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ARTICLE

Effect of Coating Method on the Survival Rate of *L. plantarum* for Chicken Feed

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Abstract

This study was designed to find the most suitable method and wall material for microencapsulation of the *Lactobacillus plantarum* to maintain cell viability in different environmental conditions. To improve the stability of *L. plantarum*, we developed an encapsulation system of *L. plantarum,* using water-in-oil emulsion system. For the encapsulation of *L. plantarum*, corn starch and glyceryl monostearate were selected to form gel beads. Then 10% (w/v) of starch was gelatinized by autoclaving to transit gel state, and cooled down at 60ºC and mixed with *L. plantarum* to encapsulate it. The encapsulated *L. plantarum* was tested for the tolerance of acidic conditions at different temperatures to investigate the encapsulation ability. The study indicated that the survival rate of the microencapsulated cells in starch matrix was significantly higher than that of free cells in low pH conditions with relatively higher temperature. The results showed that corn starch as a wall material and glycerol monostearate as a gelling agent in encapsulation could play a role in the viability of lactic acid bacteria in extreme conditions. Using the current study, it would be possible to formulate a new water-in-oil system as applied in the protection of *L. plantarum* from the gastric conditions for the encapsulation system used in chicken feed industry.

Keywords *Lactobacillus plantarum*, encapsulation stability, water in oil emulsion, gel bead

Introduction

Antibiotics are the biological medicine originated from microorganisms, which prevent bacterial growth. These are important to cure infection diseases and increase weight gain and feeding efficiency for animals. However, the problems of supplying antibiotics for domestic animal are emergence of new bacteria which have resistance to antibiotics and accumulation to human that eat the meat of animals ingested it.

Lactobacillus plantarum (*L. plantarum*) was isolated from human saliva and can be easily found in fermented food such as Kimchi, sauerkraut, cheese and stock fish. *L. plantarum* is heat-stable and active in the pH range of 4.0-6.5. It can resist the effects of bile acids in the upper small intestine when ingested, and colonizes and grows in large intestine. Being used as a probiotic, its biotherapeutic applications have been increasingly recognized (De Simone *et al.*, 1991; Lee and Salminen, 1995). It was proved that *L.* plantarum can enhance the intestinal integrity, metabolic activity of intestinal cells and stimulate immune responses (Rajam *et al*., 2012). Also, *L. plantarum* is a lactic acid bacteria (LAB) with probiotic capacity. LABs are among the most important probiotic microorganisms typically associated with the human gastrointestinal tract (GIT) (Dolly *et al.*, 2011; Gbassi *et al.*, 2009).

Recently, pro- or prebiotics are increasingly paid attention to substitute antibiotics as an animal feeding. Probiotics are defined as a live microbial feed supplement which beneficially affects the host animal by improving its microbial balance (Fuller, 1993; Havenaar and Huis in't Veld, 1992). Probiotics provide many health benefits including immune stimulation, cholesterol reduction, in-

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hibition of pathogen growth, maintenance of a healthy gut microflora, prevention of cancer, improvement in lactose utilization, prevention of diarrhoeal diseases or constipation, absorption of calcium, and synthesis of vitamins and predigestion of proteins (De Simone *et al*., 1991; Fuller, 1993; Havenaar and Huis in't Veld, 1992; Jankowski *et al.*, 1997; Lee and Salminen, 1995). In addition, it have been supplemented to diverse food products to create "functional foods or nutraceuticals" in global markets (Stanton *et al.*, 2001). To provide functional properties, the minimum level of viable bacteria is approximately 10^6 CFU/mL of product at the expiry date, and the suggested therapeutic dose is 10^8-10^9 viable cells per day. Furthermore, they contribute to the general well-being of the host, beyond inherent general nutrition due to the important symbiosis they establish with the host without any side effects caused by antibiotics (Kurmann and Robinson, 1991).

However, probiotics may not survive in sufficient number and retain their activity prior to consumptions, due to processing procedures such as oxygen stress, freezing, drying, and fermentation. Especially, it may be affect during their passage through gastro-intestinal tract (GIT) (Gismondo *et al.*, 1999; Pimentel-González *et al.*, 2009).

