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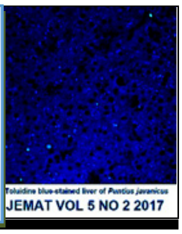

Reduction of Molybdenum by *Pseudomonas aeruginosa* strain KIK-11 Isolated from a Metal-contaminated Soil with Ability to Grow on Diesel and Sodium Dodecyl Sulphate

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ABSTRACT

Recently, molybdenum is considered as an emerging pollutant for its extreme toxicity to spermatogenesis in some organisms. Bacterial molybdate reduction to colloidal molybdenum blue (Mo-blue) forms the basis for its bioremediation. Molybdenum-reducing *Pseudomonas aeruginosa* strain KIK-11 was screened for its potential to degrade hydrocarbons and detergents. Optimal molybdate reduction to Mo-blue in this strain was supported by pH between 5.8 and 6.0, temperatures between 25 and 34 °C, molybdate concentration between 30 and 40 mM and a critical phosphate concentration of between 5.0 and 7.5 mM. The isolate was able to survive and grow on SDS and diesel. However, these compounds did not support Mo-blue production. The best electron donor source facilitating molybdate reduction is glucose, followed by galactose, fructose and citrate respectively. The process was inhibited by heavy metals such as copper (II), mercury (II) and silver (I). The bacterium was able to grow and detoxify multiple toxicants, a novel feat that is important in bioremediation.

INTRODUCTION

Pollution of the environment has increased tremendously during the last three decades due to release of man-made chemical contaminants, consequently making the environment unsafe. Countless tonnes of organic toxins and chemical pollutants are discharged into the environment yearly, requiring huge capital to clean them up. The physicochemical methods of remediation are only effective at higher concentrations of the environmental toxicant but ineffective and expensive at low concentration, making bioremediation as the only feasible and cheaper alternative [1–3].

A number of researches have linked molybdenum toxicity to reproductive failure, infertility and developmental defect to a variety of organisms, in addition to other health-related issues. Molybdenum pollution has several anthropogenic sources such as various steel and alloy industries and also from mining activity [4]. Although, the minimal concentration of molybdenum is found in fresh water and urban air (0.01 – 0.03 µg m^{-3}), however, several hundreds of parts per million (ppm) have been reported in water bodies [5] and up to 2,000 ppm was reported in soil [6]. Molybdenum is more toxic to ruminants than monogastric animals, with cow being the most susceptible causing scouring and death at relatively low levels due to hypocuprosis [7].

Recent findings indicated negative effects of molybdenum to oogenesis and spermatogenesis in several organisms [8,9]. Polluted sites often contain multiple contaminants including detergents and hydrocarbons often significantly present as co-pollutants in our water bodies. Detergents such as sodium dodecyl sulphate (SDS) and sodium dodecylbenzene sulfonate (SDBS) serve as anionic surfactants that exhibited their toxic effects to aquatic life at a very low concentration (0.0025 mg/L) [10].

Bacterial molybdate reduction to insoluble Mo-blue is a promising bioremediation tool. This phenomenon was earlier observed in *E. coli* in 1896 [11], though the first comprehensive report came up only much later (more than half a century) on *E. coli* K12 [12]. About three years later Sugio et al. [13] reported molybdate reduction by a chemolithotroph, *Thiobacillus ferrooxidans*, but their report fails to mention previous works on molybdenum reduction. Almost a decade later, the same group reported on the first molybdate reduction by a heterotrophic bacterium, *Enterobacter cloacae* strain 48 (EC 48) [14], still without citing previous works signifying the scarcity of literature on this phenomenon. Since then, numerous locally-isolated Mo-reducing bacteria have been reported [15–22] including a reducer from the Antarctic region [23]. *Klebsiella oxtoca* was the first Mo-reducer with other xenobiotic detoxifying ability reported degrade SDS [15]. These microorganisms with multi-toxicant detoxifying capacity are urgently needed for effective bioremediation as polluted sites often contain multiple toxicants [15,24].

