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The Effect of Microencapsulation on Protection of Isolated Urease-Producing *Streptococcus thermophilus* Against Stress Conditions.

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ABSTRACT

The effect of microencapsulation using sodium alginate on the tolerance of the isolated urease-producing *Streptococcus thermophilus* to select processing conditions and simulated gastrointestinal environments were studied. Ten cultures were isolated from laban rayeb (natural sour milk) on M17 agar medium from different regions in Egypt. Coccid lactic acid bacteria strains were identified as *Enterococcus*; *Lactococcus lactis* and *Streptococcus*. After purification and identification from these isolates *Streptococcus thermophilus* strains were chosen and screened for their ability to produce urease. Effect of stress conditions on the viability of microencapsulated urease-producing *Streptococcus thermophilus* was studied. The organism survived better in the protected form at high temperatures (63, 72, 80, and 90 °C) and at high salt concentrations (2%, 4%, and 6%). The free cells were completely destroyed at 90 °C whereas the microencapsulated cells reached to 3.3 log cfu/ml after 1 min. The log cycle reduction was 3 and 2, in free and encapsulated cells, respectively when incubated for 18 h with 6% (w/v) NaCl. Microencapsulation provided better protection at simulated conditions of gastric pH (2 and 4) and at high bile salt concentrations (0.5%, 1%, and 2.0%). Microencapsulation of urease-producing *Streptococcus thermophilus* in sodium alginate resulted in better survival of cells after heat treatment, at high NaCl and bile salt concentrations, and at low pH as compared to free cells. Our study demonstrated that microencapsulation of urease-producing *Streptococcus thermophilus* in sodium alginate is an effective technique of protection against extreme processing conditions and under simulated gastrointestinal environment. From the present results, urease-producing *Streptococcus thermophilus* strain was chosen for further studies to produce new therapeutic dairy products.

Keywords: Isolation, *Streptococcus thermophilus*, urease, Stress conditions, microencapsulation

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INTRODUCTION

Streptococcus thermophilus has ''Generally Recognized as Safe'' status and is extensively used for the manufacture of several important fermented dairy foods, including yoghurt and some cheese varieties (Swiss, Limburger, Brick). *Streptococcus thermophilus* also has a number of functional activities such as production of extracellular polysaccharides, bacteriocins, galactose metabolism, proteolytic system, urease activity, biosurfactant and vitamins production. In addition, it also has potential as a probiotic, as demonstrated by various health effects, transient survival, and moderate adherence in the gastrointestinal tract. Furthermore, new data on the survivability of *S. thermophilus* in the intestine has added a new dimension to its potential use as a novel probiotic functional starter for the production of a functional dairy food. Therefore, the natural diversity among *S. thermophilus* strains with respect to their capacity to produce different metabolites has the potential to be exploited beyond fermentation for lactic acid production [1]. Currently, where use of probiotic starters in the dairy industry is impeded by the limitation of slow growth in milk, and hence are used as adjunct in association with active acid producers as primary starters, *S. thermophilus* strains can be explored for their dual roles of primary as well as functional starter. The careful selection of such strains may be of paramount value in the food industry for their commercial exploitation in developing value-added functional foods.

Among urease-producing species, *S. thermophilus* is particularly interesting. Urease production is common in *S. thermophilus* [2] and, although it is important for acid stress resistance [3], it slows pH decrease in milk and cheese due to the production of ammonia [4, 5]. The urease operon of *S. thermophilus* has been recently characterized [4] and it has been found to be similar to that of the taxonomically related *S. salivarius* [6, 7]. Several factors have been claimed to affect the viability of probiotic bacteria in dairy foods such as yogurt and fermented milks, including low pH and bile salts. In order to be used as potential probiotics, dairy LAB strains need to be screened for their capacity of transit tolerance to the upper gastrointestinal tract conditions [8], these factors could be called abiotic stress. Abiotic stress is defined as the negative impact of non-living factors on the living organisms in a specific environment. Stress environments such as low temperature (5, -20 °C), high temperature (58°C), acidic pH (2.5), high osmotic pressure (75% sorbitol) and high ethanol (20%) [9]. Since uremic toxins are able to be reduced by modulating bacterial growth in the colon, which leads to reduced generation of bacterial toxins as well as by targeted adsorption of toxic end products of microbial fermentation, probiotics would seem to have the potential to reduce the concentration of intestinal nitrogenous metabolites. Although numerous studies have evaluated the functional effects of probiotics [10, 11].

