MJFAS
Malaysian Journal of Fundamental and Applied Sciences

Kumar *et al.* | Malaysian Journal of Fundamental and Applied Sciences, Vol. 18 (2022) 43-51

RESEARCH ARTICLE

Immobilization of Maltogenic Amylase in Alginate-chitosan Beads for Improved Enzyme Retention and Stability

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Abstract Maltogenic amylase (MAG1) is a potent enzyme that hydrolyzes the glycosidic bond of polysaccharides to produce malto-oligosaccharides (MOS). However, the MAG1 enzyme has poor stability and reusability, leading to inefficient MOS production. Enzyme immobilization is a promising method to solve the enzyme stability problem. Entrapment and encapsulation technique was used in this study to immobilize MAG1 because of high biocompatibility and prevention of enzyme degradation, hence lesser loss of enzymatic activity. Chitosan was used as a coating membrane on the alginate matrix, preventing enzyme leaching from the beads. MAG1 entrapped in alginate-chitosan beads showed better performance compared to alginate beads in terms of thermostability, reusability, and enzyme retention. Alginate-chitosan beads showed improvement of temperature stability of approximately 35%, 30%, and 20% of enzyme activity at a respective temperature of 30 °C, 40 °C, and 50 °C. Reusability analysis showed immobilized MAG1 can be used up to at least eight cycles with the retained activity of 80% and 70% from its initial activity for alginate-chitosan and alginate beads respectively. Enzyme leakage percentage in alginate-chitosan was 7-21%, while that in alginate was 12-35%. The overall findings envisage the promising application of alginate-chitosan beads immobilized MAG1 as a biocatalyst for MOS synthesis.

Keywords: Maltogenic amylase; enzyme immobilization; alginate-chitosan; stability; reusability.

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Received: 23 August 2021
Accepted: 21 Feb 2022

Introduction

An enzyme is a substance that performs catalytic behavior in regulating the rate of chemical reactions. An enzyme that can catalyze the hydrolysis process of the starch is called amylase. Maltogenic amylase (MAG1) is a potential catalyst to produce oligosaccharides. Oligosaccharides are carbohydrate polymers made up of two to ten monosaccharides linked by glycosidic bonds and have been widely used in the food and pharmaceutical industries. Oligosaccharide is a potential ingredient and highly demanded

functional food due to its prebiotic properties. The mildly sweet taste with mouth feeling characteristics of functional oligosaccharides makes it suitable to become a partial substitute for sugars and fats to improve food texture in the food industry [1]. MAG1 is an amylolytic enzyme belonging to glycosyl hydrolase family 13 (GH13) which is capable to hydrolyse two or three substrates such as starch, pullulan, and cyclomaltodextrins (CDs) [2]. MAG1 possesses special physicochemical and catalytic properties which are capable of hydrolyzing α-1,4- and α-1,6- glycosidic bonds and transglycosylation of oligosaccharides to form various lengths of sugar molecules [3]. Compared to typical α-amylase which hydrolyze starch efficiently, maltogenic amylase exhibits the highest hydrolytic affinity to cyclomaltodextrins as the substrates.

MAG1 is widely used as a biocatalyst in the field of bioprocess technology. Due to the large extent of commercialization of biocatalysts in industry, the reusability of biocatalyst has become a crucial factor to be considered despite its expensiveness. Besides that, the maintenance of their structural stability during any biochemical reaction is challenging because the unstable biocatalyst cannot withstand high temperatures and unsuitable pH values. So, enzyme immobilization techniques have received great attention from every industrial sector to enhance the reproducibility and stability of biocatalysts. Enzyme immobilization techniques include adsorption, entrapment, encapsulation, cross-link, and covalent bonding. The bonding interactions formed between these techniques are different. Carrier materials are one of the factors contributing to the development of immobilized enzymes in enhancing their stability and reactivity [4].

