Effect of Crosslinkers on Immobilization of β-Galactosidase on Polymethacrylate Monolith

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ABSTRACT Advances in biotechnology unfold a new frontier for the development of enzyme-catalysed bioprocess which is green and sustainable in contrast with chemical processes. Immobilization technology appears as a beneficial solution to the uneconomical cost of enzyme operation. Immobilization of enzyme via crosslinking approach has become a technology interest due to the more concentrate enzyme activity in the catalyst compared to other techniques. In this study, two types of crosslinker, glutaraldehyde and hexamethylene diisocyanate at different concentration was investigated in immobilizing β-galactosidase on polymethacrylate monolith. The enzyme activity upon immobilization was measured spectrophotometrically at 405 nm. The immobilized enzyme was further characterized using Fourier Transform Infrared Spesctroscopy (FTIR) and Zeiss Axio Fluorescence Microscope. The findings showed that the optimum enzyme activity was achieved when using 0.05% and 0.01% glutaraldehyde hexamethylene diisocyanate respectively. Beyond that concentration, a significant reduction of enzyme activity was observed. It was found that glutaraldehyde was preferable as crosslinking agent as hexamethylene diisocyanate exhibited stronger effect in reducing enzyme activity. A successful binding of β-galactosidase on polymethacrylate monolith was observed using Fourier-transform infrared spectroscopy (FTIR) and Zeiss Axio Fluorescence microscope. The outcomes of this study indicate the potential of enzyme immobilization on monolith via crosslinking method.

KEYWORDS: Polymethacrylate monolith; cross-linking; glutaraldehyde; hexamethylene diisocyanate; β-galactosidase

I Received 18 April 2018 **II Revised** 7 May 2018 **II Accepted** 14 May 2018 **II Online** 28 June 2018 **I**

INTRODUCTION

The enzyme immobilization technology has driven rapid biotechnology advancement towards green and sustainable processes. Besides able to shelter and/or stabilize enzymes against chemical and environmental attacks, and importantly the immobilized enzymes could be recovered and reused in a large scale continuous process (de Melo *et al*., 2017). Enzymes have been immobilized on various support materials including natural/synthetic polymers or inorganic materials as enzyme supports. Monoliths, such as acicular mullite (ACM) monolith, aldehyde-modified polymethacrylate, polycarbonate, polyacrytonitrile, poly(vinyl alcohol), has received great biotechnological interest due to their interconnected porous structure and exceptional properties for enzyme immobilization (Han *et al*., 2014). In addition, monolithic materials with unique three-dimensional (3D) continuous structure offer various advantages such as good permeability, fast mass transfer property, high stability and easy modification (Xin et al., 2012).

Enzyme immobilization using cross-linking technique promotes rapid enzyme immobilization. Cross-linking was one of the highly researched techniques to promote enzyme stability and reusability. Enzyme reactivity could be affected due to conformational change or is reduced by exposing to crosslinking reagents. Cross-linking is conducted by configuration of intermolecular cross-linkages between the enzyme molecules by means of bi-or multifunctional reagents (Mohamad *et al*., 2015). The crosslinker that commonly used was glutaraldehyde (GA) because it is economical and efficient and hence remains the reagent of choice for cross-linking (Satar *et al*., 2017). Besides, another crosslinker that has been introduced was hexamethylene diisocyanate (HMDI) which shared the same characteristic as bifunctional reagents with glutaraldehyde. The employment of (GA) and (HMDI) as cross-linking reagent, the adsorption of β-galactosidase on polymethacrylate monolith

will avoid a direct contact of the enzyme with the surrounding medium, it also make the reagents to reach catalytic site (Chen et al., 2013).

The purpose of this study is to study the effect of crosslinkers in immobilizing enzyme on monolith. β-galactosidase was immobilized on polymethacrylate monolith via crosslinking approach using glutaraldehyde and hexamethylene diisocyanate as crosslinking agents. The findings of study provide a useful guideline in immobilizing enzyme using cross-linking approach. The immobilized enzyme was clarified via Fourier Transform Infrared (FTIR) and fluorescence microscope.