This work report on the isolation of bacterial strain with the potential to grow on xenobiotics like SDS and diesel and reduce molybdate to Mo-blue. The ability of this bacterium to degrade diesel and SDS is novel, thus could be a candidate of choice for onsite bioremediation of these pollutants.

MATERIALS AND METHODS

Isolation of a molybdenum-reducing bacterium

A collection of soil samples (5 cm beneath the topsoil) were obtained in 2014 from polluted areas in Kano Metropolis, Kano State-Nigeria. Soil suspension containing 1 g of soil in 10 ml of sterile distilled water was prepared, from which 0.1 ml aliquot was spread immediately on low phosphate agar medium, and the plates were incubated at room temperature for 48 hours. An assortment of white and blue colonies appeared after the required incubation. A bacterial colony with highest blue colour intensity was re-streaked on LPM agar until a pure culture was achieved. Mo-reducing bacterium was screened on a low phosphate molybdate media (LPM) containing; 0.242% $Na_2MoO_4.2H_2O$ (10 mM), 1% glucose, 0.5% yeast extract, 0.3% $(NH_4)_2.SO_4$ 0.05% $MgSO_4.7H_2O$, 0.5% NaCl and 0.071% Na_2HPO_4 (5 mM) [25].

The culture media for Mo-reducing bacterium turns blue following incubation, aliquot (1 ml) from the culture was centrifuged at 10,000 × g for 10 minutes, and the resulting supernatant was scanned to get the absorption spectrum of the Mo-blue produced. Bacterial resting cells were prepared on high phosphate media (HMP) using the recipe above but with phosphate concentration increased to 100 mM. Cells cultured 1 L HMP were incubated for 48 h on an orbital shaker set at 120 rpm, after which the cells were harvested by centrifuging at 10,000 × g for 10 min. The pellet was rinsed thrice with distilled water and then resuspended in 10 ml of LPM excluding glucose.

Preparation of resting cells

The preparation and use of resting cells in a microtiter format were carried out as before [26]. Bacterial resting cells were prepared on high phosphate media (HMP) using the recipe above but with phosphate concentration increased to 100 mM. Cells cultured 1 L HMP were incubated for 48 hours on an orbital shaker set at 120 rpm, after which the cells were harvested by centrifuging at 10,000 × g for 10 min. The pellet was rinsed thrice with distilled water and then re-suspended in 10 ml of LPM excluding glucose. Cell suspension (180 µL) was added into each well of a sterile microplate and 20 µL of sterilized glucose from a stock solution (10% (w/v) was mixed to initiate Mo-blue production. The microplate was covered with a sterile sealing tape while enabling gas exchange and incubated at room temperature. Mo-blue production was measured at 750 nm using BioRad microtiter plate reader (Richmond, CA Model No. 680).

The specific extinction coefficient of 11.69 $mM.^{-1}.cm^{-1}$ was used to determine the corresponding concentration of the Mo-blue formed [27]. The effect of interacting heavy metals, silver (i), mercury (ii), lead (ii), copper (ii), chromium (vi), cadmium (ii) and arsenic (v), on Mo-blue production were ascertained using Atomic Absorption Spectrometry (AAS) standard solutions (Merck Chemical Co., Germany). The capacity of this bacterium to utilize detergents (SDS and SDBS) and hydrocarbon (crude petroleum and diesel) as electron donor source to facilitate Mo-blue production was tested at 200 mg/L, but in a maximum volume of 50 µL [28].

The ability of these xenobiotics to promote bacterial growth independent of molybdenum reduction was separately investigated. The increase of bacterial growth following 72 hours incubation at room temperature was measured spectrophotometrically at 600 nm and can even be visually observed by the increased turbidity.

