

Extraction of Low-methoxyl Pectin from Mango Peels for Immobilization of Cellulase

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Abstract

Cellulase has gained interest from an industrial standpoint due to its numerous applications. The immobilization of cellulase makes it possible to repeatedly employ it under various conditions and maintain its activity while using low-cost support materials such as pectin. This study determined the effects of calcium chloride concentration (2.0, 4.0, and 6.0% w/v) and curing time (10, 20, and 30 minutes) on the bead characteristics and enzyme activity of the immobilized cellulase. Low-methoxyl pectin from mango peels was used specifically as a support material. It was extracted using an acid-catalyzed simultaneous extraction and de-esterification technique over 65 hours at 50°C, pH 1.5, and precipitated with ethanol. The results show that cellulase can be immobilized with low-methoxyl pectin and that its immobilization efficiency in all CaCl₂ concentrations decreases as curing time increases, where a CaCl₂ concentration of 2.0% w/v at 10 minutes yields the best results, which had an immobilization efficiency of 93.14 ± 7.87 . The beads formed were not spherical, as they had a sphericity factor greater than 0.05, and their moisture content decreased as the CaCl₂ concentration increased from 90.92 ± 0.0023 to $87.90 \pm 0.0020\%$. The leakage, when tested at different periods (0, 30, 60, 90, and 120 minutes) under ambient temperature and pressure, was then determined to be relatively low—in a range of 13.3 ± 0.031 to $17.2 \pm 0.21\%$, where the initial burst of leakage may be a result of cellulase leakage on the bead surface.

Keywords: enzyme activity, immobilized cellulase, immobilization efficiency, leakage, low-methoxyl pectin

1. Introduction

Cellulase is a subject of scientific interest as it has a wide range of industrial applications, such as in the oil industry for biofuel production, the food and beverage industry for wine- and coffee-making, the chemical industry for some chemical reagents, the healthcare industry for biofilms and biosensors, the textile industry, the environmental industry for wastewater treatment, and the pulp and paper industry (Patel *et al.*, 2019). This enzyme facilitates the conversion of cellulose into sugars, yet its expense hampers industrial use, especially in the biofuel sector. Therefore, lowering enzyme cost is crucial in tackling the economic challenge of lignocellulosic biomass hydrolysis, which is a key challenge in the industry.

Since cellulases are proteins, they easily denature under certain conditions after extraction from their natural environment. This sensitivity to factors like temperature and pH makes their use costly and requires controlled conditions to maintain enzyme activity. Although there is a wide range of industrial applications for cellulase, reusability has not been achieved (Avci *et al.*, 2020). One way to address these problems could be to use enzyme immobilization to improve cellulase properties while reducing cost.

Free cellulases are soluble enzymes that are used in catalytic reactions but are not economically recoverable for reuse after such reactions. This limits their industrial application. Immobilized cellulase, on the other hand, are enzymes fixed in a specific space that maintain their catalytic capability for repeated and continuous use (Katchalski-Katzir, 1993). Immobilizing cellulase offers advantages over free cellulases: enzyme reuse is possible in bioreactors (1); simplified downstream procedures, such as recovery and purification steps, are eliminated (2); capital and operating costs are reduced due to continuous processing, diverse bioreactor setups, and decreased labor and overhead expenses (3); and enzyme stability and activity are improved in solution due to altered physicochemical and biological traits (4) (Zhang and Xing, 2011; Aguiéiras *et al.*, 2018; Avci *et al.*, 2020).

Soluble cellulase is single-use, while immobilized cellulase is cost-effective due to its reusability. Enzymes maintain their catalytic ability by binding to a support matrix via physical adsorption, ionic linkages, or covalent bonds for stability, with support material aligned to the immobilization process. Yoo *et al.* (2013) highlighted several objectives: activity maintenance, strong support, minimal changes, and low cost. Encapsulation minimizes enzyme-

support interaction while retaining high activity, but leakage and mass transfer challenges can arise. Controlling soft material breakdown is easier than desorption in adsorption. Optimal conditions mitigate leakage risks, necessitating gelling-capable support for cellulase encapsulation.

Polymeric materials like Ca-alginate, pectin, agar, polyacrylamide, and collagen are common matrices for enzyme immobilization. Solutions of enzymes and polymers mix before gelation, employing ionotropic gelation with ions like Ca^{2+} , Ba^{2+} , and Al^{3+} (Yeo *et al.*, 2001). Gel beads form in the presence of a cross-linking agent, determining bead size, rigidity, and stability. Calcium chloride is popular due to its cost-effectiveness and high encapsulation efficiency (86–100%). Natural polymers, like pectin among others, are favored for their renewability, affordability, biocompatibility, and biodegradability which preserves enzyme structure (Zdarta *et al.*, 2018). Pectin is also cost-effective, stable across pH 3.5–9.5, and thermally stable up to 100°C. Furthermore, it contains D-galacturonic acid, galacturonic acid residues, and reactive groups like carboxyl, amide, and ester (Contesini *et al.*, 2012; Sriamornsak, 2008; Sharma *et al.*, 2010).

Pectin, which is usually sourced from apples and citrus fruits, has experienced expanded manufacturing with the exploration of alternative raw materials. This benefits countries like the Philippines, which import pectin due to limited extraction technology and the unavailability of primary raw materials (Gragasin *et al.*, 2014). Abundant mangoes in the Philippines have been found to contain substantial pectin levels, extractable in a technically viable and cost-effective manner (Thakur *et al.*, 1997; Taboada and Siacor, 2013; Gragasini *et al.*, 2014). Gragasini *et al.* (2014) reported an average pectin yield of 21.65% from Philippine mango peels on a dry-weight basis.

Pectin is classified into high-methoxyl pectin (HMP) and low-methoxyl pectin (LMP) based on their degree of esterification (DE). Carboxyl groups on pectin chains can be esterified with methanol. HMP refers to pectin with over 50% of these groups esterified, while LMP is categorized as pectin with fewer than 50% esterification (Narasimman and Sethuraman, 2016).

LMP replaces HMP's sugar-gelling agent with divalent cations. It forms stable gels through ionotropic gelation, showing higher stability at low pH values and high temperatures. Pectin molecules precipitate, forming gels. "Egg-box" structures develop through bridging polyvalent cations, linking carboxyl groups in galacturonic acid chains (Ridley *et al.*, 2001). This process is

organized due to the region's linear shape, forming junction zones resembling an "egg-box," with cations as "eggs" and galacturonic acid as cavities.

Figure 1 depicts the "egg box model," showing how pectin and calcium ions form a 3D network (Braccini and Pérez, 2001). This enables cellulase encapsulation with LMP. Pectin has been used as a support material for enzyme encapsulation with lipase immobilization, as seen in Costas *et al.* (2008), and with glucosyltransferase, as seen in Contesini *et al.* (2012).