Carrier material for enzyme immobilization can be classified as organic, inorganic, and composite. The types of carrier material are important to give a strong effect to produce a highly efficient catalytic system. The carrier can protect enzymes from being damaged in harsh reaction conditions so that the enzyme can retain its high catalytic activity. Biopolymer is one of the suitable carrier materials that has been widely used in enzyme immobilizations. Biopolymer is a renewable material that is obtained from natural sources. The characteristics of biopolymers are renewable, abundant in nature, non-toxic, biodegradable to harmless products, and biocompatible, which makes them suitable to become a support for enzymes [5]. The examples of biopolymers used in enzyme immobilization techniques are chitin, chitosan, and alginate.

Sodium alginate is a widely used polymer for entrapment techniques. It is known as a biodegradable substance and a controllable porosity substance. Calcium alginate is frequently used as the carrier material for immobilized enzymes because it can increase adhesion and proliferation by cross-linking the carboxyl group of alginates with cross-linker such as calcium chloride or barium chloride. However, due to the low mechanical strength of calcium alginate, there is still enzymatic leakage occurred. Previously, a study from Nawawi, et al. [6] reported that entrapment of MAG1 in calcium alginate caused enzyme leakage which resulted in low entrapment efficiency. Hence, another method needs to be developed to prevent enzyme leakage and enhance the mechanical strength of the carrier material. Special attention should be paid to chitosan which is normally used in immobilization as a coating material to improve the mechanical stability and efficiency of the carrier material (Zdarta et al., 2018). Chitosan is another natural polymer that will provide support due to biocompatibility, biodegradability, and non-toxic. In this study, MAG1 is entrapped, localized, and protected in the alginate core matrix, while the chitosan shell encapsulated the alginate core that protects the enzyme from leakage and strengthens the beads while regulating the entry and exit of substrate and product. The present work introduces a novel chitosan-coated immobilized MAG1 in calcium alginate to improve enzyme retention and stability. To the best of our knowledge, thus far, there is no study published on the MAG1 co-immobilized in calcium alginate and chitosan. The study was conducted to develop a robust biocatalyst with enhanced enzymatic retention, low enzyme leakage as well as highly reusable that is important for industrial applications.

Materials and methods

Materials

Recombinant MAG1 [2] from *Bacillus lehensis* G1 was used throughout the study. In this study, chemicals such as sodium alginate, chitosan, and calcium chloride were purchased from Sigma Aldrich (Missouri, USA), Merck (Darmstadt, Germany), and Thermo Fisher Scientific (Massachusetts, USA).

Enzyme immobilization

Maltogenic amylase (MAG1) enzyme was expressed according to the previous method by Abdul Manas, et al. [2]. The preparation of alginate core (alginate beads) is conducted according to the method as described by Nawawi, et al. [6] where sodium alginate solution was prepared by mixing it with deionized distilled water and stirred until complete dissolution. About 2 mg/mL of maltogenic amylase enzyme was added to 2.5% (w/v) sodium alginate solution. The solution was extruded by syringe into 0.6% (w/v) calcium chloride solution for beads formation. After 30 min, the beads were washed thrice with 100 mL distilled water. Then, alginate beads were dispersed into (1%, 2%, 3% and 4%) w/v chitosan in 0.1 M acetic acid solution to encapsulate the beads and left it for another 30 min, producing MAG1 immobilized into alginate-chitosan beads. The concentration of chitosan was optimized depending on the enzyme activity and compared with the alginate beads in terms of enzyme relative activity. The immobilization was carried out at 4°C to avoid enzyme degradation.

Enzyme activity determination

The enzyme activity of MAG1 was measured using the dinitrosalicylic acid (DNS) method [7]. For free MAG1, a mixture of 100 µL of maltogenic amylase enzyme, 250 µL of 1% w/v β-cyclodextrin (β-CD) dissolved in 50 mM potassium phosphate buffer pH 7.0, and 150 µL of 50 mM potassium phosphate buffer pH 7.0 were incubated at 40°C for 10 min. For immobilized MAG1, 3 beads of immobilized MAG1 were added in 250 µL of 50 mM potassium phosphate buffer pH 7.0 and 250 µL of 1% w/v β-CD before incubation at 40°C for 10 min. After incubation, the reaction solution was added by 500 µL DNS reagent and boiled for 5 min. The mixture was cooled to room temperature, and the absorbance at 575 nm was read relative to a maltose standard using a UV-VIS spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 µmol maltose per minute under the optimum conditions.