Identification of Mo-reducing bacterium

Identification of this bacterium was performed according to the Bergey's Manual [29], while the interpretation of the result was done using ABIS online system software [30]. The method includes this bacterial ability to utilize, acetate, citrate, malonate as carbon sources, urease test with phenol red as the pH indicator, test for gelatin hydrolysis, lipase production utilizing corn oil, test for the utilization of DNAse on DNAse agar, gas production from carbohydrate metabolisms, tests for the ability of the bacterium to deaminate phenylalanine, and production of acids from sugar metabolism.

Other methods include Gram staining, 24 hour catalase and oxidase test, detection of β-galactosidase activity, detection of arginine dihydrolase activity,the hanging drop method to detect motility, nitrophenyl-β-galactoside, detection of lysine decarboxylase, detection of ornithine decarboxylase, test for hydrolysis of esculin, detection of the reduction of nitrate (NO_3) to nitrite (NO_2), hydrogen sulfide (H_2S) production through triple sugar iron agar slant and Voges-Proskauer test of acetoin production.

Statistical analysis

GraphPad Prism v 6.0 (www.graphpad.com) was used to analyse the data. To compare between means, Student's t-test or ANOVA with Tukey's post hoc test was carried out.

RESULTS

Identification of molybdenum-reducing bacterium

The bacterium was found to be Gram-negative bacillus, aerobic and motile. The ABIS online software suggested 3 identities for this bacterium with the highest homology of 98% and approximately 90 % accuracy as *Pseudomonas aeruginosa*.

Table 1. Biochemical tests for *Pseudomonas aeruginosa* strain KIK-11.

Motility	+	Utilization of:	
Hemolysis	+	L-Arabinose	d
Growth at 4 ºC	–	Citrate	+
Growth at 41 ºC	+	Fructose	+
Growth on MacConkey agar	+	Glucose	+
Arginine dihydrolase (ADH)	+	meso-Inositol	–
Alkaline phosphatase (PAL)	d	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: d indeterminate result, + positive result, – negative result

Molybdenum-blue absorption spectrum

Scanning the spectroscopic profile of Mo-blue reveals the characteristic property of the heteropolymolybdate species formed. *Pseudomonas aeruginosa* strain KIK-11 formed Mo-blue exhibited a consistent maximum peak between 860 and 870 nm and a shoulder close to 700 nm, following periodic sampling as depicted in **Fig. 1**.

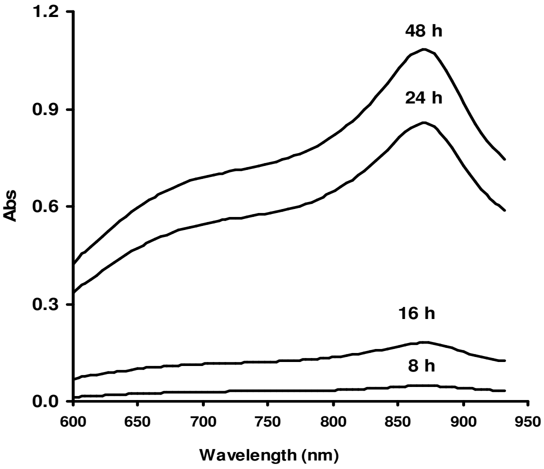

Fig. 1. Molybdenum-blue absorption spectra form *Pseudomonas aeruginosa* strain KIK-11 measured at various time intervals. LPM media was used as baseline correction.

Effect of pH and temperature on molybdate reduction

The incubation of *Pseudomonas aeruginosa* strain KIK-11 at various initial pH (5.5 to 8.0) reveals that the optimum pH for molybdate reduction to Mo-blue was between 5.8 and 6.0 (**Fig.** 2), while the optimum temperature was between 25 to 34 ºC, with no significant difference (p>0.05) when observed over a wide temperature range (20 to 50 ºC). However, temperatures exceeding 34 ºC strongly inhibits Mo-blue production by *Pseudomonas aeruginosa* strain KIK-11 (**Fig. 3**).

