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Encapsulation of *Lactococcus lactis* subsp. *lactis* on alginate/pectin composite microbeads: Effect of matrix composition on bacterial survival and nisin release

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ABSTRACT

Alginate/pectin hydrogel microspheres were prepared by extrusion based on a vibrating technology to encapsulate bacteriocin-producing lactic acid bacteria. Effects of both alginate/pectin (A/P) biopolymers ratio and physiological state of *Lactococcus lactis* subsp. *lactis* (exponential phase, stationary phase) were examined for nisin release properties, *L. lactis* survival and beads physico-chemical properties. Results showed that A/P composites were more efficient to increase beads properties than those formulated with pure alginate or pectin. Association of alginate and pectin induces synergistic effect which improves microbeads mechanical properties. FTIR spectroscopy confirms possible interactions between alginate and pectin during inter-penetrating network formation. Physiological state of bacteria during encapsulation process and microbeads composition (A/P ratio, enrichment of internal medium with nutrients) were determining factors for both bacteria viability and bacteriocin release. Of the several matrices tested A/P (75/25) with glucose-enriched M17 gave the best results when *L. lactis* was encapsulated in exponential state.

1. Introduction

The interest in application of lactic acid bacteria (LAB) in the prevention of food spoilage and foodborne pathogens growth has increased in the last twenty years (Scannell et al., 2000). Many studies have shown that LAB can reduce the presence of *Listeria monocytogenes* in meat and seafood (Budde et al., 2003; Jacobsen et al., 2003; Tahiri et al., 2009) or inhibit other foodborne pathogens such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella Typhimurium*, *Salmonella Enteritidis* and *Staphylococcus aureus* (Trias et al., 2008). Several mechanisms, such as lactic acid production, competition for nutrients or production of antimicrobial compounds, explain inhibition of spoilage or pathogenic microorganisms by LAB. Among LAB, *Lactococcus lactis* subsp. *lactis* is particularly used for food preservation because of its ability to produce bacteriocin, such as nisin, to control spoilage and pathogenic bacteria. However the possible interactions between food components and LAB decreased their effectiveness. The immobilization of LAB by encapsulation using natural polymers as proteins or polysaccharides appears as an interesting strategy to protect strain and modulate nisin release.

Encapsulation of bacteria in calcium alginate beads is one of the most studied system for probiotic immobilization and protection (Léonard et al., 2014; Madziva et al., 2005; Polk et al., 1994; Smrdel et al., 2008). Some studies focus on the interest to design composite systems by associating several biopolymers as pectin and alginate to control active components release (Jaya et al., 2008). Authors reported that an increase in pectin caused the diminution of gel barrier and increases the percentage of drug release. Moreover, morphology of alginate pectin microcapsules showed porous micro-structure and they also facilitates active components release.

Sodium alginate is a water soluble anionic polysaccharide, mainly found in the cell walls of brown algae and can be isolated from the bacteria *Pseudomonas* (Pawar and Edgar, 2012). This natural polymer possesses several attractive properties such as good biocompatibility, wide availability, low cost, and simple gelling procedure under mild conditions. Alginate composition is variable and consists in homopolymeric blocks alternating 1,4-linked β-D-mannuronic acid (M) and α-L guluronic acid (G) residues. Physical properties of alginate are dependent on the composition, sequence and molecular weight. Gel formation is driven by interactions between G-blocks which associate to form firmly held junctions due

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to divalent cations. In addition to G-blocks, MG blocks also participate by forming weaker junctions.

Pectin is one of the main structural water-soluble polysaccharides of plant cell walls. It is commonly used in the food industry as gelling and stabilizing agents. Basically, pectins are polymers of (1–4) linked partially methyl esterified α-D-galacturonic acid (Synytsya et al., 2003). Pectins gelation is driven by the interaction between the polygalacturonate chains and divalent cations and is described by the egg-box model where the divalent cations are thought to be held in the interstices of adjacent helical polysaccharide chains (Braccini and Pérez, 2001).

Therefore, the objectives of the present study were (a) to develop novel alginate-pectin hydrogel microspheres for the microencapsulation of *L. lactis* subsp. *lactis*, a lactic acid bacteria, by dripping using the vibrating technology (b) to analyze physico-chemical properties of composite microbeads (c) to evaluate the effect of polymers ratio and physiological state of encapsulated bacteria (exponential or stationary phase) on microbial survival, nisin release and antilisterial activity (d) to determine if a nutritional enrichment of hydrogel matrix by addition of synthetic media (M17) supplemented with 0.5% glucose can improve results.

2. Materials and methods

2.1. Materials

Sodium alginate from brown algae (viscosity $\leq$0.02 Pa s for an aqueous solution of 1% wt at 20 °C) and pectin from citrus peel (galacturonic acid $\geq$ 74%, Methoxy Groups $\geq$ 6.7%) were purchased from Sigma–Aldrich (France). Calcium chloride dihydrate and sodium chloride were obtained from VWR (International MgbH, Darmstadt, Germany), synthetic medium M17 from Biokar diagnostics (Beauvais, France), D (+) Glucose monohydrate from Merck (Darmstadt, Germany) and glycerol from VWR (AnalaR NORMAPUR, PROLABO, Fontenay-sous-Bois, France).