METHODOLOGY

Preparation and functionalization of polymethacrylate monolith

Monolith was prepared following the method established by Ongkudon et al., 2013). Poly(GMA-co-EDMA) monolith were prepared via free radical copolymerizations of EDMA and GMA monomers in the presence of the initiator(azobisisobutyronitrile) and cyclohexanol as the porogenic solvent. The mixture of GMA:EDMA combined with porogenic solvent at 70:30 of monomer with 1% v/v of AIBN was dissolved in 150 ml of solution . All polymerizations were performed within a confined tubular glass column with constant temperature at 60°C in a water bath for 3 hours. The solid polymer was washed with methanol overnight. After that, the polymer was washed with distilled water to removes the traces porogen. The monolith then was crushed into a powder form for enzyme immobilization study.

β-galactosidase immobilization on monolith

Approximately, 10 mg of polymethacrylate monolith was immersed into 1 ml β-galactosidase in PBS solution (1 mg/ml) and gently mixed for 2 hour to allow enzyme immobilization to takes place. Subsequently, the unbound enzyme was removed by thoroughly washing with distilled water. The immobilized enzyme was crosslinked with GA and HMDI for 1 hour at room temperature. The crosslinking of enzyme was conducted using glutaraldehye (GA) solution and hexamethylene diisocyanates (HMDI) at the different concentration of 0.01, 0.05, 0.1, 0.2 and 0.3% for 1 hour at room temperature simultaneously. The particles were collected through centrifugation and washed with water to remove unbound enzyme (Misson *et al*., 2015).

Enzyme activity assay

The activity assay of free and immobilized β-galactosidase was measured according to the procedure designated by Ansari and Husain (2012). The activity of enzyme before immobilization was used as a control study. The reaction mixture containing 0.1 ml of enzyme solution, 1.7 ml phosphate buffer solution (PBS) with pH 7.2 and 0.2 ml of 20mM 2-nitrophenyl-β-D-galactopyranoside (ONPG) was incubated at 37°C for 10 minutes. The reaction was terminated by adding 2 ml of 1M sodium carbonate (Na_2CO_3), the absorbance value was measured at 405 nm. The enzyme activity was determined from the plotted o-nitrophenol standard curve. One unit (1U) of β-galactosidase is defined as the amount of enzyme liberates 1μmole of o-nitrophenol per min under the standard assay condition. The enzyme capacity of immobilized enzyme was calculated using Eq. (1)

$$\text{Enzyme capacity} = \text{Enzyme activity} / \text{Support mass} \quad (1)$$

Characterization techniques

Fourier transform infrared (FTIR) spectra of the monolith, free β-galactosidase and β-galactosidase-loaded monolith were recorded on Agilent Technologies Cary 630 FTIR spectrometer at room temperature. The analysis was controlled using OMNIC software.

Zeiss Axio Fluorescence microscope was used to image the presence and distribution of FITC-labeled β-galactosidase on monolith particles. The labelled sample was prepared by mixing a proportion of 100 mg of enzyme with 10 μg of fluorescent isothiocyanate (FITC) in ethanol solution with a gentle mixing before immobilization on monolith before reacted for 2 hour at 4°C in dark condition. A thin layer of the monolith carrying FITC-labeled β-galactosidase was placed onto a glass microscope slide, observed and analysed using fluorescence microscope. Non-labelled enzyme on monolith was used as a negative control.

Statistical Analysis

Sample collection and analysis were conducted in triplicate. Results were analyzed using the statistical tool in MS Excel 2010. Each value corresponds to the mean of independent experiments conducted in triplicates.

