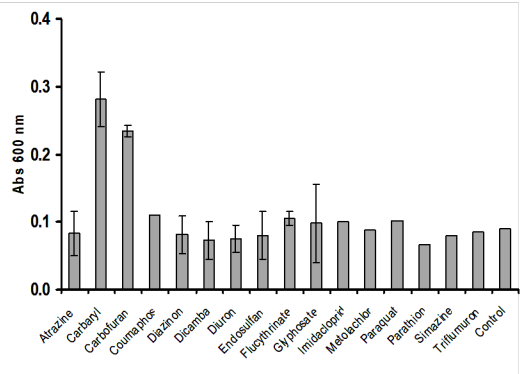

Fig. 8. Growth of *Bacillus amyloliquefaciens* strain Neni-9 on various pesticides after ten days of incubation at room temperature in a microtiter plate. The error bars indicate mean ± standard deviation of three replicates.

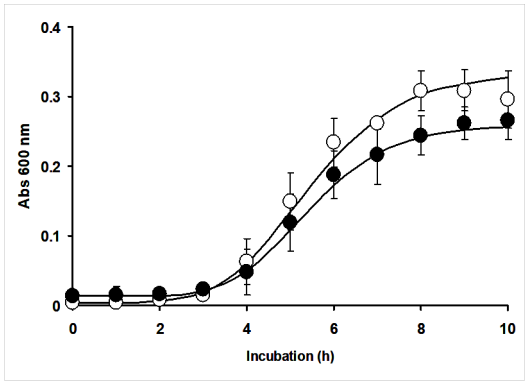

Fig. 9. Growth of *Bacillus amyloliquefaciens* strain Neni-9 on carbaryl (○) and carbofuran (●) as modelled using the modified Gompertz model (solid lines). Bacterium was incubated at room temperature in a microtiter plate. The error bars indicate mean ± standard deviation of three replicates.

DISCUSSIONS

The reduction of molybdate to molybdenum blue by bacteria was first described more than one hundred years ago in 1896 by Capaldi, and Proskauer [35]. Further isolation of Mo-reducing bacteria in the last century were reported in 1939 [36], in 1948 [37], in 1962 [38], in 1972 [39], in 1985 [13], and in 1993 [22]. Ghani et al. [22] quickly recognize the potential application of this phenomenon for the bioremediation of molybdenum. Since then, numerous Mo-reducing bacteria have been isolated. The ability of the newly isolated Mo-reducing bacterium to grow on other xenobiotics is indeed a highly sought-after property.

Two Mo-reducing bacteria from this genus have been isolated and include *Bacillus* sp. strain A.rzi [19] and *Bacillus pumilus* strain lbna [18]. The pH and temperature optima seen in this work are within the range reported for most of Mo-reducing bacteria, which range from 6.0 to 7.0, and from 25 to 37 °C [40,41]. The application of resting cells in examining molybdenum reduction is definitely an edge as every one of the Mo-reducing bacteria isolate up to now reduces molybdenum best under static or low oxygen tension circumstances, and it was first initiated in the works relating to *Enterobacter cloacae* strain 48 [22]. The Mo-blue spectrum is similar to many Mo-blue spectra from almost all of the Mo-reducing bacteria isolated to date [17,42]. The spectrum is incidentally to the spectrum of molybdenum blue from the phosphate determination method (PDM), the latter having a peak maximum near 890 nm and a characteristics shoulder at 700 nm. The latter's identity is a reduced phosphomolybdate [43,44]. Based on this, we put forward a hypothesis that bacterial reduction of molybdate to Mo-blue proceed via a phoshomolybdate intermediate [42].

Most of the Mo-reducing bacteria prefer sucrose or glucose while only *Klebsiella oxytoca* strain hkeem prefers fructose as the most optimal electron donor for reduction [10–12]. These carbon sources are easily assimilated converted to NADH and NADPH, both reducing equivalents are substrates for the Mo-reducing enzyme [24]. The substrates for chromate reductase, another similar metal, is also NADH and NADPH, and incidentally glucose is the best electron donor for cellular reduction of chromate in several bacteria [45,46].

Mo-blue production is strongly affected with high phosphate concentrations, which is seen in the concentration of phosphate utilized in LPM. In the majority of the Mo-reducing bacteria, this also hold true [10–12]. Two of the heavy metals tested which exhibit strong inhibitory response to molybdenum reduction, which are mercury and copper, inhibit many of the Mo-reducing bacteria isolate to date [10–12]. These two heavy metals are also inhibitors of bacterial chromate reduction seen in *Bacillus* sp. (Elangovan et al. 2006) and *Enterobacter cloacae* strain H01 (Rege et al. 1997). The target site of inhibition is suggested as the sulfhydryl group (Rege et al. 1997; Elangovan et al. 2006). The immobilization of the Mo-reducing bacterium especially in membrane such as a dialysis tubing has been shown to reduce the inhibitory effects of toxic cations (Halmi et al. 2014).

Isolation of multiple detoxification bacterium is very important for bioremediation bearing in mind that numerous polluted sites are often contaminated with heavy metals and organic contaminants. Previously, two Mo-reducing bacteria showed SDS-degrading capacity [16,17]. The finding in this work is novel as this is the first time a Mo-reducing bacterium showed pesticide-degrading capacity. The use of the modified Gompertz model to obtain growth parameter constants from carbofuran and carbaryl is novel to the best of our knowledge. A lot of carbofuran-degrading bacteria have been isolated from a variety of genera such as *Novosphingobium* sp. [47], *Burkholderia cepacia* [48], *Sphingomonas* sp. [49] and *Bacillus* spp., *Pseudomonas* spp. and *Rhodococcus* spp. [50]. Carbaryl-degrading microbes include *Pseudomonas aeruginosa, Micrococcus arborescens, Brachybacterium* sp. and *Salsuginibacillus kocurii* [51,52], *Arthrobacter* sp. [53], *Micrococcus* spp. [54], *Rhizobium* sp. [55] and *Rhodococcus* sp. [56].

CONCLUSION

The isolation of a Mo-reducing bacterium having the capacity to convert molybdate to molybdenum blue is reported. In addition, the bacterium exhibited the novel ability to utilize carbofuran and carbaryl as carbon sources for growth. The bacterium was tentatively identified as *Bacillus amyloliquefaciens* strain Neni-9 based on physiological and biochemical analyses. Glucose supported reduction optimally while a critical phosphate concentration was required of between 5.0 and 7.5 mM. The absorption spectrum of the Mo-blue indicated that it is a reduced phosphomolybdate similar to previously isolated Mo-reducing bacteria. Molybdenum reduction was inhibited by silver, copper, mercury and chromium at the concentration of 2 ppm. This is a novel report of a molybdenum-reducing bacterium showing pesticide-degrading capacity. In addition, the use of the modified Gompertz model was successful in describing the growth of this bacterium of the two pesticides. Works are being carried out to identify the bacterium further through 16s rRNA molecular phylogenetic analysis, to characterize pesticide-degrading capacity and to purify the molybdenum-reducing enzyme.

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