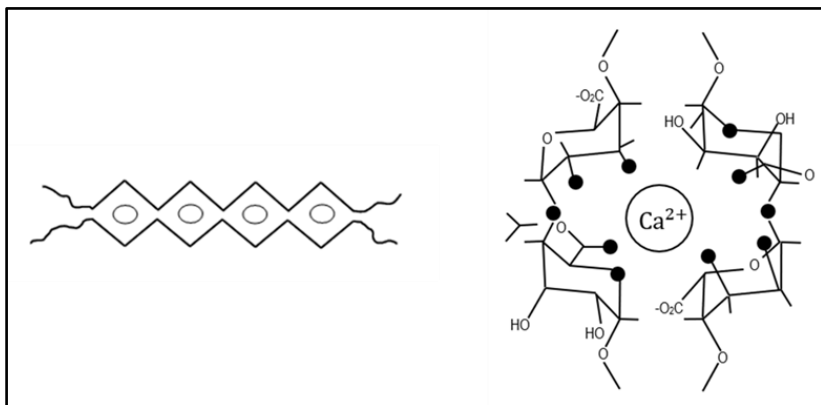


Figure 1. Schematic drawing of calcium binding and coordination of the "egg box model" (Braccini and Pérez, 2001) rendered using MS Word 2019

Cellulase's reactive functional groups, carboxylic acid, and amino groups allow immobilization where bonds form between these and the support material's functional groups, like hydrogen or peptide bonds (Lin *et al.*, 2017). Using cellulase's amino group to bond with support material is vital to maintain activity, as its carboxyl group is crucial for cellulose hydrolysis (Tata *et al.*, 2015; Zhang *et al.*, 2016). Therefore, a peptide bond could form between pectin and cellulase.

Figure 2 illustrates the pectin-cellulase immobilization mechanism. The carboxyl group in pectin bonds with cellulase's amine group through dehydration synthesis. Interactions between hydroxyl, amino, and carboxylic acid groups cause reactions, forming a peptide bond chain (Michaga *et al.*, 2021).

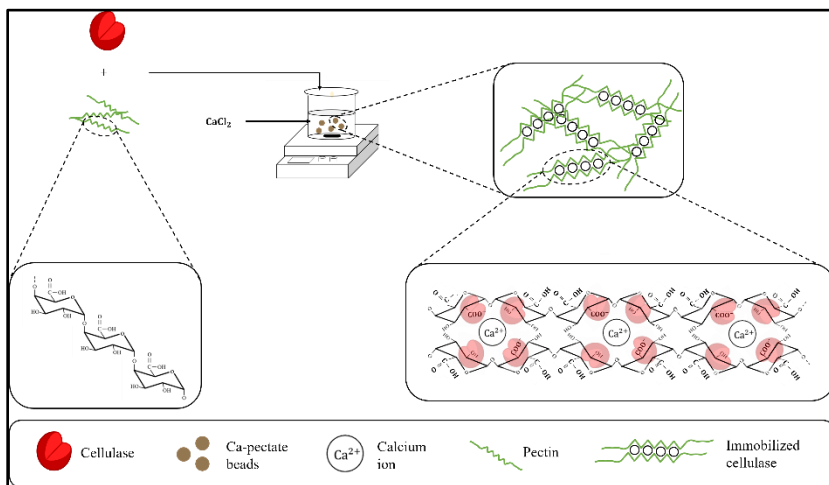


Figure 2. Expected mechanism of the immobilization of cellulase with pectin through encapsulation (Braccini and Pérez, 2001; Weng *et al.*, 2022) rendered using MS Word 2019

Among the many parameters that influences the immobilization of an enzyme, cross-linking agent concentration and curing time are vital considerations. Bead mechanical stability, size, encapsulation efficiency, yield, and enzyme activity depend on agent concentration. Bead stability hinges on support material, CaCl_2 concentrations, and curing time (Jamuna *et al.*, 1992). Curing time is crucial for gelation. It impacts bead stability, minimizes enzyme leakage and increases immobilization yield due to enhanced Ca^{2+} -polymer cross-linking. Prolonged times lead to unstable beads, reducing yield and increasing microbial leakage risk (Puguan *et al.*, 2015).

Overall, numerous aspects should be considered in the immobilization of an enzyme. Nonetheless, its success would greatly benefit various industrial applications such as bioethanol production and wastewater treatment. In bioethanol production, immobilization of cellulase offers a feasible strategy to improve enzyme stability, facilitate enzyme recovery, and enable repeated use across multiple hydrolysis cycles. This not only reduces overall enzyme cost but also enhances process sustainability. The biocompatibility and biodegradability of pectin make it particularly attractive for integration into environmentally conscious biorefinery operations. In wastewater treatment, immobilized cellulase can be employed to degrade cellulosic materials found in textile effluents, food processing waste, and pulp and paper industry discharges. Immobilized enzymes offer greater operational stability in harsh

environments, including variations in pH and temperature, and can be deployed in packed-bed or fluidized-bed bioreactors. Using immobilized cellulase may improve the breakdown of complex polysaccharides in wastewater, enhancing the efficiency of biological treatment processes and reducing solid waste accumulation.

In this study, cellulase from Cellic CTec 3 HS (Novozymes A/S, Bagsvaerd, Denmark) was immobilized using an organic support material through encapsulation. Although there are numerous studies in which cellulase was immobilized using a different support material (Avci *et al.*, 2020; Zhang *et al.*, 2016) or in which an enzyme was immobilized using pectin as a support material (Contesini *et al.*, 2012), there are currently no studies that have immobilized cellulase using LMP. Therefore, the present work i) immobilized cellulase using LMP extracted from mango peels, and ii) investigated the effects of CaCl_2 concentration (2.0%, 4.0%, and 6.0% w/v) and curing time (10, 20, 30 minutes) on the bead characteristics, enzyme activity, and the leakage of immobilized cellulase with the best immobilization efficiency.

2. Methodology

2.1 Materials

Mango peels were obtained from ProFoods International Corp., Mandaue City, Cebu. The enzyme, Cellic CTec3 HS, was provided by Novozymes, Bagsvaerd, Denmark. This enzyme formulation is a biocatalyst comprising 20–30% cellulase by weight. The following chemicals were used: 95% technical grade ethanol, 0.5 M hydrochloric acid (HCl), 70% ethanol, 1% (w/v) silver nitrate (AgNO_3), sodium chloride (99.9%; NaCl), calcium chloride (CaCl_2), trisodium citrate dihydrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$), citric acid ($\text{C}_6\text{H}_8\text{O}_7$), bovine serum albumin (BSA), phenolphthalein, sodium hydroxide (NaOH), D-galacturonic acid, carboxymethyl cellulose (CMC), sodium phosphate (Na_3PO_4), and citrate- NaH_2PO_4 buffer, which were obtained from Far Eastern Drug, Incorporated. Chemicals such as Folin-Ciocalteu reagent (2 M) and Bradford reagent were of analytical grade and sourced from Sigma-Aldrich (Singapore).

2.2 Extraction of Pectin

The extraction of pectin was based on the method proposed by Alcuirez *et al.* (2016) and Luarez *et al.* (2021), with some modifications. The simultaneous extraction and de-esterification (SED) was done to obtain LMP from mango peel powder that was pre-treated with ethanol to remove impurities like natural oils, which can interfere with the extraction of pectin. In addition, the obtained LMP was isolated and purified by alcohol precipitation.

