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Selection of Microencapsulation Method to Improve Antimicrobial Agents Production by Some *Lactobacillus* Species and *Propionibacterium thoenii* in Dairy Products

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THE AIM of this research is to select the agreeable encapsulation method to improve antimicrobial production from *Lactobacillus acidophilus, Lactobacillus reuteri, Lactobacillus rhamnosus* and *Propionibacterium thoenii.* The effect of different organic acid concentrations (1 and 2 w/v), different pH values (3, 4, 5, 6. 7 and 8), different temperature degrees (0, 7, 25, 37 and 45°C) and storage temperature on viability of encapsulated bacteria were investigated. Also, the efficiency of microencapsulated methods (alginate + NaCl, alginate + oil and *K*-carrageenan) on enhancement of antimicrobial production were studied. Microencapsulation with alginate + NaCl offered greater production in extreme conditions (low pH, low temperature and in the presence of organic acids). In addition, this method was more effective against pathogenic bacteria by enhancement of antimicrobial production, thus it may be effectively used to increase the safety and the shelf- life of dairy products.

Keywords: Microencapsulation, Antimicrobial, *Lactobacillus* spp., *Propionibacterium thoenii.*

The antimicrobial compounds of microbial origin use as a bio-preservatives can enhance the safety and extension the shelf-life of food. Many of the antimicrobial substances produced by lactic acid bacteria and propionic acid bacteria such as *Lactobacillus rhamnosus, Lactobacillus acidophilus* and *Propionibacterium thoenii* were provided to eliminate the growth of food born pathogens. The main problem concerning the batch process is the low yield of bacteriocins (Ivanova *et al.*, 2002). One of the methods applied for maintaining high cell concentration and higher bacteriocins production is microencapsulation (Sheu *et al.*, 1993). Many methods have been developed to immobilize microorganism cells. Entrapment is a process in which the cells are entrappted within the encapsulation material in order to reduce cell injury or loss (Shah & Ravula, 2000). Among the various developed methods for cell immobilization, the encapsulation in alginate or *K*-carragenan was used in many researches (Sheu *et al.*, 1993; Sadek *et al.*, 2003 and El-Shafei *et al.*, 2003). The encapsulation of the cells in capsules offers space for the cell growth and good diffusion properties. The immobilization of culture cells is a very promising tool for the improvement of processes of these microorganisms. The technology has interested in the dairy sector and the use of

immobilized starter has been proposed for a variety of fermentation such as fresh cheese, yoghurt and fermented cream (Provost *et al.*, 1985; Champagne & Cote, 1987 and Sodini-Gallot *et al.*, 1995). Therefore the present study aimed to select the preferable method of encapsulation to enhance shelf- life of dairy products and determine viability of encapsulated strains under different conditions (pH, temperatures, organic acids and storage).

Materials and Methods

Cultures

Lactobacillus reuteri B-14171; *Lactobacillus rhamnosus* B-445; *Bacillus cereus* B-3711; *Saccharomyces cerevisia* Y-2223 and *Aspergillus flavus* were provided by the Northern Regional Research Laboratory. Illinois, USA (NRRL). *Listeria monocytogenes* 598 was provided by the Department of Food Science, University of Massashusetts, Ambert MA, USA. *Escherichia coli* 0157: H7; *Staphylococcus aureus* were isolated and serologically identified by dairy microbiological Lab., National Research Centre. *Propionibacterium thoenii* P-127 was provided by Department of Food Technology, Propionibacteria culture collection, Iowa State University. *Lactobacillus acidophilus* was obtained from Chr. Hansen's Lab., Denmark. *Salmonella typhimrium; Yersenia enterocolitica* were obtained from Hungarian National Collection of Medical Bacteria, OKI, Gyaliut 2-6, H-1966 Budapest, Hungary and *Aspergillus niger* were provided by the Institute of Applied Microbiology, University of Tokyo, Japan.

Culture preparation

The pathogenic strains were routinely transferred into trypton soya broth, incubated at 37°C for 24hr. Yeast and mold strains activated in Malt extract broth (Oxoid) and incubated at 25°C for 72 hr. *Lb. reuteri, Lb. rhamnosus* and *Lb. acidophilus* were activated in DE Man Rogosa Sharpe (MRS) broth (Oxoid) and incubated at 37°C for 48hr and *P. thoenii* was activated in sodium lactate broth and incubated at 30°C for 72hr (Rehberger & Glatz, 1998).

