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## Enzymatic Conversion of Glycerol to Glyceric Acid with Immobilised Laccase in Na-Alginate Matrix

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### Abstract

In this study, enzymatic oxidation of glycerol was performed for the production of glyceric acid. The effectiveness of immobilised laccase in Na-Alginate matrix was also verified. Glycerol was incubated at 25°C with 30 mM of 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO), which acted as a mediator, in the presence of immobilized laccase from *Trametes versicolor*. Glyceric acid was quantified using HPLC, while the activity of enzyme was measured using ABTS assay. The yield of laccase activity was 90%. On the other hand, glyceric acid obtained was around 7 wt% from the total product at 24h. In conclusion, enzymatic oxidation is able to produce comparable amount of glyceric acid through a more environmental friendly method.

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**Keywords:** glyceric acid; immobilisation; laccase; oxidation of glycerol; TEMPO

### 1. Introduction

Biodiesel has emerged as an alternative sustainable source of fuel due to the great demand and consumption of petroleum. It is estimated that the global market will produce 37 billion gallons of biodiesel with an average annual growth of 42% by 2016 [1]. Glycerol (Gly), a byproduct of biodiesel, will be generated at around 10 wt% of total biodiesel production, thus approximately 4 billion gallons of glycerol will be produced in 2016 [2].

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The emanation of Gly from biodiesel has flooded the market which led to a significant decrease in price for Gly. A progressive changeover of biodiesel byproduct to high value derivatives transpired to be an inevitable necessity. The development of new applications for Gly is considered important in technological progress and would be enthusiastically welcomed by the industry.

Selective oxidation of Gly produces derivatives such as glyceraldehyde (Gald), glyceric acid (GIAC), tartronic acid and so on which serve as new chemical intermediates. Since the products are of practical value, these would address a very large chemical market [3]. In selective oxidation, primary hydroxyl groups would be oxidised to Gald, an intermediate for carbohydrate metabolism and also standard for comparison of chiral molecules (D-or L-series). GIAC can be used for treatment of skin disorders. Additionally, GIAC in its ester form with the combination of a quaternary ammonium salt can act as a biodegradable fabric softener [4]. GIAC derivatives can be used as a multifunctional building block compound [5]. From biology perspective, the enantiometric isomer D-GIAC has been reported to accelerate the oxidation of acetaldehyde and ethanol *in vivo* in rats through circumventing the rate limiting step [6]. It has liver stimulating and cholesterolytic activities in dog [7]. It also acts as trypsin inhibitors [8] and building block of bio-based polyesters [5].

Selective oxidation can also be performed by using catalyst such as Pt, Pd and Au [9,10]. However, one of the drawbacks in using the supported Pt and Pd catalysts during reaction is their deactivation increase with time. Particularly, the platinum group metals based catalyst suffer oxygen poisoning which is proportional to the oxygen partial pressure [11,12]. Low partial pressure of oxygen is typically used to limit the oxygen dissolution. Gold catalysts have better resistance than platinum based catalysts towards oxygen poisoning, allowing the use of higher oxygen partial pressure [13]. Bimetallic catalysts (featuring active sites with two or more metallic elements) are introduced to enhance activity or selectivity. Comparing Au–Pd with Au and Pd catalysts in the liquid phase oxidation of glycerol, the activity is highly enhanced by the presence of alloyed phase [14]. Preparation and impregnation of these catalysts often appear to be time consuming and higher temperature and pressure are needed as well.

A greener approach- TEMPO/laccase to oxidise glycerol is being introduced [15]. Laccase (EC 1.10.3.2: benzodiol : oxygen oxidoreductases), distributed widely in bacteria, plants, fungi and insects, is a type of multi-copper containing enzymes [16]. It can catalyse oxidation of a variety of inorganic and phenolic compounds, concomitantly reduce oxygen to water without the formation of hydrogen peroxide [17,18]. Laccases have potential in chemical synthesis [19], which are limited by its redox potential. By imitating nature, mediators are added as electron shuttles for the substrate to laccase [20]. 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) and its derivatives have been used in many areas of synthetic organic chemistry with the possible achievement of chemoselectivity in the oxidation process. TEMPO acts as mediator, in which laccases oxidise it to corresponding oxoammonium cation through one electron oxidation, thus regenerate TEMPO. The oxoammonium cation, in turn, oxidise substrate such as alcohol via heterolytic pathway [21].

