A comparison of entrapped and covalently bonded laccase: Study of its leakage, reusability, and the catalytic efficiency in TEMPO-mediated glycerol oxidation

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ABSTRACT

This article presents the comparison for reusability and leakage between entrapped and cova-lently bonded laccase and their performances towards the selective oxidation of glycerol. The reusability of immobilized laccase enzyme was studied by reacting a batch of immobilized lac-case with ABTS for 15 cycles. The investigation of the leakage of immobilized laccase was carried out by storing the immobilized laccase in acetate buffer solution for 32 days. The data show that the retained enzyme activities of entrapped and covalently bonded enzyme after being reused for eight cycles were well above 60% and the leakages after storing for a month in the acetate buffer at 4 $^{\circ}$C were well below 15%. The entrapped laccase coupled with TEMPO was found to perform better and gave a two-fold higher yield of glyceraldehyde and glyceric acid in the selective oxidation of glycerol compared to covalently bonded laccase. Hence, physical entrapment of laccase would be a suitable immobilization method in the laccase-mediated selective oxidation of glycerol.

KEYWORD

Laccase; selective oxidation; immobilization; leakage; reusability

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RESEARCH ARTICLE

A comparison of entrapped and covalently bonded laccase: Study of its leakage, reusability, and the catalytic efficiency in TEMPO-mediated glycerol oxidation

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ABSTRACT

This article presents the comparison for reusability and leakage between entrapped and covalently bonded laccase and their performances towards the selective oxidation of glycerol. The reusability of immobilized laccase enzyme was studied by reacting a batch of immobilized laccase with ABTS for 15 cycles. The investigation of the leakage of immobilized laccase was carried out by storing the immobilized laccase in acetate buffer solution for 32 days. The data show that the retained enzyme activities of entrapped and covalently bonded enzyme after being reused for eight cycles were well above 60% and the leakages after storing for a month in the acetate buffer at 4 °C were well below 15%. The entrapped laccase coupled with TEMPO was found to perform better and gave a two-fold higher yield of glyceraldehyde and glyceric acid in the selective oxidation of glycerol compared to covalently bonded laccase. Hence, physical entrapment of laccase would be a suitable immobilization method in the laccase-mediated selective oxidation of glycerol.

Abbreviations: A: Absorbance; ABTS: 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); GlAc: Glyceric acid; Gled: Glyceraldehyde; HPLC: High-performance Liquid Chromatography; ε: Extinction coefficient; TEMPO: 2,2,6,6-Tetramethylpiperidine-1-oxyl.

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1. Introduction

Oxidoreductive enzyme laccases (EC 1.10.3.2) or copper-containing polyphenol oxidase, with a molecular mass around 70 kDa, are widely distributed in fungi, plants, bacteria, and insects. They can oxidize a variety of substrates such as diamines, polyphenols, and some inorganic compounds using molecular oxygen as the electron acceptor (Piontek et al. 2002; Claus 2004). The excellent properties of laccases including high activity, selectivity, and specificity allow them to react in the chemical processes under mild environmental conditions (Koschorreck et al. 2009). The unique set of beneficial features makes laccase a very useful industrial biocatalyst. Laccases have various potential applications in different industrial processes such as pulp and paper (e.g. Novozym 51003 from Novozymes, Denmark for paper pulp delignification), textiles (e.g. IndiStar from Genencor Inc., Rochester for denim finishing), and food industries (e.g. LACCASE Y120 from Amano Enzyme USA Co. Ltd. for colour enhancement in tea) (Osma et al. 2010). The other potential uses of laccases are in the areas of biosensors, waste detoxification, effluent decolourization, nanobiotechnology, cosmetics, synthetic chemistry, and bioremediation of food industry wastewater (Mayer and Staples 2002; Rodríguez Couto et al. 2007), in which many of them were patented (Si 2001; Maupin-Furlow et al. 2013).

Laccase applications are hindered by some practical problems and the common perceptions are sensitivity to process conditions, high cost of isolation and purification, and structure instability (Krajewska 2004; Yang et al. 2016). Improvement of the enzyme features, viz. protein engineering, microbiology, and chemistry of protein, are crucial (Cirino and Arnold 2002; Reetz et al. 2006). Besides those mentioned, an apparently old technique – immobilization has been revealed as the most adopted stabilization method to overcome these limitations and allow enzyme reutilization (Hong et al. 2015). Enzyme immobilization can be categorized

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into entrapment, encapsulation, adsorption, covalent bonding, and self-immobilization (Brady and Jordaan 2009; Hong et al. 2015; Bilal et al. 2017). Entrapment is the easiest immobilization method in that it only involves the physical retention of the enzyme in the porous solid matrix with no alteration of the enzyme native structure and the procedure is less tedious than other methods (Bailey and Ollis 1986; Lu et al. 2007). Covalent attachment, on the other hand, is a method which binds the enzyme to the carrier via chemical bonds that might perturb the enzyme native structure (Durán et al. 2002). The immobilization yield depends on the immobilization technique and material. For example, Asgher et al. (2017) revealed that immobilization yield of laccase entrapped in agar–agar matrix was 79.7% while the immobilization of laccase on magnetic chitosan microspheres obtained 24% yield of protein coupling and an enzyme activity of 322.6 units per gram of support (Jiang et al. 2005). Some of the supports like carbon nanotubes have a long and complicated procedure to prepare, hence the enzyme structure may be disrupted during the immobilization process (Feng and Ji 2011). Furthermore, the stability of the immobilized enzyme highly depends on the immobilization technique.