2.2.1 Pre-treatment of Mango Peels

About 15 kg of mango peels were washed and scraped to remove any leftover flesh or pulp, blanched for five minutes at 95°C, then immersed in an ice bath to stop pectin-degrading enzymes from working. Sun drying was the method for the moist peels to become dry and brittle. Instead of using a blender (MB1002, MagicBullet, U.S.A.) at 10,000 rpm as performed by Luarez *et al.* (2021), the dried peels were milled in a Thomas Wiley® mill to generate mango peel powder, which was sieved through a 250-µm screen instead of the 180-µm screen by Alcuirez *et al.* (2016). This was done since the 250-µm screen generated mango peel powder with a particle size of around 250 µm or less.

The mango peel powder (MPP) was treated with 95% technical grade ethanol in a 250-mL Erlenmeyer flask at a ratio of 15 g mango peel powder to 150 mL ethanol. Covered flasks containing the MPP solution were inserted into a shaker (New Brunswick G25) for two hours at 150 rpm and 70°C. Nylon cloth was used to recover the pre-treated MPP, which was left to air dry overnight. The weight of the obtained MPP was recorded.

2.2.2 Moisture Content Determination

The moisture content of the pre-treated mango peel powder was evaluated using Equation 1, based on the Association of Official Analytical Chemists' standard (Chakraborty and Ray, 2011).

$$MC = \frac{m_{sample} - m_{sample,dried}}{m_{sample}} \times 100\% \quad (1)$$

where m_{sample} is the initial mass of the pre-treated mango peel powder and $m_{sample,dried}$ is the mass of the dried pre-treated mango peel powder.

2.2.3 Simultaneous Extraction and De-esterification

LMP was obtained from the pre-treated MPP. In a 2-L Erlenmeyer flask, 1125 mL of distilled water was added and mixed with 0.5 M HCl until the pH was 1.5. Approximately 75 g of pre-treated MPP was then added to the flask, which was then placed in an incubator shaker (New Brunswick G-25, New Brunswick Scientific, U.S.A.) at 50°C and 150 rpm for 65 hours. This is different from the SED time of Alcuirez *et al.* (2016), which was 48 hours, to ensure that more pectin is extracted and de-esterified. Cooling was then done by placing the flasks in a cold bath for 15 minutes. Filtration of the contents was done using a nylon cloth. The filtrate recovered was filtered again using a nylon cloth. The volume of the total filtrate collected was then recorded and used for alcohol precipitation.

2.2.4 Isolation and Purification of Pectin by Alcohol Precipitation

The collected filtrate described in Section 2.2.3 was mixed with technical-grade 95% ethanol at a filtrate-to-ethanol ratio of 1:3 by volume for 30 minutes in a large basin. Filtration was then done using a nylon cloth to separate the precipitates, where these precipitates were then wrapped in three layers of nylon cloth and submerged in 1 L of 95% ethanol. While submerged, the wrapped precipitates were compressed and mashed while submerged, and the ethanol was discarded. The process of submerging the precipitates into 1 L of 95% ethanol, compressing, and mashing was repeated a second time in the same manner. The precipitates inside the nylon cloth were then washed again, but with 1 L of 70% ethanol until washings were chloride-free.

A chloride test using 1% (w/v) silver nitrate was done to detect any chloride ions in the washings. The chloride ions were detected when white precipitates were observed upon the addition of 1% (w/v) silver nitrate. Lastly, the precipitates were washed again with 200 mL of 70% ethanol and were then weighed and placed in an oven at 40°C until the weight was constant. The final weight of the LM pectin obtained from mango peels was recorded.

2.3 Characterization of Pectin

The characterization of pectin was done by obtaining the degree of esterification (DE) content, galacturonic acid (GA) content, and molecular weight content. The DE content for the extracted pectin was necessary to ensure that the pectin obtained was LM pectin. Tests for GA content and

molecular weight content were done to further characterize the extracted pectin.

2.3.1 Degree of Esterification

The degree of esterification of dried LM pectin from SED was determined using the titrimetric technique outlined by Singthong *et al.* (2004). The sample's degree of esterification was then determined using Equation 2.

$$DE = \frac{[\alpha + (V_2 \times M_{NaOH})]}{\alpha + M_{NaOH}(V_1 + V_2)} \times 100\% \quad (2)$$

where $\alpha = V_{NaOH}M_{NaOH} \times V_{HCl}M_{HCl}$, M_{NaOH} and M_{HCl} are the concentrations (mol/L) of standardized NaOH and HCl solutions used, respectively, V_{NaOH} and V_{HCl} are the volumes of NaOH and HCl solutions added between NaOH titrations, respectively, V_1 is the amount of NaOH titrant used during the first titration, and V_2 is the amount of NaOH titrant used during the second titration.

2.3.2 Galacturonic Acid Content

The galacturonic acid content of the pectin was determined using Anthon and Barrett's (2008) spectrophotometric approach with adjustments from Siglos *et al.* (2016). To determine the solution's absorbance, a UV-Vis Spectrophotometer (UV-1700, Shimadzu Corp., Japan) set to 750 nm was used.

D-galacturonic acid solutions at various concentrations ranging from 0.02 mg/mL to 0.1 mg/mL and a reagent blank with distilled water were used to create the standard curve. These standard solutions were made from a stock solution containing 1.3 mg D-galacturonic acid per milliliter of solution. In the spectrophotometer, the absorbance of each standard solution was measured in duplicate. The net absorbance was plotted against the concentration. The absorbance of the standard solutions was subtracted from the absorbance of the reagent blank to get the net absorbance. The galacturonic acid concentration of unknown samples was determined using this standard curve. Equation 3 was then used to determine the GA content of the pectin samples.

$$\text{Galacturonic Acid Content} \left[\frac{\text{mg Gal A}}{\text{g dried pectin}} \right] = \frac{c \left(\frac{V}{V_{\text{aliquot}}} \right) V_{\text{solution}}}{m_{\text{dried pectin}}} \quad (3)$$

where C is the GA concentration (mg GA / mL solution) obtained from the standard curve, V is the volume of dilute solution (5 mL), $V_{aliquot}$ is the volume of the aliquot pectin solution, $V_{solution}$ is the volume of the pectin solution.

2.3.3 Average Molecular Weight

The intrinsic viscosity of the dried LM pectin samples was used to determine the average molecular weight of the samples. Higher viscosity means larger pectin molecular weights, which can be linked to its strong gelling ability since viscosity is a function of polymer concentration, pH, ionic strength, and molecular weight. The intrinsic viscosity of the pectin sample was determined using a capillary viscometry experiment using an Ostwald viscometer.

