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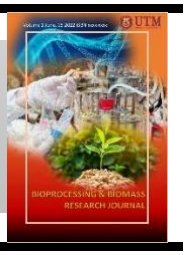

Research Article

Characterization of Maltogenic Amylase Activity Recovery: A Potential Approach for Improving Immobilization

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ABSTRACT

Cross-linked enzymes aggregate (CLEA) is a versatile carrier free-immobilization technique that has gained much attention in the development of biocatalyst technology. However, there is no precise and accurate method of using this technique that will lead to an expected outcome that meets the requirements of the industrial standard. Therefore, the objective of this study is to investigate the effect of a few methods of executing cross-linked enzyme aggregates approach using maltogenic amylase by measuring the activity recovery of the developed CLEA. Some factors that are considered in developing the methodologies are the interaction between cross-linkers and enzymes, size of the cross-linked enzyme aggregates and substrate diffusion. The addition of precipitant and the cross-linking agent steps has been manipulated and four different methodologies were developed. Based on the results, Method 2 showed the highest activity recovery (57.9%) whilst Method 4 gave the lowest activity recovery (15.7%). Method 2 is an improvised method that removed supernatant after centrifugation before proceeding to the cross-linking step. The characterization of cross-linked enzyme aggregates such as morphological characterization and Fourier Transform-Infrared Spectroscopy was also determined. In conclusion, the best and most productive preparation method was determined based on the highest activity recovery.

INTRODUCTION

Owing to the rise of development throughout the century, the advancement in enzyme-related reactions gives many benefits to various industries. With its high catalytic activities and substrate specificities, the use of enzymes in many wide ranges of applications have increased (Singh *et al.*, 2024; Lewis *et al.*, 2023; Yamaguchi *et al.*, 2018). Despite contributing to different types of industries, enzymes have their own limitations which limit their uses in industries. Without wanting to waste its efficacy, scientists have come up with a solution, enzyme immobilization, where the enzyme can be used efficiently while producing good quality end product (Cordero-Soto *et al.*, 2020; Chaturvedi *et al.*, 2021).

Maltogenic amylase (EC 3.2.1.133) is one of the amylolytic enzymes other than cyclodextrin glucanotransferase (EC 2.4.1.19), cyclodextrinase (EC 3.2.1.54) and pullulanase (EC 3.2.1.4). It roots from the α-amylase (EC 3.2.1.1) group which is one of the enzymes that are widely known for its great capability in the industry of starch hydrolysis (Wang *et al.* 2022; Guo *et al.*, 2021). Maltogenic amylase belongs to glycosyl hydrolase Family 13 (GH13). It has a distinctive multi-substrate specificity towards pullulan, cyclodextrins (CD) and starch (Abdul Manas *et al.*, 2014). According to previous study by Abdul Manas *et al.*, (2014), the expression, purification, and characterization of maltogenic amylase from locally isolated *Bacillus lehensis* G1 is used as the biocatalyst to facilitate the process of β-CD hydrolysis.

Cross-linked enzyme aggregates (CLEA) immobilization is a technique developed and improvised from many

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methodologies which have the same goal of creating enzymes with excellent yields while minimizing any unnecessary loss (Jun *et al.* 2019; Bian *et al.*, 2019). CLEA has received wide interest from a variety of industries as its main aim is to provide the simplest and lowest cost method while maximizing its efficiency. It involves two preparation steps of precipitation and cross-linking of the enzymes, many researchers have different opinions in preparing the enzyme prior to its CLEA formation (Chen *et al.*, 2023; Alves *et al.*, 2021). Since there is no definite approach of constructing CLEA, researchers have implemented different methods to enhance the efficiency of the immobilized enzymes. The type of enzyme and techniques of execution will lead to different outcomes in the study thus eventually generating different production costs (George *et al.*, 2023; Bilal *et al.*, 2021; Shakerian *et al.* 2020). Nonetheless, CLEA also has many possible factors that may be affecting the result hence the reason why optimum conditions for CLEA preparation are highly compulsory. There are many factors that affect CLEA formation such as the type and concentration of the precipitants, type and concentration of the cross-linkers, cross-linking time, and enzymes proportion. This is important in producing a thermostable enzyme and high yield (Ahrari *et al.*, 2023; Ashjari *et al.*, 2020; Bolivar *et al.*, 2022).