There are many constraints in using enzymes and the common perception is that enzymes are sensitive, unstable, not ideal as a catalyst and undesirable in most synthesis and reaction. The decrease in stability and the catalytic activity of free enzymes due to its catalytic environment seriously limit their industrial application. Immobilisation is the most adopted stabilisation method in overcoming these limitations which allows enzyme reutilisation. Laccases have been immobilised on different supports for the removal of several pollutants including dyes [22]. Entrapment is the easiest method in which it induces no structural alteration of enzymes, less tedious procedure and reduced number of chemicals used. It is just the physical retention of the enzymes in porous solid matrix [23]. As reported by Niladevi and Prema [24], immobilisation of laccase in Cu alginate has immobilisation yield of 61% while immobilization for the entrapment in PVA-alginate yields 97.4% [25].

In this paper, the oxidation of Gly with an enzyme-mediator system to obtain GIAC is shown. The study also being extended by comparing the enzymatic reaction with chemical reaction [9] and effect of different immobilisation methods in converting glycerol to GIAC. The use of immobilised laccase allows the catalyst to be separated from reaction mixture easily for recycling purposes.

### Nomenclature

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
Au	Gold
CaCl <sub>2</sub>	calcium chloride
CuSO <sub>4</sub>	copper(II) sulphate
DAD	diode array detector
Gly	glycerol
GIAC	glyceric acid
Glad	glyceraldehyde
H	hour
HPLC	high performance liquid chromatography
Min	minute
Na	sodium
PEG	polyethylene glycol
Pd	palladium
Pt	platinum
RID	refractive index detector
TEMPO	2,2,6,6-tetramethylpiperidine-1-oxyl
Wt%	weight percent

## 2. Materials and methods

Laccase from *Trametes versicolor* was purchased from Sigma Aldrich, Germany without further purification. Glycerol was obtained from Fisher Scientific. 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO), polyethylene glycol (PEG) (MW = 4000), calcium chloride (CaCl<sub>2</sub>), sodium acetate, glutaraldehyde were sought from Merck. Sodium Alginate was bought with R&M Chemicals and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was procured from Roche. All standards for HPLC were purchased from Sigma-Aldrich.

### 2.1 Laccase activity assay

Laccase activity was determined by monitoring the absorbance of ABTS oxidation at 415nm wavelength using UV-VIS spectrophotometer at room temperature (light path = 1 cm). The assay mixture for the blank consists of 1.9 ml of 0.1 M acetate buffer (pH 4.5), 0.1 ml of pure water and 1.0 ml of 0.5 mM ABTS solution. Pure water was replaced with samples for determination of the laccase activity. One unit (U) of the enzyme was defined as 1 μmol ABTS oxidised per minute under the stated assay condition.

### 2.2 Immobilisation of laccase

2.0 g of gelatine and 0.5 g of PEG was added to 100 ml of 2.0% (w/v) sodium alginate solution. 0.1 g of laccase (10U/ml) was then added and mixed thoroughly for 10 minutes at 25°C. The mixture was withdrawn by using a sterile 5 ml syringe and extruded through a 21 gauge (0.51 mm inner diameter) needle into 2.0% (w/v) CaCl<sub>2</sub>

solution, beads formed immediately when touched the surface of the solution. Beads were left unstirred to harden in the  $\text{CaCl}_2$  solution at  $4^\circ\text{C}$  for 2 hours, after which the  $\text{CaCl}_2$  solution was removed and the beads were washed twice with distilled water. Then, the beads were subsequently incubated in 100 ml of 0.6% (w/v) glutaraldehyde solution and stirred at  $4^\circ\text{C}$  for 2 hours. The beads were washed several times with phosphate buffer (pH 7) to remove the laccase which was not entrapped into the beads. The immobilised Laccase enzymes are stored at  $4^\circ\text{C}$  in acetate buffer (pH 4.5) [22].

### 2.3 Oxidation of glycerol

50 mL mixture of 100 mM glycerol, 30 mM TEMPO, and 1 g of immobilised laccase in 100 mM sodium acetate buffer (pH4.5) was incubated at  $25^\circ\text{C}$  under stirred condition. The oxidation process was run in triplicate for 24 hours and sampling was done hourly.

### 2.4 Analytical method

Quantitative analysis of the oxidation products were conducted by using a high performance liquid chromatography (HPLC) equipped with DAD and RID. A 300 mm Phenomenex Rezek ROA column was used. 3 mM  $\text{H}_2\text{SO}_4$  was used as mobile phase with  $0.5 \text{ mL min}^{-1}$  flowrate, 210 nm wavelength, injection volume of  $10 \mu\text{L}$  and the column temperature at  $75^\circ\text{C}$ . The calibration plot was obtained by linear regression of peak-area ratio against concentration. Products were identified by comparison of their retention times with standards of the respective components.

