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Use of immobilized laccase in bioremediation of phenolic compounds which causes environmental pollution

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Key words: Immobilized Enzymes; Laccase, Bioremediation, A phenolic compound, Environmental pollution, Bentonite

Abstract

The using of immobilization between laccase and bentonite by glutaraldehyde resulted to immobilized 91% of the amount of total free enzyme, the optimum pH activity of the free and immobilized laccase was 6 and immobilized enzyme was stable between 4.5-7 and its loss about 54 and 38% from original activity at pH 3 and 8 respectively, while temperature activity of free and immobilized laccase was 40 and 45°C respectively, immobilized enzyme was stable at 60°C for 15min, and it loses about 88% of its original activity at 80°C for the same time, the results was shown that immobilized laccase retained its full activity for 22 days, but it retained 89.2% of its original activity after storage for 30 days at 4°C, also, Immobilized laccase was kept all its activity after 30 consecutive used, and after 40 consecutive used keep about 75% of its initial activity. The treatment of wastewater sample with 1gm of immobilized laccase for 10, 20, 30, 40, 50, 60min lead to remove 30.17, 62.48, 90.03, 90.12, 90.13 and 90.13% of phenol compounds respectively.

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Introduction

Immobilized enzymes are used in many applications, including food industry, pharmaceutical industries, bioremediation, detergents and textiles, as well as other applications, immobilized technique was considered as one of the important methods that provides several advantages for enzymes, such as, promote stability, increase stability, improve catalytic properties and the possibility of using them more than once (Al-Soufi, 2016a). Several methods are often used to immobilized, which vary depending on the scientific basis that determines its work, covalent linkage of enzymes to supports occurs owing to their side chain amino acids, such as, aspartic acid, histidine, arginine, and degree of reactivity based on different functional groups, such as, phenolic hydroxyl, imidazole, indolyl, etc. (Datta *et al.,* 2013), Peptide modified surfaces when used for enzyme linkage results in higher specific activity and stability with controlled protein orientation (Al-Soufi, 2015).

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) is a copper-containing enzyme, also known as multi-copper blue oxidases, belong to the oxidoreductase group of enzymes that catalyzes the oxidation of a wide variety of organic and inorganic compounds by coupling it to the reduction of oxygen to water like phenol, aniline, 4-hydroxybenzoic acid and 2, 2'-azino-bis- (3-ethyl benzothiazoline-6-sulphinic acid) (ABTS) (Al-Soufi MA. 2016b). Laccase is widely found in plants and fungi, but recently it was found in some bacteria, Laccase is considered important enzyme, It can be applied extensively in many industrial fields, such as food, textile, cosmetics, paper and pulp, synthetic chemistry, pesticide or insecticide degradation, waste detoxification, removal of endocrine disruptors, soil bioremediation and biodegradation of environmental phenolic pollutant, biosensor and other applications (Kushwah *et al.,* 2014).

Bentonite is considered one of the most popular clay rocks which have exceptional adsorption properties. It is used in several fields, such us, oil well drilling, linking material for sand templates foundry metal, iron ores mining, remover of impurities and mineral contaminants, shortness colors of industrial and vegetable oils to improve their quality and its use in the manufacture of pharmaceuticals and cosmetics (Schutz *et al.,* 2013.), Montmorillonite clay is a 2:1 dioctahedral (smectites in general) which widely used as a support, is have acidic nature that provided acid sites for binding of enzymes through NH_2 group, so, It is enough to adsorption of enzymes with clays, as well, clay can be activated and linked with glutaraldehyde to make covalently bond between clay and enzyme (Al-Soufi, 2015). Due to Iraq's ownership huge amounts of bentonite clay in the Western Desert, the aim of this study was to use this substance to immobilize laccase and study some of its characteristics and its application in removal of the phenolic compounds.

Materials and methods

Place of work

All experiments were conducted in the laboratory of market research and consumer protection center, University of Baghdad, Iraq in 2017.

Source of Enzyme

Laccase (EC 1.10.3.2) was obtained from a previous study by Al-Soufi (2016b), citrate buffer 20mM pH 5 was used to dissolve of the enzyme.

Source of Bentonite

Bentonite was obtained from local markets of Baghdad, Iraq.

Estimation of protein

Protein was estimated through a method of Bradford (1976).

