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Characterization of a Molybdenum-reducing and Phenol-degrading *Pseudomonas* sp. strain Neni-4 from soils in West Sumatera, Indonesia

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ABSTRACT

Millions of tonnes of these chemicals are manufactured each year and a large quantity is determined to be contaminating the environment, making them important worldwide pollutants. The fact that they pollute the environment is a major problem on a worldwide scale. There is a continuing search for bioremediation of these contaminants employing bacteria capable of numerous detoxifications. Analysis of the bacterium yielded a preliminary identification of the organism as *Pseudomonas aeruginosa* Neni-4. Screening for the capacity of molybdenum-reducing bacteria to decolorize different polyphenols was conducted in this study. Reduction was optimum at pH 6.3 and between 25 and 40 ºC. The bacterium used glucose as the best carbon source or molybdenum reduction followed by galactose, 2-ketogluconate, and citrate in decreasing order. Phosphate between 5.0 and 7.5 mM and sodium molybdate between 15 and 20 mM maximally supported reduction. Like earlier Mo-reducing bacteria, a reduction of phosphomolybdate is seen in the absorption spectra of the Mo-blue generated. Heavy metals prevented molybdenum reduction. None of the phenolic compounds can reduce molybdenum when provided as sole carbon sources. In contrast, the bacterium was able to grow on phenol, benzoate, salicylic acid, and catechol, all of which are substances that include phenolic components. A significant bioremediation technology is this bacterium's capacity to metabolise molybdenum and thrive on poisonous phenolics.

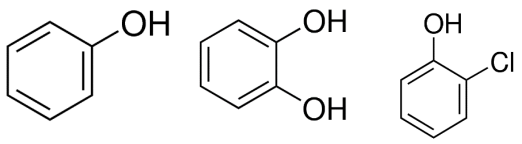
INTRODUCTION

For at least 2,000 years, molybdenum was mistaken for graphite and galena by ancient cultures, but it wasn't until 1778 that the metal's formal discovery and identification was made by Swedish chemist and pharmacist Carl Wilhelm Scheele that molybdic oxide was formally discovered and identified. Hjelm, a Swedish chemist, made the first metallic molybdenum in 1781 by heating a paste made of molybdic oxide and linseed oil in a crucible to extremely high temperatures. German chemist Bucholtz and Swede Jöns Jacob Berzelius both worked on the molybdenum chemistry in the 1800s, but it wasn't until 1895 that French scientist Henri Moissan reduced molybdenum with carbon in an electric furnace to produce a chemically pure (99.98%) metal that allowed for further study of the metal and its alloys [1–3].

At low concentrations, toxic xenobiotics can be removed by the process of bioremediation, which, over the course of time, proves to be more cost-effective than other methods, such as physical or chemical treatments. Molybdenum is one of the necessary heavy metals that are only needed in minute quantities, but in higher concentrations it may be poisonous to a wide variety of different creatures. It has a wide variety of applications in industrial settings, some of which include acting as an alloying agent, an anti-freeze component, a corrosion-resistant steel component, and a lubricant in the form of molybdenum disulfide. The extensive usage of molybdenum in industry has led to a variety of water pollution problems occurring all over the world. Some of these instances include Tokyo Bay, Austria's Tyrol, and the

Black Sea, all of which have molybdenum levels that may approach hundreds of parts per million (ppm) [4,5]. In the 1970s, it was also demonstrated that it was a substantial pollutant in sewage sludge pollution on land. This discovery came about during that decade. It has been demonstrated that even at concentrations as low as a few parts per million, molybdenum may inhibit the development of embryos and delay the process of spermatogenesis in various species, including catfish and mice [6,6–9]. In addition, molybdenum is extremely hazardous to ruminants, particularly cows, at levels of several parts per million. [10,11].

