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Degradation of Anthracene by Immobilizing Laccase From Trametes Versicolor onto Chitosan Beads and Hyacinth Plant

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are recognized as a toxic, mutagenic and/or carcinogenic compounds, and their pollution of soil and aquifer is of increasing environmentally risk. Laccases (E.C. 1.10.3.2) are phenoloxidases catalyze the oxidation of PAHs in the presence of a mediator compound and hyacinth plant. In this study laccase from Trametes versicolor was immobilized into chitosan, and the potential to oxidize anthracene in the presence of 1-hydroxybenzotriazole (HBT) was examined. Results indicated that the immobilization enhanced the stability of laccase against temperature, pH, inhibitors and loading time compared with the other cases. The immobilized laccase-mediator system was as efficient as the free enzyme for oxidizing the tested PAHs. After 24h. of incubation, immobilized laccase–HBT showed a system oxidization more than immobilized laccase without (HBT) of PAHs; Chitosan with hyacinth plant and (HBT) resulted better conversion than chitosan with or without HBT. These results indicate a new chance for applying the immobilized laccase in bioremediation.

KEYWORDS: Polycyclic aromatic hydrocarbons PAH; Immobilized laccase; Trametes versicolor; Bioremediation; HBT; Conversation ; Hyacinth plant.

الخلاصة

من المعروف أن الهيدروكربونات العطرية متعددة الحلقات (PAHs) هي مواد سامة / أو مسببة للطفرة او السرطان ، كما أن تلوثها بالتربة والمستودعات المائية الجوفية يشكل مصدر قلق بيئي كبير. انزيمات اللاكيز (E.C.1.10.3.2) هي فينولوكسيديز تحفز أكسدة PAHs بوجود مركب وسيط ومع نبات زهرة النيل. تم استخلاص انزيم اللاكيز من فطريات العفن الابيض المحمل على حبيبات الكايتوسان، وتم التحقيق في قدرته على أكسدة أنثراسين وفي وجود ١-هيدروكسي بنزوتريازول (HBT). أوضحت النتائج أن التحميل حسّن من ثبات اللاكيز تجاه درجة الحرارة والأس الهيدروجيني، والمثبطات ووقت التخزين مقارنة بالحالة الأخرى. كان نظام الوسيط المستخدم فعالا في أكسدة الـ PAHs بعد ٢٤ ساعة من الحضانة عند استخدام الانزيم الحر، أعطى الوسيط HBT-أكسدة أكثر من انزيم اللاكيز- المحمل دون (HBT) لل PAHs، وحبيبات الكايتوسان مع نبات زهرة النيل مع (HBT) أعطى نتيجة جيدة اعلى من الكايتوسان مع أو بدون HBT. تشير النتائج إلى فرصة جديدة لتطبيق انزيم اللاكييز المحمل في المعالجة البيولوجية

INTRODUCTION

One of the greatest common organic contaminants produce at industrial places are known polycyclic aromatic (PAHs). they have a harmful effect on the plants and animals because they accumulate and uptake in food on genetic defects in human such as benzo pyrene, pyrene and benz anthracene have toxic, mutagenic and/or carcinogenic properties [1]. Recently, the United Stated Environmental Protection Agency (UN-EPA) nominates sixteen type of PAHs priority pollutants who's curing in considered in human health and environmental cleanup [2]. Many studies focused on PAHs biodegradation by using the white rot fungi [3]. This method has many disadvantages; is very slow, has undesirable limitation and incomplete removal of pollutants. Enzymatic treatment was alternative solution for removing contaminated xenobiotic from the environment [4]. The white rot fungi have extracellular ligninolytic enzyme system which containing of peroxidases and laccases related to PAHs biodegradation. The benefit of the laccase can be stretched in the presence of radical mediator compounds that act as (electron shuttle) between the substrate and the enzyme [5]. Many investigations have been

