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RESEARCH ARTICLE

Microencapsulation of *Clostridium tyrobutyricum* by Spray drying Method and Its Characteristics *in-vitro*

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A B S T R A C T

Present study was conducted to improve microencapsulation process of *Clostridium tyrobutyricum* (*Ct*) by optimizing process parameters to improve its *in-vitro* characteristics over that of free cells. All process parameters including wall material (w/v concentration: modified starch 3-7%; gelatin 2-6%; maltodextrin 3-7%), sample flow rate (250-450 mLh^{-1}) and inlet air temperature (105-145°C) were analysed through single factor analysis. Response surface design test was used to develop multiple quadratic regression equations to fit the functional relationship between factors and response values and to choose the optimal conditions. The optimal conditions for maximum survival rate (82.030%) of encapsulated *Ct* were: 4% gelatin, 5% modified starch and 5% maltodextrin concentration with sample flow rate of 350 mLh^{-1} at inlet air temperature of 105°C. Encapsulation reduced the survival loss of *Ct* from 1.990 to 1.080 $lgCFUg^{-1}$ under strong acidic condition (pH 1) than free *Ct*. Survival loss of free *Ct* was 31.914% more than encapsulated *Ct* under high temperature treatment (90°C). Similarly, protected *Ct* showed higher survival rate under simulated gastric condition with long storage life. Encapsulation of *Ct* through optimized spray drying method efficiently improved its survival rate under strong acidic or high temperature environment with safe transit through gastrointestinal tract and also eradicates the technological limitations which preventing the use of many probiotic strains.

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INTRODUCTION

Probiotics are live microorganisms which offer health benefits to the host. They are supplemented in compound feed and subjected to harsh environmental conditions during feed processing which may reduce their biological activities (Ying *et al.,* 2013). Viability of probiotics could be conserved by microencapsulation (ME) (Ying *et al.,* 2013). ME improves the survival of probiotics during processing, storage or their transit through gastrointestinal tract (GIT).

Various techniques including interfacial polymerization, solvent dispersion/evaporation, coacervation or spray drying are used for ME of various products. Among these, spray drying is most common, economical and convenient technique. Spray drying has been successfully used for encapsulation of different probiotics such as *Bifidobacterium ruminantium* (O'Riordan *et al.,* 2001), *Lactobacillus paracasei* (Desmond *et al.,* 2002) and *Lactobacillus rhamnosus* (Corcoran *et al.,* 2004), but to our knowledge no data are available on *Ct* encapsulation by this method till to date. Additionally, most of probiotics do not withstand the high temperature during spray drying, which increase their survival loss during storage. This problem could be solved by using protectants in media before drying it, for example granular starch have been proved to improve the survival rate of culture media in spray drying (Crittenden *et al.,* 2001). Use of Hi-Maize starch in combination of other wall materials also enhanced the encapsulation efficiency of probiotics (Iyer and Kailasapathy, 2005). Starch could be used to improve the delivery of

metabolically active bacteria to GIT (Crittenden *et al.*, 2001). Gelatin is the wall material of choice when encapsulation is done by spray drying due to its good water-solubility, emulsification, film-formation and biodegradation characteristics (Bruschi *et al.*, 2003). Different hydrocarbon compounds are also used as wall material (Ozgur and Mustafa, 2005) which help in the development of spherical and smooth-surfaced or improve the adhesion between core and wall material (Bruschi *et al.*, 2003). Maltodextrin (MD) stimulates the growth of *Bifidobacteria* (Rycroft *et al.*, 2001) and can improve the survival of probiotic under acid conditions (Corcoran *et al.*, 2005). Similarly, gelatine-MD biopolymers protect *Bifidobacterium adolescentis* from simulated gastrointestinal conditions (Antonela *et al.*, 2010).

Compared with other methods, the spray drying process is simple, efficient and economical, and its product could be stored for long duration. Therefore, present study was conducted with aim to develop a combination for wall material containing gelatin, MD and modified starch (MS) and to optimize process parameters for encapsulation of *Ct* through spray drying technique followed by characteristics evaluation of encapsulated *Ct* under different in vitro models.

