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Effects of Mango Pectin Concentration on the Calcium Pectate Bead Properties and on the Cell Leakage of Yeast (*Saccharomyces cerevisiae*) Immobilized by Entrapment Technique

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Abstract

Immobilized yeast cells have advantages for use in industries due to their stability, viability, and productivity. As a support material, pectin is suitable because of its stability, strong mechanical attributes, and favorable absorption properties. In this study, the influence of mango pectin concentration on the CP bead characteristics and the leakage of immobilized yeast cells were determined. Results have indicated that yeast cells can be immobilized using low-methoxyl pectin (LMP) from mango peels via entrapment technique. Pectin concentration did not affect the size (3.0323 to 3.4315 mm) of the CP beads. Only 5% and 7% (w/v) pectin concentrations produced spherical beads (SF<0.05). Increasing the pectin concentration from 3% to 7% (w/v) resulted in a 36.21% decrease in swelling ratio for CP beads with yeast. The pectin concentration significantly affects the cell leakage (p-value<0.05). The results further indicate the feasibility of using locally available materials as a matrix for immobilization.

Keywords

calcium pectate beads, cell leakage, immobilization, mango pectin, yeast entrapment

INTRODUCTION

Yeast immobilization offers numerous opportunities for food and beverage industries such as the production of bread, beer, wine, sake, and cider (Bleve et al., 2016). This results from the technical and economic benefits of having immobilized yeast compared to free yeast cells (Nedović et al., 2015). Cell immobilization provides cell recovery and protection from shear stress and stability against environmental stresses such as temperature, pH, salts, inhibiting substrates and products, and organic solvents (Zhu, 2007). It improves the product yield due to high cell density and productive cell growth and enables yeast cells to have high longevity as their activity, viability, and productivity can be maintained for a prolonged period, hence enabling cell reutilization. In addition, cell immobilization provides better control and minimizes cell leakage at elevated rates of dilution during continuous operation (Kosseva, 2011).

The gel entrapment method has the most desirable attributes as such technique uses immobilization matrices that have a higher range of mechanical stability coupled with its ability to offer sufficient binding force, allow diffusion of essential molecules, prolong retention of activity, and are acquired at a lower cost. Several factors can influence the diffusion properties of the gel beads, and one example is the support material (Zdarta et al., 2018). The most common support material for entrapment of yeast is calcium alginate (Hernández et al., 2010). This material offers several benefits, including strong biocompatibility, affordability, accessibility, and can easily be prepared (Duarte et al., 2013; Sousa-Dias et al., 2021). However, drawbacks of its use include deterioration of the gel, limited diffusion properties, weak mechanical attributes, and loose gel network, which results in increased leakage (Sousa-Dias et al., 2021; Zhao et al., 2018). Calcium alginate is said to be not chemically stable and its gel is easily modified by chelating chemicals, thus its utilization in immobilization systems is limited (Bucke, 1983).

To find an alternative to calcium alginate, Navrátil et al. (2002) studied the potential of pectin in the form of calcium pectate. Similar to alginate, the ionotropic gelation of pectate is straightforward and provides a gentle physiological environment for entrapping cells. It is mentioned that Ca-pectate and Ca-alginate gel beads have similar molecular, morphologic, and diffusional properties. Ca-pectate gel beads have lower sensitivity to chelating ions and other chemical compounds which are favorable characteristics for an immobilization matrix (Navrátil et al., 2002). However, Voo et al. (2011) and Valach et al. (2006) preferred the use of calcium pectate over calcium alginate because of its mechanical strength and favorable environment for cell growth. In their study, calcium pectate exhibited lower percent breakage and lower effects of abrasion over alginate beads. Their findings indicate that calcium pectate is more suited to be used as a material for immobilization than calcium alginate due to its mechanical strength.

The properties of these calcium pectate beads are affected by both extrinsic and intrinsic characteristics of the initial pectin material (Zhao et al., 2018). Pectin is a structural polysaccharide present in plant cell walls that can be dissolved in water. It is composed of galacturonic acid chains with 300 to 1,000 units bonded by α–(1, 4) glycosidic linkages (Mellinas et al., 2020). Pectins can be classified into two groups mainly by the degree of esterification (DE): (1) high-methoxyl pectin (HMP), and (2) low-methoxyl pectin (LMP). The DE is defined as the ratio of methyl-esterified galacturonic acids and the total galacturonic acid units in the pectin molecules. LMP, having a lower degree of esterification and reduced electrostatic repulsion, enables closer packing and stronger intermolecular interactions (Thakur et al., 1997). LMP forms stable gels with divalent cations such as calcium. The gelling process of LMP occurs when calcium bonds are formed between the carboxyl groups from galacturonic acid chains that are in contact (Hotchkiss et al., 2002) as shown in Figure 1. The presence of more free carboxyl groups in LMP facilitates more ionic bonding, leading to a more robust gel structure.

