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Potential Bioremediation Charactristics of Microorganisms Isolated from Crude Oil Effluent with High Concentrations of Copper and Lead Ions

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Abstract: Rapid industrialization and urbanization have resulted in the generation of large quantities of aqueous effluents, many of which contain high levels of toxic heavy metals which can be harmful to organisms. For this reason, a number of organisms including bacteria develop processes which are able to withstand the effects of these pollutants. Crude oil effluent from the Obagi Flow Station, Total Petroleum and Exploration Nigeria (TPENG), Omoku, River State was analyzed for its copper and lead content. Results showed high levels of both metals, above EPA and CELAC recommended environmentally accepted standards. Subsequently, microorganisms were isolated from the effluent and from the effluent-contaminated soil from the site. The most successful colony was subsequently characterized and identified as *Bacillus subtilis*. Due to the fact that the Gram positive rod, *Bacillus subtilis* is known to have affinity for metal ions, the effect of pH on its metal ion sorption and accumulation was investigated. Biosorptive processes were carried out using the organism in mineral salt media containing the effluent as the sole carbon source. Effect of pH on the growth and rate of biosorption was assessed with optimum pH determined at pH 7.5-8.0. Results showed complete removal of Cu^{2+} and Pb^{2+} in the medium that showed highest growth of organism. Thus, suggesting that the organism could have a potential application as adsorptive/bioaccumulative agent in the removal/recovery of metal ions from industrial effluents before their disposal into the environment as well as in heavy metal clean-up.

Key words: Flow station · Bioremediation · Biosorption · *Bacillus subtilis* · Heavy metals

INTRODUCTION

The present day has witnessed extensive attention on the management of environmental pollution resulting from hazardous material such as heavy metals from industrial effluent. Petroleum refinery effluents are waste liquids that resulted from refining of crude oil. The effluent composed several organic and inorganic including heavy metals [1]. A number of these heavy metal compounds represent an ongoing eco-toxicological threat. The disposal of these effluents on land has become a regular practice for some industries leading to subsequent pollution of groundwater and soil of surrounding farmlands [2]. It has been reported that the amount of heavy metals pollutant generated from anthropogenic source such as industrial waste has globally outweigh those from natural sources [3]. Comprehensive analysis of Nigeria crude oil waste water effluent showed the presence of heavy metals like Cu, Pb and Zn among other organic and inorganic compounds, which are known to exert toxic effects at certain concentrations [4]. When improperly disposed, they may be taken up by plants and concentrated in certain parts such as the leaf, stem and root, which can be subsequently transferred to other food chain.

For efficient removal and cleaning of these contaminants, physical and chemical methods such as volatilization, photooxidation, chemical oxidation and bioaccumulati have been utilized [5]. However; these methods are expensive and not always effective. There has been a rising interest in the use of microorganisms for the removal and the recovery of heavy metals from sites contaminated with industrial effluent. These organisms are employed due to their ability to

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accumulate heavy metals even in very low concentration [6]. This seems to be more advantageous because of its cost effective, more efficient and easier to operate without damaging the site or its indigenous flora and fauna and also does not produce chemical sludge [7, 8 and 9]. Microorganisms tolerant to metals are often isolated from areas of high metal loading, suggesting that metal tolerance or resistance is an adaptive response to excessive metal exposure. Algae, bacteria, fungi and yeasts have proved to be potential metal biosorbents, due to their metal sequestering properties and can decrease the concentration of heavy metal ions in solution [10]. The objectives of this work include: (1) to investigate the heavy metal content of crude oil effluent from both Total Petroleum and Exploration Nigeria Limited (TPENG) Omoku flow station. (2) To isolate and characterize microorganisms that have tolerant growth rate both in flow station effluent sample and soil sample from the site contaminated by this effluent. (3) To investigate the bioremediation process of these isolated organism.

MATERIALS AND METHODS

Isolation of Crude Oil Effluent Degrading Bacteria: Crude oil effluent was collected from the analytical laboratory for Obagi Flow Station, Total Petroleum and Exploration Nig. Ltd (TPENG) at Omoku, Rivers state. Also soil sample was collected from the TPENG contaminated site, at the same flow station. The isolation of crude oil degrading bacteria was investigated under aerobic condition with crude oil as sole source of carbon. The compositions of the mineral salt media (MSM) were as follows: (g/L): 1.0 (NH_4) SO_4, 1.0 KH_2PO_4, 1.0 $Na_2HPO_4.H_2O$, 0.2 MgSO4.7H_2O, 0.01 $CaCl_2$ and 0.002 $FeCl_3$.6H2O. The pH was the adjusted to 5–8 and then amended with 1% filter sterilized crude oil (v/v) using the method of Liu *et al.* [11]. Samples were incubated in flasks on shaker at 120 rpm for 5 days at 30°C. Bacterial growth was measured by using spectrophotometer (Unico Instruments UV 2600, Japan) at 600 nm and compared with control without inoculation.

