

Evaluation of Lead Effects on Laccase Enzyme Activity in *Bacillus Subtilis* WPI



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ABSTRACT

Background: Lead (Pb) is an important environmental pollutants that play a significant role in increasing the stability of some other pollutants by changing the microbial profile of the soil. *Bacillus subtilis* WPI is an abundant bacteria existing in wastewater. Because of laccase enzyme in this bacterium, the decomposition process of aromatic pollutants in wastewaters can be facilitated. We aimed to investigate the effect of different Pb concentrations on *B. subtilis* growth and biological activity of laccase enzyme in *B. subtilis* WPI.

Materials and Methods: *B. subtilis* WPI was isolated from the paper mill industrial wastewater of Hormozgan, Iran, from March to August 2017. After purification, the growth trend of *B. subtilis* WPI as well as the activity of laccase enzyme in different concentrations of Pb was investigated based on kinetic method.

Results: Bacterial growth at Pb concentration of 400 mg/L reduced in a dose-dependent manner, and this decrease was significant at concentrations of 300 and 400 mg/L ($P < 0.001$). The level of laccase enzyme activity in the lead concentration range of 20-160 mg/L also reduced in a dose-dependent manner, which implied that the highest decrease was observed at lead concentration of 160 mg/L ($P < 0.01$). Our findings showed that there was no significant change in bacterial growth in lead concentration range of 20-200 mg/L, while a significant change was observed in the activity of laccase enzyme in the mentioned concentration range.

Conclusion: Therefore, it seems that this reduction in enzyme activity can indirectly increase the stability of aromatic oil pollutants in the environment.

Introduction

C

urrently, urbanization and industrial development in human societies have led to an ever-increase of industrial pollutants in the environment [1]. Meanwhile, heavy metals, which are the most impor-

tant environmental pollutants, in large quantities can cause a change in the balance of biological agents in the ecosystem [2]. The discharge of these elements into the environment is much higher than that produced through natural processes. Lead (Pb) as one of the most toxic elements that enter the environment through various industries, such as petrochemicals, battery pro-

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duction, crystallization, mining, lead processing, and mainly burning of fossil fuels. Lead is a metal without biological function [3, 4].

This element not only threatens human health but can also change the microbial profile of the ecosystem [5]. The increased environmental sustainability of aromatic pollutants in petroleum products can be considered due to the microbial profile change. Because of the presence of heavy metal-resistance systems and degrading enzymes for aromatic compounds in some bacteria, the bacteria have received more attention compared to other microorganisms. The metal resistance systems have been identified in many bacterial groups. *B. subtilis* is one of the most abundant bacteria in wastewater that can play a valuable role in the ecosystem. Previous studies reported that laccase enzyme affects the nature of *B. Subtilis* WPI [6].

The large distribution of this enzyme in this bacterium has converted it to an important indicator of environmental degradation [7, 8]. The laccase enzyme is a polyphenol oxidase, containing several copper atoms and has been found in many plants, fungi, insects, and microorganisms [8, 9]. Due to the environmental and economic advantages of this enzyme and the role of *B. subtilis* in its production, it was used for degrading aromatic pollutants in wastewaters [10]. In this study, we investigated the effects of various lead concentrations on the growth of *B. subtilis* WPI and the biological activity of laccase enzyme in *B. subtilis* WPI.

Materials and Methods

Sampling and isolation of *Bacillus subtilis* WPI

B. subtilis WPI was isolated from the paper mill industrial wastewater of Hormozgan, Iran, from March to August 2017. Briefly, after sampling the soil, bagasse and wastewater of the paper mill were collected from the ground of 10-15 cm soil depth in sterile plastic bags and were kept at 4°C [11-13]. To isolate the bacteria, 10 g of the sample was transferred to a 250 mL flask and mixed with 100 mL of sterile saline solution. Next, after the preparation of serial dilutions (range 10⁻¹ - 10⁻⁹) and spread culture on a nutrient agar plate (Merck, Germany), bacterial colonies were screened at the surface of the agar plate containing 0.5 mM guaiacol to determine the laccase enzyme activity.

