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Encapsulation of *Lactobacillus plantarum* in porous maize starch

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ABSTRACT

This study investigated the survival of probiotic *Lactobacillus plantarum* 299v microencapsulated in native maize starch or partially hydrolyzed maize starches after acid, bile and heat treatments. Scanning electron microscopy and confocal scanning laser microscopy confirmed that naturally present cavities and channels in native maize starch were enlarged by enzymatic hydrolysis allowing them to be filled with probiotics. The formulations using the modified starches had significantly higher initial viable cells compared to native starch after freeze-drying. Compared to free cells, the microencapsulated probiotic bacteria showed a significant improvement in acid tolerance. When comparing unmodified and modified starches, the enzymatic treatments did not significantly improve relative survival, but did result in significantly higher total probiotic numbers after exposure to acid (pH = 2.0, 1 h), bile salt (3% w/v, 4 h) and heat (60 °C, 15min). These results demonstrate that porous maize starch granules allow for a high probiotic loading efficiency and provide enhanced protection to various stressful conditions compared to free cells.

1. Introduction

In recent years, probiotics are becoming more commonly incorporated into functional foods. Health-promoting microorganisms play an important role in promotion of the gastrointestinal tract (GIT) health (Kailasapathy & Chin, 2000). *Lactobacillus plantarum* 299v is added in many food products, mainly fermented milks, because of its recognized health properties, such as improvement of irritable bowel syndrome (Niedzielin, Kordecki, & Birkenfeld, 2001) and vascular endothelial function (Malik et al., 2015). However, the applications are limited by viability of probiotic cells, which is affected by processing and storage conditions and the environment in the GIT (Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2012). In order to confer a functional effect within the body, a probiotic food should contain an adequate number of viable bacteria ($>10^7$ CFU g^{-1} of food) to exert a probiotic effect (Corona-Hernandez et al., 2013).

Various carrier material and preparation techniques are used and investigated for encapsulation of probiotics. Food-grade polymers such as alginate, chitosan, pectin, carrageenan, whey, gelatin and lipids are extensively studied to immobilize bacteria (Anal & Singh, 2007). Extrusion and emulsion techniques are commonly applied to produce calcium alginate beads in which a particularly strong molecular network can be formed to entrap cells. Although alginate hydrogel beads were found to have positive effects in protection of probiotics in a gastric environment and during storage, other polymers should be incorporated to improve stability of alginate microcapsules, as the beads formed by alginate alone have relatively low mechanical stability and entrapment of probiotics is not stable in the presence of chelating agents (Krasaekoopt, Bhandari, & Deeth, 2003; Willaert & Baron, 1996).

Starch that is slowly digestible or resistant to pancreatic amylases has a prebiotic effect which is of great interest as it is known to promote the growth of intestinal microflora and subsequently induces health benefits within the body (Topping & Clifton, 2001). Improvements in glycaemic control and bowel health are associated with the regular intake of fermentable dietary fibre (Nugent, 2005). Furthermore, selection of starch with smaller granule size, white in colour and bland flavour could impart attractive sensory characteristics for food applications.

Encapsulation is one of the best approaches to obtain a synbiotic

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effect of probiotic bacteria and enzyme resistant starch (Fuentes-Zaragoza et al., 2011). Wang, Brown, Evans, and Conway (1999) found that high-amylose maize starch enhanced the tolerance of *Bifidobacterium* to low pH and bile acids. The incorporation of starch within alginate gel beads has been widely employed to provide synergistic protection for probiotic bacteria (Chan et al., 2011; Homayouni, Azizi, Ehsani, Yarmand, & Razavi, 2008; Kailasapathy, 2006; Muthukumarasamy, Allan-Wojtas, & Holley, 2006; Sabikhi, Babu, Thompkinson, & Kapila, 2010; Sultana et al., 2000; Xing et al., 2014). However, knowledge on the use of porous granular starch as an encapsulating material is still in its infancy.

In order to obtain further functional properties and improve the performance as wall materials, starch granules can be modified into porous capsules which have industrial applications such as drug delivery, flavor entrapment etc. The presence of structural features like pores, channels and cavities in maize starch (Dhital, Shrestha, & Gidley, 2010) provides an expandable space which can be filled with bacteria after amylase digestion. The structures increase effective surface area, and facilitate a relatively higher enzymatic hydrolysis susceptibility compared to potato starch (Dhital et al., 2010). Maize starch is thus more applicable to be modified into porous capsules by enzymatic digestion than potato starch. The target core material could be physically adsorbed in the pores and cavities without any covalent bonding, and the adsorbed molecules could be completely released in a sustained pattern (Wang, Yuan, & Yue, 2015). It is also worthy of note that partially hydrolyzed maize starch has been shown to remain slowly digestible like untreated native starch (Zhang, Ao, & Hamaker, 2006), which suggests that it can be used for targeted delivery to the large intestine. The process of enzyme digestion of maize starch resulted in a 3–4 times increase of the magnitude of specific surface area (Gao, Li, Bi, Mao, & Adhikari, 2013). The porous maize starch thus could provide an ideal internal surface for adherence of the probiotic bacteria during processing. However, further studies are required to understand the effect of microstructure of porous starch on properties of microcapsules containing probiotics. More accurate delivery in the digestive tract may be subsequently achieved by manipulating preparation of starch materials.

