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Land degradation

Characterization of a Molybdenum-reducing *Burkholderia* sp. Dr.Y27 with Phenol and Acrylamide-degrading Capability

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ABSTRACT

Recent studies have shown that molybdenum is toxic to the process of spermatogenesis at concentrations of several parts per million, highlighting its significance as a toxic substance. We have previously identified a bacterium that has the ability to break down acrylamide in soils that have been contaminated. We found that this bacterium has the capability to convert the heavy metal molybdenum into molybdenum blue. The study examines the Mo-blue absorption spectra of *Burkholderia* sp. Dr. Y27, revealing a secondary peak at 700 nm and a primary peak ranging from 860 to 870 nm. It indicates that Mo-blue is probably a diminished heteropolymolybdate, aided by enzymatic reduction in media containing phosphate. The most favorable pH for molybdate reduction was determined to be approximately 6.0, while the optimal temperature range was found to be between 34 and 37°C. Multiple carbon sources were examined, and it was found that glucose, fructose, and 2-ketogluconate exhibited the greatest efficacy. The presence of heavy metals such as mercury and copper greatly suppressed the production of Mo-blue. This text discusses the potential of using bioremediation in tropical regions, specifically focusing on the ability of *Burkholderia* sp. Dr. Y27 to efficiently reduce molybdenum under optimal conditions. The results provide evidence for the capability of *Burkholderia* sp. Dr. Y27 to be a successful agent for molybdenum bioremediation, particularly in tropical settings, by optimizing factors such as pH, temperature, and carbon sources. Additional investigation is advised to examine its utilization in practical contexts.

INTRODUCTION

The coexistence of heavy metals and organic xenobiotic contaminants at polluted sites necessitates the use of diverse bacterial degraders or bacteria capable of detoxifying multiple toxicants simultaneously. Molybdenum, an essential trace metal, becomes toxic at high concentrations to various organisms. This metal is utilized in numerous industrial applications, including as an alloying agent, in automotive antifreeze, corrosion-resistant steel, and as a lubricant in the form of molybdenum disulfide. Due to its extensive industrial use, molybdenum has contributed to significant water pollution incidents globally, including in Tokyo Bay, Tyrol in Austria [1], and the Black Sea, where its concentration can reach several hundreds of ppb . Additionally, molybdenum is a notable pollutant in sewage sludge, posing a considerable health risk. Studies have shown that Molybdenum can cause considerable harm by impeding the production of sperm and halting the development of embryos in different organisms, even at concentrations as low as a few parts per million [2]. It poses significant harm to ruminant animals, particularly cows, at comparable levels [3]. Aside from heavy metals, there are also organic pollutants that are considered significant environmental contaminants. These include xenobiotics like phenol, acrylamide, nicotinamide, acetamide, iodoacetamide, propionamide, sodium dodecyl sulfate (SDS), and diesel. Every year, large amounts of these chemicals are manufactured, resulting in significant pollution of ecosystems. Bioremediation is the most cost-effective approach for eliminating these pollutants, especially when they are present in low concentrations that cannot be effectively addressed by

physical and chemical methods. Phenol, a highly toxic substance, presents significant dangers to humans and other organisms. It can cause irritation and harm to mucous membranes, skin, eyes, and the respiratory tract when exposed to its vapors. Phenolic compounds in Malaysia are classified as one of the top three scheduled wastes, with an annual waste generation of over 1000 metric tonnes. The maximum allowable phenol concentration in raw drinking water in Malaysia, as stated by the National Guidelines for Raw Drinking Water Quality, is 0.002 mg/L. Nevertheless, numerous groundwater wells and landfills in the nation exhibit phenol concentrations that surpass this threshold [4], suggesting extensive contamination.

Certain microorganisms possess the capability to break down a wide range of foreign substances, known as xenobiotics. The adaptability of these microorganisms is highly sought after in areas that are heavily contaminated, where the presence of multiple pollutants is common [5]. Heavy metals reduction coupled with xenobiotic degradation has been reported [6]. Although phenol does not support molybdenum reduction, several molybdenum-reducing bacteria are able to grow on phenol as the sole carbon in previous works [7–10].

Here, we evaluate the potential of various xenobiotics as electron donors for reduction and screen for the ability of an isolated bacterium that previously breaks down acrylamide [11] to convert molybdenum to molybdenum blue. Furthermore, we test whether the bacteria can use these xenobiotics as a carbon source for their own growth. We present here the results of our investigation into a new type of molybdenum-reducing bacterium that has the ability to biodegrade phenol, and amides found in soil that has been contaminated. Future bioremediation efforts involving amides as organic contaminants, and the heavy metal molybdenum could benefit from this bacterium's characteristics.

MATERIALS AND METHODS

Growth and maintenance of molybdenum-reducing bacterium

The bacterium [11] was grown and maintained in Low Phosphate (LPM) and High Phosphate Media (HPM) [7]. The composition of the LPM were as follows: glucose (1%), $(NH_4)_2.SO_4$ (0.3%), $MgSO_4.7H_2O$ (0.05%), yeast extract (0.5%), NaCl (0.5%), $Na_2MoO_4.2H_2O$ (0.242 % or 10 mM) and Na_2HPO_4 (0.071% or 5 mM) [12]. Bacterial reduction of molybdate is indicated by the production of blue colonies. An orbital shaker was used to reduce molybdenum in a 250 mL shake flask containing 100 mL of the aforementioned media at room temperature for 48 hours at a pH of 7.0. The shaking was done at 120 rpm. A 1.0 mL sample of the liquid culture's molybdenum blue (Mo-blue) was centrifuged at 10,000 x g for 10 minutes at room temperature in order to analyze its absorption spectra. After that, a UV-spectrophotometer (Shimadzu 1201) was used to scan the supernatant from 400 to 900 nm. To adjust for the baseline, low phosphate media was used.

