Research Article

Bioremoval and Detoxification of Anthracene by a Halophilic Laccase from *Alkalibacillus salilacus*

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Background: Polycyclic Aromatic Hydrocarbons (PAHs) as resistant compounds in the environment has received much attention in recent years due to their adverse effects on ecological health. Among the various methods studying the removal of PAHs, enzyme biotechnology is one of the most effective and appropriate method.

Objectives: In the present study, a halophilic laccase was used for bioremoval of anthracene in the presence of 1-Hydroxybenzotriazole (HBT).

Materials and Methods: Halophilic laccase from *Alkalibacillus salilacus* was tested for anthracene degradation. Residual concentration of anthracene at various concentrations of NaCl (0–4 M), incubation time, pH, solvent, and surfactants in the enzymatic reaction mixtures was determined by HPLC.

Results: The maximum removal of substrate was achieved after 72 h at 40 °C, pH 8, and NaCl concentration 1.5 M. Besides, the addition of 1% (v/v) ionic and non-ionic surfactants and 25% (v/v) of various organic solvents increased removal efficiency. The kinetic parameters K_m and V_{max} of the laccase for removing of anthracene were 0.114 μM and 0.546 μmoL. h.$^{-1}$ mg^{-1}, respectively.

Conclusions: Laccase showed the maximum removal efficiency of anthracene in the presence of 1-Hydroxybenzotriazole (HBT).

Keywords: Alkalibacillu salilacus, Anthracene, Bioremoval, Halophile, Laccase.

1. Background

Many saline environments such as natural saline lakes, salt flats, saline industrial effluents, oil fields, and salt marshes are susceptible to petroleum pollutants containing Polycyclic Aromatic Hydrocarbons (PAH)s. These compounds are known as one of the most important group of organic compounds with two or more fused aromatic benzene rings (1, 2). Although PAHs are distributed in nature by forest and prairie fires, oil seeps, volcanic activity, and exudates from trees, but several polluting anthropogenic activities include combustion of fossil fuels, municipal and urban wastewater discharge runoff, automobile engine exhausts, refinery, oil storage waste, etc., deposit significant quantities into the environment (3). They have been of global concern owing to their presence in all components of environment, hardly biodegradable, potential to bi-accumulation, and the significant health risks posed by the toxic, mutagenic, and carcinogenic properties associated with certain PAH compounds. Although considerable research efforts have been done to the search of appropriate remediation technologies to remove these contaminants from the environment or to transform them into less hazardous/ nonhazardous

compounds such as adsorption, volatilization, photolysis, and chemical oxidation, biodegradation is one of the safest and efficient way to execute this issue (4).

Various microorganisms have been found that degrade PAHs. Due to disability of conventional microorganisms to operate efficiently at saline environments, the biological treatment is possible with halophiles (5). Many bioremediation technologies on PAH-contaminated environments have employed for half past decade. Enzymatic treatment may represent extremely efficient and selective alternative to conventional bioremediation related to the use of microorganisms. Although application of enzymes may be reduced via the high cost multi steps purification processes, but recently has received considerable attention because of their unique properties such as higher reaction rates towards recalcitrant pollutants, lower sensitivity to the concentrations of contaminants, milder reaction conditions, catalysis with either narrow (chemo-, region- and stereo-selectivity) or broad specificity, coverage of a wide range of physicochemical gradients in the environmental matrix, and easy control of field application (6). Enzymes participating in the degradation of PAHs, by bacteria, fungi, and plants include cytochrome P-450 monooxygenases, oxygenases, and dehydrogenases. The ability of extracellular enzymes such as peroxidases and oxidoreductases, particularly laccases, to degrade PAHs has recently been reported from studies. Although fungi are the best-known laccase producers, bacterial laccases have been found to possess distinctive properties. For example, bacterial laccases have the ability to work excellent under alkaline conditions, and high halide tolerance (7).

2. Objectives

The aim of this work was the evaluation of a system based on the use of crude halophile laccase for the degradation of a PAH model compound, anthracene, under a variety of conditions. After the selection of the halophilic bacterial enzymes and optimization of the removal process the toxicity of the laccase-treated anthracene as the inhibitory effect on the growth of *Pseudokirchneriella subcapitata* was assessed and was compared to the untreated one.

3. Materials and Methods

3.1. Chemicals

1-Hydroxybenzotriazole (HBT), 2,6-dimethoxyphenol (DMP), anthracene, and phenanthrene were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other reagents and chemicals were of the highest purity available.

3.2. Biodegradation Screening

Six halophile bacterial strains were selected from laboratory of enzymatic technology, Department of Pharmaceutical Biotechnology, Tehran University of Medical Sciences, Tehran, Iran. Anthracene degradation ability with their crude enzymes was analyzed by HPLC. A strain with the most degradation ability was selected for further experiments.

3.3. Bacterial Strain, Culture Conditions, and Concentrated Crude Enzyme Preparation

The bacterial strain SR-079 Halo used in the present study was previously isolated from the saline water of Shoor-Mast Lake (36°08'N and 53°04'E), located in the north of Iran and identified as *Alkalibacillus salilacus* by morphological, physiological, biochemical, and molecular assays (8). Stock culture was maintained on agar slants containing modified Luria-Bertani (LB) medium: 10.0 g.L^{-1} peptone, 5.0 g.L^{-1} yeast extract, 175 g.L^{-1} NaCl, 15.0 g.L^{-1} agar, pH 7.4, incubated for 48-h at 37 °C, and then stored at 4 °C and sub-cultured every month. The cultivation was carried out in 2000-mL erlenmeyer flasks containing 500 mL modified Luria-Bertani (LB) medium contained 0.25 mM CuSO$_4$ (to induce laccase production), and 2.5 M NaCl (pH 7.4). The flasks were inoculated with 10% inoculum, which was grown in pre-culture. The cultures were harvested after 48-h incubation at 37 °C and 150 rpm, when they had reached maximum production of enzyme. For estimating enzyme production, samples of fermented media were withdrawn at regular 24-h intervals and were centrifuged at 5500×g for 20 min and the enzyme activity was assayed in the cell-free supernatant.