Therefore, encapsulation has been as a potential mechanism to enhance the viability of probiotics in the in the stomach acids or through GIT (Anal *et al.*, 2003; Anal and Stevens, 2005). Encapsulation is defined as a technology of packaging solids, liquids or gaseous materials in miniature, sealed capsules that can release their contents at controlled rates under the influences of specific conditions (Anal and Singh, 2007; Anal and Stevens, 2005). In general, encapsulation method can be used for many applications in the food and pharmaceutical industries including solubilizing water insoluble materials, masking flavours, colours or odours, extending the shelf life and protecting component. As well, encapsulation techniques have been widely applied to enhance viability of probiotic bacteria in commercial products and it can protect the active probiotics with proper coating materials such as proteins and polysaccharides (Heidebach *et al*., 2009; Krasaekoopt *et al.*, 2004; Rajam *et al.*, 2012) controlling release of probiotics in and out of microcapsules. Especially, they used for protecting probiotics from extra environments such as pH, temperature, medium composition and other factors (Shima *et al.*, 2006). For using microencapsulation technique, many coating materials have been studied in conjunction with alginate beads to enhance the protection of Lactobacillus and Bifidobacterium cells in acidic environments; these include chitosan, poly-l-lysine and whey proteins, with the former being the most well studied coating polymer (Chavarri *et al.*, 2010; Gbassi *et al.*, 2009; Krasaekoopt *et al.*, 2004; Nualkaekul *et al.*, 2012). Among the microencapsulation techniques for protecting probiotics, water in oil (W/O) emulsion system including *L. plantarum* may serve as suitable wall materials to encapsulate and protect probiotic bacteria in GIT. In addition, it may be used as a potential biocapsule to encapsulate bacterial for commercial utilization in food products or feed additives.

The aim of this work was to determine the suitability of coating agents including wall material (corn starch) and gelling agents (glyceryl monostearate or glyceryl monoolein) for formation of W/O emulsion system, and to improve the survival of the encapsulated *L. plantarum* in W/O emulsions under different temperature (37ºC and 40ºC) and pH (pH 2 and 7).

Materials and Methods

Materials

Corn starch used as wall material was purchased from Daejung Chemical & Metals (Korea). Glyceryl monostearate and glyceryl monoolein were obtained from Daejung Chemical & Metals (Korea) and TCI (Tokyo Chemical Industry Co., Japan), respectively. For preparation of water in oil emulsion system, soybean oil was purchased from Hae-phyo (Korea).

Microorganism

The bacterial strain used in this study was *Lactobacillus platarum* 10hk2 culture, provided from Milae Resources ML Research Institute (Korea). The strain was cultured on Lactobacilli MRS broth (Difco™, USA) at 37± 1ºC for 24 h and working stocks of cultures were maintained in 20% glycerol suspension frozen at -18ºC.

Concentrated cell culture preparation

Active culture for experiments were prepared by transferring one loof of cells from stock cultures to flask of MRS broth, which were incubated at growth temperature of 37±1ºC for 24 h in a shacking incubator at 90 rpm. The cells were collected by centrifugation (Centrifugal separator, 1736R, LaboGene, Korea) at 4,000 rpm for 10 min at 4ºC. After removal of supernatant, cell pellets were washed with sterile 0.9% (w/v) saline solution. There average cell concentrations of these cells were approximately 8 Log CFU/mL.

Encapsulation of *L. plantarum* through coating materials

We prepared three types of corn starch solutions (only starch solution; starch solution with monoolein; starch solution with monostearate) to confirm effect of coating materials. Corn starch solutions of 3% (w/v) with glyceryl monoglyceride of 0.1% (w/v) were mixed using stirrer for 1 h and autoclaved (JEIOTEC, Korea) and cooled at 30°C. After, the samples inoculated 5% (v/v) *L. plantarum* suspension with ratio of 100:1 (v:v) and were pour plated on MRS agar (Difco™, USA) plates after appropriate 10-fold serial dilution in 0.9% (w/v) saline solutions. Colony forming units (CFU) were enumerated manually after incubation at 37±1°C for 24 h.