Preparation of resting cells for molybdenum reduction characterization

Using resting cells in a static microplate or microtiter format, characterization studies on the reduction of molybdenum to Mo-blue were conducted, following previously established methodologies. The effects of pH, temperature, and concentrations of phosphate and molybdate were examined [13]. Cultivating the cells at room temperature from a 1 L overnight culture in High Phosphate Media (HPM) was carried out on an orbital shaker, which was adjusted to 150 rpm. The only variation between HPM and LPM was the phosphate content, which was set at 100 mM for HPM. The cells were centrifgued at 15,000 x g for 10 minutes, rinsed several times to remove any residual phosphate, and resuspended in 20 mL of glucose-free Low Phosphate Media (LPM) to get cells with an absorbance of approximately 1.00 at A600 nm.

The concentration of phosphate used in this experiment was 5 mM, as all of the Mo-reducing bacteria that were isolated from LPM demonstrated optimal growth at that concentration. Following that, 180 µL of the cell suspension was transferred to every well of a sterile microplate using a pipette. Twenty microliters of sterile glucose was added to every well to start the creation of Mo-blue. The Corning® microplate, which allows gas exchange, was sealed with sterile tape and left to incubate at room temperature. On a regular basis, we used a BioRad Microtiter Plate reader (Model No. 680) to measure the absorbance at 750 nm. The microplate reader's maximum filter wavelength was 750 nm, of which measurement must be made with reference to the specific extinction coefficient of 11.69 mM^{-1} cm^{-1}, which was used to quantify mo-blue production in the microplate format.[14].

Evaluation of xenobiotics as potential electron donors for molybdenum reduction

Utilizing the microplate format outlined earlier, the electron-donating capabilities of xenobiotics like diesel, phenol, nicotinamide, acrylamide, acetamide, iodoacetamide, Sodium Dodecyl Sulfate (SDS), propionamide, and others were demonstrated. Taking into account their overall toxicity, these xenobiotics were substituted for glucose in the low phosphate medium in this experiment, with a final concentration of 500 mg/L. Diesel was first sonicated for 5 minutes in 10 mL of media until it reached a final concentration of 0.5 g/L. Afterwards, 200 µL of the mix was added to every well of the microplate.

Reduction of molybdenum and heavy metals

We used MERCK's commercial salts or Atomic Absorption Spectrometry standard solutions to prepare seven heavy metals: lead (II), copper (II), arsenic (V), mercury (II), chromium (VI), silver (I), and cadmium (II). The bacterium was exposed to different amounts of these heavy metals in a microplate format during incubation. For one day, at 30 ºC, the microplate was left to incubate. The wavelength 750 nm was used to measure Mo-blue production, as mentioned earlier.

Evaluation of xenobiotics for bacterial growth in a manner unrelated to molybdenum reduction

This bacterium was able to grow on several aliphatic amides [15]. The microplate format was used to test the ability of diesel, phenol, and Sodium Dodecyl Sulfate (SDS) to support bacterial growth independent of molybdenum reduction. The following media were used, with a final concentration of 500 mg/L of these xenobiotics.

The following were the components of the growth medium that had a high concentration of molybdenum removed because it could hinder the growth of xenobiotics: 0.3% ammonium sulfate, 0.2% sodium bicarbonate, 0.5% magnesium chloride, 0.5% sodium bicarbonate, 0.705% sodium bicarbonate. The medium was adjusted to pH 7.0. Using a microplate reader (Bio-Rad 680), we were able to measure the increase in bacterial growth after three days of incubation at room temperature at 600 nm.

Statistical analysis

These values represent the means plus or minus the standard error. For this data analysis, we used Graphpad Prism 3.0 and

Graphpad InStat 3.05, both of which can be downloaded from the official Graphpad website at www.graphpad.com. We used either a Student's t-test or a one-way analysis of variance with post hoc analysis by Tukey's test to compare the groups. A statistically significant result was defined as $P < 0.05$.

A previously isolated bacterium identified as *Burkholderia* sp. Dr.Y27 that was used to degrade acrylamide was discovered to be able to reduce molybdenum to Mo-blue. To date, this is the fourth bacterium from this genus that has been reported to be able to reduce molybdenum to molybdenum blue (**Table 1**).

RESULTS AND DISCUSSIONS

Table 1. Characterization of Mo-reducing bacteria isolated to date, an update [16].