The concentrated crude enzyme of *A. salilacus* was produced using the method described by Moshfegh *et al.* (9). Briefly, the 48-h culture of the isolate was centrifuged at 5500×g for 20 min for removal of bacterial cells. The precipitation of cell-free supernatant was carried out by gradually adding the pre-chilled ethanol up to an 80% (v/v) saturation and stirring for 2-h. The precipitated enzyme was collected by centrifugation at 12000×g for 20 min and dissolved in a minimum volume of 50 mM phosphate buffer (pH 7.4) containing 2.5 M NaCl and 0.25 mM CuSO$_4$. The concentrated

sample was dialyzed against the same buffer overnight. All of the steps were carried out at 4 °C.

3.4. Enzyme Assay and Protein Content Determination

Activity of manganese dependent peroxidase (MnP), manganese independent peroxidase (MIP), and laccase was determined spectrophotometrically, based on the oxidation of their specific substrates at 610 nm (10). To determine manganese peroxidase activity, 0.5 mL enzyme sample was added to 0.5 mL of reaction solution containing 50 mM oxalate buffer, 1 mM $MnSO_4$, and 1 mM phenol red (pH 7.4). The enzymatic reaction was initiated by adding 1 mM H_2O_2. For manganese independent peroxidase, 1 mM EDTA was required. The absorbance was determined at 610 nm. Laccase activity was measured by monitoring the oxidation of 2,6-dimethoxy phenol as a substrate in 1mL reaction mixture containing 0.5 mL DMP (2 mM) dissolved in 50 mM of phosphate buffer (pH 7.4) and 0.5 mL of enzyme sample. The mixture was incubated at 40 °C and 120 rpm for 10 minutes. The assay was performed by an increase in absorbance at 468 nm. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmoL of substrate per minute under the assay condition. Triplicate experiments were carried out and total protein concentration was measured by the method of Bradford (11) using bovine serum albumin (BSA) as the standard.

3.5. Medium Optimization for Maximum Enzyme Production by A. salilacus

The simultaneous effect of pH and temperature was determined by the standard assay in 50 mM of phosphate buffer (pH 6.5–8) or Tris–HCl buffer (pH 9) at temperature range of 25-45 °C with 5 °C intervals. The effect of NaCl concentrations was examined at LB medium containing 1.5–3.5 M salt at pH 7.4. All treatments were in triplicate.

3.6. Removal of Anthracene Using the Concentrate Crude Laccase

The experiments were performed in 15-mL tubes with 1 mL 50 mM phosphate buffer (pH 7.4) containing 0.25 mM $CuSO_4$,5% acetone, and 0.5% Tween 80 with 1 mM HBT. Anthracene was first dissolved in acetone to increase bioavailability and added to solution at final concentration of 20 mg.L^{-1}. The concentrated crude laccase was added to each tube to a final concentration

of 28 U.mL^{-1}, and closed reaction tubes were incubated at 37 °C, 150 rpm for specified period. After incubation, acetonitrile was added to the reaction mixture for the extraction of the anthracene. The screw caps were closed tightly, and the tubes were shaken again. Each sample was centrifuged at 8000×g for 10 min and an aliquot (1 mL) of the supernatant was analyzed by high performance liquid chromatography (HPLC). 20 mg.L^{-1} of phenanthrene was added to each sample as internal standard prior to solvent extraction.

The effects of pH, temperature, time contact, salt concentration, HBT concentration, enzyme, and substrate concentration on the oxidation of anthracene were examined. Increasingly, the effect of different co solvents and surfactant on removal of anthracene was investigated. Boiled enzyme served as the control and all treatments were in triplicate.

3.7. HPLC Analysis

Residual concentration of anthracene in the enzymatic reaction mixtures was determined by an HPLC system equipped with a Smartline Pump 1000, a PDA Detector 2800 (set at 254 nm), a Degasser 5000, and Lichrospher 100 RP & C18 reverse phase column (C18, 250 × 4.6 mm), all from Knauer (Berlin, Germany). Each sample (20 μL) was injected using a Smartline Autosampler 3950 with a sample loop of 100 μL. The mobile phase was acetonitrile/methanol at a ratio of 70:30. The retention time of anthracene (at flow rate of 1 mL.min^1) was 18 min.

3.8. Effect of Different Factors on the Removal Percentage of Anthracene

3.8.1. Effect of Time

For this subject, anthracene was dissolved in acetone 5% and Tween 80 0.5% and was added to phosphate buffer containing 2 M NaCl (pH 7.4) at final concentration of 20 mg.L^{-1}. Then the reaction medium was incubated with crude enzyme (28 U.mL^{-1}) and 1 mM HBT at 37 °C for 120 h. Samples were injected to HPLC after 1, 6, 12, 24, 48, 72, 96, and 120 h after incubation.

3.8.2. Effect of NaCl Concentration

The ability of the crude enzyme at the various concentrations of salt (0–4 M) to eliminate the anthracene was studied by addition of 28 U mL^{-1} enzyme, 20 mg.L^{-1}

anthracene, 5% acetone, 5% Tween 80, and 1mM HBT to the reaction mixture, which was incubated at 37 °C and 150 rpm for 72-h.