MATERIALS AND METHODS

Probiotic bacteria, growth medium and encapsulation process: In this experiment *Ct* strain "ATCC25755" was cultured at 37°C on clostridial growth medium (w/v, % composition: 0.2 yeast extract, 2 glucose, 0.4 peptone, 0.2 $(NH_4)_2SO_4$, 0.1 K_2HPO_4, 0.1 KH_2PO_4, 0.01 $MgSO_4{\cdot}7H_2O$, 0.0015 $FeSO_4{\cdot}7H_2O$, 0.0015 $CaCl_2{\cdot}2H_2O$, 0.001 $MnSO_4{\cdot}H_2O$, 0.002 $CoCl_2{\cdot}6H_2O$, 0.0002 $ZnSO_4{\cdot}7H_2O$) as described by Zhu and Yang, (2004) under anaerobic condition and then proliferated by fermentation at same temperature with constant stirring at 150 rpm and 6.0 pH was maintained by using 30% $NH_3{\cdot}H_2O$. Fermentation process was continued for 48h and sampled out every 6h to determine the bacterial growth rate. The *Ct* obtained through fermentation was used for encapsulation and in vitro studies.

MS was mixed with *Ct* (6.50×10^9 $CFUmL^{-1}$) to prepare a homogeneous suspension and then sterilized gelatin and MD (15DE) solution were added in it followed by spray drying (BUCHI Biotechnik AG, Flawil, Switzerland). Viable count was determined by previously mentioned method of Mokarram *et al.* (2009) through agar plate counting and encapsulation rate (E-rate) of *Ct* was estimated as following:

$$\text{E-rate} = N_1/N_2\times100\%$$

Where N_1 is the no. of live *Ct* in capsules ($CFUg^{-1}$) and N_2 is the no. of live *Ct* ($CFUmL^{-1}$) in free form.

Optimization of process parameters: All process parameters: wall material (w/v concentration: MS 3-7%; gelatin 2-6%; MD 3-7%), sample flow rate (250-450 mLh^{-1}) and inlet air temperature (105-145°C) were analysed through single factor analysis. Optimization for survival rate of encapsulated *Ct* (response factor) was achieved through Design Expert Software V8.0., in which three levels (-1, 0 and +1) were selected for three different factors (A: MD concentration 4, 5 and 6%; B: sample flow rate 300, 350 and 400 mLh^{-1}; C: inlet air temperature 105, 115 and 125°C), while concentration of gelatin and MS were fixed at 4 and 5%, respectively. Response surface design test was used to develop multiple quadratic regression equations to fit the functional relationship between factors and response values, to choose the optimal conditions. In order to verify the reliability of the results, 3 parallel tests were carried out using the best conditions obtained by the above-mentioned response surface analysis method.

In vitro gastrointestinal simulation: Simulated gastric (G-solution) and intestinal solutions (I-solution) were prepared to evaluate the survival of *Ct* under these conditions in encapsulated bacteria (EB) and liquid bacteria (LB) forms. To prepare 1L G-solution, HCl (16.40mL) and gastric protease (10g) were dissolved in double distilled water, pH 1.20 was adjusted using HCl solution. Similarly, KH_2PO_4 (6.89g) and trypsin (10g) were dissolved in DD-H_2O to prepare 1L I-solution, its pH was adjusted at 7.40 with 0.4% NaOH solution. Gastrointestinal simulation was done following method of Etchepare *et al.* (2016) and samples were centrifuged at 3000×*g* for 10 min to harvest cells.

Other *in-vitro* characteristics: Cells were treated with sterilized phosphate buffers (pH 1, 2, 3, 4) to determine acid tolerance and samples were collected every hour to determine live bacterial count (Ding and Shah, 2007). Bacterial samples were treated with bile salt solution (0.30%) for 3h with constant agitation of 50 rpm and change in bacterial count was determined every hour (Liserre *et al.*, 2007). To access the effect of high temperature (60 or 90°C), samples were treated with hot water in water bath and decrease in live bacterial count was determined at 15 or 30-min intervals. Storage stability was determined according to method of Etchepare *et al.* (2016). All *in vivo* analyses were run in triplicates.

