Multilayer deposition on *B. longum* to improve probiotic stability

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Introduction

Bacteria activate protection mechanisms under starvation conditions as occurs for example during exposure to heat or during growth in late stationary phase. In latter case, bacteria produce either exopolysaccharide or protein layers. A typical example for forming such protection layer at harsh conditions represents the production of S-layer proteins.

Recent studies have shown that a novel interfacial engineering technology, based on electrostatic deposition of charged biopolymers onto the surfaces of charged oil droplets or bodies can be used to create delivery systems that could encapsulate and release active agents (Iwanaga 2008; Gu 2005). This technique is based on layer-by-layer (LbL) deposition of polyelectrolytes onto oppositely charged surfaces or colloidal particles due to electrostatic attraction. The LbL technology allows precise control over thickness and properties of the interfacial membrane. In order to simulate natural defense mechanisms in this study, bacteria were coated with layers of biopolymers using electrostatic deposition. The deposition of biopolymer layers on charged bodies can be evaluated by means of zeta potential and size measurements as well as by microscopy techniques.

Bacteria can be considered as colloidal body having an approximate size of 1 to 3 μm. As shown previously, bacteria carry negative charges on their surface when suspended in a medium of a pH ranging from 4 to 10 (Zammaretti 2003). Electrostatic deposition is obtained by mixing bacteria with a biopolymer (e.g. protein), which carries positive charges at the respective pH.

Gelatin and milk protein fractions were used as protein source in this study, gum acacia and another milk protein fraction were used as negatively charged biopolymers.

In industrial production probiotic bacteria are subjected to a combination of several stresses. Osmotic stress, dehydration of the cells or exposure to heat can have lethal effects reducing bacterial viability. In order to run an economical process a maximum amount of live bacteria should be recovered after processing and storage. Stability during processing, subsequent storage, and during passage of the consumer's stomach are important requirements (Mattila-Sandholm, 2002).

The encapsulation system must withstand harsh conditions during processing (e.g. homogenization, drying). In order to produce mechanically stable encapsulation layers, the outer biopolymer layers were cross-linked. In this study transglutaminase (TG) was used as cross-linking agent (Kellerby 2006).

This study serves as proof of principle showing that it is possible to form layers of cross-linked biopolymers around single bacteria.

Materials and methods

B. longum NCC 3001 was harvested in late stationary phase. Subsequently, the cells were washed in a 10 mM phosphate buffer solution (pH 5) at a temperature of 4 °C. In order to reduce the metabolic activity of bacteria, we kept the biomass at 4 °C for 1 h before starting the experiment. The following biopolymers were used as coating materials: Gum acacia (Instant Gum 40693, CNI, France), gelatin type A (Sigma G2625, Sigma, USA), and milk protein fractions A and B. As cross-linking agent transglutaminase (Ajinomoto Activa EB, Japan) having an optimum activity of 10 U/g at 4 °C was used.

Stock solutions of biopolymers in water were prepared at concentrations between 1 % and 2 %. The optimum addition ratio of stock solution to the bacterial suspension was determined for each biopolymer in order to reach the desired zeta potential value allowing layer-by-layer deposition. After preparation the solutions were kept at 4 °C.

The zeta potential measurements were performed in a Zetasizer (Malvern Zetasizer with Autotitrator Unit, England). The solutions/suspensions were diluted in order to obtain an optical density between 0.4 and 0.6 at 600 nm. 2 mL of solution was injected into the measuring cell and the electrophoretic mobility of the bacteria was measured three times.

Microscopic investigations were carried out using a confocal laser scanning microscope LSM 510 from Carl Zeiss (Germany). Gelatin and gum acacia were stained with fluorescein (FITC, Fluka, Switzerland) and rhodamine 6G (TRITC, Sigma-Aldrich, USA) respectively. In order to eliminate excess dye, the solutions were dialyzed.

Results and Discussion

The electrical charge (ζ–potential) of different biopolymers was measured over a pH range from 10 to 2 in order to screen materials, which are suitable to form layers around bacteria (Fig. 1). Gum acacia is uncharged at low pH (~2.2) but becomes increasingly negatively charged until the pH approaches 4.0. The net electrical charge of protein molecules is positive at low pH but becomes negative once the pH exceeds the protein's isoelectric point (Damodaran, 1996). A wide range of isoelectric points was covered in this study from 7.2 for milk protein fraction A down to 3.8 for milk protein fraction B. Gelatin has an isoelectric point of 5.2. The large range of isoelectric points gives a certain degree of freedom in choosing the layer materials. For example multi-layer capsules can be produced consisting either of proteins and polysaccharides or only of proteins.

