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Fluidized Bed Microencapsulation of *Lactobacillus Sporogenes* with Some Selected Hydrocolloids for Probiotic Bread Production

S. S. Mirzamani [a], ***A. Bassiri*** [b*], ***H. Tavakolipour*** [c], ***M. H. Azizi*** [d], ***.M. Kargozari*** [e]

[a] MSc of the Department of Food Science and Technology, North Tehran Branch, Islamic Azad University, Tehran, Iran.
[b] Assistant Professor of the Department of Chemical Technologies, Iranian Research Organization for Science and Technology (IROST), Tehran, Iran.
[c] Associate Professor of the Department of Food Science and Technology, Sabzevar Branch, Islamic Azad University, Sabzevar, Iran.
[d] Associate Professor of the Department of Food Science and Technology, Tarbiat Modares University, Tehran, Iran.
[e] Assistant Professor of the Department of Food Science and Technology, North Tehran Branch, Islamic Azad University, Tehran, Iran.

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ABSTRACT: This research investigated the encapsulated *Lactobacillus Sporogenes* resistance to simulated gastric acid condition and extreme heat treatment and the aim was the production of probiotic bread using the encapsulated probiotic. Microcapsules were produced using the *L. Sporogenes* by a two-step fluidized bed granulation and button spray coating technique with microcrystalline cellulose powder and alginate or xanthan gum as the first layer to enhance bacterial survival under gastrointestinal conditions and gellan or chitosan as the outer layer to increase heat resistance. The results indicated that encapsulation efficiency decreased with increasing the level of both alginate and xanthan and microcapsule containing 0.5% xanthan had significantly higher ($p<0.05$) encapsulation yield. In the case of acid resistance, 1.5% xanthan in the wall matrix made significantly ($p<0.05$) higher viability of the probiotics. 0.5% chitosan in the outer layer of the microcapsules caused probiotic more resistant to the heat treatment of 90°C for 15 min. Heat treatment for 30 min at 90°C decreased severely the probiotics population. Moreover, the results from SEM indicated that chitosan represented the smoother surface which is an essential factor to protect cells against environmental condition. Evaluation of encapsulated Probiotic viability in bread showed that 1.5% gellan in outer layer caused higher survivability 24 h after baking. These finding indicated that the application of alginate and chitosan in the microcapsules can protect the *L. Sporogenes* and considered as an effective method in probiotic bread production.

Keywords: *Fluidized Bed Drying, Lactobacillus Sporogenes, Probiotic Bread, Simulated Gastric Acid.*

Introduction

Functional foods that contain probiotics are continuously increasing in the global market (Amin *et al*., 2013). Various health benefits, for example on disorders relating to the gastrointestinal tract, are reported on the consumption of different probiotics (Kailasapathy & Chin, 2000). The most important probiotic microorganisms belong to the group of lactic acid bacteria (Axelsson, 1993). However, supplementation of functional foods with probiotic bacteria poses a considerable technological challenge: to provide health

*Corresponding Author: bassiri@irost.ir

benefits, the level of viable probiotic bacteria should be $>10^7$ colony-forming units (cfu) when consumed (FAO/WHO, 2001). Bread has been part of the human diet for thirty thousand years. It provides energy in the form of carbohydrates and essential nutrients, dietary fibre and phytochemicals. The development of probiotic bread presents many challenges, particularly with respect to the thermal stability of the bioactive compounds during baking, storage and passage through acidic gastric environment. With the objective of maintaining the viability of probiotics during production, storage and consumption of food to which these microorganisms were added, new technologies have been proposed and among them the microencapsulation seems to be a promising technique. The goal of microencapsulation of probiotics is to protect microorganisms from adverse conditions, enabling the arrival in the intestine at the concentration required to exert its beneficial effect (Kailasapathy, 2002, 2006; Krasaekoopt, Bhandari, & Deeth, 2003; Shah, 2000).