Enzyme assay

Free and immobilized laccase activity was determined by using ABTS as the substrate. The assay mixture contained 5mM ABTS, a 100mM sodium acetate buffer (pH 5.0), and 100µl aliquots of an appropriately diluted enzyme solution. The oxidation of ABTS was monitored spectrophotometrically by measuring the increase in the A420 (ε=36,000$M^{-1}cm^{-1}$). One unit of laccase activity was defined as the amount of enzyme required to oxidize 1µmole of ABTS per minute at 25°C (Mi and Park, 2008).

Activation of bentonite

The clay was activated as in the method of Al-Soufi (2015) by stirring with 10% 3-APTES solution in acetone (v/v) for 1h at room temperature, filtered out, washed with acetone and drying at 80°C. After that, treated with 10% aqueous glutaraldehyde solution (v/v) for 1h, filtered out, washed and dried at room temperature, and stored in 0.1 phosphate buffer, pH 6 at 5°C until used to immobilized laccase.

Immobilization of laccase

The activated clay from the previous step was mixed with equal volumes of enzyme (10mg/ml) in citrate buffer 20mM pH 5 and stirred for 1 hour at 4°C, then, centrifuged at 4°C and 5000rpm for 30min, clay was saved in the same volume of citrate buffer 20mM pH 5 (Al-Soufi, 2015).

Yield of immobilization

Immobilization yield was determined by measuring the difference between protein concentration (mg/ml) of enzyme solution that adds to activated bentonite (At_0) and same solution after stirring activated support with at 4°C for 24h (*Att*). The immobilization yield (*IY*) was calculated with the following equation (Al-Soufi, 2016a).

$$IY(\%) = \frac{At_0 - Att}{At_0} \times 100$$

Determination of pH stability

The effect of optimum pH activity and stability for free and immobilized laccase was determined within a pH range of 2-8 (pH 2-4; 50mM citrate-phosphate buffer, pH 4.5-6; 50mM sodium-acetate buffer, pH 6.5-8; 50mM Tris-HCl buffer) by using ABTS as the substrate, optimum pH for stability was determined after incubation of free and immobilized enzyme with buffer for 15min (Al-Soufi, 2016b).

Determination of thermal stability

The optimum temperature activity of the free and immobilized enzyme was determined over a temperature range of 30-80°C, at the optimum pH value and with ABTS as the substrate; while optimum temperature stability was determined after free and immobilized were incubated within limit temperature for 15min (Al-Soufi, 2016b).

Estimation of total phenolic compounds

Phenolic compound concentration before and after treating with immobilized laccase was estimated through a method of Gonzalez *et al.* (2003) by using the Folin-Ciocalteau method, and Gallic acid (3,4,5-trihydroxybenzoic acid) was used to prepare calibration curves with a concentration between 10-100mg\ml, all samples were measured by absorbance at 765nm.

Estimation of immobilized enzyme for removal of phenolic compounds

The effect of immobilized laccase activity on the removal of phenolic compounds was studied by an add 5gm immobilized enzyme (10mg/ml) (20U/mg) to the 1000ml of the sample of wastewater and incubation at 35°C and 50rpm for 30min. The reaction mixture was then analyzed for remaining phenolic concentration (%) as described above (Al-Soufi, 2016b).

Effect time of use immobilized enzyme on the removal of phenolic compounds

The effect of time was studied by an add 1gm immobilized enzyme (10mg/ml) (20U/mg) to the 100ml of the sample of wastewater and incubation at 35°C and 50rpm for 10, 20, 30, 40, 50 and 60min (Asadgol *et al.,* 2014.).

Effect of storage on enzyme activity

Free and immobilized laccase were stored for 60 days at 4°C (Al-Soufi, 2016a).

Effect of recycling immobilized laccase

Immobilized laccase was followed up after each use up to 40 times (Al-Soufi, 2016a).