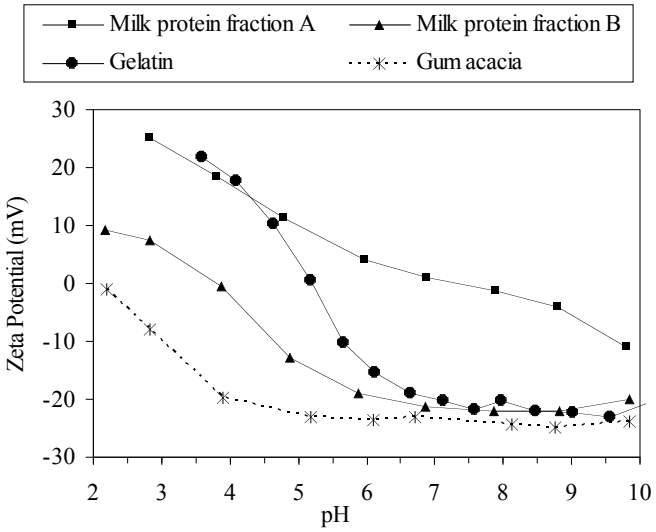

Figure 1: Influence of pH on the biopolymer electrical charge (ζ–potential).

Fig. 2 shows the ζ–potential value of a bacterial suspension before coating and after several coating steps (up to 7 layers). The electrical charge of a suspension of uncoated bacteria was approximately -27 mV. The change of the charge of the suspended particles from negative to positive after the first coating layer suggests that milk protein fraction A was adsorbed onto the surface of the bacteria. After the addition of milk protein fraction B to the suspension the ζ–potential dropped again to a negative value. Further biopolymer layers could be deposited around the bacteria (in Fig. 2 up to 7 layers). Each time the bacteria acquired a positive or negative charge, depending which milk protein fraction was added.

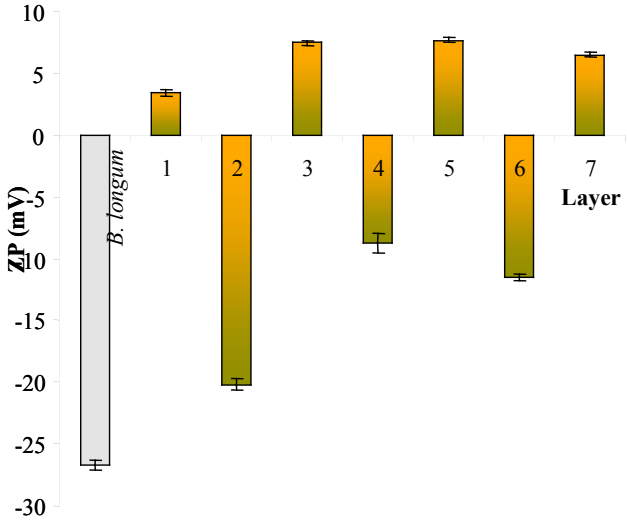

Figure 2: Zeta potential measurement of *B. longum* alternately covered by layers of milk protein fraction A and milk protein fraction B (layers 1 to 7) at pH 5. Every bar represents four independent samples and three measurements for each sample.