At the present time there are only two approaches that are able to reduce the accumulation of protein-bound uremic toxins [12]. These are the regulation of intestinal bacterial growth and metabolism (the use of probiotics and prebiotics) and the use of an adsorbent therapy (AST-120). Several studies have reported that the ingestion of the prebiotics such as inulin and lactulose is able to significantly reduce the levels of p-cresol and 15N in serum; furthermore, they also give rise to a significantly higher fecal excretion of 15N [13, 11]. In recent years, microencapsulation has also been found to be a useful tool for the stabilization of probiotic cells in functional food applications. Microencapsulation can enhance the viability of probiotic cells during processing, storage and the subsequent consumption and gastrointestinal transit [14]. Microencapsulation in which the cells are retained within an encapsulating matrix or membrane has emerged as an alternative for protection of probiotics, providing a particular and convenient micro-environment for the encapsulated microorganism, enhancing their viability, and enabling controlled release of cells in the intestinal tract [15]. Encapsulation technology has been proved to be one of the most effective ways to protect probiotics during processing and subsequent storage. Furthermore, encapsulation systems with control-released ability can deliver probiotics to a specific target and release them at required [16]. The benefits of encapsulation protect probiotics against stress conditions. Non-encapsulated probiotic microorganisms may be exposed to high temperatures [17, 18], low pH, high osmotic pressure and high levels of oxygen during processing and storage of foods [19, 20]. The survival of probiotic organisms is also affected by acid in the stomach and the bile salts in the intestine tract [21]. These microcapsules may provide a more suitable anaerobic environment for the susceptible probiotic bacteria [22, 23]. Sabikhi et al. [24] reported that microencapsulated probiotic *Lactobacillus acidophilus* LA1 survived better at high temperatures **(**72, 85, and 90 °C) and at high salt concentrations (1%, 1.5%, and 2%). The free cells were completely destroyed at 90 °C whereas the microencapsulated cells reduced by 4.14 log cycles. The log cycle reduction was 5.67 and 2.30, respectively, in free and protected cells when incubated for 3 h with 2% (w/v) NaCl. *Lactobacillus bulgaricus* was encapsulated in alginate-milk microspheres prepared by Shi et al. [16]. The tolerance of encapsulated *L.*

bulgaricus to adverse environments such as low pH (pH 2.0 and 2.5), high concentration of bile salt (1.0% and 2.0%) and long time storage (1 month), was investigated. This study showed that encapsulation could improve the tolerance of *L. bulgaricus* to adverse environments.

The aim of the present preliminary work was to isolate and identify *S. thermophilus* from traditional Egyptian fermented milk and to select *S. thermophilus* that are able to produce urease and study the effect of microencapsulation on *S. thermophilus* viability against stress conditions.

MATERIALS AND METHODS

Isolation of *Streptococcus thermophiles*

Ten samples of Laban Rayeb (Natural sour milk) were collected from different locations in Egypt. M17 agar medium (Difco) was used to isolate Streptococci. Typical colonies were picked and further purified in three successive passages on M17 agar.

Identification of *Streptococcus thermophilus* strains

Streptococcus thermophilus isolates were identified according to the criteria described by Holt et al. [25]. From these isolates *Streptococcus thermophilus* strains were screened for their ability to produce urease.

Screening of urease activity by strains

The phenol red assay described by Zotta et al. [7] and modified as follows, was used as an approximate measurement of urease activity. Specifically 0.5 ml of a fresh M17 culture of *S. thermophilus* was added to a solution containing one volume of solution A(urea, 2 g dissolved in 2 ml of ethanol and 4 ml of sterilized water) and 19 volumes of solution B (KH_2PO_4, 1 g l^{-1}, K_2HPO_4, 1 g l^{-1}, NaCl, 5 g l^{-1}, phenol red, 20 µg ml^{-1}).The suspension was incubated at 37 ºC for 1-2 h and the development of a red-violet color indicated positive urease activity.

