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Microbiological and Other Characteristics of Microencapsulation Containing *Lactobacillus acidophilus* (CICC 6075)

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In order to improve the stability and effective utilization of *Lactobacillus acidophilus,* this study was conducted to evaluate the viability and the physical properties of microencapsulated *L. acidophilus* using porous starch as a carrier. Survival assays were conducted to evaluate the retention of *Lactobacillus acidophilus* cell load during the preparation process. The encapsulation parameters that gave maximum viable cell counts were investigated and the optimum condition during the process was 10 % (w/v) wall material, 50 min of shaking time, 20 ! of the temperature, and 6.0 of pH level, respectively. In addition, SEM photos showed that the microencapsulation technology may facilitate the flow of material and all microcapsules showed similar morphologies. Result under heat treatment showed that the survival of the lactobacilli was found to increase on encapsulation and also decrease with increasing the temperature. The results suggest a higher stability of microcapsules containing *L. acidophilus* compared to the free cells. The study indicates that this microencapsulation using porous starch as carrier can be considered a useful technology to provide the protection for *L. acidophilus* and increase its stability.

Key words: *Lactobacillus acidophilus*, survival assays, physical properties, stability**.**

In recent years, there has been an increased interest in the role of probiotic bacteria in human health (Chandramouli *et al*., 2004). Probiotics could confer beneficial effects on the host by improving their intestinal microbial balance in adequate levels (Pedroso *et al*., 2012). Probiotic bacteria have been reported by other researchers to suppress the growth of pathogens and stabilize the digestive system (Kaur *et al*., 2002; Cremonini *et al*., 2001).

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L. acidophilus is one of the important probiotic microorganisms and is frequently used in food products. These organisms should remain viable because they should pass through the stomach and intestine to provide beneficial effects on human health (Fung *et al*., 2011; Chandramouli, *et al*., 2004). In recent years, microencapsulation has been studied as an alternative to improve the stability and viability of these cells because of their sensitivity to many factors including the presence of acidic media and oxygen (Fung *et al*., 2011).The International Dairy Federation has recommended that the bacteria be active and be present in the product at least till the level of 10^7 CFU/g until the product's expiration date (Ouwehand *et al*., 1998). Therefore, microencapsulation techniques have been investigated for improving the viability of LAB (Rao *et al*., 1989).

Microencapsulation techniques were successfully used to improve the survival of microorganisms in dairy products (Picot *et al*., 2004; Lee *et al*., 2004; Dembczynski *et al*., 2002). However, vacuum drying, freeze-drying and/or freeze-drying treatment in the microencapsulation process may cause injury and death of cell (Ananta *et al*., 2005). Chandramouli *et al*. (2004) studied the optimal encapsulation condition for protecting LAB in artiûcial gastric conditions. Picot and Lacroix (2004) also found that encapsulation of *Biûdobacterium breve R070* with whey protein significantly increased viable cell counts when compared to the non-encapsulated *B. breve R070* and *B. longum R023* during refrigerated storage. Moreover, according to Ding and Shah (2009), microencapsulation may improve the survival of these microorganisms, during both processing and storage. As reported by Mandal, Puniya, and Singh (2006), the survival of encapsulated *L. casei* was better at low pH, high bile salt concentration compared to free cells. Carlise B. Fritzen-Freire *et al*. (2012) also reported that microencapsulation of biûdobacteria was prepared by spray drying in the presence of prebiotics. The investigation of Pedroso *et al*. (2012) was to produce and evaluate solid lipid microparticles containing *L. acidophilus*. Microencapsulation is drawing more and more attention for being a method to improve the stability of probiotic organisms in functional food products (Semyonov *et al*., 2010).

Therefore, the objective of this study was to understand the effect of microencapsulation on the changes in survival rate of the *L. acidophilus* during preparation process. On the other hand, the morphology of the microencapsulation was also observed by SEM. The change in heat susceptibility of *L. acidophilus* during the heat treatment was also investigated.

MATERIALS AND METHODS

Materials

A probiotic culture composed of *L. acidophilus* CICC 6075 was used as the active material for the microcapsules. The encapsulating agents used were commercial porous starch (Liaoning Lida Bio-Technology Co. Ltd., Jinzhou, China); sodium propionate, calcium chloride (Chengdu Ruifeng Lier Technology Co. Ltd, Chengdu, China) and were used for the prepared process.