Experimental procedures

Cultivation and harvesting of lactobacilli and propionibacteria cells

MRS broth (Oxoid) was used to prepare the cell suspensions for lactobacilli which was inoculated with 2% (w/v) active Lactobacilli cells and incubated at 37°C for 48 hr. *P. thoenii* was activated by inoculating 2% (w/v) of its culture on sodium lactate broth and incubated at 30°C for 72 hr. Cells were harvested by centrifugation at 5000 rpm for 15 min at 4°C. The supernatant was used as bacteriocins source from cells after moderate pH to 7 using 1M - NaOH and were then heat sterilized at 72°C for 10 min. Cells were washed twice with saline and used to prepare capsules.

Preparation of microencapsulated cell culture

(Method I) Microencapsulation using alginate with NaCl: Suspension of cells was mixed with an equal volume of sodium alginate (4%, w/v). The mixture was added as drop-wise into solution of sodium chloride (0.2 mol/l) and calcium

chloride (0.5 mol/l) and magnetically stirred at 200 rpm till alginate beads were formed (Klinkenberg *et al.*, 2001).

(Method II) Microencapsulation using alginate with vegetable oil : Cells were microentrapped by mixing one part of suspended cells with four parts of sodium alginate (4%, w/v). One part of the mixture was then added drop wisely to five parts of vegetable oil (250 ml on 800 ml beaker) containing tween 80 (0.02%, v/v), and stirred at 200 rpm by magnetic stirrer until an uniformly turbid emulsion was obtained (within 10 min). Calcium chloride ~ 500 ml (0.50 M) was added quickly but gently down the side of the beaker until the water/ oil emulsion was broken. Calcium alginate beads were formed within 10 min alginate drops solidified upon contact with $CaCl_2$, forming beads and thus entrapped bacterial cells. The beads were collected by gentle centrifugation (350 rpm for 10 min). The entrapped cells were stored at 4°C until use. (Sheu & Marshall, 1993).

(Method III) Microencapsulation using K-carrageenan : This was proceeded by mixing 20 g cells (wet weight) in 1000 ml of a sterile solution of *K*-carrageenan (2%, w/v), then the mixture was added drop-wisely into KCl (3%,w/v) under agitation. *K*-carrageenan beads were formed within 10 min. (Dinakar & Mistry,1994).

Efficiency of microencapsulation methods on enhancement of antimicrobial activity using well diffusion agar

This method was carried out according to Batish *et al.* (1989) and Effat (2000). Melted agar medium was transferred to the Petri dishes and allowed to solidify. An aliquot of 0.2 ml of each pathogenic strain suspension was transferred to plates and spreaded uniformly over the agar surface with a sterile bent glass rod. Plates were dried at 37°C for 1 hr and wells of 6 mm diameter were cut, activated microencapsulated strain (24 hold) were grown in the selective media for 48 hr at 37°C for lactobacilli and at 30°C for *P. thoenii*. The cell–free supernatant of each tested strain culture was obtained after centrifugation at 5000 rpm/min at 4°C for 10 min. The supernatants (0.1 ml) were added into wells after neutralization to pH 6.0 and heating at 71°C for 10 min (Effat, 2000) the plates were then incubated at 37° for 24hr. The inhibition zone formed around each well was measured in mm. (Plockov *et al.*, 1997 and Effat, 2000). Free cell culture was subjected to the same conditions to serve as a control.

Effect of activation media pH on survival of microencapsulated strains

MRS and sodium lactate broth media were prepared, distributed as 100 ml volumes and sterilized, then adjusted to pH values of 3, 4, 5, 6, 7 and 8, using sterilized HCl solution. The media were inoculated singly with 10% (w/v) of each the tested microencapsulated strains of *Lactobacillus* and *Propionibacterium*, respectively, and then incubated at 37 °C for 48 hr. Free cells were subjected to the same conditions and used as control (Kim *et al.*, 1996). Counts were determined initially and after 48 hr using MRS and sodium lactate agar media at 37, 30°C, in that order, anaerobically for 48 and 72 hr.

Effect of acidification with different organic acid concentrations on the survival of microencapsulated strains

To determine the stability of microencapsulated cells, under acidic conditions, acidified MRS and sodium lactate broth media with 1 and 2 % (w/v) of lactic, acetic and citric acid were inoculated with 10 % (w/v) of the tested encapsulated strains as well as free cells. The tested cells were then plated immediately (initial count) on MRS agar and sodium lactate agar (Mosilhey, 2003).

Influence of incubation temperature on the viability of microencapsulated strains

To study the influence of incubation temperature on the viability of immobilized strains, MRS and sodium lactate broth media were inoculated singly with 10 % (w/v) of each the tested microencapsulated strains of lactobacilli and propionibacteria, respectively. Then incubated at 7, 25, 37 and 45°C for 24 hr. The plates were inverted and incubated at 37°C for 48 hr under anaerobic conditions.

