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Original Article

Isolation and identification of Rhodococcus and their efficacy in bioremediation of heavy metals in biomedical waste ash

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Abstract

Biomedical waste (*BMW*) ash from BMW incineration remains poses a serious threat to the ecosystem because it contains large amounts of toxic heavy metals, which are often disposed of in regular landfills or directly into the soil and can contaminate soil and groundwater. This study aimed to develop a bioremediation technique to remove the toxicity of ash before disposing of it in landfills. Bacteria belonging to the Rhodococcus genus were used in this study; one species was isolated from soil contaminated with livestock remains and another species was isolated from an agricultural area in Sabha city. The degradation ability of the bacteria was tested by measuring the concentration of the heavy metals by Atomic Absorption Spectroscopy (AAS) at different ranges of temperatures (25,30,35°C) and exposure times (2,4,6 and 8 days). The bacteria proved their ability to degrade 85% of Cadmium. The best removal rate of Cadmium was at 35 ° C on the eighth day. Our findings revealed that the *Rhodococcus* genus bacteria have high efficiency in removing Cadmium from samples of BMW ash and could be used as a potential agent to treat heavy metals contamination efficiently and reduce the negative impact of BMW ash on the environment.

Keywords **- Biomedical waste ash, Cadmium, Heavy metals, Rhodococcus, Temperature.**

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Introduction

The improper disposal of biomedical waste (BMW) has a significant impact on human health and the environment, especially in developing countries where the number of health facilities is growing and many of these healthcare facilities do not safely dispose of their medical waste. Poor management of BMW results in around 5.2 million deaths worldwide each year from waste-related diseases, such as typhoid, cholera, AIDS, and hepatitis B (HVB)[1].

In many developing countries, including Libya, little information is known about the production, management, and disposal of medical waste; however, a study that included healthcare institutions in three Libyan cities revealed that most of the evaluated institutions lacked both guidelines for the proper collection and classification of waste as well as methods for its storage and disposal. This study found that the average waste generation rate was found to be 1.3 kg/patient/day, comprised of 72% general healthcare waste (non-risk) and 28% hazardous waste. The average general waste composition in this study was: 38% organic, 24% plastics, 20% paper, sharps and pathological elements comprised 26% of the hazardous waste component [2].

In most countries, it is common for BMW to be treated by incineration, where the volume of waste can be reduced to a tenth of its original size; in addition, this method can destroy all pathogens in the waste. However, the ash generated by this method is often buried in regular landfills, which poses risks and has harmful impacts on the environment. Several studies have proven that this ash contains a high percentage of heavy metals such as Cadmium, Mercury, Arsenic, Lead, and Chromium, these heavy metals can accumulate in the food chain and pose a major threat to living organisms[3].

Although there are many studies on reducing the toxicity of incinerator ash, all of them use harmful hazardous chemicals; however, bioremediation is considered one of the safest and cleanest methods, as it is an eco-friendly technology and inexpensive [4]. Several bacterial genera have been identified that can optimally remove heavy metals, such as *Rhodococcus sp*, *Lysinibacillus*, and *Bacillus* [5].

Strains of *Rhodococcus* are important organisms, characterized by high bioprocessing efficiency and the ability to tolerate and resist heavy metals. These bacteria belong to the phylum Actinobacteria, order Actinomycetales, suborder Corynebacterineae, and family Nocardiaceae. The bacteria are characterized by their remarkable diversity in metabolic processes due to their possession of linear plasmids that carry genes encoded for a variety of enzymes, which can break down and treat a range of organic compounds. In addition, these bacteria have a special cell wall that contains mycolic acid, the bacteria also have several physiological adaptation strategies and can be isolated easily from environmental samples [6]. Therefore, this study aims to evaluate the efficiency of *Rhodococcus* in the treatment of heavy metals specifically Cadmium (Cd) and Lead (Pb) in ashes of medical waste from the AL-darn centre incinerator in Sabha city.

Materials and Methods

Samples collection

Ash samples in this study were collected from the AL-darn centre incinerator in Sabha. The bacteria samples were collected from six samples of soil contaminated with livestock residues from three different sites around the agricultural fields of Sabha city. Two samples were collected from each site, one from the surface and the other from a depth of 10 cm from the surface of the soil, and the samples were preserved in sterile plastic containers so that there would be no interference with the selected microorganism during treatment.

