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Effect microencapsulation of *Saccharomyces boulardii* on viability of yeast in vitro and ice cream

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ABSTRACT

The effect of encapsulation by alginate and alginate-chitosan on the survival of *Saccharomyces boulardii* ATCC MYA-796 in simulated gastric and intestinal juices was investigated. The survival of yeast encapsulated cells treated in ice cream production. The precentage survival of alginate and alginate-chitosan encapsulated cells treated in simulated gastric juice after 240 was 80 % and 90 % respectively, simulated intestinal juice was 80% and 85% respectively. The viability of *Saccharomyces boulardii* (Log CUF/g) in ice cream after storage time (at freezing temperatures 21 day) was 7.11, 6 and 5.25 for alginate-chitosan microcapsules treatment, alginate treatment and free cell treatment respectively. The pH values range of ice cream in three treatments at the initial storage time was 4.70-4.77. After 21 days, The pH decreased in all treatments.

1.Introduction

Probiotics have been defined as live microbes, which transit the gastro-intestinal tract, and in doing so benefit the health of the consumer. Generally, probiotic microorganisms have a therapeutic effect for improved immune system (by microbial metabolites, cell wall components and DNA) (FAO/WHO, 2002). As well as the treatment of diarrhoea (antitoxin effects) by *Saccharomyces boulardii* and *Saccharomyces cerevisiae* are the most common yeast strains that have desirable properties used in probiotic products (Nousia *et al*., 2011; Arslan *et al*., 2016) .

S. boulardii is unique probiotic and biotherapeutic yeast, known to survive in gastric acidity and it is not adversely affected or inhibited by antibiotics or does not alter or adversely affect the normal microflora in the bowl. *S. boulardii* has been utilized worldwide as a probiotic supplement to support gastrointestinal health. In recent years, by incorporating *S. boulardii* various dairy foods such as yoghurt, ultra high temperature treated (UHT) milk, acidophilus yeast milk, ice cream etc. (Hattingh, and Viljoen , 2001 ; Karaolis *et al*., 2013). Ice cream is a product with peculiar textural and organoleptic features and is highly appreciated by a very broad spectrum of consumers. Ice cream's structure and colloidal design, together with its low-temperature storage, renders it a very promising carrier for the stabilization and *invivo* delivery of bioactive compounds and beneficial microorganisms. (Soukoulis *et al*., 2014), However, temperature change, also referred to as "cold shock", during freezing and melting may cause damage such as reduction or even complete loss of metabolic activity (Mohammadi *et al*., 2011).

A method for encapsulation and stabilization of probiotic yeast as S. boulardii or bacterial strains (*Lactobacillus* spp. and *Bifidobacterium* spp.) in polymeric or biopolymeric fibers has been developed, Biopolymers are natural polymers (chitosan , alginate) that are abundantly available and extractable from natural sources and these biopolymers offer a wide range of unique applications (Abd and Niamah, 2012; Al-Manhel *et al*. 2016). In order to achieve the claimed beneficial effects of probiotic

bacteria, these specific microorganisms must be viable, active, and abundant in the product up to the expiry date (cell counts range from 10^6 to 10^9 CFU/g) (Casarotti and Penna, 2015). Microencapsulation is a process by which bioactive materials are coated with other protective materials or their mixtures (Huq *et al*., 2013). Ice cream has nutritional significance, but possesses no therapeutic properties.

The growing interest of consumers in therapeutic products has led to the incorporation of probiotic cultures into ice cream to result in a dietetic ice cream. Some studies have demonstrated that it is possible to produce probiotic ice cream using different microorganism (Ahmadi *et al*., 2012).

This study was undertaken to select a suitable for S. boulardii microencapsulation and checked for their viability, survival of the encapsulated probiotics under the stimulated gastric conditions and study was to manufacture a probiotic ice cream containing *S. boulardii* and to determine how long these yeasts would remain viable during frozen storage of the ice cream.

2. Materials and methods

2.1. Materials

2.1.1.Compounds

Sodium alginate and chitosan were purchased from (BDH, UK). Whole cow milk obtained from Animals station of Agriculture College / University of Basrah.

2.1.2. Yeast cell culture

S. boulardii ATCC MYA-796™ was obitand from Department of Food Science / College of Agriculture / University of Basrah and grow in YPD media (content: 0.3% yeast extract, 0.5% peptone and 1% glucose) at 30°C for 48 h.

2.2. Methods

2.2.1. Yeast inoculum preparation

33×10^9 CFU/ mL of S. boulardii was inoculated in 50 ml of YPD media and incubated at 30ºC at 150 rpm in a shaking incubator for 48 h. The cells were harvested by spinning them at 5,000 rpm for 10 min. The cultures were then washed twice with sterile saline solution (0.9%) and used in the microencapsula process.