reported for PAHs degradation by laccase-mediator system. The degradation was significantly higher in the presence of mediator-compounds such as (2,2-azino-bis3-ethylbenzothiazoline-6 sulfonic acid) (ABTS), N-hydroxybenzotriazole (HBT), and siring aldehyde [6]. The method for laccase immobilization for manufacturing requests a chemical interaction by the (covalent binding). The covalent binding has utilized for laccase immobilization amid most recently. The sustainable biopolymer, chitosan is considered as a perfect carrier to immobilize a protein [7]. Chitosan is preferably used due to the presence of high amount of amino group that not only improve the interaction with enzymes, and the solubility in slightly acidic solutions, mechanical strength and imperviousness to substance corruption. Moreover, chitosan is easily produce at low cost and is environmentally fascinating [8]. The utilizing of chitosan as immobilized support can diminish the defilement of debasement and improve decontamination prepare. Glutaraldehyde as cross linker usually used to the biocatalyst at chitosan beads away from an immediate contact of the enzymes with the encompassing medium, it likewise makes the reagents to reach the catalytically active site of enzyme. More recently naphthalene was significantly adsorbed while pyrene was least adsorbed by water hyacinth (Eichhornia crassipes) [9]. Autor study hard. also observed about 5% adsorption of naphthalene by E. crassipes in about 7 days in non-microbial aided treatment of PAHs contaminated soil. The oxidative possible of immobilized laccase-mediator system for response with PAHs in polluted mineral water consumes not remained studied [10]. The impartial of the current study is to estimate the possible of immobilized laccase to dissolve anthracene in the presence hyacinths plant and HBT. The beads form is spherical shape, solid with diameter (1mm) and have ability to recovery preferred because it allows better surface characterization, enabling the establishment of useful geometric parameters for reproducibility of the process and for comparisons, as well as permitting optimal packing in reactors and filtration devices. Chitosan is a linear polysaccharide resulting from the partial substitution of the N-acetyl groups present in chitin when in the presence of an alkaline solution. Its chemical structure is formed by repeating constitutional units of 2-amino-2-deoxy-β-(1,4)-Dglucose and 2-acetamide-2-deoxy-β-(1,4)-D-glucose. When chitosan is used in its natural form, it tends to agglomerate and form gels, as well as presenting other disadvantages in adsorption processes, such as solubility in acid media, which prevents it from being recycled.

MATERIALS AND METHODS

Chitosan of low molecular weight was supplied by ABCO Laboratories (Eng. Ltd., Gillingham, England). Commercial laccase from T. versicolor,1-hydroxybenzotriazole (HBT) and acetic acid was provided by Fluka Chemicals Company (Switzerland). Glutaraldehyde was purchased from Aldrich, Anthracene, 2,2-azino-bis (3-ethylbenzothiazoline- 6-sulfonic acid) (ABTS), acetone, methanol, NaOH and Tween 80 were obtained from Sigma (St. Louis), Bovine serum albumin (BSA) from Himedia compony (India), Phosphate buffer

(Darmstadt, Germany).

Chitosan Beads Preparation

One gram of chitosan powder was dissolved in

40 mL of acetic acid (1 % v/v). The solution was stirred for 1hr until a homogenous solution is obtained. The subsequent solution was carefully dropped keen on a coagulant bunch of (2M) NaOH to produce sphere-shaped beads. Then, the beads of 1- 2 mm in diameters were collected by filtration process. Thereafter, the collected beads were washed away many times by deionized water, untill partiality is accomplished, and dried at room temperature toward constant weightiness [11].

Entrapment of Tvlac in Chitosan Beads

Glutaraldehyde was utilized as a crosslinker for Laccase entrapment. One gram of chitosan beads was mixed 2 ml of glutaraldehyde-crosslinked. Then, the beads were washed with deionized water and mixed with of (46.2 u/ml) Tvlac and shake at 150 rpm for 24 hr and dried at room temperature and stored in dry conditions until use. Then, the (beads) remained washed by deionized water, dried at room temperature and kept in dry circumstances up to further use [12].