The use of laccase has been extended by the introduction of laccase-mediator system (LMS) to overcome the limitation of its redox potential. The mechanistic details of LMS catalyzed oxidations have been reported in detail (Christopher et al. 2014; Moilanen et al. 2014). To date, the abundancy of glycerol (a by-product of biodiesel production) has prompted the development of new applications. In this context, the resulting surplus of glycerol has been used as a substrate in LMS for selective oxidation (Liebminger et al. 2009) to attain a series of value added intermediates. This LMS is a greener approach compared to the conventional oxidation method with noble metals in glycerol oxidation (Porta and Prati 2004; Wang et al. 2015). The efficient utilization of glycerol with LMS, however, requires extensive studies to provide sufficient evidence for practical usage in industries. Previous studies on LMS focused more on pre-treatment of paper and pulp (Moilanen et al. 2014) and dye decolourization (Soares et al. 2001). Since oxidizing glycerol using LMS involves a series of reactions and the transfer of electrons among oxygen, laccase, mediator, and substrates, the reaction is complex. Hence, investigating the method of immobilizing laccase and its reusability in the complex reaction is vital. In an industrial setting, besides robustness of the process, enzyme reusability and its storage stability are also important factors to be considered, especially when the process is run in batch mode. Unfortunately, Arends et al. (2006) only studied the rate of the LMS in oxidizing glycerol with free laccase, while Liebminger et al. (2009) only briefly compared the same system with covalently bonded laccase in terms of stability for a long-term reaction. Therefore, this paper examines two different immobilization methods and the practical problems of both immobilized enzymes such as stability, reusability, and efficiency of immobilized laccase incorporated with TEMPO towards the selective oxidation of glycerol. It is hoped that the outcomes of the study would assist the industry in selecting a proper immobilization method for the LMS in oxidizing glycerol.

2. Materials and methods

2.1. Enzymes and chemicals

Laccase from *Trametes versicolor* ($\geq$10 U/mg) was purchased from Sigma-Aldrich, Germany without further purification. Alumina pellets and gelatine were purchased from Sigma-Aldrich, Germany. Glycerol was obtained from Fisher Scientific, United Kingdom. 2,2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO), polyethylene glycol (PEG) (MW = 4000), glutaraldehyde, calcium chloride ($CaCl_2$) were bought from Merck, Germany. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was procured from Roche, Germany while sodium alginate was provided by R&M chemicals.

2.2. Laccase immobilization

2.2.1. Entrapment by sodium alginate

Two grams of gelatine and 0.5 g of PEG was added to 100 mL of 2.0% (w/v) of sodium alginate solution. One thousand units of laccase were then added and mixed thoroughly for 10 min at 25 °C. The mixture was withdrawn using a sterile 5-mL syringe and extruded through a 21 gauge (0.51 mm inner diameter) needle into 2.0% (w/v) of $CaCl_2$ solution. Beads formed immediately when the droplets of the mixture touched the surface of the solution. Beads were left unstirred to harden in the $CaCl_2$ solution at 4 °C for 2 h, after which the $CaCl_2$ solution was removed by filtration and the beads were washed twice with distilled water. The beads were subsequently incubated in 100 mL of 0.6% (w/v) glutaraldehyde solution and stirred at 4 °C for 2 h. The beads were washed several times with phosphate buffer (pH 7) and kept at 4 °C in acetate buffer (pH 4.5). The method was adapted from Wang et al. (2008).

2.2.2. Covalent attachment on alumina support by cross-linking agent

Laccase immobilization using covalent bonding was adapted from Rodríguez Couto et al. (2007). In brief, alumina pellets were silanized at 45 °C for 20 h in a 2% (v/v) solution of aminopropyltriethoxysilane in acetone. The silanized supports were washed once with acetone and silanized again for 24 h. Then, they were washed several times with deionized water and air dried. In the second stage, the alumina pellets were treated with 2% (v/v) aqueous glutaraldehyde (50% v/v) for 2 h at room temperature, washed again with deionized water, and air dried. These supports were immersed in 100 mL of laccase solution (10 U/mL) for 48 h at room temperature. The supports were washed numerous times with 0.05 M phosphate buffer (pH 7) to remove the unbound proteins and kept at 4 °C until further use.