Bacteria	Country of origin	Unique ability	Optimal pH and tempe-rature	Best carbon source	PoO_4 (mM)	MoO_4 (mM)	Heavy metals inhibition	Auth or
Raoultella ornithinolytica A1	Iraq	Psychrotolerant	pH 7.0 and 25 °C	glucose	n.a.	10	n.a.	[17]
Enterobacter aerogenes strain Amr-18	Egypt	Acrylamide can act as electron donor. acrylamide, acetamide, and propionamide as N source for growth	6.3 and 6.8	glucose	7.5	15 – 20	Ag^{+}, Cu^{2+} Hg^{2+}	[18]
Pseudomonas sp. strain Neni-4	Indonesia	growth on phenol, benzoate, salicylic acid, and catechol	6.3 25 and 40 °C	glucose	5.0-7.5	15-20	n.a.	[7]
Pseudomonas putida strain Neni-3	Indoensia	Decolorizaton of Congo Red	6-6.5 25 to 37 °C	glucose	2.5-7.5	10-15	n.a.	[8]
Serratia sp. strain Amr-4	Egypt	Growth on the pesticides carbamates carbofuran and carbaryl	6.0 and 6.8 and between 30 and 34 oC	glucose	2.5-7.5	20-30	Ag^{+}, Pb^{2+}, Cu^{2+} Hg^{2+}	[19]
Escherichia coli strain Amr-13	Egypt	Growth on PEG 200, 300 and 600	5.5 and 8.0 30 and 37 °C	glucose	5	10-30	n.a.	[20]
Bacillus sp. strain Zeid 15		Growth on acrylamide and propionamide as well as sources of electron donor for reduction	6.0 25 and 34 °C	glucose	2.5-5	15-20	Cu^{2+}, Hg^{2+}, Ag^{+}, and Cr^{6+}	[21]
Bacillus sp. strain Neni-8	Indonesia	Growth on various PEGs such as 200, 300 and 600	6.3 and 6.5, and between 30 and 37 °C	glucose	2.5-7.5	20-30	Cu^{2+}, Hg^{2+}, Ag^{+}, and Cr^{6+}	[22]
Bacillus amyloliquefaciens strain Neni-9	Indonesia	Growth on the pesticides carbaryl and carbofuran	pH 6.3 and 6.5, 30-37 °C	glucose	5.0-7.5	20-30	Ag^{+}, Cr^{6+}, Cu^{2+} Hg^{2+}	[23]
Pseudomonas sp.	Nigeria		pH 6.5-7.5 37 °C	glucose	3.5-7.5	100	n.a.	[24]
Pantoea sp. strain HMY-P4	Nigeria		pH 6.0-8.0 35-40 ˚C	glucose	5.0	20-40	n.a.	[25,26]
Enterobacter cloacae	Nigeria		pH 6.5-7.0 35-40 °C	glucose	5.0-7.5	80-100	n.a.	[27,28]
Morganella sp.	Nigeria		pH 6.0-7.5 35 °C	glucose	3.5	40	n.a.	[29]
Pseudomonas. strain Dr.Y Kertih	Malaysia	growth on various xenobiotics- phenol, sodium dodecyl sulfate (SDS), acrylamide, acetamide, nicotinamide, propionamide, iodoacetamide, acetamide and diesel	pH 6.0- 6.3 25-40 °C.	glucose	5.0	20	Ag^{+}, Pb^{2+}, As^{5+} Hg^{2+}	[30]
Clostridium pasteurianum BC1 (USA)	USA	metallic (Mo0) nanoparticles 5–20 nm in size degradation of methyl orange	pH 6.8 n.a.	peptone	1.74	20.67		[31]
microbial electrolysis cells consortium (China)	China	Tungsten reduction and acetate biodegradation Hydrogen production	pH 3.0 22 °C	acetate	n.a.	1	n.a.	[32]
Raoultella ornithinolytica strain Mo1	Egypt		pH 6, 30 °C	glucose		20	n.a.	[33]
Raoultella planticola strain MoI	Iraq		pH 6, 30 °C	glucose		20	n.a.	[33]
Bacillus sonorensis strain Pharon3 (MK078035)	Egypt	Thermophilic bacterium	pH 7.07 52.2 °C	glucose	4.0	10		[34]
Bacillus tequilensis strain Pharon2 (MK078034)	Egypt	Thermophilic bacterium	pH 7.02 46.1 °C	sucrose	4.0	10		[34]
Bacillus sp. strain Neni-12	Indonesia	growth on coumaphos	pH 6.3 25-37 °C	glucose	5.0	15-20	Ag^{+}, Cr^{6+}, Hg^{2+}	[35]
Pseudomonas sp.	Nigeria		pH 6.5-7.0 35- 40 °C	glucose	3.5	40-60	n.a.	[36]
Burkholderia vietnamiensis AQ5-12	Malaysia	Growth on glyphosate	pH 6.25-8.0 30-40 °C	glucose	5.0	40-60	n.a.	[37]
Burkholderia sp. AQ5-13	Malaysia	Growth on glyphosate	pH 6.25-8.0 35-40 °C	glucose	5.0	40-50	n.a.	[37]
Serratia marcescens strain KIK-1	Nigeria	Decolorize various azo and triphenyl methane dyes	pH 5.8-6.5 34-37 °C	glucose	5.0	10-25	Ag^{+}, Cr^{6+}, Hg^{2+}, Cu^{2+}	[38]
Pseudomonas putida strain Egypt-15	Egypt	Growth on PEG 4000	pH 6.5 34 °C	glucose	5.0	20	n.a.	[39]
Bacillus amyloliquefaciens	Malaysia	Growth on SDS	pH 5.8-6.3 25-34 °C	glucose	5.0-7.5	30-50	Hg^{2+}, Cu^{2+} Ag^{+}	[40]
Serratia sp. strain HMY1	Nigeria	Growth on cyanide	pH 6.5-7.0 30-35 °C	sucrose	3.95	55	n.a.	[41–43]
Enterobacter sp. Strain Saw-2	Malaysia	Growth on phenol and catechol	pH 6.3-6.8 34-37 °C	glucose	5.0	15-30	n.a.	[9]
Serratia sp. strain HMY3	Nigeria	Growth on cyanide	pH 6.5 35 °C	sucrose	3.95	55-57.5	As^{3+}, Cr^{6+}, Hg^{2+}, Cu^{2}	[44]
Bacillus sp. strain Neni-10	Indonesia	Growth on dye Metanil Yellow	pH 6.3 34 °C	glucose	2.5-7.5	20	Ag^{+}, Cu^{2+}, Cr^{6+}, Hg^{2+}	[45,46]