The most common method for CLEA is by the addition of precipitant to aggregate the enzyme prior to the inclusion of cross-linker to form immobilized enzymes. The mixture is believed to still have active precipitants that can interrupt the cross-linking process afterwards. This situation has led to the question of whether higher enzyme activity can be yielded if this problem is prevented. Thus, the aim of this study is to improve the preparation of maltogenic amylase cross-linked enzyme aggregates (Mag1-CLEA) by improvising the general strategy. Each method exhibits different own properties and characteristics while having similarities in terms of function and substrate.

MATERIALS AND METHOD

Expression and purification of maltogenic amylase from *Bacillus lehensis* G1 (Mag1)

Maltogenic amylase (Mag1) has been previously isolated from *Bacillus lehensis* G1 and cloned into pET-21a (+) vector system (Novagen) before kept in *Escherichia coli* JM109 which then were re-transformed into *E. coli* BL21 (DE3) (Novagen Merck KGaA, Darmstadt, Germany) for protein expression (Abdul Manas *et al.*, 2014). Expression of Mag1 from *Bacillus lehensis* G1 was carried out by using the methodology of Pachelles (2013). *E. coli* BL21 (DE3) carrying the recombinant plasmid was cultivated overnight in sterile Luria-Bertani agar plate. Then, grown *E. coli* strains were cultured overnight in sterile Luria-Bertani (LB) broth medium containing 50 µg/mL ampicillin at 37 °C with shaking at 200 rpm in a rotary shaker. Using the ratio of 1:100, 10 mL of starter culture was grown in 1000 mL of sub-culture broth until the OD600 reached 0.4 – 0.6. Next, induction was performed using 50 mM of isopropyl ß-D-1-thiogalactopyranoside (IPTG) and further incubated at 30 °C with shaking at 200 rpm in the rotary shaker for 16 h.

Purification of recombinant Mag1 was carried out using a procedure explained by Abdul Manas *et al.*, (2014). Using AKTAprime plus purification system (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and HisTrap HP column (GE Healthcare, Little Chalfont, Buckinghamshire, UK), the purification was carried out by washing the system and column using the filtered distilled water. Then, equilibration of the system and column was done at a flow rate of 5 mL/min with 5 column volume (CV) of a binding buffer. Unbound protein was removed using a binding solution at a flow rate of 1 mL/min after the protein sample was introduced into the column. The protein was then eluted using a gradient elution method using an elution solution containing imidazole (from 0 M to 0.5 M of imidazole). All eluted protein samples such as crude, flow-through, binding and pure Mag1 solution were collected by a fraction collector.

Enzyme assay

The purified Mag1 was observed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine the purity and molecular weight of the enzyme. Protein concentration assay was determined by using Bradford assay. A total of 5 µL of protein samples and bovine serum albumin (BSA) standards were dispensed into a flat-bottomed 96 well plate. The absorbance was measured at a wavelength of 595 nm and the standard curve was plotted with the absorbance versus BSA concentration.

Enzyme assay was performed to determine the enzyme activity using 3, 5 – dinitrosalicylic acid, (DNS) method. A mixture of 100 µL Mag1 solution (1.0 mg/mL), 250 µL of 1% (w/v) β-CD dissolved in potassium phosphate buffer (pH 7, 50 mM) and 150 µL potassium phosphate buffer (pH 7, 50 mM) was incubated at 40 °C for 10 min. Then, 500 µL of DNS reagent was added into the mixture and incubated in boiling water for 5 min to stop the reaction. The absorbance (A540) was read relative to a maltose standard curve.