Isolation and purification of bacteria

Rhodococcus bacteria were detected in soil samples instantly upon sample arrival in the laboratory. Then, 0.1 ml of serially diluted (10^{-1}– $10^{-}5$) soil samples were poured on Lowenstein Jensen medium and incubated at 35 °C for 3-5 days, after that the obtained isolates were purified on glycerol agar medium prepared according to the manufacturer's instructions (ATCC). [6] [7]

Diagnosis and preservation of bacterial samples

All suspected colonies were characterized using morphology, colour and size, stained with Gram stain, acid-fast and malachite green stains, and microscopically examined. Some biochemical tests were performed to identify the bacteria including tests of catalase, oxides, urea hydrolysis, esculin hydrolysis, and nitrate reduction, in addition to the carbohydrate fermentation tests [8].

The samples were preserved in nutrient broth medium supplemented with 50% glycerol medium in sterile Eppendorf tubes at -20°C [9].

Bacterial treatment to remove heavy metals of BMW ash

Nutrient broth medium was prepared according to the manufacturer's instructions (Oxoid) in 60 glass beakers)250ml(. One gram of sterile ash sample was added to each beaker, and then a suspension of *Rhodococcus* bacteria was added to the ash, 15 beakers were left as a control and marked: (Nutrient broth medium with the ash sample without bacteria). All beakers were incubated on a rotary shaker incubator at 150 rpm at different temperatures: 25 °C, 30 °C, and 35 °C; 20 beakers for each temperature degree and at (pH = 7), for 8 days and the results were taken at intervals [3].

Analysis of the ash samples

The treated samples were examined periodically, and the content of some heavy metals specifically Cadmium and Lead were estimated in the samples using Atomic Absorption Spectroscopy (AAS) after the process of digesting heavy metals using 3HNO 65%, H2O2 and Aqua regia[10].

Statistical analysis

The statistical analysis of the results was carried out using SPSS software, version 22. The ANOVA test was performed to compare the significant differences between the means by calculating the value of the least significant difference at a significance level of 0.05.

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Results and Discussion

Isolation and identification of the isolates

One species belonging to the *Rhodococcus* genus was obtained from the surface layer of agricultural land in the Qaradeh area, and *Bacillus* insulation was obtained from other soil samples. Their appearances on the Glycerol agar were consistent with findings reported by AL- Sahlany [11]. The colonies appeared as shown in Figure 1 in cream-white colour, scattered and flat, with a mucous and non-transparent strength with circular edges the colony was 1-2 cm in size, and it is fast growing in aerobic conditions. *Rhodococcus's* isolation on the Lowenstein Jensen in Figure 2a and on the Glycerol agar in figure 2b and its colonies appeared orange with a convex circular shape, a soft surface, and edge, transparent and mucous, the colony was 0.3-0.5 mm large compared to the bacillus, which is relatively slow to grow taking about three days to grow in air conditions. These findings are consistence with the findings reported by Whitman et al. [6].

The results of microscopic tests of the isolation obtained showed the bacillus isolations; stained positive with Gram stain and appeared purple-coloured chains of bacilli as they appeared Gram-positive endospore producing and these qualities consistent with AL- Sahlany study [11] and were excluded because they were not targeted in this study.