Corsetti *et al*. (2008) stated that Probiotics including lactic acid bacteria improve the bread quality and on the other hand, oligosaccharides in the whole-wheat bread can improve the growth of probiotic bacteria (Charalampopoulos *et al*., 2002). The non-pathogenic spore forming *Bacillus* species such as *B. coagulans (lactobacillus sporogenes)* have been widely used as probiotics for both animal and human (Elshaghabee *et al*., 2017). Spore forming probiotic bacteria are heat resistant and offer a number of advantages over other non-spore formers such as *Lactobacillus* and *Bifidobacterium* species. Various methods for probiotics microencapsulation that have been reported, including spray-drying, spray-coating, extrusion, emulsification, fluidized bed drying and complex coacervation (Champagne and Fustier, 2007, Martín *et al*., 2015). Due to an optimal heat and mass transport as well as uniform temperature distribution, fluidized bed encapsulation can be carried out at lower temperatures which results in higher survival rates of encapsulated bacteria (Barbosa-Canovas & Uliano, 2004; Chua & Chou, 2003). The coating material determines the protection and targeted release properties. The most widely used matrix for microencapsulation is alginate which has been found to increase the survival of probiotics from 80 to 90% (Mandal *et al*., 2006). Inulin is a low-digestible and soluble fiber increasingly used in human food preparation. It is well known as an important prebiotic substance influencing the microbial composition of the gastrointestinal tract of the host (Frank, 2002; Zaeim *et al*., 2020). Xanthan is a bacterial heteropolysaccharide composed of pentasaccharide repeating units. (Chen and Chen, 2007; Sultana *et al*., 2000). Gellan is also a heteropolysaccharide composed of tetrasaccharide repeating units (González-Cuello *et al*., 2012). Gellan gum reported as a suitable encapsulated material for temperature-sensitive cells such as lactic acid bacteria (Camelin *et al*., 1993).

Several studies have been carried out on microencapsulation of probiotics by fluidized bed coaters. Poncelet and *et al*. (2009), used Arabic gum, skim milk and sucrose for microencapsulation of *L. casei* and *L. acidophilus* on microcrystalline cellulose (MCC) by fluidized bed coater and determined the optimum parameters for prevention of agglomeration.

Stumme *et al*. (2010), coated *L. reuteri* with shellac- sodium alginate using fluidized bed coater and showed that the survival of bacteria after acidic test at gastric simulated conditions was 0.14%. Semyonov *et al*. (2012), used fluid- bed coating method for microencapsulation of *L. paracasei*; they suggested the optimum conditions of flow rate as 3.5 mL/min at 47 °C for prevention

of agglomeration. Nug and Das (2013) compared stabilization efficiency in terms of long term ambient temperature storage viability of *Lactobacillus casei* CRL 431 by using freeze and fluidized bed drying techniques. Fluidized bed drying was able to retain 2.5 log cfu/g higher viability after 52 weeks of storage at 25°C. Zaghari *et al*. (2018) used air-suspension fluidized-bed technique for generation of core and shell microcapsules containing probiotic *Lactobacillus reuteri* cells and the efficacy of shellac and sodium alginate at different concentrations on viability of capsules in simulated gastrointestinal conditions was evaluated. The relative survival (%) of bacteria coated with sodium alginate at concentration of 1% (w/v) was significantly higher compared to other solutions under simulated gastro-intestinal conditions (pH 2.0, for 1h). Seyedain-Ardabili *et al*. (2016) encapsulated *Lactobacillus acidophilus LA-5* and *L. casei 431* with calcium alginate and Hi-maize resistant starch via emulsion technique and coated with chitosan and the encapsulated probiotics were inoculated into the bread dough and bread loaves were baked. They demonstrated that *L. casei 431* was more resistant to high temperature than *L. acidophilus LA-5*. A significant increase in probiotic survival was observed when the protective coating of chitosan was used in addition to calcium alginate and Hi-maize resistant starch. Zaghari *et al.* (2018) reported cell viability of encapsulated bacteria with 5% w/v calcium chloride in baked bread after 1 and 24h storage was 0.56% and 0.52% respectively and was significantly higher compared to sample encapsulated with 1% w/v chitosan 0.47% and 0.44% respectively. The final counts of viable cells in both samples after 1 and 24 h storage were higher than 10^6 CFU/g.