Results and discussion

Yield of immobilization

The results obtained showed that this technique led to the binding of 91% of the total free enzyme. Immobilization method need to use of binding materials, which are often an Inert polymers and inorganic materials are usually used as carrier matrices, available at reasonable prices, have high strength and stability for the reaction materials, ability to increase enzyme specificity activity and reduce product inhibition, as well as its ability to bind the highest amount of enzyme (Datta *et at.,* 2013; Al-

Soufi, 2016c), so, the amounts of enzyme which bind with carriers, are different in accordance with the carrier used, surface area and method which use immobilization (Jořenek and Zajoncová, 2015). In this subject, Liu *et al.,* (2012) observe that recovery for laccase immobilized into bimodal carbon based mesoporous magnetic composites (CMMC) was 91.0%, while, Patel, *et al.,* (2014) obtain a maximum yield and efficiency for immobilized laccase on SiO_2 nanoparticles were 75.8 and 92.9% respectively, also, it was, 62.9 and 48.7% for immobilized laccase on magnetic (nano and micro) particles (Jořenek and Zajoncová, 2015), whilst, Mureşeanu *et al.,* (2016) refer that the efficiency for immobilized laccase on Santa Barbara Amorphous (SBA-15 and SBA-15-NH_2), Hexagonal Mesoporous Silica (HMS, HMS-NH_2 and SBA-15-NH_2 cov) supports were 59.47, 67.33, 40.93, 81.86 and 75.47% respectively.

Effect of pH activity and stability

The optimum pH activity of the free and immobilized laccase was 6 (Fig.1: a) and immobilized enzyme was stable between 4.5-7 and its loss about 54 and 38% from original activity at pH 3 and 8 respectively (Fig.1: b).

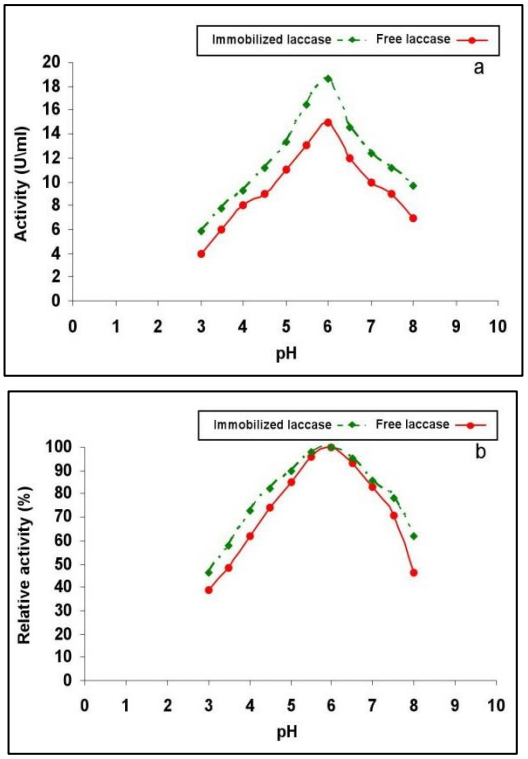


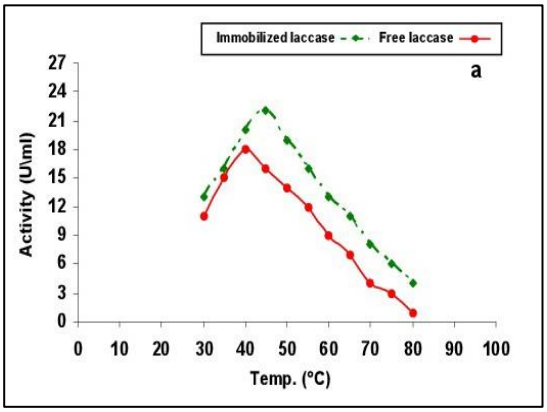
Fig. 1. Optimum pH of free and immobilized laccase, a: activity; b: stability.

Optimum pH for activity and stability is considered an important indicator for the success of enzyme immobilization, the deviation of the pH value of the immobilized enzyme may lead to inability to use it in specific. The inability to use it in specific field, thus ending the feasibility of immobilization and It becomes reliance on use of free enzyme with an economic importance of greater (Al-Soufi, 2015; Al-Soufi, 2016a), many authors have reported effect of immobilization on pH profile for enzyme, all of Liu *et al.,* (2012) and Chen *et al.,* (2015) found that optimum pH were 3.0 and 4.0 for free and immobilized laccase into (CMMC) and natural nanostructured bacterial cellulose respectively, also, Patel *et al.,* (2014) observed that the optimum pH for free and immobilized laccase on SiO_2 nanoparticles were 3.0 and 3.5 respectively.

On the other side, Jořenek and Zajoncová (2015) explain that optimum pH for free and immobilized laccase on magnetic (nano and micro) particles were 5.1, 4.7 and 4.5 respectively, while Mureşeanu *et al.,* (2016) observed that optimal pH for immobilized laccase on SBA-15, SBA-15-NH_2, HMS, HMS-NH_2 and SBA-15-NH_2 cov supports were ranged between 5.5 to 6, and it stable at pH range 4 to 7.