Preparation of Mag1-CLEA

Four different methodologies of constructing Mag1-CLEA were carried out to determine the best approach that will yield the highest reducing sugars. In the first method (**Method 1**), Mag1-CLEA was prepared as described by Nawawi *et al.*, (2020). The precipitant (ammonium sulphate) 80% (w/v) was added into 1.0 mg/mL of purified Mag1 under the agitation of 200 rpm for 1 h at 4 °C. After 1 h of orbital shaking, 0.25% (w/v) chitosan cross-linker was directly added to the mixture and the solution was shaken for another 1.5 h. The mixture was also added with 50 mM potassium phosphate buffer (pH 7.0). Then, the insoluble Mag1-CLEA was separated from its supernatant by centrifugation at 10,000 rpm for 2 min. Mag1-CLEA was then washed three times using the same buffer before it was suspended in the buffer and stored at 4 °C prior to use.

For the second method (**Method 2**), the procedure was similar with **Method 1** except for the additional step of centrifugation after the precipitation process. After the orbital shaking, the precipitated product was collected and the insoluble Mag1-CLEAs was separated by centrifugation. Then, the chitosan solution was added and shaken and the separating process of the insoluble Mag1-CLEAs by centrifugation was done before it was washed three times using the same buffer. Next, it was suspended in the buffer and stored at 4 °C prior to use.

For the third method (**Method 3**), the procedure of Method 2 was repeated except that the centrifugation step after the precipitation process was replaced with sedimentation. After 1 h of precipitation, the solution was shaken by vortex before it was let to settle down. Then, the

supernatant was discarded carefully to collect the precipitated enzymes. Later, chitosan was added, and the same protocol was proceeded as in previous methods.

In the final method (**Method 4**), 80% (w/v) ammonium sulphate precipitant, 50 mM potassium phosphate buffer (pH 7.0) and 0.25% (w/v) chitosan were simultaneously added into 1.0 mg/mL of purified Mag1 under the agitation of 200 rpm at 4 °C for 2.5 h. Next, the insoluble Mag1-CLEAs was separated by centrifugation at 10,000 rpm for 2 min. Mag1-CLEAs was then washed three times using the same buffer before it was suspended in the buffer and stored at 4 °C prior to use.

Physical characterization of Mag1-CLEA

The morphological analysis of Mag1-CLEA was performed using JSM-6390LV scanning electron microscope (SEM) (JEOL Ltd., Tokyo, Japan). It was used to analyze the surface morphology of the immobilized Mag1. The samples were dried using Eppendorf Concentrator Plus (Protein Concentrator Plus, 5305) prior to the analysis. The SEM images of Mag1-CLEA was taken at different magnifications.

The chemical composition of the immobilized enzyme was examined using Fourier Transform-Infrared spectroscopy (FT-IR) in a transmittance mode with a Perkin-Elmer Spectrum One infrared spectrometer (PerkinElmer, Ohio, USA). For Mag1 analysis, the FT-IR spectra in the range of 500-4000 cm^{-1} were recorded in order to investigate the functional groups present in the samples (Fang *et al.*, 2016; Nawawi *et al.*, 2020).

RESULTS AND DISCUSSION

Expression and purification of maltogenic amylase from *Bacillus lehensis* G1 (Mag1)

Fractions from affinity chromatography that contained Mag1 were collected, pooled, and concentrated to 8 mL (**Figure 1**). The purification was performed for 80 min and fractions were collected between 55 – 80 min.

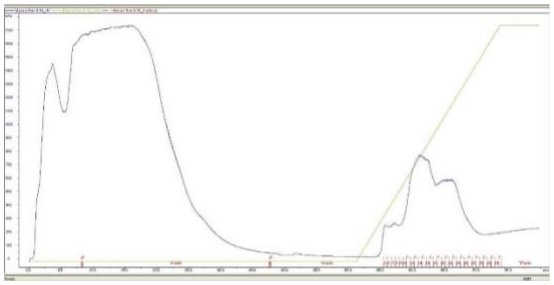

Figure 1 Affinity purification chromatogram of the recombinant protein in *E. coli* using Ni^{2+} affinity chromatography purification.