Statistical analysis: The data regarding single factor and *in vitro* analysis were analysed using the One-way ANOVA procedure of SPSS 20.0 (SPSS. Inc., Chicago, USA) and expressed as the mean±SD. Tukey test for post hoc comparisons was used to determine statistically significant differences among treatments with a significant level at $P<0.05$.

RESULTS

Optimization of process parameters: According to the results of single factor analysis, best levels of process parameters for encapsulation of *Ct* were designated as: concentration of gelatin, MS and MD were 4, 5 and 4-6%, respectively with material flow rate of 300-450mLh^{-1} at 130-150°C of temperature (Fig. 1A-E). Effect of sample flow rate, MD concentration and inlet air temperature on survival rate of encapsulated *Ct* (Y_{Ct}) was further explored by regression analysis (Table 1). The regression analysis of the response surface test, and the response value fitting equation for the experiment was given by:

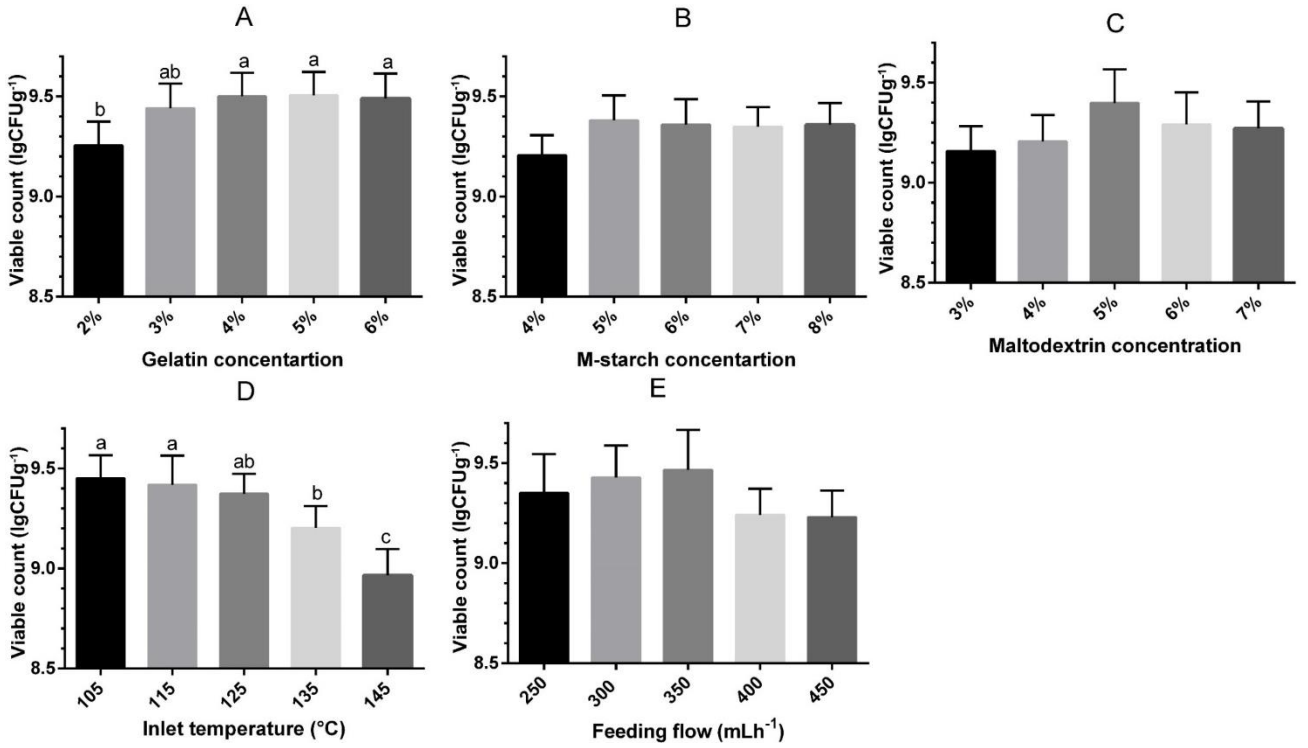

Fig. 1: Effect of different processing parameters on viable count of encapsulated *Ct.* A: Effect of gelatin concentration; B: Effect of different concentration of modified starch; C: Effect of different concentration of maltodextrin; D: Effect of varying inlet air temperature; E: Effect of different feeding flow rate. All data were presented as mean±SD, n=6. Bar having different superscripts differ significantly (P<0.05).

Table 1: The experimental results of BOX-Behnken of preparation conditions for encapsulation of *Ct* through spray drying

Run	Factors			Y_{ct}
	A	B	C	
1	4	300	115	67.08
2	6	300	115	62.47
3	4	400	115	56.42
4	6	400	115	61.29
5	4	350	105	68.34
6	6	350	105	80.23
7	4	350	125	51.63
8	6	350	125	55.94
9	5	300	105	72.21
10	5	400	105	82.38
11	5	300	125	66.41
12	5	400	125	60.47
13	5	350	115	75.28
14	5	350	115	76.19
15	5	350	115	76.01
16	5	350	115	76.08
17	5	350	115	75.86

A: Maltodextrin concentration (%); B: Sample flow (mLh^{-1}); C: Inlet air temperature (°C); Y_{Ct}: Survival rate of encapsulated *Ct.*

$$Y_{Ct}=75.88+2.06A-0.95B-8.59C+2.37AB-1.90AC-4.03BC-10.20A^2-3.87B^2-1.65C^2$$

Where Y_{Ct} is the survival rate (%) of encapsulated *Ct* and A, B and C are MD concentration (%), intake flow (mLh^{-1}) and inlet air temperature (°C), respectively.