RESULT AND DISCUSSION

Enzyme Binding on Monolith

Prior to the crosslinking procedures, the β-galactosidase enzyme was bound on monolith surface through physical binding. Enzyme possesses functional groups, such as amino (-NH_2), carboxylate (-COOH), thiol (-SH) and hydroxyl (-OH) groups, that could interact with functional groups from monolith (Zhang *et al*., 2011). Meanwhile, polymethacrylate monolith was functionalized with epoxide groups as a binding platform for the β-galactosidase. The successful binding of the enzyme was confirmed by observing under fluorescence microscope. The enzyme was initially labelled with Fluorescein isothiocyanate (FITC), a dye regularly used for antibody fluorescence labelling (Sert *et al*., 2017). FITC has been known to exhibit green excitation under fluorescence microscope as shown in Figure 1b. Monolith without enzyme under the same fluorescence condition was used to portray the fluorescence background (Figure 1a). As can be seen, the FITC-labelled-β-galactosidase emanated demonstrated excitation image indicating the presence of homogenously distributed enzyme on the monolith surface. Negative control of the monolith-β-galactosidase assembly which was prepared without fluorescence excitation was used a comparison (Figure 1c).

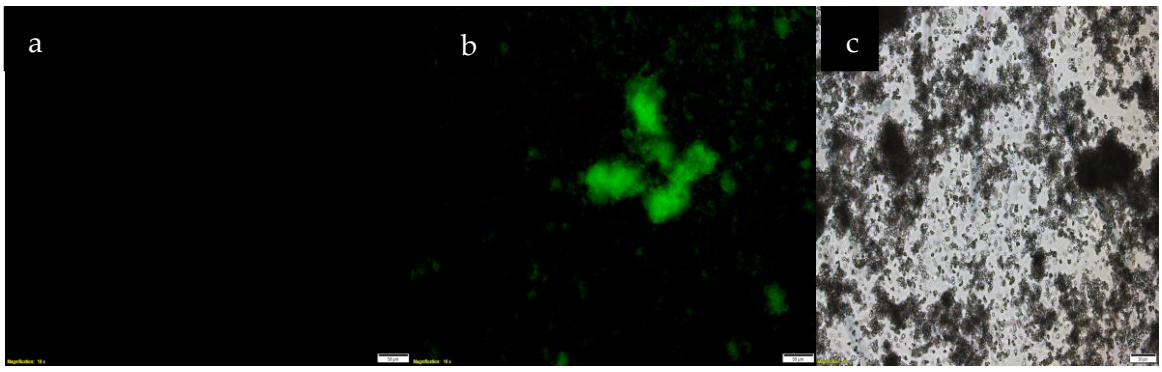

Figure 1. Photomicrographs of FITC-tagged enzyme using fluorescence microscope: (a) monolith fluorescence background, (b) FITC-monolith-β-gal fluorescence image excitation, (c) negative control monolith-β-gal without fluorescence excitation

Effect of cross-linkers in Enzyme Immobilization

Upon adsorption of the enzyme, crosslinking agent, either glutaraldehyde (GA) or hexamethylene diisocyanate (HMDI) from 0.01 to 0.3% concentration, was applied to crosslink the enzyme molecules on monolith surface. Figure 2 and 3 show the effect of different concentration of GA and HDMI on enzyme activity respectively. For the GA, as the concentration of GA increased from 0.01 to 0.05%, a remarkable increase of the enzyme activity was observed from 0.69 (U) to 0.8 (U). However, further increased the concentration up to 0.2%, the enzyme activity has decreased significantly into 0.4 U only (Figure 2a). The enzyme capacity also exhibited a similar trend with the enzyme activity (Figure 2b). After crosslinking with glutaraldehyde, the intermolecular crosslinking between the nucleophilic groups of the adsorbed β-galactosidase and the compact layer of neighbouring epoxy groups on the support were exhibited (Mateo et al., 2000). The additions of glutaraldehyde as a crosslinker amend the low reactivity of epoxy groups toward unbound β-galactosidase by reacts rapidly with N-terminal amino group that located in reaction site which is lysine. Nevertheless, when the concentration of glutaraldehyde was too high, rapid inactivation of enzyme was elevated decreasing the enzyme activity and capacity (Chen et al., 2013). The enzyme inactivation was due to the some distortion and deformation of enzyme when exposed to high concentration of glutaraldehyde. This might happen during crosslinking process.