Sudhakar and Maini (2000) found that the peels of Totapuri mangoes contain 20.8 wt. % (dry basis) of pectin. Similarly, Gragasin et al. (2014) extracted LMP from Philippine carabao mango peels with a yield of 21.65 wt. % (dry basis). These yields are comparable to the highest yield of 24.7 wt. % (dry basis) from the 26 organic food wastes that were studied by Müller-Maatsch et al. (2016), indicating that mango peel contends as a good pectin source, comparable to other sources. In addition, this supports the need to use this mango waste resource effectively as a pectin source, particularly in tropical regions, such as the Philippines, where extensive mango cultivation is done.

LMP can form a polymer network with calcium ions in the form of gel beads (CP beads) via the extrusion process. The bead structure encloses the cells in a cage-like matrix while still allowing osmotic

movements of substrates and products (Rathore et al., 2013; Sriamornsak, 1998). The CP beads can be characterized by their swelling ratio, mean particle diameter, and sphericity. The swelling behavior of the calcium pectate beads indicates its calcium-binding stability. According to Partovinia and Vatankhah (2019), the size and sphericity of the beads are essential elements in mass transfer phenomena. Beads with smaller diameters tend to improve mass transfer efficiency and cell activity as they provide a higher surface volume ratio (Panesar et al., 2007; Voo et al., 2015). Moreover, beads with high sphericity offer uniform mass transfer, uniform cell distribution, and high stability. Zhao et al. (2018) mentioned that the average size and sphericity beads are influenced by the DE and pectin concentration.

Pectin concentration has a significant influence on the CP beads' properties. It also serves as a significant parameter that affects the leakage of the entrapped cells (Nazos & Ghanotakis, 2021). Leakage minimization is crucial as it leads to increased cell density, enhanced reusability, and improved fermentation capacity of the immobilized system. With that, this study aims to produce calcium pectate beads of varying pectin concentrations to be used as an immobilization matrix in the entrapment of yeast (*Saccharomyces cerevisiae*). Specifically, the study determines the effect of different mango pectin concentrations (3%, 5%, 7% w/v) on the obtained calcium pectate beads in terms of size, sphericity, swelling behaviour, and the leakage of the immobilized yeast cells.

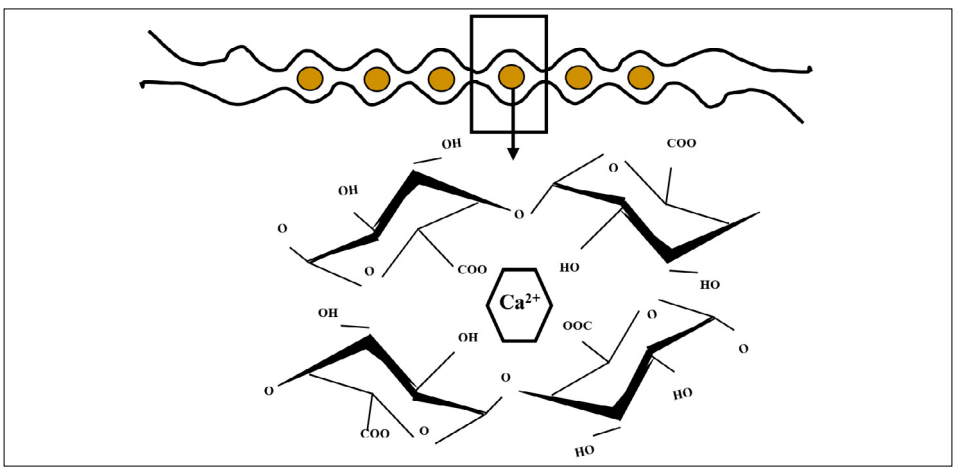

Figure 1. *Schematic representation of the "egg box" model as a mechanism of pectin gelation by calcium ions binding to polygalacturonate sequences of low-methoxyl pectin*

METHODS

Material

The low-methoxyl pectin was extracted from mango peels collected from a mango processing plant in Mandaue City, Cebu Philippines. Dry yeast of *Saccharomyces cerevisiae* strain (Eagle Instant Dry Yeast) was purchased at local stores in Cebu, Philippines. The following chemicals were used: 95% technical grade ethanol and 70% ethanol; 0.5 M hydrochloric acid (HCl) and sodium chloride (99.9% purity $NaCl$) from Ajax Finechem; calcium chloride (99.9% purity $CaCl_2$); trisodium citrate dihydrate ($Na_3C_6H_5O_7$) from Scharlau; and phenolphthalein and sodium hydroxide ($NaOH$) from HiMedia Laboratories. Analytical grade chemicals such as Folin-Ciocalteu reagent (2 M), D-galacturonic acid, and Bradford reagent were sourced from Sigma-Aldrich (Singapore).

Methods for Pectin Preparation

Pre-treatment of Mango Peels

Mango peels were rinsed, scraped to remove any leftover pulp, and were blanched (95°C) for 5 minutes, and soaked immediately in a cold bath for 2 minutes. The wet peels were sun-dried until the dried peels became brittle. The dried peels were powdered using a Thomas Wiley mill to obtain mango peel powder (MPP) with a size of 180 to 250 μm. Next, the defatting of MPP was done to remove lipids that may interfere with the pectin extraction process. The MPP was treated with 95% technical grade ethanol to dissolve the lipids in MPP with a 100 g MPP to 1 L ethanol ratio. The defatting was done in an incubator shaker (New Brunswick 25) at 150 rpm and 70°C for 2 hours. The defatted MPP was filtered using a taffeta cloth and was air-dried until the remaining ethanol was removed.