The mathematical model was analysed by analysis of variance to calculate the coefficient of correlation (R^2) and the coefficient of determination (R^2Adj), (Table 2). The model was significant (P<0.01) and coefficient of determination (R^2Adj at ~ 0.8549) designated that 85.49% variations in the survival rate of encapsulated *Ct* were due to independent variables. Additionally, three-dimensional response surface of the secondary regression equation (Fig. 2) supported the results of analysis of variance i.e. the interaction between the independent variables on the survival rate of encapsulated *Ct* was not significant (P>0.05).

The regression equation can be analysed to determine the optimal conditions at: 4% gelatin, 5% MS and 5% MD concentration with sample flow rate of 350 mLh^{-1} at inlet air temperature of 105°C, under these parameters the survival rate of encapsulated *Ct* was maximum (82.030%). The optimal conditions obtained by the experiment were carried out in 3 parallel tests, and the average survival rate of encapsulated *Ct* was 81.560% (viable count = 9.320±0.26 $lgCFUg^{-1}$), which was basically in line with the theoretical optimization results.

***In-vitro* gastrointestinal simulation:** According to the results of gastrointestinal simulation overall survivability of EB (65%) was better than LB (62%), (Fig. 3-A). Average loss in survival rate of EB and LB under simulated gastric solution (pH 1.2) was about 4 and 10%, respectively at contact time of 2h. Bacteria were further treated with simulated intestinal solution (pH 7.4) for further 4h. Reduction in survival rate of *Ct* was more rapid in simulated intestinal solution than gastric solution. Under simulated intestinal conditions survival rate of EB was decreased from 95 to 65% while that of LB from 90 to 62%.

Acid tolerance: Results of acid resistance study of *Ct* (EB vs. LB) are presented in Fig. 3-B, which shows that encapsulation enhanced the tolerance of *Ct* against strong acidic conditions. Average no. of *Ct* in EB and LB at the start of acid treatment were 9.140 $CFUg^{-1}$ and 9.430 $CFUmL^{-1}$, respectively, which were reduced to 8.060 $CFUg^{-1}$ and 7.440 $CFUmL^{-1}$ after 4h acid treatment at pH 1. So, encapsulation reduced the survival loss of *Ct* from 1.990 to 1.080 $CFUg^{-1}$ than free *Ct*.