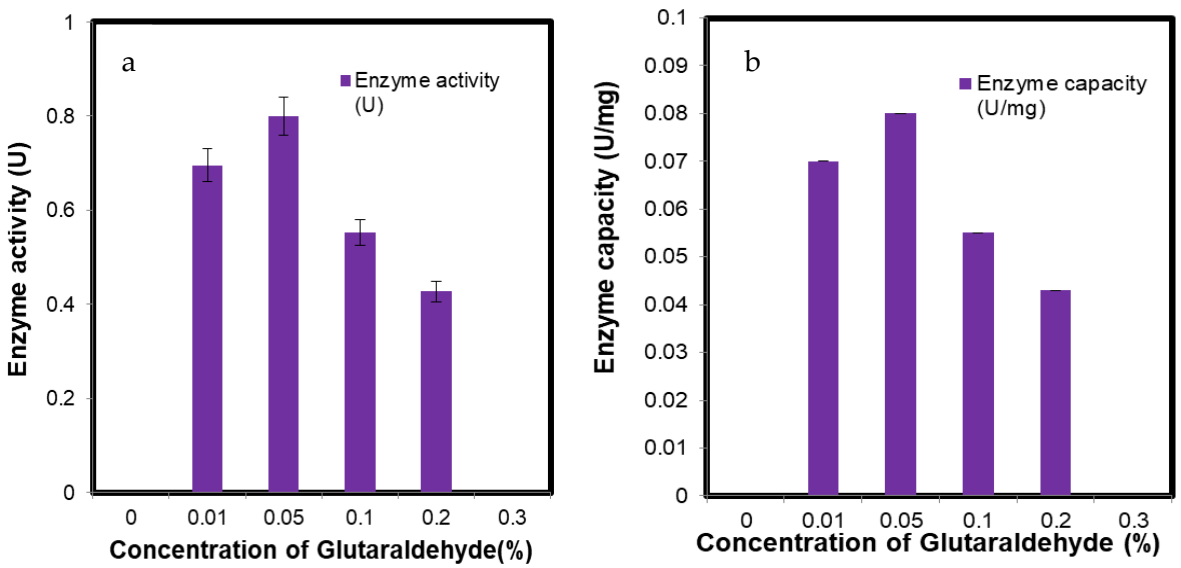

Figure 2. Effect of glutaraldehyde (GA) on the immobilized (a) enzyme activity and (b) enzyme capacity. Error bars represent the standard deviation of triplicate samples.

Meanwhile, for the HDMI, the highest activity was obtained at the lowest tested concentration (0.01%) with the enzyme activity determined at 0.151 U. Extending the concentration up to 0.3 % yielded around 0.06 to 0.08 enzyme activity, approximately 50% lower than using 0.01% concentration of HDMI. The findings indicate HDMI has a strong effect on the crosslinking in which the activity was significantly affected even at low concentration (0.05%). The enzyme activity reduction (Figure 3a) was probably due to the alteration of the unique structure of enzyme upon immobilization. The enzyme capacity (Figure 3b) also demonstrated a similar trend of reduction with the increased concentration of crosslinker. An approximate of enzyme capacity was found when using HDMI at concentration ranging between 0.05-0.1% or 0.2-0.3%. By comparing the effect of GA and HDMI in immobilizing enzyme via crosslinking technique, the GA capable to obtain higher enzyme activity which is about 81% greater than HDMI.