Morphological and chemical composition analysis

Immobilized maltogenic amylase in alginate and alginate-chitosan beads were freeze-dried at −70 °C under vacuum for 4 h and sputter-coated with platinum before examination using scanning electron microscopy (SEM) (JSM-6390LV, JEOL Ltd., Tokyo, Japan). The chemical compositions of immobilized MAG1 were observed using Fourier-transform infrared (FTIR) spectroscopy (PerkinElmer, Ohio, USA). Potassium bromide (KBr) powder was mixed with the freeze-dried beads and compressed into discs. The absorbance percentage was observed at 500–4000 cm^{-1}.

Stability analysis

The thermal stability was evaluated by pre-incubating the immobilized MAG1 into alginate and alginate-chitosan beads in 50 mM potassium phosphate buffer (pH 7.0) without substrate at different temperatures, 30 °C, 40 °C, 50 °C for 10 min until 50 min [8]. The relative enzymatic activity was determined by a DNS assay with MAG1 immobilized into alginate beads as control.

Enzyme leaching analysis

Developed beads were incubated in 50 mM potassium phosphate buffer (pH 7) for 30 min intervals for each cycle. Enzyme leaching analysis was measured by Bradford assay [9] with Bovine serum albumin (BSA) as a standard and absorbance reading was measured at a wavelength of 595 nm using a UV-VIS spectrophotometer. The enzymatic activity was determined and the percentage of leaching was calculated by using Equation (1) [10].

$$L\,(\%) = \frac{Free\ enzyme\ activity}{Enzyme\ activity\ in\ beads\ used\ in\ leaching\ analysis} \times 100\% \quad (1)$$

Reusability of immobilized MAG1 enzyme

The reusability of the immobilized enzyme was evaluated by the repeated use in the β-CD hydrolysis reaction. After each cycle of reaction, the immobilized MAG1 were washed with distilled water and used for the next reaction using a fresh reaction medium. The enzyme activity for the first cycle was set as 100% and measured as relative activity.

Results and discussion

Optimization of alginate-chitosan beads

Factors affecting the formation of alginate core such as sodium alginate concentration, calcium chloride concentration, and curing time have been optimized previously by Nawawi, et al. [6]. In immobilizing MAG1 into alginate-chitosan beads, the concentration of chitosan was optimized as chitosan had a significant protective role against external damages in immobilized MAG1 with a layer-by-layer approach. This sustained the permeation of small molecules, while it trapped larger molecules. Figure 1 shows the best concentration of chitosan shell was 3% w/v. The 3% chitosan developed the suitable membrane which allows substrate β-CD to diffuse through and prevent enzyme leaching from the beads. When the chitosan concentration was increased over 3% w/v, β-CD diffusion was limited due to the compact structure of the chitosan membrane, thus reducing enzyme activity. In contrast, if chitosan concentration was reduced below 3% w/v, there were more porous structures formed on the coated layer of chitosan causing the enzyme to easily leach out. Few studies of enzyme immobilization reported that 2.5%, 3%, and 2% (w/v) of chitosan were the optimum concentration for enzyme laccase, α-amylase, and metalloprotease, respectively [11-13].