Pseudomonas sp. strain 135	Malaysia	Growth on acrylamide, acetamide and propionamide acrylamide can support Mo-blue production	pH 6.0-6.3 25-40 ºC	glucose	5.0-7.5	15-25	Ag^{+}, Cu^{2+}, Cd^{2+}, Hg^{2+}	[47]
Serratia marcescens strain DR.Y10	Malaysia	Growth on acrylamide, propionamide and acetamide	pH 6.0-6.5 30-37 °C	glucose	5.0	10-30	Ag^{+}, Cu^{2+}, Cr^{6+}, Hg^{2+}	[48]
Pseudomonas aeruginosa strain KIK-11	Malaysia	grow on diesel and SDS	pH 5.8-6.0 25-34 ºC	glucose	5.0-7.5	30-40	Ag^{+}, Cu^{2+}, Hg^{2+}	[49]
Serratia sp. *strain* MIE2	Malaysia		pH 6.0 27 to 35ºC	sucrose	3.95	20	Hg^{2+}, Zn^{2+}, Cu^{2}	[50,51]
Bacillus sp. strain khayat	Malaysia	Growth on SDS and diesel	pH 5.8-6.8 34 ºC	glucose	5-7.5	10-20	Ag^{+}, As^{3+}, Pb^{2+}, Hg^{2+}, Cu^{2+}	[52]
Burkholderia sp.strain neni-11	Indonesia	Growth on acrylamide	pH 6.0-6.3 30-37 ºC	glucose	5	15	Ag^{+}, Cr^{6+}, Hg^{2+}	[53]
Enterobacter sp. strain Aft-3 (Pakistan)	Pakistan	Growth on azo dye	pH 5.8-6.5 37 ºC	glucose	5	20-25	Ag^{+}, Cu^{2}, Hg^{2+}	[54]
Klebsiella oxytoca strain saw-5	Malaysia	Growth on glyphosate	pH 6.3-6.8 34 ºC	glucose	5	20-30	Ag^{+}, Cd^{2+}, Cr^{6+}, Hg^{2+}, Cu^{2+}	[55]
P. aeruginosa strain Amr-11	Egypt	Growth on phenol	pH 6.3-6.8 34 ºC	glucose	2.5-7.5	20-30	Ag^{+}, As^{3+}, Pb^{2+}, Cd^{2+}, Cr^{6+}, Hg^{2+}, Cu^{2+}	[56]
Klebsiella oxytoca strain Aft-7	Pakistan	Growth on SDS	pH 5.8-6.3 25-34 ºC	glucose	5-7.5	5-20	Ag^{+}, As^{3+}, Pb^{2+}, Cd^{2+}, Cr^{6+}, Hg^{2+}, Cu^{2+}	[57]
Enterobacter sp. strain Zeid-6	Sudan	Growth on Azo dye Orange G	pH 5.5-8.0 30-37 ºC	glucose	5	20	Ag^{+}, Pb^{2+}, Hg^{2+}, Cu^{2+},	[58]
Pseudomonas putida strain Amr-12	Egypt	Growth on phenol catechol	pH 6.0-7.0 20-30 ºC	glucose	5.0-7.5	20-30	Ag^{+}, Cr^{6+}, Hg^{2+}	[59]
Enterobacter sp. Strain Neni-13	Indonesia	Growth on SDS	pH 6.0-6.5 37 ºC	glucose	2.5-5.0	15	Ag^{+}, Cd^{2+}, Hg^{2+}, Cu^{2+}	[60]
Bacillus sp. strain Zeid 14	Sudan	Growth on amides and acetonitrile acrylamide can support reduction	pH 6.0-6.8 25-34 ºC	glucose	5.0-7.5	10-20	Ag^{+}, Cd^{2+}, Cr^{6+}, Hg^{2+}, Cu^{2+}	[61]
Klebsiella oxytoca strain DRY14	Malaysia	Growth on SDS	pH 7.0 25 ºC	glucose	5	25-30	Ag^{+}, Pb^{2+}, Cd^{2+}, Cr^{6+}, Hg^{2+}, Cu^{2+}	[62]
Bacillus pumilus strain lbna	Malaysia		pH 7.0-8.0 37 ºC	glucose	2.5-5	40	As^{3+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Cr^{6+}, Hg^{2+}, Cu^{2+}	[63]
Bacillus sp. strain A.rzi	Malaysia		pH 7.3 28-30 ºC	glucose	4	50	Cd^{2+}, Cr^{6+}, Cu^{2+}, Ag^{+}, Pb^{2+}, Hg^{2+}, Co^{2+}, Zn^{2+}	[64]
Pseudomonas sp. strain DRY1	Antarctica		pH 6.5-7.5 15-20 ºC	glucose	5	30-50	Cd^{2+}, Cr^{6+}, Cu^{2+}, Ag^{+}, Pb^{2+}, Hg^{2+}	[65]
Klebsiella oxytoca strain hkeem	Malaysia		pH 7.3 30 ºC	fructose	4.5	80	Cu^{2+}, Ag^{+}, Hg^{2+}	[66]
Pseudomonas sp. strain DRY2	Malaysia		pH 6.0 40 ºC	glucose	5	15-20	Cr^{6+}, Cu^{2+}, Pb^{2+}, Hg^{2+}	[67]
Acinetobacter calcoaceticus strain Dr.Y12	Malaysia		pH 6.5 37 ºC	glucose	5	20	Cd^{2+}, Cr^{6+}, Cu^{2+}, Pb^{2+}, Hg^{2+}	[68]
Enterobacter sp. strain Dr.Y13	Malaysia		pH 6.5 37 ºC	glucose	5	25-50	Cr^{6+}, Cd^{2+}, Cu^{2+}, Ag^{+}, Hg^{2+}	[69]
S. marcescens strain Dr.Y9	Malaysia		pH 7.0 37 ºC	sucrose	5	20	Cr^{6+}, Cu^{2+}, Ag^{+}, Hg^{2+}	[12]
Serratia sp. strain Dr.Y8	Malaysia		pH 6.0 37 ºC	sucrose	5	50	Cr^{6+}, Cu^{2+}, Ag^{+}, Hg^{2+}	[70]
5Serratia sp. strain DrY5	Malaysia	The 1st purification of Mo-reducing enzyme	pH 7.0 37 ºC	sucrose	5	30	Cu^{2+}	[71–75]
Serratia marcescens strain DRY6	Malaysia		pH 7.0 35 ºC	sucrose	5	15-25	Cr^{6+}, Cu^{2+}, Hg^{2+}	[76]
Enterobacter cloacae strain 48	Malaysia		pH 7.0 30 ºC	sucrose	2.9	20	Cr^{6+}, Cu^{2+}	[77]
Escherichia coli K12	n.a.		pH 7.0 30-36 ºC	glucose	5	80	Cr^{6+}	[78]