Hydrocarbons, such as oil, grease, and phenolics, rank first among the scheduled industrial wastes, just behind heavy metals. [12]. Accidents are another source of pollution. For example, the capsize of the 533-ton Indonesian tanker MV Endah Lestari in 2001, which was carrying 18 tonnes of fuel and 600 tonnes of phenol, contaminated Indonesian and Malaysian coastal waters and killed thousands of fish and cockles raised in 85 offshore cages [13]. In addition to being harmful to humans, phenol and phenolic compounds are also dangerous for many other creatures. [14]. The mucous membranes, skin, eyes, and respiratory tract are caustic to their vapours. A third-degree burn can result from prolonged skin contact with dermatitis. The liver and kidneys can be damaged as a result of long-term exposure. Hydrophobicity and the production of phenoxyl radicals contribute to the substance's toxicity [15]. It's well-known that it pollutes the world. There are various coal mines in Sumatra that might be a source of phenolic pollution [16].



(a) Phenol (b) Catechol (c) 2-chlorophenol

Fig. 1. The structure of some toxic phenolic compounds.

A wide range of microorganisms are capable of degrading a selection of xenobiotics and removing heavy metals simultaneously, and their adaptability is highly sought after in contaminated areas. A few examples are the decrease of chromate and the biodegradation of phenols [17,18]. This study examines the capability of a newly isolated Mo-reducing bacterium isolated from polluted soil to thrive on a wide range of phenolic chemicals, including phenol, in the presence of several antibiotics. Because most bioremediation takes place in water or soil with environmental oxygen EO concentrations lower than 20% EO and other acceptors such as nitrate started to be used. We purposefully employ static growth or circumstances, and this may be easily accomplished in a microplate environment with lower oxygen concentrations than aerobic conditions (0.10 percent environmental oxygen, or EO). In this paper, we present a newly discovered molybdenum-reducing bacterium that has the ability to flourish on a wide variety of phenolic compounds that may be found in contaminated soil and that we were able to isolate. Both the heavy metal molybdenum and phenolic compounds might be remedied using this bacterium's properties.

MATERIALS AND METHODS

Isolation of Mo-reducing bacterium

In January 2009, soil samples were gathered from a polluted site in the Indonesian state of Pariaman, Sumatera (5 cm deep from the topsoil) by the late Dr Neni Gusmanizar. Soil was mixed with sterile tap water to create a suspension. After incubating at room temperature for 48 hours, the soil suspension was transferred onto agar plates that contained low-phosphate medium (pH 7.4). These were the components of the low phosphate medium: glucose (1%), (NH4)2.SO4 (0.3%), MgSO4.7H2O (0.05%), yeast extract (0.5%), NaCl (0.5%), Na2MoO4.2H2O (0.242 percent or 10 mM), and Na2HPO4 (0.071 percent or 5 mM) [19]. Molybdate reduction is shown by the production of blue colonies. Pure culture was obtained by isolating and reseeding the colony that produced the most intense blue colour. Molybdenum reduction in liquid medium above (at pH 7.0) was carried out in a 250 mL shake flask culture carried out at room temperature for an incubation period of 48 h and shaken on an orbital shaker set at 120 rpm. The phosphate concentration was raised to 100 mM. Centrifuged at 10,000xg for 10 minutes at ambient temperature, the molybdenum blue (Mo-blue) absorption spectra of the liquid culture above were investigated. A UV-spectrophotometer was used to scan the growth medium supernatant from the wavelengths of 400 to 900 nm (Shimadzu 1201). It was used as the baseline adjustment for the low phosphate medium.

Partial identification of the isolated strain

A variety of standard methods were used to determine the strain's biochemistry and phenotype, including colony shape, gramme staining, the size and colour of the agar colonies, motility, oxidase activity (for 24 hours), arginine dihydrolase (ADH), ONPG (beta-galactosidase), catalase activity (for 24 hours), ornithine decarboxylase (ODC) and lysine decarboxylase [20]. Bacterial identification was carried out using the ABIS online system [21] as before [22].