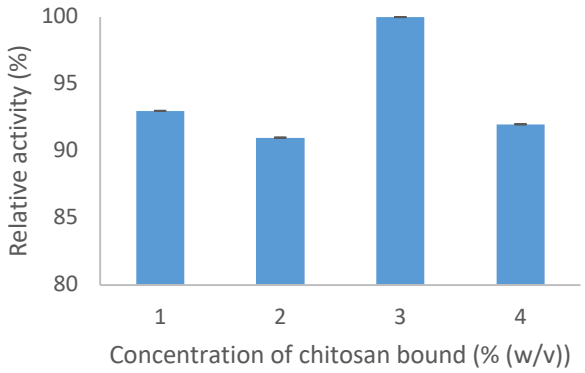

Figure 1. Optimization of chitosan concentration on immobilized MAG1 on 2.5% (w/v) sodium alginate, 0.6% (w/v) calcium chloride and 30 min curing time. Relative activity was calculated by enzyme assay and the error bars represented the standard deviation of triplicate experiments.

Physical characterization and chemical composition analysis

The cross-section of alginate and alginate-chitosan beads were characterized by scanning electron microscopy as represented in Figure 2(a) and Figure 2(b) respectively. Experimentally, MAG1 was entrapped and presented in alginate core as illustrated in Figure 2(a) and Figure 2(b). Based on the morphology, the structure of alginate beads [Figure 2(a)] was observed to be rough and porous that similarly described by Nawawi, et al. [6]. This morphology structure was observed evenly distributed on the whole bead. In contrast, alginate-chitosan beads [Figure 2(b)] developed two distinct structures consisting of a rough outer structure of chitosan layer and porous structure of alginate core, similarly as viewed by Dhillon, et al. [14]. The distinct morphology structure of alginate-chitosan beads proved the successful coating of chitosan on the outer surface of alginate beads.

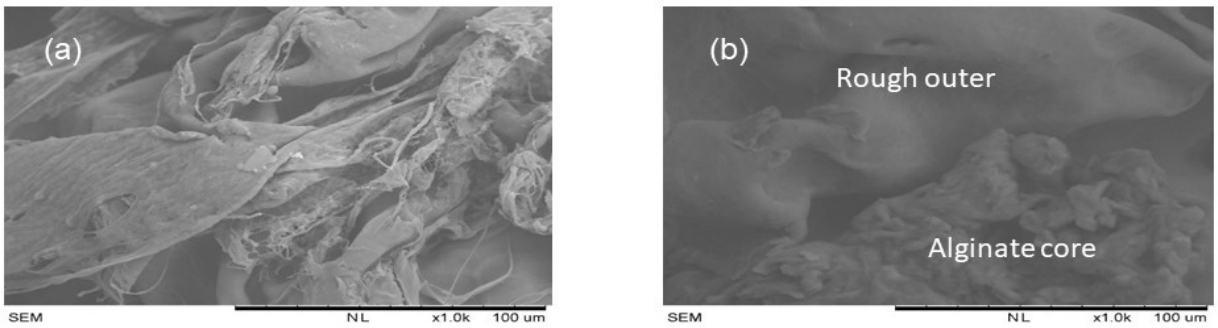

Figure 2. Morphology of immobilized (a) MAG1 in alginate beads for x1000 magnification and (b) immobilized MAG1 in alginate-chitosan beads for x1000 magnification

The chemical compositions of entrapped MAG1 in alginate and alginate-chitosan beads were observed using FTIR analysis as shown in Figure 3. The complexed material of alginate-chitosan has a more intense band at about 3294.42 cm^{-1} and 3327.21 cm^{-1} which is caused by intermolecular bond formation between –OH and $–NH_2$ group in chitosan and –C=O and –OH groups of calcium alginate. This band indicates an increase in the concentration of NH_2 groups which may be due to less enzyme leakage and more enzyme entrapped in the beads. However, alginate-chitosan beads have a lower peak compared to alginate beads due to the unavailability of –OH and $–NH_2$ groups of chitosan that are unable to form a bond with calcium alginate. These results corroborate those found by Kulig, et al. [15] and Pereira, et al. [16].