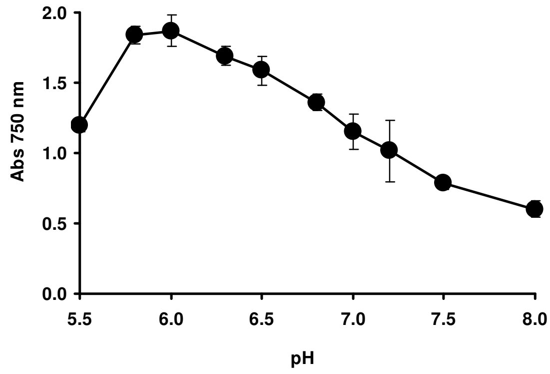

Fig. 2. Effect of pH on Mo-blue production by *Pseudomonas aeruginosa* strain KIK-11. Error bars indicate the mean ± SD of triplicates.

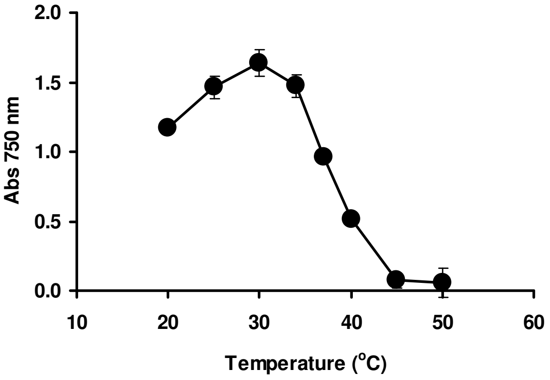

Fig. 3. Effect of temperature on Mo-blue production by *Pseudomonas aeruginosa* strain KIK-11. Error bars indicate the mean ± SD of triplicates.

Effect of electron donor sources on molybdate reduction

Studying the effect of electron donor sources reveals that glucose was the best followed by galactose, fructose and citrate supporting Mo-blue production (**Fig.** 4). Other carbon sources promoted growth but did not support Mo-blue production.

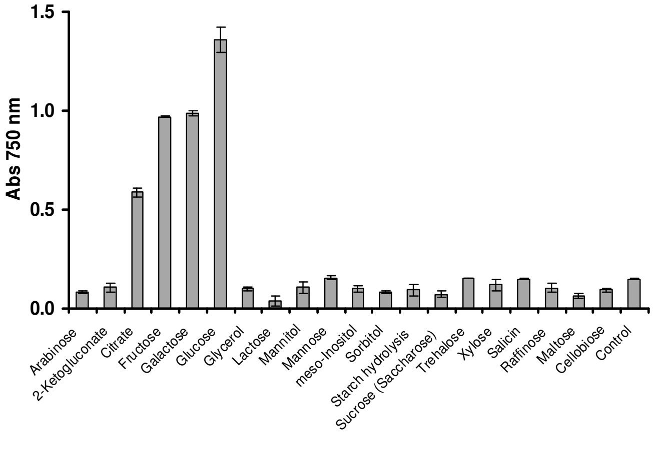

Fig. 4. Effect of various electron donor sources (1% w/v) on Mo-blue production by *Pseudomonas aeruginosa* strain KIK-11. Error bars indicate the mean ± SD of triplicates.

Effect of molybdate and phosphate on molybdate reduction

Investigating the effect of molybdate and phosphate concentration in strain KIK-11 shows that Mo-blue production was linear up to 20 mM, attaining optimal reduction between 25 and 40 mM (**Fig. 5**). Molybdate concentration higher than 40 mM was inhibitory, with a complete cessation of Mo-blue production at 70 mM molybdate. Similarly, optimal phosphate concentration supporting Mo-blue production appears to be narrow, ranging from 5.0 to 7.5 mM. Phosphate concentrations higher 15 mM was strongly inhibitory and Mo-blue production completely cease at a concentration higher than 30 mM (**Fig. 6**).