Preparation of microencapsulated bacteria

Cells were trapped in sodium alginate according to the procedure of Klinkenberg et al. [26]. Elliker broth media Oxide (500 ml) was inoculated with 5% (v/v) fresh active culture of *S. thermophilus* and incubated at 37 ºC for 18h. Cells were harvested aseptically by centrifugation (8400 ×g, 20 min, 4 ºC). The pellets were suspended in dilution buffer to a concentration of 4 g dry weight cells l^{-1}. Re-suspended cells were mixed with an equal volume of 4 % (w/v) sodium alginate, yielding a final cell concentration of 2 g dry weight cells l^{-1}, which was used in the experiments. The mixture of alginate and cells was added drop wise into a sterile solution of sodium chloride (0.2 mol l^{-1}) and calcium chloride (0.05 mol l^{-1}). Sodium chloride was used in the gelling solution to ensure a homogeneous polysaccharide concentration throughout the beads. To ensure complete gelling, the beads were stirred for at least 40 min in this solution.

Effect of stress conditions on the viability of microencapsulated probiotic urease-producing *Streptococcus thermophiles*

Thermal resistance

Thermo-tolerant capacity of each of *S. thermophilus* culture and beads was determined in water baths at different temperatures, adapting the methodology reported by Petäjä [27] and Anderson et al. [28]. The test tube method of Donnelly et al. [29] was used to determine heat resistance of both microorganisms and beads of *S. thermophilus*. One tenth of ml of 24 old culture (after the come up period) was inoculated into 10 ml of sterilized cow's milk in screw-capped tubes and were heated in a thermostatically water bath. 63º C for 0, 10, 15, 20, 25 and 30 min, 72 º C for 0, 0.5, 1, 3, and 5 min 80 and 90º C for 0, 0.5, 1, 2.5, and 5 min and then samples were cooled in ice bath. Plate counts using M17 agar (oxoid) at 37°C for 48h were used to determine the survivals. Rates for thermal inactivation of each bacterium were determined graphically by plotting the log10 cfu/ml of surviving cell population versus heating time. A line was drawn through the date points and D-values were obtained from the slope of the best lit line [30].

Salt tolerance

M17 media (Difco, USA) containing different concentrations as 2, 4 and 6 % of added salt (NaCl) and inoculated with 2% (v/v) cultures and beads (w/v) incubated at 37°C for 18h. Rates for NaCl tolerance of each bacterium culture and beads were determined graphically by plotting the $\log_{10}$cfu/ml of surviving cell population at different concentration of NaCl.

3-Acid and bile salt resistance

Resistance to acidic conditions was tested according to Iyer [1]. The pH of M17 broth was adjusted to pH 2.0, pH 4.0, and pH 6.0 with 1 M HCl and with pH 7.0 as control. Survival was evaluated using the log phase cultures (8 log cfu mL^{-1}) by plating on M17 agar, after 30, 60, 90, and 120 min, of incubation at 37 ºC in acidic M17 broth. Bile salt tolerance was tested using supplemented M17 broth with 0.5%, 1%, 2% w/v of bile salt (Himedia Laboratories Pvt. Ltd, Mumbai, India) and without supplement as a control were inoculated with actively growing bacteria. Survival was evaluated using log phase cultures (8 log10 cfu ml^{-1}) by plate count on M17 agar, after 60, 120, 180 min of incubation at 37 ºC in M17 broth containing bile salts according to Iyer [1].

Measurement of number of bacteria

The number of bacteria was assayed using the plate method. M17 Agar (Difco) with aerobic incubation at 37°C/72h was used for enumeration of *S. thermophilus*.

RESULTS AND DISCUSSION

Ten coccid lactic acid bacteria (LAB) isolates were classified into the genera *Enterococcus, Streptococcus, and Lactococcus* based on their morphology and biochemical characters .Table (1) shows the percentage distribution of different genera of LAB. Of the cultures, 40 % belonged to the genus *Streptococcus.* Also, (Table 1) reveals that *Enterococcus, and Lactococcu* were 20% & 40% respectively. The results of identification are presented in (Table 2). Four strains of the identified cultures were *Streptococcus thermophilus.* Giraffa et al. [31] isolated forty strains of *Streptococcus thermophilus* from dairy products and identified using phenotypic and genotypic criteria. Also, El-Sharoud et al. [32] isolated a total of 170 lactic acid bacteria cocci from traditional Egyptian dairy products. Physiological characterization of these isolates preliminarily identified 101 of them as potential Enterococcus spp. and 69 isolates as potential *Streptococcus thermophilus*. The majority of *Streptococcus thermophilus* isolates were recovered from Kariesh cheese (52 isolates), followed by Laban Rayeb (16 isolates) and Zabady (1 isolate).