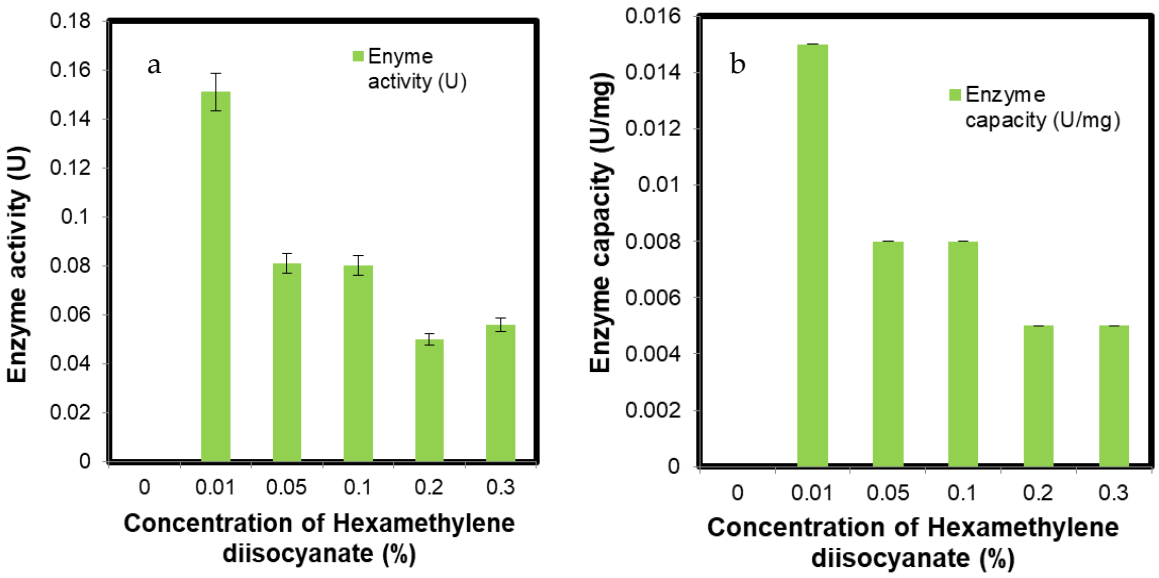

Figure 3. Effect of hexamethylene diisocyanate (HMDI) on the immobilized (a) enzyme activity and (b) enzyme capacity. Error bars represent the standard deviation of triplicate samples.

Characterization of immobilized enzyme on monolith

The crosslinked β-galactosidase on monolith surface was further characterization using FTIR analysis. FTIR was known as a powerful tool to assess the formation of protein-surface interaction by detecting the bond formation generated by the wavelength and intensity of IR radiation (Kong and Yu, 2007). Figure 4 and 5 show the FTIR spectra of free β-galactosidase, polymethacrylate monolith and β-galactosidase-loaded monolith crosslinked using GA and HDMI, respectively.

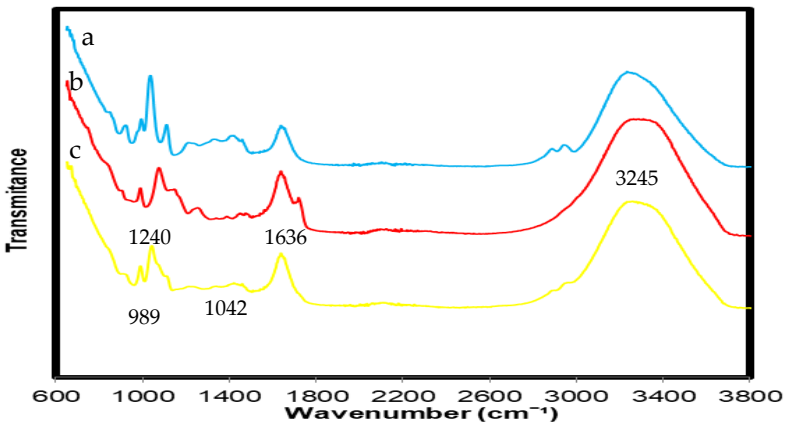

Figure 4. FTIR spectra of a) β-galactosidase, b) polymethacrylate monolith, c) Crosslinked β-gal and monolith with GA.