The pectin sample's intrinsic viscosity was assessed using the procedure performed by Kar and Arslan (1999), with modifications particularly on the pectin-buffer solution where instead of heating the mixture to 20°C as performed by the aforementioned study, the pectin-buffer solution was immediately mixed for 12 hours in an ambient shaker to ensure adequate dissolution. For viscosity measurements, 5 mL of the prepared solutions were pipetted into the Ostwald viscometer, and a timer was used to record the flow times for each solution and solvent. A 10-mL Gay-Lussac pycnometer was used to test the densities of the prepared solutions and the solvent. The specific viscosity was then determined using Equation 4.

$$\eta_{sp} = \frac{\eta \cdot \eta_s}{\eta_s} = \frac{t_1 \rho_1}{t_2 \rho_2} - 1 \quad (4)$$

where η is the viscosity of the solution (Pa·s), η_s is the viscosity of the solvent (Pa·s), t_1 and t_2 are the time taken by the solution and solvent, respectively, to flow in the viscometer (s), and ρ_1 and ρ_s are the density of the solution (kg/m³) and of 0.1 M sodium phosphate buffer, respectively. The intrinsic viscosity $[\eta]$ was estimated by obtaining the y-intercept of the linear least squares fit for a specific set of data points by plotting $\left(\frac{\eta_{sp}}{C}\right)$ vs. C (concentration of pectin solution (mg/mL)). The Mark-Houwink-Sakurada equation (Equation 5), which in general describes the polymer's (e.g., pectin, gelatine, xanthan gum, etc.) intrinsic viscosity and molecular weight through an empirical relationship, was used to calculate the molecular weight of the pectin extracted from mango peels (Alinsug *et al.*, 2024).

$$[\eta] = k \cdot MW_{ave}^a \quad (5)$$

From Equation 5, k and a are constants that depend on the nature of the molecule, solvent type, and temperature. The conditions used for the study of Alcuirez *et al.* (2016), which is one of the bases for the present study, were assumed to be valid. Therefore, the values for $k = 2.34 \times 10^{-5}$ and $a = 0.8224$.

2.3.4 Characterization of Enzyme

The characterization of enzyme, specifically on the determination of the protein content, was achieved using the colorimetric technique developed by Bradford (1976). Equation 6 was used to calculate the total amount of protein contained in the enzyme solution.

$$P_{T,i} = P_{unknown\ sample} \cdot DF \quad (6)$$

where $P_{T,i}$ the total concentration of protein (mg/mL), $P_{unknown\ sample}$ is the protein concentration in the diluted sample as measured by the calibration curve (mg/mL), and DF is the dilution factor (mL/mL).

2.3.5 Formation of calcium-pectate beads

The formation of calcium pectate beads was carried out by ionotropic gelation based on a procedure described by Jeong *et al.* (2020) and Nawaz *et al.* (2015) with slight modifications particularly on the pectin solution (10% w/v), pectin solution to cellulase solution ratio (1:5), a dropping height of 6 cm, and the varying CaCl_2 concentrations (2.0, 4.0, and 6.0% w/v) and curing time (10, 20, and 30 minutes). Following the curing process, the beads were then filtered and rinsed in distilled water to remove the excess calcium ions. The drying conditions for the beads were based on the study of Bourgeois and co-authors (2006) where the beads were oven dried at atmospheric pressure for two hours at 40 °C.

2.4 Characterization of calcium-pectate beads

2.4.1 Size and shape analysis

The shape and size analysis of the Ca-pectate beads was done by examining randomly selected five beads per sample. The average size of the beads was

then computed from the five randomly selected beads per batch sample. A dimensionless shape indicator known as the sphericity factor (SF) was calculated using Equation 7 (Voo *et al.*, 2011).

$$SF = \frac{(d_{max}-d_{per})}{(d_{max}+d_{per})} \quad (7)$$

where d_{max} is the maximum diameter at the bead centroid (mm) and d_{per} is the perpendicular diameter to d_{max} measured at the bead centroid (mm). Both diameters are measured using ImageJ to minimize errors. A bead was considered spherical if the calculated SF was < 0.05 and perfectly spherical if $SF = 0$. Similarly, if SF approached unity, the bead was considered to have an elongated shape.

2.4.2 Moisture Content

The beads were dried in a microwave oven at atmospheric pressure for two hours at 40°C. The bead's weight loss during the drying process was then calculated using Equation 8.

$$Moisture\ Content = \frac{(w_0-w_t)}{w_0} \times 100\% \quad (8)$$

where w_0 and w_t are the weights of the beads before and after the drying process, respectively.

2.4.3 Swelling Ratio

Hydrolysis of cellulose by cellulases is mostly carried out in an aqueous system. Hence, the swelling behavior of the Ca-pectate beads was investigated. The characterization of this behavior was done based on the procedures outlined by Kowalski *et al.* (2019). The swelling ratio was calculated using Equation 9.

$$SR = \frac{(M_w-M_D)}{M_D} \quad (9)$$

where SR is the swelling ratio at time t (g H₂O/ g dry beads), M_w is the weight of the swollen beads at the defined time t (g), and M_D is the weight of the dry beads (g).

2.5 Characterization of Immobilized Enzyme

2.5.1 Enzyme assay for free and immobilized cellulase

The assay for free and immobilized cellulase was done according to the CMC method described by Ghose (1987). For the substrate and standard, 1% (w/v) carboxymethyl cellulose (CMC) and glucose were used, respectively. Enzyme activity for free and immobilized cellulase was determined using Equation 10 and 11, respectively.

$$\text{Free cellulase activity (U/L)} = \frac{\text{reducing sugar content (mg)} \times N \times 1000}{0.18 \times t \times V} \quad (10)$$

$$\text{Immobilized cellulase activity (U/g)} = \frac{\text{reducing sugar content (mg)} \times 1000}{0.18 \times t \times g} \quad (11)$$

where N is the dilution ratio, t is the reaction time, V is the volume of added cellulase solution during the measurement of free cellulase activity, and g is the amount of added immobilized cellulase. The free cellulase activity was expressed in terms of protein concentration. Therefore, the specific activity of free cellulase was acquired and described as the enzyme unit per milligram of protein (U/mg). This was achieved by acquiring the quotient of free cellulase activity (U/L) and the concentration of protein (mg/mL).

2.5.2 Immobilization Yield

The information presented in determining immobilized yield, relative activity, and efficiency was based on the method of Savitha *et al.* (2020). Immobilization yield is one way to determine the success of enzyme immobilization. It is described below in Equation 12:

$$\text{Immobilization Yield (\%)} = \frac{C_i - C_f}{C_i} \times 100\% \quad (12)$$

where C_i is the protein concentration introduced for immobilization and C_f is the protein concentration present in the calcium chloride solution and water used for washing. The determination of the final protein concentration present in the calcium chloride solution and water used for washing is the same as discussed in Section 2.5.1.

2.5.3 Relative Activity

The relative activity can be determined from the immobilized enzyme activity compared to the free enzyme activity. This demonstrates the success of the immobilization process as a whole. The free and immobilized enzyme activity was determined as discussed in Section 2.5.1. This activity will be calculated using Equation 13, where the activity of the immobilized enzyme is compared to that of the free enzyme activity.

$$\text{Relative activity (\%)} = \frac{\text{activity of immobilized enzyme}}{\text{activity of free enzyme}} \times 100\% \quad (13)$$

2.5.4 Immobilization Efficiency

The utilized protein binding capacity of the matrix/support is known as immobilization efficiency. The percentage of total protein bound to the matrix indicates how well it immobilizes the protein. This is calculated using Equation 14.

$$\text{Immobilization Efficiency (\%)} = \frac{\text{relative activity}}{\text{immobilization yield}} \times 100\% \quad (14)$$

2.5.5 Leakage of immobilized cellulase from beads

The leakage of the encapsulated enzyme was determined using the method of Mong Thu and Krasaekoopt (2016). The utilized beads were obtained from the experimental setting with the best immobilization efficiency. The enzyme activity was obtained as described in Section 2.5.1, and the leakage was calculated using Equation 15:

$$\text{Leakage (\%)} = \frac{\text{enzyme activity in deionized water}}{\text{enzyme activity in beads used in leakage assay}} \times 100\% \quad (15)$$

2.6 Statistical Analysis

A two-way analysis of variance (ANOVA) was conducted to determine the significance of extraction conditions and was further analyzed using post-hoc analysis at a level of confidence of $p < 0.05$.