The plates were incubated at 25-35°C for 96 h [14]. The isolation and screening methods were used several times to obtain a pure specimen that produces only one strain containing laccase enzymes. By considering physiological and biochemical properties, the morphology of the strain that produces the laccase enzyme was studied.

Biochemical tests were performed to detect *B. subtilis* bacteria including oxidase, catalase, amylase, mannitol, lactose, glucose, citrate, xylose, and sucrose [12].

Determining bacterial growth at different times

First, bacterial suspension was performed according to the half-McFarland standard (equal to 1.5×10⁸ colony forming units (CFU /mL) and was inoculated into 20 mL of Tryptic Soy Broth (TSB). Next, the samples were incubated in a shaker incubator at 37°C. The bacterial growth assay was investigated at various times (from 1 to 10 days) using a spectrophotometer at a wavelength of 600 nm [15].

Optimization of growth conditions of *B. subtilis* bacteria in TSB medium

The effects of temperature, growth time, and different concentrations of Copper (Cu) as a co-factor required for bactericidal activity and production of laccase enzyme were investigated.

Bacterial growth rate at different temperature

The bacterial growth capability was investigated at temperatures of 25°C, 30°C, 35°C, 40°C, and 45°C. The bacteria growth was measured over a period of 5 days using a spectrophotometer at a wavelength of 600 nm [15].

Optimizing laccase enzyme activity

The concentration of copper sulfate in the environment is another factor affecting the growth, activity, and production of laccase enzymes in *B. subtilis* bacteria [16, 17]. Therefore, different concentrations of copper sulfate (CuSO₄) (0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, and 0.5 mM) and control sample (without adding copper sulfate) were incubated for five days. The activity rate of the laccase enzyme at these concentrations was measured at 436 nm wavelength using two measurements of the optical absorbance over three minutes interval with 2, 2-azino-bis (3- ethylbenzothiazoline- 6-sulfonic acid) Sigma-Aldrich (ABTS) substrate. Eventually, the enzyme activity was determined in accordance with the following equation [18].

$$\text{Enzyme activity rate} = \text{Optical Density (OD)} \times 60 \times (\text{enzyme value} / 1150) \times 0.371$$

Effect of different concentrations of lead on the growth of *B. subtilis* WPI

To obtain the optimum concentration of lead Pb(NO₃)₂, the effects of this metal at concentration range of 40-400 mg/L on the growth process of *B. subtilis* bacteria were

evaluated. Briefly, 1 ml of bacteria suspension was incubated in 20 mL of TSB medium containing 40 mg/L, 100 mg/L, 200 mg/L, 300 mg/L, and 400 mg/L of lead. Moreover, non-Pb containing medium was used as the control. All of the mediums were kept in a shaker incubator at 150 rpm and a temperature of 35°C and their OD was measured at a wavelength of 600 nm for five days.

Effect of different concentrations of lead (Pb) on laccase activity

The lead concentrations of 160 mg/L, 80 mg/L, 40 mg/L, and 20 mg/L were introduced into bacterial culture medium and the activity of laccase enzyme was measured daily for five days. Based on the results of microbial culture, there was no change in the growth rate of the bacteria at concentration range of 20-160 mg/L.

Statistical analysis

All experiments were performed in triplicates, and the data analyzed using the variance (ANOVA) followed by the Tukey's test in SPSS V. 21. All the results are expressed as Mean±SD. The level of significance was defined as P-values less than 0.05.

Results

Identification of *B. subtilis* WPI

The results obtained from the biochemical tests were for the identification and confirmation of *B. subtilis* WPI.

Optimal conditions for bacterial growth

The results obtained for *B. subtilis* WPI growth within 10 days of incubation indicated that the highest and

lowest growth rates on 5 and 10 days were 2.6×10^4 and 0.73×10^4 CFU/mL, respectively (Figure 1A). Moreover, this bacterium had the best growth rate at 35°C and the highest light absorption at this temperature was 0.35. The lowest growth rate was observed at 25°C and was 0.21. As shown in, Figure 1B there was a significant difference in the maximum bacterial growth between 25-35°C ($P < 0.05$).

