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MICROENCAPSULATION OF *LACTOBACILLUS ACIDOPHILUS* 5 WITH ISOMALTO-OLIGOSACCHARIDE

Chan, L.Y.[1] and Pui, L.P.[1]✉

[1]*Department of Food Science with Nutrition, Faculty of Applied Sciences, UCSI University, No. 1, Jalan Menara Gading, UCSI Heights, 56000 Cheras, Kuala Lumpur, Malaysia.*
✉*puilp@ucsiuniversity.edu.my*

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ABSTRACT

Co-extrusion microencapsulation of *Lactobacillus acidophilus* 5 (La-5) was performed using isomalto-oligosaccharide (IMO) as prebiotic, alginate as shell material and chitosan as the coating. The optimization of alginate (1.3% (w/v) to 1.7% (w/v)) and IMO concentration (1.0% (w/v) to 5.0% (w/v)) was evaluated based on bead size and microencapsulation efficiency of La-5. Subsequently, the chitosan-coated alginate with or without IMO were subjected to sequential digestion. It is found that 3.0% (w/v) IMO and 1.5% (w/v) alginate were the optimal concentration based on microencapsulation efficiency (MEE). The morphology of the beads containing IMO was found to be smooth and spherical, with diameter of 622.00 µm. The addition of IMO and chitosan are effective in protecting La-5 under gastric conditions but not effective in protecting the viability of La-5 under intestinal digestion.

1.Introduction

In recent years, research on probiotics as nutraceuticals and functional food has received increasing attention globally. Probiotics are bacteria defined as "live microorganisms in which when administered in adequate amounts confer a health benefit on the host" (Siang et al., 2019). Probiotics are used as food supplements as they can enhance the immune system and improve protection in terms of gastrointestinal health against pathogens (Chaikham et al., 2012). In addition, consumption of probiotics has been associated with several health benefits such as boosting immune function, maintenance of mucosal integrity, treat atopic and allergic diseases (Liserre et al., 2007).

Some probiotics might lose viability in the gastrointestinal tract since they are sensitive to low acidic conditions, presence of trypsin, pepsin, and bile salts in the stomach (Sahadeva et al., 2011). Hence, to have beneficial effects to the body, the probiotics must survive through these harsh conditions (Martín et al., 2015).

Prebiotics are non-digestible food components that are stimulating the growth of beneficial colonic bacteria of the host (Siang et al., 2019). Probiotics incorporated with prebiotic in functional foods are known as synbiotics (Roberfroid, 1998). With prebiotic consumption, it is reported that it can prevent colon cancer, lower cholesterol levels, and reduce diarrhea (Patel and Goval, 2012). Fructooligosaccharides (FOS), galactooligosaccharides (GOS), inulin, and lactulose are common prebiotics incorporated into food.

Isomaltooligosaccharide (IMO) are one of the emerging prebiotics, naturally found in fermented food such as soy sauce, miso, or sake and honey (Gourineni et al., 2018). Health claims of IMO reported includes the activation of the immune system, improving liver and

kidneys function, enhancing the resistance to diseases as well as improving lipid metabolism (Li et al., 2009).

Microencapsulation is a process that encapsulates sample with an encapsulating matrix or membrane (Krasaekoopt et al., 2004). It protects probiotic bacteria against the harsh condition in the digestive tract (Etchepare et al., 2016). In microencapsulation, the semipermeable membrane surrounds the liquid core. This allows the excretion of secondary metabolites with penetration of oxygen supply to reach entrapped live probiotic bacteria.

Microencapsulation has been a prominent method for protecting probiotic from harsh conditions (Ozyurt and Ötles, 2014). Studies on the encapsulation method of probiotic includes extrusion, spray-drying, and emulsion techniques (Chew et al., 2019; Gandomi et al., 2016). Co-extrusion method can produce uniform and smaller size beads as compared to extrusion technique (Krasaekoopt et al., 2004). However, there are fewer reports on application of co-extrusion technique to encapsulate probiotic as compare to extrusion techniques (Silva et al., 2016; Olivares et al., 2017). In our previous work, co-extrusion microencapsulation of probiotic such as *Lactobacillus plantarum* 299v, *Lactobacillus rhamnosus* GG, *Lactobacillus acidophilus* NCFM and *Bifidobacterium animalis* subsp. lactis BB-12 (Lai et al., 2020; Ng et al., 2019; Siang et al., 2019, Yee et al., 2019; Yong et al., 2020).