3. Results and Discussion

3.1 Pectin Characterization

Mango peel pectin extracted by the SED process has been presented in multiple studies (Alcuirez *et al.*, 2016; Luarez *et al.*, 2021). After the simultaneous extraction and de-esterification of the mango peel pectin, the moisture content, degree of esterification, galacturonic acid content, and molecular weight were determined. A summary of the extracted pectin's characteristic composition and comparison with other literature values is presented in Table 1.

Table 1. Characteristics of mango peel pectin extracted by SED

Pectin Characterization			References
Degree of Esterification (%)	Galacturonic acid content (mg GA/g pectin)	Molecular weight (g/mol)	
42.5	448.96	~2000	Alcuirez <i>et al.</i> (2016)
47.52 ± 0.25	286.3025 ± 27.8367	12,330 ± 2797	Luarez <i>et al.</i> (2021)
47.06 ± 4.30	339.7571 ± 11.9208	38,183.89	In this study

In this study, the SED time was the same as in the study of Luarez *et al.* (2021), which was 65 hours, and the moisture content of the dried mango peel powder ($8.1910 \pm 0.004\%$) is similar to their study ($5.0 \pm 0.3\%$). This indicates that longer SED time was needed as compared to the study of Alcuirez *et al.* (2016), which had a moisture content of $14.9 \pm 0.6\%$ and SED time of 45 hours.

Additionally, the amounts of free GA molecules that are available for cross-linking with the divalent cation are indicated by the DE and GA content. The GA content is the amount of pectin in terms of galacturonic acid, the main component of the biopolymer. The degree of esterification from the different literature presented in Table 1 that extracted pectin using the SED process falls within the range for LMP, which is less than or equal to 50% (Narasimman and Sethuraman, 2016). The GA content in this study falls within the values acquired by Luarez *et al.* (2021) and Alcuirez *et al.* (2016), which are 286.3025 ± 27.8367 mg GA/g pectin and 448.96 mg GA/g pectin, respectively.

The pectin molecule's chain length was determined by its molecular weight, and it should not be less than 2800 g/mol since a shorter chain length inhibits gel formation (Fraeye *et al.*, 2010). Compared to commercial pectin, which has a GA and molecular weight of 650 mg GA/g pectin and 100,000–300,000 g/mol pectin, respectively, the pectin characteristic in this study is much lower (Yapo and Koffi, 2014). Nonetheless, the extracted pectin used in this study was low-methoxyl and was still able to form into beads with a calcium chloride solution.

3.2 *Ca-Pectate Beads Characterization*

Mono-dispersed and spherical calcium-pectate beads can be formed from LMP with calcium chloride as its cross-linking agent through extrusion dripping. This phenomenon, ionotropic gelation, is caused by the interaction of the negatively charged carboxyl groups of LM pectin and the positively charged counter ion of the cross-linking solution (Günter *et al.*, 2014; Sriamornsak *et al.*, 2008). The characteristics of the instantaneously formed gel beads analyzed in this study include size, shape, moisture content, and swelling behavior.

3.2.1 Size and Shape

The size and form of the beads can also be affected by characteristics related to the actual execution. The cured blank beads differ visually and physically from the beads with encapsulated enzymes—the blank beads were bigger and more rigid. The average maximum diameter of the former ranged from 3.5 cm to 5.7 cm, whereas the latter ranged from 3.2 cm to 3.7 cm. Moreover, they were darker than the beads with encapsulated enzymes.

The effect of curing time and calcium chloride concentration on the calculated sphericity factor of the beads is presented in Figure 3. From the randomly selected bead samples, the calculated sphericity factor values were all above 0.05, indicating that the beads formed were irregularly shaped (e.g., some were elongated, and some had spherical bodies with tails).

As shown in Figure 3, the trends are uniformly decreasing for both blank beads and beads with encapsulated enzymes. The extent of cross-linking of the polymer matrix is dependent on the hardening time of the beads (Smrdel *et al.*, 2008). In this case, as the curing time increases from 10 to 30 minutes, the sphericity factor decreases from 0.34 ± 0.03 to 0.07 ± 0.05 . In general, this

would indicate that the shape of the beads becomes circular the longer they are submerged in the cross-linking solution as a result of prolonged exposure of the polymer matrix to the CaCl_2 solution. Smrdel *et al.* (2008) studied the influence of selected parameters on the size and shape of alginate beads prepared by ionotropic gelation and found out that longer hardening time or curing time was responsible for the improved sphericity of the beads. However, the effect of curing time to the size and shape of the beads in this study was not significant with $p = 0.40520$ ($p > 0.05$). Indicating that the effects are most likely due to another factor.

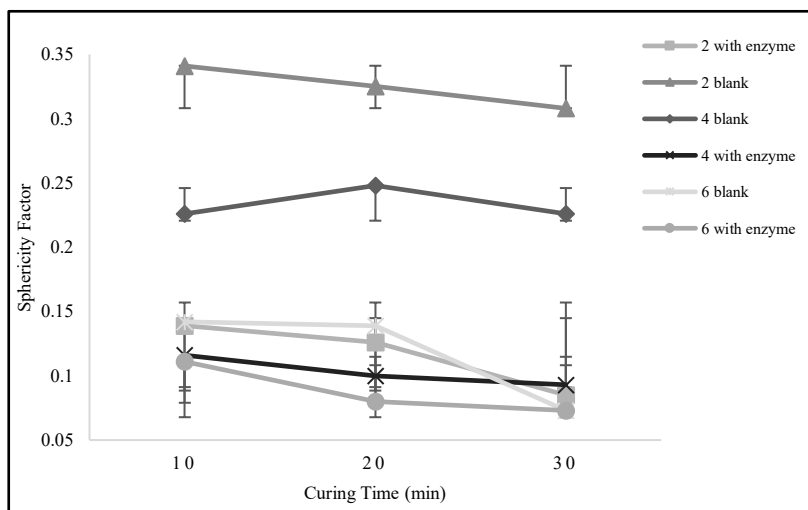


Figure 3. Effect of curing time (10, 20, 30 minutes) and CaCl_2 concentration (2%, 4%, 6% w/v) on the sphericity factor of cured blank beads and beads with encapsulated enzymes

With this in mind, the prepared beads with the highest cross-linking concentration (6.0% w/v CaCl_2) have the lowest sphericity factor (0.07 ± 0.05) for both blank beads and beads with encapsulated enzymes where an increased CaCl_2 concentration led to a significant decrease in sphericity factor with $p = 1.802 \times 10^{-7}$ ($p < 0.05$). Hence, the beads are more spherical when formed with higher CaCl_2 concentration, which signifies increased Ca^{2+} ion concentrations leading to the rapid entrapment and stabilization of the beads (Chen *et al.*, 2006; Lee *et al.*, 2013). Moreover, the uniformity of formed beads can be attributed to the high cross-linking solution, which produces beads with a more homogeneous network (Chen *et al.*, 2006).