Simultaneous Extraction and Deesterification

To extract and, at the same time, deesterify pectin from mango peel powder (MPP), simultaneous extraction and deesterification (SED) were used. This method was adapted from the studies of Sayed et al. (2022) and Koubala et al. (2008) where pectin of near low-methoxyl range was obtained using an acidic solvent. The acid simultaneously extracts and lowers the degree of esterification (DE) of pectin. Modifications in extraction time were done to effectively lower the DE and obtain LM pectin. Four 2-L Erlenmeyer flasks were prepared, and 1150 mL water was added to each flask. This water was acidified to a pH of 1.5 using 0.5 M HCl and 130 g of MPP was added to each flask. The addition of MPP changed the pH, and then the pH was readjusted back to 1.5 using the same acid. The sealed flasks were put in an incubator shaker (New Brunswick G25) operated at a temperature of 50°C at 150 rpm, with each having an operating time of approximately 50 hours to get a degree of esterification of below 50%. After shaking with the set time, the contents in the flask were filtered and the filtrate containing the pectin was collected.

Precipitation, Isolation, Purification and Drying of Crude Pectin

To separate pectic substances from the filtrate collected after SED, the filtrate was mixed with 95% technical grade ethanol using a 1:3 volume ratio of filtrate to ethanol. The mixture was mixed thoroughly for 30 minutes. As pectin is insoluble in ethanol, pectic substances are precipitated. After precipitation, the isolation of pectin was done by filtration using a taffeta cloth. The pectin was purified by washing thrice with 200 mL ethanol. The extracted pectin was oven-dried at 40°C.

Analysis of Obtained Mango Pectin

The characteristics of the extracted mango pectin were determined analytically in terms of its degree of esterification (DE), galacturonic acid (Gal-A) content, and average molecular weight. The DE was determined via titrimetric analysis using the methods of Singthong et al. (2004). The Gal-A content of the obtained dried crude pectin was measured by the spectrophotometric approach presented by Anthon and Barrett (2008). In determining the molecular weight, intrinsic viscosity measurement was used using the methods specified in the study of Kar and Arslan (1999).

Preparation of pectin solutions

The methods for solution preparation were adapted from Zhao et al. (2018) and Damayanti et al. (2021) with modifications in yeast preparation. The yeast concentration in all pectin solutions was 2% dry weigh yeast/volume of solution (Kostov et al., 2010). Each of the three 250 mL-Erlenmeyer flasks was

added with 70 mL 0.9% NaCl aqueous solution. A sufficient amount of the extracted pectin was added to the three flasks to produce 3%, 5%, and 7% (w/v) pectin solutions. The flasks were subjected to the incubator shaker for 10 minutes at 37°C. After pectin dissolution, the contents of each of the flasks were transferred into three different 100 mL volumetric flasks. To be used in each pectin solution, 2 grams of instant dry yeast were hydrated using 20 mL 0.9% NaCl aqueous solution at 38°C (Pereira et al., 2014). The hydrated yeast was added to each of the flasks. Using the same NaCl solution, each flask was filled to the 100 mL mark, which produced 3%, 5%, and 7% (w/v) pectin solutions. Additionally, pectin solutions (3%, 5%, and 7% w/v) without the addition of yeast were also prepared using the same procedures mentioned above.

Preparation of calcium pectate beads

Immobilization of yeast by entrapment is expected to occur in this step upon gelation of the pectin solution to form calcium pectate (CP) beads. The method for pectin gelation to form CP beads is a one-step process adapted from Bokkhim et al. (2018) with modifications on the needle size, dropping height, and $CaCl_2$ concentration used. The experimental setup for CP bead formation is shown in Figure 2.

The prepared pectin solutions were extruded dropwise at 0.33 mL/min using a 21G needle (0.819 mm O.D. & 0.514 mm I.D.) using a syringe pump (CA08-100) into a 0.2 M $CaCl_2$ solution (gelling bath) stirred at low speed (60 rpm) at ambient temperature and pressure. The falling height was 10 cm to guarantee the formation of spherical beads. The initial depth of the gelling solution was set also to be 10 cm. The CP beads were collected from the gelling bath after 30 minutes of gelling (Bokkhim et al., 2016). The collected CP beads were washed with distilled water to remove any impurities present while being filtered with a taffeta cloth.