This study aims to produce microencapsulated probiotic bacteria, *Lactobacillus acidophilus* 5, using the co-extrusion technique. The effectiveness of prebiotic and co-extrusion microencapsulation in protecting La-5 from the gastrointestinal condition were investigated.

2. Materials and methods

2.1. Materials

2.1.1. Samples

Lactobacillus acidophilus La-5 was purchased from Bio-Life, Malaysia. All the chitosan and sodium alginate were of food-grade, while the chemicals and reagents were of analytical grade.

2.2. *Microencapsulation of LA-5 using co-extrusion technique*

Microencapsulation of La-5 was carried out using Büchi Encapsulator B-390 (Büchi, Switzerland) through the co-extrusion method as described by Ng et al. (2019) with modification. The core fluid (comprised of La-5 suspended in PBS with or without IMO) and shell fluid (sodium alginate solution) were added into two separate pressured bottles connecting to the Büchi Encapsulator B-390. During the microencapsulation process, core fluid and shell fluid were pumped simultaneously through the concentric nozzles with a diameter of 200 μm (inner nozzle) and 300 μm (shell nozzle) by the air pressure of 600 mbar to give a core-shell fluid stream. The vibration frequency of the nozzle was set at 300 Hz, with an amplitude of 3 and a voltage of 1.5 kV.

2.2.1. Optimization of Alginate

The optimization process was carried out using different concentrations of alginate from 1.3% (w/v) to 1.7% (w/v), with the concentration of calcium chloride fixed at 2.0% (w/v). The alginate beads were determined based on the microencapsulation efficiency of probiotics and bead size.

2.2.2. Optimization of Isomalto-oligosaccharide (IMO)

The optimization process was carried out using different concentration of IMO from 1.0% (w/v) to 5.0% (w/v), with a concentration of alginate fixed at 1.5% (w/v) (Siang et al., 2019). The optimum concentration of IMO was determined based on bead size and the viable cell counts in colony-forming unit per milliliter (CFU/mL) (Equation 1). Microencapsulation efficiency was calculated using Equation 2.

Colony forming unit (CFU/mL) = (Average number of colonies)/(Dilution factor × volume plated) (1)

Microencapsulation efficiency (%) = ($Log_{10}N$/ $Log_{10}N_0$) x 100 (2)

Where N represents the number of microencapsulated probiotics released from beads, and N_0 represents the number of probiotics in the initial microbial suspension.

2.2.3. Morphology and size of bead

The morphology and mean diameter of 10 randomly selected beads were determined and size measured using an optical microscope (Olympus, Japan), with x100 magnification, fitted with a micrometer scale (Lai et al., 2020).

2.3. *Sequential digestion of La-5*

Sequential digestion of La-5 was adapted from method reported by Yee et al. (2019) with slight modification. About 1 g of beads or 1 mL of free cells were added to 15 mL falcon tube (BD FalconTM, USA) containing 9 mL of sterile SGJ at pH 2.0. Simulated gastric juice (SGJ) consists of 3.5 mL of hydrochloric acid (Merck KGaA, Germany), 1 g of sodium chloride (R&M Chemicals, UK) in 500 mL distilled water, with pH adjusted to 2.0. It is then autoclaved at 21°C for 15 min, before adding 1.6 g of pepsin (Chemsoln, India).

The mixture was then incubated at 37°C and agitated gently at 150 rpm and for 1 hour and 2 hours in the incubator. After 1 hour and 2 hours exposures, SGJ was removed by centrifugation at 4200 rpm, 4°C for 10 min to test for the viability of La-5. After incubation in SGJ for 2 hours, the beads or free cells were transferred into 9 mL of SIJ. Simulated intestinal juice (SIJ) was prepared, according to Yong et al. (2020), with modification. SIJ consisted of 3.4 g potassium dihydrogen phosphate (Bendosen, Germany), 95 mL of sodium hydroxide (Merck KGaA, Germany) in total of 500 mL solution, adjusted to pH 7.5. It was then sterilized at 121°C for 15 min before adding 3 g of bile salt (Chemsoln, India)