Table 1: The percentage distribution of different genera of coccid (LAB)

Genera	No	%
Streptococcus	4	40%
Lactococcus	4	40 %
Enterococcus	2	20%

Table 2: identification of 4 Streptococcus isolates

Species	No	(%)
Streptococcus thermophilus	4	(100%)
Urease - producing Streptococcus thermophilus	2	(50%)

Out of 4 strains belonging to Streptococcus screened for urease production, only one *S. thermophilus* strain scored positive. Zotta et al. [7] found that some strains of *S. thermophilus* produce urease.

Screening for urease activity

Urease production is common in *S. thermophilus* strains which it can change the color of indicator to red-violet color when incubated at 37 ºC for 1-2 h. Zotta et al. [7] found that some strains of *S. thermophilus* produce urease. Also, Mora et al. [2] found that urease production is common in *S. thermophilus* and, although it is important for acid stress resistance [3].

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Effect of stress conditions on the viability of microencapsulated urease-producing *Streptococcus thermophilus*

Influence of heat treatments

The control (culture) and encapsulated *S. thermophilus* were exposed to high temperatures of 63, 72, 80, and 90 °C at different times and examined for their survival explained in (Fig. 1). The minimum lethality values were observed at 63 ºC at different times which the viable counts of culture reached to 6.60, 6.23, 5.30, 4.47 and 4.30 log cfu/ml after 10, 15, 20, 25 and 30 min and reached to 8.55, 7.84, 7.47, 7.07 and 6.41 log cfu/ml after10, 15, 20, 25 and 30 min for the beads at the same temperature. The control (culture) *S. thermophilus* were very sensitive to heat, their numbers reducing from 9 log cfu/ml to 3 log cfu/ml at 72 °C/10min and 2 log cfu/ml at 80 °C/2min. The counts reached to zero at 90 °C/1min. The corresponding numbers of the encapsulated organisms whose initial count was 9 log cfu/ml were 5 log cfu/ml at 72 °C/10min., 4.9 log cfu/ml at 80 °C/2min., and 3.3 log cfu/ml at 90 °C/1min. Our results confirm previous findings of Sabikhi et al. [24] who studied the effect of microencapsulation using sodium alginate and starch on the tolerance of probiotic *Lactobacillus acidophilus* LA1 to selected processing conditions and simulated gastrointestinal environments. The organism survived better in the protected form at high temperatures (72, 85, and 90 °C). The free cells were completely destroyed at 90 °C whereas the microencapsulated cells reduced by 4.14 log cycles. Also, Mosilhey [18], and Kim et al. [17] suggested that microencapsulation using alginate and alginate-chitosan may enhance thermal resistance of the bacteria. Sheng-Yao et al. [33] studied the effect of heat on the survival microencapsulated *Lactobacillus kefiranofaciens* M1 using mixture of sodium alginate, gellan gum and skim milk. The viable cell counts remained constant at 5×10^7 cfu/g after heating from 25 °C to 75 °C and holding at 75 °C for 1 min. The viable cell counts were reduced to 10^6 cfu/g and 10^5 CFU/g after 8-week storage at 4 °C and subsequent heat treatment with simulated gastrointestinal fluid test (SGFT) and bile salts, respectively.