Microencapsulation of *L. plantarum* through hydrogel in oil system

L. plantarum was microencapsulated by mixing culture concentrated with corn starch as wall material and glyceryl monostearate as gelling agent. First, 10% (w/v) Corn starch and 0.1% (w/v) glyceryl monostearate were mixed with distilled water and stirred until complete dissolution. After, the mixture was autoclaved and cooled to 60°C. The freshly harvested cell concentration of 10% (v/v) was mixed with solution of corn starch and glyceryl monostearate. The water-in-oil (W/O) emulsions were prepared by emulsifying a continuous phase (W) made up by corn starch solutions containing 8 Log CFU/mL into an dispersed phase (O) made up by corn oil, and the ratio of dispersed phase to continuous phase was set at 2:1 (w:w). Emulsification was carried out with the high speed homogenizer (ULTRA-TURRAX® T25, IKA® Labotechnik, Germany) at 11,000 rpm for 5 min. To collect of pure hydrogel microcapsules, the W/O emulsions were centrifuged at 800 rpm for 10 min at 4°C, and then the samples were washed with 0.9% (w/v) saline solutions to eliminate soybean oil residue. Pure hydrogel microcapsules of five gram were diluted with 20 mL of 0.9% (w/v) saline solutions, and the samples were stored at 4°C prior to use.

Analysis of thermal properties

Differential scanning calorimetry (DSC) studies were used by a DSC 200F3 apparatus (Netzsch-Geraetebau Gmbh, Germany) to confirm the optimal gelling agent. Monoglycerides of two types such as monoolein and monostearate were determined by DSC. Temperature calibration was performed using indium. The samples were weight at an accuracy of 3±0.01 mg and were hermetically sealed in an aluminum pan. Each sample was scanned from 20°C to 200°C with a heating rate of 20°C/min under nitrogen gas injection. Through the DSC analysis, we confirmed melting point of each monoglyceride.

Microscopic observation

Wall materials and entrapped *L. plantarum* into the W/O emulsions were observed with optical microscope (Olympus CX31RTSF, Japan).

Survival of free and encapsulated *L. plantarum*

To determine the viable counts the entrapped *L. plantarum*, hydrogel microcapsules and non-entrapped control cells culture were stored at 37°C for 0, 24, 48 h. Cell counts were calculated from the colonies on lactobacilli MRS agar plates after 48 h incubation at 37°C under anaerobic conditions, and thus expressed as colony-forming units per mL (CFU/mL).

For confirming of pH of *L. plantarum*, 1 mL of hydrogel microcapsules and 1 mL of non-entrapped control cells culture were inoculated by separate into 9 mL pH 2 and pH 7 solutions. Samples were incubated at 37°C and 40°C and taken at different time intervals to determine the complete release of encapsulated *L. plantarum* by plating as described previously.

Viscosity measurement

Initially, one gram of hydrogel microcapsules was poured into the CP50-2 plate of a Rheometer® (Anton Paar, Austria). Hydrogel microcapsules were stored at 37°C and 40°C. After 0, 1, and 2 h, the microcapsules were sampled and analyzed using with CP50-2 probe with shear rate of 50/s for 20 min.

Statistical analysis

The data were analyzed by using one-way analysis of variance with storage periods. An analysis of variance was performed on all the variables using the General Linear Model (GLM) procedure (SAS 9.3, SAS Institute, USA). Differences among the means were compared using Tukey's Studentized Range (HSD) Test ($p<0.05$).