Preparation of bacterial resting cells

The effects of pH, phosphate, temperature, and sodium molybdate concentrations on molybdenum reduction to Mo-blue were studied statically employing the resting cells form in a microplate or microtiter format, as had been described earlier [23]. Growth was carried out on an orbital shaker at room temperature and shaken at 120 rpm in a 1-L overnight culture in High Phosphate medium (HPM). The phosphate content was set at 100 mM for the HPM. Centrifuged at 15,000 x g for 10 min and then the bacterium was washed of its pellet several times using sterile deionized water and then the bacterial cells were resuspended in LPM without the addition of glucose to a cellular suspension having an absorbance values of about 1.00 when measured at 600 nm, and then resuspended in 20 ml of LPM. All of the Mo-reducing bacteria that have been identified so far thrive best on low phosphate media at a concentration of 5 mM phosphate, so that's what we utilised here. Molybdate reduction was shown to be severely inhibited at higher doses [19,24–38]. Then each well of a sterile microplate was filled with 180 uL of sterile solution. Each well received 20 uL of sterile glucose from a stock solution, which sparked the creation of Mo-blue. The tape was sealed with Corning® microplate, a sterile sealing tape that enables gas exchange. The microplate was kept at room temperature during the incubation process. A BioRad (Richmond, CA) Microtiter Plate reader was utilized to measure absorbance at 750 nm at predetermined intervals (Model No. 680).

Effect of heavy metals on molybdenum reduction
Seven heavy metals, including lead, arsenic, copper, mercury, silver, chromium, and cadmium, were obtained from commercial salts or MERCK standard solutions. At various concentrations, the bacterium was exposed to heavy metals in a microplate format. At the same wavelength of 750 nm as previously, the quantity of Mo-blue was measured.

Screening of molybdenum reduction and independent growth using phenolics
Molybdenum reduction using various phenolic compounds as electron donors was tested using phenolic compounds at the final concentration of 200 mg/L in a volume of 50 uL [39]. Then 200 uL of the medium (LPM) was added into the microplate wells with 50 □L of resting cells suspension. It was cultured for three days at room temperature, and Mo-blue production was measured at 750 nm as before. Using phenolics as a carbon source for growth only, rather than for Mo-reduction, a second set of experiments was carried out. The pH of the media was brought up to 7.0. At 600 nm, the bacterial growth rate increased.

Statistical analysis
In order to do the data analysis, GraphPad Prism version 7.0 (trial version) was employed. Analysis of variance with post-hoc Tukey's test or Student's t-test were used to compare groups. The significance level was set at p0.05.

RESULTS AND DISCUSSION

Identification of molybdenum reducing bacterium
The bacterium shows properties such as being Gram-negative, was motile, and was a short rod-shaped organism. Identification of the bacterium was carried out by culturing, morphological, and biochemical assays (**Table 1**). The ABIS online software [21] programme provided three choices for the bacterial identification, with *Pseudomonas aeruginosa* having the highest homology (99 percent) and accuracy (88 percent). Molecular identification techniques based on the comparison of the 16srRNA gene will be required in the future to identify this species further. In honour of the late Dr. Neni Gusmanizar, the bacterium is now provisionally named as Pseudomonas sp. strain Neni-4. Examples of Mo-reducing bacteria from this genus are *Pseudomona*s sp. strain DRY2 [31] and the Antarctic bacterium *Pseudomona*s sp. strain DRY1 [35] that have been reported previously.

Table 1. Biochemical tests for *Pseudomonas* sp. strain Neni-4.