The aim of the present work was to develop a microencapsulation process for *Lactobacillus sporogenes* (IBRC-M 10807) by a two-step fluidized bed granulation at bottom spray mode with the Wurster device coater to enhance the resistance to acid conditions and to increase the heat stability during bread baking.

Materials and Methods

- *Bacterial strain*

The bacterial strain *L. sporogenes* IBRC-M 10807 was obtained from Iranian Biological Resource Center (IBRC) located in Alborze province,Iran. These probiotic Gram-positive, rod shaped aerotolerant and heterofermen tative lactic acid bacteria exhibit optimal growth at 37 °C. Cell growth analysis were measured by absorbance at 600 nm (OD_{600}).

- *Encapsulation materials*

Microcrystalline cellulose (Avicel Ph-200; FMC Bio Polymer, Brussels, Belgium) as encapsulation matrix was employed according to Estaphan *et al*. (2008), Piar *et al*. (2013) and symonovo *et al*. (2012), either 10% (w/v) Inulin (Sigma-Aldrich, Stainhein, Germany) was added to the cell suspension to protect cells from thermal damage. An aqueous alginate (Sigma-Aldrich, Stainhein, Germany) and xanthan gum (Sigma-Aldrich, Stainhein, Germany) was used as coating material, to offer resistance against acid conditions and the second group of coating containing chitosan and gellan to improve the heat stability of microcapsules.

- *Growing conditions and culture preparations*

L. sporogenes IBRC-M 10807 were pre-cultured in 9 mL de Man-Rogosa-Sharpe (MRS) broth (pH 6.2) at 37 °C, using a cryo-culture. For inoculation, 9 mL pre-culture aged 24–48 h was added to 200 mL MRS-broth and cultivated in a 37°C agitating flask culture. The cells were harvested after 24 h by centrifugation (Eppendorf, 5702 R,

Germany) at 5000 ×g for 10 min at 4°C. For the fluidized bed drying process the resulting cell pellets were re-suspended in 34 mL Ringer solution to a viable count of 5 × 10^8–10^9 CFU mL^{-1} Followed by the addition of 10% (w/v) of inulin (Sigma-Aldrich, Stainhein, Germany), was added to the cell suspension. To prevent cooling off, the cell suspension was stored an hour at 37°C before drying. Cells dried in order to investigate their resistance to acid conditions were stored at room temperature (18–20°C). cell growth analysis were measured by absorbance at 600 nm (OD_{600}).

- ***Microencapsulation***
- ***Pre-tests***

In various pre-tests the fluidization pressure, atomization pressure and volumetric flow rate of the microencapsulation process were varied to examine their influence on process conditions, especially on the particle development (data not shown). For the pretests Microcrystalline cellulose powder was applied as a drying matrix as well as Inulin and Maltodextrin in water for granulation(Semyonov et al, 2012). Drying processes at the temperature of 30°C caused the fluid bed to become unstable and collapsed. In contrast, at a drying temperature of 37°C the drying process was stable and great thermal destruction of bacteria was minimized. Survival of bacteria increased at the temperature of 62°C.

- ***Fluidized bed granulation***

10 g of Microcrystalline cellulose powder was sieved once to eliminate existing agglomerates and to ensure a homogenous powder texture (75µm), before adding to the fluid bed granulator as a dry matrix. The slurry, consisting of Ringer solution, bacteria and inulin, was added to a 80 °C pre-heated fluid bed granulator using a peristaltic pump (Petro Gas Ausrüstungen Berlin KG, Berlin, Germany) and atomized by a pneumatic nozzle (d= 0.5 mm) in button spray position, applying an atomization pressure of 6 bar. The drying temperature was 37°C. According to the drying temperature, the spraying flow rate was adapted to 0. 5 mL/min which resulted in a drying time of 15–20 min. dependent on the used saccharide additive of the slurry. Fluidization of the microcrystalline powder was finally achieved by a fluidization air pressure of 4 bar and the slurry was sprayed onto the powder for granulation (Figure 1).