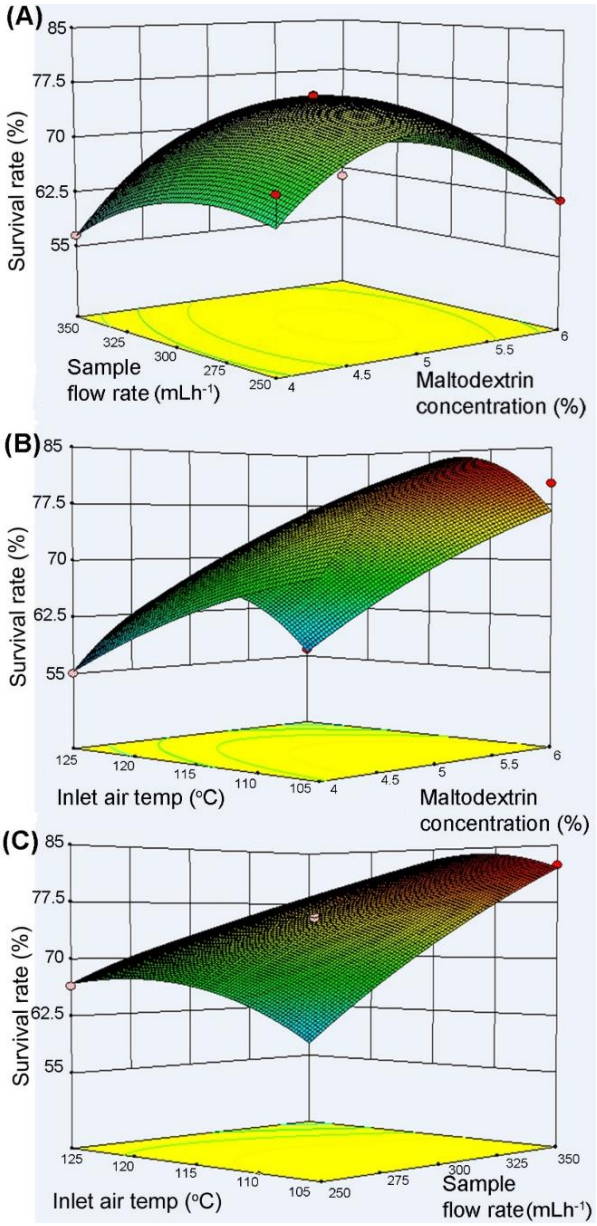

Fig. 2: Response surface plot showing interactive effects of different variables on survival rate of encapsulated *Ct*. A: The effect of maltodextrin concentration and sample flow rate; B: The effect of maltodextrin concentration and inlet air temperature; C: The effect of sample flow rate and inlet air temperature.

Table 2: The ANOVA of BOX-Behnken Design of preparation conditions for encapsulation of *Ct* through spray drying

Source	Degree of freedom	Sum of squares	Mean squares	F-value	*P*-value	Significance
A	1	33.87	33.87	2.74	0.1421	
B	1	7.24	7.24	0.58	0.4694	
C	1	590.13	590.13	47.68	0.0002	**
AB	1	22.47	22.47	1.82	0.2198	
AC	1	14.36	14.36	1.16	0.3171	
BC	1	64.88	64.88	5.24	0.0558	
A^2	1	438.13	438.13	35.4	0.0006	**
B^2	1	63.00	63.00	5.09	0.0587	
C^2	1	11.44	11.44	0.92	0.3684	
Model	9	1278.02	142.00	11.47	0.002	*
Residual	7	86.63	12.38			
Pure Error	4	0.51	0.13			
Corrected total	16	1364.65				

**P<0.001, highly significant ; *P<0.05, significant. Coefficient of correlation (R^2): 93.65%. Coefficient of determination (R^2 Adj): 85.49%.