Determination of Enzymatic Activity

Laccase activity was assayed by measuring the speed of oxidiation in the presence of a suitable redox mediator, 2,2_-azino-bis (ethylbenzothiazoline-6-sulfonic acid) (ABTS) at the room temperature. Acetate (0.1M and pH 4.6) containing 0.02U of laccase and 1mM ABTS was add for a final-volume –up to- (0.2mL). The oxidization reaction of ABTS was followed by determining the absorbance at 405 nm or at 420 nm (ε 405 = 36 000 M-1cm-1). One unit of the enzyme activity was outlined the quantity of laccase that converted one µmol of substrate per minute into product [13].

Buffers and reagents

Bradford's reagent

The Bradford assay is a colorimetrical method used for quantifying proteins. This assay is based on a shift of the maximum absorption Coomassie from 470 nm to 595nm when protein binding at acidic pH. The protein concentration of the unknown sample is determined according to calibration. The colorimetrical reaction depends on the content of aromatic and basic amino acids. To prepare 100 mL of Bradford chemical agent, 10mg of Coomassie brilliant blue G was dissolved in 5mL of ethanol. After 1 hr of stirring, 10 mL of phosphoric acid was added and diluted the mixture up to 100 mL by distilled water [14].

Phosphate buffer saline PBS

PBS was ready to use by dissolving one pill in 100 mL of distilled water. The pH was adjusted to be 7.2 and autoclaved for 10 min at 100 ˚C.

Bovine serum albumin BSA

A stock of BAS was prepared by dissolving 6mg 1 mL of PBS, and adjust pH to 7. This solution was used to prepare series of the required working solutions.

Determination of protein

The stock solution of BSA was used to prepare series of diluted solutions (0.025-12mg/mL). in PBS (pH 7.2), A volume of 50µL of each diluted sample was added to 950µL of Bradford reagent mixed thoroughly and left for 10 minutes at room temperature, the optical density measured in duplicates at 595nm. The measurement was done. A standard curve was plotted between the concentrations against optical density of the standard protein, the protein concentration was calculated from the equation curve as seen in Figure 1 [15].

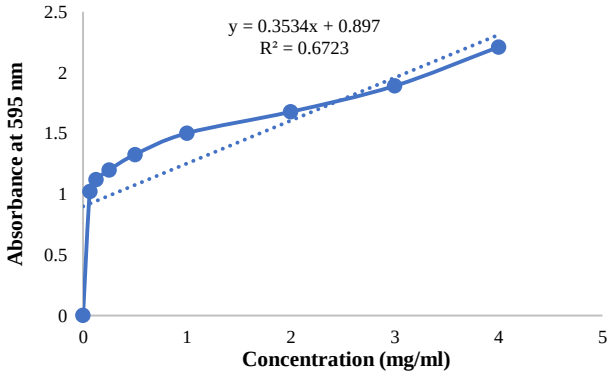


Figure 1. Calibration curve of BSA for quantifying concentration.

Stability of laccase

The effect of pH

Laccase activity was estimated using 0.02U from T. versicolor. Acetate buffer 0.1M was used to study the effects of four pH degrees (3.6, 4.6, 5.6, and pH 6.6).The enzyme samples were incubated at 30 ºC at various incubation times. The oxidation activity of laccase against 1mM ABTS, as a substrate, was determined by measuring the absorbance at 405 nm in ELISA reader.

The effect of temperature

The ideal temperature for laccase activity was measured by 0.02U of T. versicolor laccase in 0.1M acetate buffer (pH 4.6).The reaction was carried as previously described under five temperatures (20- 30- 40-50- 60 ˚C) .