Table 1 shows the summarization of Mag1 purification table. In crude samples, the enzyme activity was 36.7 U/mL with a protein concentration of 7.8 mg/mL. After purification, the yield of Mag1 recovered was 50.9 % and its specific activity increased 2.2-fold compared to the crude fraction. Furthermore, the purified Mag1 was also resolved as a single band with an estimated size of 68 kDa, as shown in **Figure 2**.

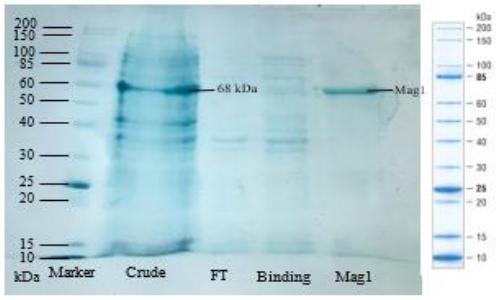

Figure 2 SDS-PAGE of crude and purified Mag1. The purified Mag1 was the eluted Mag1 with Ni^{2+} affinity chromatography purification whilst the crude was Mag1 lysate before enzyme purification. The size of Mag1 is approximately 68 kDa.

Preparation of cross-linked enzyme aggregates maltogenic amylase using different methods

In **Method 1** (standard method), the highest Mag1-CLEAs activity recovery was 63.7% while the lowest is 6.7%, with their first wash enzyme activity of 0.1 U/mL and 0.1 U/mL, respectively (**Figure 3**).

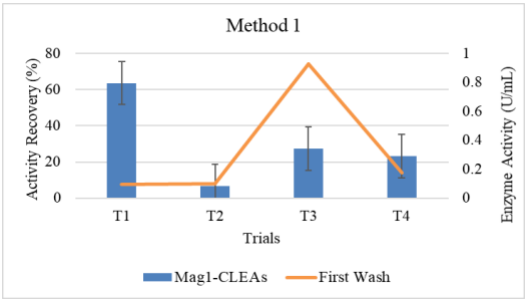

Figure 3 Activity recovery of Mag1-CLEA for Method 1. Error bars represent the mean standard deviations.

Next, **Method 2** was expected to have a higher recovery activity compared to Method 1 since all existing precipitants were discarded before the cross-linking process. As shown in **Figure 4**, the highest activity recovery of Method 2 gave the results of 65.8% (Trial 1). Meanwhile, the lowest is Trial 3 which produces only 22.7 % of Mag1-CLEA activity. Thus, it can be concluded that Method 2 has higher activity recovery compared to Method 1. Low activity recovery of Method 2 in Trial 3 could be due to low precipitation activity. Some of the enzymes may have been discarded together with unreacted precipitants thus resulting in low cross-linking and activity recovery. The second reason could be because of the broken structure of Mag-CLEA after the centrifugation step. Even though the breaking process was done carefully, Mag1-CLEA structure could still be very tender and sensitive. Hence, this step can result in broken Mag1-CLEA structure before the cross-linking process.

Table 1 Purification table of Mag1

Fractions	Volume (mL)	Enzyme Activity (U/mL)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Purification fold
Crude	40.0	36.7	1469.1	311.3	4.7	100.0	1.0
Purified	8.0	93.5	747.9	70.7	10.9	50.9	2.2

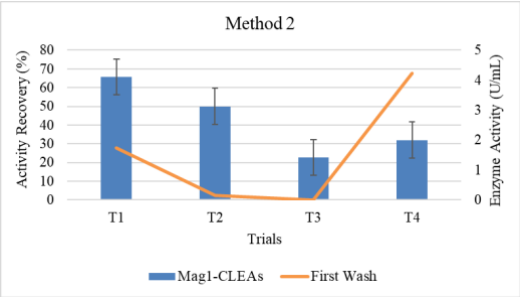

Figure 4 Activity recovery of Mag1-CLEA for Method 2. Error bars represent the mean standard deviations.