Results and Discussion

Morphological properties of bulky mixture between *L. plantarum* and starch

To encapsulate the *L. plantarum* for the chicken feeds within gelatinized starch, the morphological comparison of each used samples was needed to distinguish the structural properties. In the present study, corn starch was

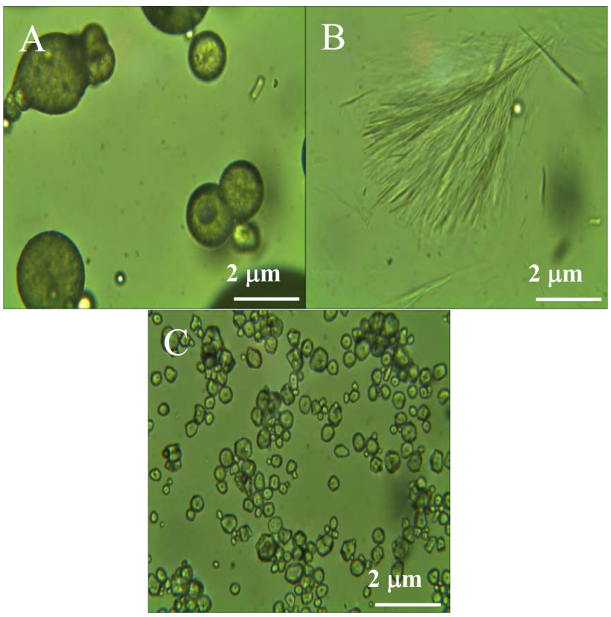

Fig. 1. Structures of (A) corn starch, (B) glyceryl monoolein and (C) glyceryl monostearate as a raw material.

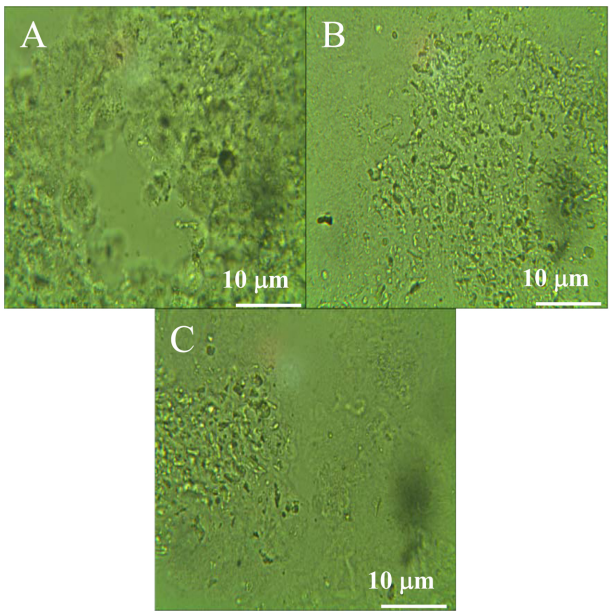

Fig. 2. Optical microscopic observation of *L. plantarum* mixed into gelatinized starch matrix (A) without gelling agent (glyceryl monostearate or glyceryl monoolein), (B) with glyceryl monostearate and (C) with glyceryl monoolein as a gelling agent.

selected as a wall material for the encapsulation of *L. plantarum* due to its hydrophilic properties. For the formation of gel form, glyceryl monostearate or glyceryl monoolein was mixed into the gelatinized corn starch as a gelling agent. Sultana *et al.* (2000) postulated that the addition of starch to an alginate mixture increased recovery of encapsulated cells without altering its sensitive to acid. Fig. 1 shows the optical microscope observation of non-gelatinized corn starch, glyceryl monoolein, and glyceryl monostearate. The granule of non-gelatinized corn starch and glyceryl monostearate can be clearly seen in the suspension with distilled water (Fig. 1A and C). On the other hand, the glyceryl monoolein were not visible clearly, but the fibrous form can be observed (Fig. 1B).

In order to develop the formulation of encapsulation for *L. plantarum,* the gelatinized corn starch was mixed with or without glyceryl monoolein or glyceryl monostearate. Their structure was observed by optical microcopy and shown in Fig. 2. Comparing to control (without gelling agent), the structures of matrix prepared with gelling agent showed relatively small and uniform particles regardless of type of gelling agent. However, all images showed the irregular form, namely, the rounded shape or coating membrane could not be seen at the simple mixing treatment *L. plantarum* and starch irrespective gelling agent. From these observations, the coating membrane was not clearly shown in the bulky mixture between gelatinized starch and *L. plantarum*. A similar result was reported by Sultana *et al.* (2000). They demonstrated the positive role of incorporation of resistant starch and glycerol into the alginate mix during encapsulation. Therefore, we have attempted to manufacture the round shape of capsule in order to produce the obvious membrane of gelatinized starch on the surface *L. plantarum*. W/O emulsion formation was applied into this system and it will be described in followed section.

