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Original Research Article

Optimization of *Clostridium tyrobutyricum* encapsulation by extrusion method and characterization of the formulation

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Abstract

Purpose: *To optimize the process parameters for the encapsulation of Clostridium tyrobutyricum (Ct) and to determine its in vitro characteristics.*
Methods: *The process parameters, including the concentration of the wall and hardening material, Ct to gelatin ratio and hardening time, were studied by single factor analysis, while optimization was performed by orthogonal experimental design for the encapsulation rate of Ct.*
Results: *Optimal conditions exhibited by orthogonal experimental design at a 92.17 % encapsulation rate with a viable count of 9.61 ± 0.06 lgCFU/g were: 6 % modified starch, 3 % sodium alginate, and 2 % $CaCl_2$ at a Ct to gelatin ratio of 1:1 with a hardening time of 30 min. The survival rates of encapsulated Ct were higher than free Ct in simulated gastric (6.22 %) and intestinal juices (15.55 %). Reduction in viable counts of Ct at 90 °C were higher for free cells (44.76 %) than encapsulated cells (28.09 %) after 30 min of heat treatment. Correspondingly, encapsulation boosted the capacity of Ct to withstand the strong acidic conditions of the stomach and improved the storage properties of Ct.*
Conclusion: *The results suggested that extrusion is a good technique for the encapsulation of Ct, as it enhances the viability of Ct during their transit through the gastrointestinal tract. Furthermore, encapsulation is favorable for Ct if planned for use in formulations where high temperature treatment is required.*

Keywords: *Encapsulation, Acid resistance, Bile salt tolerance, Clostridium tyrobutyricum, Extrusion, In vitro simulation, Temperature tolerance*

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INTRODUCTION

Probiotics are live microorganisms that have beneficial effects on the host when fed in adequate amounts. Although probiotics have many physiological functions, such as killing harmful bacteria, improving digestion and absorption, and enhancing growth, productivity and intestinal immune function [1], there are many problems associated with their application as feed additives. These include low survival of live bacteria during processing and

transportation; difficulty withstanding low pH in the stomach; and difficulty in resisting adverse environmental conditions during feed processing, such as heating, pelleting and storage. Therefore, it is an interesting topic of research for nutritionists to resolve these challenging situations.

Microencapsulation (ME) is an important technology that not only improves the resistance of probiotics to adverse environments but also safely transfers them to the target site in the intestine to play a specific role. In recent years, reports on probiotic ME enhancing the survival rate of *Lactobacillus* up to 10^6 CFU/mL in simulated gastric fluid have been published [2]. Encapsulation of lactic acid bacteria using calcium alginate as a wall material significantly improves heat resistance and gastrointestinal survival [3]. Annan *et al* [4] found a significantly higher survival rate of *Bifidobacteria* in encapsulated form than as free bacteria (FB) in simulated gastrointestinal fluids.

Extrusion is a simple technique used for ME of different materials. Bajracharya *et al* [5] observed a 99 % encapsulation rate of pig-derived lactic acid using sodium alginate (Na-alginate; 2 %) with $CaCl_2$ solution (0.1 mol/L) as the hardening fluid at 45 °C through the extrusion method with 70 % more bioavailability under simulated gastric fluid (pH 2) than in the unprotected form. In most ME techniques, alginate is used as a wall material due to its nontoxic nature and acceptance as a food additive. However, huge variation in the dose rate of Na alginate is quoted in the literature [6–8]. A similar situation was reported in the case of $CaCl_2$ concentration and hardening time of the capsules [9–11]. Although encapsulation significantly improves the survival of probiotics in different situations, huge variation in the concentration and type of wall materials and lack of consideration of all parameters make it difficult to select the best parameters.

This study was designed to optimize the process parameters for microencapsulation of *Ct* at maximum rate with more viable bacteria and *in vitro* characterization of prepared microcapsules under different conditions.

EXPERIMENTAL

Bacteria, growth medium and preparation of cell suspension

Clostridium tyrobutyricum (*Ct*) ATCC25755 was grown on clostridial growth medium (CGM) at 37 °C under anaerobic conditions and propagated through fermentation at 37 °C with continuous stirring at 150 rpm and 6.0 pH. A bacterial growth curve was developed by measuring the absorbance (600 nm) every 6 h. The composition of CGM was the same as that used previously [9]. The cell suspensions obtained through fermentation were used either directly (free cells) in analysis or for ME.