Effects of different concentrations of lead metal on the growth of *B. subtilis* bacteria

As shown in Figure 2, there was no significant change in the bacterial growth at the lead concentration range of 20-200 mg/L. However, at lead concentrations of 300 and 400 mg/L, the bacterial growth rate significantly decreased compared to the control ($P < 0.001$).

Effects of different concentrations of lead metal on the activity of laccase enzyme in *B. subtilis*

The activity of the laccase enzyme was evaluated in a safe dose range of lead (20-200 mg/L). As shown in Figure 3, with increasing lead concentration (within the concentration range mentioned above), the activity of the laccase enzyme decreased in a concentration dose-manner, and this decrease was significant at concentrations of 80 and 160 mg/L ($P < 0.001$). Effect of various copper sulfate (CuSO_4) concentrations on the production of laccase enzymes.

Our findings showed that there was a significant difference in the activity level of laccase enzyme in 0.2 mM of copper sulfate compared to the control ($P < 0.001$). Therefore, this amount (0.2 mM) of copper sulfate was chosen as the optimum concentration for the study of laccase enzyme activity (Figure 4) ($P < 0.001$).

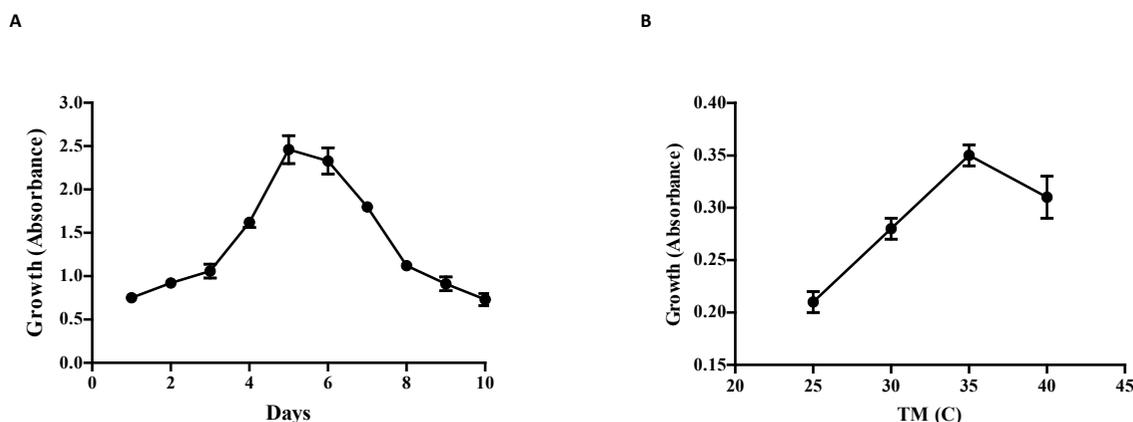


Figure 1. A: Measuring growth kinetics of *B. subtilis* WPI during 10 days of incubation at 600 nm measurements; B: Measuring growth kinetics of *B. subtilis* WPI at various temperatures

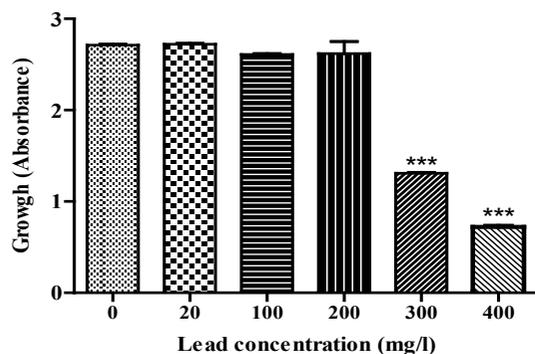


Figure 2. Growth changes in *B. subtilis* after five days of exposure to different lead concentrations

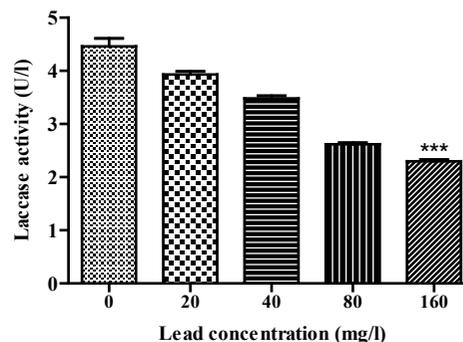


Figure 3. Changes in the activity of laccase enzyme in *B. subtilis* after five days of exposure to different lead concentrations