3.8.3. Simultaneous Effect of pH and Temperature

The effect of pH and temperature on the anthracene removal rate was studied by changing the aqueous phase pH of reaction mixture between a range of 5 to 9 using different buffer systems (sodium citrate, pH 5–6; phosphate buffer, pH 6–8;Tris–HCl, pH 8–9) containing 1.5 M NaCl, simultaneously in front of different temperatures (20–50 °C) and by applying the anthracene concentration 20 mg.L^{-1}, enzyme concentration 28 U.mL^{-1}, HBT dose 1 mM, acetone 5%, Tween 80 0.5%, and incubation time 72-h.

3.8.4. Simultaneous Effect of Enzyme and Substrate Concentration

1-mL solutions containing enzyme concentration of 7-28 U.mL^{-1}, 5, 10, 20, 50, 100 mg.L^{-1} anthracene, acetone 5%, Tween 80 0.5%, and HBT 1 mM in phosphate buffer (contained 1.5 M NaCl) at a temperature of 40 °C, and pH 8 was shaken at 150 rpm for 72-h.

3.8.5. Oxidation of Anthracene in Different Solvents and Surfactants

Solvents with different log Pow values including acetone, DMSO, Chloroform, and Ethyl acetate were selected to investigate the effect of organic solvents on the removal of anthracene. For these test, 25% of the solvent was added to the reaction mixture containing 21 U.mL^{-1} crude laccase, 20 mg.L^{-1} anthracene, 0.5% Tween 80, 5% acetone, 50 mM saline phosphate buffer (containing 1.5 M NaCl), and 1 mM HBT. The mixture was incubated at 40 °C for 72-h and shaking at 150 rpm. The mixture contained boiled enzyme served as control. After incubation, samples were extracted with chloroform and ethyl acetate from the aqueous phase and then were dried with anhydrous sodium sulphate column and were evaporated using a rotary evaporator. Residual anthracene was diluted with acetonitrile and was injected to HPLC.

To study the effect of surfactant, cetyl trimethyl ammonium bromide (CTAB), Tween 80, and Triton X-100 were selected as surfactants and crude enzyme was incubated with 10 mM CTAB, 1% of other selected surfactants, and 5% acetone at 40 °C for 72-h. Boiled enzyme considered as control.

3.8.6. Oxidation of Anthracene Using Laccase-Mediated System

The removal of anthracene in the presence of HBT was evaluated by introducing different concentrations of mediator (0, 1, 5, and 10 mM) to the reaction mixture. All experiments were carried out in triplicate and reaction mixture without HBT containing heat-activated enzyme was used to check of removal.

3.9. Kinetic Studies

The kinetic parameters of the enzymatic removal of anthracene were obtained after the determination of velocity for different concentrations of substrate. Next, a Michaelis-Menten curve was drawn by plotting the obtained velocity (V) against the anthracene concentrations (S). K_m (Michaelis constant) and V_{max} (maximal velocity) were then calculated using the Lineweaver-Burk transformation of the Michaelis-Menten equation.

3.10. Toxicity Assay

A toxicity assay was performed to evaluate the toxic effect of anthracene and its metabolites on the growth of *P. subcapitata* based on OECD 201 method. Toxicity tests measured parameters indicating population development: growth of the freshwater alga *P. subcapitata* in a 3-day batch culture. Algal toxicity tests were performed in BG-11 medium. The culture was supplemented with various concentrations of anthracene which were previously dissolved in dimethyl sulfoxide (DMSO) at a concentration of 0.5 mL.L^{-1}. This dose also added to the control culture. Control solutions (concentration of 0.0 μg.L^{-1}) were conducted to confirm the accuracy of the test. An initial cell concentration of approximately 104 cells.mL^{-1} from a late log phase culture was grown in a 50-mL Erlenmeyer flasks with 10 mL of culture medium. The culture was incubated at 25 °C, with (12:12) light: dark conditions. Growth was monitored by counting the cells with lam under optical microscope every 24-h over a 3-day period. The growth inhibition and toxicological data (EC) were estimated based on OECD 20l protocol. This process was also performed for the metabolites of the concentrations to determine EC range.

4. Results

4.1. Production of the Extracellular Enzyme

The strain SR-079 with the most anthracene degradation

was selected among 6 halophilic isolates for further studies. Based on the morphological and physiological assessments, the cells of the isolate were motile, aerobic, spore-forming, gram positive, rods, and with round, smooth, and creamy colonies on LB agar medium containing 12% NaCl according to Samaei *et al.* (8) study. The isolate grew well in optimum condition including 12% NaCl, pH 8.0, and temperature 40 °C (8).

4.2. Effect of Time on the Removal of Anthracene

The time course of anthracene degradation by concentrated crude laccase of *A. salilacus* was monitored during a period of 5 days. 51.6% decrease in anthracene presence was observed within the three experimental days. In the following days, their content continued decreasing; however, this decrease was not as intensive as in the initial period. Maximal degradation was at 120-h, however, there was no significant difference