Preparation of microencapsulated cells

The bacterial suspensions were prepared as the procedures described by Mandal, Puniya and Singh (2006) with modiûcations. A freeze-dried ampoule of *L. acidophilus* was activated in chalk litmus milk (37 °C, 24 h) and maintained in a refrigerator (7 °C) before being sub-cultured monthly. The culture was reactivated by transferring 2-3 times in MRS broth and the cells were harvested (80 mL) by centrifugation at 2000 *g* for 10 min at 4 °C. The cells were washed twice before resuspending them in 5 mL normal saline. The final cell concentration was adjusted to 1.0×10^{11} cfumL^{-1}.

L. acidophilus cells were encapsulated in porous starch and sodium alginate matrix as described by Mandal *et al*., (2006) with some modifications. The 50 mL cell suspension with 10^8 cfu/ml *L. acidophilus* was transferred into a sterilized beaker. The porous starch were also prepared and added into this sterilized beaker. Then this solution was shocked by ultrasonic wave for a certain minutes in order to the free cell absorbed absolutely by porous starch. The pH values of this solution were adjusted by using NaOH and HCl. Sodium alginate with 2 % concentration was added after absorption. Soybean oil (10 mL) containing 0.2 % Tween 80 (emulsiûer) was taken in a beaker and to this porous starch-alginate-cell mixture was added dropwise while stirring magnetically. After 5 min, a uniformly emulsion was obtained to which 0.1 M calcium chloride (100 mL) was quickly added for hardening of microcapsules and breaking the emulsion. The capsules were harvested by centrifuging at 350 g for 10 min at 4 °C and washed twice with distilled water. The beads were separated by filtration using Whatman filter paper, transferred to a sterile Petrdish and precooled in a refrigerator (-40°C) for 4 h. Then the obtained material was freeze drying under -58 °C by freeze drying machines for 24 h. To determine the effect of prepared process on the viabilities of the microorganisms, cell counts were performed before the microencapsulation process.

Enumeration of probiotic cells

The viable *L. acidophilus* cells were counted by the pour-plate technique on MRS agar

according to the method reported by Grosso & Fávaro-Trindade (2004) with some modifications. For *L. acidophilus*, MRS agar was supplemented with lithium chloride (0.1 %), L-cysteine (0.05 %) and aniline blue (0.01 %). Serial dilutions were prepared with a 2 % sodium citrate solution. The plates were incubated in anaerobiosis with the anaerobic system at 37°C for 72 h. The plating was performed in duplicate. For the enumeration of probiotics in the microparticles, however, a pre-heated sodium citrate solution at 48 °C was used in order to melt the fat matrix and promote the release of the cells during serial dilutions.

Optimization of encapsulation parameters

The encapsulation parameters such as porous starch concentration (6 %, 8 %, 10 %, 12 % w/v), shaking time (20 min, 30 min, 40 min, 50 min or 60 min), temperature(15°C, 20°C, 25°C, 30°C, 35°C or 40°C) and pH values (5.0, 5.5, 6.0, 6.5 or 7.0), initial cell load (10^7,10^8,10^9 CFU/ml), were optimized for their efficacy in increasing the viability of encapsulated bacteria in simulated gastric conditions.

Morphology of microencapsulation

Morphology of microencapsulation was characterized by scanning electron microscopy (SEM). The samples were placed on the SEM stubs using a two-sided adhesive tape and then analyzed using a JSM-7500F SEM at a voltage of 5 kV acceleration after Pt sputtering.

Survival of microencapsulated cells under heat treatments

Tolerance of encapsulated *L. acidophilus* to heat treatment (50°C, 55°C, 60°C or 65°C for 20 min) was studied using distilled water (pH 6.4±0.2) as a suspending medium. One gram of microcapsules or 1mL of the free cell suspension (10^{10} cells mL^{-1}) was transferred in test tubes containing 10mL of distilled water. After the heat treatment, the content was cooled to room temperature and viable cells were enumerated as described in Section 2.2.4. (Mandal *et al*. 2006)

Statistical analysis

The tests were carried out in triplicate analyzed using SPSS 13.0 software (SPSS Inc.). The one way analysis of variance procedure followed by least significant difference test was used to determine the significant difference ($p<0.05$) between treatment means.

RESULTS AND DISCUSSION

Effect of wall material concentration on *L. acidophilus* survival

The choice of the prepared technology of microencapsulation is critical because the microencapsulation of probiotic microorganisms is designed to increase its stability and viability (Pedroso *et al*., 2012). The higher survival of *L. acidophilus* in products is important and advantageous for the powder products. Therefore, the effect of wall material concentration on the survival of *L. acidophilus* during processing was studied and the result was shown in Fig. 1. The survival *L. acidophilus* was improved with increasing in wall material concentration. There was no effect of *L. acidophilus* cell load on the retention at high concentrations of wall material, but the retention of cell increased when the wall material concentration increased from 6 %(w/v) to 10 %(w/v), which is the highest with the concentration at 10 %. There was no significant increase in viable cell numbers of capsules when the wall material concentration was further increased to12 % (w/v).