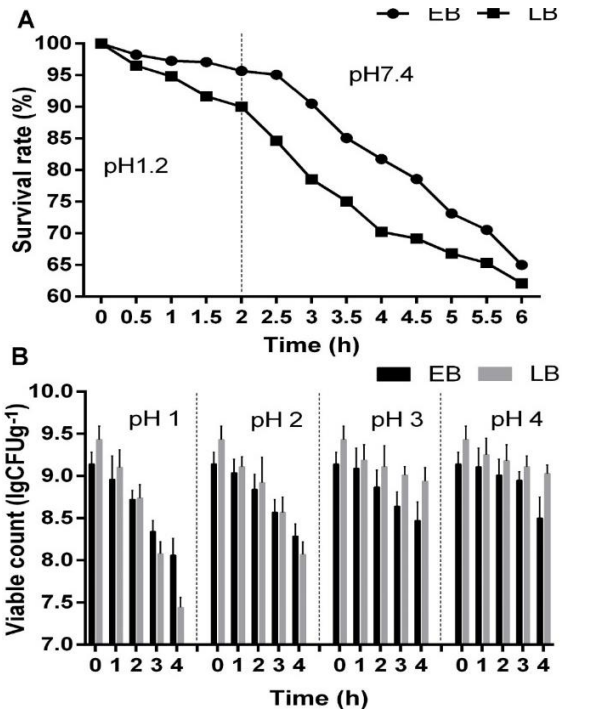

Fig. 3: In vitro characteristics of encapsulated bacteria (EB) vs. liquid bacteria (LB). A: Survival rate (%) of *Ct* under simulated gastric and intestinal fluid, *Ct* was treated with simulated gastric fluid for first 2h at pH 1.2 and then with intestinal fluid form 2-6h at pH 7.4; B: Acid tolerance, change in viable count with the passage of time at varying pH 1-4. All data were presented as mean±SD, n=6.

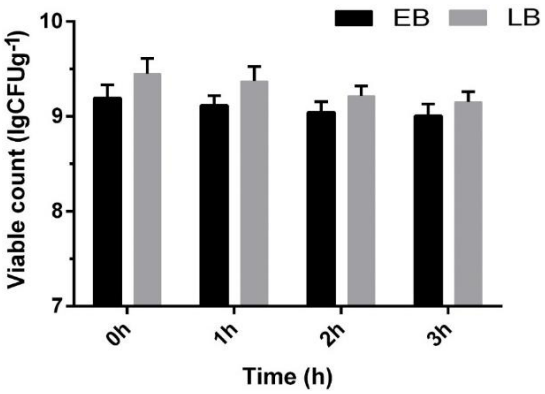

Fig. 4: Survival of *Ct* under bile salt (3%) treatment. EB: Encapsulated bacteria; LB: liquid bacteria. All data were presented as mean±SD, n=6.

Bile salt resistance: As showed in Fig. 4, encapsulation also improved the survival rate of *Ct* in bile salt treatment. Reduction in viable count of *Ct* was higher in free form (3.0×10^9CFUmL^{-1}) than encapsulated form (1.9×10^9 CFUg^{-1}), after 3h of bile salt reaction with *Ct*.

Effect of heat treatment: Heat tolerance of EB and LB at 60 and 90°C presented in Fig. 5. High temperature was found to be lethal for free bacteria and encapsulated *Ct* survived well at that temperature. Average loss in EB and LB were 31.58 and 44.760%, respectively at 90°C after 30 min of heat treatment.

Storage stability: *Ct* was stored at 25°C for 60 days and change in its viable count was observed with the passage of time to evaluate its survival kinetics during storage (Fig. 6). Initial viable count of EB and LB were 9.15±0.18 lgCFUg^{-1} and 9.23±0.14 lgCFUmL^{-1}, respectively. At the

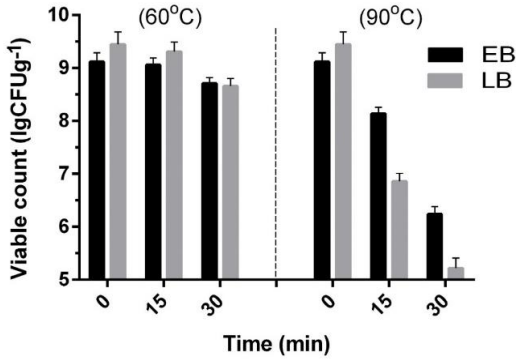

Fig. 5: Effect of heat treatment (60 and 90°C) for different intervals of time on survival of *Ct.* EB: Encapsulated bacteria; LB: liquid bacteria. All data were presented as mean±SD, n=6.