The mixture was then incubated at 37°C for 1 hour and 2 hours with constant agitation at 150 rpm in an incubator. After incubation, the mixture was centrifuged at 4200 rpm, 4°C for 10 min, and the SIJ discarded. Before cell enumeration, the filtered beads were washed with sterile PBS to remove the excess SIJ solution. 9 mL of 4.0% (w/v) tri-sodium citrate (Merck KGaA, Germany) was added and followed by vortexing to release bacteria from retrieved beads. For cell enumeration, an aliquot of 0.1 mL of the mixture was spread on the MRS agar plate and incubate at 37°C for 24 hours. The viable cell counts for microcapsules and free cells were expressed as logarithm colony forming unit per gram (log CFU/g) and logarithm colony forming unit per milliliter (log CFU/mL), respectively. Survivability (%) of probiotic after exposure to SGJ and SIJ was calculated using equation 3.

Survivability (%) = $Log_{10}N_t$/ $Log_{10}N_0$ x 100 (3)

N_t is the number of viable cells in the free cell (CFU/mL) or beads (CFU/g) after exposure to SGJ or SIJ, and N_0 is the number of viable cells in the free cell (CFU/mL) or beads (CFU/g) at 0 hours.

2.4. *Statistical Analysis*

All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation. Data were analyzed using MINITAB 16 (Minitab Inc, Pennsylvania, USA). One-way analysis of variance (ANOVA) was carried out, with Tukey's HSD test to determine the significant difference set at $p \leq 0.05$.

3.Results and discussions

3.1. Optimization of concentration of alginate and IMO

The effect of alginate concentrations on size of bead produced and microencapsulation efficiency *Lactobacillus acidophilus* 5 (La-5)

were exhibited in Table 1. Chitosan and calcium chloride concentration were fixed at 0.4% (w/v) and 2.0% (w/v), respectively. It was found that there was no significant difference ($p>0.05$) on beads size among beads produced with different alginate concentration

From Table 1, it was observed that the microencapsulation efficiency increases when alginate concentration increased from 1.3% (w/v) to 1.5% (w/v), with the maximum microencapsulation efficiency (91.26%) at 1.5% (w/v). However, with further increase, the microencapsulation efficiency decreases slightly (87.90%) at alginate concentration 1.7% (w/v).

This is in agreement with Lotfipour et al. (2012), who reported that encapsulation of *Lactobacillus acidophilus* with alginate concentration 1.5% (w/v) to 2% (w/v) and calcium chloride concentration at 2.5% (w/v), have high microencapsulation efficiency at 98%.

Table 1. Effect of different concentration of alginate on bead size and microencapsulation efficiency of chitosan-coated microencapsulated La-5

Alginate (% (w/v))	Calcium chloride (% (w/v))	Diameter (μm)	Microencapsulation efficiency (%)
1.3	2.0	481.00 ± 88.6^{a}	83.39 ± 0.80^{c}
1.4	2.0	490.00 ± 94.4^{a}	84.70 ± 0.61^{c}
1.5	2.0	425.00 ± 70.4^{a}	91.26 ± 0.45^{a}
1.6	2.0	408.00 ± 141.8^{a}	89.58 ± 0.68^{ab}
1.7	2.0	411.00 ± 110.7^{a}	87.89 ± 0.67^{b}

$^{a-c}$Means ± standard deviations followed by different superscript letters within the same row are significantly different at $p \leq 0.05$, according to Tukey's test.

According to Mandal et al. (2010), increased alginate concentration could lead to higher encapsulation efficiency. The cross-linking of sodium alginate with calcium chloride produces a tight junction between the guluronic acid residues (Rajinikanth et al., 2003). The further increase of alginate concentration will then lead to increase in number of the cross-linking points formed, as there is greater availability of active calcium-binding sites in the polymeric chains (Mandal et al., 2010).

Table 2, on the other hand, shows the effects of different concentrations of isomalto-saccharide (IMO) on produced bead size and microencapsulation efficiency of La-5. The concentration of alginate was fixed at 1.5% (w/v).