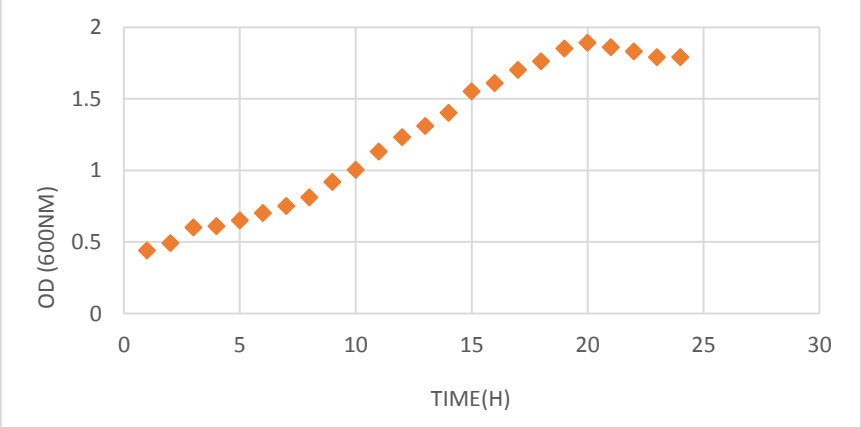

Fig. 1. *L. sporogenes* growth curve.

- ***Coating***

In order to increase the resistance against simulated gastric acid condition and heat resistance,after granulation microcapsules were coated by button spray respectively with an aqueous sodium alginate or xanthan gum solutions in the fluid bed granulator and chitosan or gellan. Only granules containing Inulin as thermo-resistant were used for coating. All parameters applied were the same as in the granulation step. Particles were coated for 15 to 20 min. The final chitosan or gellan coated beads were kept in 0.1% peptone solution and stored at 4˚C.

- Encapsulation Efficiency (EE)

Uncoated alginate and xanthan beads contain encapsulated *lactobacillus sporogenes* were disintegrated in sodium citrate 0.1 M for 10 min and stirred. Thereafter, samples respectively were diluted, pour plated in MRS agar, plates were incubated at 37˚C for 2 days and the encapsulated bacteria enumerated. Encapsulation yield was calculated by the following equation:

$$EY= \frac{N}{N0} \times 100$$

Where N is the number of viable encapsulated *lactobacillus acidophilus* inside the beads and N0 is the number of free viable ones before encapsulation.

- Resistance to acidic conditions

Simulated gastric juice were prepared freshly. A simulated gastic juice was prepared by suspending 3mg/mL pepsin (sigma Aldrich, Germany) in sterile saline and adjusted the pH to 3 with 1 mol/ l HCL. 1 g and 1 mL encapsulated beads (4˚C stored) and free cells respectively were washed twice with sterile saline before being resuspended in simulated gastric juice. Resistance was assessed in terms of viable colony count and enumerated after incubation at 37 ˚C for 2h. The viable count in simulated gastric juice was determined (Sathyabama, *et al.*, 2014).

The surviving cells were determined by direct plate count and samples were serially diluted in phosphate buffered saline (PBS) (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) instead of Ringer solution to maintain a steady pH-value. The percentage of surviving bacteria was calculated by the following equation [%]:

$$\text{Surviving rate}= \frac{N2}{N1} \times 100$$

Where N_2= Number of viable cells (CFU/g) after exposure to simulated gastric juice

N_1= Number of viable cells (CFU/g) before exposure to simulated gastric juice

Data are expressed as mean values of triplicate order determinations

- Survival of encapsulated L. Sporogenes under heat treatments

As described by Sabikhi *et al.* (2010) the encapsulated probiotics were subjected to heat treatment to determine the thermal stability. In order to evaluate the viability of encapsulated *L.* sporogenes, heating was carried out on 1 g microcapsules in 10 mL of distilled water, the content was then cooled to room temperature and enumerated the cell counts of probiotics.

