LWT - Food Science and Technology 81 (2017) 274–280

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Effect of xanthan-chitosan-xanthan double layer encapsulation on survival of *Bifidobacterium* BB01 in simulated gastrointestinal conditions, bile salt solution and yogurt

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ARTICLE INFO

Article history:
Received 9 November 2016
Received in revised form
24 January 2017
Accepted 1 April 2017
Available online 5 April 2017

Keywords:
Xanthan and chitosan
Encapsulation
Bifidobacterium BB01
Gastro-intestinal tract
Storage stability in yogurt

ABSTRACT

Xanthan-chitosan hydrogels have been rheologically characterized in the simulated gastrointestinal conditions while the studies on the applicability of the system for anaerobic bacterial *Bifidobacterium* BB01 encapsulation in yogurt are scarce. The aim of this work was to investigate the effect of a new encapsulation system (xanthan-chitosan-xanthan) on the viability of *B. bifidum* BB01 in yogurt during 21 days storage at 4 °C and 25 °C, respectively. Chitosan was applied as an inner layer to coat the xanthan-probiotic and xanthan was again used as an outer layer for coating chitosan-xanthan-probiotic particles. Moreover, the probiotic survival under the simulated gastrointestinal conditions and bile salt solution were investigated. The results indicated that xanthan-chitosan-xanthan microcapsules (XCX) and xanthan-chitosan microcapsules (XC) significantly ($p < 0.05$) improved the cell survival of *Bifidobacterium BB01* in yogurt during 21 days storage at 4 °C and 25 °C when compared to free cells. All the microcapsules showed higher cell survival of probiotic in the simulated gastric fluid (SGF) and bile salt solution in comparison to free cells. The XC microcapsules exhibited better release profile than XCX microcapsules in the simulated intestinal fluid (SIF). The results of this study suggested that the new encapsulation systems XCX and XC were effective to improve bacterial survival both during yogurt storage and in the gastrointestinal condition.

1. Introduction

Currently, a growing interest in certain lactic acid bacterial strains called "probiotics" has been shown to provide specific-health benefits when consumed as food supplements or as food components (Sanders & Marco, 2010; Sohail, Turner, Coombes, Bostrom, & Bhandari, 2011). The international dairy federation recommends that the minimum concentration of probiotic should be around 10^6–10^7 CFU/mL at the end of product's shelf life (Champagne, Ross, Saarela, Hansen, & Charalampopoulos, 2011; Ding & Shah, 2008; Kailasapathy & Chin, 2000; Lopez-Rubio, Gavara, & Lagaron, 2006; Maukonen et al., 2006). Besides that, probiotic bacteria need to survive harsh factors encountered in the gastro-intestinal tract and keep viable in high concentrations to keep microbial balance on the host (Kailasapathy & Sureeta, 2004; Papagianni & Anastasiadou, 2009; Rodrigues et al., 2011). A number of approaches have been adopted to improve survival of probiotic bacteria, but limited success has been achieved.

Encapsulation system has been developed to enhance the survival of probiotic bacteria consequently (Goin, 2004; Kanmani et al., 2011; Liserre, Ines-Re, & Franco, 2007). In this swelling-controlled-release system, solute transport through the polymer network is controlled by several physico-chemical phenomena such as gel layer formation, polymer water uptake, and polymeric chain relaxation (Kim, La Flamme, & Peppas, 2003; Krasaekoopt, Bhandari, & Deeth, 2003; Llabot, Manzo, & Allemandi, 2004). Encapsulation in specialized wall material has been successfully shown to protect live bacterial cells in many fermented dairy products (Champagne et al., 2011; Ei-salam & Ei-shibiny, 2012; Ding & Shah, 2007; Krasaekoopt et al., 2003; Sheehan, Ross, & Fitzgerald, 2007).

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http://dx.doi.org/10.1016/j.lwt.2017.04.005
0023-6438/

Among the available techniques for immobilizing living cells, the entrapment in chitosan beads has been frequently used for the immobilization of probiotic bacteria because of its ease of handling, low cost, non toxic to the cell being immobilized, and it is an accepted food additive (Krasaekoopt, Bhandari, & Deeth, 2006; Murata, Toniwa, Miyamoto, & Kawashima, 1999; Dumitriu & Chornet, 1997). Xanthan gum, an anionic polyelectrolyte, consisting of a cellulosic backbone with side chains of two mannose and one glucuronic acid on every second glucose residue, can form polyelectrolyte complex (PEC) gels with chitosan (Chellat et al., 2000; Jannson, Kenne, & Lindberg, 1975; Melton, Mindt, Rees, & Sanderson, 1976; Richardson & Ross-Murphy, 1987). Xanthan and chitosan are capable of forming physically crosslinked hydrogels with reversible ionic linkages (Argin, Kofinas, & Lo, 2009; Berger et al., 2004; Chu, Kumagai, & Nakamura, 1996; Magnin, Lefebvre, Chornet, & Dumitriu, 2004). These hydrogels have relatively high enzymatic resistance and pH-sensitive swelling characteristics (Argin, Kofinas, & Lo, 2014). Thus, xanthan and chitosan complex has a good potential for the targeted delivery, and the controlled release of encapsulated products for oral administration (Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2011).