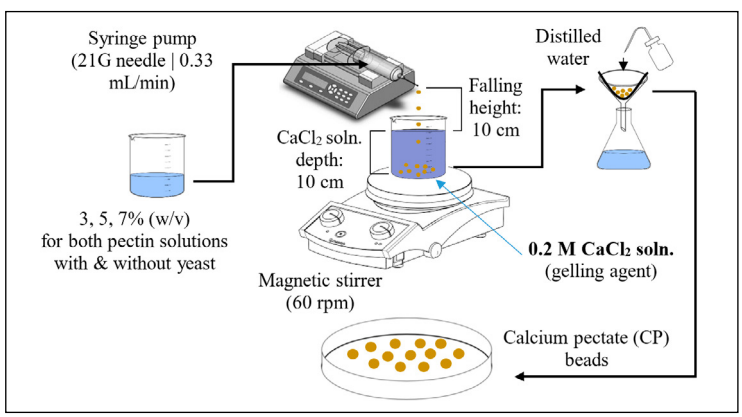

Figure 2. *Experimental setup for the entrapment of yeast cells in calcium pectate (CP) beads*

Calcium pectate bead characterization

Size and sphericity

The size and sphericity of the CP beads with and without immobilized yeast were characterized in terms of mean particle diameter and sphericity factor (SF) (Zhao et al., 2018). An image of 30 CP beads from each trial of different pectin solutions was captured together with a piece of ruler to serve as a reference to

the measurement. The maximum diameter (d_{max}) of the CP beads and the diameter perpendicular to that (d_{perp}) were measured using the AutoCAD measuring tool. The obtained actual diameter measurements were used in calculating the mean particle diameter and the SF of the CP beads using Equation 1 and Equation 2, respectively. CP beads exhibiting a sphericity factor below 0.05 were categorized as spherical.

$$mean\ particle\ diameter = \frac{d_{max} + d_{perp}}{2} \qquad \text{Equation 1}$$

$$sphericity\ factor\ (SF) = \frac{(d_{\max}) - (d_{perp})}{(d_{\max}) + (d_{perp})} \qquad \text{Equation 2}$$

Swelling behavior

The swelling behavior of the CP beads with and without yeast was characterized in terms of swelling ratio at different incubation times (Zhao et al., 2018). Two grams of CP beads from each trial of different pectin solutions were dried at 38°C for 20 hours and weighed to obtain its dry weight (W_d). The CP beads were incubated in the incubator shaker with 50 ml distilled water. The samples were weighed after one, three, and five days of incubation to obtain (W_t). Then, the swelling ratio was determined using Equation 3.

$$swelling\ ratio\ = \frac{W_t - W_d}{W_d} \times 100\% \qquad \text{Equation 3}$$

Cell leakage determination of immobilized yeast in calcium pectate beads

The leakage determination of immobilized yeast involved (1) the total cell count of immobilized cells in CP beads and (2) the cell count of leaked cells in sterilized water where these CP beads are submerged and shaken. Both cell counts were done via hemocytometry specifically using a Neubauer-improved hemocytometer. The cell counting of yeast was done with a basis of 1g CP beads each made from varying pectin concentrations (3%, 5%, 7% w/v). The ratio of the two cell counts determined the cell leakage. It was expressed as percent cell leakage as in Equation 4.

$$\%\ cell\ leakage\ = \frac{leaked\ cell\ count\ per\ gram\ CP\ beads}{immobilized\ cells\ per\ gram\ CP\ beads}\ \text{x}\ 100\% \qquad \text{Equation 4}$$

To facilitate the counting of the total immobilized cells, the entrapped yeast cells were freed from the CP beads. One gram of CP beads was washed with a sterile saline solution. The CP beads were completely dissolved in 160 mL sterile 0.1 M sodium citrate with continuous stirring for 10 minutes at room temperature and pH 6 (Kongruang & Wonganu, 2009). When sodium citrate is added to the calcium pectate beads, it binds to the calcium ions, breaking the cross-linking bonds that hold the beads together, and causing them to dissolve, forming a yeast suspension. A small amount of the yeast suspension ($10\ \mu L$) was pipetted and loaded in a Neubauer-improved hemocytometer.

For the cell count of leaked yeast, 1g of CP beads was soaked in a 12.5 mL sterile distilled water using an Erlenmeyer flask. The flask was covered with its cork and shaken using an incubator shaker at 250 rpm, 30°C at different incubation times of 24, 48, 72, and 92 hours. After the shaking duration, a small amount ($10\ \mu L$) of the water (where the beads are shaken) was pipetted and loaded in a Neubauer-improved hemocytometer.

RESULTS AND DISCUSSIONS

Pectin Characterization

One of the intrinsic properties of pectin is its DE which indicates the extent to which the Gal-A units are esterified with methanol in the pectin molecule. LMP is commonly used in the food industry as a gelling agent (Jha & Kumar, 2019). The extracted pectin in this study has 42.5% DE and falls within the low-methoxyl range, which suggests that it may have gelling properties. Another important parameter for pectin is its molecular weight, which determines its chain length and can affect its functional properties. The pectin molecule's chain length should not be less than 2800 g/mol, as shorter pectin chains with low molecular weights could not form gel beads (Capel et al., 2005). Additionally, the extracted pectin has a molecular weight of 41900 g/mol, which is well above the suggested value by Fraeye et al. (2010).