Preliminary Test for Degradation Ability: The determination of degradation capabilities of the bacteria isolates was carried out by growing them on crude oil effluent plates containing petroleum oil 0.5% (v/v). The incubation of the plates was carried out at 30°C for 18 hours. The strain showing the highest oil degradation capability was selected as a good candidate for extensive study.

Determination of Copper and Lead: The crude oil effluent obtained was digested using the wet oxidation method and concentration of copper and lead in was determined using Atomic Absorption

Spectrophotometer (Varian Spectra Aa200, Japan)

Identification of Bacteria: The ability of the bacteria to grow in presence of crude oil as sole source of carbon in growth media was used as basis for selection. The colonies with higher growth rates were selected for further experiments and identified using Biolog Gen III (Biolog Inc., USA) identification system.

Preparation of Innocula: The aliquot portion (0.1 mL) of the overnight nutrient culture of each strain and mixed consortium was washed twice in physiological saline solution (0.87% NaCl, pH 7.2) and suspended in the same to optical density of 0.1 (OD600) [12].

Biodegradation Assay: The overnight culture containing both individual and mixed bacterial consortiums at the log phase of growth were added to 250mL conical flasks, each containing 100mL of sterile mineral salts medium with (0.2% v/v) crude oil effluent [13]. The experiment was carried out in triplicate. The controls constitute uninoculated flasks c, accounting for abiotic losses. The flasks were incubated at 22°C for specified time intervals of (7, 14 and 21 days). The determination of the residual concentrations of Cu and Pb was carried out using AAS.

RESULTS

Analysis of Crude Oil Effluent: The result of lead and copper ions present in the effluent sample (Table 1) showed a value of 24.8 and 8.64 mg/L respectively, which is considered quite high to reach water source or soil.

Isolation and Identification of Bacteria Isolate: A total of 5 cultures of bacteria were isolated from the crude oil effluent while 6 cultures were isolated from the soil contaminated with the effluent. However, only one isolate (*Bacillus subtilis)* from the crude effluent showed highest number of colonies while two isolate (*Bacillus subtilis* and *Psuedomonas*) (Figure 1 & 2) from the contaminated soil were selected for further screening. The morphological and biochemical characteristics of isolates from effluent sample showed a rod with rounded shape, presence of central endospore and gram positive stain.

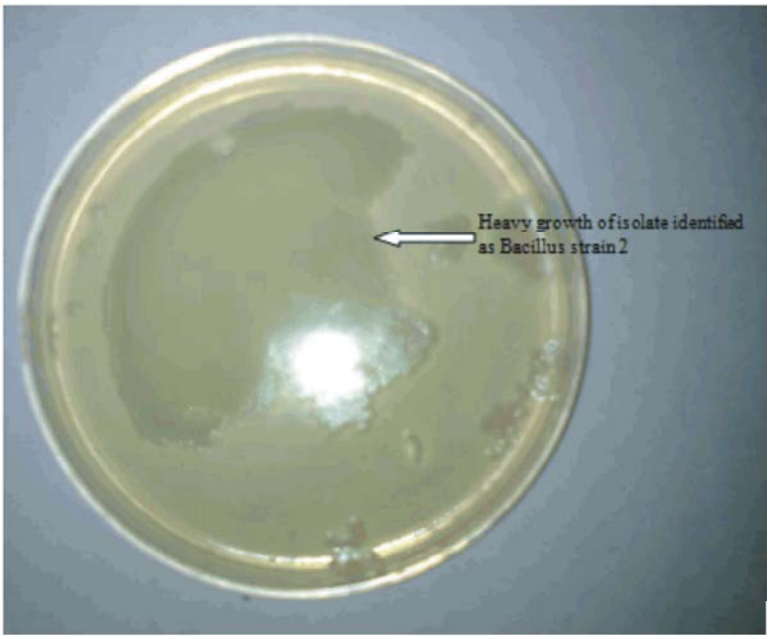

Fig. 1:

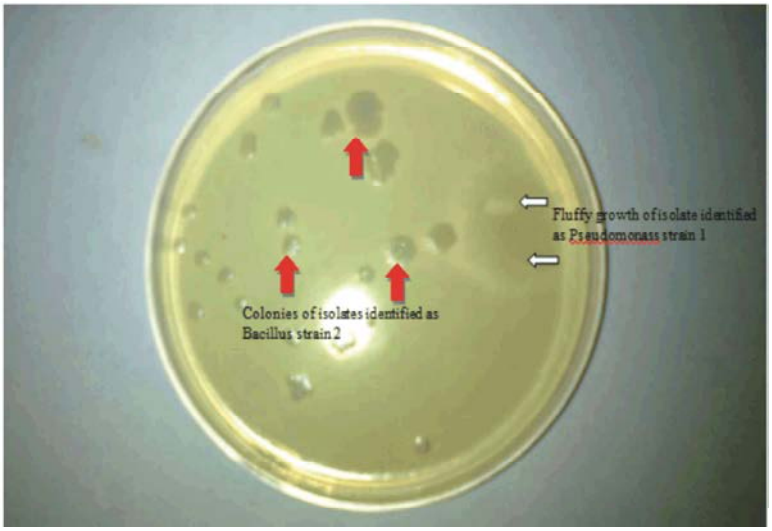

Fig. 2:

Table 1: Concentration of metals in flow station petroleum sample

Metal	Concentration mg/l
Copper (Cu)	8.64 ± 0.02
Lead (Pb)	24.8 ± 0.03

*Mean ± SD of duplicate experiments

Table 2: Morphology of microbial isolates from crude oil effluent sample

		Colony Morphology				
Colonies	Av. Number of Colonies obs/plate	Edge	Elevation	Texture	Colour	Tentative Name
1	5 ± 0.11	Serrated	Flat	Dry	Milky	*Bacillus Str. 1
2	8 ± 0.13	Entire	Raised	Dry	Milky	*Bacillus Str. 2

*Identified as Bacillus

Table 3: Morphology of Microbial Isolates from the Soil contaminated by crude oil effluent Sample

		Colony Morphology				
Colonies	Av. Number of Colonies obs/plate	Edge	Elevation	Texture	Colour	Tentative Name
1	3 ± 0.01	Serrated	Flat	Dry	Milky	*Bacillus Str. 1
2	5 ± 0.1	Entire	Raised	Dry/fluffy	Milky-yellow	**Pseudomonas Str 1
3	7 ± 0.01	Entire	Raised	Dry	Milky	*Bacillus Str. 2

Identified as Bacillus ** Identified as Pseudomonas

Table 4: Morphological and Biochemical Characteristics of isolates from Effluent sample

Test.	Result
Aeration	Aerobic
Shape	Rod with rounded end
Arrangement	Pairs and chains
Endospores	Present (central)
Gram stain	+
Motility	+
Catalase	+
Capsule	-

Key: + = Positive reaction; - = Negative reaction

Table 5: Residual Metal ion Concentrations after 38 days of incubation with *Bacillus subtilis*

Group	Initial Conc. (mg/100ml) At Time, t = 0	Conc. of Cu (mg/100ml) At Time, t = 38	Conc. of Cu (mg/100ml) At Time, t = 38
A (Cu)	0.86	Not detected	
B (Pb)	2.48	-	Not detected
C (Cu+Pb)	3.34	Not detected	Not detected

*Not detected may mean that the metal ions have been completely adsorbed or removed by the microorganism or that the instrument's sensitivity could not detect the remaining concentration

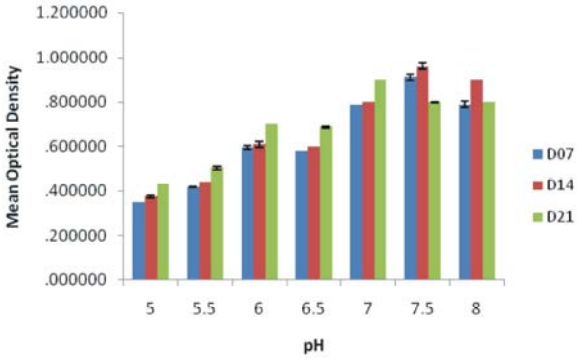

Fig. 3(a)