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Molybdenum absorbance spectrum

The Mo-blue absorption spectra of *Burkholderia* sp. Dr. Y27 showed a shoulder at about 700 nm and a maximum peak in the infra-red range of 860 to 870 nm, with a median at 865 nm, as represented in **Fig. 1**. Due to its complex structure and abundance of species, determining the Mo-blue's identity is no easy matter [79]. Isopolymolybdate and heteropolymolybdate are two types of molybdenum complexes, and Mo-blue is a reduced byproduct of one of these. It has been suggested by Campbell et al. [78] that the Mo-blue seen in molybdenum reduction by *E. coli* K12 is actually phosphomolybdate in its reduced form, but no one has yet explained how this happens. Because the conversion requires strong reducing agents and acidic conditions, it is not feasible to form isopoly Mo-blue from molybdate itself using biologically based reducing agents. Based on the results of the phosphate determination method using ascorbic acid, it is more likely that heteropoly Mo-blue is formed by enzymatic reduction or biologically based reducing agents [80]. It was postulated that the phosphomolybdate intermediate is required for microbial molybdate reduction in phosphate-containing media. The pH drops as bacteria grow, which converts molybdate to this structure. In other words, molybdenum reduction to Mo-blue involves chemical and biological processes. Assuming this mechanism is successful, the Mo-blue absorption spectra produced by this bacterium should be very similar to those seen when determining phosphate levels. Specifically, there was a shoulder at around 700 nm and maximum absorption in the 860–870 nm range in the measured spectrum. Peak absorption at 880–890 nm and a shoulder at 700–720 nm are typical features of the Mo-blue spectra obtained by the phosphate determination method [81]. This requirement is satisfied by all Mo-blue spectra observed in other bacteria, as demonstrated earlier [79]. In this study, the absorption spectra results provide credence to the theory by suggesting a comparable spectrum. The phosphomolybdate species must be precisely identified using n.m.r and e.s.r. because of the compound's complex structure. The scanning spectroscopic profile analysis of heteropolymolybdate species, on the other hand, is a more convenient and widely used technique for spectrophotmetric characterization [82]. Despite Mo-max blue's absorption wavelength being 865 nm, measurements taken at 750 nm—

roughly 30% lower—were sufficient for regular monitoring of Mo-blue production due to the significantly higher intensity compared to cellular absorption at 600-620 nm [13]. The production of Mo-blue has been previously monitored using a variety of wavelengths, including 710 nm [77] and also 820 nm [78].

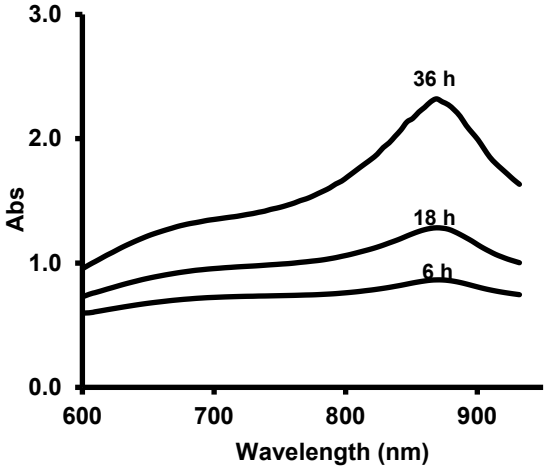

Fig. 1. An absorption spectrum of Mo-blue from *Burkholderia* sp. Dr. Y27 scanned over time.