Anthracene Degradation by the Laccase-Mediator System

Chitosan entrapped Tvlac was used to oxidize 86µM anthracene prepared dissolved in 10 mL of acetone and added to a solution of 5mL Tween 80, for increasing PAH bioavailability, in 50ml of distilled water. The PAH was homogenized by ultrasonic bath and diluted to a final volume of 200mL with distilled water. Chitosan beads (250mg) were immersed in 5mL of anthracene, in the presence of 100mM HBT as synthetic mediator. The beads were

incubated at 30 ˚C and oxidation is determined by HPLC.

FTIR Analysis

Fourier transformation of infrared (FTIR) spectra were investigated by Perkin- Elmer Fourier transformation of infrared spectrophotometer (model 2000) over a wavenumber range of 400-4000 cm^{-1}.

HPLC Analysis

The quantification of polycyclic aromatic hydrocarbon was performed by HPLC on a Kontron 422 equipped with a Jasco 2070 Plus ultraviolet-visible detector and a Prosphere reverse-phase C18 (5 µm, 15 cm × 4.6 mm ID) column. The injected sample was prepared by mixing 1mL sample with 8mL of methanol/water (4:1, v/v) and eluted by methanol/water (4:1, v/v) with a flow rate, 0.1mL/min. The eluate was detected at 270nm. The retention times of anthracene were 12.2and 14.6 min. The proportion of each PAH oxidized was estimated from the change between each PAH level of the tested sample and that of the equivalent control.

RESULTS AND DISCUSSION

Stability of free laccases

Effect of pH on the activity of immobilized laccase

The pH effect on the activity of immobilized laccase was examined in a (pH) range (of 3.6 - 6.6) at 30 ˚C. At prearranged time intervals, suitable aliquots of the enzyme were reserved and their activities examined at the ideal pH. The ideal pH used for immobilized laccase was (4.6). The optimum pH for immobilized enzymes and bulk solution usually has unequal partitions of H+ and OH-concentration due to electrostatic leads to displacement in (pH) activity profile. Furthermore, the pH profile of immobilized method preserved enzyme activity in a widen pH rang. These results perhaps approved the stabilization of laccase on the surface of beads. Figure 2 shows the laccase absorbance at different pHs, where the highest absorbance was in pH (4.6) in (0, 2, 4, 24and48 h) [16].

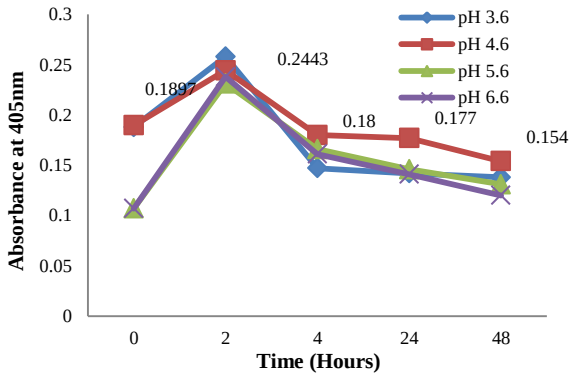


Figure 2. The stability of laccase in different pHs at different times.

Effect of temperature on the activity of immobilized laccase

The effect of temperature on the immobilized laccase activities is showed in Figure 3 where the optimum temperature for best immobilizing laccase appeared at (30 ˚C) after 24h where the results proved that the immobilized laccase could with, stand at higher temperature conditions. The shifting in temperature is affected by changing the chemical and physical properties of the immobilized enzyme. The covalent bond formation through amino groups of immobilized laccase might also reduce in activation energy of the molecule to suitably rearrange the conformation of substrate binding [17].