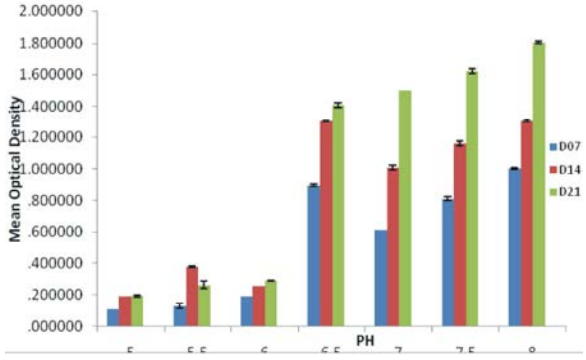

Fig. 3(b)

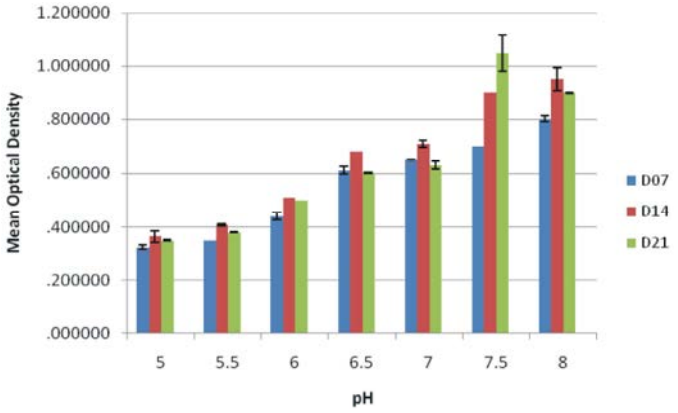

Fig. 3(c)

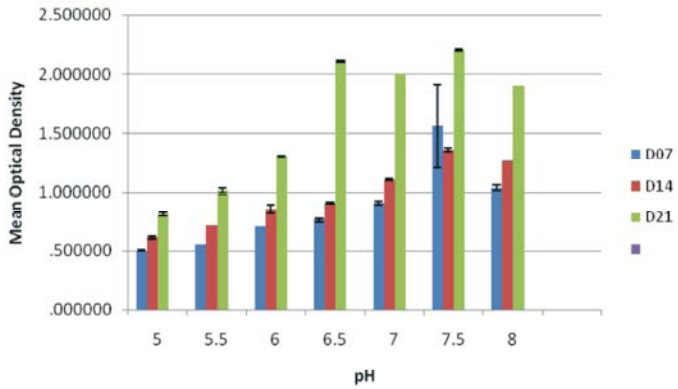

Fig. 3(d)

Oil Biodegradation Behaviour of *Bacillus strain* in Different P^{H} Media: The growth of the organism in media contaminated with lead, copper and then mixed consortium with varying pH was investigated (Fig. 3a-3d). The result showed that the optimum growth of the organism ranges from pH of 7.5-8.0. The growth profile of the isolated organism showed that the organism grow better in effluent contaminated by individual metals than mixed consortium. The residual metal ion concentration after 38 days incubation showed that the metal ions were completely removed from the media (Table 4).

Recovered/Accumulated or Sorbed Copper and Lead ions from the Bacterial Biomass: The amount of copper and lead ions and the mixed ion from the bacteria biomass was investigated (Table 5). The result showed that approximately 62% of copper ion, 59% of lead ion and 52% of the mixed ions were recovered from the bacteria biomass, indicating a significant difference between the recovered ion in bacteria biomass and the removed ion from the media. These differences could be from the washing of the biomass prior to determination of the metal ions.

DISCUSSION

The recent time has witnessed increased discharge of industrial effluent into the environment due to rising population and rapid industrialization. One major public and environmental problem is pollution of soil and aquatic system with heavy Metals from industrial effluent. The removal of heavy metals from industrial effluent using conventional methods are often costly and less efficient, therefore, biological removal considered as a cheap treatment method, which proved high capability for pollutants removal [14]. Microorganisms tolerant to metals are often isolated from areas of high metal loading, suggesting that metal tolerance or resistance is an adaptive response to excessive metal exposure [15]. The concentration of lead and copper ion on the crude oil effluent investigated in this study showed higher value than the recommended standard. The implication of this is that when this effluent is not treated before discharge into the environment it could result in the contamination of soil, ground water and other surface water bodies. When these metals bioaccumulate in the food chain and get consumed by man, they could

be toxic. This underlines the danger of improperly disposed waste effluent. The relative abundance of these microorganisms in crude effluent suggests that they could be the most competent bacteria for treating such metal ion- contaminated effluents. Being Gram positive also indicates great potential in serving as biosorbents of metal ions in effluent sample. Also being motile suggests increased rate of circulation in a medium and could have an advantage over a non-motile organism in a metal- contaminated effluent sample as it could have a faster rate of metal mop-up as a bioremediating organism. Crude oil effluent being waste water from crude oil is expected to have traces of the mixture of hydrocarbons found in crude oil. Synergistic or antagonistic interactions leading to subsequent inhibitions in the growth of microorganisms might not be ruled out.