The Gal-A content is also an important characteristic of pectin, as it indicates the amount of free GA molecules that are available for crosslinking with divalent cations such as calcium ions. The pectin in this study has a Gal-A content of 340.05 mg GA/g pectin. Compared to commercial pectin, which has a GA of around 650 mg GA/g pectin and a molecular weight ranging from 100,000 to 300,000 g/mol pectin, the commercial pectin has a higher GA content and molecular weight than the pectin extracted in this study (Yapo & Koffi, 2014). However, the pectin was still able to form beads when combined with a calcium chloride solution. The characteristics of the mango pectin extracted in this study being it is a low methoxyl pectin with considerably high molecular weight and moderate galacturonic acid content, make it a promising candidate for further investigation in applications such as cell immobilization.

Characterization of Calcium Pectate Beads

Size and Sphericity

The size and sphericity of the calcium pectate (CP) beads affect several important parameters, such as mass transfer, nutrient supply, and mechanical stability (Damayanti et al., 2021), which influence the effectiveness and sustainability of the immobilized cells (Zhao et al., 2018). Hence, accurate measurement and control of the size and sphericity of the CP beads is essential to achieve optimal results in cell immobilization applications of pectin. Controlling the size and shape of the CP beads is difficult as several factors affect these properties including pectin concentration, $CaCl_2$ concentration, gelling time, extrusion rate, and nozzle diameter (Lee et al., 2014). In this study, the $CaCl_2$ concentration, gelling time, extrusion rate, and nozzle diameter were set constant with only the pectin concentration varied in three different values. The produced CP beads with and without yeast are shown in Figure 3 and Figure 4 with their mean particle diameter tabulated in Table 1.

The visual characteristics of the CP beads in Figure 3 and Figure 4 suggest that there is no significant difference in size between the CP beads formed with and without yeast from the three pectin concentrations. The mean particle diameter of the CP beads varied from 3.0885 ± 0.0498 mm to 3.4315 ± 0.0353 for those with yeast and 3.0323 ± 0.117 mm to 3.1130 ± 0.150 for those without yeast. Additionally, there is no observed trend in the mean particle diameter of the CP beads upon increasing the pectin concentration. In line with this, the One-way ANOVA resulted in high p-values (p-value>0.05) which indicate that there is no correlation between the pectin concentration and the mean particle size of the CP beads with yeast ($F(2) = 2.741485$, $P = 0.142656$) and without yeast ($F(2) = 0.618086$, $P = 0.570068$).

On the other hand, the CP beads with and without yeast from 5% w/v and 7% w/v pectin solutions exhibit a more spherical shape unlike the CP beads formed from 3% w/v pectin solution. This visual observation is consistent with Figure 5, in which the effect of pectin concentration on the (SF) of CP beads is shown.

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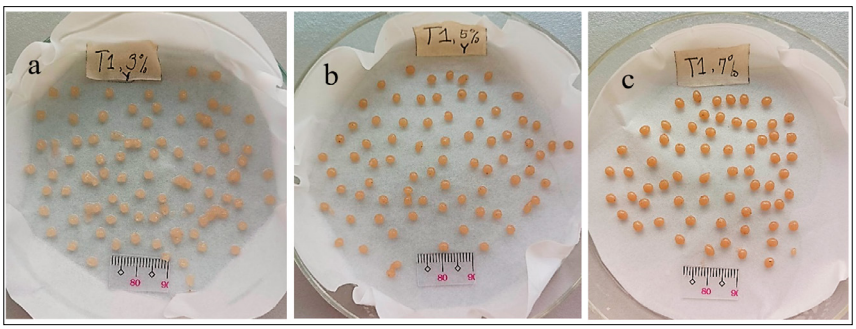

Figure 3. *CP beads with yeast formed from (a) 3% w/v, (b) 5% w/v, (c) 7% w/v pectin solution using 0.2 M $CaCl_2$ at 60 rpm, 0.33 mL/min, 21G needle, and cured for 30 minutes*

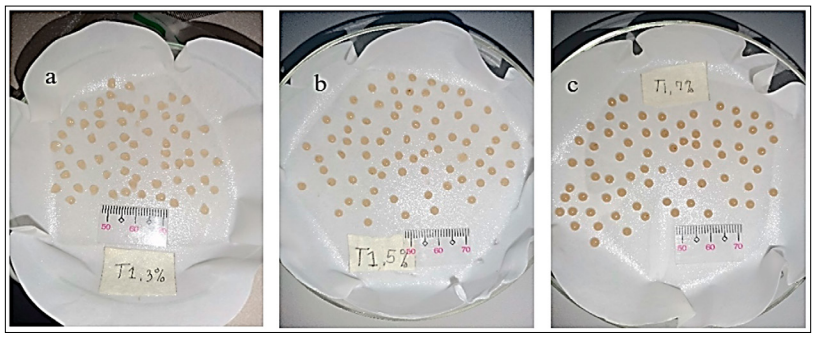

Figure 4. *CP Bead without yeast formed from (a) 3% w/v, (b) 5% w/v, (c) 7% w/v pectin solution using 0.2 M $CaCl_2$ at 60 rpm, 0.33 mL/min, 21G needle, and cured for 30 minutes*

Table 1. *Mean particle diameter of CP beads with and without yeast*

Pectin concentration (% w/v)	Mean particle diameter (mm)	
	CP beads with yeast	CP beads without yeast
3	3.1417 ± 0.328	3.0323± 0.117
5	3.0885 ± 0.0498	3.0410± 0.0772
7	3.4315 ± 0.0353	3.1130± 0.150