Effect of pH and temperature on molybdate reduction to Mo-blue measured at 750 nm

Burkholderia sp. Dr.Y27 was incubated at different pH ranging from 5.5 to 8.0 using Bis-Tris and Tris.Cl buffers (20 mM). Analysis by ANOVA indicates that the optimal pH for molybdate reduction to Mo-blue is around 6.0, as this point shows the highest absorbance at 750 nm. The activity decreases significantly beyond pH 6.5, indicating reduced efficiency of the reduction process at higher pH levels (**Fig. 2**). The effect of temperature (**Fig. 3**) was observed over a wide range of temperature (20 to 60 °C) with an optimum temperature ranging from 34 °C to 37 °C with no significant different ($p>0.05$) among the values measured as analysed using ANOVA. Temperatures higher than 37 ºC were strongly inhibitory to Mo-blue production from *Burkholderia* sp. Dr.Y27.

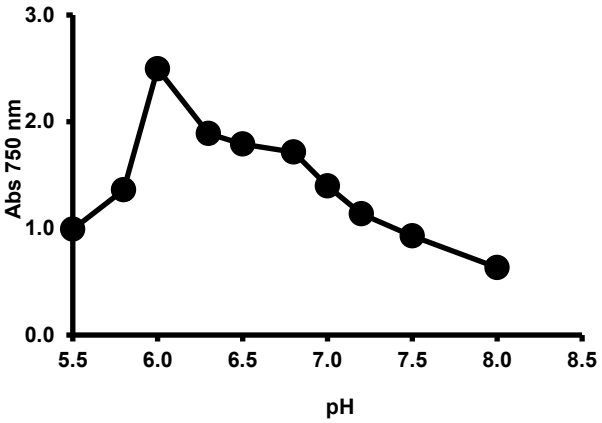

Fig. 2. Effect of pH on molybdenum reduction by *Burkholderia* sp. Dr.Y27. For exactly 72 hours, the bacteria's resting cells were placed in an ideal environment within a microtiter plate. n = 3, the error bars show the mean ± standard deviation.

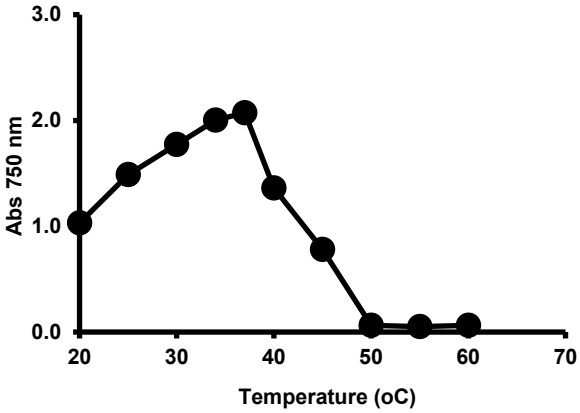

Fig. 3. Effect of temperature on molybdenum reduction by *Burkholderia* sp. Dr.Y27. For exactly 72 hours, the bacteria's resting cells were placed in an ideal environment within a microtiter plate. n = 3, the error bars show the mean ± standard deviation.

Because molybdenum reduction is an enzyme-mediated process, changes in temperature and pH can influence protein folding and enzyme activity, which in turn can inhibit molybdenum reduction. Tropical countries, such as Malaysia, with annual average temperatures of 25 to 35 oC, provide ideal circumstances for bioremediation [76]. Thus, *Burkholderia* sp. Dr.Y27 may have potential as a molybdenum soil bioremediation agent both in this region and others in the tropics. Between 25 and 37 ºC is the best range for most Mo-reducers (**Table 1**) as they are isolated from tropical soils with a few psychrotolerant reducer isolated from Antarctica and Iraq showing an optimal temperature supporting reduction of between 15 and 20 ºC and less than 25 °C, respectively [65].

Because of its neutrophilic nature, *Burkholderia* sp. Dr.Y27 displays an optimal pH range that allows it to support molybdenum reduction. Neutrophils can thrive in environments with a pH range of 5.5 to 8.0, which is one of their defining features. An essential note about molybdenum reduction in bacteria is that the ideal pH for the process is slightly acidic, falling somewhere between 5.0 and 7.0 (**Table 1**). Presumably, the formation and stability of phosphomolybdate prior to its reduction to Mo-blue are significantly impacted by acidic pH. Therefore, the ideal reduction happens when the stability of the substrate and the activity of the enzyme are both optimized [79].

Effect of electron donor on molybdate reduction

Glucose, fructose, 2-ketogluconate, mannose, sucrose, L-arabinose, mannitol, xylose, meso-inositol, trehalose, and citrate were the best electron donors for supporting molybdate reduction, in descending order, according to **Fig. 4**. Molybdenum reduction was not supported by other carbon sources like glycogen, methyl-mannoside, D-melezitose, inulin, starch, and D-turanose, perhaps due to the inability of this bacterium to metabolize these substrates. In the presence of carbon sources in the medium, the bacteria could utilize metabolic pathways like glycolysis, Kreb's cycle, and electron transport chain to produce NADH and NADPH, which are substrates that donate electrons. The electron-donating substrates for molybdenum reducing enzymes are NADH and NADPH [83,84].

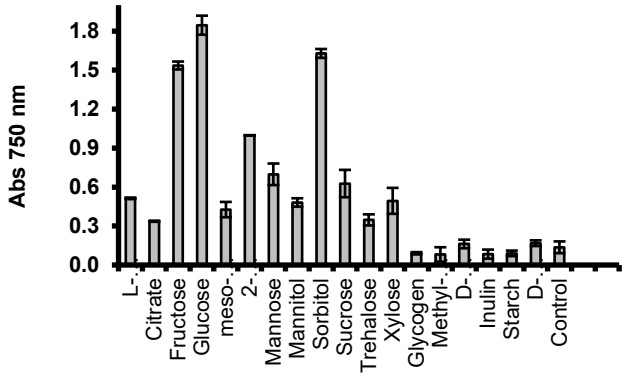

Fig. 4. Inhibition of molybdenum reduction by various electron donor sources (1% w/v). A variety of electron donors and 10 mM molybdate were added to low phosphate media in which *Burkholderia* sp. Dr.Y27 was cultured. For exactly 72 hours, the bacteria's resting cells were placed in an ideal environment within a microtiter plate. n = 3, the error bars show the mean ± standard deviation.

