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Research article

Effect of lead on biofilm formation by environmental isolates of *Bacillus* spp.

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ABSTRACT

Biofilm formation is one of the biggest challenges of scientist. Role of heavy metals in forming biofilm is not clear enough. Here, the effect of lead on biofilm formation by *Bacillus* spp. isolated from soil in terms of biofilm formation and remove was studied. In present study, 10 isolates of *Bacillus* spp were isolated from soil. The ability of all isolates to form biofilm was evaluated. The effect of lead on biofilm formation was studied by adding lead (pb) before forming biofilm. In another experiment the lead was added after biofilm formation to study the effect of lead on biofilm remove. The current study, showed the ability of all studied isolates to form biofilm. Maximum biofilm formation by *Bacillus* spp isolate number 8 (B8) followed by B1 and B3. The lowest biofilm formation was found in case of isolate 4 (B4). The lead (50 ppm) reduced biofilm formation by B8, B1 and B3 isolates when the lead used before biofilm formation ($P < 0.05$). In another experiment the lead (50 ppm) was added after biofilm formation, it was observed that the biofilm formation was higher when the lead added (after biofilm formation) as compared with control (serial distilled water) and the difference was significant ($P<0.05$). It can be concluded that the lead effect negatively on biofilm formation and positively on stability of biofilm.

Keywords: *Bacillus*, Biofilm, Heavy metals, Lead.

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INTRODUCTION

Heavy metals are environmental pollutants that are produced by anthropogenic activities, such as mining and smelting, as well as through other sources of industrial waste. Heavy metals contaminate drinking water reservoirs and freshwater habitats and can alter macro- and microbe-ological communities [1]. The mechanisms of heavy metal toxicity to bacterial cells by inducing oxidative stress and interfering with protein activity [2].

Biofilms are problematic in a broad range of areas, and specifically in the food, environmental, and biomedical fields [3]. Biofilms formed by members of the *Bacillus* genus [3]. As *Bacillus* species are ubiquitously present in nature. The biofilm formed by thermo-resistant *Bacillus* species which rapidly grow and form biofilm. Thus, biofilms formed by *Bacillus* species is the major type of hygiene problems in different field of life [4].

Electrolyte concentrations have important impacts on biofilm formation [5]. Heavy metals can affect on biofilm formation directly on electro-static interactions and indirectly via physiology-dependent attachment processes by acting as important enzyme cofactors [6]. In spite of the potentially important role, the effect of heavy metals on bacterial adhesion and biofilm formation has barely been studied. Mg^{2+} was shown to influence adherence to surfaces

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in *Pseudomonas* spp. [7]. The Mg^{2+} affects on swarming and biofilm formation [8]. Accordingly to Dunne and Burd, heavy metals significantly enhanced *in vitro* adhesion of *Staphylococcus epidermidis* to plastic [9]. The present study, focused on the effect of lead on biofilm formation by *Bacillus* before and after formation.

MATERIALS and METHODS

Collection and Preparation of Soil Sample

Forty Soil sample were collected (from January 2016 till March 2016) from different regions of Baghdad, Iraq. Four gram of each samples were collected using clean and sterile plastic container. All samples were transferred to laboratory under sterile conditions [10].

Isolation of *Bacillus* spp

One gram of each soil samples was added to 10 ml of nutrient broth and incubated at 37 oC for 18 h. After the incubation 100 µL of the supernatant of each tube containing suspension of soil and culture media were inoculated onto nutrient agar plates and incubated at 37°C for 24 h. The plates were examined and the suspected colonies were stained by Gram staining method. The Gram-positive, rod-shaped, spore forming bacilli were selected for additional identification tests.

