RESEARCH ARTICLE

Efficiency of Purified Laccase from *Pseudomonas* Spp. as Bioremediator Agent in Gasoline Contaminated Soils

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ABSTRACT

Petroleum and petrochemical pollution, such as petroleum hydrocarbons (PHCs), is one of the most important environmental threats today due to its global dispersion. Contamination by these chemicals degrades the global environment and reduces biodiversity significantly. So that *Pseudomonas fluorescence, Pseudomonas aeruginosa*, and *Pseudomonas putida* isolated from contaminated soils with hydrocarbons could produce laccase with higher productivity by *P. putida.* SSF conditions with rice bran were better than with wheat bran in the induction of laccase production. The degradation of hydrocarbons with purified laccase increased with time and the maximum degradation level was appeared after 12 days then declined at the peak with time, therefore; the potential employment of microbes as biological instruments might provide a more cost-effective and efficient way to reduce waste and save natural resources, could be a viable answer to one of modern society's most pressing issues.

Keywords: Laccase, Petroleum hydrocarbons, *Pseudomonas putida*.

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INTRODUCTION

One of the most important current issues is environmental contamination caused by oil and PHCs. PHCs are organic pollutants that are of major concern because of their toxicity and widespread distribution over the planet. Diesel-range hydrocarbons (DRHs) and gasoline-range hydrocarbons (GRHs) are the two types (GRHs). Longer chain alkanes and hydrophobic compounds, such as polycyclic aromatic hydrocarbons, are examples of DRHs (PHCs). Hydrocarbons such as ethylbenzene, benzene, xylenes, and toluene are examples of GRHs.[1]

Laccases (p-benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are multicopper proteins that belong to the blue-copper protein family and are frequently found in plants, fungi, and bacteria.[2] They are a kind of enzyme that catalyzes the oxidation of ortho- and para-diphenols, aminophenols, polyphenols, polyamines, lignins, and aryl diamines, as well as various inorganic ions.[3] Laccases have gotten a lot of interest from academics in the recent decade because of their broad substrate specificity and ability to oxidize a wide spectrum of phenols and polyphenols. They're also important in the detoxification of textile effluents and bioremediation.[4]

The goal of this study was to see how reliable natural substrates like rice bran and wheat bran are for increasing laccase enzyme synthesis in *Pseudomonas* spp. during solid-state fermentation versus liquid fermentation. In addition, laccase has been partially purified and is being used as a bioremediator.

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MATERIALS AND METHODS

Samples Collection

To perform the work, 23 samples of gasoline contaminated soil were collected from Baghdad gas stations and packed in sterile plastic bags before being transported to the lab.

Screening of Laccase production under LSF Condition

Qualitative Analysis

One gram of soil sample was mixed with 99 mL of distilled water, shacked for 30 minutes, and then allowed to settle for 30 minutes. We made serial dilutions ranging from 10^{-2} to 10^{-6}. Each sample was inoculated onto nutritional agar with 0.02% 1 mM guaiacol and incubated for 24 hours at 30˚C.[5] Laccase producers were identified by the appearance of a reddish-brown oxidation zone on agar plate media.

Identification of Laccase Producers

On the established colonies, morphological and biochemical tests were performed.[6] In addition to the Vitek 2 system to confirm these isolates.

Quantitative Analysis

The selected isolates were inoculated with nutrient broth supplemented with 0.02% of 1 mM guaiacol and incubated at 30°C for 24 hours. Laccase activity was measured after centrifugation at 8000 rpm for 30 minutes.

Laccase Activity and Protein Contents

The oxidation of 1 mM guaiacol was used to determine laccase activity. 300 liters of 1 mM guaiacol, culture filtrate, and 0.2 M sodium acetate buffer made up the reaction mixture (900 µL) (pH 4.5). The quantity of enzyme that oxidized 1 mol of guaiacol per minute was defined as one unit of enzyme activity. The enzyme activity was measured in units per milliliter (U/mL).[6] Protein contents were carried out using a bovine serum albumin standard and the Bradford technique[7] with the absorbency measured at 595 nm.

