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Preliminary Study towards Enhanced Crude Oil Biodegradation Reveals Congeneric Total Peroxidases with Striking Distinctions

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Abstract

Peroxidases (POXs) are the key extracellular enzymes produced by crude oil degrading microbes. Knowledge of optimum conditions for POXs activity is crucial for providing effective environment for bioremediation. In this study, physicochemical properties of POXs produced by *Actinomyces israelii* and *Actinomyces viscosus* during growth on crude oil were studied. The POXs exhibited similarities in activity and stability with striking differences in response to two divalent metal ions. The POXs from both species had optimum pH of 7.0 and were very stable over a narrow pH range (6.0 - 8.0). The POXs demonstrated similar thermostability exhibiting relative residual activity of 62% at 50˚C after 30 min incubation and 45% residual activity at the same temperature after 60 min despite the fact that POXs from *A. viscosus* and *A. israelii* had optimum temperatures of 50˚C and 40˚C, respectively. The POXs from *A. viscosus* and *A. israelii* were greatly activated by Fe^{2+} at 5.0 and 10.0 mM. The enzymes were both strongly inhibited by Cu^{2+}, Mg^{2+} and Hg^{2+}. Surprisingly, these congeneric POXs demonstrated striking differences in their response to Ca^{2+} and Mn^{2+}. POX from *A. viscosus* was activated by Ca^{2+} and Mn^{2+} exhibiting relative activity of 136% and 106% at 5 mM, respectively. In contrast, POX from *A. israelii* was strongly inhibited by Ca^{2+} and Mn^{2+} exhibiting 62.5% relative activity in the presence of 5 mM of each metal ion. Increasing the concentration of Ca^{2+} and Mn^{2+} led to further activation of POX from *A. viscosus* and inhibition of POX from *A. israelii.* Results provide deeper insights into functional properties of studied POXs from closely related microbes. The physicochemical properties are very similar; however, notable differences provide a strong basis for structural characterization of these congeneric enzymes.

Keywords

***Actinomyces israelii*, *Actinomyces viscosus*, Bioremediation, Congeneric Enzymes, Crude Oil, Peroxidases**

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1. Introduction

Crude oil spills have caused destruction of farmlands, contamination of water bodies and loss of biodiversity [1] [2]. Crude oil is a natural, heterogeneous mixture of hydrocarbons, consisting mainly of alkanes with different chain lengths and branch points, cycloalkanes, mono-aromatic and polycyclic aromatic hydrocarbons. Some compounds contain nitrogen, sulfur and oxygen with trace amounts of phosphorus while presence of heavy metals such as nickel and vanadium has also been reported [3] [4]. Many polycyclic aromatic hydrocarbons (PAHs) in crude oil are highly toxic, mutagenic and carcinogenic to microorganisms and higher systems including humans [5].

Among the different remediation techniques, bioremediation is the most advantageous due to its cost effectiveness and environmental friendliness [6] [7]. This has led to screening for various microorganisms with high crude oil degrading efficiency. Actinobacteria have been reported as useful microbes in the bioremediation of soils contaminated with crude oil and derivatives [8]-[10]. A wide variety of microbes initiate biodegradation of polyaromatic and aliphatic hydrocarbons by co-metabolism using extracellular and intracellular enzymes of which peroxidases play a crucial role [11] [12]. Enzymes involved in the degradation of PAHs are oxygenase, dehydrogenase and lignolytic enzymes comprising peroxidases and laccases [13]. Peroxidases (POXs) have been considered useful in the degradation of organic pollutants, especially, poly aromatic hydrocarbons [14]-[16]. Peroxidases are redox enzymes which are found in all forms of life where they play diverse roles. It is quite surprising that peroxidases isolated from fungi and plants have been extensively studied while actinobacterial peroxidases have been unexplored despite their diverse metabolic capabilities which represent large resource for biocatalysis [17].

It is very important to note that the microorganisms used for bioremediation secrete extracellular enzymes which initiate the degradation of the crude oil hydrocarbons. Knowledge of the optimum conditions for the activity of secreted enzymes is envisaged to provide effective environment for bioremediation by the crude oil degrading microbes. Since peroxidases are the key extracellular enzymes involved in crude oil biodegradation, it is highly pertinent to determine physicochemical properties of the peroxidases produced by crude oil degrading microbes.