Result above showed that the cell load capacity depended on the concentration of wall material. This result indicated that the effect of the concentration of wall material on the cell retention was existed. On the other hand, wall material with 10 % concentration showed higher *L. acidophilus* retention than the other concentration of wall materials. This was consisted with the result reported for liquid flavors by other researchers

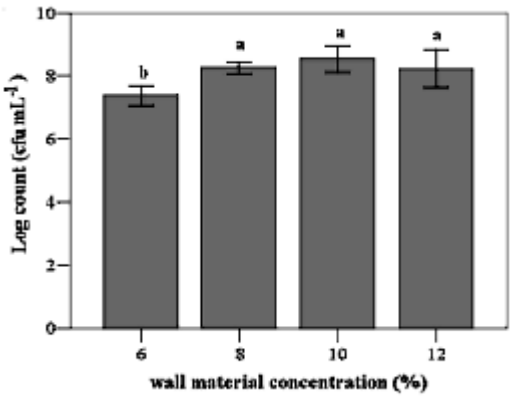

Fig. 1. Effect of different concentration of wall material on the viability of *L. acidophilus* microcapsules. Mean bars with different letters (a-b) for different wall material concentration differ significantly $p<0.05$

(Soottitantawat *et al*., 2005). As reported by Lee & Heo (2000), the death rate of *B. longum* entrapped in alginate beads decreased proportionately with increased capsules size and alginate concentration. This result may be due to the micropores in the porous starch, which was also can be shown in Fig. 5. The higher wall material condition increased the micropores for free cell load resulting in the increasing cell retention during processing. Therefore, these micropores in the porous starch acts as the protector of free cells and wall material of cell load efficiently retaining *L. acidophilus* cell.

Effects of shaking time on *L. acidophilus* survival

The effect of the shaking time on the survival of *L. acidophilus* was showed in Fig. 2. As can be seen, the survival of *L. acidophilus* increased as the shaking time increased. There was 1.2-log increase in viable cells of encapsulated *L. acidophilu* with increasing the shaking time from 20min to 50 min. The highest in encapsulated cell number of *L. acidophilu* was observed at 50 min of shaking time, compared to a 2.6-log decrease in the free cells at 20 min of shaking time. The shaking time during microcapsule preparation influenced the encapsulation efficiency. Shocks between microcapsules in the reaction medium could lead to the dispersion of porous starch and free cell in the solution; this may be due to the increasing cell load in the micropore in the wall material.

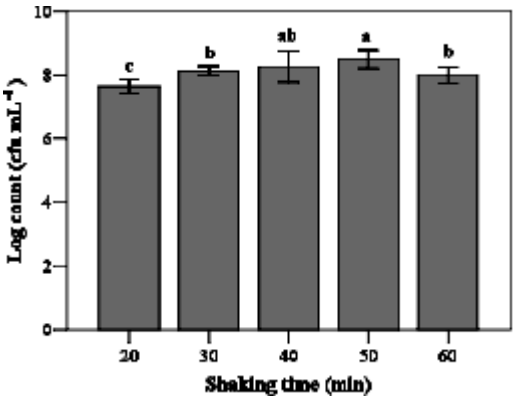

Fig. 2. Effect of shaking time on the viability of *L. acidophilus*. Mean bars with different letters (a-c) for different shaking time differ significantly $p<0.05$.

Effects of temperature on *L. acidophilus* survival

Effect of the temperature on the survival of *L. acidophilus* during prepared processing was investigated and shown in Fig. 3. Survival of the lactobacilli was found to decrease with increasing the prepared temperature. There was 1.0-log decrease in viable cells of encapsulated *L. acidophilu* at 15°C of the low prepared temperature compared to a 3.7-log decrease for encapsulated cells at the temperature of 40°C. The highest retention of cell was observed when the temperature was at 20°C. There was significant effect of the temperature during process on the retention of *L. acidophilus* cell load, but the retention of cell decreased when the temperature during the prepared process was 25°C. There was significant decrease in viable cell numbers of capsules when the wall material concentration was further decreased to 35°C.