Production of Laccase under SSF Condition

In separate flasks, 2 g of wheat bran and rice bran were moistened with 100 mL of Mineral Basal Salt Solution (MBSS) containing (g/L): peptone 3, dextrose 10, K_2HPO_4 0.4, KH_2PO_4 0.6, $MnSO_4$ 0.5, $FeSO_4$ 0.0005, and $ZnSO_4$ 0.01.[5] The medium had a moisture content of 20% (w/v) at the start. The flasks were disinfected, cooled to room temperature, and injected with the chosen bacterial isolates, which were then incubated for 24 hours. Flasks were completely mixed with 0.05 M sodium phosphate buffer (pH 8) under shaking conditions after incubation and centrifuged at 10000 rpm for 10 minutes at 4°C. The laccase activity of the resulting supernatant was determined.

Partial Purification of Laccase

After centrifugation, the supernatant was concentrated using fractioned precipitation of 20–80% ammonium sulfate saturation. Flasks were suspended in 0.05 M sodium phosphate buffer (pH 8) and centrifuged for 10 minutes at 10000 revolutions per minute. After centrifugation, the sample with the highest enzyme activity was dialyzed extensively against the same buffer and used for future research.

Removal of Hydrocarbons Content from Contaminated Soil by Laccase

A total of 20 grams of hydrocarbon-contaminated soil were combined with 40 mL of pure laccase. The laccase was replaced with distilled water and incubated at 30°C for 24 hours as a control. The generated liquid solution from the soil was provided after centrifugation for 10 minutes at 6000 rpm. Every two days, the number of hydrocarbons in the soil following contact with pure laccase or in control was measured by combining the identical amounts of soil and toluene. The hydrocarbon-dissolved toluene was measured at 410 um absorbance after centrifugation.[8] Optical density for control-optical density for test), optical density for control x 100 was used to calculate the percentage of hydrocarbon degradation.

RESULTS AND DISCUSSION

Identification of Producer Isolates of *Pseudomonas* spp. for Laccase

After culturing contaminated soils with hydrocarbons on nutrient agar supplemented with guaiacol and identifying the grown colonies, the results revealed 11 isolates of Pseudomonas out of 23 collected samples. These isolates included 2 (18%), 6 (55%), and 3 (27%) isolates that belonged to *Pseudomonas fluorescence, Pseudomonas aeruginosa,* and *Pseudomonas putida,* respectively, as reported in Figure 1. Many bacterial strains capable of decomposing petroleum hydrocarbons have been identified, including *Pseudomonas, Acinetobacter,* and *Mycobacterium.*[9] This capacity is ascribed to the existence of genes and enzymes that utilize chemical complexes contained in petroleum as important energy sources.

Laccase Production under LSF and SSF Conditions

According to the qualitative screening for laccase production, the diameter of reddish-brown oxidation zones on agar plates ranged between 15–29 mm, and P. putida revealed *the best productivity* isolates, as shown in Table 1. So *P. putida* isolates were chosen as the best producers for laccase and subjected to LSF and SSF conditions. The results revealed

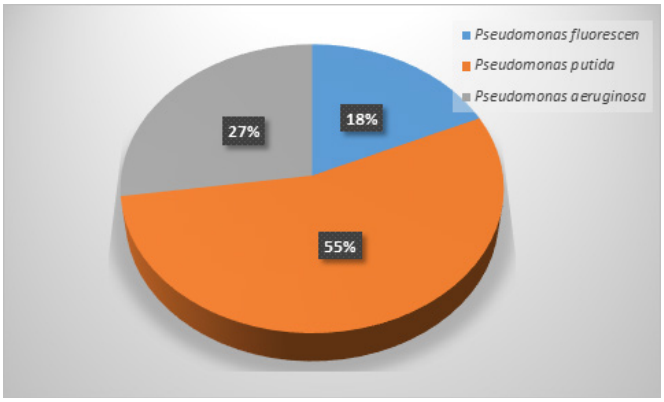

Figure 1: Percentage of laccase producers isolates of *Pseudomonas* spp.

Table 1: Productivity of laccase under LSF and SSF Conditions by *P.* spp.