In this study, whole cells of *Actinomyces israelii* and *Actinomyces viscosus* were used to degrade crude oil and the activity of total extracellular peroxidases (POXs) produced by the organisms during biodegradation was determined. Physicochemical properties of POXs from the two species of the same genus, *Actinomyces*, were investigated with the aim of determining the optimal conditions for the activity of the enzymes for effective use of the actinobacteria in crude oil degradation. Comparative analysis of the exhibited physicochemical properties of the congeneric POXs was carried out.

2. Materials and Methods

2.1. Materials

Media components were products of Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade and obtained from Sigma-Aldrich.

2.2. Microorganism, Culture Preparation and Peroxidase (POX) Production

The microorganisms used for this study were isolated from soil samples of crude oil spill site in Ogoniland, Niger Delta region of Nigeria. The isolation and identification of isolates were done by the Biotechnology Unit of Federal Institute of Industrial Research, Lagos based on methods described in Bergey's Manual of Systematic Bacteriology [18]. The isolates used for this study were identified as *Actinomyces viscosus* and *Actinomyces israelii*. They were maintained on nutrient agar slants and stored at 4˚C. The seed inoculum was prepared by growing a loopful of slant culture in nutrient broth containing peptone (5 g/L), NaCl (5 g/L), beef extract (1.5 g/L) and yeast extract (1.5 g/L) at pH 7.4. The seed culture was incubated at 30˚C for 24 h at 180 rpm in a shaking incubator (Stuart, UK) and used as inoculum (5% v/v) for crude oil based medium (COBM). The COBM contained 1% crude oil as sole carbon source, NH_4NO_3 (2.0 g/L), KH_2PO_4 (0.2 g/L), $KH_2PO_4 \cdot 12H_2O$ (0.2 g/L), NaCl (0.8 g/L), KCl (0.8 g/L), $CaCl_2 \cdot 2H_2O$ (0.1 g/L), $MgSO_4$ (0.2 g/L) and $FeSO_4 \cdot 7H_2O$ (0.002 g/L), pH 7.0. Cultures grown on COBM were incubated at 30˚C for 144 h at 180 rpm. At the end of the cultivation period, the broths were centrifuged at 10,000 rpm for 20 min at 4˚C. The clear supernatants were recovered and assayed for total POX activity. The total peroxidase activities produced from *Actinomyces viscosus* and *Actinomyces israelii* were 1.03

U/L and 1.54 U/L, respectively (unpublished data). The crude enzyme solution was subjected to further studies.

2.3. Assay of Total Peroxidase (POX) Activity

Total POX activity was determined according to the method of Hunter *et al*. [19] with slight modifications. POX activity was determined via oxidation of 0.24 mM 2,2'azino-di-[3-ethyl benzothiazoline-6-sulphonic acid] (ABTS) buffered with 50 mM sodium acetate buffer pH 5 in the presence of 5 mM H_2O_2 at 414 nm for 5 min in a UV/Visible spectrophotometer. The reaction mixture (3 mL) was made up of 1 mL ABTS solution, 1 mL enzyme solution and 1 mL H_2O_2 solution. Controls were set up as the reaction mixture without crude enzyme; 50 mM sodium acetate buffer pH 5 (1 mL) replaced the enzyme solution. One unit (U) of peroxidase activity was defined as the amount of enzyme oxidizing 1 µmol ABTS per minute at pH 5.0 and 30˚C with a molar extinction coefficient for the ABTS radical cation of 31,100 $M^{-1}\cdot cm^{-1}$ at 414 nm.

2.4. Investigation of Physicochemical Properties of Total POXs from *A. viscosus* and *A. israelii*

2.4.1. Effect of pH on Total POX Activity and Stability

The effect of pH on activity of the crude total POX was determined over a pH range of 5.0 - 9.0. The pH was adjusted using 50 mM of the following buffer solutions: sodium acetate (pH 5.0 - 5.5), sodium citrate (6.0 - 6.5), Tris-HCl (pH 7.0 to 8.5) and glycine-NaOH (pH 9.0), respectively. POX activity was determined using the standard assay method earlier described. pH stability of total POX was determined by incubating the crude extract with relevant buffer solution for 24 h before assay of the residual activity which was done according to the standard assay procedure.