## 3. Results and discussions

Methods of immobilisation affected the amount of laccase immobilised and influenced the final activity remained. This is due to the different in procedures of immobilisation and chemicals used, which in turn determined the types of interaction between laccase and support. The lengthiness of the procedures also contributes to the possible loss of enzyme during the process. Table 1 shows the activity of laccase after immobilisation in Na-Alginate matrix in this study was 90%. Activity of laccases, which was being immobilised using other immobilisation methods, was also depicted in Table 1. It could be seen that some of the immobilization yield were high but some were moderate.

Table 1. Immobilisation methods and yield.

Author	Immobilisation method	Material	Immobilisation yield (%)
[26]	covalent bond	Chitosan	52.2
[27]	entrapment	Alginate solution cross-linked by $\text{CuSO}_4$	74.6 - 146.8
[28]	encapsulation	LbL technique (assembly of differently charged polyelectrolyte layers) on manganese carbonate ( $\text{MnCO}_3$ )	75
[29]	adsorption	Chitosan-g-PEI	72
[30]	covalent bond	Green coconut fiber	50,70
Current study	entrapment	Na-Alginate	90

Time series profiles of the reaction are depicted in Figure 1. It can be seen from Figure 1 that in the early stage of reaction, Gald was the only product. This implied that selectivity of TEMPO is highly towards primary alcohol. Nearly 30% of Gly was converted. The concentration of Gald increased until it reached its maximum at 24h. After 3h of incubation, GIAC was detected in the reaction mixture. The concentration of GIAC increased steadily throughout the period of incubation. The final amount of GIAC obtained is about 7 wt %. In this oxidation process, Gald became substrate for TEMPO besides the initial reactant, which is Gly. GIAC was obtained as a result of further oxidation from Gald. The oxidation of Gly does not stop at GIAC, it can lead to other organic acids such as tartronic acid, mesoxalic acid, formic acid etc. However, the concern of this paper is only GIAC, therefore the products appeared in extended time after 24 h were not included.

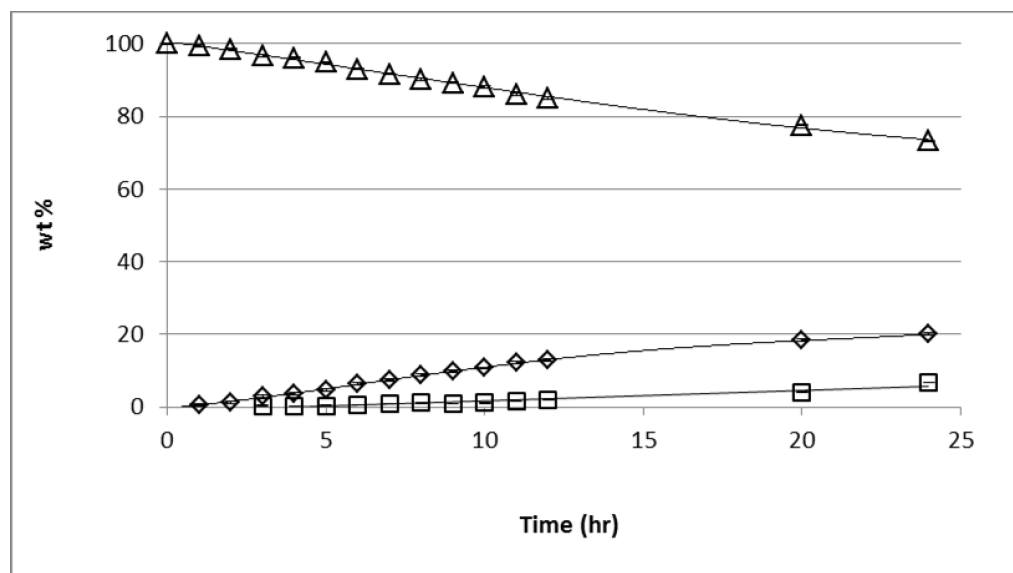


Fig 1. Oxidation of 100mM glycerol by 30mM TEMPO with immobilized Laccase and formation of product ( $\Delta$ :glycerol; $\diamond$ :glyceraldehyde; $\square$ :glyceric acid)

In general, nitroxyl radical plays an important role in selective oxidation of alcohol. Due to its high reaction rate and high selectivity, nitroxyl radical is employed for the modification of high molecular mass polysaccharides too [31]. Conventional methods such as manganese dioxide, dimethyl sulfoxide activated by oxalyl chloride and silver oxide are based on chromium reagents which are toxic and only applicable to lab scale usage [15]. As a result, nitroxyl radicals such as TEMPO and its derivatives that are proven to be effective in selective oxidation of alcohols [15,32] has drawn attention as a substitution. In addition, TEMPO can be recovered from reaction mixture by extracting it with organic solvents [33], thus giving an extra advantage.