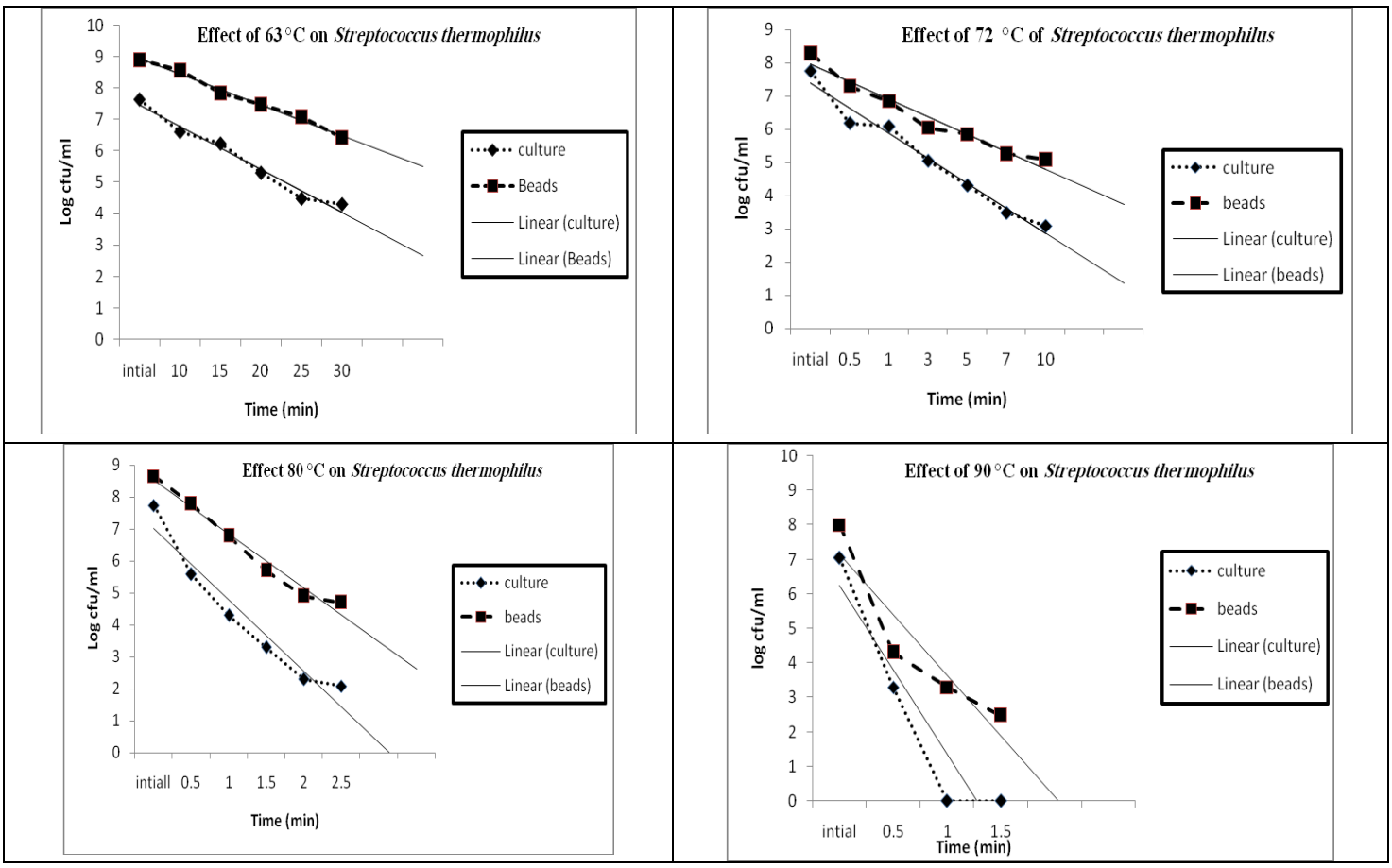

Figure 1: Survival of non-encapsulated and microencapsulated Urease-producing *Streptococcus thermophilus* after exposure to 63, 72, 80, 90 °C at different times.

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Survival of microencapsulated and non-microencapsulated Urease - producing *Streptococcus thermophilus* in different NaCl concentrations

The effect of salt concentrations (2, 4 and 6 %) on the viability of *S. thermophilus* after 18h of incubation at 37 ºC is illustrated in (Fig. 2). The counts of culture (control) decreased steadily during incubation in 2, 4 and 6% NaCl concentrations and after 18h reached 6.39, 6.30, and 5.94 log cfu/ml respectively. The numbers decreased by 2 log cycles 2% and 3 log cycles in 6% NaCl compared to the initial count (8.65 log cfu/ml). But in the case of microencapsulated bacteria using sodium alginate the viability decreased from 9.92 log cfu/ml (initial count) to 8.39, 7.47 and 7.20 log cfu/ml after 18h for 2, 4 and 6% NaCl respectively, which the number of decreased by 1 & 2 log cycle in 2% & 6% NaCl respectively. Also, the initial count of free cells (8.46 log cfu/ml) decreased to 7.38, 6.44 and 5.25 log cfu/ml at 2, 4 and 6% NaCl after 18h of incubation at 37 ºC. Our results confirmed the data obtained with Sabikhi et al. [24], who studied the effect of high salt concentrations on the survival of free cells and protected cells of *L. acidophilus* LA1 .The respective initial counts of 9.13 and 7.44 log cycles in free cells and protected cells reduced to 3.46 and 5.14 log cycles with increasing NaCl content and time of incubation. The rate of decrease of the numbers was greater in the free cells than in the protected cells. Guinee and Fox [34] noted that salt content greatly affects the degree of survival and activity of *L. acidophilus* and bifidobacteria in cheese matrix. Gomes et al. [35] reported that survival of *L. acidophilus* decreased with increasing sodium chloride levels greater than 3% (w/w). The reductions in water activity and increase in osmolarity may be the reasons for decreased resistance and survival during storage [36]. Despite the lower viability of free *L. acidophilus* in salt solutions during storage, cells protected with combinations of gum Arabic with whey protein, soy protein, or soy milk remained for longer periods [18], suggesting that microencapsulation may be an alternative to deliver probiotics in lightly salted products.