2.4.2. Effect of Temperature on Total POX Activity and Stability

The activity of total POX was determined in the reaction mixture at different temperatures ranging from 30˚C to 70˚C according to the standard assay procedure earlier described. Stability of total POX at different temperatures was carried out by prior incubation of the enzyme solution at temperatures ranging from 40˚C to 70˚C over a period of 30 to 180 min before determination of residual activity following the standard assay procedure.

2.4.3. Effect of EDTA and Divalent Metal Ions on Total POX Activity

Effect of 5 and 10 mM of metal chelating agent, ethylene diamine tetra acetic acid (EDTA) on POX activity was determined. Also, effects of divalent metal ions (Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Hg^{2+}) on POX activity were evaluated at 5 and 10 mM. Each metallic chloride was added to the reaction mixture at the optimum temperature and pH determined for the enzyme and POX activity was determined in comparison with controls according to the standard assay procedure.

3. Results and Discussion

3.1. Effect of pH on Total POX Activity and Stability

The total POXs from *A. viscosus* and *A. israelii* were active over pH range of 4.0 to 9.0 exhibiting above 60% relative activity over pH 6.0 to 8.5 with optimum pH at 7.0 (**Figure 1(a)**). Some studies have reported optimum pH for POXs from fungi in the range of 4.0 - 7.0 [20]-[22] while there are reports showing pH optimum in range of 2.0 - 5.0 for fungal POXs [23]-[25]. In contrast, recently reported bacterial POXs from *Pseudomonas* sp. SUK1 and *Bacillus* sp. VUS had optimum pH of 3.0 [26] [27]. Our present studies show that actinobacterial POXs have optimum pH of 7.0. POXs from both *A. viscosus* and *A. israelii* were very stable between pH 6.0 and 8.0 exhibiting maximum stability at pH 7.0 with 64% and 77% relative residual activity at pH 6.0 and 8.0, respectively (**Figure 1(b)**). Rodrıguez-Couto *et al*. [28] reported POXs from white rot fungus, *Phanerochaete chrysosporium* which exhibited great stability only at pH around 4.5. Also, POX from *Pleurotus sajor caju* MTCC-141 was stable and active around pH 3.0 [29]. The actinobacterial POXs under study demonstrated unique stability around neutral pH.

3.2. Effect of Temperature on Total POX Activity and Stability

The optimum temperature for the activity of total POX from *A. viscosus* was 50˚C. There was a decline in