2.2.2. Microencapsulation of yeast cells

A 3.0% of Alginate solution was autoclave sterilized at 121oC for 20 min and stored at 4^{o}C. After washing, the cells were suspended in 5 mL of sterile` distilled water and mixed with 20 mL of 2% (w/v) sodium alginate for at least 30 min. The cell suspension was placed in a sterile syringe and injected through a 0.11 mm needle into sterile 0.05 M $CaCl_2$. The 0.11 mm needle was used to produce beads with a diameter of 1-2 mm. Moreover, stirring with a magnetic stirrer was applied during dropping to produce spherical beads, and the distance between the needle and the surface of the solution was controlled to approximately 1 to 2 cm to avoid formation of flat beads. After 30 min gelification in $CaCl^2$, the beads were rinsed with, and then kept in, sterile water at 4°C. (Krasaekoopt *et al*., 2004).

2.2.3. Preparation of alginate-chitosan microcapsules entrapping yeast cells

Chitosan solution (0.5% w/v) was prepared by the addition of chitosan to acetic acid solution (0.1M) with stirring until dissolution (~1 h). The calcium alginate microcapsules were immersed in 100 ml of chitosan solution and shaken at 100 rpm for 40 min on an orbital shaker for coating and then filtration. The chitosan-coated beads were washed and kept in sterile water at 4ºC. (Krasaekoopt *et al*., 2004).

2.2.4. Preparation of simulated gastric and intestinal juices and inoculation of cells

The simulated juices were prepared according to Brinques et al. (2011). Simulated gastric juices were prepared by dissolving pepsin (Riedel-DeHaen Hannover) in sterile sodium chloride solution (0.5% w/v) to a final concentration of 3.0 g/L and adjusting the pH to 1.5 with hydrochloric

acid. Simulated intestinal juices were prepared by suspending pancreatin (BDH) in sterile sodium chloride solution (0.5%, w/v) to a final concentration of 1 g/L with 4.5% bile salts (Oxoid, UK) and adjusting the pH to 8.0 with sterile NaOH (0.1 M). Both solutions were filtered for sterilization through a 0.22 µm membrane. *S. boulardii* inoculated to the simulated gastro-intestinal juice individually in three different forms, non-encapsulated, encapsulated with calcium alginate and encapsulated with alginate and chitosan. Then one gram of freshly encapsulated yeast samples or 1 mL of cell suspensions (free cells) were gently mixed with 10 mL of sterile simulated gastric juice or sterile simulated intestinal juice and incubated at 37 °C for 60,120, 180 and 240 min. Surviving yeast were enumerated by pour plate counts in YPG agar aerobically incubated at 30°C for 48 h. the survival percentage of yeast was calculated by the following formula:

Survival of yeast cells % = Final Log. (CFU/ mL) / Control Log. (CFU/ mL) × 100

2.2.5. Preparation of ice cream

Ice cream mix was prepared by mixing ingredients and then heated to 80°C for 30 sec. Mixes were cooled to 5°C and aged overnight at the same temperature. After ageing, the mix was heat treated to 80°C for 30 sec and cooled to 37°C. Yeast probiotic (*S. boulardii*) were inoculated into ice cream mix at the rate of 5% (33×10^9 CFU/ mL) and incubated at 30°C for 5 h. The ice cream was filled in 50 ml food grade paper cups, covered with food grade lids and stored at -18°C (Pandiyan, 2010). The probiotic ice cream was prepared using the different treatments as shown below: Treatment **I**: Ice cream prepared with free cell of S. bolardii Treatment **II** : Ice cream prepared with sodium alginate microcapsules entrapping yeast cells. Treatment **III**: Ice cream prepared with alginate-chitosan microcapsules entrapping yeast cells.

2.2.6. Yeast viability

Frozen fermented ice cream was thawed and then diluted. The viability of free and microencapsulated yeast cells was enumerated by pour plate count method. To determine the probiotic viability count, the entrapped probiotics were released from the microcapsules. One gram of the ice cream samples transfers to 9 mL of sterile peptone water and then homogenized for 15sec in a vortex mixer. The success of the encapsulating formula was tested. Thus, One-gram sample of microcapsules was diluted in sterile peptone water and then an aliquot of 100 mL was discharged into potato dextrose agar plates and incubated at 30ºC for 24–72 h. The population of S. boulardii was then quantified and expressed as the number of colony-forming units (CFU) per gram (CFU/g) Samples were taken at 0, 1, 7, 14 and 21days of storage (Zamora-vega *et al.*, 2012). The pH of the samples was measured with a pH meter (SD-300, Germany).