Motility	+	*Utilization of:*	
Hemolysis	+	L-Arabinose	–
Growth at 4 ºC	–	Citrate	+
Growth at 41 ºC	+	Fructose	+
Growth on MacConkey agar	+	Glucose	+
Arginine dihydrolase (ADH)	+	meso-Inositol	–
Alkaline phosphatase (PAL)	–	2-Ketogluconate	+
H_2S production	–	Mannose	–
Indole production	–	Mannitol	+
Nitrates reduction	+	Sorbitol	–
Lecithinase	–	Sucrose	–
Lysine decarboxylase (LDC)	–	Trehalose	–
Ornithine decarboxylase (ODC)	–	Xylose	–
ONPG (beta-galactosidase)	–	Starch hydrolysis	–
Esculin hydrolysis	–		
Gelatin hydrolysis	+		
Starch hydrolysis	–		
Oxidase reaction	+		

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

In this study the microplate format was employed to expedite characterization work and collect more data than the conventional shake-flask technique [23,40]. Ghani et al. initiated the use of resting cells under static circumstances to study bacterial molybdenum reduction [25].

Molybdenum absorbance spectrum
The identification of the Mo-blue is difficult because to its complicated shape and several species. Mo-blue is a reduction compound of isopolymolybdate and heteropolymolybdate, two types of molybdenum complexes. The identification of the Mo-blue compound is tricky because it has an intricate structure and exists in many species. It has been suggested by Campbell et al. [24] that Mo-blue, which was detected during the reduction of molybdenum by *E. coli* K12, is phosphomolybdate in its reduced form, although no credible explanation was provided.

Due to the need for powerful reducing agents and acidic circumstances, biologically based reducing agents cannot produce isopoly Mo-blue. Heteropoly Mo Blue synthesis by biologically-based reductants, such as ascorbic acid or enzymatic reduction, is more likely to occur than the ascorbic acid-based phosphate determination technique. (Hori et al., 1988). In other words, molybdenum is reduced to Mo-blue by both chemical and biological processes.

If this technique is used, the absorption spectra of the Mo-blue that is produced by this bacterium should display a spectrum that is very similar to the one produced by the method used to determine phosphate. To be more specific, the spectra that was seen had a maximum absorption in the range of 860 to 870 nm and a shoulder at around 700 nm (Fig. 2). The Mo-blue spectrum, which was obtained using the phosphate determination technique, typically exhibited a maximum absorption in the range of 880 to 890 nm and a shoulder in the range of 700 to 720 nm [41].

Previous research has demonstrated that the entirety of the Mo-blue spectra produced by other bacteria conform to this criterion. In this study, the result from the absorption spectrum unmistakably suggests a spectrum that is comparable, which consequently gives proof that the hypothesis is correct. Because of the intricate nature of the compound's structure, n.m.r. and e.s.r. must be utilized in order to arrive at an accurate identification of the phosphomolybdate species [42].

On the other hand, spectrophotometric characterisation of heteropolymolybdate species, which involves examining the scanning spectroscopic profile, is an approach that is less laborious and more widely recognized [43–46]. Although the highest wavelength for Mo-blue absorption was 865 nm, measurements at 750 nm were adequate for normal Mo-blue production monitoring because the intensity achieved was much greater than cellular absorption at 600-620 nm. Despite the fact that the reading at 750 nm was around 30% lower, this was the case. Earlier measurements of Mo-blue production used wavelengths such as 710 nm.[25] and 820 nm [24].

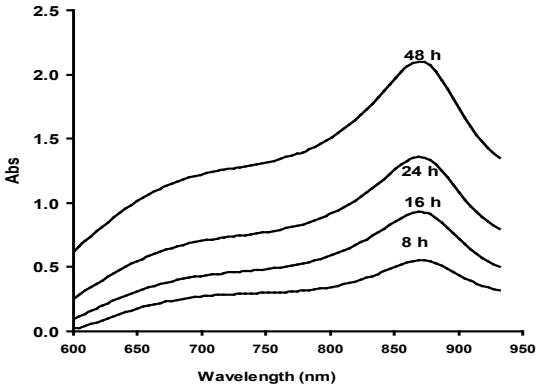


Fig. 2. Scanning absorption spectrum of Mo-blue from *Pseudomonas* sp. strain Neni-4 at different time intervals.