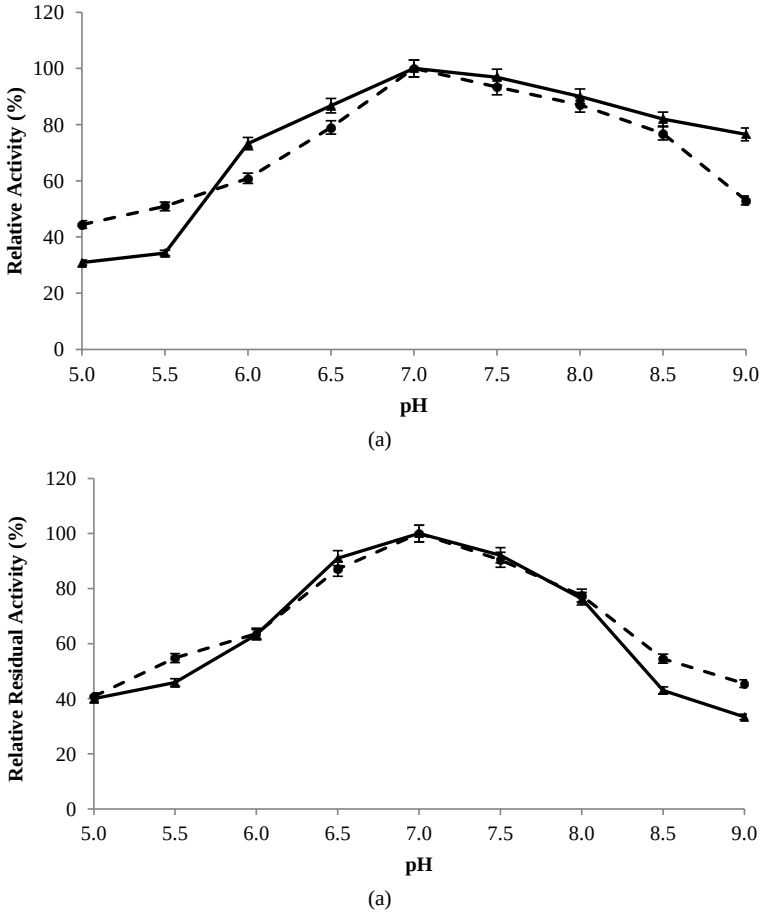
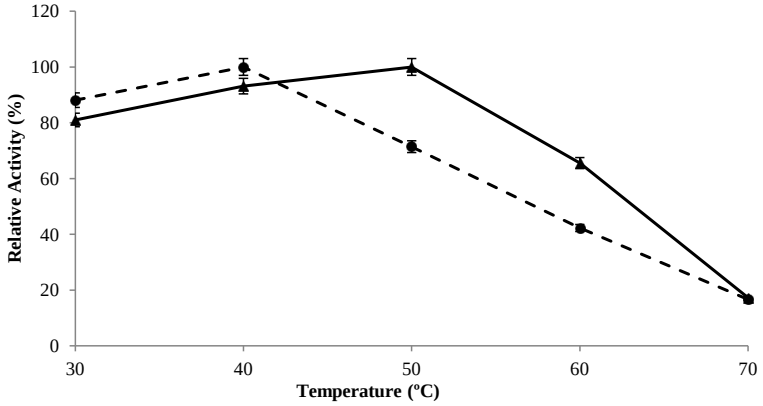
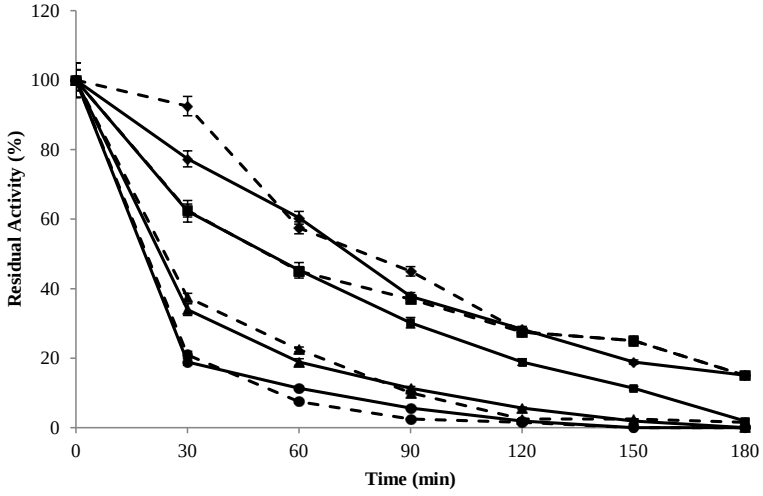


Figure 1. (a) Effect of pH on activity of POXs from *Actinomyces israelii* (- - -) and *Actinomyces viscosus* (—); (b) Effect of pH on the stability of POXs from *A. israelii* (- - -) and *A. viscosu s* (—). Maximum activity was expressed as 100%. Symbols and bars represent mean values and standard deviations of triplicate determinations.