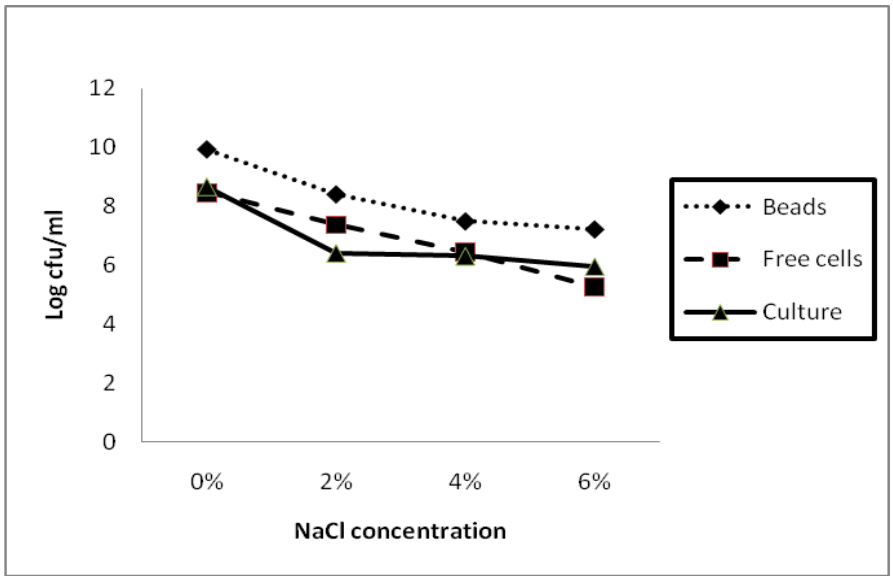

Figure 2: Survival of microencapsulated and non-microencapsulated *Streptococcus thermophilus* in different NaCl concentrations.

Effect of pH values on the survival of *Streptococcus thermophilus*

One of the major problems in efficacy of probiotic food is low survival of the organisms in the gastric pH. The microencapsulated culture of *Streptococcus thermophilus* was tested for survival in different pH values (2, 4, 6, 7) at different time (zero, 30, 60, 90 120 min). The results expressed in (Fig. 3). Illustrate that the survival of *Streptococcus thermophilus* was lower at lower pH ranges and decreased further as the incubation period increased. Microencapsulated *Streptococcus thermophilus* survived in greater numbers and for longer duration as is evident from the graph. In general, the total viable counts decreased in all tested pHs with the time progress. The most observed decrease was at pH 2.0 followed by pH 4.0 and pH 6.0 respectively. It should be mentioned also that the decline was more rapid for culture than for microencapsulated cells. At the pH 2 the viable counts of the culture was declined from initial count (7.49 log cfu/ml) to reached to (4.68, 3.69, 2.20