To determine the effect of storage at low temperatures, additional two sets of the inoculated media were prepared and stored at 0 and 7°C for 4 weeks. The counts were estimated intervally, using the same previos technique (Mosilhey, 2003).

Results and Discussion

Efficiency of microencapsulation methods on the enhancement of antimicrobial production

The data obtained (Fig. 1) showed that microencapsulated strains with different techniques appeared to be inhibitory with a variable spectrum activity against pathogenic bacteria, yeasts and moulds.

The antagonistic activity of microencapsulated strains were higher than that of the free strains (control), especially by the method I (alginate + NaCl). Microencapsulated *Lb. acidophilus* and *P. thoenii* (Method I) have the most inhibitory effect against *Y. enterocolitica, B. cereus,* and moulds tested, respectively compared with the control and other methods (Fig. 1-B). Similar results were obtained by El-Kholy *et al.* (2006), who found that *P. thoenii* P.127 produced metabolites with antimicrobial activity against coliform yeast and mould. Also, Effat (2000) recorded that the anti-fungal substance produced by *Lb. reuteri* could be used as a bio-preservative against yeasts and moulds. Generally, our results accorded with the results obtained by Sadek *et al.* (2003), who concluded that metabolites of entrapped *P. thoenii* proved to be more effective against *B. cereus, Sac. cerevisia* and *A. niger.* On the other hand, microencapsulated of cell in gel matrix of alginate has been shown to offer many advantages for biomass and metabolite production compared with the free strains. Moreover, the foregoing results are in accordance with those reported by El-Kholy *et al.* (2003) who added immobilized *Lb. reuteri* and *Lb. rhamnosus* to milk and used this milk in manufacturing soft cheese and resulted in a pronounced reduction in numbers of the pathogens, total bacterial count, total psychrotrophic bacteria, mould and yeasts. So, applying it as a strategy can increase the safety and the shelf-life of soft cheese.

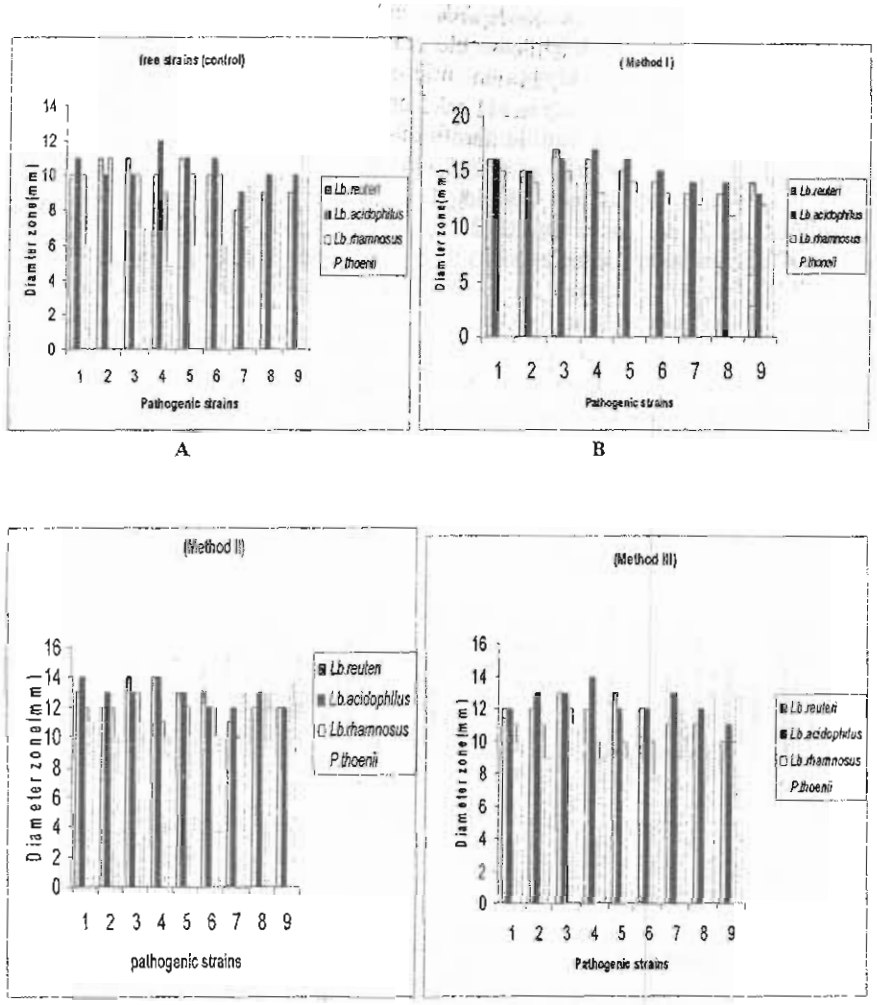

Fig. 1. Efficiency of microencapsulation methods on enhancement of antimicrobial production by same *Lactobacilli* species and *P.thoenii* against:
1- Staph. aureus 2- S.typhimrium 3- L. monocytogenes 4-Y. enterocolitica 5- E. coli 6- B. cereus 7- Sac. cerevisia 8- A. flavus 9- A. niger.