between 72-h and 120-h and hence, 72-h was selected as the optimal time (**Fig. 1A**).
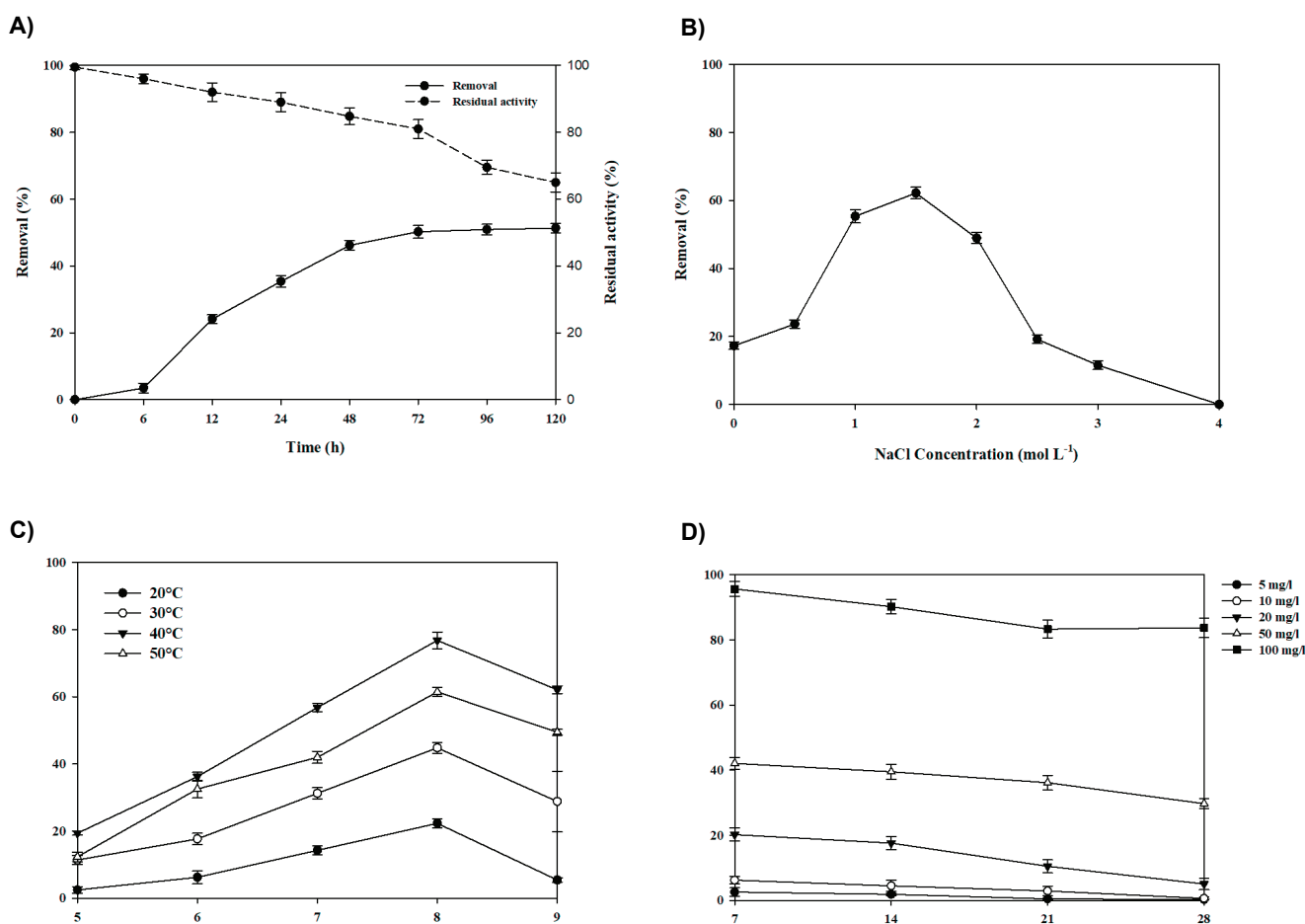
4.3. The effect of Salt Concentration

As shown in **Figure 1B**, the biodegradation rate of anthracene reached 56.7%, 63.4%, 50.1% in medium containing 1, 1.5, and 2 M NaCl, respectively.

4.4. Simultaneous Effect of pH and Temperature

The effect of pH and temperature on the removal percentage of anthracene is shown in **Figure 1c**. The optimum pH and temperature were 8 and 40 °C at 72-h. Under the optimal conditions, anthracene was oxidized 76.8% as illustrated in **Figure 1C**.

4.5. Simultaneous Effect of Enzyme and Substrate Concentration

The effect of enzyme concentration on the removal of

Figure 1. Effect of incubation time **A)**, different salt concentrations **B)**, pH and temperature **C)**, and enzyme and the substrate concentrations on anthracene removal by the laccase **D)**.

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different concentrations of anthracene was shown in **Figure 1D**. As the enzyme concentration increased, the removal of anthracene increased in each concentration, while further enhancement of enzyme (to 28 U.mL^{-1}) demonstrated no significant influence on the residual concentration of anthracene. Optimum concentration for enzyme and anthracene was a point that had maximum removal rate with minimum enzyme loading. In this case, for initial concentration of anthracene of 20 mg.L^{-1} and laccase activity of 21 U mL^{-1}, maximum removal rate was obtained, and the anthracene removal was about 61.5%.