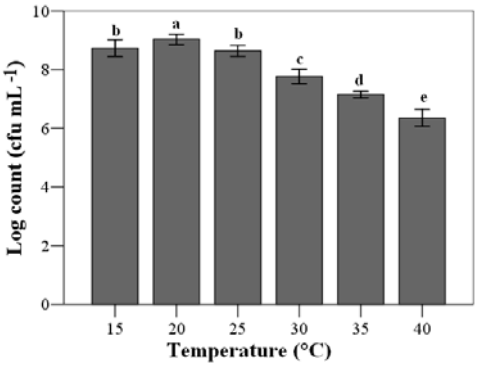

Fig. 3. Effect of temperature on the viability of *L. acidophilus*

Mean bars with different letters (a-c) for different temperature differ significantly p"ÿ0.05. This result indicated that the temperature during the prepared processing was significant influenced the free cell survival. This result was also reported by other researchers. According to the investigation conducted by Mandal, *et al*.(2006), free cells in distilled water (9.20 log cfu mL^{-1}) were drastically reduced to 5.55, 4.93 and 3.98 log cfu mL^{-1} on heat treatments at 55, 60 or 65°C for 20min, respectively.

Effects of pH values on *L. acidophilus* survival

Effect of pH values on the survivals of encapsulated *L. acidophilus* cell during prepared processing was shown in Figure 4. At pH 6.0 levels of *L. acidophilus* remained high retention load of *L. acidophilus* cell. There was 2.3-log decrease in viable cells of encapsulated *L. acidophilu* at low pH (pH 5.0) compared to a 2.5-log decrease in the encapsulated cells at high pH (pH 7.0). There was

significant effect of *L. acidophilus* cell load on the retention at pH increased during process. The retention of cell increased when the pH level was increased from 4.5 to 6.0 during the prepared process. The highest retention of cell was observed when the pH level was at 6.0 levels.

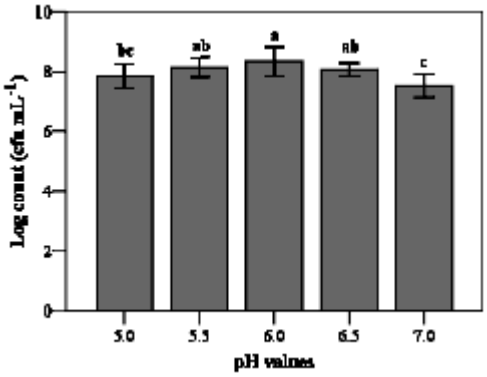

Fig. 4. Effect of pH values on the viability of *L. acidophilus*. Mean bars with different letters (a-c) for different pH level differ significantly $p<0.05$

There was no significant increase in viable cell numbers of capsules when the pH level was further decreased to 7.0. The certain pH levels in the reaction medium could lead to sodium alginate wrap up the free cell around porous starch. The pH level in the solution also affected the survival of cell. As the result reported by Mandal, *et al*. (2006), the simulated colonic pH was also influenced the survival of cell (Picot and Lacroix, 2004).

Morphological characterization

Morphological characterization of *L. acidophilus* microencapsulation was carried by SEM (Fig.5). After vacuum freeze-dried process, a solidified, white and cooled free-flowing powder was collected. The SEM photos revealed that the microencapsulation displayed spherical shapes with many micropore. (Pedroso *et al*., 2012). This shape was also observed by Savolainen *et al*. (2002) and Saénz *et al*. (2009). The SEM revealed the microcapsules produced with prebiotics showed similar morphologies. This result is interesting because this format facilitates the flow of material, although it is not only shape that determines flowability. As shown in Fig.5, the external surfaces showed many micropore, which is fundamental for guaranteeing higher protection. As seen in Fig. 1, an increased wall material concentration in the prepared solution, as increase in the retention of *L. acidophilus* cell, correlated with an increased presence of cell load.