Effect of activation media pH on the survival of microencapsulated strains

The effect of activation media pH on the survival of microencapsulated strains is shown in Fig. 2. It could be observed that the survival count of strains was affected with pH values and the pronounced effect was at the extreme pH 3.0. Microencapsulated cells in alginate + NaCl (Method I) (Fig. 2B) gave high survival rate followed by the microencapsulated cells by Method II (Fig. 2C).

Also, microencapsulated *Lb. acidophilus* had high viable count followed by *Lb. reuteri* at pH 3.0 and 4.0 while viable count of *Lb. rhamnosus* and *P. thoenii* disappeared at pH 3.0. In addition, microencapsulated cells in Method I and Method II gave high survival rate at pH 5 compared with microencapsulated cells in Method III (Fig. 2D). A similar finding were reported by El-Shafei *et al.* (2003) who found that free cells of *B. bifidium* in MRS broth incubated for 3 hr at pH 3.0 decreased by almost 1.8- log cycle. On the other hand, counts of encapsulated cells decreased by 0.52 - log cycle, indicating that encapsulated *B. bifidium* was more resistant at pH 3.0. Besides, Mandel *et al.* (2006) found that survival of the encapsulated *Lb. casei* NCDC.298 was better at low the hilly acidic pH (1.5), as compared to free cells, also the survival of lactic acid bacteria was especially affected by environmental low pH. On the other hand, Shah & Jelen (1990) and Lankaputhra & Shah (1995) reported that although, *Lb. acidophilus* tolerates acidity, a rapid decrease in their numbers had been observed under acidic conditions both in *vitro* and in *vivo*.

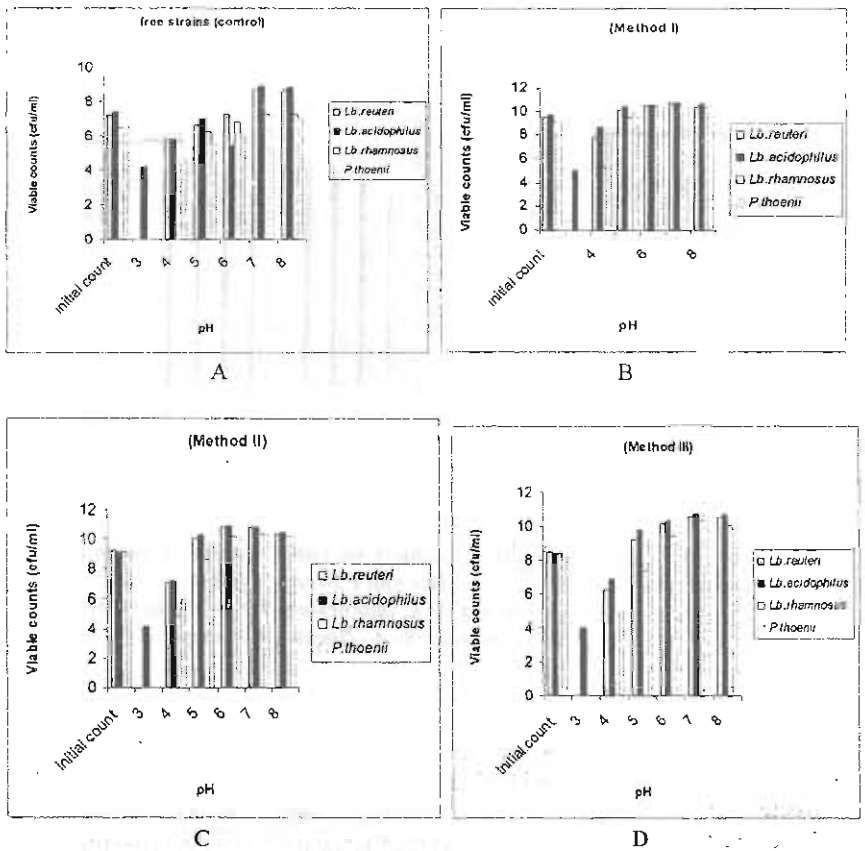

Fig. 2. Effect of activation media pH on the survival of microencapsulated strains at 37°C.