3.Results and discussions

3.1. The survival *S. boulardii* in simulated gastric juice

The survival of the free cells and encapsulated *S. boulardii* in simulated gastric juice after being kept for 240 min is shown in Figure 1. Food stays in the stomach for 2 to 4 hours, while the liquids can only stay about 20 minutes. pH of gastric juice simulates affected the survival of both the free and encapsulated cells of *S. boulardii*.

The encapsulated *S. boulardii* survived well in simulated gastric conditions compared to the free yeast cells and the encapsulated by alginate-chitosan was best than with alginate. The percentage survival of encapsulated *S. boulardii* in gastic juice after 3h was 89% and 92%, for alginate microcapsules method and alginate-chitosan microcapsules method while the percentage was 80% free yeast cells. After 240 min. the difference between alginate and alginate-chitosan microcapsules was more clearly in

results. Whereas the yeast in the alginate-only microcapsules loss of percentage survival to 82%, the chitosan-mixed alginate microcapsules was 90%.

Alginate mixture remains stable structurally in acidity environments when the pH decreases as the viscosity of alginate solutions increase and the carboxylate groups in the alginate soutions backbone become protonated lead to form hydrogen bonds, which increased bindings yeast cells (Lee and Mooney, 2012).Encapsulation, either by alginate or by alginate/chitosan provide the best protection in the case of yeast cells encapsulated compared with non-encapsulated cells (free cells). This can be attributed to the polysaccharides of chitosan acting as a buffer, reducing the activity of the acid. It does protective effect to yeast cells.

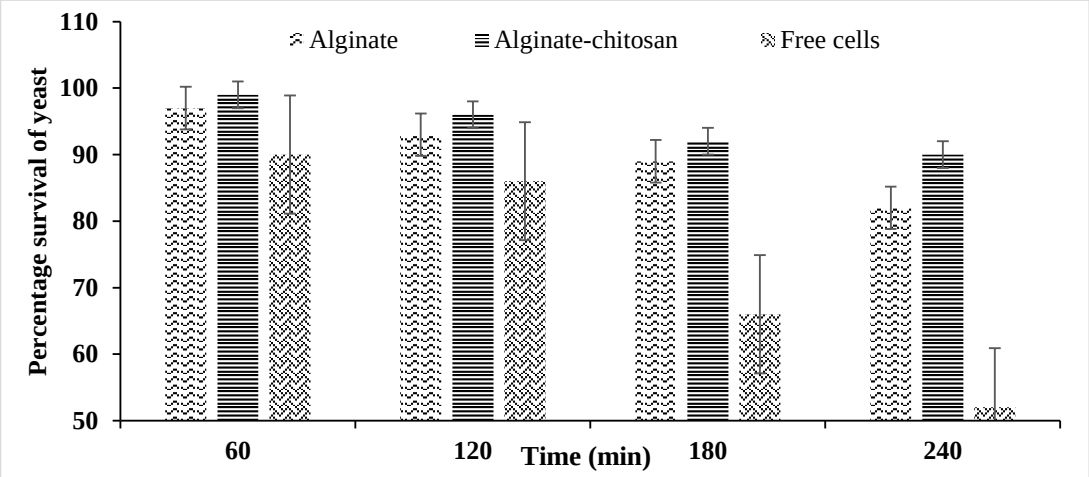

Figure 1. The percentage survival of yeast in simulated gastric

3.2.The survival *S. boulardii* in simulated intestinal juice

Bile salt tolerance is one of the basic characteristics of the probiotic to survive in the small intestine. Survival of encapsulated and free cells of S. boulardii after exposure to 4.5% bile salt for 240 min. This reflects the time of the stay of food in the small intestine is shown in Figure 2. The percentage survival of encapsulated *S. boulardii* was 80% and 85% after 240 min for alginate microcapsules method and alginate-chitosan microcapsules method while percentage survival of S. boulardii free cells was 67%. It was noted that the survival of alginate encapsulated and alginate-chitosan encapsulated *S. boulardii* reduced to somewhat compared with S. boulardii free cells. Mixed the alginate microcapsulation with chitosan more improve the viability of *S. boulardii* (Cook *et al*., 2011). The results of the study agreed with the (Dikit *et al*, 2015) who studied the effect of encapsulation on the survival of *Lactobacillus plantarum*.

pH-base in the small intestine affecting the survival of *S. boulardii* cells. Microcapsulation of *S. boulardii* cells does to prevent the effect of the acid and bile salts. Chitosan polysaccharide chitosan is positively charged, and therefore, it is not suitable for encapsulated yeast when it is used by itself. A mixture of chitosan with other biological substances usually soluble in solvents such as alginate. Alginate is a

negatively charged polysaccharide and best substance for growth and microcapsulation of probiotic microorganisms. Crosslinked three-dimensional made of chitosan and alginate provides much better for microcapsulation process (Baysal *et al*., 2013).