DSC

The thermal properties of glyceryl monoolein and glyceryl monostearate were presented in Fig. 3. The melting point of glyceryl monoolein and glyceryl monostearate was presented at 42.1ºC and 187.5ºC. When glyceryl monostearate selected as a immolibizer (gelling agent), we expected that bulky mixture of the coated *L. plantarum* can maintain the solid form at the room temperature. Glyceryl monoolein initiate to melt from 30ºC, whereas glyceryl monostearate still remained solid state at the same temperature. From these results, it was expected that glyceryl monostearate was proper as a gelling agent for the protection of *L. plantarum* at chicken body temperature of around 40-41ºC. Therefore, glyceryl monostearate was selected not only as a gelling agent but also as an emulsifier for the stabilizer in the W/O emulsion system. Relevant studies have shown that melting point of glyceryl monoolein and glyceryl monostearate for the pure form

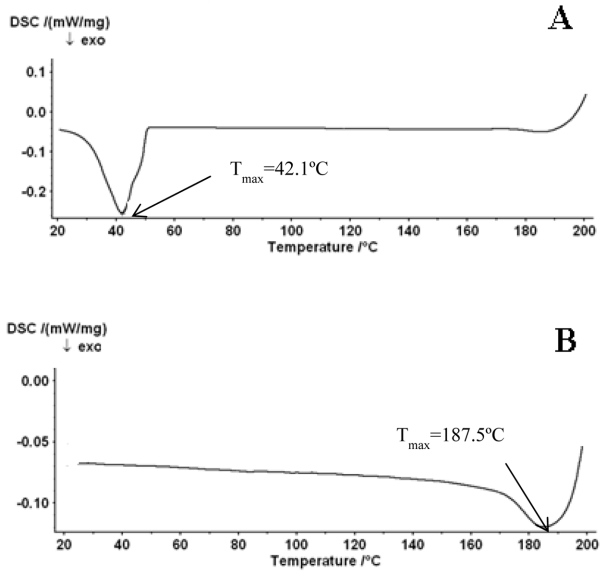

Fig. 3. Thermal properties of (A) glyceryl monoolein and (B) glyceryl monostearate, melting point of glyceryl monoolein and glyceryl monostearate were 42.1 and 187.5°C.

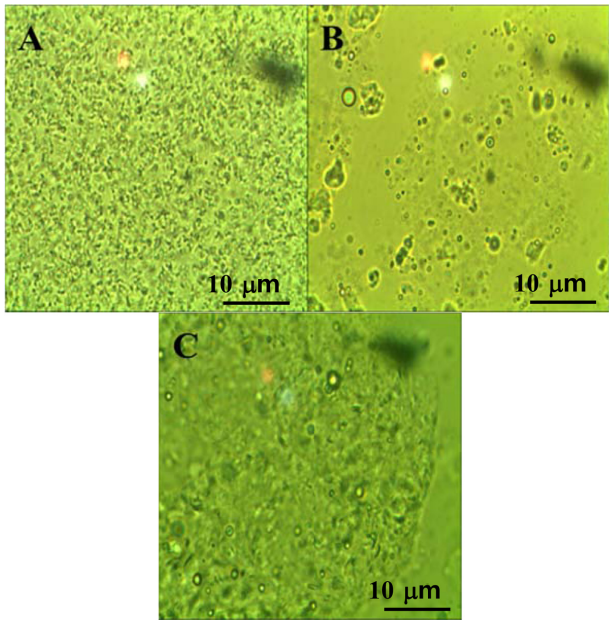

Fig. 4. Morphological structure of (A) *L. plantarum* suspension without starch, (B) starch bead without *L. plantarum* and (C) *L. plantarum* coated within starch gel by W/O emulsion.

was 36°C and 81.5°C respectively (Ganem-Quintanar *et al.*, 2000; Raymond and Cornish, 1968). The melting point of glyceryl monostearate was higher than that of glyceryl monoolein. This might be the reason of the different type of fatty acid of the monoglyceride.

