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The ability of alginate matrix containing isomalt as an encapsulated agent to protect *Lactobacillus acidophilus* FNCC 0051 during storage

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Abstract

This study was carried out to measure the ability of various Na-alginate concentration to protect an encapsulated *Lactobacillus acidophilus* FNCC 0051 in the synbiotic candidate containing 3% (w/v) of isomalt as prebiotic during storage. The viability of encapsulated *Lactobacillus acidophilus* FNCC 0051, beads characteristics (diameter and texture of beads), the count of released cells, pH and total acid of milk as beads carrier showed a significant ($\alpha = 0.05$) difference. Higher concentration of Na-Alginate resulted in higher effectiveness of alginate matrix protection to the encapsulated *Lactobacillus acidophilus* FNCC 0051 up to 20 days of storage time. Matrix consisting of 2% alginate and 3% isomalt can produce beads with high cell viability in the amount of 9,3 log cfu/g for 20 days at 5°C. This study indicated that alginate combined with isomalt can be an effective matrix protect the bacterial cells during storage.

Introduction

Synbiotic is a combination of probiotic and prebiotic which can provide human health benefits. Synbiotic product is usually milk-based products. Synbiotic candidate is a term used to express similar synbiotic product that has not been through consumer acceptance examination. Probiotics can be defined as living microorganisms consumed to improved the balance of the host intestinal microflora in the digestive tract and contribute to the improvement of the health status of the host (Weichselbaum, 2009). Probiotics had to survive in the digestive tract and able to colonizes, thus requiring a dose of at least 10^6-10^7 cfu/ml of the probiotics in the product to be consumed. (ISAPP, 2005). *Lactobacillus acidophilus* FNCC 0051 may help to maintain healthy microflora of the intestine by increasing acidity of the gut and killing the pathogens because of their anti-microbial known activities against pathogenic bacteria and fungal microorganisms (Harti *et al.*, 2012).

Many studies have shown low viability of probiotics in dairy products during storage. One approach that has been adopted to improve survival of probiotic bacteria is providing probiotic living cells with a physical barrier against adverse environmental conditions called cell encapsulation or cell immobilization as well as cell entrapment. Cell immobilization entraps bioactive materials within a polymeric membrane. The polymer/capsule protects the immobilized materials from harsh environmental condition without detrimentally affecting its physiological properties. Huezo *et al*. (2011) reported on the activation of immobilized cells on wet beads that leads to metabolism and better survivability.

Among the available techniques for immobilizing living cells, entrapment in calcium alginate beads has been frequently used for immobilization of lactic acid bacteria. Alginate is an accepted food additive, easy to handle, low cost, biodegradable and it does not involve toxic solvents during preparations (Sheu and Marshall, 1993). Moreover, combination of alginate with carbohydrates can increase the ability to protect bacterial cells and to diffuse micronutrients and metabolites into and out of beads (matrix trappers). Carbohydrates, such as isomalt (polyols) can also act as prebiotic agents (Livesey, 2003).

Cummings *et al.* (2001) report that combination of alginate with prebiotics, such as polyols, can increase the ability to protect bacterial cells by filling hollow spaces of Ca-Alginate gel matrix resulting in a dense gel matrix containing bacterial cells which are metabolically active (Jankowski *et al*.,1997). Klingeberg *et al.* (2004) stated that isomalt can be degraded by *Bifidobacteria* and used for growth and multiplication as well as *Lactobacteria*. Some studies have shown that the incorporating both prebiotics and alginate coating materials may better protect probiotic in food systems and the gastrointestinal tract due to synbiosis (Chen *et al*., 2005; Nazzaroa *et al*., 2009; Okuro *et al*., 2013; Krasaekoopt and Watcharapoka, 2014; Haghshenas *et al*., 2015).

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Entrapment of *Lactobacillus acidophilus* bacterial cells with Na alginate and isomalt is expected to increase the viability of bacteria during storage. The study was to measure the ability of various Na alginate concentration to protect an encapsulated *Lactobacillus acidophilus* FNCC 0051 in the synbiotic candidate containing 3% (w/v) isomalt as prebiotic during storage.

Materials and Methods

Materials

Na-Alginate (Zigma A2033), bacterial cultures of *Lactobacillus acidophilus* FNCC 0051, Full Cream Milk (Ultra High Temperature Milk), distilled water, 0.85% NaCl solution (Riedel - de Haen brands 31.434), 0.1 M of Na citrate, 1% $CaCl_2$ solution, 0.1% of peptone water (MERCK 1.07224), 96% alcohol. The medium used for microbiological analysis were de-Man, ROGOSA, Sharpe bouillon/MRS broth and MRS agar (Pronadisa Cat 1215.00 and Paint 1043.00), Bacto Agar (MERCK 214 010).