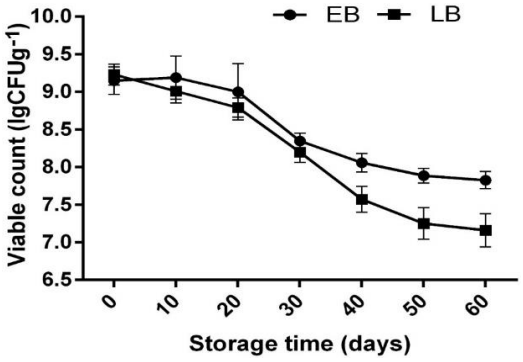

Fig. 6: Storage kinetics of *Ct* at 25°C for a period of 60 days. EB: Encapsulated bacteria; LB: liquid bacteria. All data were presented as mean±SD, n=6.

end of storage period decrease in viable count was higher in LB (2.07 $lgCFUg^{-1}$) than EB (1.32 $lgCFUmL^{-1}$). Results showed that encapsulated *Ct* can survive more efficiently under storage conditions than in free form, because average loss in viable count of LB was about 55% more than EB.

DISCUSSION

Microcapsules produced through spray drying using gelatin as wall material are good carries for different food materials (Shu *et al.,* 2006), which also provide great mechanical support and thermal consistency (Gomez-Guillen *et al.,* 2011). Correspondingly, results of present study exhibited that gelatin has protective effect on *Ct*, but this effect was limited to use of gelatin up to 4%. Using higher concentration of gelatin might increase the viscosity of emulsion which disturb particle size distribution (Shu *et al.*, 2006) and become the source of under-dried particles (Ozgur and Mustafa, 2005), which negatively affect the encapsulation process. MS has low viscosity which reduce the energy consumption for drying process and decrease the cost of production. Sultana *et al*. (2000) reported that use of MS for encapsulation of *Bifidobacteria* and *Lactobacillus* improved their survival rates in simulated gastrointestinal solution and similar effect was found in present study. MD can promote the growth of probiotics e.g. *Bifidobacteria* (Rycroft *et al.,* 2001), enhances their heat resistance (Antonela *et al.,* 2010) and acid tolerance (Corcoran *et al.,* 2005; Antonela *et al.,* 2010). In literatures limited data are available on use of MD as wall material in ME process. According to the results of single factor analysis, positive relationship was observed between survival rate of *Ct* and MD concentration, might be due to increase in the protection of bacteria by thickening of wall but further increase in concentration (>5%) might increase the viscosity of the wall material, which was not conducive to the spray formation or uniform distribution of the bacteria (Ozgur and Mustafa, 2005; Shu *et al.,* 2006), which negatively affect the survival of *Ct.*

Optimal flow rate was $350mLh^{-1}$, lower flow rate might be enhanced the water evaporation and excessive dehydration due to hot air exposure for a relatively long time cause the death of *Ct*. Higher flow rate was also detrimental for *Ct* might be due to insufficient evaporation or too high moisture in the capsules. The low air temperature causes incomplete drying and sticking of product with walls of drying box which decrease the yield, on the other hand high inlet temperature lead to reduced survival rate of probiotics. At temperature <125°C wall material showed some protective effects but at higher temperature (>125°C) the insulation of the wall and heat absorption due to water evaporation, cannot resist the excess heat transferred to the microcapsule lead to rapid inactivation of encapsulated *Ct,* due to dehydration (Peighambardoust *et al.,* 2011). Similar, results were found by Arslan *et al.* (2015), during encapsulation of *Saccharomyces boulardii* through spray drying. Additionally, high inlet temperature disturbed the equilibrium between film-formation and rate of water evaporation leading to break the wall of microcapsules and reduced the ME efficiency (Shu *et al.,* 2006).