2.3. Selective oxidation of glycerol by immobilized laccase coupled with TEMPO

The reaction was performed in an incubator with the orbital shaking system. The reaction mixture (total volume = 50 mL) consisted of 100 mM of glycerol, 30 mM of TEMPO, and 0.1 g of immobilized laccase in 100 mM of sodium acetate buffer (pH 4.5). The stirring speed was set at 180 rpm. The reaction temperature and pH were controlled at 25 °C and pH 4.5, respectively. One hundred microlitres of sample were taken hourly for the first 3 h and the final sample at the 24th h. Nine hundred microlitres of 10% (v/v) of acetic acid was added into the sample immediately to stop the reaction. Analysis of samples was conducted as described in the Section 2.4.4.

2.4. Analytical methods

2.4.1. Spectrophotometric enzymatic assay

Laccase activity was determined using ABTS as substrate and performed spectrophotometrically with UV–VIS spectrophotometer (light path = 1 cm) at room temperature. The oxidation of ABTS by laccase was monitored at 415 nm with a molar absorption value of 36,000 M^{-1} cm^{-1}. The assay mixture for the blank consisted of 1.9 mL of 0.1 M of acetate buffer (pH 4.5), 0.1 mL of pure water, and 1.0 mL of 0.5 mM of ABTS solution. In free enzyme assay, pure water was replaced by 0.1 mL of laccase, while 1 g of immobilized laccase was used to replace pure water in the immobilized enzyme assay. One unit (U) of the enzyme was defined as 1 μmol ABTS oxidized per minute under the stated assay condition.

2.4.2. Enzyme reusability test

The reusability of immobilized laccase was studied for 15 cycles. Three sample vials, each containing approximately 0.1 g covalently bonded laccase enzyme, were supplemented with 1.9 mL of buffer solution and 1.0 mL of ABTS solution. The immobilized laccase was allowed to catalyze the oxidation of ABTS for 75 min (Mohd Zain et al. 2010). The immobilized laccases were filtered, washed, and placed into a new batch of ABTS solution for the second cycle. The experiment was repeated to test the reusability of entrapped laccase by substituting the covalently bonded laccase with the entrapped enzyme. The activity of laccase was assayed as described in the Section 2.4.1. The retained activity was calculated using Equations (1) and (2).

$$\text{Unit of enzymes}_{(n)} = \frac{\text{Absorbance of mixture}}{\varepsilon} \quad (1)$$

$$\text{Retained activity } (\%) = \frac{\text{Unit of enzyme}_{(n)}}{\text{Unit of enzyme}_{(n=1)}} \quad (2)$$

2.4.3. Leakage test

Leakage test was carried out for a period of 32 days for both covalently bonded and entrapped laccase. Immobilized laccases were stored in acetate buffer (pH 4.5) and a sample of the buffer solution was collected every day for the first week and then every 5 days thereafter. One hundred microlitres of the sample buffer solution were added to 1.9 mL of 0.1 M of acetate buffer. One millilitre of ABTS solution was added and the activity of laccases was assayed as described in the Section 2.4.1. The sample analysis was repeated three times to obtain an average value. In the period of study, all samples were stored at 4 °C. The percentage of leakage was calculated based on Equations (3) and (4).

$$\text{Units of enzyme leaked } (U) = \frac{\text{Absorbance}}{\varepsilon} \times \text{dilution factor} \times \text{volume of buffer } (\text{ml}) \quad (3)$$

$$\text{Leakage } (\%) = \frac{\text{Units of enzyme leaked}_{(d)} - \text{Units of enzyme leaked}_{(d=1)}}{\text{Units of enzyme leaked}_{(d=1)}} \times 100\% \quad (4)$$

2.4.4. Analysis of oxidation products

Glycerol oxidation products were analyzed by high performance liquid chromatography (Agilent 1260 Infinity Liquid Chromatography, United State) equipped with DAD and RID. A Phenomenex Rezek ROA column (300 × 7.8 mm) was used with 3 mM of H_2SO_4 as a mobile phase. The temperature of column and RID were fixed at 75 and 35 °C, respectively. DAD wavelength was set at 210 nm. Ten microlitres of sample was injected into the system with 0.5 mL/min of flow rate. Identification of components was performed by comparison of their retention times with the standards.

2.5. Data analysis

All experiments were performed in triplicates and their mean values were taken into consideration for calculation.

3. Results and discussion

3.1. Yield and morphology of immobilized laccase

Immobilization yield was determined as described in the Section 2.3 in which the yield for both entrapment and covalent bonding method were 90 and 94%, respectively. Figure 1 shows the structural morphologies of alumina pellet only and immobilized laccase on alumina pellet. Under 5k magnification, the surface morphology of the alumina pellets was considerably altered. A noticeable roughness on the surface of alumina pellets after enzyme immobilization is possibly due to the laccase that was randomly bound and filled the alumina pellets (Figures 1(c,d)). Micropores in a range of 200–400 nm were reduced to approximately 100 nm in 50k magnification (Figures 1(a,b)), suggesting that laccase was immobilized on the surface and covered a part of the pores. In general, these results demonstrate that the laccase enzyme was effectively immobilized on the surface of alumina pellets.