Cell imobilization

Na-alginate solution with various concentrations (1, 1.5, and 2% w/v) and sterile isomalt (3% w/v) was added with 2 mL of starter culture of *Lactobacillus acidophilus* FNCC 0051, resuspended in MRSB and homogenized. The solution was dropped using a syringe into 200 mL of 1% cold $CaCl_2$ (5±2°C) and allowed to stand in the refrigerator (5±2°C) for 15 minutes. Beads were washed with 0.85% NaCl solution (3 times of washing @ 100 mL), then 30 grams of beads filled in the sterile cup, added with 100 ml of UHT milk, and stored in a refrigerator (5 ± 2°C) for 0, 10, and 20 days.

Experimental design

Factorial Randomized Block Design was applied with three replication. Concentrations of Na-Alginate consisting of three (3) levels [1%, 1.5% and 2% (w/v)] and storage times consisting of three (3) levels [0, 10, and 20 days]. The results were analyzed through Analysis of Variance Technique (ANOVA) using cohort version 6.1 (costat-2003) to determine the level of significant.

Total plate count

Milk that has been store aseptically was filtered to separate the milk and beads. Beads that were separated from the milk were washed three times with 100 ml of 0.85% of NaCl solution, and used for the testing of the viability of immobilized cells. Three grams of immobile cells (beads) were weighed and dissolved in 27 mL Na citrate 0.1 M. An aliquot was taken for determination of the viable cell counts, diluted (1:10, v/v) with 0.1% (w/v) sterile peptone water and mixed uniformly with a vortex mixer. Serial dilutions were prepared and viable numbers enumerated using spread plating on MRS agar and colonies counted after 48 h incubation at 37°C.

The number of free cells detached from the beads was determined by serial dilutions using 0.1% (w/v) sterile peptone water. The viable numbers of free cells enumerated using spread plating on MRS agar and colonies were counted after 48 h of incubation at 37°C.

Beads diameter

Diameter of beads was measured by a micrometer. Measurement was repeated 10 times every sample. Sampling as much as 5% of the total beads (± 10 pieces of beads).

Beads texture

Measurements refer to Huezo *et al.* (2011) with modifications. Texture of beads was measured using Texture Profile Analyzer "Stable Micro Systems TA-XT2i Texturometer models" with Cylindrical Probe Acrylic (35 mm of diameter), automatic detection, 8 grams of force and 1 mm of distance. Sample compression 30% with 0.5 mm of speed. Parameters to be measured were hardness, cohesiveness, and springiness. The sample measurement repeated 10 times by sampling 5% of the total beads (± 10 pieces of beads).

Result and Discussion

Viability of immobilized Lactobacillus acidophilus FNCC 0051

The effect of Na alginate concentration and storage time on viability of immobilized *Lactobacillus acidophilus* FNCC 0051 in the beads were determined by calculating the total plate count (TPC) and expressed as log 10 cfu/g of beads. Analysis of variance showed that the interaction ($\alpha = 0.05$) of the two factors (Na-alginate concentration and storage time) significantly influence the viability of immobilized *Lactobacillus acidophilus* FNCC 0051. The results of Duncan Multiple Range Test on the viability of immobilized *Lactobacillus acidophilus* FNCC 0051 illustrated in Figure 1.

Figure 1 showed that greater concentration of Na-alginate leads to the higher viability of immobilized bacterial cells. The higher Na-alginate would increase the density of cells trapping matrix which increased the effectiveness of bacteria encapsulation

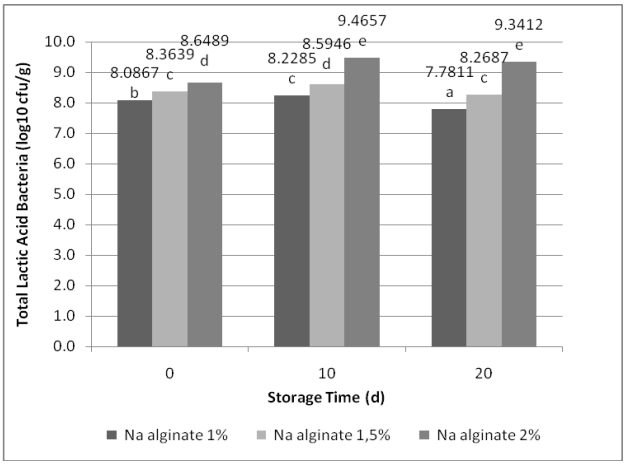

Figure 1. The effect of Na-alginate concentration and storage time on viability of immobilized *Lactobacillus acidophilus* FNCC 0051