Effect of Temperature Activity and Stability

Temperature activity of free and immobilized laccase was 40 and 45°C respectively (Fig.2: a), and immobilized enzyme was stable at 60°C for 15min, and its loss about 88% of its original activity at 80°C for the same time (Fig.2: b).



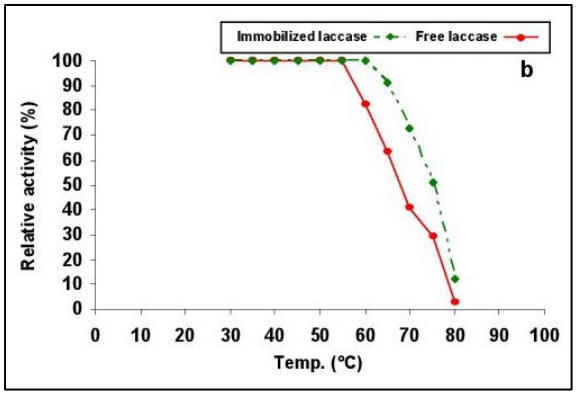


Fig. 2. Optimum temperature of free and immobilized laccase, a: activity; b: stability.

Increase of temperature lead to reduced enzyme activity due to thermal inhibition, so the folds of the enzyme molecule and content of amino acids at high temperatures will be exposed to medium which leads to enzyme denaturation, so, the improvement of immobilized enzyme resistance against temperature perhaps due to a reduction in molecular movement and conformation changes by binding into support materials (Al-Soufi, 2015).

The optimum temperature values of laccase are considerably depending on the source of enzyme, support materials and the method of immobilization; therefore, many studies were referring to this case. For instance, Liu *et al.,* (2012) refer that both free and immobilized laccase into (CMMC) was maximal activity at 45°C, also Patel *et al.,* (2014) observe that the optimum temperature for free and immobilized laccase on SiO_2 nanoparticles was 40 and 45°C respectively, But, Chen *et al.,* (2015) found that optimum temperature of free and immobilized laccases on natural nanostructured bacterial cellulose was 50 and 60°C respectively, while, Mureşeanu *et al.,* (2016) pointed out that optimal temperature for laccase immobilized on SBA-15, SBA-15-NH_2, HMS, HMS-NH_2 and SBA-15-NH_2 cov supports was ranged between 44.85 to 49.85, and laccase immobilized on SBA-15 or HMS is more stable at 39.85 to 79.85 temperature range.

Effect of time of the removal of phenolic compounds from wastewater

The treatment of wastewater sample with 1gm of immobilized laccase for 10, 20, 30, 40, 50, 60min lead to removed 30.17, 62.48, 90.03, 90.12, 90.13 and 90.13% of phenol compounds respectively (Fig.3).

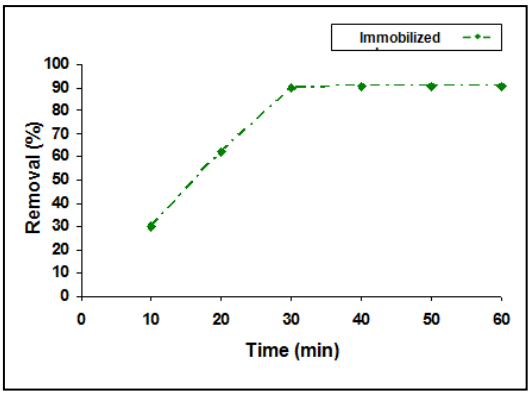


Fig. 3. Effect of time of the removal of phenolic compounds from wastewater by immobilized laccase.

In general, laccase could be used for the detection and removal of phenolic compounds in wastewater samples and other solutions (Kushwah *et al.,* 2014). In this subject, Chakroun *et al.,* (2010) use 0.3U/ml laccase for removal 2,4-dichlorophenoxyacetic acid, 4-chlorophenol, *o*-cresolor and catechol to get 21, 28, 100 and 100% removal of pollutants respectively after 24h of incubation, while, Asadgol *et al.,* (2014) observe that 5U/ml of laccase could to remove 80 and 59.7% of phenol and bisphenol A respectively after 30 minutes of treatment of phenolic pollutants, also, Al-Soufi (2016b) found that the treatment of apple juice with 10U\mg of laccase for 10, 20, 30, 40, 50, 60min led to removed 28.42, 60.93, 88.03, 89.37, 90.01 and 90.04% of phenol compounds respectively, whilst, Naghdi *et al.,* (2017) refer that immobilized laccase on oxygen functionalized nanobiochars through mineral acids using for degradation of carbamazepine exhibited 83% and 86% removal in spiked water and secondary effluent, respectively.