- Preparation of bread with encapsulated l. Sporogenes

Bread was prepared according to Zhang *et al*. (2018) containing wheat flour (100 g), sugar (4g), fine salt (1.5 g), instant yeast (1 g), non-salted butter (3g), and UHT skimmed milk (65g). 1 g of microencapsulated probiotic was added and the ingredients were mixed using a blender. The dough was divided into 60 gram pieces then allowed to proof at 40°C with 85 % RH. The samples were baked at 180°C for 15 min in an oven (Changdi®CRTF30W,

China). 98 % estimated starch gelatinization was the criteria for time and temperature selection of baking. A thermocouple was used for measurement of bread surface and core temperature. Bacterial enumerations determined before and after 24 h of baking.

- ***Scanning electron microscopy***

In order to observe the microcapsules morphology by scanning electron microscopy, the encapsulated samples were fixed in stubs and coated with a fine layer of gold. Observations were made using the scanning electron microscopy (Tescan, Miram, England) at an accelerating voltage of 15 kV (Mokarram *et al.*, 2009).

- ***Statistical methods***

The data were subjected to one-way analysis of variance (ANOVA) and the significance of the difference between means was determined by Duncan's multiple range tests ($p<0.05$) using Statistical Package for the Social Science (SPSS, version 16.0)Values are expressed as mean of triplicate determinations ±standard deviation.

Results and Discussion

- ***Growth kinetics***

The bacteria indicated the highest resistance in the mid-log phase and stationary phase under the environmental harsh condition. *L. sporogenes* was monitored until the time to reach the mid-log and stationary phase. The time to reach the mid-log phase was approximately 4 h and to reach stationary phase was 19- 20 h (Figure1). these time were 4 h and 14 h respectively in the research designed by Kim *et al*. (2001). This minor difference between the results might have been attributed to different environmental condition. Several factors limit bacterial growth and the main factor is the media acidity which reported that it is generated by cell growth itself (Rallu *et al.,* 1996). bacterial strain, the amount of oxygen and nutritional components are the other growth limiting factor.

- ***Survival of L. sporogenes after fluidized bed granulation and coating***

In order to verify the efficacy of fluidized bed technology for microencapsulation of probiotics, survival of *L. sporogenes* IBRC-M was determined after the encapsulation process illustrated in Table 1. In pre-tests a drying temperature of 37°C was defined to be the optimal for this process (data not shown).The results showed that during preparation of alginate beads, encapsulation yield had a slight decrease ($p>0.05$) with increasing the alginate concentration. Microcapsules produced with Xanthan exhibited the same trend as Alginate however, there was a wide range of encapsulation yield from 0.04 to 22.85% which it was significantly higher ($p<0.05$) for the 0.5% of Xanthan.

This result is in contrast with lotfipour *et al.* (2012) and Abbaszadeh *et al*. (2014) who reported an increase in EY as the concentrations of Alginate and Xanthan increased. Furthermore, encapsulation yield was higher for the microcapsule produced with 0.5% xanthan as compared to the same concentration of alginate.