and not detect log cfu/ml) after 30, 60, 90 and 120 min of incubation at 37 ºC compared with microencapsulated strain which the viable count decreased from initial count (7.90 log cfu/ml) to reach to (4.30, 3.18, 2.69 and 2.62 log cfu /ml) after the same time respectively. But at pH 7 the viable count of culture approximately stable when the viable count of microencapsulated strain increased from initial count (8.84 log cfu/ml) to reach to (8.95, 9.69, 9.90 and 10.0 log cfu/ml) after 30, 60, 90 and 120 min respectively. These findings agree with several earlier reports. Shi et al. [16] investigated the tolerance of encapsulated *L. bulgaricus* to adverse environments such as low pH (pH 2.0 and 2.5), high concentration of bile salt (1.0% and 2.0%) and long time storage (1 month). This study showed that encapsulation could improve the tolerance of *L. bulgaricus* to adverse environments. Trabelsi et al. [37] studied the survival rates of free and microencapsulated *L. plantarum* TN8 during exposure to artificial gastrointestinal conditions. The encapsulated cells exhibited significantly higher resistances to artificial intestinal juice and artificial gastric juice. Also, Kim et al. [17] studied the microencapsulated *L. acidophilus* ATCC 43121 with sodium alginate and the effects of microencapsulation on the changes in survival rate of the *L. acidophilus* ATCC 43121 during exposure to artificial gastrointestinal. Therefore, non-encapsulated cells were completely destroyed when exposed to artificial gastric juice (AGJ) of pH 1.2 and 1.5, while the treatment diminished the viable count of encapsulated samples only by 3 log. Encapsulated cells exhibited a significantly higher resistance to artificial intestinal juice (AIJ) and heat treatment than non-encapsulated samples. Survival of free and encapsulated *B. bifidum* and *L. gasseri* in SGJ, Chandramouli et al. [38] and Iyer and Kailasapathy [39] have shown that only the microencapsulated probiotics were able to maintain viability in gastro-intestinal conditions. Microencapsulation of probiotics in alginate beads has previously been tested for improving viability of probiotic bacteria in simulated gastric conditions [40, 41, 42].

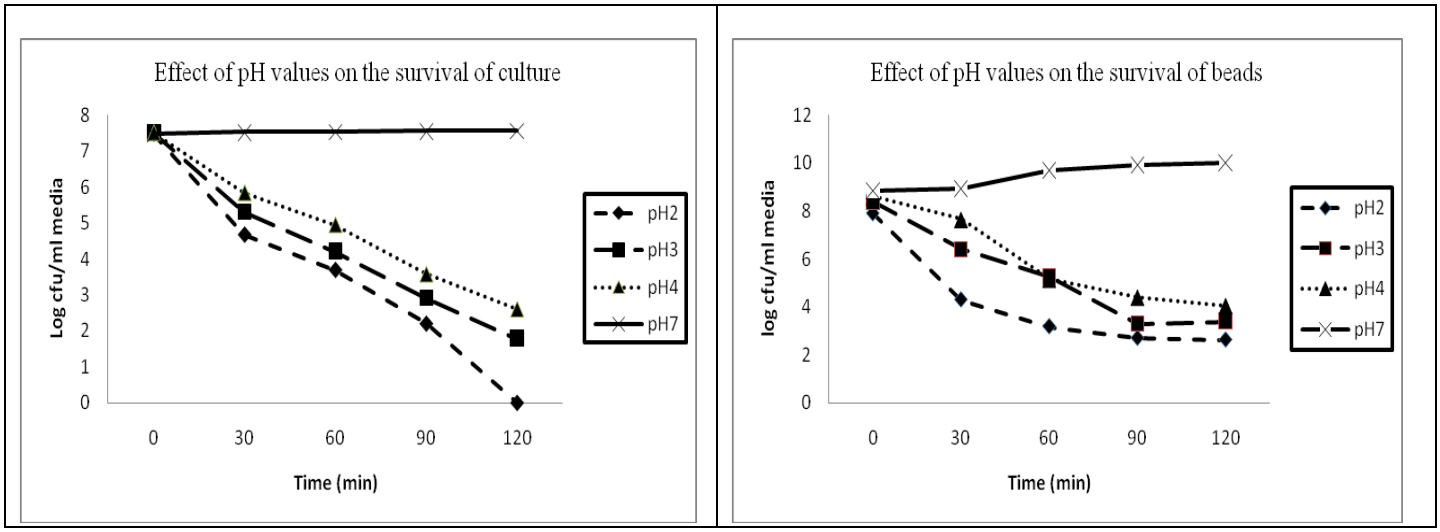

Figure 3: Effect of pH values on the survival of microencapsulated and non-encapsulated *Streptococcus thermophilus* at different times.

Effect of high bile salt concentration on the survival of microencapsulated *S. thermophilus*.