3.2. Reusability of immobilized laccase

The retained activities of covalently bonded and entrapped laccase are illustrated in Figure 2. The percentage of retained activity was calculated with respect to the initial activity. It can be observed that the retained activity of entrapped laccase was slightly higher compared to the covalently bonded laccase for the first eight cycles of reactions (65.1 and 62.4%, respectively). However, the retained activities for covalently bonded and entrapped laccase after being reused for 15 cycles were 45.7 and 33.5%, respectively. These results are better than the one obtained by Asgher et al. (2017) where they reported that the retained activity of laccases entrapped in gelatin after eight cycles of reactions were 22.8%. In this study, the retained activity for covalently bonded laccase after eight cycles of reuse was also comparable to 60% as reported by Diao et al. (2002), who covalently immobilized the laccase on the activated carboxylated polyvinyl alcohol carrier. Hence, the reusability of the laccase enzymes immobilized using these two methods in this study is high.

The disintegration of alginate beads might be one of the reasons that lower the retained activities at the end of the 15th cycle of reaction. Possible reasons for disintegration to occur are poor immobilization handlings such as the presence of bubbles in the beads and the pH of storage buffer. Since sodium alginate dissolves slowly in water, air tends to enter the solution during the stirring process, causing bubbles to form in the solution. The formation of bubbles might also occur if the alginate solution is not properly withdrawn using the syringe. When bubbles are present in the beads, the chance of leakage increases as the polymerization and cross-linking bonds are weak due to the obstruction of bubbles. According to Babu et al. (2010), alginate beads are stable at low acidic pH but undergo swelling and disintegration at intestinal pH (pH 6–7.4). Since beads are highly dependent on the pH of the medium, phosphate buffer with a pH of 7 might not be a good storage buffer and causes the disintegration (Sabyasachi 2017).

A smaller diameter of beads could promote mass transfer and minimize bead disintegration or rupture due to the formation and accumulation of gas. However, bead size is limited by the viscosity of the solution and the diameter of the syringe needle. Alginate beads are formed immediately when calcium is introduced to the alginate in the soluble form. The gels produced are usually inhomogeneous and have a structure that is stronger at the exterior than that of the interior. This is because when alginate and calcium ions were in contact with each other, gelation occurs. Since the alginate solution was introduced as a droplet into the high calcium ions solution, gelation on the surface of the droplet was faster than that inside the droplet. Gelation of alginate solution inside the droplet require diffusion of calcium ions through the gel (Roopa and Bhattacharya 2008; Bhushan et al. 2015; Hong 2016).

Gelation or the strengthening of gel depends on diffusion rate of calcium ions through the gel. In this study, the gelation kinetic was extremely rapid, observed by an instantaneous sol-gel transition at the