Effect of acidification with some organic acids on the survival of microencapsulated strains

The effect of acidification with some organic acids on the survival of microencapsulated strains was shown in Fig. 3. Generally, the viability of all tested strains decreased in the presence of organic acid, however this decrease was not more than 1 log cycle of total viable count for all microencapsulated strains compared with free strains (Fig. 3A). Microencapsulated *Lb. reuteri* and *Lb. acidophilus* had higher viability reaching the highest viable counts when exposed to organic acids comparing with the other tested strains. The most marked effect, in the presence of 2% (w/v) lactic acid followed by the 2% (w/v) acetic acid and citric acid, respectively. It is worthy to mention that the decline was more rapid for free cells than for microencapsulated cell. On the other hand, microencapsulated cells within alginate + NaCl showed more resistance to organic acids compared to the other encapsulated materials. The presented data confirmed those obtained by Mosilhey (2003), where he reported that the total viable counts of microencapsulated *Lb. acidophilus* during the refrigerated storage in lactic, acetic and citric acids solution in pH ranged from 3-5 decreased overtime in the order of pH 3.0, 4.0, and 5.0 compared to the control (pH 6.4). Also, the drop was more rapid for free cells than for microencapsulated cells. A similar observation were recorded by Nighswonger *et al.* (1996) and showed that the antimicrobial effect of the diacetyl, acetic acid and lactic acid present in the cultured buttermilk could be responsible for the decline of viability. According to this by the free *Lb. acidophilus* exhibited a higher loss in cell viability than for microencapsulated cells in the presence of organic acids (acetic and citric) during refrigerated storage.

Organic acids such as lactic, acetic and citric acids are commonly used in food manufactures mainly as anti-microbial preservatives or acidulates in variety of food products. Many factors influence the effectiveness of organic acids as anti-microbial, the most important is undoubtedly the pH of the food. Many investigators (Siragusa & Dikson, 1992; Doores, 1993 and Conner *et al.*, 1997) demonstrated that the activity of organic acids was related to pH and that the undissociated form of the acid is primarily responsible for its anti-microbial activity.

Influence of incubation temperature and period on the viability of microencapsulated strains

The effect of incubation temperature on survival of microencapsulated strains are shown in Fig. 4. It could be observed that survival counts of strains were affected with incubation temperature. Microencapsulating strains showed stable viable counts and survivability of strains compared with free strains which decreased at 0°C and 7°C, specially *P. thoenii* which, completely disappeared at these degrees. Microencapsulated cells within alginate + NaCl (Method I) and alginate + oil (Method II) gave more stable rate comparing with encapsulated cell in *K*-carrageenan (Method III). The drop in the counts of microencapsulated strains during storage (1 month) at 0°C and 7°C was slower than of the non-encapsulated counter parts. Microencapsulated *Lb. acidophilus* and *Lb. reuterii*

were more resistant to low temperature (0°C, 7°C) and the higher temperature (45°C) comparing with *P. thoenii*, which disappeared at 0°C. (Fig. 5 and 6). These results accorded with those obtained by Mosilhey (2003), who exposed free *Lb. acidophillus* to 37, 45, 50, 55 and 60°C for 30 min. He reported that 53°C was considered as the sub-lethal temperature for *Lb. acidophilus*, because cells were still growing, while 60°C was a lethal temperature. On the other hand, the results for microencapsulated cells demonstrated more thermo-tolerant cells at 60°C comparing to free cells. Microencapsulated *Lb. acidophilus* were less thermo- tolerant at 63°C and 65°C. In this respect, Laeroix *et al.* (2004) recorded that microencapsulated technique gave high cells density and very high volumetric productivity and high process stability (Physical and biological) over long fermentation periods, improved resistance to contamination, uncoupling of biomass and metabolite productions, stimulation of production and secretion of secondary metabolites, physical and chemical protection of the cells comparing with the free cells.