The role of TEMPO as mediator in enzymatic oxidation with the presence of immobilised laccase is outlined in Figure 2. In this case, TEMPO acted as electron shuttle, being oxidised by laccase, diffused away from enzymatic pocket and oxidized glycerol (substrate) which could not directly entered and reacted with laccase. Therefore, with the presence of mediator, the range of substrate is being extended, oxidation which is not available to enzyme can be performed [34].

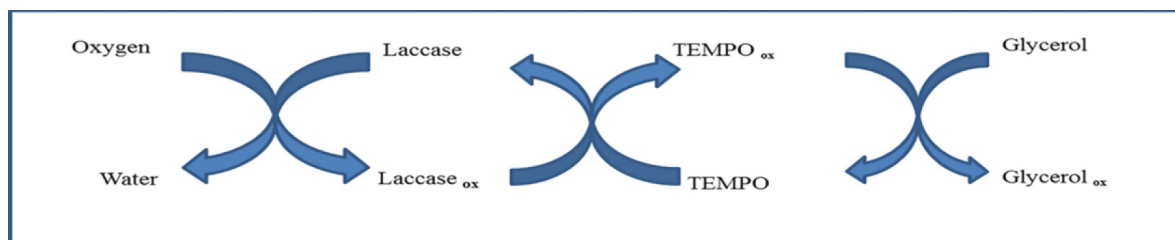


Fig 2. The role of a mediator of the enzymatic activity

The current study was an example of oxidation by TEMPO/laccase in which Na-Alginate as a support for laccase seemed to be a promising immobilisation method. The oxidation products by TEMPO/immobilised laccase in Na-Alginate within 24 h of reaction were shown in Figure 3. Comparing with Figure 4 which was reported by Liebinger *et al.* [15] (activity recovery on carrier was 68%) glyceric acid obtained by immobilised laccase on Na-Alginate was just about half of it. One of the reasons was due to difference in immobilisation method. Immobilisation of laccase in Na-Alginate was an entrapment method while immobilisation of laccase on Alumina pellet was a covalent bond technique. TEMPO had more contact with laccase in the covalent bond technique than entrapment since laccases were bonded on the surface covalently which made the oxidation reacted faster to desired product. However, laccases were trapped in the gel matrix in entrapment technique which caused diffusion limitation. TEMPO will have to take longer period of time to diffuse and react with laccase and substrate as described earlier. Thus, longer reaction period is required to obtain desired product. Another possible reason might be the difference in redox potential for laccase species. Liebinger *et al.* [15] used the *Trametes hirsute* while *Trametes versicolor* was being used in this study. Each and every laccase has its own intrinsic redox potential, the more positive the potential, the greater the species' affinity for electrons and tendency to be reduced. Immobilisation by using Na-Alginate not only cheaper than the compared method (immobilisation on Alumina pellet), it also took only half a day for a complete immobilisation rather than a week for the compared method.