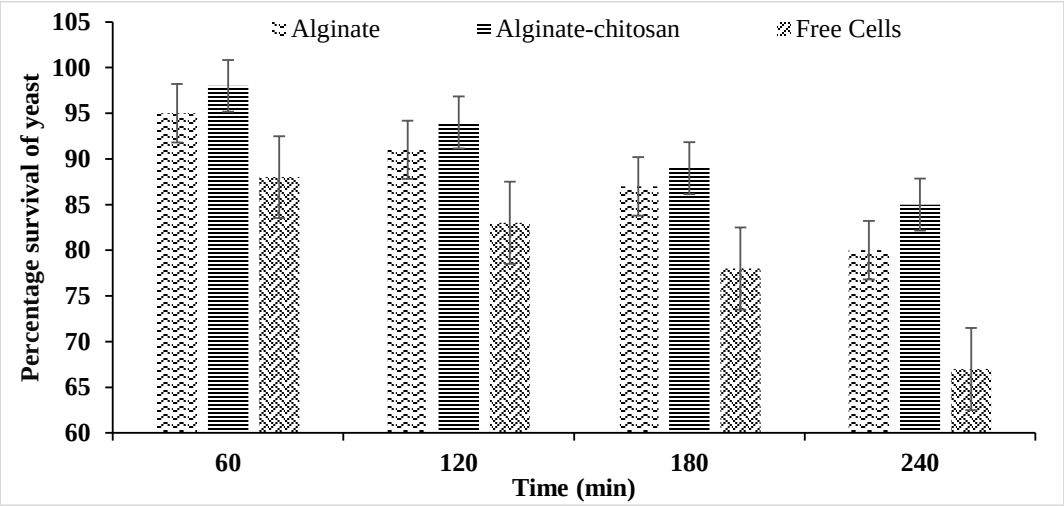

Figure 2. The percentage survival of yeast in intestinal environment

3.3. The viability of *S. boulardii* in ice cream

S. boulardii grew to high numbers in ice cream mix. Even the high solids level of the ice cream mix did not prevent growth of either free cells or microcapsule when a high percentage (4%) of inoculum was used. Viable cell count of *S. boulardii* (free cells) decreased from 8.55 to 5.25 log CFU/g due to encapsulation process; Changes in viable counts of S. boulardii in ice cream mix due to freezing and during frozen storage at -18°C are presented in Figure 3. Results showed that there was an approximately 3.3 Log cycle decrease in the count of probiotic cells in the free form immediately after freezing for 3 week compared with the encapsulated probiotic cells decreased 2.51 and 1.51 Log cycle in Treatment **II** and Treatment **III** respectively. This indicates that freezing had destructive effects on probiotic cells most probably due to the freezing injury of cells. During the freezing process, the cells of probiotics can be lethally injured by damage to their cell walls or membranes caused by thawing, and the mechanical stresses (generated by mixing and incorporating oxygen into the mixture, during the manufacture of ice creams) of ice crystals forming in the external medium or inside the cells, by cold injuries and temperature decrease shock to the cells, by condensation of solutes in the extracellular/intracellular medium, or by dehydration of the cells. All these factors may result in even lower viability, (Akin *et al*. 2007; Mohammadi *et al*., 2011).

According to several findings, encapsulation is a useful alternative to increase the survival rate of probiotic bacteria in ice cream and fermented frozen dairy desserts. This result agrees with Ahmadi *et al*. (2012) has found that the viable counts of free probiotics decreased from ~9.55 to ~7.3 Log CFU/g after 60 days of frozen storage while that of encapsulated cells merely decreased less than 1 Log cycle. Encapsulation with alginate microbeads protected the probiotic cells against injuries

in the freezing stage as well as, during frozen storage. Homayouni *et al*. (2008) reported that the survival of *L. casei* and *B. lactis* were monitored during the product's storage for 180 days at -20˚C. The viable cell number of *L. casei* and *B. lactis* in the free state in prepared ice cream mixture was 5.1×10^9 and 4.1×10^9 CFU/mL at day one and after 180 days storage at -20˚C, these numbers were decreased to 4.2×10^6 and 1.1×10^7 CFU/mL respectively. When encapsulated the mentioned probiotic bacteria in calcium alginate beads, the probiotic survival raised at a rate of 30% during the same period of storage at the same temperature. Akin *et al*. (2007) and Heydari *et al*. (2012) reported a similar observation. Encapsulation within alginate microbeads and alginate-chitosan protected the probiotic cells against injuries in the freezing stage (Figure 3) as well as, during frozen storage. Viable number of encapsulated *L. acidophilus* was merely decreased less than 1 Log cycle after 60 days of frozen storage.