Table 2. Effect of different concentration of isomalto-oligosaccharide (IMO) on bead size and microencapsulation efficiency of chitosan-coated microencapsulated La-5

IMO (% w/v)	Alginate (% w/v)	Diameter (μm)	Microencapsulation efficiency (%)
1.0	1.5	599.00 ± 87.1^{a}	87.93 ± 0.27^{b}
2.0	1.5	610.00 ± 103.7^{a}	84.75 ± 1.114^{b}
3.0	1.5	622.00 ± 87.4^{a}	94.42 ± 1.42^{a}
4.0	1.5	587.00 ± 123.3^{a}	91.84 ± 2.57^{a}
5.0	1.5	539.00 ± 111.5^{a}	86.71 ± 0.57^{b}

$^{a-c}$Means ± standard deviations followed by different superscript letters within the same row are significantly different at $p \leq 0.05$, according to Tukey's test.

From Table 2, it was found that there was no significant difference ($p>0.05$) among the beads size when produced with different IMO concentration. Studies by Haghshenas et al. (2015) and Ng et al. (2019) found that size of beads is not affected by increasing the prebiotic concentration. The bead produced in this study, which ranges from 539 to 622 μm, was in agreement with the work of Yee et al. (2019), who reported that the bead size of microencapsulated *Lactobacillus acidophilus* NCFM were in the range of 543 to 613 μm.

During microencapsulation, the addition of prebiotics will influence the size of bead, as observed in Table 2, as compared to Table 1. The diameter of beads without prebiotics was smaller than the beads with prebiotics for all encapsulated probiotics (Krasaekoopt and Watcharapoka, 2014).

From Table 2, it was also observed when the IMO concentration increases, the microencapsulation efficiency increased, with 3% (w/v) IMO showed the maximum microencapsulation efficiency (94.42%). La-5 produces oligo 1-6 glucosidase enzymes to hydrolyze IMO from the sucrase-isomaltase complex into D-glucose. IMO helps in stabilizing and increase probiotics resistance in gastrointestinal conditions.

3.2. Morphology, size and microencapsulation efficiency of bead

Figure 1 shows the size and shape of microcapsules measured with a scale micrometer, while bead size and microencapsulation efficiency of with and without IMO addition are shown in Table 3. From Figure 1, it was found that the bead produced was white and surrounded by a thin layer of the membrane. It was noted that the shape of the beads was generally spherical, with some oval shaped.

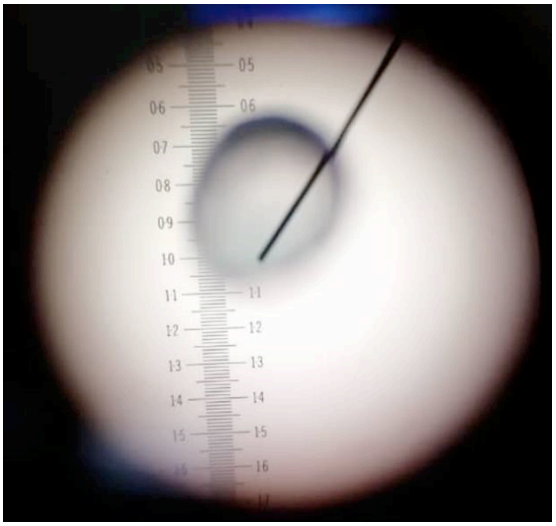

Figure 1. Shape and size of microcapsules measured with a stage micrometer (Ladd Research model 75545) using an optical microscope (Olympus model CX31)

Table 3. Effect of different concentration of isomalto-oligosaccharide (IMO) on bead size and microencapsulation efficiency of chitosan-coated microencapsulated La-5

Probiotic	Prebiotic	Diameter (μm)	Microencapsulation efficiency (%)
La-5	-	532.00 ± 0.07^{b}	95.32 ± 1.25^{a}
La-5	IMO	616.00 ± 0.09^{a}	92.51 ± 0.32^{b}

[a-b]Means ± standard deviations followed by different superscript letters within the same row are significantly different at $p \leq 0.05$, according to Tukey's test.