Several studies have reported the probiotic microencapsulation by using chitosan, alginate, xanthan, starch gelatin and vegetable gum to provide protection to *Bifidobacteria* and *Lactobacilli*. Nualkaekul showed that the encapsulation of *Lactobacillus plantarum* cells within single and double chitosan coated alginate beads enhanced considerably cell survival in the highly acidic pomegranate juice (Nualkaekul, Lenton, Cook, Khutoryanskiy, & Charalampopoulos, 2012). To our best knowledge, no work has been carried out on supplementation of yogurt with xanthan-chitosan-xanthan double encapsulated probiotic *Bifidobacterium bifidum (B. bifidum)*. The use of *B. bifidum* BB01 in probiotic applications may reduce the chances of acute diarrhea and the risk of *E. coli* infections, and contributes to the maintenance of vaginal homeostasis. Therefore, *B. bifidum* BB01 were encapsulated in single-layer (xanthan-chitosan, XC) and double-layer (xanthan-chitosan-xanthan, XCX) structure by extrusion in this study (De Castro-Cislaghi, Silva, Fritzen-Freire, Lorenz, & Sant'Anna, 2012; Chen et al., 2014). The aim of this study was to assess the ability of the encapsulation beads (XC and XCX) to improve the survival of *B. bifidum BB01* during exposure to simulated gastrointestinal solution, bile salt solution and in yogurt under different temperature (4 °C and 25 °C).

2. Material and methods

2.1. Microorganism

B. bifidum BB01 was provided by College of Life Science & Engineering, Shaanxi University of Science & Technology. The strain was reactivated in MRS medium (20 g glucose, 10 g peptone, 4 g yeast extract, 8 g beef extract, 2 g Dibasic Ammonium Citrate, 2.2 g K_2HPO_4, 3 g sodium acetate, 0.2 g $MgSO_4$, 0.04 g $MnSO_4$, 1 mL Tween 80, pH of 6.2-6.4) and incubated at 37 °C for 24 h.

The cells were harvested by centrifugation at 4000g for 10 min at 4 °C and then washed twice before re-suspending them in 5 mL normal saline. The final cell concentration was adjusted to 1.36×10^9 CFU/mL.

2.2. Microencapsulation

The chitosan solution (0.7% w/v, pH 5.3, Xingcheng Biological Co., Ltd., Jiangsu, China) was prepared, sterilized by autoclaving (120 °C for 15 min) and cooled to 38–40 °C. The xanthan (Zhongxuan biochemistry Co., Ltd., Shandong, China) was dissolved in deionized water to obtain 0.7% (w/v) stock solution, followed by sterilizing at 110 °C for 10 min. The *B. bifidum BB01* culture containing 1.36×10^9 CFU/mL was suspended in xanthan solution to a final concentration of 14% (v/v). The suspensions were extruded into chitosan matrix by a 450 μm nozzle syringe according to Chen (Chen, Song, Wan, Wang, & Shu, 2014). After 40 min magnetic stirring, the microcapsules (XC) were collected, rinsed with sterile saline solution, filtered and sealed in sterilized conical tubes. The 0.1% (w/v) xanthan solution was added and mixed with above mentioned XC microcapsules for 30 min. Finally, the xanthan coated xanthan-chitosan beads were formed and washed with sterile saline solution for three times. Thus, the free *B. bifidum BB01*, xanthan-chitosan encapsulated *B. bifidum BB01* cells (XC), and xanthan-chitosan-xanthan encapsulated *B. bifidum BB01* cells (XCX) were obtained for further evaluation of survival and storage stability.

2.3. Encapsulation yield

To confirm the viable count of the XC encapsulated *B. bifidum* BB01 beads and XCX encapsulated *B. bifidum BB01* beads, 1.0 g of fresh microcapsules were dispersed in 9 mL of simulated intestinal fluid (SIF), being shaked at 37 °C for 120 min, 210 rpm. The SIF was the modified KH_2PO_4 buffer (50 mM, pH 7.5). The buffer was prepared by dissolving 6.8 g of KH_2PO_4 in 250 mL of deionized water followed by the addition of 0.2 mol/L NaOH until pH 7.5. The final volume was adjusted to 1 L with deionized water followed by the addition of 400 mL trypsin solution (0.025 g/mL).

The bacterial suspensions were serially diluted ten-fold by sterile saline solution and spread on MRS medium, incubated at 37 °C for 48 h. The viable count was calculated. Each experiment was repeated for three times and the results are presented in mean ± standard deviation (SD). The encapsulation yield was calculated as following Eq. (1):

$$\text{Encapsulation yield} = \frac{NE \times ME}{N0 \times V} \times 100 \quad (1)$$

where N_0 (CFU/mL) is the viable count in cell suspension, V (mL) is the volume of cell suspension for encapsulation, N_E (CFU/g) is the viable count in microcapsules and M_E (g) is the mass of microcapsules. The qualified microcapsules were freeze-dried stored and sealed in tubes for up to 28 days for *in vitro* experiments.

2.4. Survival of free B. bifidum BB01 cells, XC encapsulated B. bifidum BB01 cells, and XCX B. bifidum BB01 cells in vitro

2.4.1. pH stability

One gram of microcapsules or 1.0 mL of free suspended cells were added to test tubes containing 9 mL simulated gastric fluid (SGF) (1% w/v, HCl at pH 1.2) and incubated at 37 °C at 210 rpm for 2 h. At pre-determined time (0, 30, 60, 90, and 120 min), wet microcapsules were removed from the SGF by centrifugation for 10 min, washed with saline solution, then suspended in 9 mL SIF for 2 h. The survival of encapsulated or free suspending cells was determined as shown in Section 2.3.

2.4.2. Bile stability

One gram of microcapsules or 1.0 mL of free suspended cells were added to test tubes containing 9 mL simulated (1%, w/v) OX bile Salt (Aoboxing Biological Co., Ltd., Beijing, China) and incubated at 37 °C for 2 h. After pre-determined time (0, 30, 60, 90, and 120min), the beads were collected by centrifugation for 10 min, washed with saline solution, and then dissolved in 9 mL SIF for 2 h. The viability of encapsulated or free suspending cells was