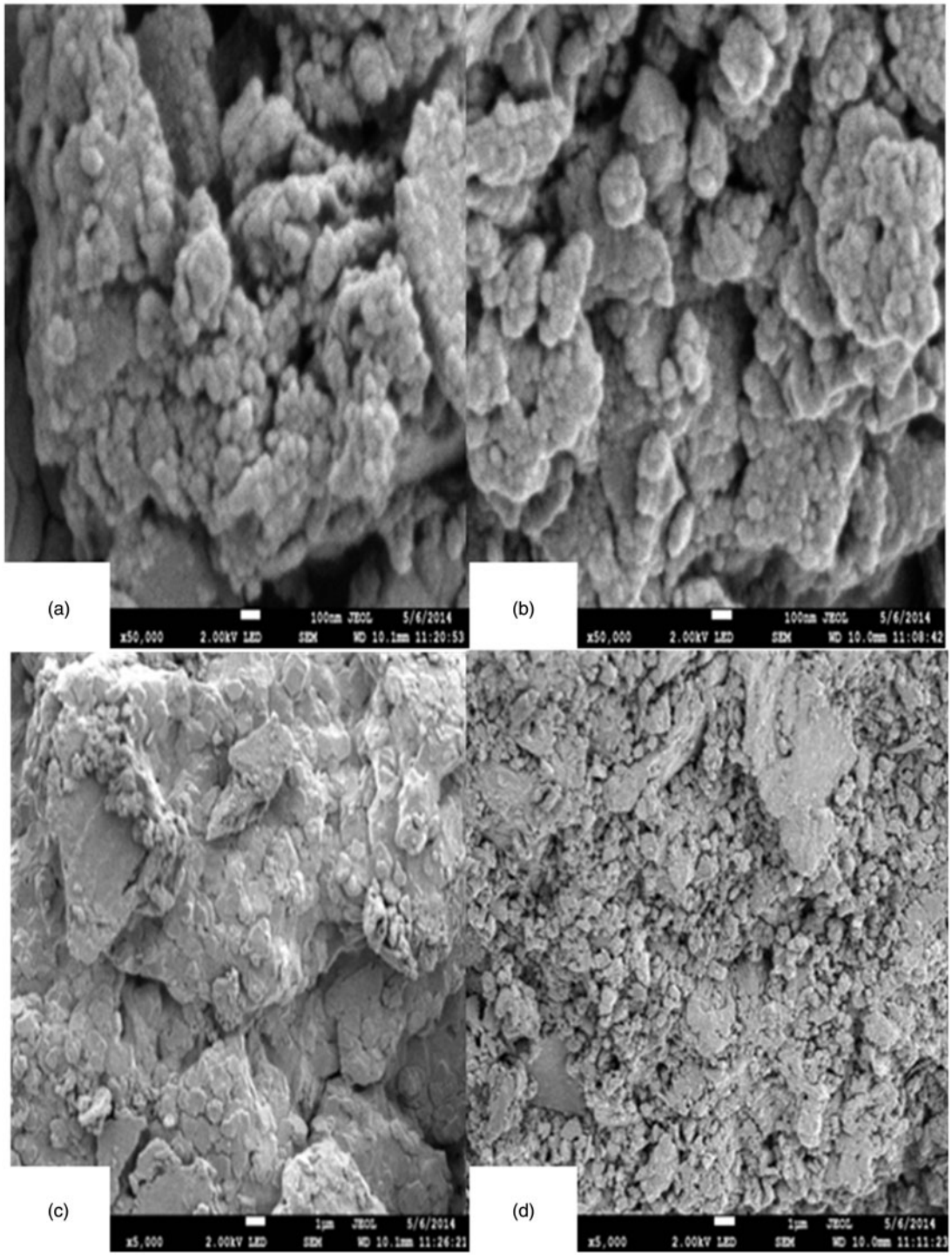

Figure 1. FESEM image in 50k magnification for (a) alumina and (b) immobilized laccase on Alumina pellet; in 5k for (c) alumina and (d) immobilized laccase on Alumina pellet.

surface of the alginate. Glutaraldehyde (GA) was used as a crosslinker for the enzyme to make the enzyme molecules aggregate and thus reducing their leakage (Kumar et al. 2017). Bhushan et al. (2015) demonstrated an improvement in the immobilization efficiency and a 2-fold increase of activity by using GA as the crosslinker. They revealed that the entrapped enzyme after crosslinking with GA was stable with reduced diffusion characteristics. Besides that, different alginate sources also affect the mechanical properties of alginate gel. Mancini et al. (1999) studied the mechanical properties of several guluronic and mannuronic alginate gels at different concentrations by using stress relaxation and uniaxial compression method. They reported that mannuronic alginates formed softer and more elastic gel than that of guluronic alginates

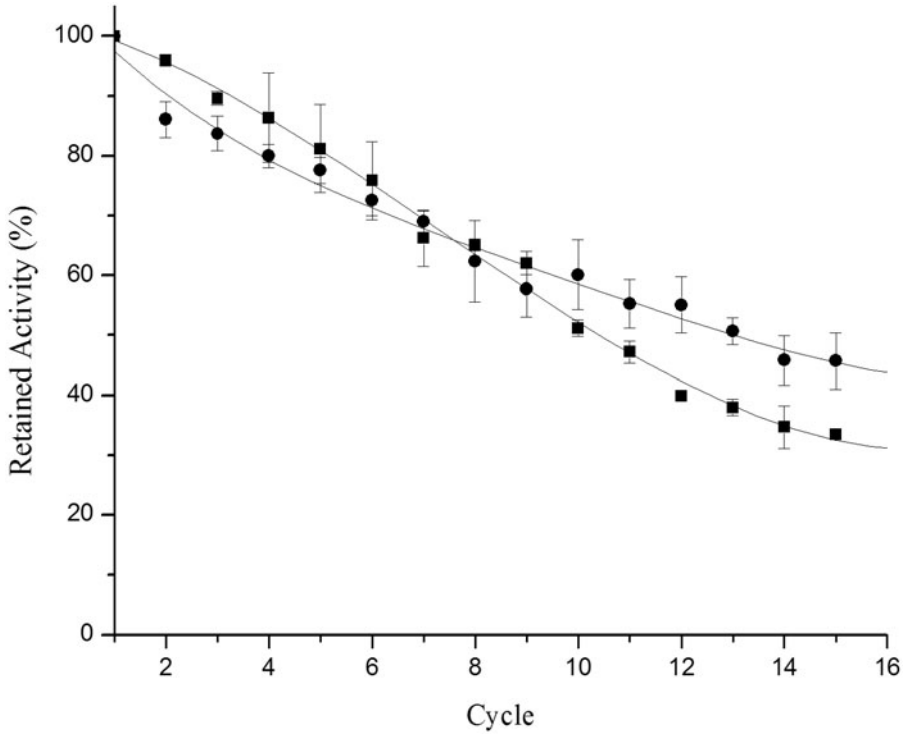

Figure 2. The retained activities of entrapped and covalently bonded laccase for 15 cycles. Data were reported as the average of three replicates (symbols: ■= entrapment; ●= covalent bond).