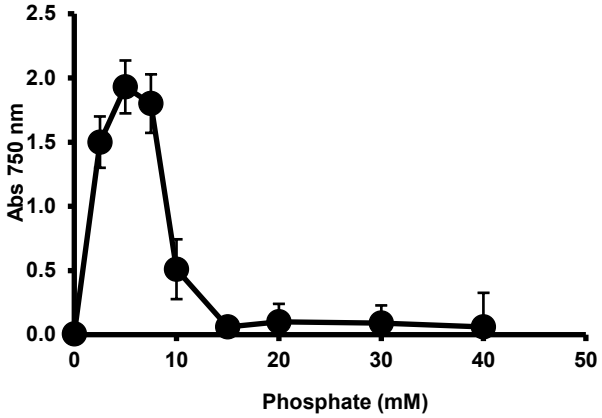

Fig. 5. The effect of phosphate concentration on molybdenum reduction by *Burkholderia* sp. Dr.Y27. For exactly 72 hours, the bacteria's resting cells were placed in an ideal environment within a microtiter plate. n = 3, the error bars show the mean ± standard deviation.

Effect of phosphate and molybdate concentrations to molybdate reduction

Because both phosphate and molybdate anions inhibit bacterial Mo-blue production (**Table 1**), determining their optimal concentrations is important for molybdenum reduction. **Fig. 5** shows that the ideal phosphate concentration was 5-7.5 mM, and that concentrations higher than that were highly inhibitory to reduction. Since phosphomolybdate is only stable in acidic environments, it stands to reason that a high phosphate concentration would reduce its stability. This is because phosphate buffers are more effective under high phosphate concentrations. Furthermore, for some reason, high phosphate makes the phosphomolybdate complex itself unstable [85–87]. For optimal reduction, none of the molybdenum-reducing bacteria that have been discovered thus far require phosphate concentrations greater than 5 mM (**Table 1**).

The newly-isolated bacterium reduced molybdenum concentrations as high as 60 mM with reduced Mo-blue production, according to studies that examined the effect of molybdenum concentration on molybdenum reduction. **Fig. 6** shows that the ideal concentration range for reduction was 10–50 mM. The strain would be able to reduce molybdenum pollution at high concentrations if it reduced to an insoluble form at this concentration. According to the literature, *Pseudomonas* sp. strain Dr.Y2 requires a minimum of 15 mM of molybdenum for optimal growth [81], whilst the highest molybdenum required for optimal reduction was 80 mM in *E. coli* K12 [78] and *Klebsiella oxytoca* strain hkeem [66]. The ideal molybdate concentrations, which typically range from 20 to 50 mM, could be utilized by other Mo-reducing bacteria to produce Mo-blue (**Table 1**). Actually, molybdenum pollutant levels as high as 2000 ppm, or around 20 mM, have been found in the environment. [88].

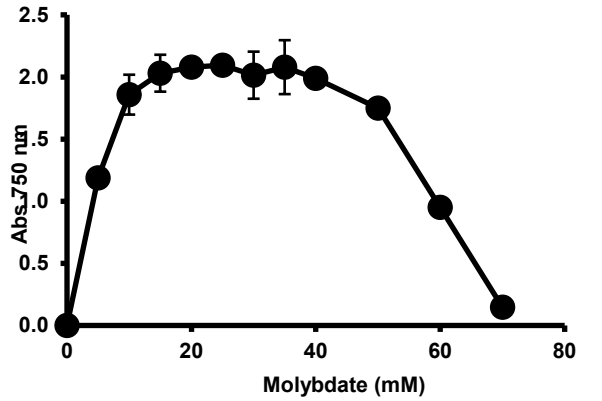

Fig. 6. The effect of molybdate concentration on molybdenum reduction by *Burkholderia* sp. Dr.Y27. For exactly 72 hours, the bacteria's resting cells were placed in an ideal environment within a microtiter plate. n = 3, the error bars show the mean ± standard deviation.

Effect of heavy metals

Fig. 7 shows that when different metals were added to *Burkholderia* sp. Dr. Y27 at a concentration of 1 ppm, the Mo-reducing activity of the bacteria was inhibited to varying degrees. Mercury and copper inhibited Mo-blue production by 45.5 and 16.3%, respectively. Heavy metals and other metal ions having inhibitory effects are a big issue in bioremediation. Screening and isolating bacteria with a wide range of metal resistance capabilities is, thus, crucial. Shukor et.al. [89] proved that mercury was found to be the physiological inhibitor to molybdate reduction, while other metal ions may be pseudo inhibitors. A review of the heavy metal types that inhibited molybdenum-reducing bacteria revealed that poisonous heavy metals inhibit nearly all of the reducers (**Table 1**).

Enzymes typically have their sulfhydryl groups targeted by heavy metals like copper, cadmium, silver, and mercury [90]. It is well-known that chromate inhibits enzymes like glucose oxidase [91]. When heavy metals are bound to an enzyme, it rendered it incapable of reducing metals.