Literature reports emanating from various authors have shown that pH level is main prevailing factor in biosorption/bioaccumulation efficiency by different organisms [16, 17 and 18]. It has been shown that low pH affect the network or chemistry of the cell wall as well as its physiochemistry and the hydrolysis of the heavy metals [19]. At low pH values, lead ions compete with hydrogen ions at the binding sites of the microbial cells. Lead ions can precipitated out at higher pH values (pH > 8.0), due to the high hydroxyl ion consumption in the medium [20]. From the results of this work, the highest growth rates was observed in all the groups at alkaline pH of 7.5-8.0, which agrees with the evidence that the optimal pH range for bioremediation by bacteria is 6.0-8.5. With increase in pH, there will be a resulting increase in negative charge on the surface of the cell which favoured electrochemical attraction and adsorption of metal [21] hence the incremental growth of the bacteria isolates at the high pH favored the adsorption of metal ions.

The preferences for higher molecular weight element in the growth of bacteria organisms have been reported [22 and 23]. Indeed, the observed results showed the highest growth rate in order of Group D (without any metal salt) > Group B (containing Pb) > Group C (containing Cu and Pb) > Group A (containing Cu). The probable preferences for these elements might depend on the genetic constitution of the organism, physiological conditions in the system, chemistry of the metals and prevailing environmental conditions such as pH. The better growth rate observed in Group C (Cu + Pb) than in Group A (Cu only) suggests that copper and lead are co-metabolites, whereby the presence of one metal enhances the resistance of the organism to the other metal.

The residual metal ions not-detected in this study may mean that the metal ions have been completely adsorbed or removed by the microorganism. It has been established that the walls of gram-positive bacteria are efficient metal chelators [21]. Thus, the peptidoglycan in *B. subtilis*, contains a carboxylic group (glutamic acid), which can act as the major site of metal deposition. The quantity of copper and lead recovered from the harvested microbial cells shows that the organism played a vital role in the metal removal, either through biosorption or bioaccumulation mechanisms or both. The quantity of metal ion absorbed by organism is expected to increase appreciably with increase in the number of absorbing bacterial cells.

A comparison between the residual metal ions and the recovered metal ions as (Table 6) showed appreciable differences between the two. The quantity removed was not equal the quantity recovered. We anticipate that some metal ions might have been lost during the isolation process of the bacterial biomass. However *Bacillus subtilis* is a better removal agent than a recovery agent. This organism when run through several cycles in an activated sludge containing metal-contaminated aqueous effluents could lead to the removal of the metals.

These results suggest that better results could be achieved when *Bacillus subtilis* is added in consortium with the other organisms isolated in the biosorption/bioaccumulation of metals in aqueous effluents. The performance could also be enhanced by the addition of a suitable carbon source which the organisms would metabolize to have a sustained growth and consequently lead to increased quantity of metal bioabsorbed/bioaccumulated.

Table 6: Recovered/Accumulated or Sorbed Copper and Lead ions from the Bacterial Biomass

		Recovered from Biomass		
Group	Initial Conc. (mg/100ml) At Time, t = 0	Conc. of Cu (mg/100ml) At Time, t = 38	Conc. of Cu (mg/100ml) At Time, t = 38	Diff
A (Cu)	0.86	0.53 ± 0.02	-	0.33
B (Pb)	2.48	-	1.47 ± 0.01	1.01
C (Cu+Pb)	3.34	0.53 ± 0.02	1.47 ± 0.01	

In conclusion, this study proves that *Bacillus subtilis* is capable of utilizing hydrocarbon and metal contaminants and may be employed in the future for the removal of copper and lead metals from industrial waste effluents. A major advantage in the use of microorganisms in metal ion removal/recovery is that the organisms package and sequester the metals such that they are not released back into the environment and when properly disposed e.g. by burying, does not pose threat to man and animal. This is as against the harmful waste products encountered in the use of physico-chemical methods. The use of microorganisms in biosorption/bioaccumulation of metals holds a great future. This method is cheaper, more sufficient, more environmentally friendly and easier to handle.

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