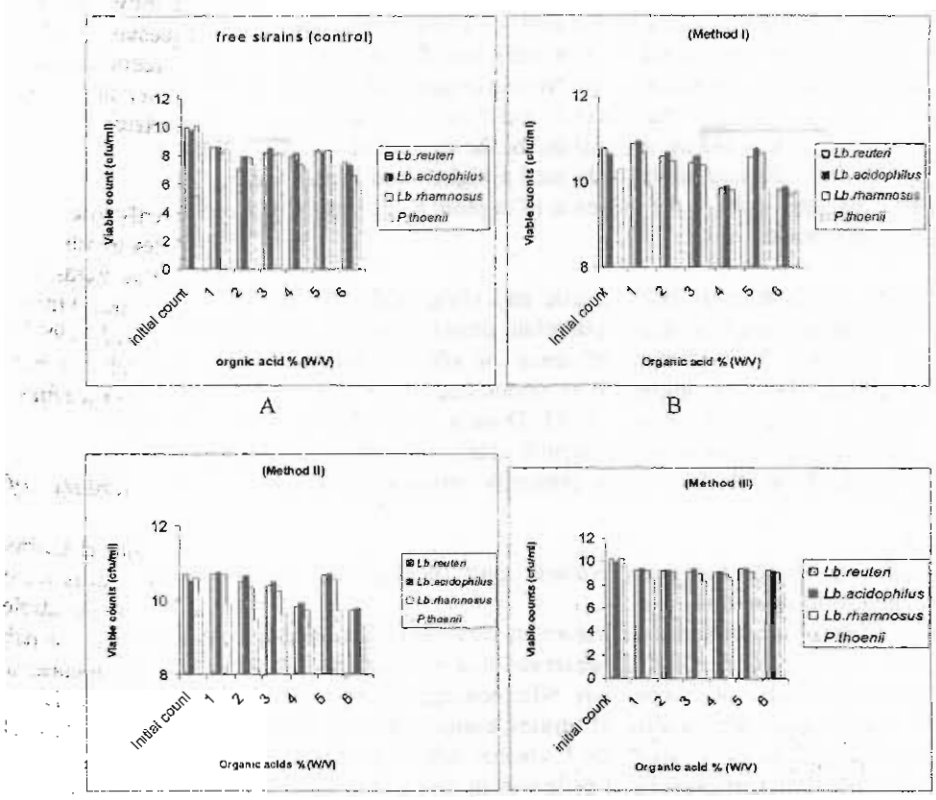

Fig. 3. Effect of organic on survival of microencapsulated strains at 37°C/48 hr.
1- lactic acid 1% 2- lactic acid 2% 3- lactic acid 1% 4- lactic acid 2%
5- citric acid 1% 6- citric acid 2% .

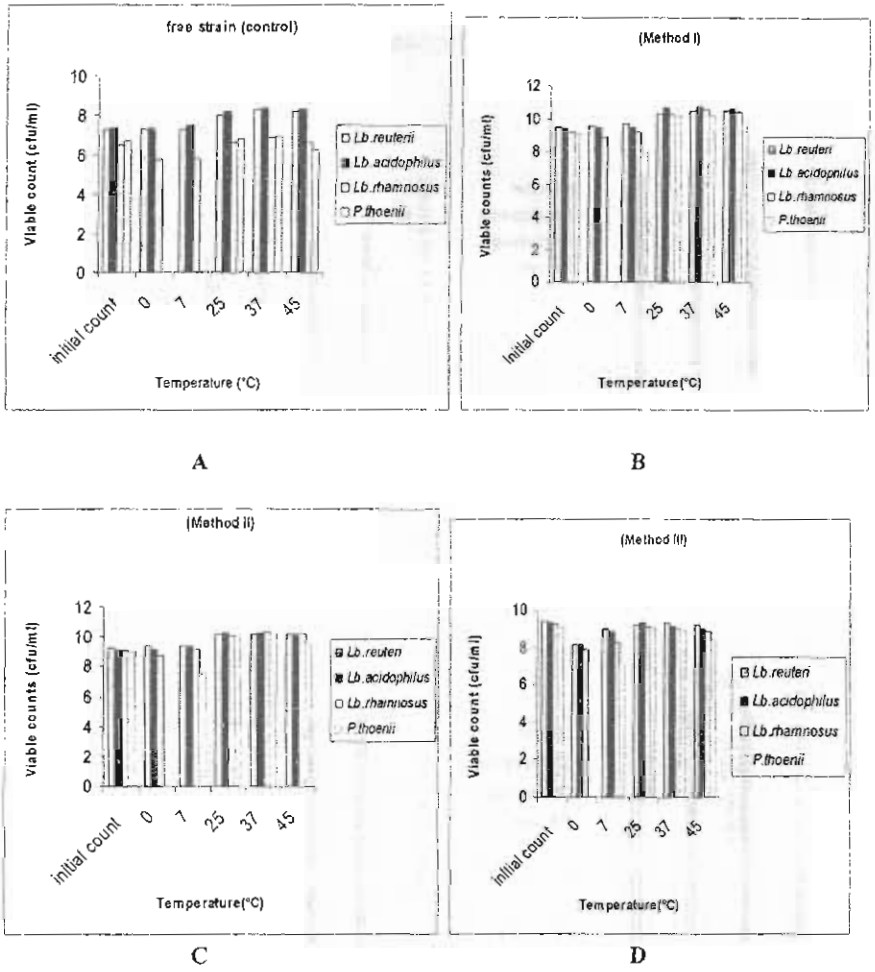

Fig. 4. Influence of incubation temperature on viability of microencapsulated strains.