Table 1. Effect of fluidized bed technology on encapsulation yield of probiotics

	Alg			XG		
Concentration	**0.5**	**1**	**1.5**	**0.5**	**1**	**1.5**
Encapsulation yield%	79.1±22.4[a]	77.56±10.74[a]	74.5±4.77[a]	22.85±0.98[a]	1.77±0.18[b]	0.04±0.007[b]

- *Resistance to acidic conditions*

The impact of simulated gastric condition of high acid on viability of encapsulated *L. sporogenes* is presented in Table 2. Viability of bacteria was significantly (p<0.05) affected by the concentration of xanthan. In simulated gastric condition, increasing the level of xanthan concentration resulted in a significant (p<0.05) increase in bacterial viability therefore, 1.5% of xanthan represented the highest viability of 60.19%. Viability of bacteria was significantly (p<0.05) affected by the concentration of xanthan. In fact, bacterial viability decreased with increase alginate concentration.

Comparison of the capsule type and concentrations in order to determine the optimized encapsulation condition showed that the 1.5% xanthan presented the highest *L. sporogenes* viability. Chandramouli *et al.* (2004) and Iyer *et al.* (2005) proved that only microencapsulated probiotics were able to tolerate the gastric acid condition. The results of our research were in line with Chandramouli *et al.* (2004).They stated that higher survivability of *L. sporogenes* in the presence of high acid condition acquired by Immobilization of bacteria in alginate beads. They also reported that higher concentration of sodium alginate is capable of better protection of bacteria however, inordinate increasing may result to heterogeneous capsule followed by an increased viscosity. An improved survivability of *Bifidobacterium longum* encapsulated with calcium alginate in the gastric condition has been reported by Lee and Heo (2000). Moreover, only one logarithmic cycle reduction of immobilized *L. acidophilus and B. lactis* immediately and after 2 hours exposure of high acid condition has been presented by Favaro-Trindade.and Grosso (2002). Trindade and Grosso (2000) reported that the population of immobilized *L. acidophilus and B. lactis* at zero time and pH of 1 was unaltered whereas in the same condition of pH the viability of free cells were affected significantly. On the contrary, Sultana *et al.* (2000) concluded that alginate beads will not be able to protect encapsulated probiotics in high acid condition.

- *Viability of L. sporgenese in heat treatment:*

Table 3 have shown the evaluation of encapsulated probiotics resistance to temperature at 90°C for 15 and 30min. The results showed that the encapsulated probiotics by the concentration of 0.5% of chitosan had the highest viability (15.22%) for 15 min heat treatment. The probiotics viability decreased with increasing the chitosan concentration. The chitosan coated probiotics had also higher viability than the probiotics coated with the same concentration of gellan. Moreover, population of encapsulated probiotics drastically decreased with duration of heating time.

Table 2. Impact of simulated gastric acid on viability of alginate and xanthan encapsulated probiotic

	Alg			XG		
Concentration	**0.5**	**1**	**1.5**	**0.5**	**1**	**1.5**
Probiotic viability%	58.43±1.53^{a}	30.52±2.92^{b}	24.21±0/16^{b}	4.516±0.01^{a}	40.33±1.7^{b}	60.19±0.32^{b}

Table 3. Viability of encapsulated probiotics affected by heat treatment at 90°C for 15 and 30 min

Microcapsulation matrix &concentration	**0.5% Ch**	**1% Ch**	**1.5% Ch**	**0.5% Gel**	**1% Gel**	**1.5%Gel**
Viability % in 15 min heat treatment	15.23±1.89^{a}	5.67±0.2^{b}	0^{d}	0^{d}	0^{d}	0.25±0.07^{d}
Viability% in 30 min heat treatment	0^{a}	0.07±0.0069^{a}	0^{a}	0^{a}	0^{a}	0.2±0.006^{a}

In the same study, Fareez *et al.* (2015) reported that the application of chitosan in the microencapsulation of *L. plantarum* LAB12 caused higher survival rate of probiotic in the lethal heat stress. The dense matrices of the Chitosan coated beads probably considered the main reason (Chitprasert *et al.*, 2012).