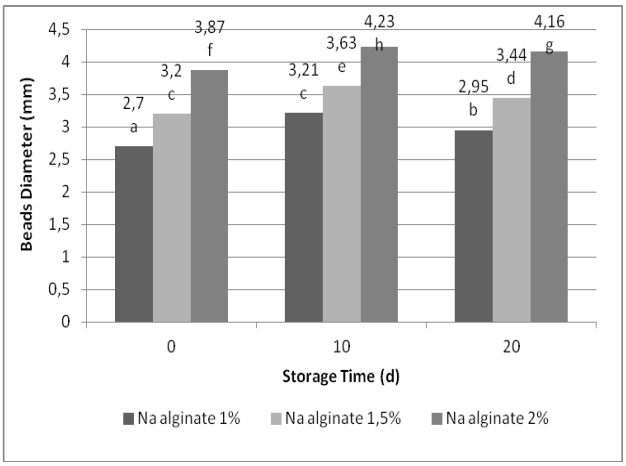

Figure 2. The effect of Na-alginate concentration and storage time on beads diameter

during storage. Castilla *et al.* (2010) stated that the efficiency of encapsulation increased significantly with increasing concentration of biopolymers. Lee and Heo (2000) also showed that the mortality rate of Bifidobacteria longum encapsulated in calcium alginate beads decreased proportionally with increasing concentration of alginate. Another research on the effect of sodium alginate concentration (0, 2, 3 and 4%) on the viability of *Lactobacillus casei* NCDC 298 at pH 1.5 demonstrated the viability increased with increasing concentration of alginate and the highest viability was obtained from the usage of 4% alginate (Mandal *et al*., 2006).

Beads diameter

The effect of Na-alginate concentration and storage time on the beads diameter were determined by a micrometer and expressed in mm. Based on the analysis of variance, there was significant effect ($\alpha = 0.05$) of interaction between Na-alginate concentration and storage time to the beads diameter. Figure 2 showed that higher concentration of Na-alginate used contributed to the smaller changes in the matrix trappers resulting during storage. The beads diameter increased until 10 days of storage, while the extension of storage time until 20 days resulted in decreased of beads diameter. This result is consistent with the research of Lee and Heo (2000), who states that greater concentration of matrix trappers caused the smaller changes of beads during storage. If there is a change during storage, matrix gel is strong enough to protect the immobilized cells. The decrease of beads diameter which occurs at the end of storage in accordance with the research of Ari *et al.* (2010). Their result was that the lactic acid as metabolite produced by *Lactobacillus casei* is a chelating compound which causes releasing of Ca^{2+} from the alginate binding, partially dissolving the beads. Reduced Ca^{2+} in the gel matrix causes the beads not able to maintain its structure, producing beads with smaller diameter.

Gel matrix from Na alginate and $CaCl_2$ has a beads structure which are easily broken and porous surface. Addition of isomalt as a prebiotic aims to improve bacterial cells protection by filling cavities of Ca-alginate gel matrix and produces a more dense of gel matrix containing metabolically active bacteria cells (Jankowski *et al*., 1997). However, the alginate-isomalt matrix still have pores that allow for diffusion of nutrients and metabolites into and out of the beads. That can cause structure changes of the gel matrix to becomes more tenuous with the enlarged pores of beads. Rokka and Rantamaki (2010) said that the alginate gel matrix has sufficient elasticity that allows the development associate with the imbibitions (water absorption) which cause an enlargement of the beads diameter. In addition, an increase in diameter may occur because of the growth bacterial cells (Koyama and Seki, 2004). Bacteria at low storage temperatures (5 ± 2°C) still growth with a slow rate of metabolism (Ray, 2001).

Beads texture

Beads texture influenced by concentration of Na-alginate as a gel matrix constituent. Encapsulation efficiency of gel matrix can be improved by adding the prebiotic that can fill cavities of calcium matrix. Sultana *et al.* (2000), in their research stated that starch prebiotics filled cavities of alginate matrix and helps to strengthen the beads structure. Texture tested include hardness, cohesiveness, and springiness of beads. During storage, the calcium alginate gel matrix will be softened (Krasaekoopt *et al.*, 2003), causing the beads become brittle and breakable, thus

Table 1. The effect of Na-alginate concentration on beads textures

Na-alginate concentration (% w/v)	Beads textures		
	Hardness (g)	cohesiveness	springiness
1	2.6916[a]	0.1299[a]	0.2539[a]
1.5	81.1614[b]	0.2523[b]	0.5001[b]
2	129.7754[c]	0.6138[c]	0.6964[c]

Means bearing (n=3) different superscripts between treatments differ significantly (α=0.05)

not allowing to test the beads texture. Therefore, the texture measurements was performed when the beads were formed, in order to determine the initial alginate gel texture and the result in this study to be presented in Table 1.