3.2.2 Moisture Content and Swelling Behavior

The drying of the beads was done after the specific curing time to analyze the water content and swelling ratio of the beads. The data analysis for blank beads and beads with encapsulated enzymes are shown in Table 2.

Table 2. Water content and swelling ratio analysis of cured (10, 20, and 30 minutes) blank beads and beads with encapsulated enzymes in 2.0, 4.0, and 6.0 w/v% of calcium chloride solution

CaCl ₂ solution (% w/v)	Curing time (min)	Moisture content (%)		Swelling ratio (%)	
		Blank	With encapsulated enzymes	Blank	With encapsulated enzymes
2	10	90.78 ± 0.004	90.92 ± 0.002	98.97 ± 0.033	43.48 ± 0.043
	20	90.61 ± 0.001	90.15 ± 0.009	83.32 ± 0.065	60.52 ± 0.164
	30	94.37 ± 0.020	90.69 ± 0.006	228.35 ± 0.84	64.89 ± 0.142
4	10	89.81 ± 0.009	88.54 ± 0.016	90.33 ± 0.125	115.86 ± 0.13
	20	90.79 ± 0.003	88.61 ± 0.007	52.00 ± 0.152	89.45 ± 0.033
	30	86.83 ± 0.006	88.59 ± 0.013	37.09 ± 0.047	74.43 ± 0.320
6	10	88.37 ± 0.011	88.13 ± 0.007	5.601 ± 0.131	65.34 ± 0.192
	20	85.99 ± 0.003	89.61 ± 0.006	28.48 ± 0.070	103.18 ± 0.26
	30	89.61 ± 0.012	87.90 ± 0.002	57.46 ± 0.219	56.18 ± 0.213

Moisture content was determined by drying the beads and measuring their weight before and after the water inside the beads dried off. As the concentration of the cross-linking solution increases, the moisture content values for both blank beads and beads with encapsulated enzymes significantly decrease ($p < 0.05$; $p = 3.5254 \times 10^{-06}$). The drying process increases bead deformation owing to water loss, which weakens the matrix gel structure, causing shrinkage and collapses in the bead surface (Sampaio *et al.*, 2019). When the dried beads were soaked in water and shaken for 90 minutes, they hydrated and swelled, although, visually, the swollen beads were smaller relative to their form after ionotropic gelation.

The lowest cross-linking solution (2.0% w/v CaCl₂) produced beads with a significantly higher swelling ratio, the highest being $228.35 \pm 0.84\%$ ($p < 0.05$; $p = 1.9628 \times 10^{-05}$). In comparison with the beads formed in higher concentrations of calcium chloride solution, the highest SR from the lowest CaCl₂ solution can be attributed to its weaker matrix gel structure. Therefore, beads that are formed at a higher cross-linking solution concentration are characterized by a lower rate of water absorption (Kowalski *et al.*, 2019). This trend was also observed by Günter *et al.* (2014), who reported that the reduced

swelling of their calcium pectate beads was due to increased calcium cross-linking.

The swelling behavior of the beads were also evaluated where an increase in curing time decreases the moisture content of the beads. In general, this is because an increase in curing time can lead to a thicker calcium pectinate shell/membrane due to the cross-linked pectin structure formed that is accompanied by an increase in the mechanical strength of the beads formed (Sriamornsak *et al.*, 2008). However, the effect of curing time to the moisture content of the beads in this study was not significant with $p = 0.40520$ ($p > 0.05$). This is also seen when the trends observed in Table 2 have variations in results. Additionally, the stability of the beads increases when a long time is given for the cross-linking between calcium ions and the polymer matrix. Hence, when the curing time is increased, the swelling ratio significantly decreases ($p < 0.05$; $p = 0.0137$). The stronger gel-matrix achieved with the longer curing time provides less porosity in the beads.

Structure and integrity of the beads are indeed essential, especially in the immobilization of enzymes, especially in their industrial applications. While the ionotropic gelation process produced beads that were visually uniform and structurally stable during laboratory handling, no quantitative evaluation (e.g., compression, rupture force, or resilience tests) was conducted to assess their mechanical robustness under simulated industrial conditions. As a result, the suitability of the prepared beads for large-scale or long-term biocatalytic operations remains to be verified.

3.3 Enzyme Activity Characterization

An enzyme's overall catalytic abilities are referred to as its activity. When dealing with enzyme immobilization, enzyme activity is an important factor to consider. The characterization of the cellulase activity was divided into two categories: free cellulase activity and immobilized cellulase activity. The free cellulase activity was determined as a baseline to evaluate the success of the enzyme immobilization, which was represented by three parameters: immobilization yield, relative activity, and immobilization efficiency.

3.3.1 Free Cellulase Activity

Free cellulase activity is a measure of the catalytic activity of cellulase enzymes in a solution, typically expressed as the amount of glucose produced

per unit time. The protein concentration of the enzyme solution is also determined through a standard protein assay (the Bradford assay) to normalize the enzyme activity with respect to enzyme concentration (Roberts and Gibb, 2013).

The resulting value of free cellulase activity per unit of protein concentration, which is the specific free cellulase activity, was used as a baseline for the characterization of enzyme activity. The specific free cellulase activity obtained in this study was 9874.89 U/g.

3.3.2 Immobilized Enzyme Activity

The effect of curing time and calcium chloride (CaCl₂) concentration was investigated on the activity of the immobilized enzyme in order to determine the best conditions for the immobilization of cellulase as presented in Figure 4.

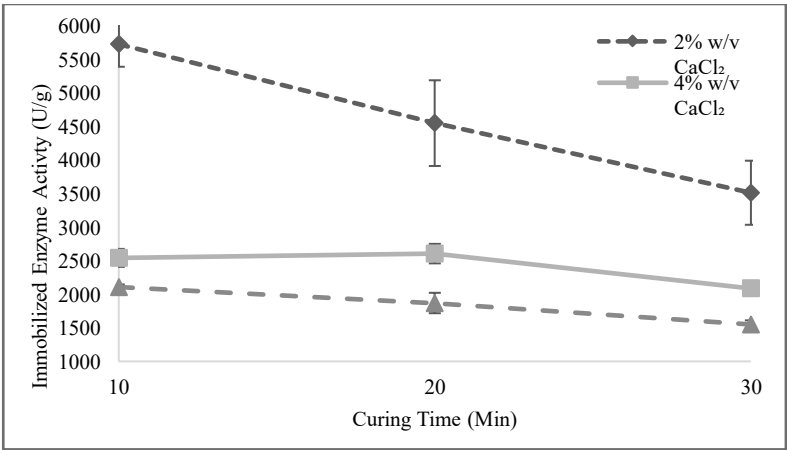


Figure 4. Effects of curing time and CaCl₂ concentration on the activity of immobilized enzymes in beads

As presented in Figure 4, increasing the concentration of CaCl₂ solution led to a significant decrease in the activity of encapsulated cellulases in beads ($p < 0.05$; $p = 3.67406 \times 10^{-13}$). Additionally, a significant decrease in the activity of encapsulated cellulases in beads was seen as the curing time of the beads increased ($p < 0.05$; $p = 2.54016 \times 10^{-6}$). Based on this, the best condition in which the immobilized enzyme acquired the highest enzyme activity was a CaCl₂ solution concentration of 2.0% (w/v) and a curing time of 10 minutes.