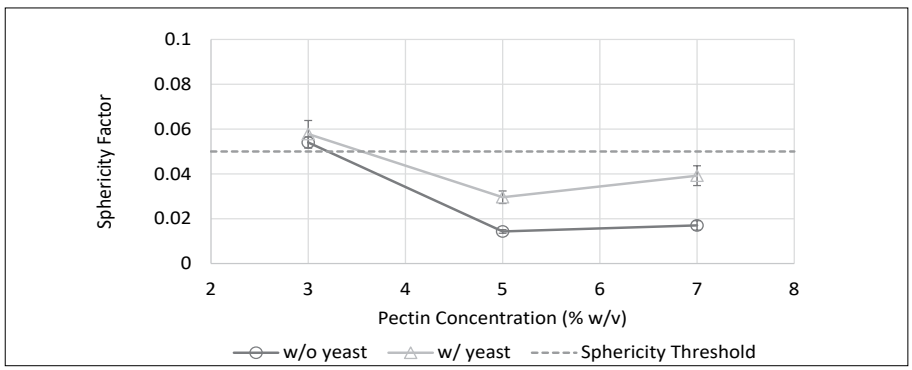

Figure 5. *Effect of pectin concentration (% w/v) on the sphericity factor of CP beads formed using 0.2 M $CaCl_2$ at 60 rpm, 0.33 mL/min, 21G needle, and cured for 30 minutes*

As observed in Figure 5, only CP beads formed from high pectin concentrations (5% w/v and 7% w/v) exhibit a spherical shape (SF < 0.05). The SF of the CP beads are SF = 0.058 ± 0.0060 (with yeast) and SF = 0.054 ± 0.0025 for the 3% w/v pectin concentration; SF = 0.030 ± 0.0028 (with yeast) and SF = 0.014 ± 0.0008 (without yeast) for the 5% w/v pectin concentration; and SF = 0.039 ± 0.004 (with yeast) and SF = 0.017 ± 0.002 (without yeast) for the 7% w/v pectin concentration. The observed differences in the shape of the gel beads can be explained by the variation in viscosity that resulted from different pectin concentrations. According to the studies, by Bokkhim et al. (2018) and Voo et al. (2015), increasing the polysaccharide concentration will result in an exponential increase in the viscosity of the solution – the viscosity increased by 253% by increasing the pectin concentration from 5% w/v to 7% w/v. These studies also found that to obtain spherical gel beads, the viscosity of the polysaccharide solution must be high enough to counteract any shape distortion that may occur when the beads enter the gelling bath. Hence, the 5% w/v and 7% w/v pectin solutions with and without yeast used in this study likely had a high enough viscosity to produce spherical CP beads, while the 3% w/v pectin solution did not.

The correlation between pectin concentration and SF observed in the study is further supported by the results of the One-Way ANOVA. The statistical analysis revealed significant differences in SF between CP beads made from different pectin concentrations with yeast [$F(2) = 9.670691$, $P = 0.013273$] and without yeast [$F(2) = 128.5727$, $P = 0.0000119$]. These findings suggest that pectin concentration has a significant effect on the SF of CP beads, regardless of the presence of yeast.

Swelling Ratio

The swelling behavior of the CP beads is another important parameter to consider as it relates to the intrinsic rigidity and the degree of cross-linking of the gel network (Sriamornsak & Kennedy, 2008), and it can influence its ability to absorb essential nutrients and the integrity of the immobilized system (Zhao et al., 2018). The swelling ratio of the CP beads at different incubation periods were obtained and plotted in Figure 6.

The swelling ratio (SR) of the CP beads varies with pectin concentration and incubation period as observed in Figure 6. The CP beads made with the highest pectin concentration (7% w/v) have the lowest SR at all incubation periods (1, 3, and 5 days). Conversely, the CP beads made with the lowest pectin concentration (3% w/v) have the highest SR at all incubation periods. This trend is due to the differences in the degree of cross-linking between pectin molecules and divalent ions during ionotropic gelation, which depends on the pectin concentration (Sriamornsak, 1998). The same observation was made in the study of Sriamornsak and Kennedy (2010), where the relative weight change of alginate gel beads decreased by 77% after increasing the concentration of the alginate solutions from 1.5% w/v to 5% w/v.

The CP beads with and without yeast exhibit a rapid increase in swelling ratio on day 1, followed by a gradual decrease in the rate of increase on days 3 and 5. This swelling behavior has been observed in previous studies by Günter et al. (2014) and Sriamornsak & Kennedy (2008). The initial upsurge in the swelling ratio on day 1 is due to the high concentration of water molecules in the surrounding medium, which readily diffuse into the beads. However, as the beads continue to absorb water, the concentration gradient between the beads and the medium decreases, resulting in a slower rate of water uptake. The increasing density of the gel network in the beads also creates resistance to further swelling, contributing to the decrease in the rate of increase in swelling ratio (Günter et al., 2014). Additionally, as the beads swell and their size increases, the distance for water to diffuse into the beads also increases (Sriamornsak & Kennedy, 2008).