by the asymptotic stress relaxation constant. Since the source of sodium alginate was unable to be identified for this study, it is presumed to be guluronic alginate as revealed by the experimental results. Another possible reason for the decrease in the retained activity is the degradation of laccase for both immobilization methods. Enzyme at its optimum temperature might not be stable for a long period as well. The exposure time would be another factor that influences the efficiency of the enzyme at a certain temperature (Hong 2016).

3.3. Leakage of immobilized laccase

Figure 3 illustrates the leakage of both covalent bonded and entrapped laccase enzymes increased over time. The leakages at day 32 were 14.5 and 13.9% for covalent bonded and entrapped laccase enzyme, respectively. Since the leakage for both immobilization methods is less than 15% in a month, it could be considered as low leakage. The low leakage level obtained might be due to the application of optimum storage temperature of 4 °C, which is suitable to be used to store both immobilized laccase enzymes. Storage temperature of 4 °C was in agreement with the investigation by Moreno-Pérez et al. (2016). In their study, they reported that the immobilized laccase still retained 95–100% of its initial value after 10 days of storage at 4 °C. At this temperature, laccase is inactive and hence prolonging its shelf life.

On the other hand, entrapped laccase has low leakage because the enzyme is physically restricted within a network or matrix. With GA, the leakage is presumed to be minimized, yet, leakage of the enzyme through large pores of the matrix over a long period is still possible. Some of the enzymes gradually diffuse towards the gel outer shell and eventually leak into the medium. Mancini et al. (1999) suggested that the swelling of the beads might affect the diffusivity of certain molecules through the pore of the beads.

Fernandez-Lorente et al. (2015) reported that most soluble enzymes have high stability with high concentrations of PEG. With this interesting antecedent, the fixation of PEG on enzyme surface would be a viable stabilization strategy as in the case of enzyme entrapment in this study. In contrast, the enzyme immobilized through covalent binding provides a strong and stable enzyme attachment, thus reducing the detachment of enzyme from the support. As a consequence, the enzyme leakage is low for both methods.

3.4. Oxidation of glycerol by immobilized laccase coupled with TEMPO

Valuable chemical intermediates produced from the selective oxidation of glycerol include glyceraldehyde,

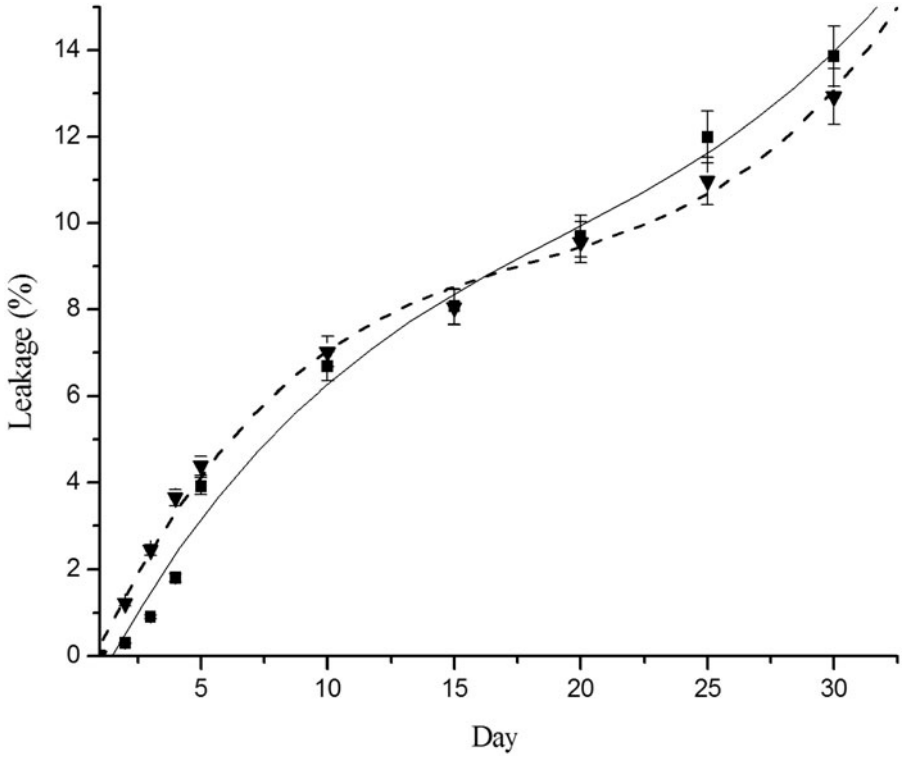

Figure 3. The leakage of laccase from its respective support over a period of 32 days. Data were reported as the average of three replicates (symbols: ■ = covalent bond; ▼ = entrapment).