The analysis of the immobilized enzyme in terms of immobilization yield, relative activity, and immobilization efficiency is presented in Table 3.

The amount of calcium ions present influences enzyme inhibition. Wang *et al.* (2012) investigated the effect of various metal ions, including Ca^{2+} , on the activity of cellulase and have observed an increase in cellulase activity for a calcium ion concentration of less than 1 mM. A similar inhibitory effect of a higher concentration of CaCl_2 on the enzyme activity was also discussed by Yadav (2012). Another possible reason for the reduction in immobilized cellulase activity is the limitation of the diffusivity of the substrate due to the excess gelation of calcium chloride that is formed on the surface of the bead (Wang *et al.*, 2015; Zhan *et al.*, 2013). Therefore, the condition in which the immobilized enzyme acquired the highest enzyme activity was a CaCl_2 solution concentration of 2.0% (w/v) and a curing time of 10 minutes. Nonetheless, although a reduction in enzyme activity was observed following immobilization, the immobilization of cellulase using low-methoxyl pectin is still a viable option for industrial applications given that the biocatalyst has remained active and stable, which is advantageous for continuous use in enzyme reactors. This indicates that the main objective of enzyme immobilization, which is to enhance the economics of biocatalytic processes by reusing the enzyme in various reaction cycles, is achieved (Guzik *et al.*, 2014).

Table 3. Immobilization yield, Relative activity, and Immobilization efficiency analysis of cured (10, 20, and 30 minutes) beads with encapsulated enzymes in 2, 4, and 6 w/v% of calcium chloride solution

CaCl ₂ conc. (% w/v)	Curing time (minutes)	Immobilization yield (%)	Relative activity (%)	Immobilization efficiency (%)
2	10	62.34 ± 0.578	58.06 ± 2.78	93.14 ± 7.87
	20	62.22 ± 0.555	46.13 ± 5.29	74.14 ± 13.79
	30	62.11 ± 0.424	35.61 ± 3.95	57.34 ± 10.12
4	10	64.60 ± 0.833	25.77 ± 1.36	39.89 ± 2.40
	20	64.72 ± 1.05	26.41 ± 1.47	40.81 ± 3.08
	30	63.92 ± 0.481	21.15 ± 0.901	33.08 ± 1.47
6	10	65.28 ± 0.278	21.35 ± 4.36	33.05 ± 0.023
	20	65.28 ± 0.481	19.01 ± 1.31	29.38 ± 0.054
	30	65.06 ± 0.975	15.77 ± 0.434	24.67 ± 0.082

The immobilization yield was determined in terms of the protein concentration of the enzyme, relative activity was determined in terms of the activity of the

enzyme, and immobilization efficiency was determined in terms of the immobilization yield and relative activity. In this case, the concentration of calcium chloride (CaCl_2) considerably affects these three parameters. As the concentration of the CaCl_2 solution increases from 2.0% (w/v) to 6.0% (w/v), the immobilization yield also increases, while the relative activity and immobilization efficiency significantly decreases. These parameters presented in Table 3 determine the success of the cellulase immobilization.

3.3.3 Immobilization Yield

The immobilization yield pertains to the protein concentration present during the process of immobilization. As the CaCl_2 concentration increases, the immobilization yield significantly increases ($p < 0.05$; $p = 0.0328$). The maximum immobilization yield was 65.28% when the concentration of calcium chloride was 6.0% (w/v).

Li *et al.* (2019) acquired the maximum immobilization yield of 57.2% for cellulase at a concentration of 3.5% (w/v) for the cross-linking solution concentration. Nawaz *et al.* (2015) studied the effect of varying the sodium alginate concentration on the entrapment immobilization of maltase and found that 4.0% sodium alginate yielded the highest percent immobilization yield of maltase. The decrease in immobilization yield could be attributed to enzyme leakage during the washing process, which was caused by the formation of soft, fragile beads with large pores due to the low concentration of the cross-linking solution.

3.3.4 Relative Activity

As presented in Table 3, as the calcium chloride concentration increases, the relative activity significantly decreases ($p < 0.05$; $p = 7.017 \times 10^{-12}$). The maximum relative activity is $58.06 \pm 2.78\%$, acquired at a curing time of 10 minutes and 2.0% (w/v) CaCl_2 concentration. These conditions are considered the optimum immobilization conditions in this study.

The acquired result for the relative activity can be correlated with the study of Viet *et al.* (2013), who found that increasing the curing time results in a decrease in relative activity. In their study, as the curing time reached 30 minutes, the immobilized enzyme activity drastically decreased to 84.89% as enzymes were continuously lost. Andriani *et al.* (2012) acquired the same optimum cross-linking solution concentration at 2.0% (w/v) for a relative

activity of 60%. The reason for the decrease in relative activity with increasing CaCl_2 concentration is that excessive calcium chloride would gel on the surface of the bead, reducing the substrate's diffusivity and, consequently, the relative activity of the immobilized enzyme (Bradford, 1976). This might also be attributable to the reduced pore size of beads, which prevented the substrate from penetrating the beads (Andriani *et al.*, 2012).

3.3.5 Immobilization Efficiency

Similar to relative activity, as the calcium chloride concentration increases, the immobilization efficiency generally decreases. The maximum immobilization efficiency obtained in this study was $93.14 \pm 7.87\%$, with a curing time of 10 minutes and a CaCl_2 concentration of 2.0% (w/v).

A similar result for the effect of calcium chloride on immobilization efficiency was evident in the study of Geethanjali and Subash (2013). In their study, the highest immobilization efficiency was 45%, with a cross-linking solution concentration of 2% (w/v). Beyond this optimal condition, it was observed that there was a gradual decrease in immobilization efficiency. Sharma *et al.* (2010) acquired a maximum efficiency of 98–99% for the immobilization of amylase utilizing calcium chloride (CaCl_2) as the cross-linking agent with a concentration of 2% (w/v). A higher concentration of a cross-linking solution leads to an inhibitory effect and may be attributed to a smaller pore, which results in reduced immobilization efficiency (Geethanjali and Subash, 2013; Wang *et al.*, 2012; Dey *et al.*, 2003). A gel matrix should have pores large enough for the substrate and product to easily permeate into and out of while keeping the enzyme in the microenvironment of the beads (Dey *et al.*, 2003).

3.4 Leakage of Immobilized Enzyme

Enzyme leakage is a common problem that is faced when dealing with the encapsulation of enzymes. The leakage of the encapsulated cellulase that had an experimental setting that achieved the best immobilization efficiency (2.0% w/v CaCl_2 with a curing time of 10 minutes) was determined and is shown in Figure 5.