Stock cultures of *L. lactis* subsp. *lactis* ATCC 11454, a nisin producing-strain, *Micrococcus flavus DSM 1790* and *L. monocytogenes CIP 82110* were kept frozen (−80 °C) in synthetic media enriched with 30% glycerol (M17 broth for the LAB and TSB broth (Biokar diagnostics, Beauvais, France) for the others strains).

2.2. Microbeads preparation

Alginate and pectin solutions (1% (w/w)) were prepared with sterile physiological water (9% sodium chloride, VWR Belgium) or with sterile M17 broth supplemented with 0.5% D (+) glucose. Preliminary studies indicate a positive effect of addition of 0.5% glucose on *L. lactis* growth and nisin production (data not shown).

L. lactis culture was regenerated by transferring a loopful of the stock culture into 10 mL of M17 broth and incubated at 30 °C overnight. A 10 μL aliquot from overnight culture was again transferred into 10 mL of M17 broth and grown at 30 °C to exponential or stationary phase of growth (6 and 48 h respectively). *L. lactis* cells were collected by centrifugation (20 min, 4 °C, 5000 rpm) and diluted to obtain a target inoculum in microbeads of 10^5 CFU mg^{-1}.

Alginate/pectin hydrogel microspheres were made using the Encapsulator B-395 Pro (BÜCHI Labortechnik, Flawil, Switzerland). In this study, five polymers ratios (A/P) were selected: 100/0; 75/25; 50/50; 25/75; 0/100. The encapsulator technology is based on the principle that a laminar flowing liquid jet breaks up into equal sized droplets by a superimposed nozzle vibration. The vibration frequency determined the quantity of droplets produced and was adjusted at 1200 Hz to generate 1200 droplets per second. The flow rate was 3 mL min^{-1}. A 120 μm diameter nozzle was used for the preparation of beads. Droplets fell in 250 mL of a sterile $CaCl_2$ solution (100 mM) continuously stirred at 150 rpm to allow microbeads formation. The beads were maintained in the gelling bath for 15 min to complete the reticulation process and then were filtered and washed with buffer solution (9% sodium chloride).

2.3. Physico-chemical characterization of microbeads

2.3.1. Size

The mean distribution of beads was measured using a laser light scattering particle size analyzer Mastersizer S (Malvern Instruments Ltd. UK) equipped with a He–Ne laser, a beam of light of 360 nm. The system was able to determine particles in size ranging from 0.05 up to 900 μm. Measurements were achieved in ten replicates for each system. Results were reported as the volume weighted mean globule diameter D (4,3) in μm:

$$D(4,3) = \sum n_i d_i^4 \Big/ \sum n_i d_i^3 \quad (1)$$

Where n_i was the number of particles; d_i was the diameter of the particle (μm). The D (4, 3) was chosen instead of D (3, 2) since it is very sensitive to the presence of small amounts of large particles. All tests were run in triplicate.

2.3.2. Morphology

The beads just prepared were observed under an optical microscope (Olympus AX70, Japan) equipped with a camera (Olympus DP70). Dp controller software (version 2.1.1) was used for taking pictures.

Beads size and shape were also determined by using a QICPIC™ analyzer (Sympatec MgbH, Clausthal-Zellerfeld, Germany). The analyzer was directly connected to the reactor and made measurements every 5 min during 60 min. The liquid with beads was pumped into the reactor, passed through the measuring cell, and images were captured and recorded. The results analysis provided 2D, 3D particle, and then determined several size and shape parameters. The diameter of a circle of equal projection area (EQPC) was calculated. It identified the diameter of a circle with the same area as the 2D image of the particles. As different shaped particles may have the same EQPC, other parameters were used to characterize the particles. The sphericity was defined as the ratio between the EQPC perimeters with the real particle perimeter. The convexity provided information about the harshness of the particle. A particle with smooth edges had a convexity value of 1 whereas a particle with irregular ones had a lower convexity (Burgain et al., 2011). All tests were run in triplicate.

2.3.3. Mechanical stability

To investigate mechanical stability of alginate, pectin and alginate/pectin composite microbeads, individual beads were compressed between two parallel plates.

A rotational rheometer Malvern Kinexus pro (Malvern Instruments, Orsay, France) with a plate-and-plate (20 mm) geometry was used.

A force gap test was used to compress the microbeads in a droplet of water from 200 to 1 μm with a linear compression speed of 1 μm s^{-1}. The gap and the normal force being imposed were measured simultaneously at the upper plate. At least three replicates were considered for each type of microbeads.

2.3.4. FTIR spectroscopy

Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra of freeze-dried beads were acquired using a Tensor 27 mid-FTIR Bruker spectrometer (Bruker, Karlsruhe, Germany) equipped with an ATR accessory (128 scans, 4 cm^{-1} resolution,