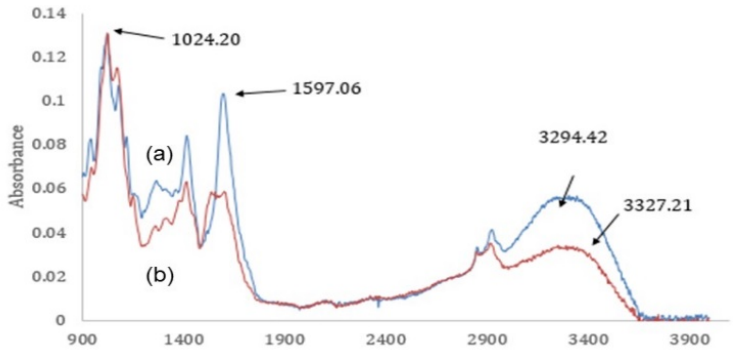

Figure 3. Fourier-transform infrared (FTIR) spectroscopy of immobilized MAG1 in (a) alginate, labeled in blue, and (b) alginate-chitosan, labeled in red.

The reading of 1597.06 cm^{-1} for alginate bead is also within the absorption range which is usually seen between 1649 cm^{-1} and 1652 cm^{-1} and 1558–1598 cm^{-1}, corresponding to C-O stretching (amide I) and N-H bending (amide II) respectively, which was confirmed by Smitha, et al. [17], Batista, et al. [18], Brena, et al. [19], Leceta, et al. [20]. Thus, the overlapping of the band created a strong peak which was 1597.06 cm^{-1}. As chitosan was not involved in cross-linking with the alginate beads, the peak does not exist within the range of 1649 cm^{-1}, 1652 cm^{-1}, and 1558– 1598 cm^{-1} for alginate-chitosan beads.

It was identified that there was a spectrum of the produced biocatalytic immobilization system that contains signals characteristic for MAG1 which was successfully deposited on the surface of the support material. This was proven by the presence of a peak at 1415.75 cm^{-1} in alginate beads, which indicated that there were changes in the intensity of signals, which is also supported by Zdarta, et al. [21]. Besides, absorption bands were observed at 1024.20 cm^{-1} shows the characteristics of the polysaccharide structure [17, 18].

Thermal stability analysis

Generally, the immobilized MAG1 was more stable due to a higher resistance against thermal denaturation compared to the free MAG1. Experimental results in Figure 4 confirmed that the immobilization process namely entrapment and encapsulation improved the thermal stability of MAG1. Thermal denaturation occurred at the high temperature and caused unit activity to drop. It was seen that alginate-chitosan beads retained the highest activity at 30 °C, 40 °C, and 50 °C after 50 min of incubation, with relative activity of 88%, 85%, and 78% respectively. Similarly, immobilized inulinase which immobilized onto alginate-chitosan beads had retained 86.5% activity at 50°C even after 4 hr [22]. The increased stability observed with alginate-chitosan beads was probably due to the solid core that reduces the mobility of the enzyme, thus shielding it from the effects of the environment. Besides, alginate-chitosan beads have a protective layer that can protect MAG1 from high temperatures and also decrease the conformational flexibility for the immobilized enzyme [23]. The coating can reduce the permeability of the beads, therefore increasing the stability under harsh conditions, such as high temperature [24, 25]. Alginate beads retained lesser relative activity which was 83%, 75%, and 60% at temperatures 30°C, 40°C, and 50°C after 50 min, respectively as compared to alginate-chitosan beads. As there was no boundary coated layer near the surface of alginate beads, the substrate concentration at the surface may differ from the substrate concentration in the bulk fluid, which potentially does not withstand higher temperatures for a longer period. The free MAG1 was inactive and decreased sharply as observed in Figures 5, 6, and 7 which showed relative activity of 48%, 43%, and 40% at temperatures 30°C, 40°C, and 50°C, respectively after 50 min. Studies had discussed that immobilized MAG1 increased the stability of enzyme compared to free MAG1 because the multipoint attachment led to an improved denaturation resistance of the immobilized MAG1 against a range of temperature changes. Hence, the immobilization method preserved the MAG1 enzymatic activity.