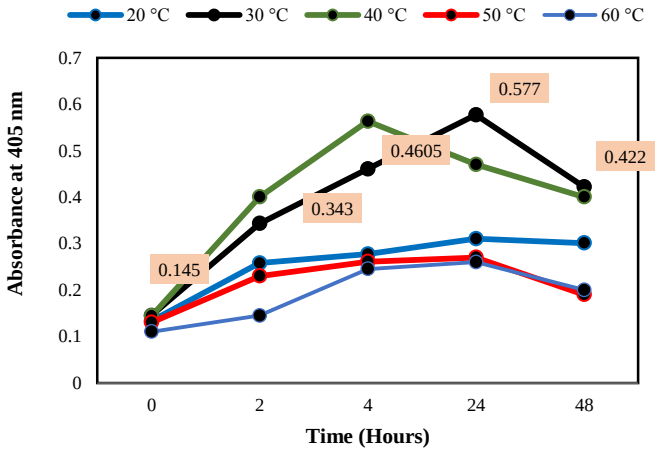


Figure 3. The stability of laccase in pH (4.6) at different temperatures.

HPLC Analysis

Anthracene degradation

The results in Figure 4 indicated that chitosan with laccase in the presences of (HBT) showed the highest degradation to anthracene when compared with that without (HBT) and within

(6 and 24 h). Several of these peroxidases are stronger oxidants than laccase, but their dependency on high dosage of hydrogen peroxide practically is not suitable [18]. Laccases are reliant only on atmospheric oxygen and are more stable than peroxidases. Xenobioticshas have been shown using intact fungal cultures and lonely laccase in soluble form. The substrate range of laccase can be further extended by the presence of radical mediator compounds that act as "electron shuttle" between the enzyme and the substrate. The degradation of PAHs by laccase-mediator system was reported to be considerably higher in the existence of mediator compounds such as (HBT) [19]. The laccases are copper-containing oxido-reductive enzymes that catalyzes a one-electron oxidation of a broad range of polyphenols and aromatic substrates. The effect of laccase is shown in Figures 4-11.

FTIR analysis

The FTIR spectra chitosan immobilized laccase before and after cross-linking with glutaraldehyde were determined and showed in Figure 12-A. Chitosan is formed by the deacetylation of chitin, in the role of immobilized transporter contains an (amino group). The cross-linker glutaraldehyde interacted with the amino group of chitosan to form a Schiff base. Generally, the reaction process between the amino group and an aldehyde group is modified by the effect of the electrophilicity of the carbonyl group in the aldehyde. The results showed that chitosan spectra involved a band of around 1651 cm^{-1}, corresponded to the in-plane bending vibration of ($-NH_2$). Glutaraldehyde pretreated with chitosan has displayed two bands, one at 1657 cm^{-1} that could be attributed to an imine bond (N=C) .The second at 1599 cm^{-1} could be related to the contribution of an ethylene bond (C=C).These results confirmed an overlapping matching to –NH and –C=N– extending in the newly shaped Schiff base (Figure 12-B). On the other hand, the carbonyl peak at

1599 cm^{-1} has disappeared upon creation of the imine bond (Figure 12-C). These results confirmed the attendance of covalently-bound enzyme onto the chitosan beads, which agreed a previous study [20].