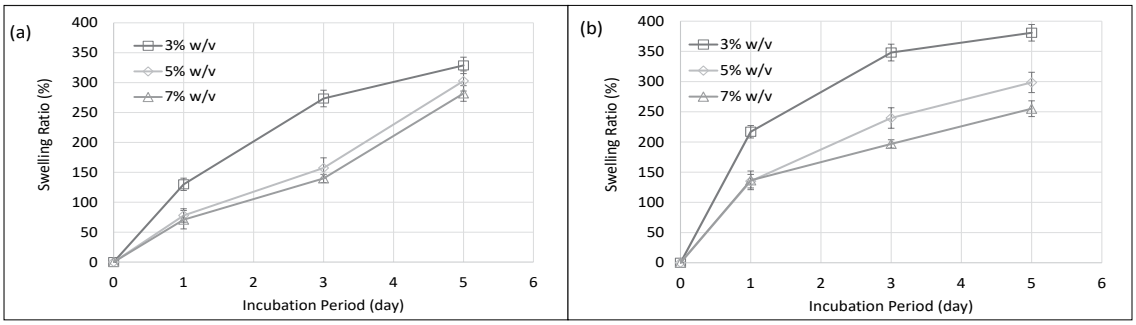

Figure 6. *Swelling Ratio (%) of CP beads (a) with yeast and (b) without yeast over incubation periods (1, 3, and 5 days) at 37°C*

The two-way ANOVA revealed that both pectin concentration and incubation period significantly impacted the swelling ratio (SR) of calcium pectate (CP) beads. In particular, the results showed that the pectin concentration and incubation period had a significant effect on the SR of both CP beads with and without yeast, as evidenced by the very low p-values [F(2) = 29.67385, P = 2E-06 for CP beads with yeast and F(2) = 95.6167, P = 2.58E-10 for CP beads without yeast for pectin concentration; F(2) = 184.7066, P = 1.01E-12 for CP beads with yeast and F(2) = 141.2611, P = 9.92E-12 for CP beads without yeast for incubation period. These findings indicate that both factors are significant determinants of the SR of CP beads. Moreover, the interaction between pectin concentration and the incubation period was also found to be significant: [F(4) = 4.032438, P = 0.016584 for CP beads with yeast and F(4) = 2.988935, P = 0.046896 for CP beads without yeast]. This suggests that the impact of the interaction between the two factors (pectin concentration and incubation period) on the SR of CP beads is significant.

Cell leakage of yeast in calcium pectate beads

One of the major challenges in immobilizing cells is the occurrence of cell leakage from within the beads, which can reduce the efficiency and effectiveness of the immobilization process. In this study, the effect of pectin concentration in calcium pectate beads on the cell leakage of immobilized yeast was investigated. The immobilized system was subjected to a 250-rpm agitation in the incubator shaker over a 96-hour time duration and leaked cells were obtained at a 24-hour interval. The resulting leakage is shown in Figure 7. Cell leakage is expressed as % cell leakage which is calculated as the ratio of leaked cells and the total immobilized cells per mass of CP beads.

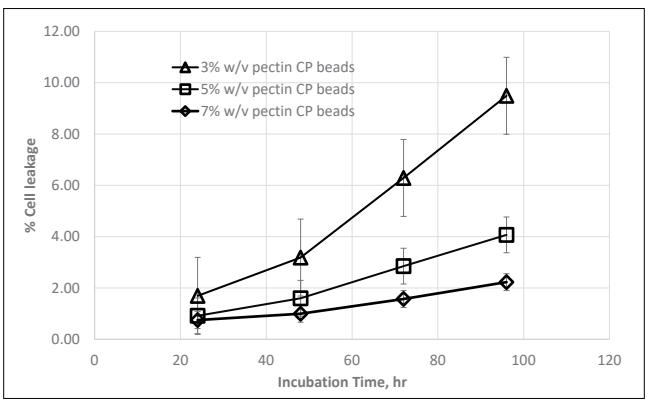

Figure 7. *Percent cell leakage of yeast in calcium pectate beads for pectin concentrations of 3%, 5%, and 7% submerged in 12.5 mL sterile water and shaken in an incubator shakerat 250 rpm and 30°C*

The results indicated that the cell leakage of the immobilized yeast varied with the pectin concentration and the incubation time. The highest pectin concentration used in the study, 7% w/v, resulted in the lowest cell leakage percentages, ranging from 0.75% to 2.23% over the 96-hour incubation period. In contrast, the lowest pectin concentration used, 3% w/v, resulted in the highest cell leakage percentages, ranging from 1.69% at 24 hours to 9.49% at 96 hours. The intermediate pectin concentration, 5% w/v, resulted in intermediate cell leakage percentages, ranging from 0.91% to 4.07% over the 96-hour incubation period.