4.6. Enzyme Crude Production by A. salilacus and Medium Optimization

The isolate produced extracellular enzyme (laccase, manganese peroxidase, and manganese independent peroxidase) in the LB culture. The growth curve and the extracellular enzyme activity were shown in **Figure 2A**. The start of the production of all extracellular enzymes was at the beginning of the exponential phase of growth and reached their maximum level in the early-stationary phase (48-h). The bacterium showed a very low activity of MnP (0.36 U.mL^{-1}) and MIP (0.26 U.mL^{-1}). The dominant enzyme was laccase with an average activity of (0.40 U.mL^{-1}). The laccase activity increased about 2.25-fold after adding CuSO$_4$ (**Fig. 2B**). Copper had been reported to be a promising inducer for laccase (12). Moreover, produced laccase had optimum activity at the pH 8, temperature 40 °C, and NaCl concentration 2.5 M (**Fig.2C, 2D**).
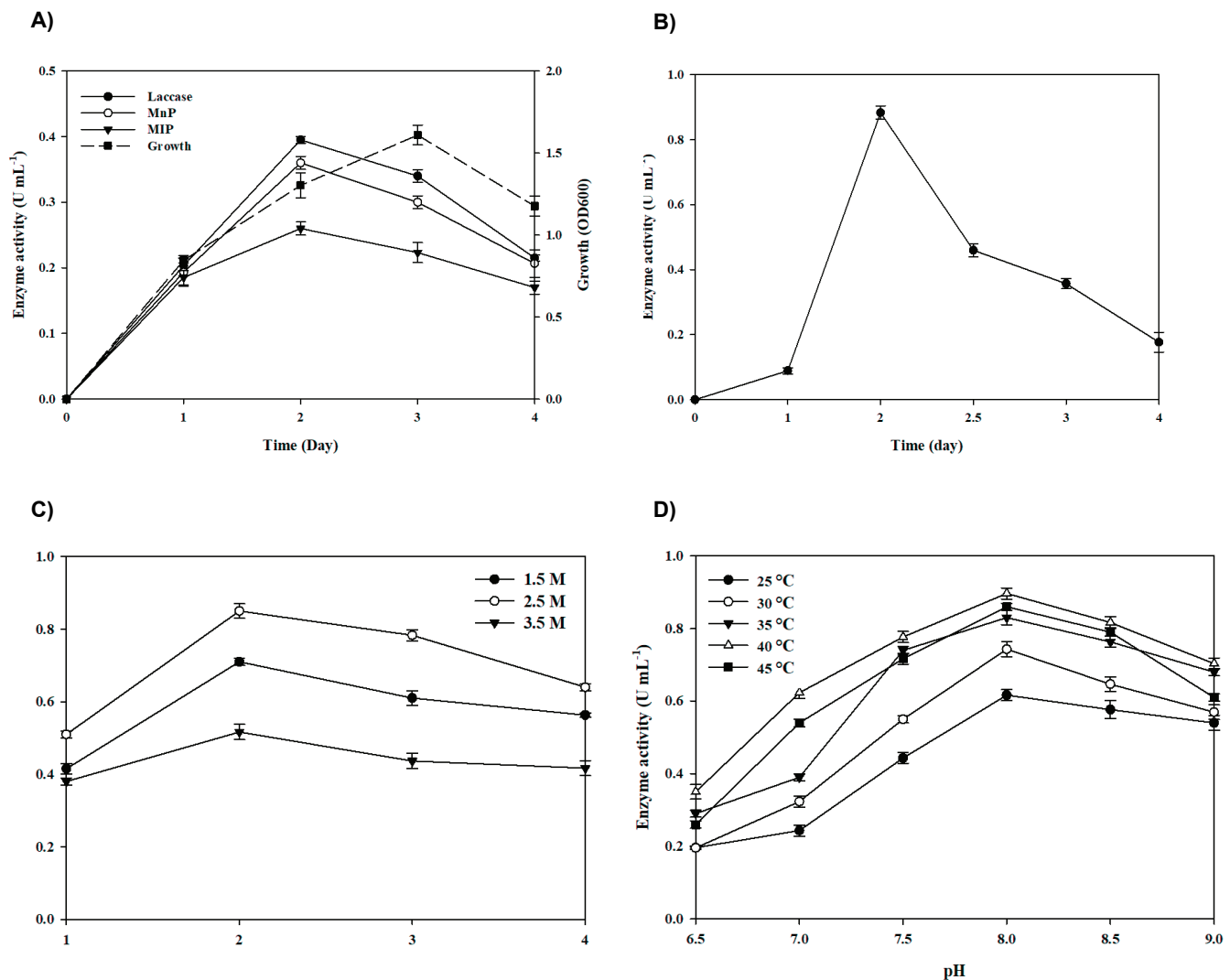
Figure 2. MnP, MIP, and laccase activity **A)**, Enhanced production of the laccase by CuSO4 as an inducer **B)**, Effect of the salt concentrations on the laccase production **C)**, Effect of pH and temperature on the laccase production **D)**.

## 4.7. Oxidation of Anthracene Using Laccase-Mediated System

Figure 3A manifests that in the presence of HBT, the removal of anthracene significantly enhanced, which was more efficient than laccase alone. However, in higher concentration of HBT (5 mM and 10 mM), there was no enhancement from 1 mM and it negatively affected the removal rate of PAHs (14).

## 4.8. Oxidation of Anthracene in Different Solvents and Surfactants

The laccase from *A. salilacus* was shown the ability to tolerate in high percentage (25% v/v) of different solvents and 0.5% Tween 80 as demonstrated in **Figure 3B, 3C**. Adding surfactant has positive effect on improving the stability of enzyme and reducing the inhibitory effect of solvents. The percentage of anthracene removal increased in order with DMSO>acetone> ethyl acetate> chloroform. None of these solvents inhibited the laccase in 25% (v/v) over 72-h. From these results, laccase from *A. salilacus* can be applied in many enzymatic treatments and biological processes due to its resistance in the present of organic solvents.