Microencapsulation and optimization

The cell suspension (6.5×10^9 CFU/g), modified starch (M-starch) and sterilized Na-alginate solution were mixed evenly and extruded into $CaCl_2$ solution to produce microencapsulated bacteria (MEB) and solidified at room temperature. The viable count was determined by a previous method [10], and the microencapsulation rate (ME rate) was calculated as in Eq. 1.

ME rate = C1/C2 x 100 …… (1)

where C1 is the viable count (CFU/g) in microcapsules and C2 is the viable count (CFU/mL) of FB.

All process parameters (Table 1) were studied by single factor analysis ANOVA to determine the effects of individual parameters on the ME rate. An orthogonal experimental design was used to obtain the best combination of all factors to achieve an optimal ME rate [27,28].

In vitro characterization of microencapsulated *Ct*

Encapsulated bacteria were evaluated by a two-stage *in vitro* assay. Gastrointestinal simulated solutions were prepared following the method of Yaqoob *et al.* [9], and simulation was performed according to the procedure of Etchepare *et al* [11].

Table 1: Factors studied in singe factor and orthogonal experimental design

Na-alginate (w/v)	M-starch (w/v)	$CaCl_2$ (w/v)	*Ct.*: gelatin (v:v)	Hardening time (min)
1	4	1	1:01	10
2	5	2	1:02	20
3	6	3	1:03	30
4	7	4	1:04	40

Bile salt resistance was evaluated by treating MEB or FB (1 g or 1 ml) with bile liquid (9 ml of 0.3 % pig bile salt solution) at 37 °C for 3 h with constant agitation (50 rpm) [12].

MEB and FB were treated with hot water at 60 or 90 °C, and changes in the number of live bacteria were counted at different intervals of time to examine their resistance to changes in temperature [3]. Acid resistance and storage kinetics were studied by following the methods of Ding and Shah [13] and Etchepare *et al* [11], respectively. All *in vitro* analysis were run in triplicates.

Statistical analysis

Data regarding the effects of individual factors on the ME rate and *in vitro* characteristics were analyzed by one-way ANOVA in SPSS 20.0 and expressed as the mean ± SD. To determine statistically significant differences among treatments, Tukey's test was used ($p < 0.05$).

RESULTS

Bacterial growth curve

Figure 1 shows that 0–12 h was the adaptation period followed by the growth phase (12 – 24 h) of *Ct,* while the growth curve at 24 – 36 h indicated that the total number of bacteria was no longer growing, and the viability of the entire flora gradually decreased, which might be due to the depletion of nutrients, accumulation of toxic products and decline in pH.

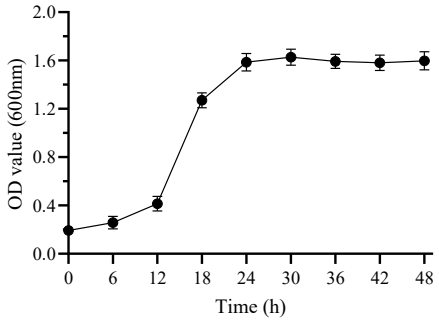

Figure 1: Bacterial growth curve

Optimized microencapsulation process

The ME rate under different experimental conditions is shown in Figure 2 a-e. The maximum ME rate (77.31 %) with the highest number of live bacteria (3.8×10^9 CFU/g) was obtained by hardening the microcapsules for 30 min (Figure 2 a). A further increase in hardening decreased the ME rate (10.09 %) and viable counts (15.79 %). Figure 2 b shows that the ME rate was increased by increasing the concentration of M-starch, and the maximum ME rate (89.60 %) was obtained by using 7 % M-starch. When the concentration of Na-alginate was less than or equal to 3%, a positive relationship was observed between the Na-alginate concentration and ME rate, reaching a maximum rate of 87.98 % at a concentration of 3 %, but a further increase in the concentration of Na-alginate negatively affected the yield of the product (Figure 2 c). Similarly, the ME rate was also increased by increasing the $CaCl_2$ concentration up to 2 % (Figure 2 d). The highest ME rate (78.44 %) was obtained by using 2% $CaCl_2$ solution, and a further increase in its concentration decreased the ME rate. At 4 % $CaCl_2$, a 14.63 % reduction in the ME rate was observed. Single factor analysis of the *Ct* to gelatin ratio showed that 1:3 was the best ratio for the peak ME rate (78.28 %) under the conditions of this study (Figure 2 e).