A two-way ANOVA was conducted to determine if there is a significant relationship between the pectin concentration, incubation time, and cell leakage of CP beads. The result shows that the pectin concentration significantly affects the cell leakage (p-value = 6.7×10^{-21}). Moreover, the incubation time indicated a significant effect on the leakage (p-value = 1.4×10^{-21}). The interactions of these two independent variables were shown to be statistically significant as well (p-value = 3.7×10^{-15}). With small p-values, the findings show that the leakage of yeast cells is strongly affected by the pectin concentration within the time duration indicated. Lower pectin concentrations lead to a higher cell leakage, while higher pectin concentrations help reduce cell leakage. The leakage observed during the incubation period may be due to the gradual degradation of the pectate matrix, leading to the release of immobilized cells.

The resulting leakage trend in this study is consistent with previous studies on immobilization of other cell types using various polymers, such as alginate (Many et al., 2019), as shown in Table 2. The smallest matrix concentration of alginate beads, 1% w/v, showed the highest cell leakage. Both the 5% w/v alginate and pectin beads have comparable leakage after 32 and 48 hours, respectively.

Table 2. *Comparison of the leakage of alginate and pectin beads, their concentrations, bead shape, and specifications in the cell immobilization process*

Entrapment Matrix	Matrix concentration	Incubation time (hours)	Cell loading (cells/ mL solution)	% cell leakage	Reference
Alginate	5% w/v	216	2.0×10^7	0.22%	(Soo et al., 2017)
Alginate	-	24	1.0×10^9	< 1%	(Callone et al., 2008)
Alginate	1% w/v	32	-	12.2%	(Many et al., 2019)
	3% w/v	32	-	3.9%	
	5% w/v	32	-	1.3%	
Pectin	3% w/v	24	1.2×10^9	1.69%	[in this study]
	5% w/v	24	1.2×10^9	0.91%	
	7% w/v	24	1.2×10^9	0.75%	
Pectin	3% w/v	48	1.2×10^9	3.19%	[in this study]
	5% w/v	48	1.2×10^9	1.60%	
	7% w/v	48	1.2×10^9	0.99%	

At similar matrix concentrations and incubation times, the comparison between alginate and pectin shows that pectin beads have a slightly lower percentage of cell leakage per gram of beads compared to alginate beads. However, it should be noted that the data presented in this table is limited and may not represent the overall performance of pectin and alginate matrices.

Abdul Manaf et al. (2021) mentioned that the cell leakage of the beads is attributed to the bead disintegration and stability over time, which is affected by the concentration of the matrix as well as cell loading. Their study stated that the cell loading as well as the matrix concentration were the most significant elements contributing to low cell leakage. In terms of cell loading, Soo et al. (2017) used a

significant cell loading of 2.0×10^7 cells per mL solution as shown in Table 2. Conversely, this study and the study of Callone et al. (2008) used higher cell loading of around 10^9 cells per mL solution. Low cell loading is associated with low cell density and the beads are less congested with cells, leading to a reduced leakage even if the study of Soo et al. (2017) had the longest incubation time implemented.

Sevda and Rodrigues (2014) used 1% to 5% (w/v) alginate concentrations as a matrix in immobilizing *Saccharomyces cerevisiae* for wine production and mentioned that lower matrix concentrations (1% to 3% w/v) had the problem of cell leakage while the beads with higher alginate concentrations showed good mechanical properties as opposed to the low concentrations. Moreover, the pore structure of the beads is affected by the composition of the matrix material and matrix concentration. Increasing the concentration of the matrix reduces the bead's pore size, which results in a decreased cell leakage. This trend between matrix concentration and cell leakage agrees with the results of this study using 3% to 7% (w/v) pectin concentrations as well as in the study of Many et al. (2019) using 1% to 5% (w/v) alginate concentrations as shown in Table 2.

CONCLUSION

Pectin concentration has no significant impact on the size of CP beads but does play a role in their sphericity. Specifically, pectin concentrations of 5% w/v and 7% w/v were found to produce spherical CP beads (SF<0.05), while a concentration of 3% w/v did not (SF>0.05). The higher pectin concentrations (5% w/v and 7% w/v) likely had a high-enough viscosity which resulted in the formation of spherical CP beads. Conversely, the increase in pectin concentration from 3% w/v to 5% w/v was associated with a 30.15% decrease in swelling ratio for both CP beads with and without yeast, while increasing the pectin concentration further from 5% w/v to 7% w/v resulted in a 9.01% and 11.16% reduction in swelling ratio for CP beads with and without yeast, respectively. From this, it can be concluded that at higher pectin concentrations, the swelling ratio of the CP beads will likely be lower than that of CP beads from lower concentrations.

For cell leakage, results showed that the leakage of the immobilized yeast varied with pectin concentration and incubation time, with the highest pectin concentration (7% w/v) resulting in the lowest cell leakage percentages ranging from 0.75% to 2.23% over the 96-hour incubation period. The study found that lower pectin concentrations (3% w/v) lead to higher cell leakage (9.49%), whereas higher pectin concentrations reduce cell leakage. By adjusting pectin concentrations, this study provides possible ranges in improving the properties of calcium pectate beads and in reducing cell leakage of yeast. The results of this study can serve as a future reference to enhance stability, and economic viability and improve the overall efficiency of the immobilization process using locally available material in applications such as food and beverage production.

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