Characteristics of alginate beads was determined by the type and composition of biopolymers which were used. Sheu and Marshall (1993) reported that concentration of Na-alginate will affect the structure of the beads. The greater concentration of Na-alginate to be used, the hardness of beads increased. The isomalt will fill a gap in the junction zones of gel matrix. The isomalt has a great viscosity, so if its solution solidifies, it will give a firm texture of the beads. Increased of Na-alginate concentration will increase the number of sides that bind to Ca^{2+}, so that solid gel formed as a result of a crosslink (Chandramouli *et al*., 2004). The increasing of Na-alginate concentration, more fluid can be trapped in the matrix which caused the higher hardness, cohesiveness, and springiness of the beads (Table 1).

Total plate count of released cells in milk

In this study, total plate count of the released cells, which separated out into the milk as beads carrier during storage, were counted as free cells. Table 2 showed mean value of total plate count of free cells (log_{10} cfu/ml) and changes of pH and total acid during storage. The highest number of free cells resulted by interaction of 1% Na-alginate and 20 days of storage time, whereas the lowest number of free cells resulted by interaction of 2% Na-alginate without storage (0 day). The changes in strengthen and robustness of beads during 20 days of storage caused the immobilized cells separated out into the milk.

During storage, metabolites accumulation of immobilized cells and free cells metabolism produced acid and cause a pH decrease. The presence of H^+ from the acid would substitute Ca^{2+} and caused gel matrix of Ca-alginate to be degraded and become more loose, resulting in the increased number of released cells (Krasaekoopt *et al*., 2003). In addition,

Table 2. The free cells, pH and total acid in milk as beads carrier during storage

Na-alginate concentration (% w/v)	Storage Time		
	0 day	10 days	20 days
		free cells (log 10 cfu/ml)	
1	4.86 [d]	5.50 [f]	5.97 [g]
1.5	4.47 [c]	4.83 [d]	4.96 [e]
2	3.79 [a]	3.94 [b]	3.98 [b]
		pH	
1	6.48 [h]	6.30 [c]	6.16 [a]
1.5	6.44 [g]	6.34 [e]	6.25 [b]
2	6.48 [h]	6.41 [f]	6.32 [d]
		total acid (%)	
1	0.12 [d]	0.13 [e]	0.20 [g]
1.5	0.10 [c]	0.12 [d]	0.16 [f]
2	0.07 [a]	0.09 [b]	0.12 [d]

Means bearing (n=9) different superscripts between treatments differ significantly (α=0.05)

increasing of beads diameter during storage was due to the diffusion of milk nutrients into the beads, leading to a decrease in the density and robustness of the gel matrix. The greater concentration of the trapper matrix material would produce stronger physical characteristics of beads and also able to withstand the changes that occur during storage to reduced the number of free cells (Chandramouli *et al*., 2004).

Milk acidity during storage

The acidity of milk as a carrier expressed as pH and total acid. The mean value of pH and total acid (%) of milk caused by interaction between Na-alginate concentration and storage time are presented in Table 2. The pH decrease followed by an increase in total lactic acid during storage. However, a decreasing of pH was not always proportional with an increasing of total acid (%), because the pH value depends on the degree of acid ionization in the product, while the total acid concentration depends on the amount of acid that is dominant in a product.

The differences of a decrease in pH and an increase in total acid during storage could be due to both of the immobilized and released cells still active during storage, although their metabolism are very slow in refrigerator (temperature ± 5°C). The greatest changes in pH and total acid were from the combination of 1% Na-alginate and 20 days of storage, because gel matrix as the cells trapper was not capable to protect the *L. acidophillus* FNCC0051 cells during the storage. Usage of 2% Na-alginate and the storage time of 20 days would result in a small decrease in pH and increase in total acid (%) because of high strength and density of beads which reduce the number of detached cells and reduce the rate of

nutrients diffusion and accumulation of metabolites (lactic acid) by both immobilized cell and free cells (Lee and Heo, 2000; Chandramouli *et al*., 2004 ;. Sheu and Marshall, 1993). This is in accordance with the report of Chandramouli *et al*. (2004), that the greater concentration of matrix material for trappers, so the beads physical characteristics also getting stronger.

Conclusions

The lowest level of Na-Alginate concentration increased the number of released cells, decreased pH value and increased total acid. Higher concentration of Na-Alginate resulted in higher effectiveness of alginate gel matrix protection to the encapsulated *Lactobacillus acidophilus* FNCC 0051 up to 20 days of storage time. Matrix consisting of 2% alginate and 3% isomalt can produce beads with high cell viability in the amount of 9,3 log cfu/g for 20 days at 5°C. This study indicated that alginate combined with isomalt can be an effective matrix protect the bacterial cells during storage.

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