The high concentration of bile salts in the intestine is one of the major hurdles in the survival of organisms in the gastrointestinal tract. Culture and microencapsulated *S. thermophilus* were subjected to different concentrations of bile salts (0.5%, 1%, and 2%) and incubated for different intervals of time to assess whether microencapsulation improved resistance to bile salts. The data of survival of microencapsulated cells in bile salt concentrations are represented in (Fig. 4). From the initial counts of 8.90 log cfu/ml, the numbers declined steadily as the bile concentration and time of incubation increased. The rate of decrease was greater in the non-encapsulated cells. The survival obtained at 0.5% bile salt during the time it took to conduct the initial plating showed a close relative numbers as compared to the initial cell count for capsules cells. Considerable viability was observed up to 90 min reached 8.36 log cfu/ml; and up to 180 min reached 8.3 log cfu/ml compared with culture. In the case of 2% bile concentration, the microencapsulated cells showed only a little decrease (1 log cycle) as compared to the initial cell counts. They could retain the recommended therapeutic-minimum numbers which reached to 7.07 log cfu/ml after 90 min; and reached to 7.0 log cfu/ml after 180 min compared with culture at the same time. Encapsulation has been reported to provide protection

to probiotic bacteria during transit through the human gastrointestinal tract [43, 44]. Several studies have documented the tolerance of lactic acid bacteria to bile [45, 46, 47]. Most of the results indicate the necessity of identifying bile-tolerant strains of probiotics if they are to be used as dietary adjuncts. The results obtained during the present study are in accordance with the observations of other researchers [48, 49]. Chandramouli et al. [38] found that encapsulation of *L. acidophilus* in alginate significantly increased the viability in 1% bile salt. In contrast, Trindade and Grosso [50] also reported that immobilization of *Bifidobacterium bifidum* and *L. acidophilus* in calcium alginate beads was not effective in protecting the cells from 2% and 4% bile salt. Our study demonstrated microencapsulation using alginate may be an effective way to increase the survival of *S. thermophilus* in bile solution.

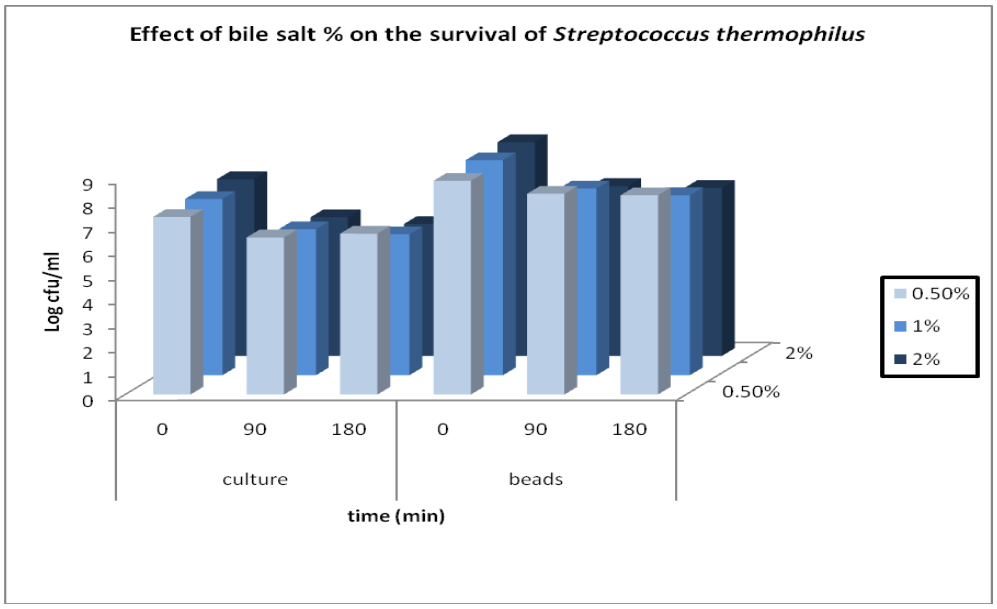

Figure 4: Effect of bile salt concentrations on the survival of microencapsulated and non-encapsulated *S. thermophilus* at different time.

CONCLUSION

In conclusion, the microencapsulation of urease-producing *Streptococcus thermophilus* in sodium alginate resulted in better survival of cells after heat treatment, at high NaCl and bile salt concentrations, and at low pH as compared to non-microencapsulation cells. Also, improve the bacterial survival in simulated gastric environment, and allow viable cells to reach a beneficial level as probiotic. However more research is needed to concentrate on production of new therapeutic dairy products with adding microencapsulated urease - producing *Streptococcus thermophilus.*

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