Effect of pH and temperature on molybdate reduction

Pseudomonas sp. strain Neni-4 was subjected to an incubation process at several pH levels, spanning from 5.5 to 8.0, utilizing Bis-Tris and Tris.Cl buffers (20 mM). According to the findings of an ANOVA study, the optimal range for pH throughout the reduction process was between 6.0 and 6.5. When the pH was lower than 5, there was a significant inhibition of reduction (**Fig. 3**). The influence of temperature (shown in **Fig. 4**) was seen throughout a wide range of temperatures, from 20 to 60 degrees Celsius; the optimal temperature ranged from 30 to 37 degrees Celsius. ANOVA analysis revealed that there was no significant difference ($p>0.05$) between the values that were recorded. Temperatures higher than 37 degrees Celsius have a significant negative impact on the formation of Mo-blue from *Pseudomonas* sp. strain Neni-4.

The rate of an enzyme-catalyzed reaction increases as the temperature rises, as is the case with many chemical processes. Nevertheless, at high temperatures, the enzyme denatures and ceases to operate, so the rate decreases again. The rate of enzyme activity increases in direct proportion to the rise in temperature. It is at this temperature that the enzyme's optimal activity is achieved. With each subsequent rise in temperature, the enzyme's active region changes its shape, resulting in a rapid decline in activity and enzymes denaturation. The active site shape of an enzyme can also be altered by pH changes. The optimal pH for each enzyme varies. For an enzyme, the ideal pH relies on the environment in which it performs its function. Small intestine and stomach enzymes have different pH optimums, for example. In this case, the enzyme's optimal pH of 8 results in maximum activity. As the enzyme's active site changes shape due to the increased pH, its activity plummets dramatically [47,48].

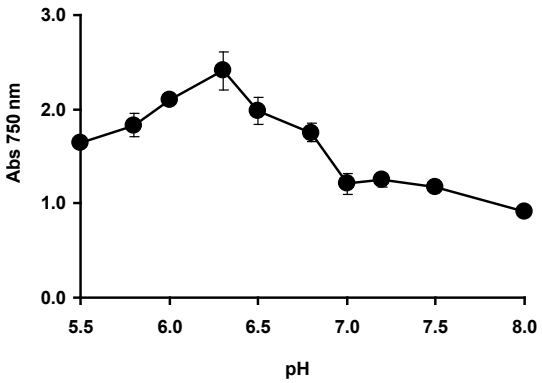


Fig. 3. Molybdenum reduction at various pHs Data is mean ± standard deviation of triplicates.

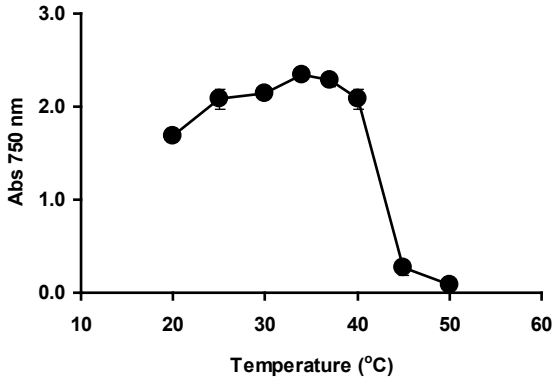


Fig. 4. Molybdenum reduction at various temperatures. Data is mean ± standard deviation of triplicates.

Temperature and pH affect folding of proteins and enzyme activity, which can lead to the suppression of molybdenum reduction, which is an enzyme-mediated process. Molybdenum reduction, on the other hand, necessitates the presence of both of these elements. In a country like Malaysia, where the yearly average temperature is between 25 and 35 degrees Celsius, having the optimal conditions for bioremediation would be advantageous [27]. As a result, *Pseudomonas* sp. strain Neni-4 has the potential to be a candidate for molybdenum soil bioremediation not only in the immediate area but also in other tropical nations. The ideal temperature for the most majority of the reducers is anywhere between 25 and 37 degrees Celsius. [19,27,28,30–34,36–38,49] since they have been isolated from tropical soils, with the exception of the lone psychrotolerant reducer, which has been isolated from Antarctica and shows that the best temperature for sustaining reduction is between 15 and 20 degrees Celsius [35].