The encapsulated cellulase that had the best immobilization efficiency in this study had low leakage (in the range of 13.3 ± 0.031 to $17.2 \pm 0.21\%$). The leakage for the first 30 minutes was evident; after that, it increased slightly for

the next 30 minutes, then proceeded to increase linearly for the next 60 minutes.

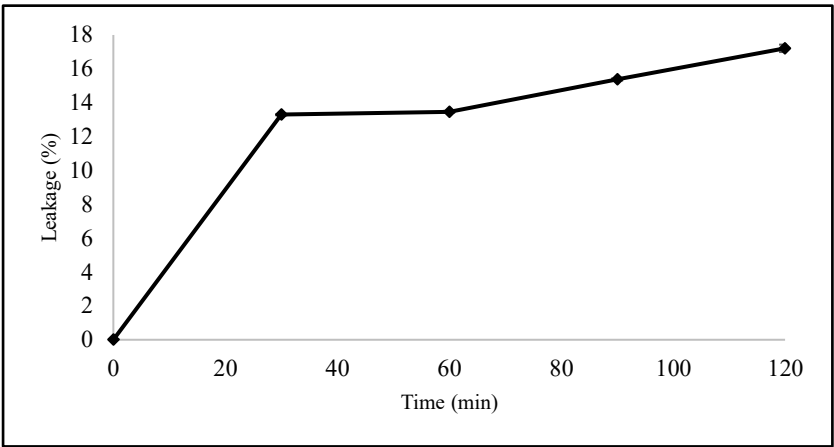


Figure 5. Leakage of encapsulated cellulase at 2% CaCl₂ with a curing time of 10 minutes

The sudden increase in leakage from the first 30 minutes may have been a result of the leakage of cellulase found on the bead surface (Sivakumar *et al.*, 2011). A comparison of the leakage results on multiple studies is presented in Table 4.

Table 4. Leakage of immobilized enzymes

Enzyme	Support material	Temperature	Leakage time	Leakage (%)	Reference
Cellulase	Pectin	Ambient temperature	120 minutes	13.3 ± 0.031 - 17.2 ± 0.21%	This study
Cellulase ^a	Sodium Alginate	40 °C	30 minutes / cycle ^b	9.0 – 39.1%	Li <i>et al.</i> (2019)
Lipase	Alginate	Ambient temperature	120 minutes	6.2 - 20.6%	Mong Thu and Krasaekoopt (2016)
Protease	Alginate	Ambient temperature	120 minutes	6.7 - 17.6%	Mong Thu and Krasaekoopt (2016)

^aleakage was based on the reusability of the immobilized enzyme subjected to CMC hydrolysis for 30 minutes at different cycles.

^breaction time

As presented in Table 4, multiple studies have reported the leakage results from different enzymes immobilized in different support materials using CaCl_2 as a cross-linking agent. Li *et al.* (2019) immobilized cellulase using sodium alginate and performed a CMC hydrolysis for 30 minutes using the immobilized cellulase. The leakage rate of the cellulase was high (9.0–39.1%) after three reused cycles. The sudden increase in leakage was because of the gradual increase of pore size after repeated use, resulting in increased leakage of cellulase (Li *et al.*, 2019). Furthermore, Mong Thu and Krasaekoopt (2016) encapsulated protease and lipase using alginate as the support material, then performed a leakage analysis for 120 minutes under ambient temperature and pressure. The leakage for protease was in the range of 6.7–17.6%, while the leakage of lipase was in the range of 6.2–20.6%. An initial burst in leakage was evident, which followed a gradual increase in leakage that could be observed from all of the studies presented. It was also reported that the initial burst of leakage may have resulted from the leakage of cellulase on the bead surface (Sivakumar *et al.*, 2011).

Another reason for the leakage of the enzyme would be from the bead porosity attributed to the low concentration of CaCl_2 , making the matrix less dense. Although the leakage in this study is low, reducing the molecular weight cutoff of membranes or the pore size of solid matrices can prevent enzyme leakage (Liu, 2017). Immobilization methods could also be combined to minimize or prevent enzyme leakage (Long *et al.*, 2019).

4. Conclusions and Recommendation

The effects of the curing time and the concentration of the calcium chloride solution on the characteristics of the beads and the enzyme activity were investigated in this study. It was found that cellulase can be immobilized with pectin as the support material through the encapsulation method. LMP (DE = $47.06 \pm 4.30\%$) was able to gel out and form beads where cellulase was encapsulated for the immobilization process.

The prepared beads had a sphericity factor above 0.05, which indicates that no perfectly spherical beads were formed. As the calcium chloride concentration increased from 2.0 to 6.0% (w/v), the moisture content and swelling ratio values for both blank beads and beads with encapsulated enzymes decreased from $94.87 \pm 0.0199\%$ to $87.90 \pm 0.0020\%$ and $228.35 \pm 0.8394\%$ to $5.60 \pm$

0.1311%, respectively. Moreover, the longer curing time was attributed to the stronger gel matrix of the bead.

An increase in CaCl_2 concentration from 2.0 to 6.0% (w/v) decreased the relative activity from 58.06 to 15.77% and immobilization efficiency from 93.14 to 23.47%, while it had little effect on the immobilization yield, which increased slightly from 62–65%. On the other hand, an increase in curing time from 10 to 30 minutes decreased the relative activity and immobilization efficiency for the CaCl_2 concentrations of 2.0, 4.0, and 6.0% w/v. In addition, an increase in curing time had no significant effects on the immobilization yield since the values obtained were proximate.

The best immobilization efficiency condition—2.0% (w/v) CaCl_2 solution and 10 minutes curing time—was used to investigate the leakage of the immobilized cellulase, and it was discovered that the encapsulated cellulase had low leakage, ranging from 7.2 to 11.4%.

The effect of calcium chloride concentration on the immobilization yield and moisture content of the beads was statistically significant, whereas the effect of curing time was statistically insignificant. Therefore, it is recommended to conduct the immobilization process using other sets of calcium chloride concentrations while utilizing a fixed curing time that would obtain higher relative enzyme activity. Moreover, further improvement on the understanding and applicability of the immobilized cellulase system can be achieved by performing additional analyses such as a scanning electron microscopy (SEM) for a more detailed morphological characteristic of the beads, assays involving a wider range of substrate concentration to explore a wider range of enzyme-substrate interaction, a dynamic light scattering (DLS) to assess the size distribution and aggregation behavior of enzyme-polymer complexes in solution prior to gelation, and even an FTIR spectra of pre- and post-immobilization of cellulase to show interaction sites of functional groups.

It is also recommended to perform another immobilization technique (e.g., cross-linking) on top of encapsulation to improve immobilized enzyme activity. In addition, the reusability of immobilized cellulase is one of the important characteristics of enzyme immobilization, as it directly addresses one of the main issues of the use of enzymes, which is reusability. Therefore, a reusability analysis should be conducted on immobilized cellulase. Lastly, assessment on the mechanical strength of the calcium-pectate beads were not performed due to limitations on availability of instrumentation and resources,

thus it is recommended for future studies that characterizations concerning the mechanical strength of the beads are performed.

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