Next, **Method 3** also requires the supernatant to be removed after the precipitation process but using the technique of sedimentation without any centrifugation. Method 3 is expected to have a lower activity recovery of Mag1-CLEA compared to Method 2. Based on **Figure 5**, the highest activity recovery is at Trial 3 which is 90.2% while the lowest is at Trial 2 of 24.4%. The difference between all trials is very significant. In this method, low recovery of enzyme activity is could be mainly from the loss of precipitated enzyme due to the supernatant removal before the cross-linking process.

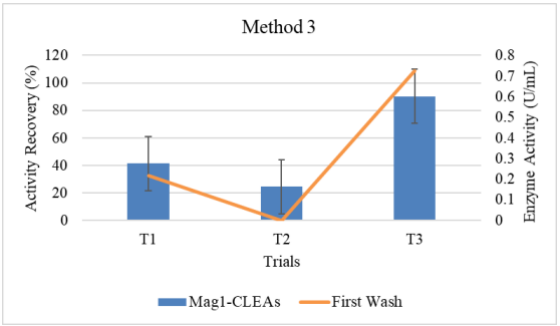

Figure 5 Activity recovery of Mag1-CLEA for Method 3. Error bars represent the mean standard deviations.

Lastly, **Method 4** requires pure Mag1 to be added simultaneously with the solution of precipitating agent and cross-linker, which was expected to give the lowest Mag1 production among all methods in this study. This is due to the first step in constructing CLEA which is the precipitation process has been disturbed with the presence of cross-linker agent in the same solution. The precipitating agent could not properly react with the pure enzyme (Jailani *et al.* 2022). When the precipitation process is disrupted, the amount of precipitated enzymes will be low thus interrupting the formation of CLEA. It can be seen that the highest recovery of Mag1-CLEAs is 106.0% while the lowest is 14.7% (**Figure 6**). Although all these trials were performed using the same method, there are also existing factors that made these differences. The low Mag1-CLEA recovery activity could be due to the disruption of cross-linking agent during precipitation process which resulted in low production of precipitated enzymes and hence cross-linking products.

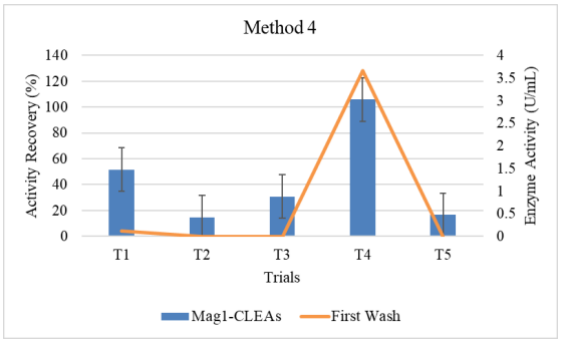

Figure 6 Activity recovery of Mag1-CLEA for Method 4. Error bars represent the mean standard deviations.

Comparison of methodologies for Mag1-CLEA development

The activity recovery for all methodologies was summarized and compared in **Figure 7**. The descending order of activity recovery is found to be from Method 2, Method 3, Method 1 and finally Method 4.

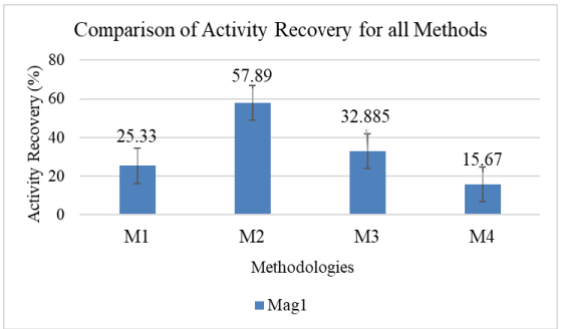

Figure 7 Activity recovery for all methods producing Mag1-CLEA. Error bars represent the standard deviations.

The highest activity recovery is found to be from Mag1-CLEA developed using Method 2. The precipitant and cross-linker were added separately which resulted in a complete process of precipitation and cross-linking. The addition process of centrifugation after the precipitation was proven to not be a factor that can cause the structural changes that increase the substrate diffusion limitation, but in fact, aided the cross-linking process. The

compacted enzyme after centrifugation was fragmented before the cross-linking process, which eases the cross-linker, chitosan to cross-link the enzyme to be the Mag1-CLEA. Hence, the reason Method 2 gave the highest activity recovery among the procedures.