As a neutrophil, the optimal pH range for molybdenum reduction by Pseudomonas sp. strain Neni-4 may be seen in the bacteria. At contrast to other organisms, neutrophils are able to thrive in a pH range of 5.5 to 8.0. For the best molybdenum reduction in bacteria, an acidic pH of between 5.0 and 7.0 is ideal. This is an important finding on bacteria's molybdenum reduction [24,25,27–38,49]. In the past, it has been hypothesized that an acidic pH plays a significant part in the synthesis and stability of phosphomolybdate before it is reduced to Mo-blue. This notion has been supported by evidence. Therefore, the best reduction is achieved by striking a balance between the activity of the enzyme and the stability of the substrate [50].

Effect of electron donor on molybdate reduction

To support molybdate reduction, glucose was found to be the best electron donor among those that were tested followed by sucrose, adonitol, mannose, mannitol, myo-inositol, maltose, glycerol, d-sorbitol, salicin, trehalose, and xylose. Finally, xylose was found to be the worst electron donor (**Fig. 5**). Molybdenum reduction could not occur with the use of other carbon sources. Nearly all Mo-reducers utilize glucose as the best electron donor [19,27,28,30–34,36–38,49]. Glucose is processed through glycolysis, the Kreb's cycle, and the electron transport chain, producing NADH and NADPH that are substrates for molybdenum reduction [49,51].

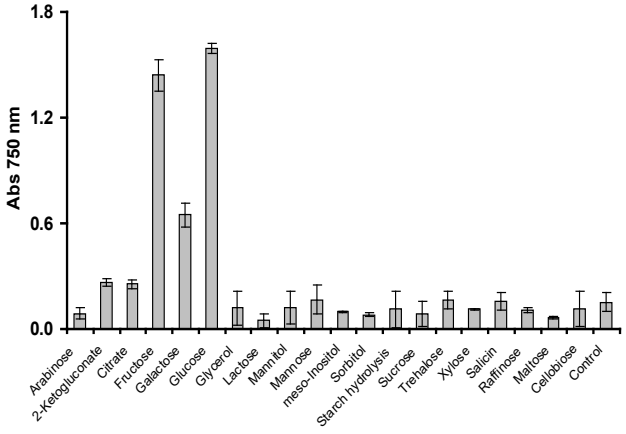


Fig. 5. Molybdenum reduction at various carbon sources. Data is mean ± standard deviation of triplicates.

Reduction at various phosphate and molybdate concentrations

It is vital to determine the amounts of phosphate and molybdate that enable effective molybdenum reduction since it has been demonstrated that both phosphate and molybdate hinder the synthesis of molybdenum blue in bacteria [24,25,27–38,49,52–57]. Phosphate was shown to be most effective at a concentration of 5 millimoles per liter (mM), with greater quantities being highly reduction-inhibitory (**Fig. 6**). It was hypothesized that a high concentration of phosphate might hinder phosphomolybdate stability due to the fact that the complex demands acidic conditions, and the stronger the buffering capacity of the phosphate buffer, the higher the concentration of phosphate that is utilized. In addition to this, the phosphomolybdate complex is inherently unstable if exposed to high levels of phosphate via a process that is not well understood [43,44,58].

For optimum reduction, each and every molybdenum-reducing bacteria that has been discovered up to this point requires a phosphate concentration of no more than 5 mM [24,25,27–38,49,52–57]. According to research conducted on how the concentration of molybdenum affected the process of molybdenum reduction, the newly isolated bacterium tolerate and reduce molybdenum even at 60 mM, but at the expense of a reduction in Mo-blue synthesis. The most effective decrease was achieved at concentrations between 20 and 40 mM (**Fig. 7**). Reduction at this level is shared by many reducers [19,27,28,30–34,36–38,49].