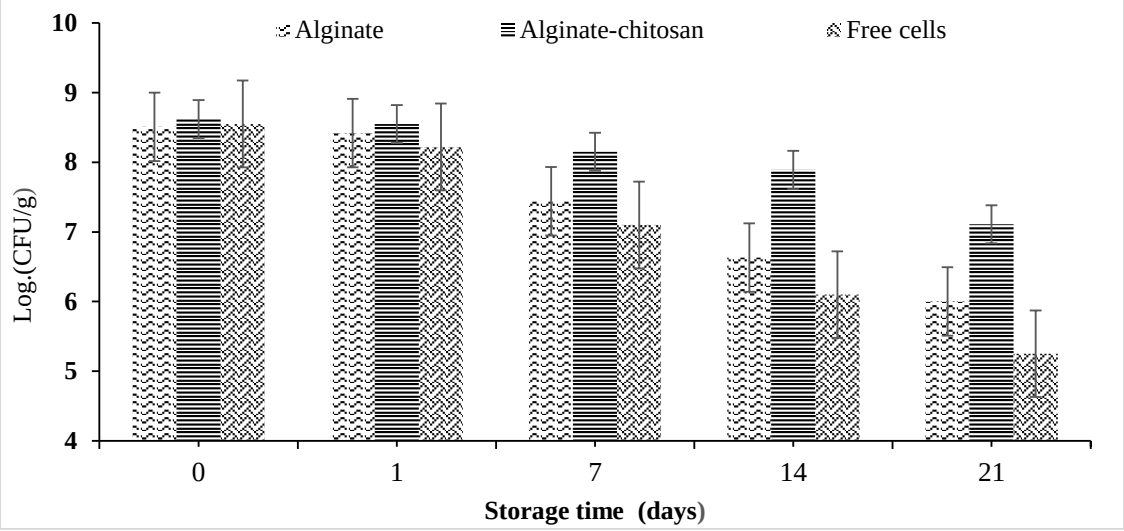

Figure 3. Viability of free and encapsulated *S. boulardii* (Log CFU/g) in ice cream during storage periods at freezing temperature.

3.4. pH of ice cream

Table 1. Show that the pH values of three ice cream treatments after process was between 4.70-4.77. The values decreased after storage for up to 4.20 in alginate-chitosan microcapsules treatment, 4.45 in alginate microcapsules treatment and 4.45 in free cell treatment. The reduced pH because of the ability of yeast to ferment sugars in the ice-cream mixture.

S. boulardii either by acid hydrolysis or by producing enzymes that cleave the sucrose into glucose and fructose and glucose utilization rate was much faster than the fructose and sucrose (Kurtzman *et al*., 2011). *S. boulardii* is acetate produce from sugar utilization and anther carbohydrates (Anjum *et al*., 2010) . The high viability cells of S. boulardii in alginate-chitosan microcapsules treatment led to increased acidity and reduced pH compared with other treatments

.

Table. 3 pH of ice cream treatment

Cell	pH				
	Time (day)				
	0	1	7	14	21
Alginate microcapsules	4.77 ± 0.03	4.72 ± 0.04	4.62 ± 0.01	4.53 ± 0.02	4.45 ± 0.01
Alginate-chitosan microcapsules	4.70 ± 0.03	4.65 ± 0.01	4.55 ± 0.02	4.43 ± 0.00	4.20 ± 0.05
Free cell	4.75 ± 0.05	4.70 ± 0.01	4.63 ± 0.01	4.60 ± 0.10	4.54 ± 0.06

±: Standard division (SD)

4. Conclusions

Microcapsulation during the emulsion technology was an effective way to increase the survival of probiotics yeasts as they pass during digestive tract conditions of of warm-blooded animals. Adding chitosan when using encapsulation techniques can improve the survival of *S. boulardii* in simulated gastric juice and intestinal juice. Probiotic ice cream can be manufactured using encapsulated *S. boulardii* to ferment ice cream mix. Coating alginate microcapsules with chitosan further improve the viability of S. boulardii during storage time of ice cream production. The probiotic yeast *S. boulardii* can be well incorporated into ice cream based foods to develop functional and therapeutic foods.

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