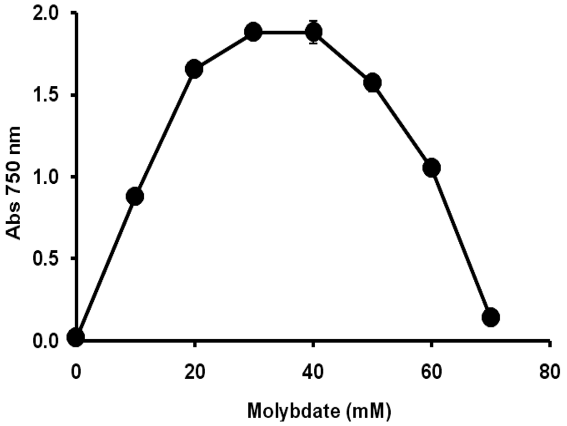

Fig. 5. Effect of molybdate concentrations on Mo-blue production by *Pseudomonas aeruginosa* strain KIK-11. Error bars indicate the mean ± SD of triplicates.

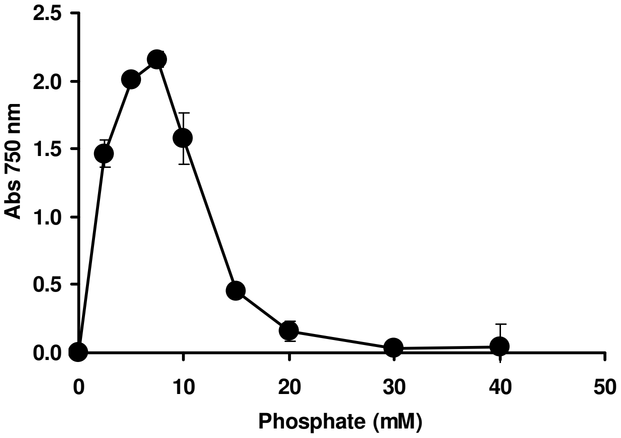

Fig. 6. Effect of phosphate concentrations on Mo-blue production by *Pseudomonas aeruginosa* strain KIK-11. Error bars indicate the mean ± SD of triplicates.

Effect of heavy metals

Heavy metals such as silver, copper and mercury at 2 ppm, inhibited molybdate reduction to Mo-blue by 27, 29 and 35% respectively, whereas, arsenic, cadmium, chromium and lead did not inhibit Mo-blue production (**Fig.** 7).

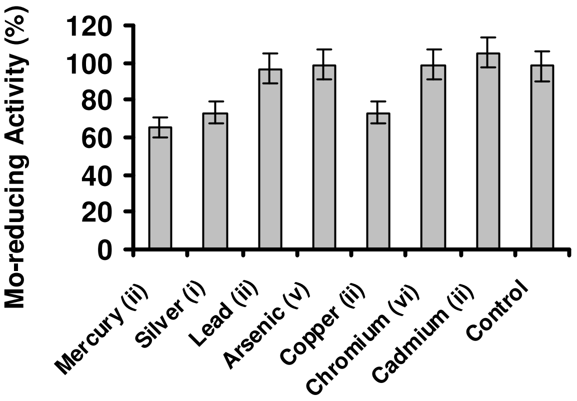

Fig. 7. Heavy metals effect on Mo-blue production by *Pseudomonas aeruginosa* strain KIK-11. Error bars indicate the mean ± SD of triplicates.

Hydrocarbon and pesticides as electron donor sources for growth and molybdenum reduction

Screening of some xenobiotics like detergents SDS, SDBS, tergitol NP9, witconol 2301, tergitol 15S9, benzethonium chloride, benzalkonium chloride and hydrocarbons diesel and crude petroleum as electron donor source for molybdate reduction failed to give a positive result (Mo-blue). However, strain KIK-11 was able to grow on SDS and diesel (**Fig. 8**) as the media became turbid following incubation.