Morphological properties of encapsulation of *L. plantarum* by W/O emulsion formation

The aqueous phase and oil phase was mixed at the ratio of 2:1 (v:v) to disperse aqueous phase droplet into the oil continuous phase with the addition of glyceryl monostearate. Their morphological structure was shown in Fig. 4. *L. plantarum* without starch showed the suspended dispersion in distilled water (Fig. 4A). The empty starch gel did not present any *L. plantarum*, whereas the starch hydrogel as a dispersed phase contained *L. plantarum* surrounded gelatinized starch membrane. According to Muthukumarasamy *et al.* (2006), the shape of microcapsules produced by various methods such as extrusion was almost round shape with stable W/O/W emulsion. They reported that lactic acid bacteria encapsulated with alginate and starch can be protected from the acidic condition (Muthukumarasamy *et al.*, 2006).

Effect of pH and temperature on viability of *L. plantarum*

Viabilities of *L. plantarum* during storage period at acidic and neutral conditions at 37°C are depicted in Fig. 5. Initially, viable count of *L. plantarum* in control was estimated about 9.5 Log CFU/mL, and no general changes in the viability was shown during 6 h storage. In the case of capsuled sample, *L. plantarum* tended to decrease with increasing time, and significant decrease was obtained after 6 h storage ($p<0.05$). Under the acidic condition (pH 2), drastic decrease in the *L. plantarum* count was observed regardless of capsulation. Both treatments showed complete inactivation within 2 h. However, capsuled sample showed more resistance (2 h) under extreme pH condition comparing to 1 h of untreated *L. plantarum*. Meanwhile, both treatments exhibited higher viability at neutral condition, where *L. plantarum* count was decreased after 1 h ($p<0.05$) then the decrease in the count was gradual. In particular, capsuled *L. plantarum* exhibited highly stable at the neutral condition. Shima *et al.* (2009) reported that the survival rate of *L. acidophilus* incorporated in the inner-water phase of a W/O/W emulsion was highest under the model digestive juice containing the bile acids. They reported that the viability of free lactic acid bacteria dispersed in the juice was rapidly decreased, then, a colony on the MRS broth agar plate was not detected in 1.33 h. Chandramouli *et al.* (2000) observed that *L. acidophilus* encapsulated by calcium alginate can be significantly survived at gastric condition (pH 2) than free cells.

At the elaborated incubation temperature (40°C), the viability of *L. plantarum* showed similar pattern to those obtained at 37°C (Fig. 6). For control, both treatments