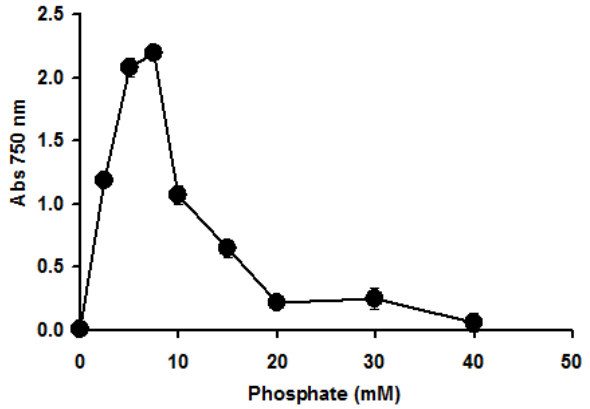


Fig. 6. Molybdenum reduction at various phosphate concentrations. Data is mean ± standard deviation of triplicates.

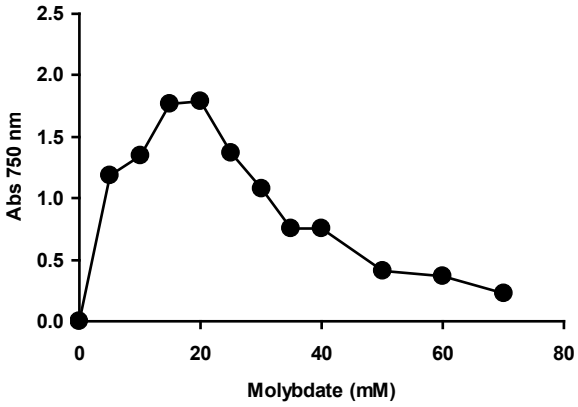


Fig. 7. Molybdenum reduction at various molybdenum concentrations. Data is mean ± standard deviation of triplicates.

Effect of heavy metals

Molybdenum reduction was inhibited by Pb (II), Hg (II), Cd (II), Cu (II) and Ag (I) at 2 ppm by 64.3, 61.1, 53.1, 36.8 and 27.7 %, respectively (**Fig. 8**). Bioremediation is complicated by the inhibitory effects of other metal ions especially heavy metals. This necessitates the screening and extraction of metal-resistant microorganisms. Molybdate reduction is inhibited by mercury, as previously indicated. A review of the heavy metals that inhibited Mo-reducing bacteria found that hazardous metals inhibited nearly all of the reducers [59]. Heavy metals usually target sulfhydryl group of enzymes [60]. Nearly all of the Mo-reducing bacterium isolated to date are affected strongly by heavy metals [24,25,27–38,49,52–57].

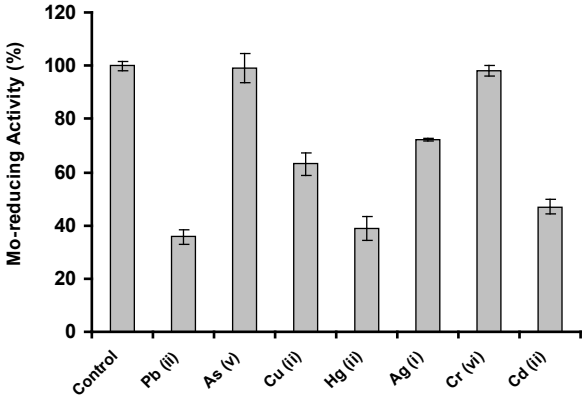


Fig. 8. Molybdenum reduction in the presence of various heavy metals. Data is mean ± standard deviation of triplicates.