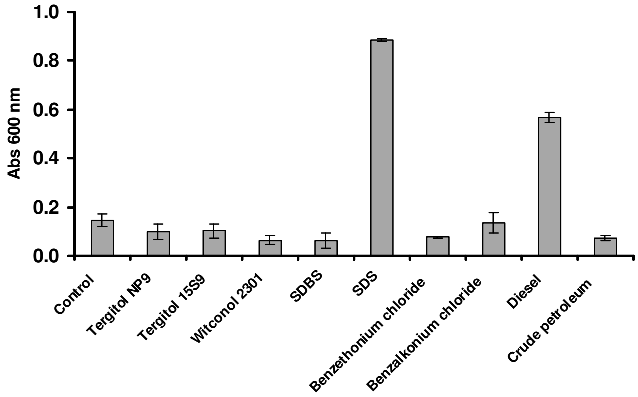

Fig. 8. The growth of *Pseudomonas aeruginosa* strain KIK-11 on xenobiotics independent of molybdenum reduction. Error bars indicate mean ± standard deviation of three replicates.

DISCUSSION

In solution, molybdenum (6+) or Mo^{6+} exists as molybdate ions $[MoO_4]^{2-}$ [31] which under acidic conditions combine to form a basket-like structure that includes the polyions $Mo_{12}O_{37}^{2}$, $Mo_8O_{26}^{4-}$ and $Mo_7O_{24}^{6-}$ [32]. The polyions upon combination with numerous anions such as arsenate, phosphate, silicate, sulphate and tungstate form arsenomolybdate, phosphomolybdate, silicomolybdate, sulphomolybdate, and tungstomolybdate, respectively [33]. These heteroatoms are positioned at the centre of the basket-like structure. In the presence of reducing agents or bacterial reducing activity would convert these polyions into Mo-blue.

This occurrence is a ubiquitous feature of molybdenum chemistry [31,33]. Microbial molybdate reduction generally involved two pathways; the first involve fractional oxidation state between 6+ and 5+ to form Mo-blue, a colloidal product with an intense blue colouration and the second uses sulphide to reduce molybdenum (Mo^{6+}) to insoluble MoS_2 (Mo^{4+}) as found *Desulfovibrio desulphuricans* [34]. Previously, this pathway leading to Mo-blue production was reported in *Thiobacillus ferreoxidans* (now *Acidothiobacillus ferreoxidans*) [35]. However, subsequent work by Yong et al. [36] demonstrated that molybdate reduction by this genus is chemically mediated by ferrous ions supplemented in the acidified growth media. It has been established that reducing agents such as ascorbic acid and ferrous ions can reduce phosphomolybdate to Mo-blue, a basis for the phosphate determination method [37]. During the last decades, research on microbial molybdate reduction to Mo-blue has been limited to local Malaysian and Antarctic isolates involving both Gram negative and positive bacteria (**Table 2**). The purification of the enzyme responsible for this process was only recently successful [19].

Table 2. List of Mo-reducing bacteria and their optimal conditions for reduction of the metal.