activity at higher temperatures with the enzyme having a relative activity of 65.5% at 60˚C (**Figure 2(a)**). Similar result was obtained for POX from *P. chrysosporium* which was optimally active at 55˚C [25]. The enzyme showed a drastic loss of activity at 70˚C exhibiting only 17% relative activity due to heat denaturation. Total POX from *A. israelii* had optimum temperature of 40˚C while it exhibited relative activity of 71% and 42% at 50˚C and 60˚C, respectively. The enzyme also had a drastic loss of activity at 70˚C similar to what was observed on POX from *A. viscosus* showing about 17% relative activity. Similar finding was reported on the bacterial POX from *Pseudomonas* sp. SUK1 which was optimally active at 40˚C [26]. Lower optimum temperatures of 25˚C and 35˚C have been reported for POXs from *B. pumilus* and *Paenibacillus* sp. [22] while POX from *Loweporus lividus* MTCC-1178 was optimally active at 24˚C [29]. The difference in optimum temperature of the POXs under study could be related to some structural differences in the total peroxidases. Total POXs from *A. viscosus* and *A. israelii* showed high stability between 40˚C and 50˚C which decreased with time (**Figure 2(b)**). The enzymes exhibited about 60% residual relative activity after 60 min incubation at 40˚C and 45% relative residual activity at 50˚C after 60 min incubation, respectively. Activity of total POXs from both species was completely lost at 70˚C after 150 minutes of incubation. Similarly, POXs from *Rhizoctonia* sp. SYBC-M3 and *P. chrysosporium* were totally inactivated at 65˚C [30] [31]. In another report, POX from the white-rot fungus, *Irpex lacteus* showed high stability between 30˚C and 40˚C [32]. Rodríguez-Couto *et al*. [28] reported POXs from *P. chrysosporium* which were most stable between 32˚C and 34˚C. High thermostability is an attractive and desirable characteristic of an enzyme for a variety of industrial and biotechnological applications [33]-[35]. The total POXs under study demonstrated higher thermostability than most of the earlier reported POXs. This suggests that the two actinobacteria under study have potential for use in bioremediation and industry as producers of thermostable POXs.



(a)



(b)

Figure 2. (a) Effect of temperature on activity of POXs from *A. viscosus* (—) and *A. israelii* (---); (b) Effect of varied temperature (40˚C (♦), 50˚C (■), 60˚C (▲) and 70˚C (●) on stability of POXs from *A. viscosus* and *A. israelii* over 30 to 180 min pre-incubation period at Maximum activity was expressed as100%. Symbols and bars represent mean values and standard deviations of triplicate determinations.

3.3. Effect of EDTA and Metal Ions on Activity of Total POXs

EDTA inhibited the activity of the total POXs at 5 mM and completely inhibited the enzyme activity at 10 mM. Strong inhibition of POXs by EDTA had been reported in previous studies [36]-[38]. This inhibition is due to metal chelating characteristic of EDTA which makes metal ions that are co-factors for the activity of POXs unavailable to the enzymes. The activity of total POXs from both species under study was enhanced greatly in the presence of 5 and 10 mM Fe^{2+} (**Table 1**). The POXs from *A. viscosus* and *A. israelii* were activated 22-fold in the presence of 10 mM Fe^{2+} while activation in the presence of 5 mM Fe^{2+} was 7- and 8.6-fold for POXs from *A. viscosus* and *A. israelii*, respectively. Asgher *et al*. [36] and Cheng *et al*. [38] had reported activation of POXs by low concentration of Fe^{2+} while strong inhibitory effects of Fe^{2+} on POXs have also been reported in the presence of 1 and 10 mM Fe^{2+} [38] [39]. Our results have demonstrated that Fe^{2+} is a possible cofactor for the POXs under study and suggested that the binding of Fe^{2+} stabilizes and activates the enzymes. However, total POXs from *A. viscosus* and *A. israelii* were both strongly inhibited by Cu^{2+}, Mg^{2+} and Hg^{2+} (**Table 1**). Similar inhibition of activity was reported for POXs in the presence of Cu^{2+}, Mg^{2+} and Hg^{2+} in the studies by Cai *et al*. [30] and Praveen *et al*. [37]. The inhibitory characteristic of Hg^{2+} on most enzymes is due to its high affinity for thiol groups in proteins which leads to inactivation of the enzymes [40]. It is interesting to note that despite the fact that total POXs from *A. viscosus* and *A. israelii* exhibit many similar physicochemical properties as congeneric enzymes, they also demonstrated striking differences in their response to Ca^{2+} and Mn^{2+}. The activity of

Table 1. Effects of divalent metal ions and EDTA on peroxidase activity.