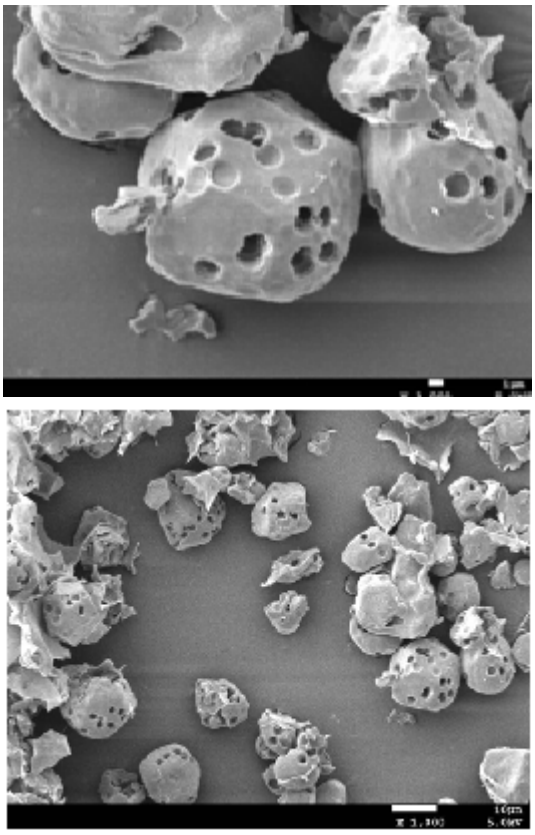

Fig.5. Morphology of microcapsules observed by SEM

This result was due to the increasing micropore with the increasing porous starch in the prepared solution. However, *L. acidophilus* cells were not visible clearly by SEM. This result was also consisted with the research reported by Carlise B. Fritzen-Freire *et al*. (2012) and Pedroso *et al*. (2012).

Survival of encapsulated cells after heat treatments

Effect of heat treatments on the survival of free cell and encapsulated cells was investigated and shown in Fig. 7a and 7b. As shown in Figure 7a, free cells in distilled water (9.10 log cfu mL^{-1}) were drastically reduced to 6.05, 5.52, 4.21 and 3.0 log cfu mL^{-1} on heat treatments at 45, 55, 60 or 65°C for 20 min, respectively (Fig. 7a). Survival of the lactobacilli was found to increase on encapsulation

and also with increasing concentrations of wall material in capsules. On the other hand, as shown in Fig. 7b, encapsulated cells in distilled water (9.15 log cfu mL^{-1}) were reduced to 8.01, 7.46, 6.73 and 6.0 log cfu mL^{-1} on heat treatments at 45, 55, 60 or 65 for 20min, respectively (Fig.7b). The low temperature of heat treatment might have led to the higher survival of *L. acidophilus* (Fig. 7b).

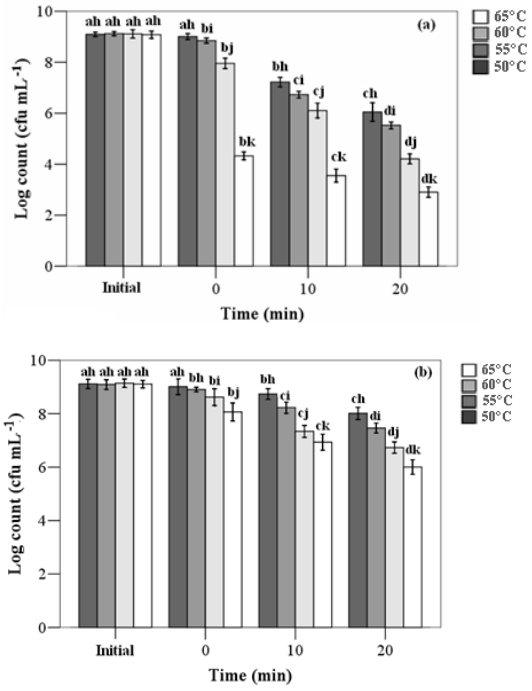

Fig.7. Effect of different encapsulating agents on the viability of free *L. acidophilus* cell (a) and microcapsules containing *L. acidophilus* (b) at 50°C, 55°C, 60°C and at 65°C. Mean bars with different letters (a-c) at same temperature for different heating period differ significantly *p*"ÿ0.05. Mean bars with different letters (h-k) for same heating period in different temperature differ significantly $p<0.05$.

This result showed that the heat treatment significant influenced the survival of free cell compared to the cell in the microencapsulation. Result showed that survival of the lactobacilli was found to increase on encapsulation. According to the result reported by Mandal *et al*. (2006), under heat treatment, the survival increased proportionately with increasing alginate concentrations without affecting the release of entrapped cells in solution of colonic pH. The study indicates that this microencapsulation using porous starch as carrier can be considered a useful technology to provide the protection for *L. acidophilus*.

CONCLUSIONS

Microencapsulated *L. acidophilus*, using porous starch as carrier was produced in order to protect the probiotics. The best conditions are 10% (w/v) of wall material, 50 min of shaking time, 20°C of the temperature and 6.0 of pH level, respectively. SEM photos indicated that all microcapsules showed similar morphologies. The survival of lactobacilli was found to increase on encapsulation and decrease with increasing the temperature. This indicates that microencapsulation using porous starch as carrier can be considered a useful technology to provide the protection for *L. acidophilu*.

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