Bead produced using sodium alginate generally has a smooth surface (Solanki et al., 2013). This is important as bead with a broken surface (protrusion of cell) will lower the survivability of encapsulated cells because the chances of the free cell in the bead expose to the external environment and unable to protect encapsulated probiotic (Krasaekoopt and Watchapoka 2014).

The size of beads produced without prebiotic was smaller (532.00 μm) as compared to those with prebiotic (616.00 μm) (Table 3). Different concentrations and viscosity of alginate, calcium chloride solution, and the size of the nozzle could influence the difference in bead size (Solanki et al., 2013). High sodium alginate viscosity resulted in larger size of beads, as the higher coaxial air flow rate is needed to cut the droplet (Bhujbal et al., 2014). Also, increment in bead size can improve the stability of bead due to cross-linkage formation between divalent ions and alginate bead (Bhujbal et al., 2014).

Furthermore, the production of beads with micron-size create a smooth texture when it is added into food product (Fahimdanesh et al., 2012; Zanjani et al., 2012). Large beads size gives better protection on probiotics, but it will affect the sensory properties when it is used for consumption. While for the size of beads smaller than 100 μm, will prevent the coarse texture from being detected in mouth (Zanjani et al., 2017).

The beads sphericity may prevent the problem of cell overgrowth in encapsulated beads (McMaster et al., 2005). The range between 200 μm to 3000 μm of beads size can protect probiotic against harsh conditions (Heidebach et al., 2012). However, the study of showed that the co-extrusion technique is useful in producing beads which are smaller than the extrusion technique. Co-extrusion method produces bead size with smaller and more consistent in size, as compared to extrusion method (Krasaekoopt and Watchapoka 2014).

From Table 3, the microencapsulation efficiency of La-5 without IMO is higher (95.32%), as compared to the ones with IMO (92.51%). This is in agreement with the work of Ng et al. (2019) where the encapsulated beads without FOS has microencapsulation efficiency of 97%, as compared to beads with FOS (93%), as the prebiotic serves as food for probiotic to improve its growth.

3.3. Sequential digestion for free cell encapsulated La-5 with and without IMO

Sequential digestion is the continuous incubation of cell or beads in simulated gastric juice (SGJ) and subsequently incubate in simulated intestinal juice (SIJ) to mimic the human gastrointestinal condition (Minekus et al., 2014). 1 to 2 hours are required to digest the food in the stomach and followed by the partially digested food entering through the small intestine is 1.6 to 4.8 hours. However, the duration may be varied due to factors such as eating time, type of food intake, body type, and body size (Hellmig et al., 2006). Figure 2 shows the viability and survivability of free cells, encapsulated La-5 with and without IMO under sequential digestion.