Bacteria	Optimal pH and Temperature for reduction	Optimal C source	Optimal Molyb-date (mM)	Optimal Phosphate (mM)	Heavy Metals that inhibit reduction	Author
Klebsiella oxytoca strain Aft-7	5.8 and 6.3 25 and 34°C	Glucose	5-20	5-7.5	Cu^{2+}, Ag^{+}, Hg^{2+}	[38]
Bacillus pumilus strain lbna	7.0-8.0 37°C	Glucose	40	2.5-5	As^{3+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Cr^{6+}, Hg^{2+}, Cu^{2+}	[18]
Bacillus sp. strain A.rzi	7.3 28-30 °C	Glucose	50	4	Cd^{2+}, Cr^{6+}, Cu^{2+},Ag^{+}, Pb^{2+}, $Hg^{2+,}$ Co^{2+},Zn^{2+}	[17]
Serratia sp. strain Dr.Y8	6.0 37 °C	Sucrose	50	5	Cr, Cu, Ag, Hg	[39]
S. marcescens strain Dr.Y9	7.0 37 °C	Sucrose	20	5	Cr^{6+}, Cu^{2+}, Ag^{+}, Hg^{2+}	[25]
Serratia sp. strain Dr.Y5	7.0 37 °C	Glucose	30	5	n.a.	[40]
Pseudomonas sp. strain DRY2	6.0 40 °C	Glucose	15-20	5	Cr^{6+}, Cu^{2+}, Pb^{2+}, Hg^{2+}	[20]
Pseudomonas sp. strain DRY1	6.5-7.5 15-20 °C	Glucose	30-50	5	Cd^{2+}, Cr^{6+}, Cu^{2+},Ag^{+}, Pb^{2+}, Hg^{2+}	[23]
Enterobacter sp. strain Dr.Y13	6.5 37 °C	Glucose	25-50	5	Cr^{6+}, Cd^{2+}, Cu^{2+}, Ag^{+}, Hg^{2+}	[41]
Acinetobacter calcoaceticus strain Dr.Y12	6.5 37 °C	Glucose	20	5	Cd^{2+}, Cr^{6+}, Cu^{2+}, Pb^{2+}, Hg^{2+}	[21]
Serratia marcescens strain DRY6	7.0 35 °C	Sucrose	15-25	5	Cr^{6+}, Cu^{2+}, Hg^{2+}*	[42]
Enterobacter cloacae strain 48	7.0 30 °C	Sucrose	20	2.9	Cr^{6+}, Cu^{2+}	[14]
Escherichia coli K12	7.0 30-36 °C	Glucose	80	5	Cr^{6+}	[12]
Klebsiella oxytoca strain hkeem	7.3 30 °C	Fructose	80	4.5	Cu^{2+}, Ag^{+}, Hg^{2+}	[16]

The bacterium was tentatively identified as *Pseudomonas aeruginosa* strain KIK-11, though more advanced technique like molecular identification may be required to further identify this species. Previously, two strains from this genus including *Pseudomonas* sp. strain DRY1 [23] and *Pseudomonas* sp. strain DRY2 [20] were reported to reduce molybdate to Mo-blue. Similarly, isolates from this genus are known to metabolically degrade both SDS and diesel [43–52]. The use of bacterial resting cells present work to characterize molybdate reduction is not new as it was earlier reported in EC 48 [14]. It has been employed also in other metals reduction like chromate [53] and selenate [54], in addition to biodegradation of organic compounds like diesel [55] and phenol [56].

The absorption spectrum of the Mo-blue produced by strain KIK-11 classically resembles ascorbate-reduced phosphomolybdate during phosphate determination method [37], with a characteristic maximum absorption peak between 880 and 890 nm shoulder at 710 nm [57]. The Mo-blue spectra of all the previously isolated molybdenum-reducing bacteria revealed similar characteristics, and particularly resembles the ascorbate-reduced phosphate determination method, hinting at the probable formation of phosphomolybdate intermediate [32,57,58].

Phosphomolybdate formation in a bacterial culture media is believed to be the result of acidification of the media by metabolism of sugars that occur under low oxygen tension [59]. Sidgwick [60] and Kazansky and Fedotov [61] established the fact that reduced phosphomolybdate (between 6+ and 5+) or Mo-blue has a Keggin structure with the general formula of $[XM_{12}O_{40}]^{n-}$, in which M is the addenda atom like tungsten and molybdenum, and X is any heteroatom such as P^{5+}, Si^{4+}, or B^{3+}. It was similarly reported during reduction of chromate from 6+ to 3+ by *Pseudomonas ambigua* [62], an intermediate species Cr^{5+} is formed, thus indicating that presence of intermediate species is not unique to molybdenum reduction.

The optimum pH supporting molybdate reduction in this bacterium could also protect the phosphomolybdate structure since the complex is unstable at alkaline pH [63]. Most molybdenum-reducing bacteria showed sub-neutral pH range from pH 5.0 to 7.0 as the optimal pH for reduction [15–19,22,23,64]. The temperature range supporting optimal Mo-blue production in this bacterium makes it suitable for bioremediation in the tropical region. The vast majority of molybdenum-reducing bacterium exhibit optimal temperature range from 25 to 37 °C [15–19,22,64].