In the research designed by Kim *et al.* (2008) and Sabikhi *et al.* (2010) it was found that encapsulated *L. acidophilus* had significantly higher heat stability than non-encapsulated ones. Also, Hannoun and Stephanopoulos (1986) stated the same result about encapsulated lactobacilli. The results in this research were consistent with Ding and Shah (2007) who stated that in longer incubation, encapsulated probiotics behaved similar to non-encapsulated ones and survivability had a severe decline. Abbaszadeh *et al.* (2014) expressed that although chitosan improved heat stability of alginate-encapsulated *L. rhamnosus* however its protective effect is greater in the higher temperature than low temperature.

- ***Morphological characterization of the micro particles using scanning electron microscopy***

Alginate microcapsules incorporated with different level of gellan and chitosan captured under SEM are presented in Figure 2. Morphological analysis showed that capsules could not be describe in a regular shape and size.They also appeared in a porous surface which attributed to the drying process followed by loss of water during the process. According to Seyedain-Ardabili *et al*. (2016), alginate microcapsule coated with gellan had no significant difference in the surface properties as compared to the chitosan coated microcapsule. However, the addition of chitosan made the external surface a slightly smoother with the smaller pores size. Fujiwara *et al.* (2013) had some reports about the chitosan ability of modifying shrinkage and bead permeability, making smooth surface, improving stability and keeping the spherical shape of microcapsule. Fritzen-Freire *et al.* (2012) stated that the Smooth surface has an important role for cells maintenance in adverse environmental conditions. Zaiem *et al.* (2020) reported a slight roughness of the Ca-alginate network was thoroughly covered by chitosan through the double-stage procedure and a smoother surface is obtained.

- ***Viability of L. sporogenes in bread:***

Viability of encapsulated probiotic evaluated 24 h after baking is presented in Table 4. Ratio of enumerated encapsulated probiotics 24 h after baking indicated that gellan at the concentration of 1.5% caused the highest viability (p<0.05). However, all of the samples inoculated with encapsulated probiotics disreganding what encapsulation material and concentration, maintained probiotics viability 24 h after baking even in a small extent. The final counts of viable cells in both samples after 24 h storage were higher than 10^6 CFU/g.

Cell viability of encapsulated bacteria with 1.5% w/v gellan in baked bread after 24 h storage was 12.15% and was significantly higher as compared to other samples encapsulated.

In the study by Arslan-Tontul *et al.* (2019) *L. acidophilus* encapsulated with different microcapsules, protected by heat transfer inhibition of microcapsules during the plain cake production but not protected the free cells. Altamirano-Fortoul *et al.* (2012) indicated that although in the short term storage (24 h), encapsulated *L. acidophilus* endured the viability reduction, the survival revealed that immobilization of probiotics may be an applied method to achieve functional breads. Zanjani *et al*. (2012) investigated the evaluation of encapsulated *L. casei* viability during 4 weeks' storage of cream filled cake revealed that microencapsulation significantly

influenced probiotics viability by protecting them in the microcapsule matrix. Hosseininezhad *et al.* (2018) evaluated the time of storage that reduced microbial population of the probiotic bread. Zaghari *et al.* (2018) reported cell viability of encapsulated bacteria with 5% w/v calcium chloride in baked bread after 1 and 24h storage and indicated this to be 0.56% and 0.52% respectively that was significantly higher as compared to sample encapsulated with 1% w/v chitosan where the results were 0.47% and 0.44% respectively.

Conclusion

The results properly describe the positive effect of encapsulation in probiotics survivability under environmental adverse condition. Encapsulated *lactobacillus sporogenes* in alginate (1%) microcapsule could better tolerate the simulated gastric acid condition as compared to other encapsulation materials and concentration. Moreover, incorporated chitosan (0.5%) as an outer layer improved probiotic survivability in the extreme heat treatment**.** Moreover, morphological analysis indicated the alginate (1%) microcapsule had the smoother external surface when integrated with chitosan as an outer layer. Therefore, microencapsulation considered as a protective method for probiotics that provide condition to utilize them in the bread production. The final counts of viable cells