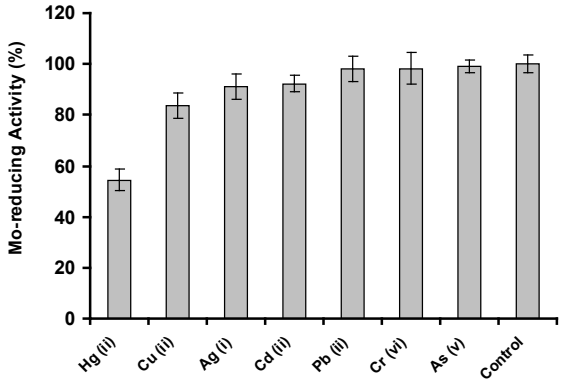

Fig. 7. The effect of metals on Mo-blue production by *Burkholderia* sp. Dr.Y27. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 72 hours. Error bars represent mean ± standard deviation (n = 3).

A novel approach to molybdenum reduction and autonomous growth using xenobiotics as electron donors

So far, nearly every strain of molybdenum-reducing bacteria has been able to convert molybdenum into molybdenum blue using easily assimilable carbon sources such as glucose and sucrose while very few could use other non-easily assimilable carbon sources as sources of electron donors. The exception is *Enterobacter aerogenes* strain Amr-18, where acrylamide can be used as an electron donor for Mo-bleu production (**Table 1**). The potential of different xenobiotics to facilitate molybdenum reduction was investigated. While other xenobiotics failed to support molybdenum reduction, acrylamide did so at a lesser efficiency than glucose (**Fig. 8**). Xenobiotics like phenol could be utilized as electron donors in chromate reduction [6] but was not found to be a source of electron donor for molybdenum reduction in this study. The bacterium was able to grow on phenol, acrylamide, acetamide and propionamide as a carbon sources and energy for growth (**Fig. 9**). Bacteria that could degrade phenol include *Pseudomonas* species [92–95], Bacillus brevis [96], *Alcaligenes* sp. [97], *Ochrobactrum* sp. [98], *Acinetobacter* sp. [99] and *Rhodococcus* species [100].

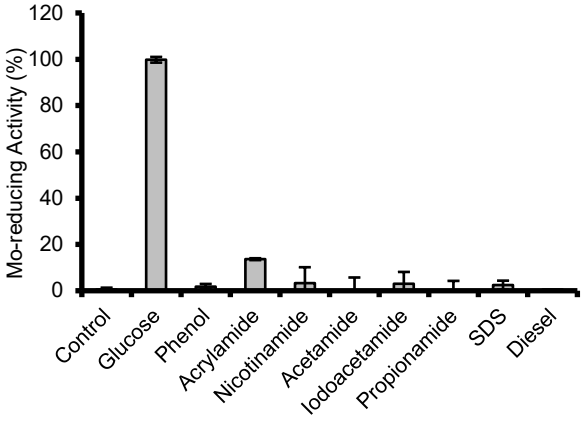

Fig. 8. Mo-blue reduction by xenobiotics at 10 mM in low phosphate media. Glucose was the positive control. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 72 hours. Error bars represent mean ± standard deviation (n = 3).

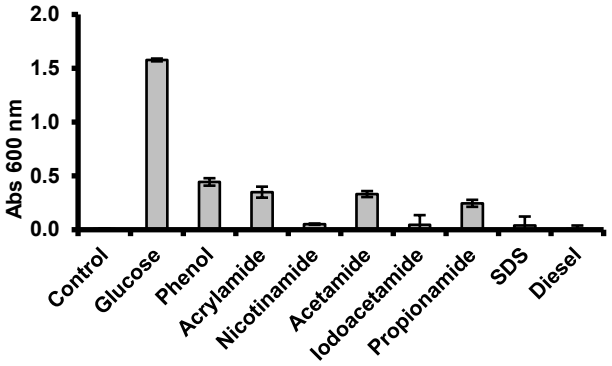

Fig. 9. Growth of *Burkholderia* sp. Dr.Y27 on xenobiotics independent of molybdenum reduction. Glucose was the positive control. Resting cells of the bacterium were incubated in a microtiter plate under optimized conditions for 72 hours. Error bars represent mean ± standard deviation (n = 3).

CONCLUSION

The study shows that *Burkholderia* sp. Dr. Y27 can efficiently convert molybdate to Mo-blue under ideal conditions. The absorption spectrum of the converted product exhibits a peak at 860-870 nm and a smaller peak at 700 nm. The pH that yielded the best reduction results was approximately 6.0, while the temperature range that produced optimal outcomes ranged from 34 to 37°C. Multiple carbon sources, particularly glucose, fructose, and 2-ketogluconate, greatly facilitated the reduction of molybdate. The process was hindered by heavy metals, specifically mercury and copper. The results emphasize the possible use of *Burkholderia* sp. Dr. Y27 for molybdenum bioremediation, particularly in tropical environments with appropriate temperature conditions. Additional investigation is necessary to comprehensively comprehend the biochemical mechanisms and practical implementations in real-world settings. This bacterium possesses a valuable characteristic for bioremediation as it has the desirable capability to detoxify various toxic substances. Malaysia is contemplating enacting a law to ban the importation of foreign microbes and genetically modified organisms (GMOs) for the purpose of using them in xenobiotic bioremediation. In light of this, we believe that our locally obtained isolate shows great potential for effectively treating polluted land and water bodies in Malaysia. The bacterium is currently undergoing active purification efforts to isolate the molybdenum-reducing enzyme it produces.

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