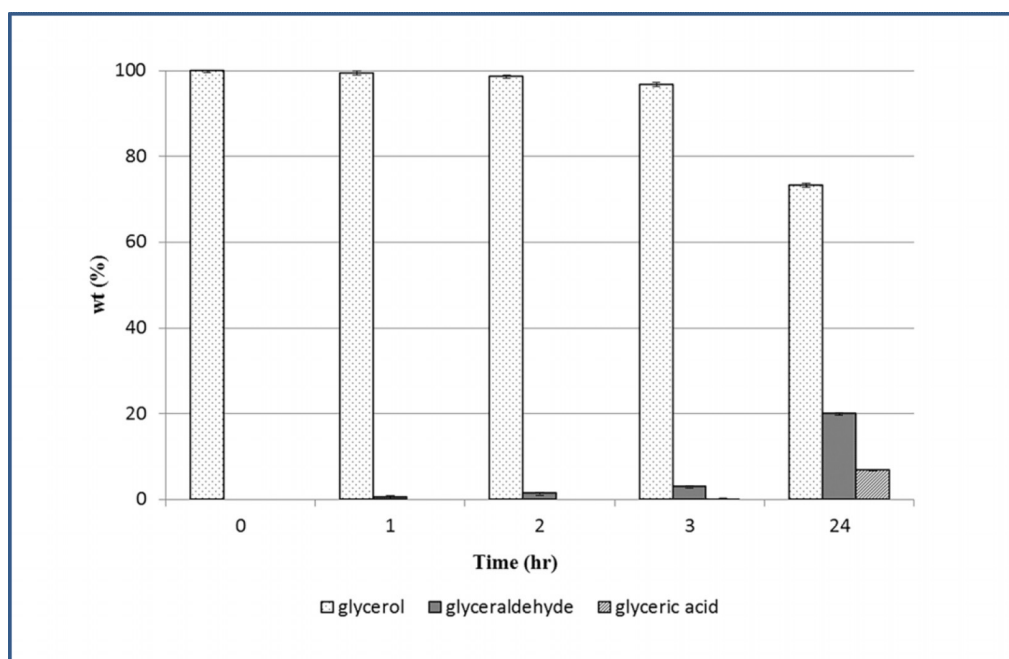


Fig 3. Oxidation of glycerol by 30mM TEMPO in the presence of immobilised laccase in Na-Alginate

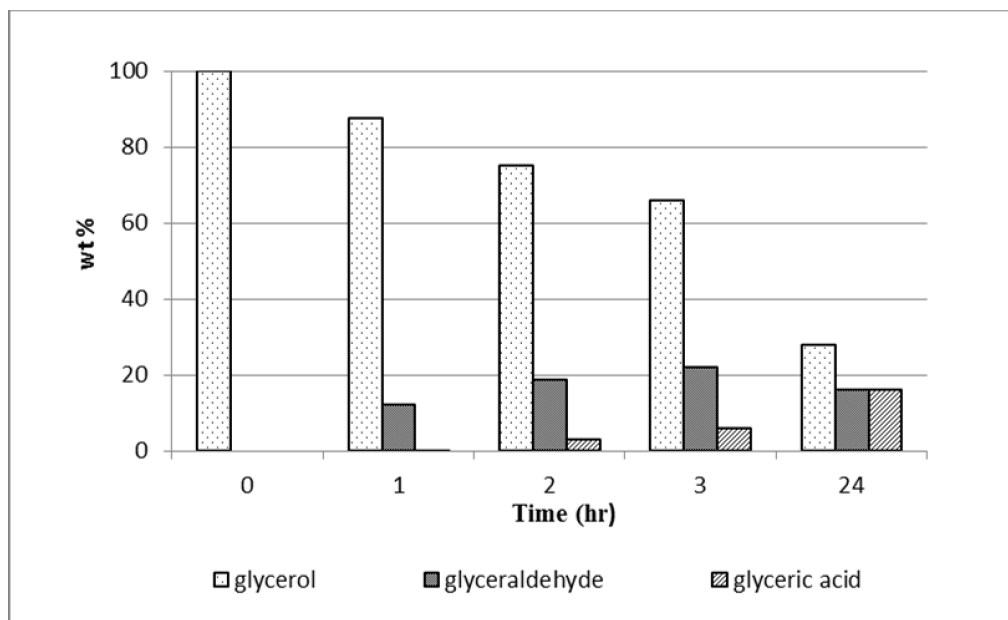


Fig 4. Oxidation of glycerol by 30mM TEMPO in the presence of immobilised laccase on Alumina pellet [15].

A study involved catalytic reaction which was reported by Porta and Prati [9] was used to compare with current enzymatic study. The catalytic oxidation in which 1% Au/C was used with graphite (Lonza HSAG300) as the support in the following reaction condition,  $T = 60\text{ }^{\circ}\text{C}$ , 10 wt% of glycerol,  $\text{NaOH/glycerol} = 1\text{ mol/mol}$ ,  $\text{glycerol/Au} = 500\text{ mol/mol}$ , the yield for GIAC was about 2.5 mole %, which is almost the same with the oxidation of Gly by using 30mM TEMPO in the presence of immobilised laccase on Na-Alginate at 24h. The reaction was slow for TEMPO/laccase system, which might be due to the reaction that was not optimised yet (the reaction was run under ambient temperature and pressure).

#### 4. Conclusion

Laccase from *Trametes versicolor* was successfully immobilised in Na-Alginate. The TEMPO/ laccase system for oxidation of glycerol to glyceric acid was reliable, achieved nearly 7 wt% of the total product at the end of reaction time. This study proved that laccase was an effective and environmental friendly regeneration oxidant compared to other stoichiometrically used chemicals. Optimisation is recommended for future study.

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