Metal ions	Concentration (mM)	*Relative activity (%)	
		Actinomyces viscosus	***Actinomyces israelii***
Ca^{2+}	5	136.36 ± 0.08	62.50 ± 0.53
	10	151.82 ± 0.03	12.50 ± 0.02
Fe^{2+}	5	697.27 ± 0.04	858.75 ± 0.02
	10	2236.36 ± 0.04	2158.75 ± 0.03
Cu^{2+}	5	50.91 ± 0.02	57.50 ± 0.02
	10	18.18 ± 0.02	25.00 ± 0.03
Mg^{2+}	5	27.27 ± 0.01	8.13 ± 0.01
	10	13.64 ± 0.02	6.25 ± 0.03
Mn^{2+}	5	106.36 ± 0.01	62.50 ± 0.01
	10	124.54 ± 0.02	50.47 ± 0.02
Hg^{2+}	5	9.09 ± 0.01	12.50 ± 0.02
	10	5.45 ± 0.02	1.25 ± 0.03
EDTA	5	7.04 ± 0.02	12.50 ± 0.01
	10	0.00	0.00

*Relative activity of peroxidase (POX) at each concentration of metal ion was expressed as % in comparison to the activity of POX of control without metal ion. Data in the table are mean values of triplicate determinations.

total POX from *A. viscosus* was enhanced by Ca^{2+} 1.4- and 1.5-fold at 5 and 10 mM (**Table 1**). Surprisingly, total POX from *A. israelii* was inhibited by Ca^{2+} exhibiting 62.5% and 12.5% relative activity in the presence of 5 and 10 mM Ca^{2+}, respectively. Inhibitory effect of Ca^{2+} on POX activity is quite rare though it was reported by Cheng *et al*. [38] on POX from white-rot fungus, *Schizophyllum* sp. F17 which exhibited 89% relative activity in the presence of 1 mM Ca^{2+}. In another study, Ca^{2+} had insignificant effect on POX activity [41]. Similar to our results on activation of total POX from *A. viscosus* by Ca^{2+} is the report by Praveen *et al*. [37] on POX from *Stereum ostrea*. The activation of POXs has been attributed to the role played by the calcium binding sites on proximal and distal domains of the POX structure [42]. Ca^{2+} has earlier been reported to contribute to structural stability of heme POXs [43]. Peroxidases undergo a catalytic cycle during which electron transfer results in the reduction of H_2O_2 to water [17]. The inhibition of total POX from *A. israelii* by Ca^{2+} was found to be concentration dependent because the relative activity of the enzyme was lower in the presence of 10 mM Ca^{2+} than with 5 mM. In fact, activation of POX from *S. ostrea* reported by Praveen *et al*. [37] was higher at lower concentration of Ca^{2+} (133% at 1 mM) than with higher concentration of Ca^{2+} (111% at 10 mM). The disparity in response of the total POXs under study to Ca^{2+} remains unclear. Interestingly, Mn^{2+} also enhanced activity of POX from *A. viscosus* but inhibited the activity of POX from *A. israelii* at 5 and 10 mM (**Table 1**). The activity of total POX from *A. viscosus* was enhanced 1.2-fold in the presence of 10 mM Mn^{2+} while total POX from *A. israelii* was strongly inhibited at same concentration exhibiting 50% relative activity. Janusz *et al*. [44] reported in a review that Mn^{2+} enhances manganese peroxidase activity and inhibits lignin peroxidase activity. Cheng *et al*. [38] in their studies recorded inhibition of POX from *Schizophyllum* sp. F17 at 0.1 and 1 mM Mn^{2+} and activation at 10 mM. Activity of POX from *A. israelii* was inhibited at both 5 and 10 mM Mn^{2+}. The differences in total POXs from these two related species under study suggest that the properties of total POXs produced from *A. viscosus* and *A. israelii* could be related to the dominant POX in the crude total POX. This further explains that the mechanism of oxidation by the total POXs is not the same in the presence of metal ions.

4. Conclusion

Effects of metal ions on the POXs suggest that the presence of metal ions in oil contaminated sites could either promote or hinder bioremediation process. The total POXs under study from the same genus, *Actinomyces*, but different species, were found to possess very similar properties. However, there was a very clear distinction between the total POXs in their tolerance to Ca^{2+} and Mn^{2+}. The differing characteristics could be related to difference in flexibility of the active sites of individual POXs constituting the total POX. Detailed purification and characterization of these congeneric POXs are in progress to provide further insights into their mechanism of action. The physicochemical properties of total extracellular POXs from *A. viscosus* and *A. israelii* have estab-

lished the optimum conditions under which the actinobacteria under study could be effective for bioremediation purposes.

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