On the other side, Sheu & Marshall (1993) reported that about 40% more lactobacilli survival freezing of ice cream when they were entrapped in calcium alginate than when they not entrapped. Moreover, encapsulation protected the microorganisms in batch-frozen and continuously frozen ice cream mixers (Sheu *et al.*, 1993). Also, Kim *et al.* (2007) found that when exposing the non-encapsulated and encapsulated *Lb. acidophilus* ATCC43121 to 65°C for 30 min, the bacterial counts decreased from 2.0×10^7 to 3.5×10^4 and from 1.2×10^7 to 1.2×10^5 cfu/ml, respectively. This substantiated that *Lb.acidophilus* ATCC43121 immobilized on alginate micro-particles showed high heat stability than the non-encapsulated.

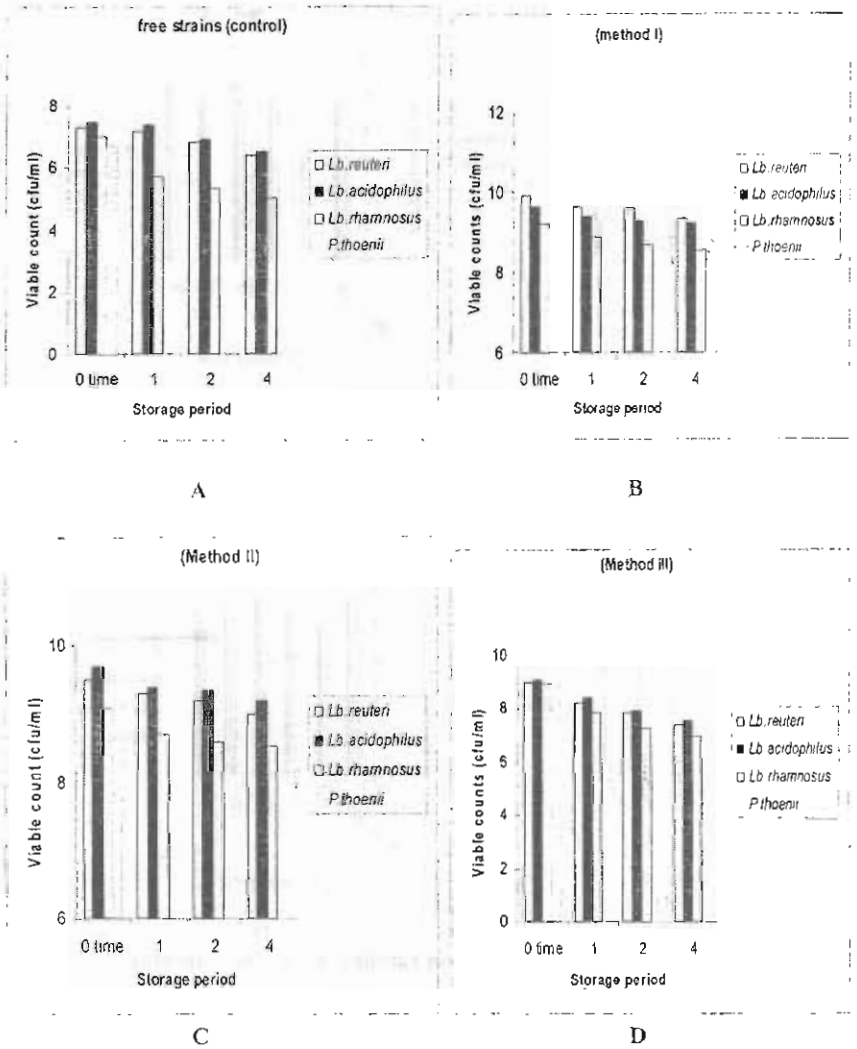

Fig. 5. Viability of microencapsulated strains during storage at 0°C.

Conclusion

From the forgoing results, it could be concluded that microencapsulated strain with alginate +NaCl have high resistant to extreme conditions as low pH, low and high temperature degrees and in presence of organic acids. In addition, this method maintained the cell concentration for production of antimicrobial substances and to be more effectively against pathogenic bacteria. Consequently, it could be expected that the microencapsulated cells are able to improve the shelf-life, quality and safety of some dairy products.