Moreover, the laccase-catalyzed removal of anthracene was studied in the presence of 1% (v/v) surfactants, Tween 80, Triton X-100, and (10 mM) CTAB. It was found that the addition of Tween 80, Triton X-100, and CTAB enhanced the removal of anthracene to 93.8%, 88.2%, and 81.2%, respectively, in this experiment.
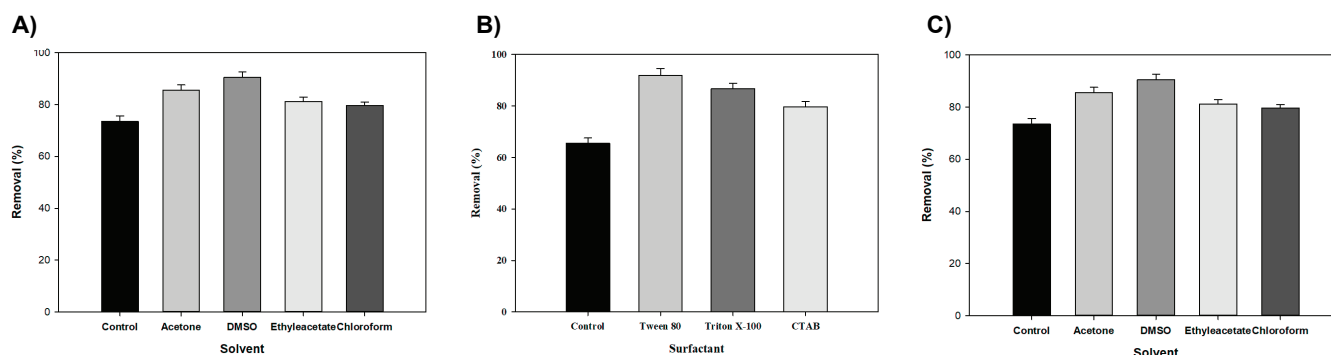
Figure 3. Effect of HBT on anthracene removal catalyzed by laccase **A)**, different solvents **B)**, and different surfactants on the laccase-assisted removal of anthracene **C)**.
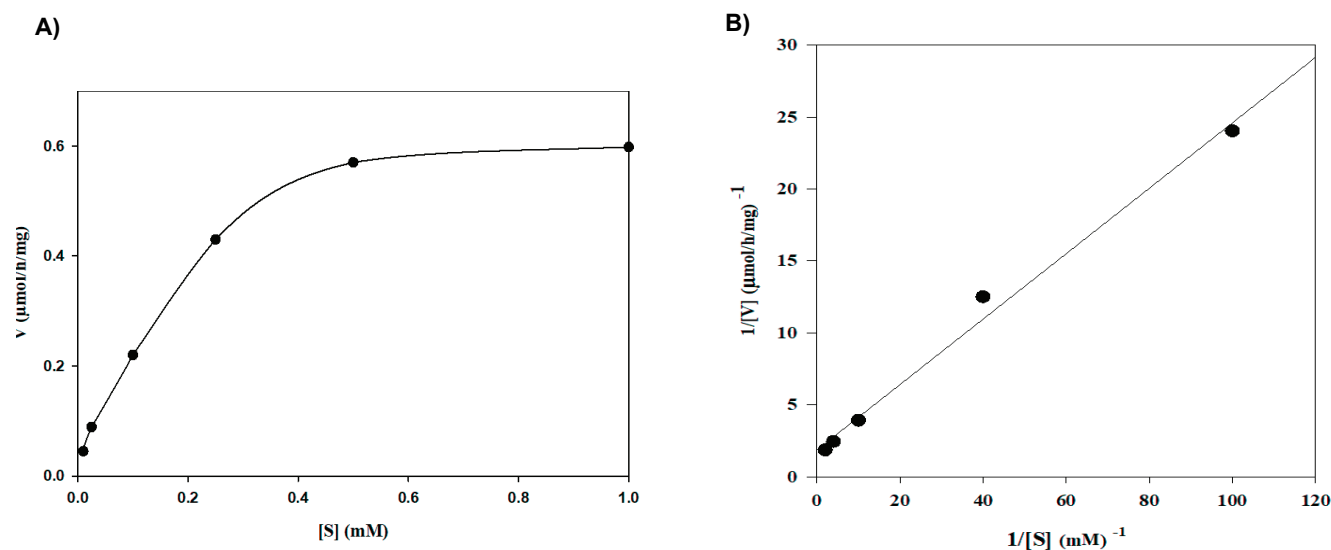
Figure 4. Kinetic study of the removal reaction, Michaelis-Menten **A)** and Lineweaver-Burk plots **B)**.
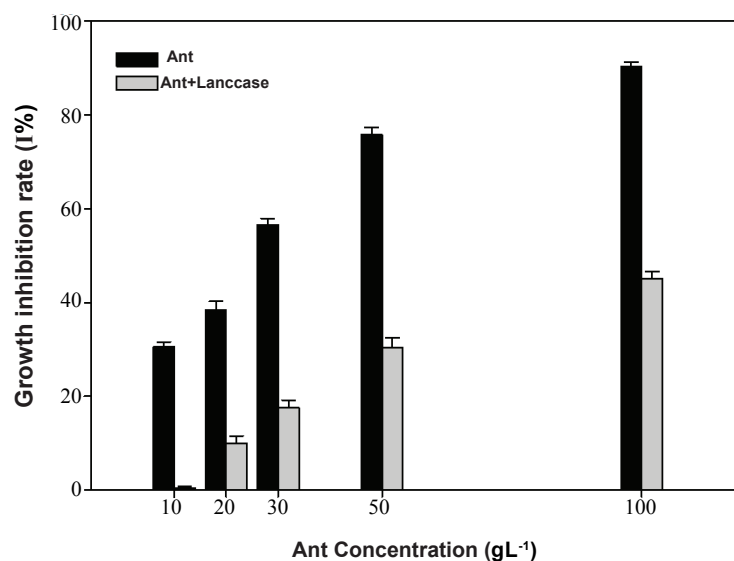
Figure 5. Influence of anthracene and the laccase-converted metabolites on P. subcapitata cell growth.