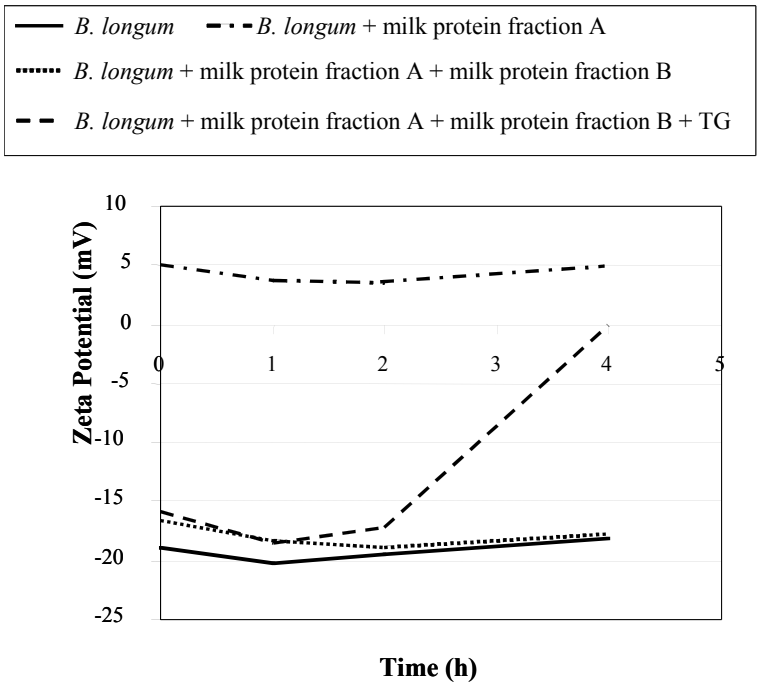

Figure 3: Zeta potential as a function of time for *B. longum* covered with different protein layers and cross-linked with transglutaminase (TG) at 4°C, pH 6.

In this study transglutaminase was used to crosslink the outer biopolymer layer in order to improve the mechanical stability of the encapsulation system. The propensity of transglutaminase to interact

with a protein substrate depends on the protein's structural conformation that influences the accessibility of its glutamine and lysine residues (Kellerby 2006). In order to identify whether the cross-linking reaction was taking place zeta potential of the bacterial suspensions was monitored over time. As shown in Fig. 3, both the pure bacterial suspension and bacteria covered with different biopolymer layers have a constant ζ–potential value over time. However, when transglutaminase (TG) was added, the ζ–potential value increased from a negative value towards zero, showing that a reaction was taking place.

Confocal microscopy was used to confirm that single bacteria could be coated by biopolymer layers. As shown in Fig. 4 single *B. longum* bacteria were covered by gelatin (in green). The gelatin can only be found around the bacteria and does not dissolve in the medium showing the effectiveness of the process.

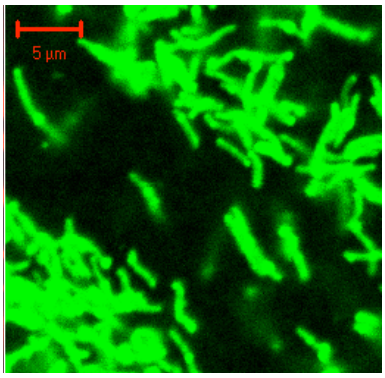

Figure 4: Confocal laser scanning microscopy image of *B. longum* covered with gelatin stained with fluorescein (FITC).

Conclusion

It was shown that single bacteria can be covered with several biopolymer layers. Protein layers were successfully cross-linked with transglutaminase in order to improve the mechanical stability of the encapsulation system. The aim of the work is to improve probiotic stability during process, storage and passage through the stomach. To study the efficacy of the encapsulation system in protecting the bacteria, stability tests are on-going in liquid and in dry state where the viability of encapsulated bacteria is compared to an uncoated reference.

Bibliography

T. Mattila-Sandholm et al. (2002) *Technological challenges for future probiotic foods.* International Dairy Journal 12 173-182

S. Damodaran (1996) *Amino acids, peptides and proteins* in Food Chemistry, O.R. Fennema, Dekker, New York

Y. Gu et al. (2005) *Production and Characterization of Oil-in-Water Emulsions Containing Droplets Stabilized by Multilayer Membranes Consisting of β-Lactoglobulin, ι-Carrageenan and Gelatin.* Langmuir 21(13) 5752-5760

D. Iwanaga et al. (2008) *Stabilization of Soybean Oil Bodies Using Protective Pectin Coatings Formed by Electrostatic Deposition.* J. Agric. Food Chem. 56(6) 2240-2245

S. Kellerby et al. (2006) *Lipid oxidation in a menhaden oil-in-water emulsion stabilized by sodium caseinate cross-linked with transglutaminase.* J. Agric. Food Chem. 54 10222-10227

P. Schär-Zammaretti et al. (2003) *The cell wall of lactic acid bacteria: Surface constituents and macromolecular conformations*. Biophysical Journal 85(6) 4076-92