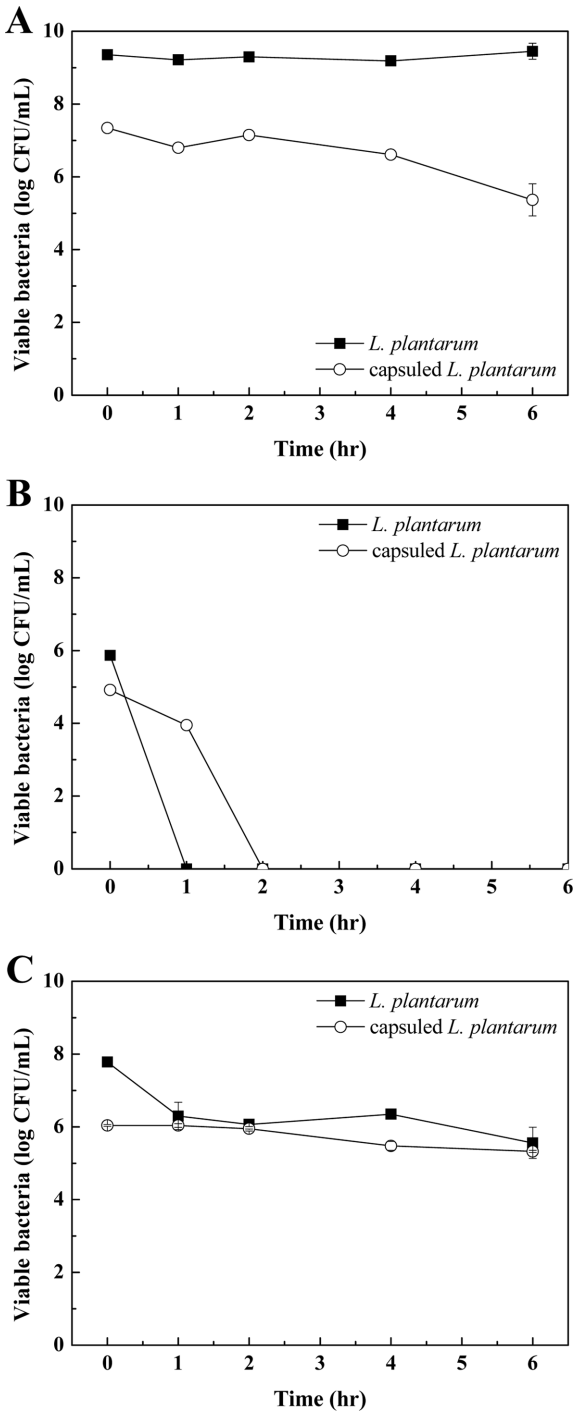

Fig. 5. Effect of pH on the viability of *L. plantarum* stored at (A) control, (B) pH 2 and (C) pH 7 under 37°C.

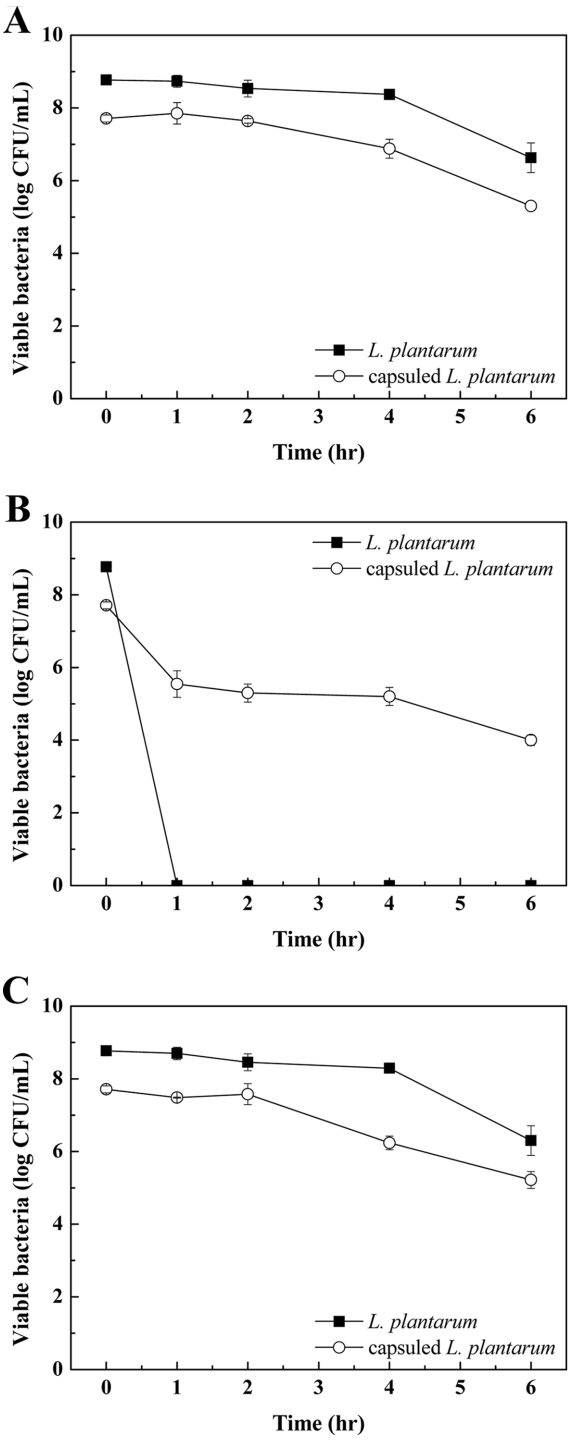

Fig. 6. Effect of pH on the total viable count of *L. plantarum* stored at (A) control, (B) pH 2 and (C) pH 7 under 40°C.