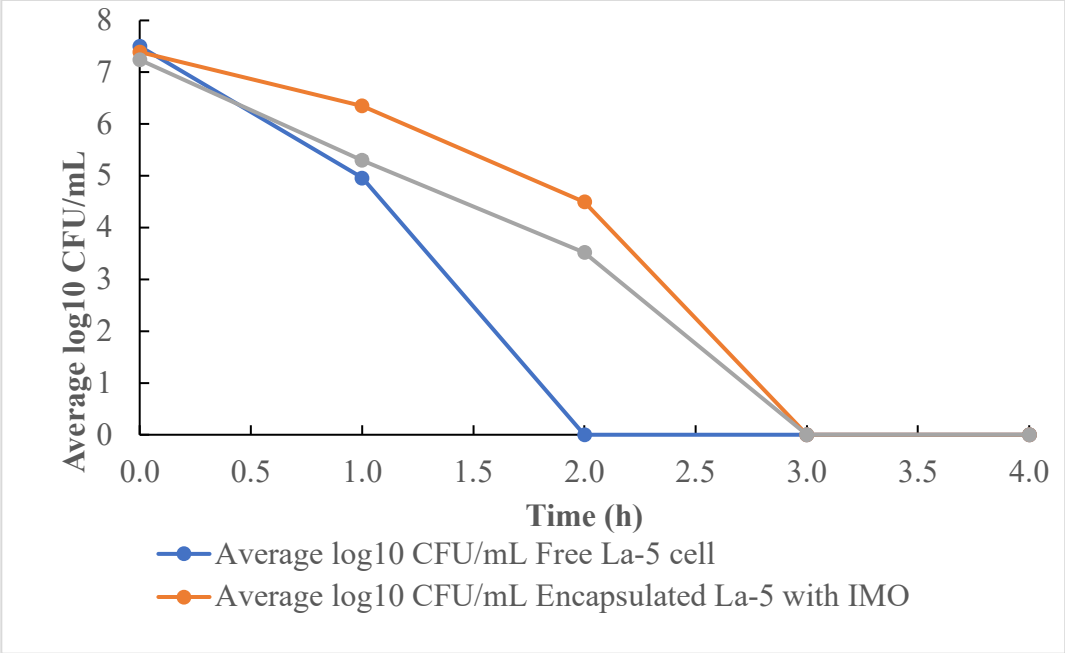

Figure 2. Average $\log_{10}$ CFU/mL and survivability of free cell encapsulated La-5 with and without IMO under sequential digestion.

From Figure 2, after exposure to acidic conditions, the viability of the La-5 free cell decreased notably compared to encapsulated La-5 with and without IMO. The viability of free La-5 cells dropped drastically by 33.83% from 100% (7.50 log CFU/mL) to 66.17% (4.96 log CFU/mL) after 1 hour of incubation in SGJ, followed by drastic reduction was below the detection limit (≤ 2 log10 CFU/mL) after exposure to 2 hours of simulated gastric juice (pH 1.2). This is because stomach probiotics in free form were easily damaged by stomach acid (Shi et al., 2013). Gebara et al. (2013) reported that La-5 cells reduced 3.54 log units after exposure to 5 hours of simulated gastric juice (pH 3.0) and simulated intestinal juice (pH 7.0).

The viable cell count of encapsulated La-5 without IMO showed a greater reduction, with viable cells count of encapsulated La-5 without IMO decreased significantly from 100% (7.24 log CFU/mL) to 73.25% (5.30 log CFU/mL) after 1 hour of exposure and to 48.63% (3.52 log CFU/mL) after 2 hours of exposure in SGJ.

On the other hand, the viable cells count of encapsulated La-5 with IMO reduced slightly from 100% (7.39 log CFU/mL) to 85.95% (6.35 log CFU/mL) after 1 hour of exposure in SGJ. However, after 2 hours of exposure in SGJ, the viable cell count dropped significantly to 60.92% (4.50 log CFU/mL).

Generally, encapsulated La-5 with IMO has best survivability, followed by encapsulated La-5 without IMO and lastly free La-5 cells. Alginate beads improve the physical and chemical characteristics of beads. The structure of the beads is stronger and denser, protecting probiotics and minimizing the exposure in gastrointestinal tract (Zhou et al., 1998; Krasaekoopt et al., 2004).

Chavarri et al. (2010) reported that alginate-chitosan were able to enhance microencapsulated *Lactobacillus gasseri* and *Bifidobacterium bifidum* maintained cell concentration at about 10^7 CFU/mL as compared to free cells, which decreased drastically in simulated gastric conditions after 2 hours of incubation. The electrostatic interactions between chitosan and alginate

happens when chitosan binds to alginate forms a strong membrane of the beads (Gaserod et al., 1998).

On the other hand, when subjected to SIJ, all 3 cells form (free cells, encapsulated La-5 with and without IMO) have 0 viability. Alginate is stable in low pH solution, which hydrochloric acid (HCl) presents in gastric juice but swelling in a weakly base condition (Annan et al., 2008). Krasaekoopt et al. (2004) also reported that encapsulated *Lactobacillus acidophilus* shows lower resistance in the presence of 0.6% (w/v) bile salt solution. Hence, further enhancement of encapsulation material is needed.

4. Conclusions

In this study, *Lactobacillus acidophilus* 5 (La-5) was microencapsulated with IMO as prebiotic. 1.5% (w/v) alginate and 3.0% (w/v) IMO was selected as optimized parameters, with 622.00 μm bead size and highest microencapsulation (94.42%). When subjected to SGJ, both IMO and chitosan have enhanced the survivability of La-5 in gastric conditions but not in intestinal conditions. Further studies are required to enhance the effect of IMO and chitosan in protecting La-5 from intestinal juice to confer health benefits for humans.

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