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

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**Abstract**

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 mL/h) 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 mL/h 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 (NH₄)₂SO₄, 0.1 K₂HPO₄, 0.1 KH₂PO₄, 0.01 MgSO₄·7H₂O, 0.0015 FeSO₄·7H₂O, 0.0015 CaCl₂·2H₂O, 0.001 MnSO₄·H₂O, 0.002 CoCl₂·6H₂O, 0.0002 ZnSO₄·7H₂O) 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% NH₃·H₂O. 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 (6.50×10⁹ CFU/mL⁻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 described method of Etchepare et al. (2016) through spray drying technique followed by characteristics evaluation of encapsulated Ct under different in vitro conditions. 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, KH₂PO₄ (6.89g) and trypsin (10g) were dissolved in DD-H₂O 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 (Yc) 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:

E-rate = N₁/N₂×100%

Where N₁ is the no. of live Ct in capsules (CFUg⁻¹) and N₂ is the no. of live Ct (CFUml⁻¹) in free form.
The mathematical model was analysed by analysis of variance to calculate the coefficient of correlation (R²) and the coefficient of determination (R²Adj), (Table 2). The model was significant (P<0.01) and coefficient of determination (R²Adj) 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 mL/h 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 CFU/mL¹, respectively, which were reduced to 8.060 CFU/g⁻¹ and 7.440 CFU/mL after 4h acid treatment at pH 1. So, encapsulation reduced the survival loss of Ct from 1.990 to 1.080 CFU/g⁻¹ than free Ct.

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

| Run | A | B | C | Y.  |
|-----|---|---|---|-----|
| 1   | 4 | 300 | 115 | 67.08 |
| 2   | 6 | 300 | 115 | 62.47 |
| 3   | 4 | 400 | 115 | 56.42 |
| 4   | 4 | 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 (mL/h); C: Inlet air temperature (°C); Y.  Survival rate of encapsulated Ct.

Y Ct=75.88+2.06A-0.95B-8.59C+2.37AB-1.90AC-4.03BC-10.20A²-3.87B²-1.65C²

Where Y Ct is the survival rate (%) of encapsulated Ct and A, B and C are MD concentration (%), intake flow (mL/h) and inlet air temperature (°C), respectively.
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    | 0.0559  |              |
| A²     | 1                | 438.13         | 438.13       | 35.4    | 0.0006  | **           |
| B²     | 1                | 63.00          | 63.00        | 5.09    | 0.0587  |              |
| C²     | 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 | 16            | 1364.65        |              |         |         |              |

**P<0.001, highly significant; *P<0.05, significant. Coefficient of correlation (R²): 93.65%. Coefficient of determination (R² 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 from 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.

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 (3.0×10⁹ CFU/mL) than encapsulated form (1.9×10⁹ CFU/g), 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 lgCFU/g and 9.23±0.14 lgCFU/mL, respectively. At the
end of storage period decrease in viable count was higher in LB (2.07 lgCFUg) than EB (1.32 lgCFU mL^{-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; Lisserre 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 logCFUg⁻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 spraying 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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