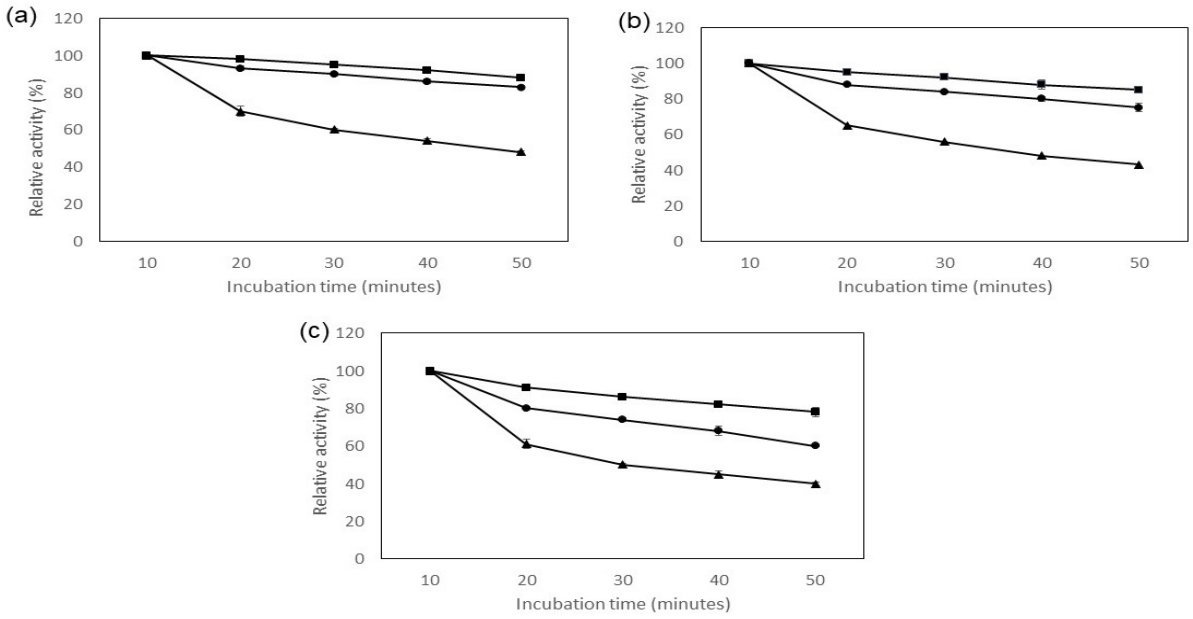

Figure 4. Temperature stability of immobilized MAG1 alginate (●), alginate-chitosan (■), and free MAG1 (▲) at (a) 30°C, (b) 40°C, and (c) 50°C. Relative activity was calculated by enzyme assay. The error bars represented the standard deviation of triplicate experiments.

Enzyme leaching analysis

MAG1 was encapsulated within alginate-chitosan beads to create an intracellular environment for the enzymes, preventing them from leaching out or coming into direct contact with the external environment. However, there was a possibility that enzyme leaching occurred on immobilized MAG1 after the 8th time

of repeated usage. The leaching analysis of immobilized MAG1 after the 8th cycle for each interval of 30 min for alginate and alginate-chitosan beads is shown in Figure 5. For MAG1 entrapped in alginate beads, leaching activity ranged from 12-35%. The initial burst leaching of enzymes might have been caused by the leaching of enzymes near the bead surface. Alginate at the surface has a very low content of negative charges and cannot interact strongly either with calcium or positively charged chitosan [26]. The leaching of encapsulated alginate coated with chitosan was quite low in the range of 7-21%. There was a clear decline in the leaching of encapsulated enzymes in alginate-chitosan beads, enhancing the exchange of sodium ions from glucuronic acid with the divalent cations during gelation [27]. This resulted in a denser matrix structure and a reduction in leaching. The chitosan-coated could reduce the pore size of the alginate gel matrix and improve the thickness of the gel membrane between the amine group and the carboxyl group of alginate [28]. Similarly in a study, the encapsulated protease onto calcium alginate-chitosan beads and calcium alginate-xanthan gum beads had a low leaching percentage of 8.1% and 6.2% [10].