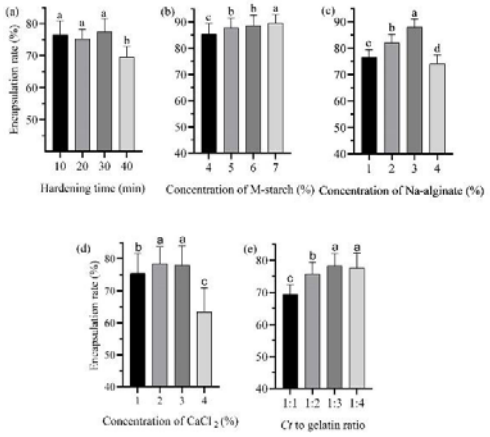

Figure 2: (a) Effect of hardening time, (b) concentration of modified starch, (c) concentration of sodium alginate, (d) concentration of calcium chloride, (e) proportion of *Ct* and wall material on microencapsulation rate of *Ct*. All data were presented as mean ± SD, n = 6. Different letters on the top of bars shows statistical difference ($p<0.05$)

According to the results of the orthogonal design test, the order of four key factors affecting the ME rate was Na-alginate concentration > *Ct* to gelatin ratio > $CaCl_2$ concentration > M-starch concentration. The best combination of parameters was 6 % M-starch, 3 % Na-alginate, 2 % $CaCl_2$, and *Ct* to gelatin ratio of 1:1 with a hardening time of 30 min, which were validated by a follow-up experiment, and the highest ME rate (92.17 %) and viable counts (9.61 ± 0.06 LgCFU/g) were observed at these selected parameters compared to 16 combinations studied previously (Table 2).

Table 2: The orthogonal design and ME rate of *Ct*. at four levels of four different factors

No.	Factors				ME rate (%)
	Na-alginate (w/v)	M-starch (w/v)	$CaCl_2$ (w/v)	*Ct.*: gelatin (v:v)	
1	1	4	1	1:1	67.2
2	1	5	2	1:2	72.6
3	1	6	3	1:3	75.4
4	1	7	4	1:4	69.5
5	2	4	2	1:3	82.3
6	2	5	1	1:4	74.6
7	2	6	4	1:1	90.7
8	2	7	3	1:2	66.8
9	3	4	3	1:4	78.3
10	3	5	4	1:3	83.9
11	3	6	1	1:2	78.1
12	3	7	2	1:1	88.7
13	4	4	4	1:2	68.4
14	4	5	3	1:1	75.5
15	4	6	2	1:4	81.3
16	4	7	1	1:3	74.6
Value					
k1	71.18	74.05	73.63	80.53	
k2	78.60	76.65	81.23	71.48	
k3	82.25	81.38	74.00	79.05	
k4	74.95	74.90	78.13	75.93	
R	11.08	7.32	7.60	9.05	

Note: k1–k4 values are average rate of ME for each factor at levels 1-4, respectively; R is the range for each factor. A larger R value indicates a greater effect of the factor on the process

In vitro gastrointestinal simulation

The results showed that ME protected the bacteria from the strongly acidic and basic conditions of the stomach and intestine, respectively. The survival rates of MEB and FB at pH 1.2 were 96.25 and 90.03 %, respectively, after 2 h of contact time. After simulated gastric solution, bacteria were placed in simulated intestinal solution (pH 7.4), and after 4 h Of treatment, their survival rates were 85.80 and 70.25 % for MEB and FB, respectively (Figure 3).

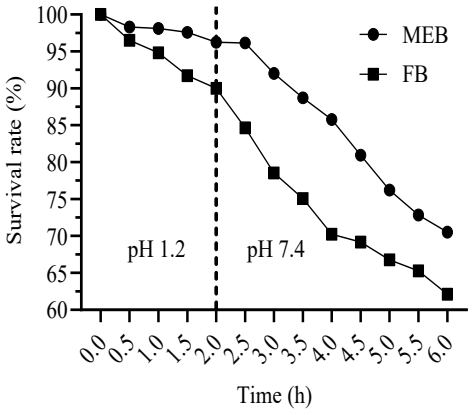

Figure 3: *In vitro* characteristics of MEB vs. FB. Effect of simulated gastric and intestinal fluid. All data were presented as mean ± SD, n = 6

Acid tolerance

Results show that *Ct* was less resistant to a strongly acidic environment, and ME improved its tolerance because at low pH (1 – 4), the survival rate of MEB was higher than that of FB (Figure 4). After 4 h of acid treatment, the decrease in the number of live MEB was 1.05 LgCFU/g, whereas FB decreased by 1.99 LgCFU/mL.