Biofilm formation

Biofilm formation Overnight cultures of *Bacillus* spp (B1, B2, B3, B4, B5, B6, B7, B7, B8, B9 and B10) in 2 ml of Tryptose soy broth (TSB) (Himedia) and washed three times with phosphate buffer saline and one time with fresh TSB, and bacterial count was adjusted to be 10^7 c.f.u/ml. Two hundred microlitter of standardized inoculums (10^7 c.f.u/ml) were added to the wells of sterile flat-bottom polystyrene tissue culture plates, and incubated at 37°C for 18 h in a closed and humidified plastic container.The medium was then discarded, and non adherent cells were removed by washing three times with sterile PBS (0.1 M, pH 7.2). Spectrophotometric method was followed to check the level of biofilm formation. Slime and adherent bacteria were fixed by incubating for 30 min at 60°C and then stained with Hucker crystal violet (0.4%) for 5 min. After thorough washing with distilled water to remove excess stain, the plates were dried for 30 min at 37°C. The extent of biofilm was determined by measuring the absorbance of stained adherent film upon treatment with acetone:ethanol (30 : 70) at a wavelength of 490 nm [11].

Effect of lead on biofilm formation and remove

Same procedure of biofilm formation was followed but 50 µl of 100 ppm of lead was add to the 50 µl TSB (2x), (final level of lead was 50 ppm) in wells of test, while in the wells of control, PBS (0.1 M, pH 7.2) was added instead of lead. Then, the level of biofilm was measured in test and control wells. In another experiment the procedure of biofilm was followed but after overnight incubation the wells were washed to remove the non adherent bacteria, in some wells sterile distilled water was added (control) and in other wells for each isolate the 100 µl of distilled water (50 ppm of lead) was added (test). After incubation for 18 h the level of biofilm was measured by spectrophotometric method.

Statistical analysis

All the experiments were carried out in triplicate and all values have been taken as mean value and standard deviation calculated. The differences between test and controls were analyzed by using Student's *t*_test calculated by employing Origin 8.0 version Software. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Spectrophotometric method was followed to check the ability of different isolates of *Bacillus* to form biofilm. The present study proved the ability of all studied isolates to form biofilm but in different level the highest ability to form biofilm was found in case of isolate B8 followed by B1 and B3. The lowest ability to form biofilm was observed in case of isolate B4 (**Fig 1**).

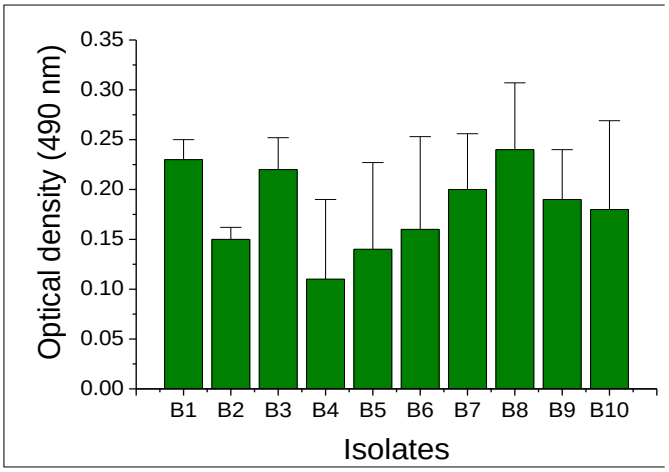

Fig 1. Biofilm formation of ten isolates of *Bacillus* on microtiter plate, the highest value of biofilm formation was found in case of isolates B1, B3 and B8.

The highest three isolates (B1, B3 and B8) were used in further experiment to evaluate the effect of lead on biofilm formation and remove. **Fig 2** shows the effect of lead on biofilm formation. In this experiment the lead was added before biofilm formation. The result showed that lead was reduced biofilm formation significantly ($P < 0.05$).

The results of **Fig 3** showed the effect of lead after biofilm formation (removing the biofilm). It was found that the lead was enforced of biofilm formation. The results showed that the level of biofilm after adding the lead was higher than the level of biofilm after adding the distilled water.