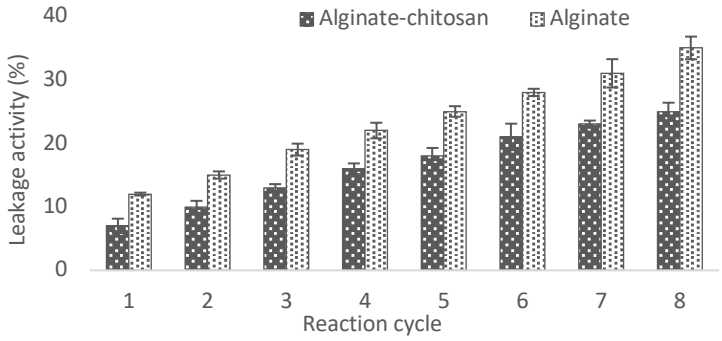

Figure 5. Leaching analysis of immobilized MAG1 after 8th cycle for each interval of 30 min alginate (●) and alginate-chitosan (■). The error bars represented the standard deviation of triplicate experiments.

Reusability

Figure 6 shows the relative activities of the alginate beads and alginate-chitosan beads for 8 consecutive usages. The free MAG1 can be used only once due to poor enzyme recovery and poor stability while immobilized MAG1 can be used up at least 8 times. MAG1 in alginate-chitosan beads retained 80% relative activity while MAG1 in alginate beads retained 70% relative activity. As observed, immobilized alginate beads have retained lesser enzymatic activity which was 10% than that of immobilized alginate-chitosan beads. This is because the core and shell of the structure of the alginate-chitosan bead prevent the motility of the enzyme out of the immobilized cell and thus enzyme leakage is avoided.

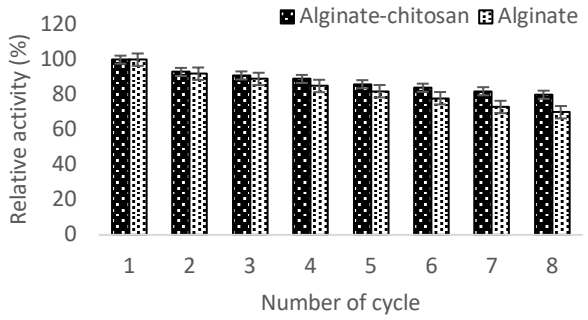

Figure 6. Reusability of immobilized MAG1. The error bars represented the standard deviation of triplicate experiments.

Similar results were observed from other immobilization studies where alginate beads were coated with chitosan to prevent enzyme leakage by using a different enzyme [22, 29]. Hence, it reveals that immobilized MAG1 has good operational stability. On the study of immobilization of α-amylase onto the calcium alginate, it retained its activity after the 5th cycle [30]. As reported by Abd Rahim, et al. [31], α-amylase which was immobilized on calcium alginate had retained 51.77 % activity after the 7th cycle.

Conclusions

Encapsulation represents the best alternative to preserve MAG1 enzymatic activity for MAG1 immobilization. Chitosan and sodium alginate are used due to their biocompatibility, renewable source, and abundance in nature. Alginate-chitosan beads could be used up to eight cycles of reaction and retained 80% relative activity with leakage activity of 7-21%. This research had successfully developed a novel chitosan-coated immobilized MAG1 in alginate. The coating provides a protective layer that creates a microenvironment and gives inclusion to immobilized MAG1, improving the enzyme stability and preventing enzyme leakage. This improves the enzymatic performance in terms of reusability and stability which are crucial factors for its application.

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

Funding statement

This study was financially supported by Universiti Teknologi Malaysia and the Ministry of Higher Education Malaysia's Fundamental Research Grant Scheme [Grant no. FRGS/1/2020/TK0/UTM/02/6].

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