The second highest recovery of Mag1-CLEA is from Method 3. In this method, the supernatant was discarded manually and carefully after the precipitation process. This step removed any remaining precipitants from the precipitated enzymes without affecting the formation of enzyme aggregates during the cross-linking process, which is similar to Method 2. Since the supernatant was discarded manually, the aggregates were found to be larger than CLEA particles in Method 2. However, the precipitated enzymes after the precipitation were vaguely visible and hence it was quite difficult to remove the supernatant. This has resulted in the precipitated enzymes accidentally discarded along with the precipitating agent in the suspended solution. The remaining precipitated enzymes were cross-linked during the cross-linking process and have a larger enzyme structure because it is not compressed and compacted. The large structural conformation of Mag1-CLEA has low substrate diffusion limitation and therefore increased the substrate conversion into products.

Method 1 which is the standard method has the third highest activity recovery. The highlight of this method is the cross-linking agent was added to the mixture of enzymes and precipitants without removing the supernatant from the precipitation process. During the addition of cross-linkers, the carry-on supernatant solution remained in the solution, which may interrupt the cross-linking process hence affecting the activity recovery.

Last but not least, Method 4 has the lowest activity recovery in suspended solution among all the procedures proposed in this study. This is because the precipitant and cross-linking agent were added simultaneously to construct Mag1-CLEA. When both precipitant and cross-linker were added together, they might interact and interrupted with each other's functions as they acted on the same enzymes at the same time and hence both processes could not be performed properly and completely (Jailani *et al.*, 2022). This is why the fourth method has the lowest activity recovery.

Physical characterization of Mag1-CLEA

Morphological analysis

Figure 8 showed SEM images of Mag1-CLEA developed using different methodologies (Method 1 – 4). According to the SEM images, Mag1-CLEA appeared as a fine and uniform-structured in a small size of aggregates. This is in accordance with the findings of Nawawi *et al.*, (2020).

SEM images for Method 2 (Figure 8c and d) showed the Mag1-CLEA structures particles with a very wide gap among the aggregates. Next, for Mag1-CLEA from Method 3 (Figure 8e and f), it can be seen that there were aggregates with a huge gap of pores between the particles. These SEM images validated the activity recovery results of Mag1-CLEA from Method 2 and 3 which showed the highest and second highest catalytic activity recovery, which are 57.9% and 32.9%, respectively.

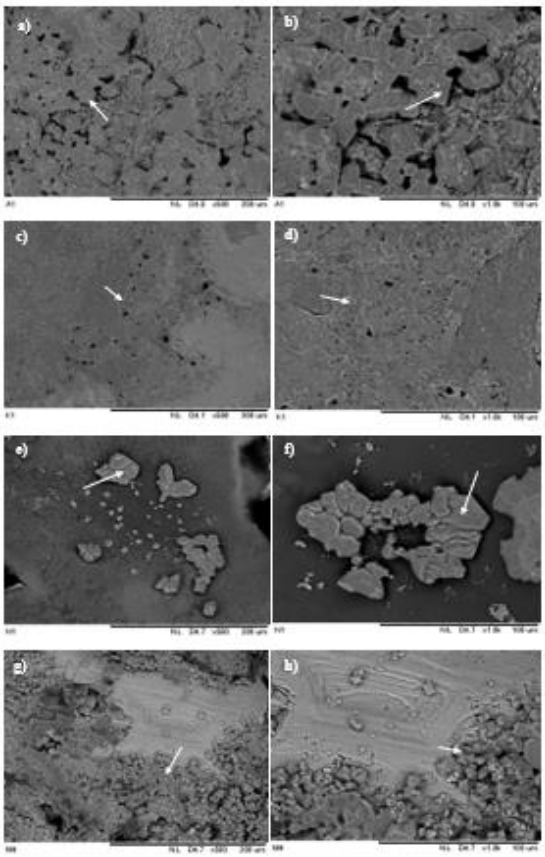

Figure 8 Scanning electron microscope images of Mag1-CLEA developed using different methods. (a) Method 1 at 500x, (b) Method 1 at 1000x, (c) Method 2 at 500x, (d) Method 2 at 1000x, (e) Method 3 at 500x, (f) Method 3 at 1000x, (g) Method 4 at 500x and (h) Method 4 at 1000x. Bar represents 10µm in all figures. The arrow indicated the enzyme aggregates of CLEA.