Effect of storage on enzyme activity

Immobilized laccase retained its full activity for 22 days, but it retained 89.2% of its original activity after storage for 30 days at 4°C (Fig.4).

Storage stability is an extremely important parameter for immobilized enzymes (Al-Soufi, 2016b), so, many research referred to this parameter. Daâssi *et al.,* (2014) reported that free and immobilized laccase on

Ca-alginate beads retained about 22.4 and 82.7% of their initial activities, respectively at the end of the 20 days of storage at 4°C, also, Ilk *et al.,* (2016) refer that free and immobilized laccase on nanocomposites lost 82 and 35% respectively of its initial activity over stored 4°C for a 30day, On the other side, Chaudhary *et al.,* (2016) observe that immobilized laccase on PVA membrane which stored at 4°C; showed no noticeable loss of activity during its use for 15 days.

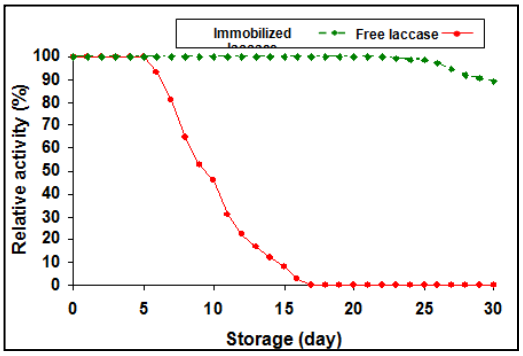


Fig. 4. Storage stability of immobilized laccase.

Effect of recycling immobilized laccase

Immobilized laccase was kept all its activity after 30 consecutive used, and after 40 consecutive used, immobilized enzyme kept about 75% of its initial activity (Fig.5).

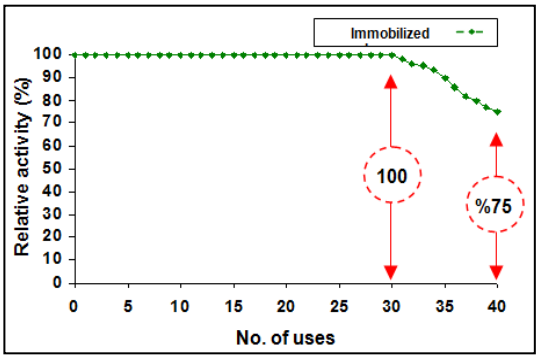


Fig. 5. Recycling effect of immobilized laccase activity.

Enzyme cycles are one of the important economic factors when considering the immobilization of enzyme to give it a clear perception of the efficiency of support materials for immobilization (Al-Soufi, 2015), so, many research have been calculated immobilized laccase cycles, all of Liu *et al.,* (2012) , Patel *et al.,* (2014) and Ilk *et al.,* (2016) found that immobilized laccase on (CMMC), SiO_2 nanoparticles and nanocomposites retained 50, 82.6 and 77% respectively of its activity at the end of 10 cycles of use, whilst, Chen *et al.,* (2015) pointed out that immobilized laccase on natural nanostructure bacterial cellulose by adsorption and by adsorption plus cross-linking was retained 69 and 23% respectively of original activity after 7 cycles, also Mureşeanu *et al.,* (2016) observe that immobilize laccase on SBA-15, SBA-15-NH_2, HMS, HMS-NH_2 and SBA-15-NH_2 cov. supports have 100 of its original activity and after the 5th reaction cycle the conversion reached about 58% of the SBA-15-NH_2 cov and 25% of HMS-NH_2 sample, respectively, while, Naghdi *et al.,* (2017) refer that immobilized laccase on oxygen functioned nanobiochars through mineral acids preserved 70% of the initial activity after 3 cycles.

Conclusions

This study show that possibility to immobilized laccase with bentonite by glutaraldehyde and able to use the immobilized enzyme for many times without any loss of its activity.

Recommendations

Use immobilized laccase for bioremediation of phenolic compounds from wastewater with highly efficient.

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