Phenolics as potential sources of electron donors for the reduction of molybdenum and for independent growth

The use of phenolics as electron donors to assist molybdenum reduction did not provide any favorable findings in the screening process. Nevertheless, the bacterium as able to cultivate on phenolic chemicals such as phenol, benzoate, salicylic acid, and catechol (**Fig. 9**). Bacteria capable of decomposing phenol and phenolics are ideally suited for use in phenol cleanup due to cost considerations. Research into the microorganisms that are responsible for the biodegradation of phenol and other phenolic compounds has been going on for a very long time all over the world. Bacteria that could degrade phenol and phenolic compounds include *Pseudomonas* spp. [61–64], *Bacillus brevis* [65], *Alcaligenes* sp. [66], *Ochrobactrum* sp. [67], *Acinetobacter* sp. [68,69] and *Rhodococcus* species [70]. Every one of these degraders possesses a one-of-a-kind set of characteristics, such

as the capacity to withstand high concentrations of phenol, resistance to salt and heavy metals, and the capability to thrive in environments with either severe pH levels or temperatures. Bioremediation is the preferred method of phenol degradation because there are numerous microorganisms that are capable of breaking down phenol. According to the findings, this bacterium could be an effective bioremediation agent for polluted sites that are also contaminated with heavy metals and xenobiotics. Phenol degradation and heavy metal detoxification are two things that relatively few microorganisms have been shown to be capable of.

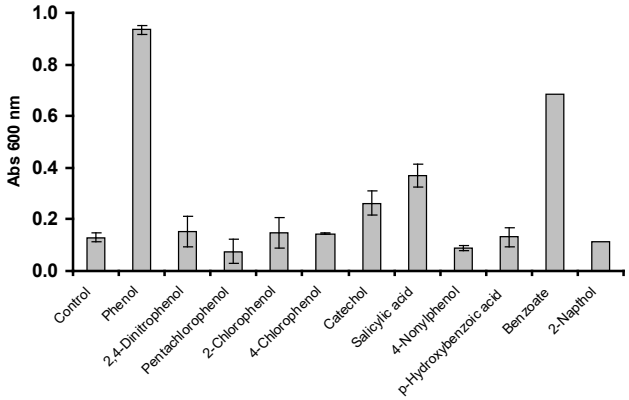


Fig. 9. Growth of *Pseudomonas* sp. strain Neni-4 on xenobiotics independent of molybdenum reduction. Error bars represent mean ± standard deviation (n = 3).

CONCLUSION

The phenolic chemicals phenol, benzoate, salicylic acid, and catechol have been successfully degraded by a Mo-reducing bacterium that was isolated locally in Indonesia and has the unique capacity to biodegrade these compounds. The ideal conditions for the bacteria to convert molybdate to Mo-blue are a pH of 6.3 and temperatures ranging from 25 to 40 degrees Celsius. In order of decreasing effectiveness, the electron donor's fructose, galactose, 2-ketogluconate, and citrate were used to assist molybdate reduction. Glucose was shown to be the most effective electron donor. A phosphate content of between 5.0 and 7.5 mM and a molybdate concentration of between 15 and 20 mM are two additional conditions that must be met. The Mo-blue that was created had an absorption spectrum that was comparable to that of a prior Mo-reducing bacteria and was strikingly similar to that of a reduced phosphomolybdate. At a concentration of 2 ppm, the reduction of molybdenum was blocked by heavy metals. The use of phenolics as electron donors to assist molybdenum reduction did not provide any favorable findings in the screening process. In spite of this, the bacterium was able to cultivate itself on phenol, benzoate, salicylic acid, and catechol—all of which are phenolic chemicals. The capacity of this bacteria to detoxify numerous toxicants is a desirable quality, and as a result, the bacterium is an essential instrument for bioremediation. At the moment, efforts are being made to purify the molybdenum-reducing enzyme that was produced by this bacterium as well as to describe research on the biodegradation of phenolics in greater detail.

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