showed a steady decrement for 4 h incubation, thereafter the decrease was drastic. Although capsuled *L. plantarum* showed same pattern to uncapsuled sample, the count of capsuled sample was lower about 1.5 Log CUF/mL than those of uncapsuled. At acidic condition (pH 2), *L plantarum* was deactivated after 1 h incubation, while capsuled sample showed stable and minor decrease with incubation time. The viability of *L. plantarum* at neutral condition was similar to the counterpart of controls.

Effect of temperature on rheological properties of starch bead

The viscosities of starch bead collected at varying incu-

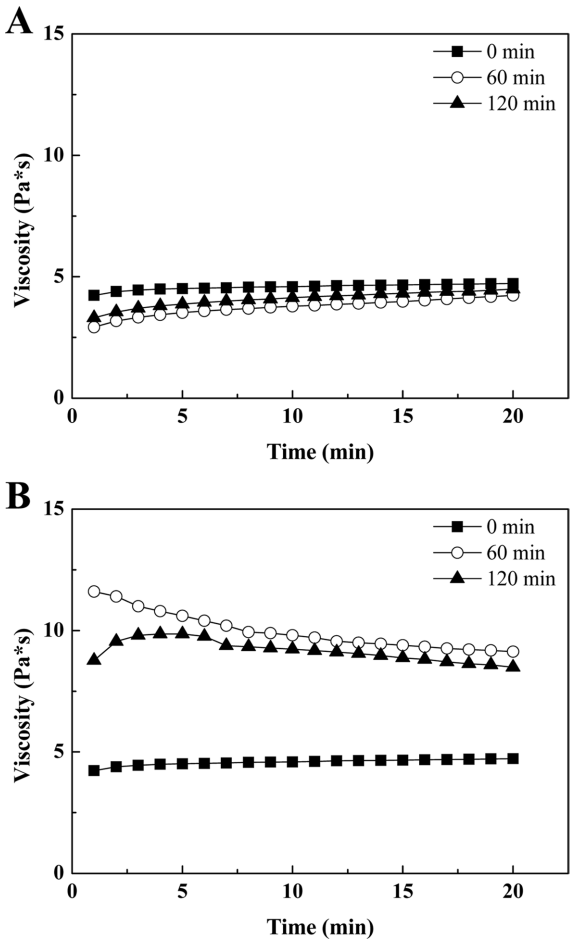

Fig. 7. The viscosity curve of starch bead measured at (A) 37°C and (B) 40°C.

bation times at 37°C were similar to one another and ranged 3-4 Pa·s (Fig. 7A). On the other hand, the viscosities of starch bead at 40°C were different depending on incubation time. Initially (0 min), starch bead showed similar viscosity to those found at 37°C. However, the viscosity of bead incubated at 40°C for 60 min had about twice higher than that incubated for 0 min. The viscosity of starch bead incubated for 120 min tended to decrease slightly, still the viscosity was higher than that for 0 min.

Conclusions

Encapsulated starch gel bead of *L. plantarum* was produced by water in oil (W/O) emulsion system with glycerol monostearate and corn starch. Encapsulated bead gel showed round shape with around 20 mm of diameter containing of *L. plantarum*. Lactic acid bacteria in the water phase at W/O emulsion system significantly persisted representing higher viability in the acidic condition. The results of this study can be concluded that corn starch as a wall material and glycerol monostearate as a gelling agent can play a role as a coating material for lactic acid bacteria from the extreme condition after encapsulation. Therefore, this formulation might be applied into the chicken feed industry instead of antibiotics to improve the health of chicken.

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