Reducing equivalents, NADH and NADPH are required by the Mo-reducing enzyme as electron donors during molybdenum reduction [19,27], which are produced from the metabolism of sugars involving pathways like glycolysis, TCA cycle, hexose monophosphate shunt and the electron transport chain. The utilization of simple assimilable sugars like glucose and sucrose as electron donor sources supporting molybdate reduction is highly reported (**Table 2**).

Pseudomonas aeruginosa strain KIK-11 reduces as high as 60 mM molybdate but with reduced potency. The lowest optimal molybdate concentration reported was 15 mM by *Pseudomonas* sp. strain Dr.Y2 [20], whereas, 80 mM molybdate was the highest observed in *E. coli* K12 [12] and *Klebsiella oxytoca* strain hkeem [16]. However, most molybdenum reducers require moderate molybdate concentrations [15–23]. The ability of strain KIK-11 to effect reduction at high molybdate concentrations is undoubtedly a benefit to bioremediation of molybdenum contaminated sites with elevated levels. The best site to test the efficiency of this bacterium for molybdate reduction would be New Mexico, where the molybdenum level was reported to reach as high as 2,000 ppm [6].

It has been generally observed that nearly all molybdenum reducers including strain KIK-11 are affected in a similar fashion by toxic heavy metals (**Table 2**). Heavy metals like mercury and copper are strong inhibitors of bacterial chromate reduction as found in *Bacillus* sp. [65] and *Enterobacter* *cloacae* strain H01 [66]. These metal ions bind irreversibly to amide, amine, carboxyl, phosphoryl and sulfhydryl groups in the chromate reductase, thereby inactivating the enzyme. Soils with elevated levels of these toxicant metals could be supplemented with calcium carbonate, phosphate, magnesium hydroxide and manganese oxide to reduce the ions solubility and to allow bioremediation to proceed [67,68]. Immobilization of the bacterium in dialysis tubing could offer a better alternative to the toxicity of these metals [69].

Previously, two molybdenum-reducers with SDS-degrading potential have been isolated [38,47], which is a positive development towards finding a more reducers having the multi-toxicant degrading capacity. A number of SDS-degrading bacteria from various species are reported in the literature [46,70–75] including a cold-adapted bacterium [76].

SDS as detergent kills microbial population by stripping the outer lipopolysaccharide layer in Gram-negative bacteria thereby depolarization of the cell membrane and ultimately alters with the absorption of important nutrient [77]. Similarly, diesel is toxic to microorganism at high concentration [78], due to the solvent effect that damages bacterial cell membrane. Several genera like *Aeruginosa*, *Aeromonas*, *Acinetobacter*, *Bacillus*, Flavobacterium, *Klebsiella*, *Micrococcus*, *Proteus*, *Pseudomonas*, *Serratia* and *Staphylococcus* were reported as hydrocarbon utilizers [52,78–81]. Hydrocarbon and SDS-tolerant bacteria like this bacterium have a unique cell wall and cell membrane compositions that enable them to assimilate SDS and diesel much more efficiently.

CONCLUSION

A bacterium with the ability to degrade SDS and diesel in addition to molybdate reduction has been isolated from metal contaminated soil from Nigeria and tentatively identified as *Pseudomonas aeruginosa* strain KIK-11. This bacterium displayed a wide range of optimum temperature and molybdate concentration, but with a narrow range of pH and phosphate concentration that support Mo-blue production. Glucose, galactose and fructose were the best electron donor sources for the reduction. Molybdenum reduction in this bacterium was significantly inhibited by copper (II), mercury (II) and silver (I). The ability of strain KIK-11 to degrade xenobiotics as well detoxify heavy metals suggests that it will be a useful candidate for bioremediation of polluted sites. Research is still underway to purify molybdenum-reducing enzyme and characterize the SDS and diesel degradation in more detail, which may include quantification of residual diesel and SDS using the methylene blue assay and gas chromatography, respectively.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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