SEM images from Method 4 (Figure 8g and h) showed a very compacted structure with less pores between aggregates, which indicated intense limitation of substrate diffusion or substrate accessibility problem, that decreases their catalytic activity. This is the same as the structure of Mag1-CLEA from Method 1 (Figure 8a and b) which showed compacted structure with a low pore gap between aggregates.

Chemical composition analysis

The chemical compositions of chitosan and Mag1-CLEAs developed using four different methodologies were performed and observed using Fourier Transform-Infrared spectroscopy (FT-IR) in a transmittance mode with a Perkin-Elmer Spectrum One infrared spectrometer (PerkinElmer, Ohio, USA) (**Figure 9**). The peaks located within the range of 1640 cm^{-1} and 1530 cm^{-1} for all Mag1-CLEA. According to Carbonaro and Nucara, (2010), the amide I area (C=O stretching vibration) and the amide II region (N-H bending and C-N stretching) were represented by these bands, respectively, hence verified the presence of Mag1 in the samples. These methods were also used by Ernest *et al.*, (2013) and Nawawi *et al.*, (2020) for their observations of amylase samples contained in saliva and Mag1-CLEAs-Tween 20 and Mag1-p-CLEAs, respectively.

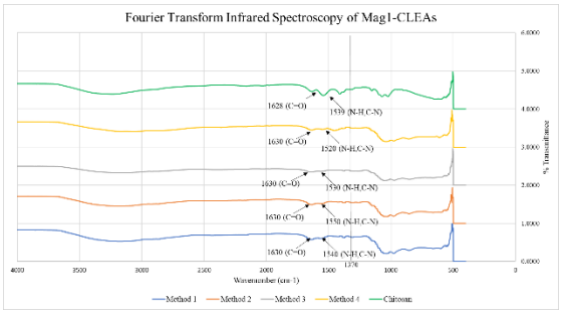

Figure 9 Fourier-transform spectroscopy of chitosan and Mag1-CLEAs developed using Method 1, Method 2, Method 3, and Method 4.

Furthermore, the region of amide I and II can also be used as an indicator for enzymes conformational changes detection (Rabolt *et al.*, 1998; Soleimani, Khani and Najafzadeh, 2012). Based on the position of amide I, it shifted from 1628 cm^{-1} in the cross-linker, chitosan to 1630 cm^{-1} for all four methods. Meanwhile, for amide II band, the shifts were significantly because for chitosan the peak is at 1539 cm^{-1} and shifted to 1520 cm^{-1}, 1530 cm^{-1}, 1550 cm^{-1} and 1540 cm^{-1} for Method 1, Method 2, Method 3, and Method 4, respectively. The structural alteration of Mag1-CLEA was revealed by these spectrum features and it in accordance with the different development techniques. Finally, 1018 cm^{-1} of wavenumbers of chitosan was detected which represented the C-OH stretch of alcohol of chitosan, where all Mag1-CLEAs did not have these bands (Nawawi *et al.*, 2020).

CONCLUSION

The improvised methodology of cross-linked maltogenic amylase immobilization is developed in this research. The best and most efficient preparation method is determined according to the highest recovery activity of the developed CLEA. From the result, the highest activity recovery is found from Method 2 followed by Method 3, Method 1 and finally Method 4. The morphological characterization concluded that the larger pore between the aggregates contributed to the higher activity recovery determination. This study showed that higher activity recovery of CLEA can be achieved by a simple modification of the process.

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