Isolate	*Reddish-brown oxidation zones*	*Laccase activity (U/mL) in LSF*	*Laccase activity (U/mL) in SSF (using rice bran)*	*Laccase activity (U/mL) in SSF (using wheat bran)*
P. putida 1	20	15.5	26.1	24.8
P. putida 2	26	20.3	31.6	30.1
P. putida 3	25	18.3	30.9	28.3
P. putida 4	21	16.8	29.3	26.2
P. putida 5	29	23	37	32
P. putida 6	28	21	35.9	30.5
P.fluorescence 1	19	-	-	-
P. fluorescence 2	22	-	-	-
P. aeruginosa 1	15	-	-	-
P. aeruginosa 2	16	-	-	-
P. aeruginosa 3	15	-	-	-

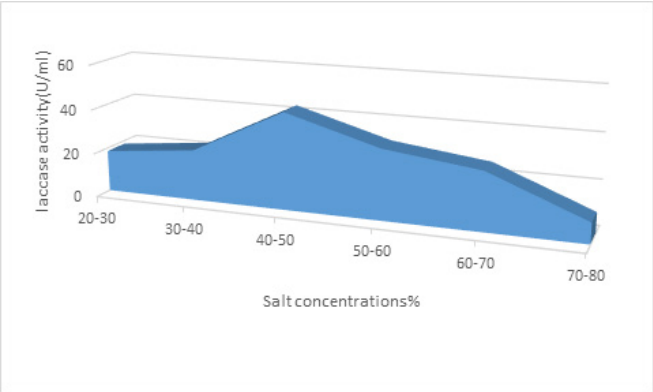

Figure 2: Partial purification of laccase by ammonium salts

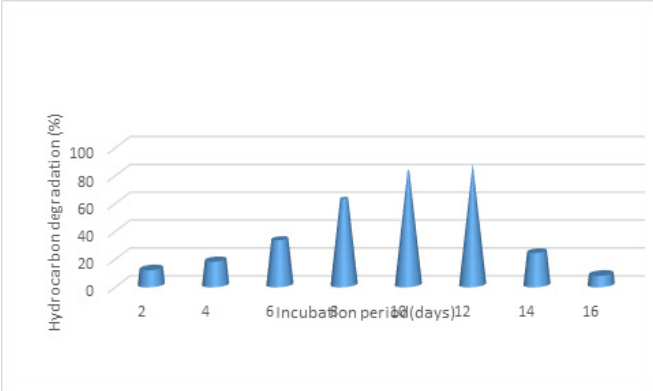

Figure 3: Efficiency of degradation of hydrocarbons by purified laccase

that SSF Conditions were better than LSF conditions since they increased the productivity of *P. putida*[5] to 32U/mL in comparison with liquid fermentation, in which the production reached 23 U/mL.

On the other hand, in SSF conditions, rice bran was better than wheat bran in the induction of laccase production, as recorded in Table 1. Because the most sugars found in rice bran are xylose and arabinose, the main component of rice bran was expected to be arabinoxylan. When those carbon sources decreased, laccase synthesis was induced by lignin and phenolic compounds present in rice bran, leading to increased laccase production.[10] Furthermore, the nutritional requirements of the organisms are the primary determinant of a substrate's compatibility. Another investigation in which starch-free wheat bran was employed as a carbon source for high laccase yield.[11]

Partial Purification of Laccase

After growing *P. putida*[5] on the nutrient agar supplemented with guaiacol, the formed clear supernatant was subjected to fractionation with ammonium sulfate at different saturation percentages after centrifugation. Maximum laccase activity at 40–50% saturation of salt reached 43.5 U/mL (Figure 2). $(NH_4)_2SO_4$ precipitation followed by DEAE-cellulose chromatography was used to purify laccase from *P. putida* with a yield of 4.45%, as revealed by S Fuentes, *et al*.[12]

Removal of Hydrocarbons Content from Contaminated Soil by laccase

After incubating purified laccase with hydrocarbons contaminated soil and measuring the absorbency of toluene after mixing, the results showed that the degradation of hydrocarbons increased with time, and the maximum degradation level appeared after 12 days then declined the peak with time, as demonstrated in Figure 3. So bioremediation has emerged as an alternative approach to oil-contaminated area cleanup, in which microbial colonies play a crucial role in the process, either by direct pollutant degradation or through interaction with other microorganisms added.

The variety and abundance of microorganisms found in polluted environments have a direct impact on the effectiveness of the restoration strategy used. Once the PHC elimination process has begun, the pace at which the pollutant can be absorbed by the microorganism is determined by the availability of the free contaminant and its capacity to enter the organism's membrane.[13]

CONCLUSION

The degradation of hydrocarbons with purified laccase increased with time, and the maximum degradation level was appeared after 12 days and then declined at the peak with time, therefore; the potential employment of microbes as biological instruments might provide a more cost-effective and efficient way to reduce waste and save natural resources, could be

a viable answer to one of modern society's most pressing issues.

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