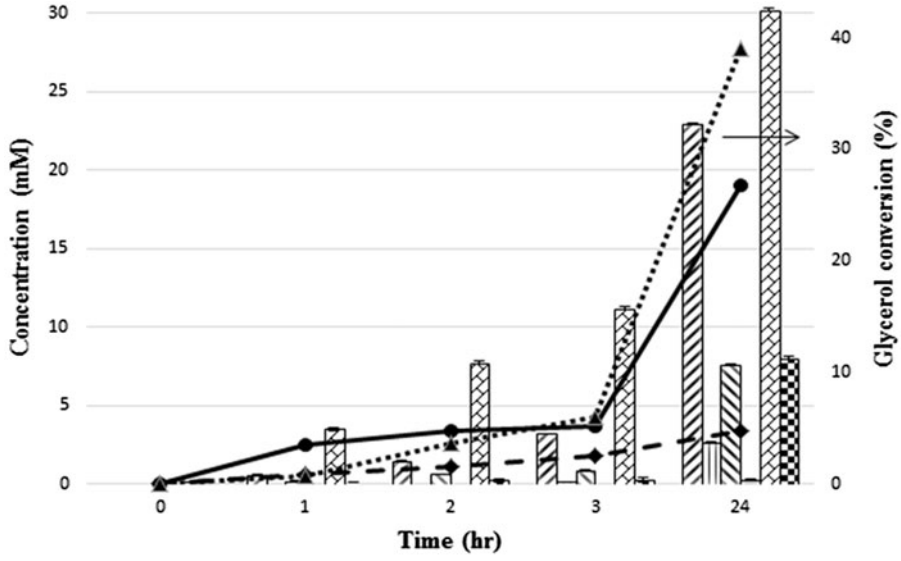

Figure 4. Conversion of glycerol (free enzyme: ▲; entrapped laccase: ●; covalent bonded laccase: ◆) and concentration of the oxidation products (free enzyme: ▨ = Gled, ▩ = GlAc; entrapped laccase: ▨ = Gled, ▥ = GlAc; covalent bonded laccase: ▧ = Gled, ▤ = GlAc). Data were reported as the average of two replicates for entrapped and covalent bonded laccase; average of three replicates for free enzyme.

glyceric acid, tartronic acid, and mesoxalic acid (Werpy and Petersen 2004). Primary hydroxyl groups would be selectively oxidized to glyceraldehyde (Gled) which is an intermediate for carbohydrate metabolism and standard for the comparison of chiral molecules (D- or L-). Further oxidation would yield glyceric acid (GlAc). It is used in treating some skin disorders. In addition, GlAc in its ester form associated with a quaternary ammonium salt can be used as a biodegradable fabric softener (Behr et al. 2008).

Figure 4 illustrates the conversion of glycerol as well as the concentration of the oxidation products for