The findings in Figure 4 show the bands appeared between 800 and 1000 cm^{-1} at 989 cm^{-1} was due to the epoxy ring of plane vibration which confirmed the presence of epoxide group and hence GMA (Ur Rahman *et al*., 2011). Moreover, the increase of the intensity of C-O stretch mode happens between 1000 and 1200 cm^{-1} from 1034 cm^{-1} to 1042 cm^{-1} which indicates that the cross-linking reaction has occurred (Hu et al., 2011). There are also a single band between 1200 and 1600 cm^{-1}. The absorption bands at 1420 cm^{-1} are amino group due to N-H bending. Next, another band at 1636 cm^{-1} was assigned to C=O stretch vibration of peptide linkages produce by amide I (Zhang et al., 2015). Furthermore, the slight peaks between 2800 and 3000 cm^{-1} coincide to the C-H stretch modes of the crosslink molecules (Hu *et al*., 2011). The wide absorption band at 3000 and 3600 cm^{-1} in the spectra can be attributed to the O-H stretching mode of absorbed water (Ur Rahman *et al*., 2011).

Meanwhile, the FTIR spectra for HDMI-crosslinked enzyme exhibited a comparable trend of spectra (Figure 5). The band formed at 3245 cm^{-1} indicates the O-H group and 1636 cm^{-1} represent C=O stretch vibration. The presence of C=O groups was formed from the isocyanate reaction (Welsh *et al*., 2002). Both of them showed similar result from the figure 4 and confirmed that β-gal was successfully immobilized on polymethacrylate monolith. Besides, the band which demonstrated the presence of epoxide group at 989 cm^{-1} also appeared. The band at 1388 cm^{-1} and 1075 cm^{-1} shows the bending of O-H which suggested that the cross linking reaction has occur (Zia *et al*., 2015).

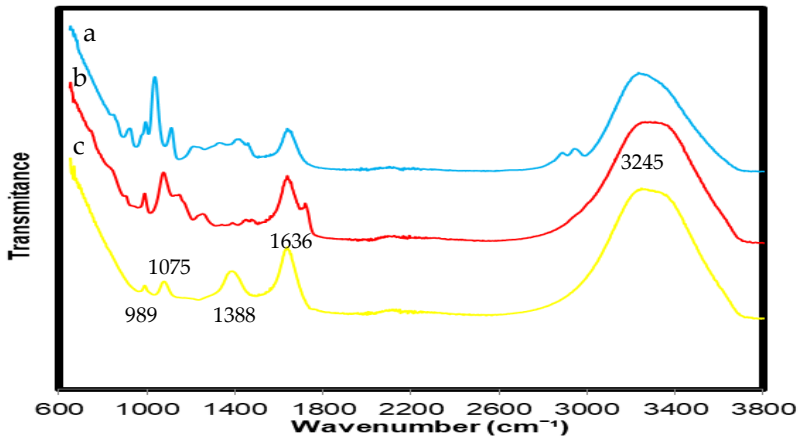

Figure 5. FTIR spectra of a) β-galactosidase, b) polymethacrylate monolith, c) Crosslinked β-gal and monolith with HMDI.

From the figure, the band formed at 3245 cm^{-1} indicates the O-H group and 1636 cm^{-1} represent C=O stretch vibration. The presence of C=O groups was formed from the isocyanate reaction (Welsh *et al*., 2002). Both of them showed similar result from the figure 3 and confirmed that β-gal was successfully immobilized on polymethacrylate monolith. Besides, the band which demonstrated the presence of epoxide group at 989 cm^{-1} also appeared. The band at 1388 cm^{-1} and 1075 cm^{-1} shows the bending of O-H which suggested that the cross linking reaction has occur (Zia *et al*., 2015).

CONCLUSION

Two types of crosslinkers at different concentration with different type of crosslinkers were successfully investigated in this work. The finding revealed both crosslinkers have the potential to immobilize enzyme on monolith surface. Based on the result, glutaraldehyde was the best crosslinker with maximum enzyme activity 0.8 U at 0.05% and higher than hexamethylene diisocyanate that has lower enzyme activity even at its highest which is 0.151 U at 0.01%.

ACKNOWLEDGEMENTS

This research was funded by Universiti Malaysia Sabah under the research grant SLB0124--SG-2016: research grant scheme: Immobilization of β-galactosidase on Monolith for Production of Galacto-oligosaccharides.

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