In this study, modified maize starches from different enzymatic treatments were employed as wall materials to encapsulate *L. plantarum* 299v. The morphologies of modified wall materials and probiotic starch capsules were characterized. Furthermore, stability of the probiotic strain in microcapsules was investigated by exposure to simple stimulated GIT conditions and under mild heat treatment.

2. Materials and methods

2.1. Preparation of probiotic culture

A probiotic strain *L. plantarum* 299v was obtained from a commercial probiotic capsule (IBS Support, Ethical Nutrients, Brisbane Australia) and confirmed as the correct species using 16s rDNA sequencing (Sreekumar, Al-Attabi, Deeth, & Turner, 2009). The strain was grown in de Man, Rogosa and Sharpe (MRS; Oxoid Ltd, UK) broth at 37 °C for 24 h and then harvested by centrifugation at 4400 × *g* for 10 min. The cell pellet was washed twice and resuspended with sterile 0.2 M sodium phosphate buffer (pH = 6.3) to obtain concentrated (approximately 10^{10} CFU mL^{-1}) probiotic organisms.

2.2. Preparation of porous starch

Pancreatic α-amylase (PA [A6255 Sigma]), pancreatin (P [P-1750 Sigma]), fungal α-amylase (FA [10065 Sigma]) were purchased from Sigma-Aldrich, USA. The three enzymes and two treatment times (30 min and 120 min) were applied to modify native maize starch (22.2% amylose, Penford Australia Ltd., Australia). The starch slurry (5% w/v) was prepared with PBS (Sigma-Aldrich, USA) buffer and enzymes (0.5 unit per mg of starch) was mixed with the starch suspension. A control was prepared without addition of enzymes. The samples, in 50 mL Falcon tubes, were continuously stirred with a magnetic stirrer bar at 250 × *g* during the incubation time in a water bath maintained at 37 °C. After 30 and 120 min, porous starches were harvested by centrifugation of the tubes at 4000 × *g* for 5 min. The residue was washed three times with excess of ethanol to remove soluble sugars and the residual enzymes. Finally, starch sediments were transferred to petri plates and vacuum-oven dried overnight at 40 °C. Two batches were prepared for each treatment. These wall materials after preparation are referred to as P30, P120, PA30, PA120, FA30, FA120 and Native. The degree of hydrolysis of starch as measured by released maltose was 18–22% for 30 min and 36–41% for 120 min hydrolysis. The degree of hydrolysis was however not significantly different among enzymes at each time point.

2.3. Encapsulation of L. plantarum cells

L. plantarum cells were encapsulated in the prepared maize starches according to the method described by Lahtinen, Ouwehand, Salminen, Forssell, and Myllärinen (2007) with slight modifications. The bacterial culture (6 mL) was transferred into sterile tubes containing 2.0 g starch and stirred at 600 × *g* for 3 h using an orbital shaker (Labtek, Australia). Then the mixture was allowed to settle for 2 h. The supernatant was carefully pipetted off. The sediment was placed in a petri plate and pre-cooled in a freezer (−20 °C) for 4 h before being freeze-dried overnight. For the coating material, gelatinized starch was prepared by heating native starch (2% w/v) in water for 15 min on a hotplate until it formed a gel. After cooling to room temperature, the gel solution was gently mixed with the freeze-dried powder (3 mL g^{-1} starch). Gelatinized starch coated porous starch granules was recrystallized at −20 °C overnight followed by freeze-drying for 24 h. After that, the microcapsules were collected and maintained in sterile 10 mL tubes at 4 °C prior to testing. The encapsulation process was conducted in duplicate, separately using two batches of prepared starches.

2.4. Determination of viable bacteria

The number of viable *L. plantarum* was counted by the spread-plate technique on MRS agar. The microcapsules (0.10 g) were first added into 0.9 mL peptone water (0.1% w/v) containing pancreatin (0.5 unit/mg starch). The pancreatin was added to hydrolyze the starch releasing the encapsulated bacteria. The plates were rotated on an orbital shaker (Labtek, Australia) at 600 × *g* for 15 min. Serial dilutions were made with peptone water (0.1% w/v) and 0.1 mL samples from each of three consecutive dilutions were spread onto MRS agar. The agar plates were incubated at 37 °C for 36 h under anaerobic conditions generated by AnaeroGen 3.5 L (Oxoid Ltd). Colony forming units (CFU) were enumerated and recorded for plates on which 15–300 colonies can be viewed. The loss in bacteria viability was calculated as follows:

Reduction of viable bacteria = $\log N_0 - \log N$, where, N_0 and N are viable count (CFU g^{-1} or CFU mL^{-1}) before and after treatments (acid, bile and heat), respectively.

2.5. Resistance to acid and bile salt

Acid and bile salt stress survival experiments of micro-encapsulated and free probiotic bacteria were carried out in