4.9. Kinetic Studies

Figure 4A, 4B shows the time course of the different concentrations of anthracene during 24-h and Lineweaver-Burk plot, respectively. First-order and Michaelis-Menten kinetic models were applied for the kinetic study of *A. salilacus* laccase to fit the experimental data. The linear relation between the initial velocity and substrate concentration resulted in Michaelis-Menten constant (K_m) 0.114 μM and maximum removal rate (V_{max}) 0.546 μmoL.h.$^{-1}$mg^{-1}. See Toxicity results and discussion sections in supplementary.

4.10. Toxicity

Enzymatic removal considered effective provided that the toxicity of the formed metabolites diminishes (15). Toxicity assessment on *Pseudokirchneriella subcapitata* was conducted by counting the cells after exposure to the anthracene and its metabolites during 72-h. Generally, growth inhibition of algal considerably decreased after the treatment of anthracene by laccase. Enzyme catalyzed (EC) of the anthracene data before and after degradation with crude enzyme is presented in **Figure 5**. As demonstrated in **Figure 5**, the inhibition at concentrations of 20 and 30 μg.L^{-1} was 39.7% and 57.6%, respectively, while the algal growth only was inhibited 11.1% and 18.7% by metabolites of these concentrations. Moreover, no toxicity was observed on *P. subcapitata* for 0.1% DMSO as negative control.

5. Discussion

The results showed that our laccase is active in the neutral to alkaline pH which was of much important and more suitable for industrial applications, since fungal laccases are functional at neutral and acidic pH and lose their activities at alkaline conditions. Our laccase demonstrated high activity at different concentration of NaCl in experiments and can be categorized as halophile enzyme (17). The enzyme exhibits advantageous over most of the fungal laccases which are inactive at concentrations higher than 100 mM NaCl. Therefore, it can be applied for wastewater and effluent treatments, bleaching, oil fields, etc., where there are possibilities for high salt contents (16).

The enzymatic degradation rates depend on many factors including the sources of laccases (bacteria, fungi, etc.), pH, temperature, substrate concentration, chemical structure of the compound, chemical partitioning in growth medium, and the presence of substrates/ mediators, cofactors, inhibitors, and organic acids (18, 19). Wu *et al.* (20) reported the transformation of PAHs by fungal laccase in soil and reaction mixture in the presence and absence of ABTS as a mediator. 24.9 and 72.3% of anthracene and 29.57% of benzo(a)pyrene were oxidized by laccase in the absence of ABTS, however, the addition of 1mM ABTS significantly enhanced the transformation of anthracene and benzo(a) pyrene to 100%. Besides, laccase transformed several PAHs efficiently and the removal rates from reaction mixture were different in some cases (20). As it was

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shown in (**Fig. 1A**), the removal percentage in this way is much faster than conventional degradation by bacteria or fungi, which took several weeks (13).

Although high NaCl concentration can increase halophile laccase activity (5), there is an inverse relationship between salinity and anthracene solubility (21). In this case, 1.5 M was selected as an equilibrium point between degradation and solubility. As a result, reduced removal of anthracene at higher and lower concentration of 1.5 M can be due to the scant interaction between enzyme and substrate or deactivation of enzyme, respectively.

Our results were also in agreement with previous studies (7, 22). In the study which conducted by Khlifi R. *et al.* (23), the optimum temperature for laccase from *T. trogii* in decolorization of a textile industry effluent was observed at 50 °C. This laccase was stable for 24-h at 50 °C; however, it lost 90% of its activity at 60 °C. Aktas *et al.* (24) showed the effect of temperature on laccase catalyzed polymerization of catechol. In this study at the range of (25–60) °C, enzyme activity increased to 45 °C, while enzyme activity was very low at low temperatures. In another study by Ceylan *et al.* (25), the effect of temperature on removal of 1-naphtol by laccase from *Trametes versicolor* was investigated. The enhancement in temperature from 20 to 35 °C, increased the removal rate from 6.98 mM min⁻¹ to 9.08 mM min⁻¹, while further increase in temperature decreased the degradation rate to 5.03 mM min⁻¹. Rahmani *et al.* (26) used free and immobilized laccase for maximum removal of sulfathiazole and sulfamethoxazole at 50 °C.

Electrostatic properties of the protein surface and the reaction center are affected by changing in pH value. Compared to fungal laccases, bacterial laccases have a lot of priority, such as activity and stability at high pH and temperatures. Xuanzhen *et al.* (22) optimized the reaction conditions for oxidation of benzo(a) pyren by laccase from *Trametes versicolor* at 4 and 40 °C, respectively. The optimum pH and temperature for benzo(a)pyren oxidation was. Fang *et al.* (7) revealed that Lac15 as a bacterial laccase can catalyze syringaldazine at pH 6.5–9.0 with an optimum pH of 7.5. In addition, held *et al.* (27) represented the decolonization of the common textile dyes at pH 9 and 60 °C with laccase containing *Bacillus subtilis* SF spores. However, there are some bacterial laccases that the optimum pH for them is in acidic ranges. Zeng *et*

al. (18) manifested the high amount of transformation of anthracene (80%) and benzo(a)pyrene (97%) by the bacterial laccase at optimum pH 5 and 60 °C. Working at non-optimum conditions decreased the removal rate that can be due to the enzyme inactivation or dearth of contact between enzyme and substrate.