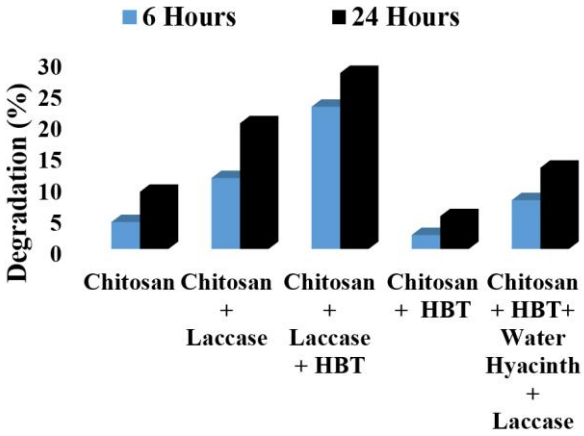
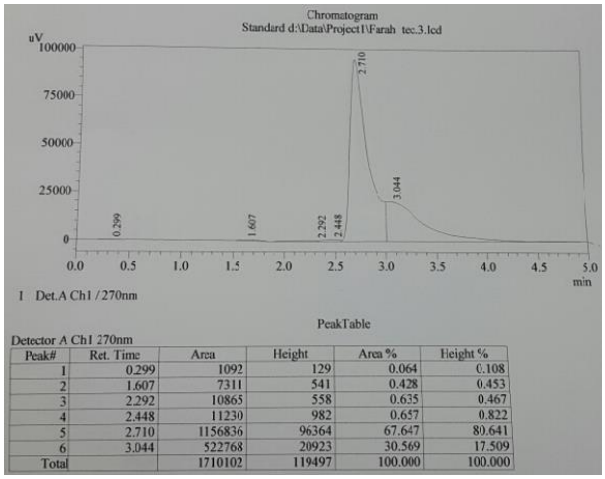
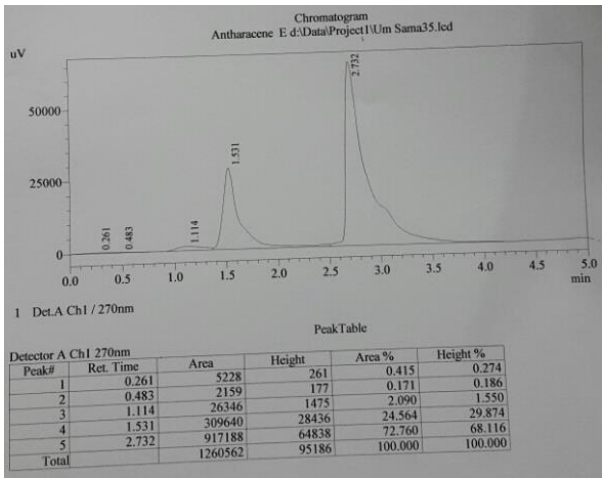


Figure 4. Degradation of (86 µM) anthracene by chitosan entrapped. Laccase in the presence of (100mM) HBT at 30 ˚C and pH (4.6) within (6 and 24 h), analyzed by HPLC.



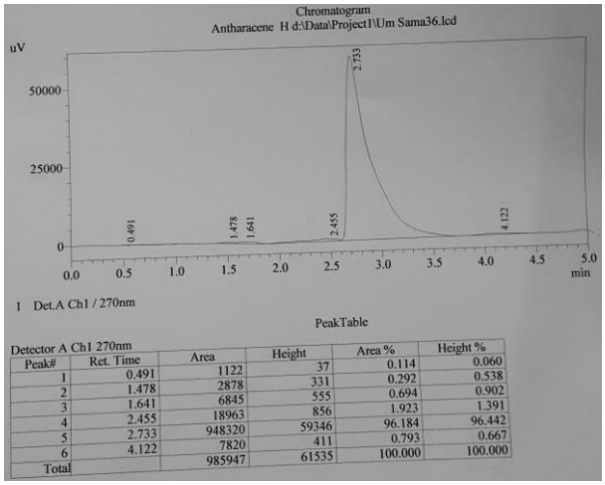
Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.299	1092	129	0.064	0.108
2	1.607	7311	541	0.428	0.453
3	2.292	10865	558	0.635	0.467
4	2.448	11230	982	0.657	0.822
5	2.710	1156836	96364	67.647	80.641
6	3.044	522768	20923	30.569	17.509
Total		1710102	119497	100.000	100.000

Figure 5. HPLC analysis of standard anthracene area (86 µM/ml).



Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.261	5228	261	0.415	0.274
2	0.483	2159	177	0.171	0.186
3	1.114	26346	1475	2.090	1.550
4	1.531	309640	28436	24.564	29.874
5	2.732	917188	64838	72.760	68.116
Total		1260562	95186	100.000	100.000

Figure 6. HLPC analysis of chitosan + Laccase + HBT , 6 h at 30 ˚C.