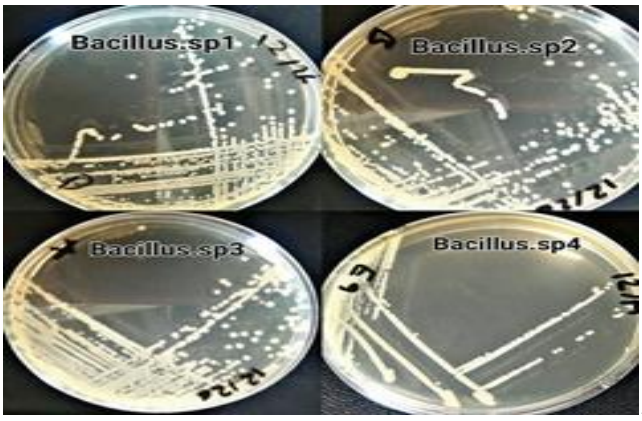
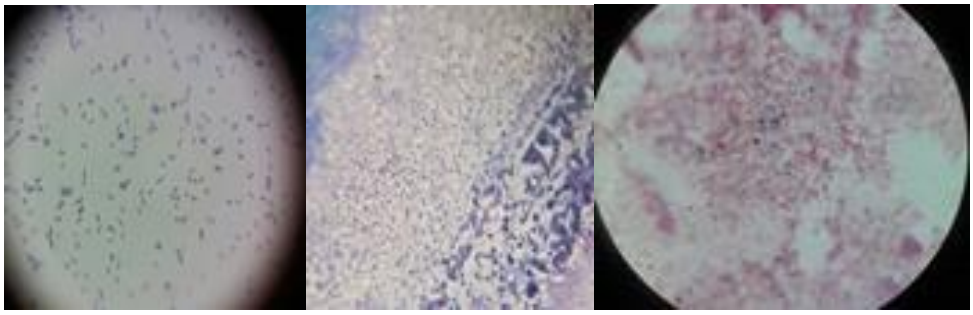


Figure 1. *Bacillus spp.* on the Glycerol agar

Rhodococcus's isolation is shown in Figure 3 and Table 1 (stained with a Gram stain). It appeared as short positive purple bacilli, and by Malachite green, it was found to be non-endospore. The Ziehl-Neelsen for this bacteria was positive where bacillus appeared in purple and is considered an advantage for the Nocardiaceae family of the genus *Rhodococcus* because it contains a high percentage of fatty and waxy substances where its cellular wall consists of mycolyl-arabinogalactan-peptidoglycan complex and these qualities were stated in a study by Whitman et al [6].

(a) **(b)**

Figure 2. *Rhodococcus* bacteria on (a) Lowenstein jensen (b) Glycerol agar



(a) **(b)** **(c)**

Figure 3. Microscopic examination of *Rhodococcus* bacteria using; (a) Gram stain, (b) Ziehl-Neelsen stain and (c) Malachite green stain.

The biochemical tests performed on *Rhodococcus* isolations are shown in Table 1. The tests showed variable results between positive and negative. This variability was because of the respiratory enzymes that were positive for catalase and negative for oxidase indicating that they had worked on the H2O2 detector hydrolysing H2O2 into water and oxygen. On the other hand, these enzymes were unable to turn the colour of the detector-saturated filtration sheet into purple when adding bacteria as the bacteria did not have the cytochrome oxidase as a hydrogen receptor. The bacteria were also positive for the Esculin hydrolysis test, which shows its ability to hydrolysis Esculin in the presence of bile salts.

As for the hydrolysis of urea, the results were negative and there is no change in the colour of the medium, which confirms that the bacteria are not consumed urea because they do not have the urease enzyme. The bacteria gave a negative result for nitrate reduction because they do not have nitrate reductase,

while they were able to ferment all types of sugars tested in this study as shown in Table 1. These results were consistence with the classification of bacteria by Whitman et al and Zhang et al [12] [6]. Furthermore, *Rhodococcus yunnanensis* a species of Nonpathogen present in agricultural soils and have the same qualities.

Table 1. Morphological, Microscopic, and biochemical characteristics of the Rhodococcus isolates. (+) denotes the presence of growth and (–) denotes the absence of growth.

Morphological test	***Rhodococcus***
Shape	Rods/cocci
Form	Irregular
Colour	Orange
Microscopic tests	
Gram staining	+
Malachite green	-
Acid fasting	+
Biochemical test	
Catalase	+
Oxidase	-
Nitrate reduction	-
Esculin hydrolysis	+
Urea hydrolysis	-
Carbohydrate fermentation	
Glucose	+
Sucrose	+
Lactose	+
Maltose	+
Mannitol	+
Ribose	+

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Heavy metal analysis of BMW ash