Discussion

Biofilm formation is one of big challenges for scientist in the different field. Most of bacterial isolates have a good ability of biofilm [12]. This phenomenon makes bacteria resistant to high level of antibiotic. In the nature the biofilm may block the tubes of water supply and swages. Several factors affect on biofilm formation, such as physical and chemical factors [13]. The affect of heavy metals on biofilm formation was studied clearly previously [2]. Many previous studies focused on the effect of heavy metals on biofilm formation [14]. In current study, the level of biofilm formation was

evaluated in different isolates of *Bacillus* isolated from soil. The results showed that all isolates formed biofilm but in different levels. The current study showed that the lead reduced the biofilm formation when the lead added in the first step of biofilm formation but when the biofilm formed, the lead enforce the biofilm formation.

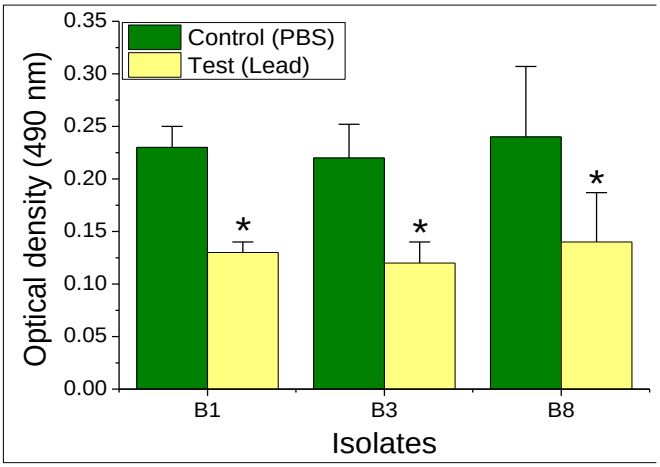

Fig 2. Effect of 50 PPM of lead on biofilm formation of three isolates of *Bacillus* into microtiter plate. The lead was added at the zero time of biofilm formation. Asterisks indicate the significant difference from control (P<0.05).

Biofilm and adhered bacteria are more resistant to heavy metals as compared to planktonic bacterial cells. The resistance of biofilm to heavy metals 2 to 600 time fold than planktonic cells [2]. Heavy metals is a toxic chemical that is why affect negatively o the bacterial growth and biofilm formation and it is cofactors that important in metabolism of bacterial cells that is why it can affect positively on biofilm formation [4].

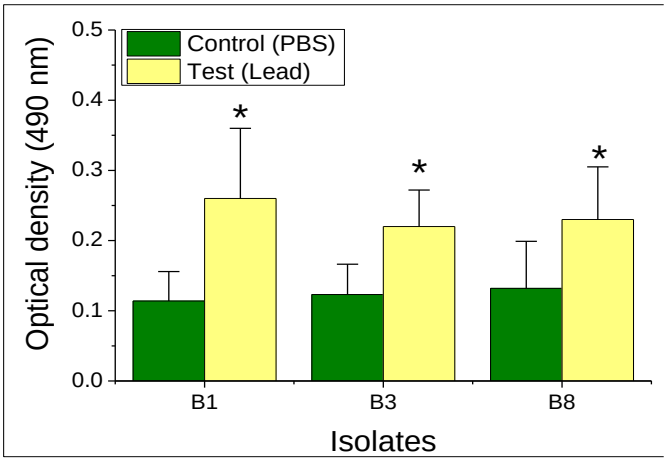

Fig 2. Effect of 50 PPM of lead on biofilm formation of three isolates of *Bacillus* into microtiter plate. The lead was added after forming of biofilm. The lead was added after 18 h of biofilm formation film. The lead was enforced of biofilm. Asterisks indicate the significant difference from control (P<0.05).

This explains the stability of biofilm after exposing to lead as compared with biofilm treated with distilled water. The study in this field very scanty and required a lot of work. In our laboratory, many projects are going on to find out the role of heavy metals on biofilm formation. From this study, it can be concluded that lead affect negatively on biofilm formation in the first stages and positively on the biofilm body in the last stages of biofilm formation.

Conflict of interest

The authors declare that they have no conflict of interests.

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