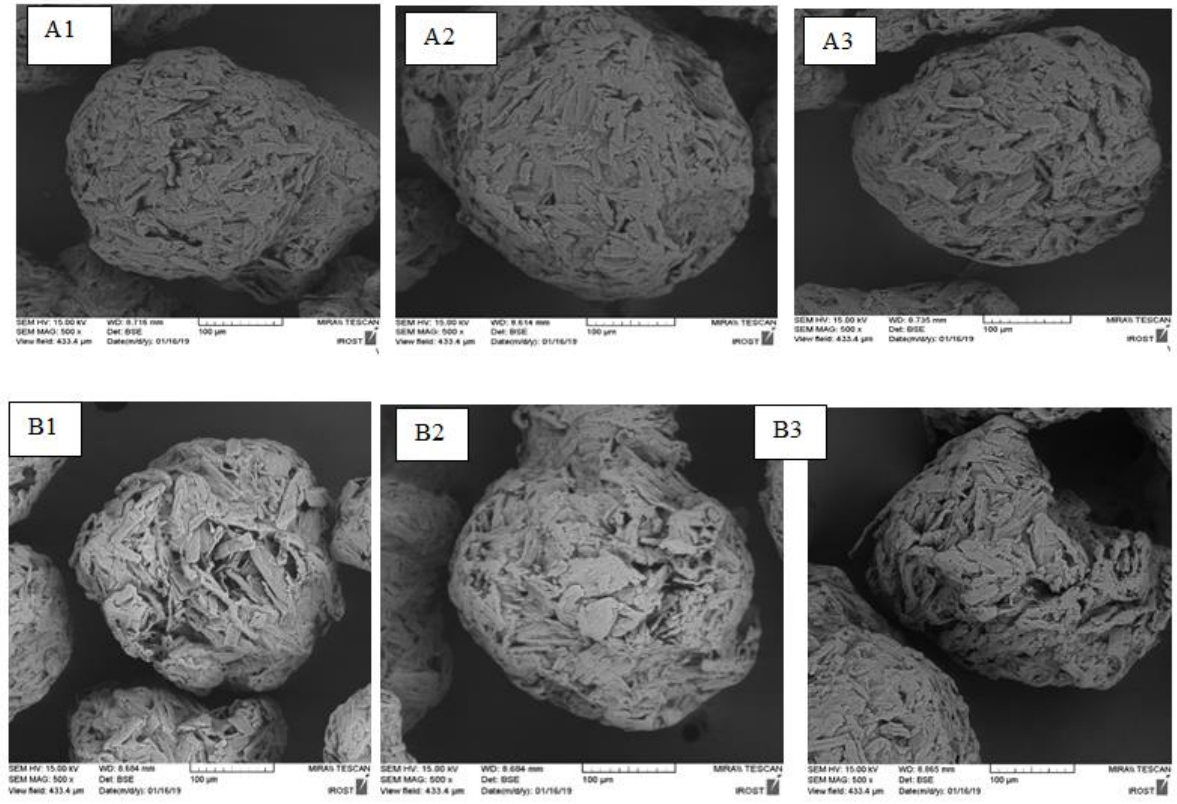

Fig. 2. SEM image of microcapsule contains of A1:1% Alg: 0.5% Gel, A2: 1% Alg: 1% Gel, A3: 1% Alg: 1.5% Gel, B1: 1% Alg: 0.5% Ch, B2: 1% Alg: 1% Ch, B3: 1% Alg: 1.5% Ch.

Table 4. Viability of probiotic 24 h after baking

Microcapsulation material&concentration	0.5% Ch	1% Ch	1.5% Ch	0.5% Gel	1% Gel	1.5% Gel
Viability%	0.0014 ± 0.00007^{a}	0.72 ± 0.016^{a}	0.19 ± 0.05^{a}	0.002 ± 0.0002^{a}	0.014 ± 0.003^{a}	12.15 ± 0.97^{b}

in 1.5% gellan sample as an outer leyer after 24 h storage were higher than 10^6 CFU/g.

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