The results of the AAS for the ash samples of the medical waste incinerator of the AL-darn centre showed the absence of lead metal which may be due to the type of the materials burned in the centre, which were mostly non-metallic medical materials such as cotton, gauze, and napkins. The results of the Cadmium metal by the AAS showed different Cadmium concentrations in the ash samples. Figure 4 shows the efficiency of *Rhodococcus* bacteria in the degradation of Cadmium metal at different temperatures with exposure time (2.4.6 and 8 days). The results showed that the removal rate was higher at 35°C on the eighth day of exposure, while the lowest removal rate was at 25°C on the fourth day at 21.97%. This is consistent with Samarth et al study [13]. The peptidoglycan layer with Teichoic and Teichuronic acids plays the key role in the process of binding to the heavy metals and this gives the many gram-positive bacterial species the ability to uptake heavy metals from their solutions and soils in different ways and mechanisms and at different removal rates. Rostom et al [14] noted that temperature has a significant impact on the vital treatment of heavy metals through its effect on the metabolic activity of bacteria. They revealed that the activity of metabolism of bacteria increases by increasing the temperature to the optimum temperature and this is consistent with our study where the maximum absorption of Cadmium was at 35°C.

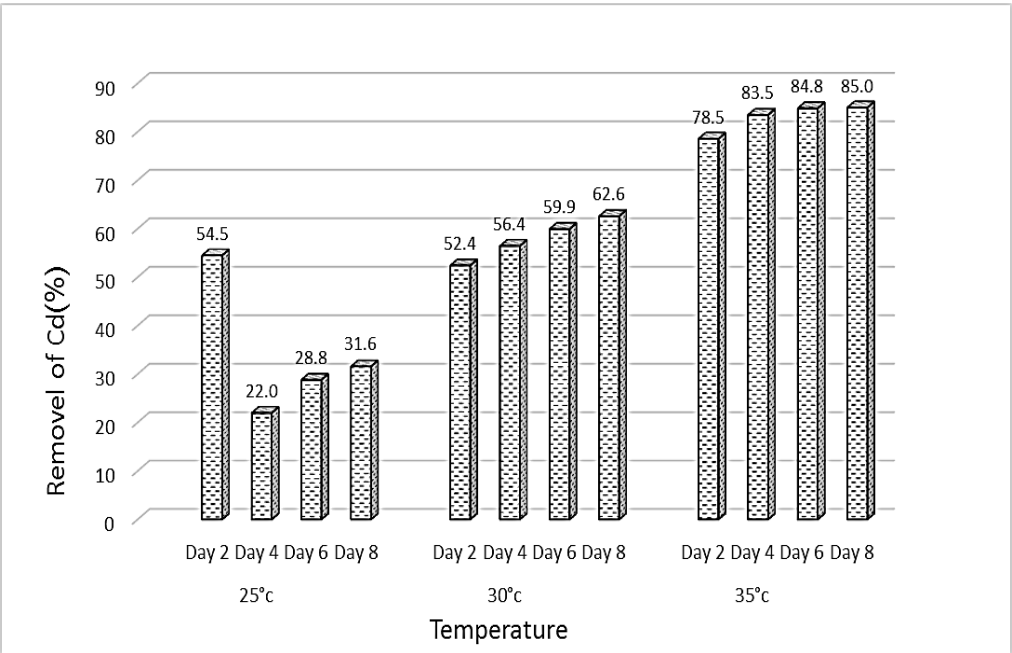

Figure 4. Cadmium uptake by R. yunnanensis as detected by AAS

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The results of the variance analysis as shown in Table 2 show statistically significant differences between temperatures and exposure time on bacteria where the probability value was lower than the indication level (0.05).

Table 2. The differences between averages of variables

Variable	Arithmetic mean	F	P-value
Day	0.497	585.645	0.00
Value	0.010	11.495	0.00
Day*Value	0.010	11.731	0.00

Conclusions

In this study, *Rhodococcus* bacteria showed high efficiency as a biological tool in removing Cadmium form of samples of BMW ash. The best removal rate was 85% on the eighth day at 35°C, while the lowest removal rate was 21.97% at 25°C on the fourth day. The use of *Rhodococcus* in the bioremediation of BMW ash would be an environmentally friendly and cost-effective alternative to traditional methods of removing heavy metals. We recommend conducting further studies using different environmental conditions such as different concentrations of pH, salinity, and a carbon source to determine the optimal condition of degradation.

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