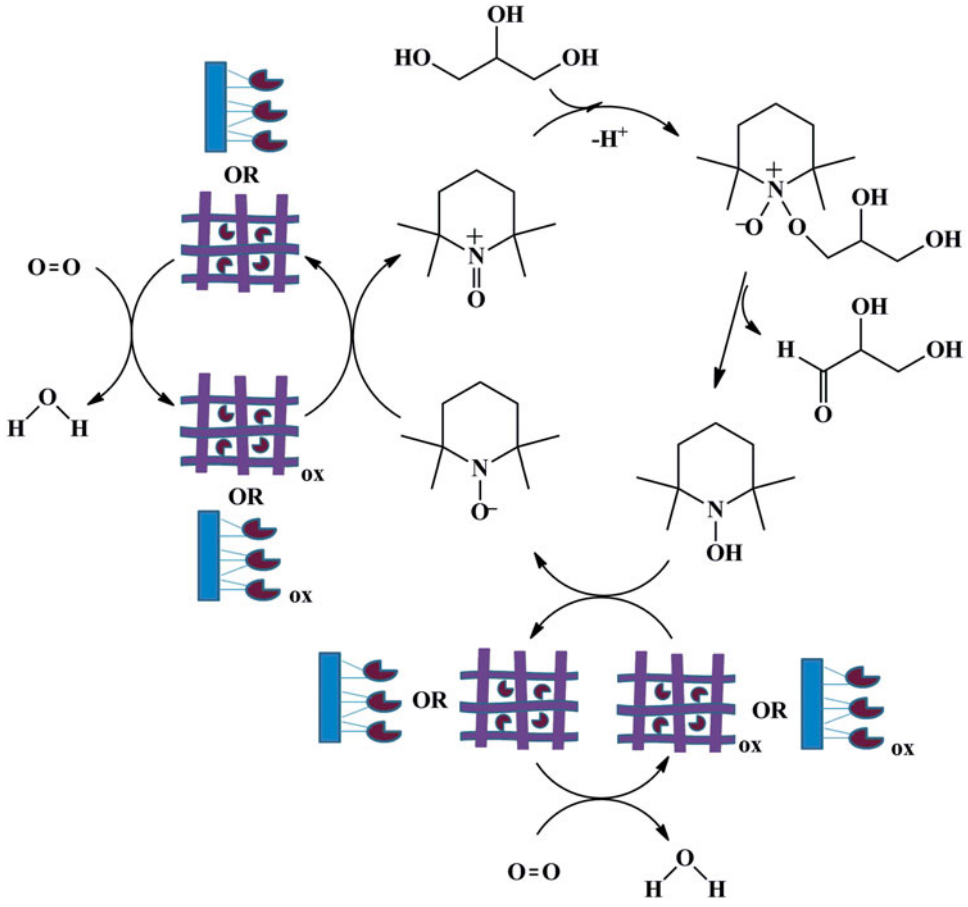

Figure 5. Proposed reaction mechanism on oxidation of glycerol by immobilized laccase/TEMPO system.

the interval studied. In this study, the reaction was only performed up to 24 h, so only the products attained within the reaction period were reported. It can be seen that free laccase performed the best after 24 h of reaction, with nearly 40% glycerol conversion and the highest amount of Gled and GlAc attained. This result is in agreement with Liebminger et al. (2009) who reported that the conversion of glycerol is higher for free enzyme than immobilized enzyme within the same reaction period. Free enzyme is homogeneous with the reactants in the solution, thus eliminating all the possible mass transfer resistances. This is noticeable from the glycerol conversion and the products concentrations achieved by the free enzyme for the first 3 h of reaction in Figure 4. The glycerol conversion of free enzyme was lower compared to the entrapped laccase, but the products concentrations achieved were higher. This is possibly due to the mass transfer resistance experienced by the entrapped laccase, where the products of oxidation required some times to transport out from the alginate beads and detected in the external reaction mixture.

Figure 4 shows that entrapped laccase had better performance than covalently bonded laccase, achieving nearly 27% of glycerol conversion and the final concentrations for Gled and GlAc were 2-fold greater than that of covalently bonded laccase. The Gled concentration attained by the entrapped laccase at 24 h of reaction is comparable to the one obtained by free enzyme. However, the GlAc achieved was only a quarter of that attained by the free enzyme. The efficiency of the immobilized laccase coupled with TEMPO in the reaction with glycerol was highly dependent on the active site of laccase as laccase would regenerate TEMPO. Figure 5 shows the proposed reaction mechanism, which reveals the reaction between immobilized laccase, TEMPO, and substrate. Unexpectedly, the entrapped laccase with TEMPO gave a high concentration of Gled and GlAc. The diffusion limits of the entrapped laccase did not seem to be a barrier for TEMPO to react with laccase. Covalently bonded laccase in which the laccase was bonded on the surface of the support was supposed to have a better interaction with TEMPO in oxidizing glycerol due to lesser mass transfer resistance. Nonetheless, this is not the case.

During covalent bonding of laccase onto the alumina support, free amino group of the laccase is used for

bonding and this includes the N-terminal of the enzyme. According to Morozova et al. (2007), a decreased contribution of free electron pair from nitrogen atom would cause an increase in the length of the Cu–N bond and reduce the redox potential of the T1 site of laccase enzyme. Since T1 site of the laccase is the primary acceptor of electron from the reducing substrate, it determines the catalysis efficiency of the enzyme. Therefore, the performance of covalently bonded laccase was not as good as entrapped laccase which was only physically retained in the alginate beads.

Immobilization by entrapment seems to be a more suitable immobilization technique compared to covalent bonding in coupling with TEMPO to oxidize glycerol into Gled and/or GlAc. The procedure of laccase entrapment is less tedious and more environmentally friendly, and a complete immobilization only took around half a day compared to the covalent method that took a week. Moreover, it consumed less energy as it could be conducted at room temperature (25 °C).

4. Conclusions

Laccase immobilized by entrapment was compared with the covalently bonded laccase on alumina pellets. The immobilization yield of laccase through covalent bonding was 94% which was higher than that of the laccase immobilized through entrapment (90%). Both immobilization methods are very satisfactory as the result of the analysis show that the reusability was high and the difference of the reusability as well as leakage between both methods of immobilization were small. Although immobilization by covalent bonding has higher immobilization yield and reusability, entrapment method is preferred due to its simple and short time preparation method at room temperature. Most importantly, the entrapped laccase incorporated with TEMPO in selective oxidation of glycerol could attain a higher Gled and GlAc than covalently bonded laccase coupled with TEMPO. Further experimental work can be carried out in optimizing the pore size of the alginate beads to reduce its mass transfer resistance.

Disclosure statement

No potential conflict of interest was reported by the authors.

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