ME is good technique to improve the stability of probiotics during their transit through GIT (Guerin *et al.,* 2003; Iyer and Kailasapathy, 2005; Liserre *et al.,* 2007). In current study, survival rate of encapsulation *Ct* were 6% more than free *Ct* in simulated gastric conditions at contact time of 2h. No data is available in literature about ME of *Ct,* but ME improved the survival of *Lactobacilli* (Le-Tien *et al.,* 2004) *Bifidobacteria* (Guerin *et al.,* 2003) and *Lactobacillus acidophilus* (Krasaekoopt *et al.,* 2004) under simulated gastric conditions. It has also been proved that combination of different wall materials protects the probiotics (*Lactobacillus plantarum*) more efficiently than single wall under such conditions (Rajam and Anandharamakrishnan, 2015). Similarly, results of present research proposed that, MD in combination of gelatin and MS are good wall material which shields the effect of simulated gastric conditions.

During in vitro bile salt tolerance experiment minute different in the survival rate of *Ct* was observed in encapsulated or free form, it means *Ct* might has strong capacity to resist bile salt. Encapsulation enhance the survival of *Ct* under simulated gastric conditions but not under simulated intestinal conditions or bile salt condition, might be due to different behaviour of capsule under acidic and alkaline conditions (Zhao *et al.,* 2015). Conflicting results are also mentioned in literature about encapsulated probiotics and their survival under bile salt conditions (Guerin *et al.,* 2003). The contradiction could be due to concentration of bile salt solution, type of wall material or probiotics used.

Results of acid resistance study exhibited that encapsulation material has enough capacity to protect the *Ct* from strong acidic conditions. As mentioned earlier that

gelatin in combination with other material produced strong capsules which protect the core form harsh environmental conditions (Krasaekoopt *et al.*, 2003; Arslan *et al.*, 2015). Microcapsules produced by spray drying at high temperature (125°C) are more stable under acidic conditions, might be due to impervious structure of wall, spray dried at high temperature (Arslan *et al.*, 2015). Encapsulation enhanced the survival of *Ct* under high temperature environment and survival rate of encapsulated *Ct* was about 32% more than free *Ct* at 90°C. Similarly, viability of probiotics in encapsulated form was 62% more than free form at 65°C (Ding and Shah, 2007). It could be concluded that combination of different wall material used in this experiment presented great protection to *Ct*.

Encapsulation also improved the survival rate of *Ct* under storage conditions, and the bacterial count after storage time was enough (>6 $lgCFUg^{-1}$) to serve for health benefits of consumer. Higher microbial count after storage period may be attributed to several factors including type of encapsulation, wall material, inherent resistance properties of strain and low moisture content of the particles. Correspondingly, Bustamante *et al.* (2015) purposed that spray drying enhanced the survival rate of *Lactobacillus acidophilus* during 45d of storage. Similarly, improved shelf life (45 days) of phenolic compounds was observed by encapsulation using MD through spray drying method (Nunes *et al.*, 2015).

Conclusions: In conclusion, optimized process parameters for ME of *Ct* through spray drying were corresponded to combination of wall material (w/v concentration: 4% gelatin, 5% MS and 5% MD) with sample flow rate of 350 mLh^{-1} at 105°C inlet air temperature, in order to obtain 81.560% survival rate of encapsulated *Ct*. Protected *Ct* can survive more efficiently under strong acidic and high temperature conditions with enhanced storage stability as compared to free *Ct*.

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Authors contribution: MQW conceived and designed the whole scheme of the experiments. BW, MUY and XP conducted the experiments. ZPX, WJS, LJL, YYJ, WJT, GW and HDW helped, analysed and interpreted the experimental data. JBZ and DBL helped to perform the spying drying experiment. MUY prepared the initial manuscript. MQW further revised and copy-edited the manuscript. All authors read and approved the final manuscript.

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