Discussion

The temperature is an effective factor for inducing and/or inhibiting enzymatic reactions participating in bacterial growth [19-20]. In the current study, the reduction of bacterial growth rate at 40°C can be due to the loss of metabolic activity of the cell, and subsequent delay in the bacterial division. In the study of Shukur et al. 35°C was reported as the optimal temperature for the growth of *B. Subtilis*, and this is in agreement with our findings [21]. However, in another study, the highest activity of the laccase in *B. subtilis* WD 23 was observed at 25°C [22].

Bacillus species were identified as the most abundant gram-positive bacteria in the soil and were used for the study of laccase enzyme activity under optimum conditions. Meanwhile, copper is one of the most important factors affecting the production and activity of laccase enzyme. In the present study, the optimum concentration of copper to induce laccase activity was determined to be 0.2 mM. In line with our findings, a similar study on

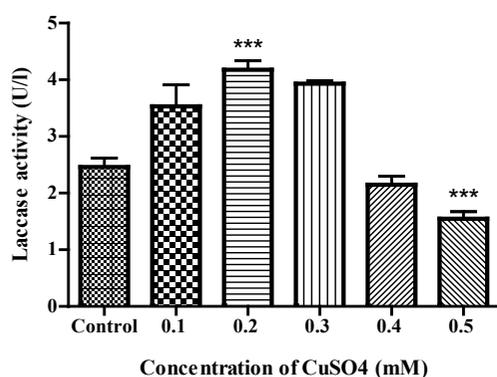


Figure 4. The activity of laccase enzyme in *B. subtilis* WPI at different concentrations of copper

the effect of different concentrations of copper sulfate on the growth of *B. subtilis* WPI showed that 0.2 mM of this compound was chosen as the optimum concentration for stimulating laccase enzyme activity [23].

Moreover, Shukur et al. in their study on *Bacillus* B16 showed that 0.2 mM of copper sulfate could stimulate the production of spore laccase enzyme and increase its amount to 6.6 Unit/mL [21]. Regardless of the bacterial species, the laccase enzyme appears to have kinetically similar responses to copper, which may be due to the structural similarity of the enzyme in different species of *B. subtilis* bacteria, and this plays an important role in the degradation of aromatic pollutants in petroleum products [24].

Based on the results of Logascus et al. this bacterium has a relative resistance to Pb exposure [25]. Our findings demonstrated that there was no significant decrease in the growth of this bacterium at the concentration range of 20-200 mg/L. The resistance of *B. subtilis* bacteria to Pb exposure in the abovementioned concentration range may be attributed to the collaboration of PbrA transfer protein and PbB phosphatase enzyme, which increases the resistance of this bacterium to Pb exposure [26]. This can lead to the formation of Pb-resistant species in the ecosystem. Concerning this, Zhang et al. showed that Pb-resistant strains in Pb-contaminated soils increased compared to uncontaminated soils [27]. However, bacterial growth significantly reduced at Pb concentrations higher than 200 mg/L, which may be attributed to sudden shock and lack of sufficient opportunity to adapt to Pb exposure at high concentrations. Laccase is a polyphenol oxidase containing several copper atoms [28].

Conclusion

The results of this study indicated that heavy Pb metal at concentrations above 20 mg/L had a decreasing trend on the activity of laccase enzymes in *B. subtilis* WPI, and increasing Pb concentration to 160 mg/L, laccase activity rate was reduced by half in the samples compared to the controls. Therefore, releasing these metals into the industrial wastewater, such as paper mills, which is a main source of *B. subtilis* WPI, will not only eliminate this strain in the ecosystem but also reduces laccase enzyme activity causing the sustainability of other pollutants in the environment.

One of the most important limitations of this study was the preparation of *B. subtilis* WPI.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles were considered in this article.

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Authors contribution

Designing the experiments, writing the manuscript, conducting the experiment and data analysis, participating in the initial draft, revision of the manuscript, reading and approving the final manuscript: Parisa Mehrabi Moghadam, Hassan Mahmoudi.

Conflict of interest

The authors declared no conflict of interests

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