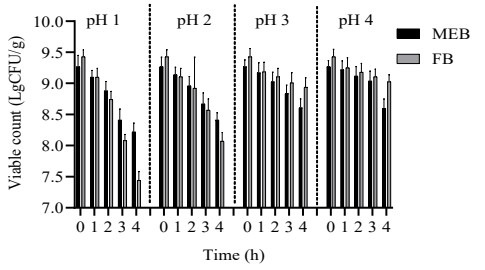

Figure 4: Acid tolerance of MEB vs. FB. All data were presented as mean ± SD, n = 6

Temperature tolerance

The effect of temperature treatment showed that ME significantly improved the resistance ability of *Ct* against high temperature (90 °C) (Figure 5). The reduction in the viable count of MEB after 15 and 30 min of treatment at 90 °C was 8.74 and 28.09 %, respectively, while that of FB was 27.41 and 44.76 %, respectively.

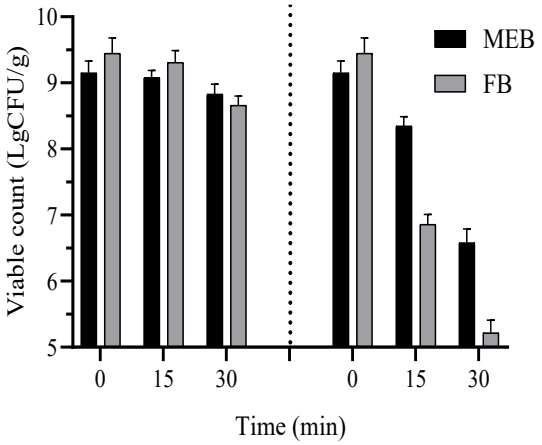

Figure 5: Effect of heat treatment on MEB vs. FB. All data were presented as mean ± SD, n = 6

Bile salt tolerance

The results of the 0.3% bile salt treatment revealed that ME had no effect against bile salt tolerance (Figure 6). The survival of MEB after 1, 2 and 3 h of bile salt treatment was 99.60, 98.13 and 96.95 %, respectively, of the initial count, while that of FB was 99.15, 97.50 and 96.86 %, respectively, under the same conditions.

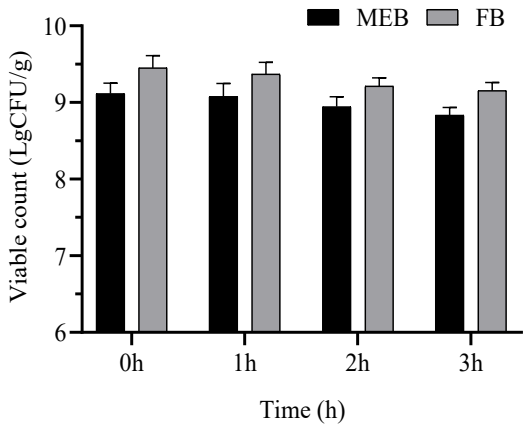

Figure 6: Comparative survival of MEB vs. FB in bile salt solution. All data were presented as mean ± SD, n = 6

Storage kinetics

Storage kinetics of *Ct* in microencapsulated or free form are presented in Figure 7. The initial concentrations of MEB and FB were 9.11 ± 0.29 LgCFU/g and 9.23 ± 0.14 LgCFU/mL, respectively. Samples were stored at 25 °C, and the total reduction in viable count was lower in MEB (1.56 LgCFU/g) than FB (2.07 LgCFU/mL), meaning ME enhanced the shelf life of *Ct*.

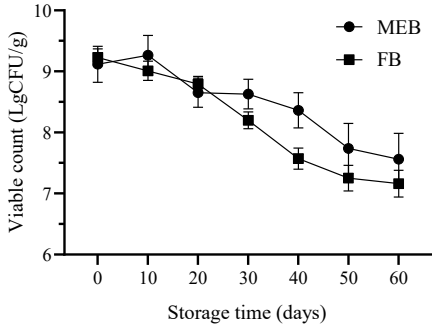

Figure 7: Storage kinetics of MEB vs. FB at 25 °C for storage time of 60 days. All data were presented as mean ± SD, n = 6

DISCUSSION

The results showed that 30 min was sufficient for hardening of microcapsules, as cited previously [2]. A shorter hardening time might be inadequate for the complete reaction between Na-alginate and $CaCl_2$; on the other hand, prolonged hardening might be the reason for the entry of some calcium ions into the microcapsule. The use of M-starch was in line with Sultana *et al* [7], and the maximum ME rate was obtained by using 3 % Na-alginate. A possible reason might be the formation of a thinner capsule wall at lower concentrations of Na-alginate. When the bacteria to gelatin ratio was less than 1:3, the encapsulation rate increased with an increasing ratio. At a higher proportion of gelatin to bacteria, more wall material resulted in thicker capsules, which enhanced the protection of bacteria and the rate of encapsulation.