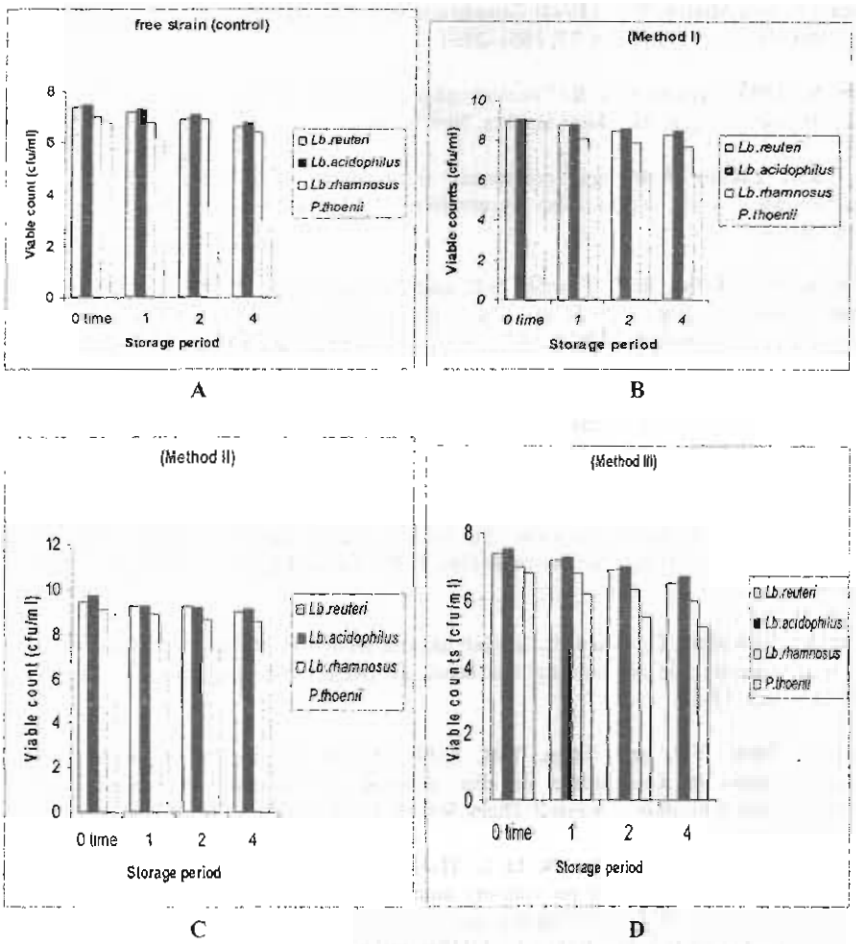

Fig. 6. Viability of microencapsulated strains during storage at 7°C.

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اختيار طريقة كبسلة لتحسين إنتاج المواد المضادة للميكروبات من بعض سلالات اللاكتوباسليس وبكتريا حمض البروبيونيك فى بعض منتجات الألبان

سمير عبد الواحد الجيزاوى ، أسامه محمود شرف* ، الفت سيد بركات ، فاطمة احمد فتحى* وهدى سمير السيد*

قسم الميكروبيولوجيا الزراعية – كلية الزراعة – جامعة القاهرة و*قسم الألبان – المركز القومى للبحوث – القاهرة – مصر.

الهدف من هذا البحث هو اختيار انسب طريقه للكبسله وذلك بغرض تحسين أنتاج المواد المضادة للميكروبات المرضية من بكتريا:
Lactobacillus acidophilus, Lactobacillus reuteri, Lactobacillus rhamnosus, Propionibacterium thoenii.

وأيضا دراسة تأثير بعض العوامل مثل الأحماض العضوية بتركيزات ١و٢٪ (وزن/حجم) , وتأثير درجات pH مختلفة (٣، ٤ ، ٥ ، ٦ ، ٧ ، ٨) ودرجات الحرارة المختلفة (١٠، ٧ ، ٢٥ ، ٣٧ ، ٤٥م°) ودرجات حرارة التخزين على حيوية البكتريا المكبسلة . وكذلك دراسة كفاءة طريقة الكبسلة (الألجينات مع NaCl ، الألجينات مع الزيت ، الكابا_ الكارجينات) على تحسين انتاج المواد المضادة للميكروبات المرضيه. ووجد أن أفضل طريقة للكبسله باستخدام الألجينات مع NaCl حيث أن كبسلة البكتريا بهذه الطريقة أدى إلى زيادة قدرتها على تحمل الظروف الغير عاديه (انخفاض الpH وانخفاض وارتفاع درجات الحرارة وفى وجود الأحماض العضوية) اضافه إلى انها أدت إلى تحسين إنتاج المواد المضادة للميكروبات المرضية. وتعتبر طريقة الكبسلة باستخدام الألجينات مع NaCl مناسبة لتحسين إنتاج المواد المضادة للميكروبات المرضية وبغرض زيادة درجة الأمان ومدة حفظ منتجات الألبان.