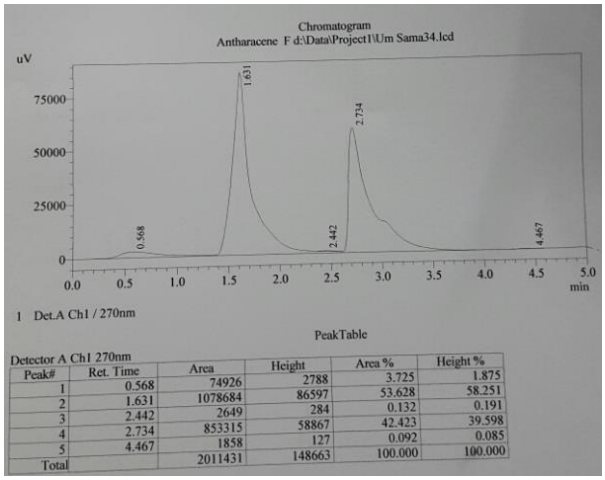


PeakTable

Detector A Ch1 270nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.491	1122	37	0.114	0.060
2	1.478	2878	331	0.292	0.538
3	1.641	6845	555	0.694	0.902
4	2.455	18963	856	1.923	1.391
5	2.733	948320	59346	96.184	96.442
6	4.122	7820	411	0.793	0.667
Total		985947	61535	100.000	100.000

Figure 7. HPLC analysis of chitosan + laccase 24 h at 30 ˚C.

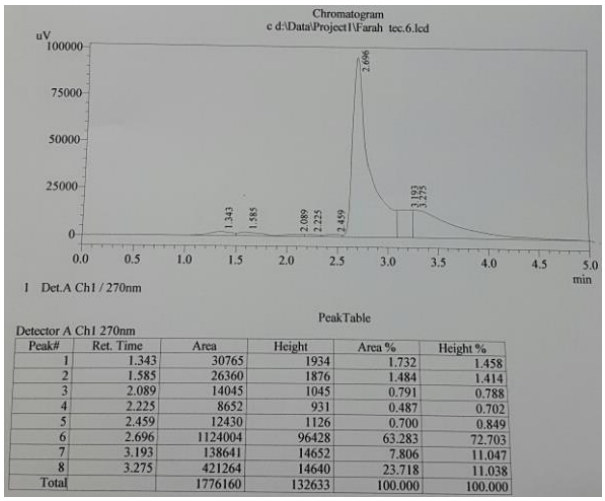


PeakTable

Detector A Ch1 270nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.568	74926	2788	3.725	1.875
2	1.631	1078684	86597	53.628	58.251
3	2.442	2649	284	0.132	0.191
4	2.734	853315	58867	42.423	39.598
5	4.467	1858	127	0.092	0.085
Total		2011431	148663	100.000	100.000

Figure 8. HPLC analysis of chitosan + laccase + HBT , 24 h at 30 ˚C.

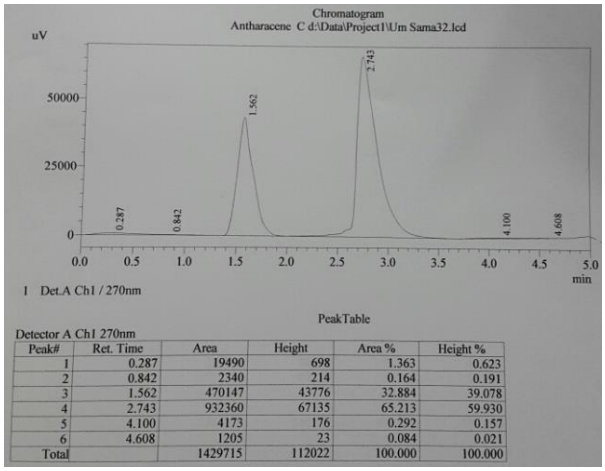


PeakTable

Detector A Ch1 270nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	1.343	30765	1934	1.732	1.458
2	1.585	26360	1876	1.484	1.414
3	2.089	14045	1045	0.791	0.788
4	2.225	8652	931	0.487	0.702
5	2.459	12430	1126	0.700	0.849
6	2.696	1124004	96428	63.283	72.703
7	3.193	138641	14652	7.806	11.047
8	3.275	421264	14640	23.718	11.038
Total		1776160	132633	100.000	100.000