In the present study, a positive relationship was found among the ME rate, viability count and concentration of $CaCl_2$ used up to 2 %; a further increase in its concentration negatively affected the ME rate. This might be due to the limited number of calcium ions bound with Na-alginate. Similarly, Mandal *et al* [14] suggested that the use of Na-alginate in the ME of *Lactobacillus casei* and hardening of its capsules in 0.1 M $CaCl_2$ significantly improved its survival at low pH and high bile salt concentration when compared to FB. In this study, the combination of M-starch and Na-alginate to form a composite wall effectively protected the bacteria and improved their encapsulation efficiency compared with a single wall of Na-alginate.

The survival rate of FB after 2 h in the simulated stomach fluid was 90.03 % and that of MEB was 96.25 %, which indicated that ME improves the survival of *Ct*. Correspondingly, Iyer and Kailasapathy [15] suggested that the use of Hi-maize starch, chitosan and alginate to

encapsulate *Lactobacillus acidophilus* increased its resistance to the GIT environment. Similarly, *Bifidobacteria* [14,16–18] and *Lactobacilli* [6,19] showed higher viability with alginate encapsulation when incubated in simulated gastric fluid. Results of the present study showed that *Ct* is less resistant to strong acids, and encapsulation improved its tolerance, as suggested by previous studies [20,21]. This was due to the barrier effect of the microcapsule wall on acid, which prevents it seeping into the microcapsule. Previous studies on *Lactobacillus casei* [14] and *Lactobacillus acidophilus* [2] also support the results in this study. In addition, the M-starch added to the wall has a strong adsorption effect and indirectly enhances the tolerance of the internal *Ct* to the acidic environment. However, contrasting results have also been reported in the literature, showing that alginate ME does not effectively protect microorganisms at low pH [7,20,22].

According to the results of the present study, ME has no protective effect against bile salt treatment, as reported by Yaqoob *et al* [9]. Similarly, higher mortality of encapsulated *Bifidobacteria* [16], *Bifidobacterium bifidum* and *Lactobacillus acidophilus* [19] was seen after bile salt treatment. Contradictory findings have been reported in the literature, in which encapsulation could significantly enhance probiotic resistance to bile salts [2,17]. The inconsistency could be attributed to the use of different encapsulation parameters, methods and types or strains of probiotics.

Encapsulation enhanced the tolerance of bacteria against high temperatures of 65 and 90 °C, as reported by Ding and Shah [13] and Yaqoob *et al* [9], respectively. This is similar to the results obtained in this study. The present results are also supported by the study of Mandal *et al* [14], which reported that high-temperature treatment drastically reduced the viable counts of *Lactobacillus* in free form and that alginate encapsulation increased its viability under such conditions. Higher survival of encapsulated *Ct* might be due to slower diffusion of water in the capsule at high temperature.

The storage kinetics results are in line with previous findings [6,23]. In another study, viable FBs were observed after storage at room temperature for 7 days, but little change in counts was observed by encapsulation of the same bacteria for up to 14 days [24]. The increase in survival rate might be due to reduced moisture contents in encapsulated form because probiotics survive longer in a dried environment than under liquid conditions [25,26].

CONCLUSION

The findings of this study indicate that the best process parameters/conditions for encapsulation of *Ct* by the extrusion method are 6 % M starch and 3 % Na-alginate at a *Ct* to gelatin ratio of 1:1 (v/v) with a 30 min hardening time in 2 % $CaCl_2$ solution. Encapsulated *Ct* produced under these conditions survive better in simulated GIT conditions with higher resistance to harsh environmental conditions and comparatively longer shelf life than free *Ct*.

DECLARATIONS

Acknowledgement

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Conflicts of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Minqi Wang designed the study, Junbiao Zou and Dongbi Leng conducted the extrusion experiment, Bin Wang, Xun Pei and Muhammad Umar Yaqoob performed the *in vitro* analysis, while Zhiping Xiao, Wanjing Sun, Yuyue Jin, Lujie Liu, Wenjing Tao, Geng Wang and Haidong Wang analyzed and interpreted the data. Muhammad Umar Yaqoob prepared the manuscript, and Minqi Wang critically reviewed the manuscript, which was then approved by all authors.

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