Rahmani *et al.* (26) reported the influence of laccase concentration on removal of SMZ and STZ using free and immobilized laccase. Removal percentage increased significantly with increasing in laccase activity from 0.1 U.mL⁻¹ to 0.8 U.mL⁻¹. The same was observed in Ashrafi et al. (28) and Asadgol *et al.* (29) studies, which reported that pollutants removal percentage enhanced by increasing laccase activity. Moreover, substrate concentration in aqueous phase has inevitable effect on removal efficiency. If the enzyme concentration was kept constant and the amount of substrate increased, the removal rate increased until reached maximum, then adding more substrate was led to no more enhancement in removal rate. Mohan *et al.* (30) reported that removal rate was high in dye concentration up to 30 mg.L⁻¹, while removal rating decreased at higher concentrations.

It has been widely demonstrated that the redox mediators extend the capacity of the laccase system to oxidize compounds with a higher redox potential. A case in point, the normal standard redox potential of PAHs ranges between 1.3 and 1.8V/NHE (31), while the redox potential of laccase is only around 0.8V/NHE (32). Nonetheless, laccase can oxidize anthracene with aid of HBT, which has a high redox potential (1.1V), forming cation radicals from the reaction between laccase and HBT, and these radicals co-oxidize the anthracene. However, high concentration of HBT can have destructive effects and destabilize the laccase (28). Our results were in agreement with the result by Johannes *et al.* (33) who found that transformation of anthracene increases rapidly at the present of different mediators. In another study by Zeng *et al.* (18) which investigated the decolorization of synthetic dyes by crude laccase, the removal rate decreased at HBT concentration higher than 1mM.

It is known that PAHs are hydrophobic; therefore, the development of an efficient degradation system for polyaromatics requires cosolvents and surfactant to increase bioavailability of substrate. To choose an appropriate solvent, the effect of the solvent on solubility of the substrate and stability of the enzyme should be

considered. The addition of organic solvents can affect some enzymes and cause some inherent problems like inactivation of the enzymes, since the solvent can affect the hydration shell of the enzyme molecule, necessary to maintain the native conformation (34). For example, in the study of Keum *et al*. (35), in which laccases from *Trametes versicolor* and *Pleurotus ostreatus* were tested to degrade hydroxy PCBs, they reported that laccase activity decreased rapidly in more than 10% acetone, acetonitrile, and DMSO. Deformation of the enzyme structure or substitution of water molecules at the active sites of the enzymes by organic solvents can be resulted in instability of laccase and its disability to remove pollutants. Using immiscible solvents for enzymatic reactions has many advantages, for example, a hydrophobic substrate is located in organic solvents layer and allows the enzyme to use high concentration of substrate without inhibition. However, there are some inherent problems e.g. low reaction rate due to the low rate of mass-transfer across the interface (36). In contrast, it was shown by Lu *et al*. (16) that 10% acetone and 20% DMSO have positive effect on laccase activity of an alkaline resistant and organic solvents tolerant laccase from *Bacillus licheniformis*LS04. In addition, 59% of laccase activity remained at 30% concentrations of different organic solvents. Since salt has the effect of reducing water activity, halophilic enzymes can act as biocatalysts in aqueous/organic and non-aqueous media (36).

Moreover, the results of laccase-catalyzed removal of anthracene in the presence of 1% (v/v) surfactants are consistent with the previous researches (37, 38). In the study of Dodor *et al*. (37), in which they investigated the influence of (0.1–5%) Tween 80 on the stability of free and immobilized laccase and oxidation of anthracene and benzo(a)pyrene, the results indicated that Tween 80 increased the activity of the free laccase 100 times higher than control (without Tween 80). The positive effect of Tween 80 could be due to the increasing the bioavailability of PAHs and stability of the enzyme. Samaei *et al*. (8) reported that the addition of Tween 80 (0.1–0.5) %, Triton X-100 (0.1–0.5) %, and CTAB (1–10 mM) enhanced the stability of the lipase from A. salilacusSR-079 Halo. From these results, 1% Tween 80 was selected for anthracene removal.

The oxidation mechanism of laccase is a multi-step process that the slowest step will be the limiting step in the degradation process. The reduction of mediator

(HBT) is a fast reaction according to the kinetic constants (39), while anthracene degradation is limiting step due to the fact that the enzymatic reaction is more rapid than the oxidation of anthracene (34). The values were similar to those obtained by other authors. Liu *et al*. (40) showed that the K_m value of *Trametes versicolor* laccase for anthracene removal reaction is 0.68 mM, and chemically modification of laccase can decrease K_m value, which results in increment of oxidation rate.

Algae are sensitive microorganisms and the basis of many food chains; therefore, they are appropriate for toxicity tests. Pagnout *et al*. (41), reported the biodegradation of pyrene, fluoranthene, and phenanthrene by *Mycobacterium* sp. strain SNP11 and toxic effect of these PAHs and their dead-end metabolisms on bacteria, algae (*Raphidocelis subcapitata*), and crustaceans. The obtained results showed a disappearance or very significant reduction of (Geno) toxic potential after biodegradation.

According to obtained results, this system is expected to be effective in bioremoval of non-phenolic compounds; however, this requires further experiments to evaluate the efficiency of process in actual samples.

6. Conclusion
The aim of the present study was to investigate the elimination of anthracene by the laccase from halophilic *Alkalibacillus salilacus*. Laccase showed the maximum removal efficiency in the presence of HBT. Anthracene is an appropriate substrate for the isolated laccase according to the kinetic parameters of the laccase-catalyzed removal reaction. Furthermore, micro toxicity test with *Pseudokirchneriella subcapitata* represented that the produced metabolites did not have inhibitory effect on algae growth.

Conflict of interest
The authors indicate no potential conflict of interest.

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