Figure 9. HPLC analysis of chitosan without laccase + HBT , 24 h , at 30 ˚C.

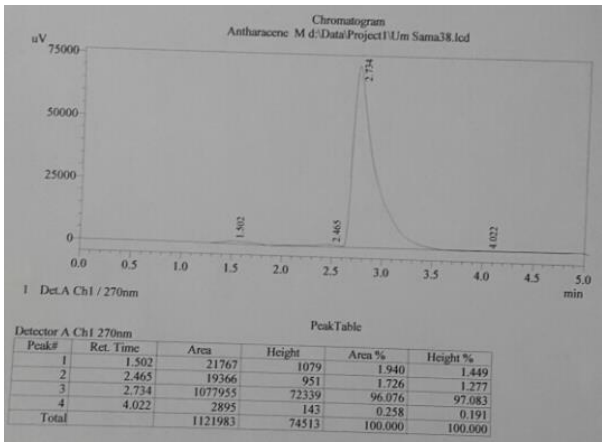


PeakTable

Detector A Ch1 270nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.287	19490	698	1.363	0.623
2	0.842	2340	214	0.164	0.191
3	1.562	470147	43776	32.884	39.078
4	2.743	932360	67135	65.213	59.930
5	4.100	4173	176	0.292	0.157
6	4.608	1205	23	0.084	0.021
Total		1429715	112022	100.000	100.000

Figure 10. HPLC analysis of chitosan + water hyacinth + HBT , 24 h , at 30 ˚C.



PeakTable

Detector A Ch1 270nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	1.502	21767	1079	1.940	1.449
2	2.465	19366	951	1.726	1.277
3	2.734	1077955	72339	96.076	97.083
4	4.022	2895	143	0.258	0.191
Total		1121983	74513	100.000	100.000

Figure 11. HPLC analysis of chitosan alone , 24 h , at 30 ˚C.

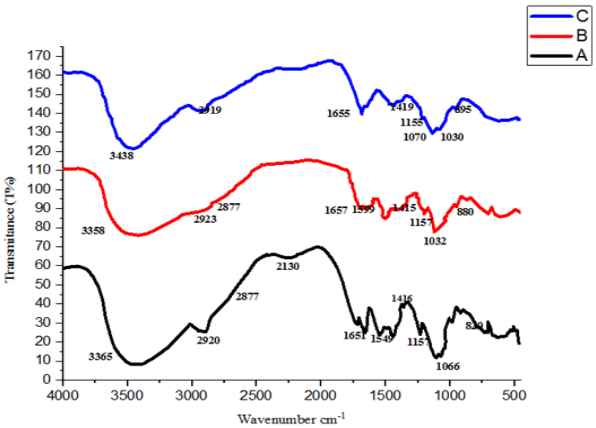


Figure 12. FTIR Spectra of: (A) Chitosan beads; (B) Chitosan beads with glutaraldehyde and(C) Chitosan beads Immobilized laccase enzyme.

CONCLUSIONS

This study showed that immobilized laccase (from T. versicolor) on chitosan was able to highly catalyze degradation of anthracene in presence of an oxidative mediator, such as HBT. Compared with immobilized laccase in

the absence of HBT the degradation of immobilized laccase with hyacinth plant was higher after 24 h than that included HBT or did not. By studying the contamination of wastewater by (PAHs), the consequences vacant showed different chances for applying of immobilized laccase in bioremediation.

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