2014 4th International Conference on Biotechnology and Environment Management
IPCBEE vol.75 (2014) © (2014) IACSIT Press, Singapore
DOI: 10.7763/IPCBEE. 2014. V75. 13

Characterization of S-layer Proteins Synthesized under Stress Conditions in a Probiotic Strain of Lactic Acid Bacteria

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Abstract. S-layer proteins are surface proteins present on bacterial cell surfaces with unique properties like recrystallization and uniform pore size between 2-8 nm. These properties make them applicable in various fields of nano-biotechnology. With their protective roll, purified S-layers are stable toward different sub-optimal conditions. In this research over producing of S-layers at the cell surface of a probiotic lactic acid bacteria under stress conditions was evaluated. The crude S-layer proteins were extracted and characterized by proteomic approaches. It was shown that S-layers could be over-expressed at acid stress in cell surface of *Lactobacillus casei* strain.

Keywords: s-layers; surface proteins; Lactobacillus *casei*; Nano-biotechnology.

1. Introduction

Crystalline S- layers are monomolecular arrays of protein and glycoprotein subunits present on all bacterial cell surfaces with few exceptions. It is made up of single protein with molecular weight in the range of 40-200 kda. S-layer proteins. The S-layers play an important role in the maintenance of cellular functions of bacteria. Contributing to approximately 15% of total bacterial proteins, with uniform pore size (2-8 nm) and recrystallization property (synthesis in a rate of 500 subunit per second) leads to proposing wide applications of S-layers in many fields of nano-biotechnology [1].

In industrial processing, bacteria are exposed to several stressors that may result in the physiological and biochemical changes that impact on performance. Considering that purified S-layers are stable toward non-physiological pH, radiation, temperature and other kind of stressors, a protection role against hostile factors has been proposed for these superficial structures [2]. Members of the *Lactobacillus casei* group are important in dairy processing either as adjuncts that are added to fermented milk products or as adventitious microflora. They contribute to the flavour and functionality traits of final products, such as aged cheeses and yogurt, including exhibiting probiotic activity. Responses to environmental stress in bacteria can include generalized stress responses and induction of condition-specific pathways [3]. These responses can have several impacts, besides regeneration of correctly folded functional proteins, including S-layers, which aid cell recovery and survival. This paper addresses the evidences of over-expression of S-layer proteins when *Lb. casei* strains exposed to acid stress.

2. Materials and Method

2.1. Bacterial growth condition

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An overnight culture of *Lb. casei* was inoculated in 50 ml of MRS buffered at either pH 6.5 (normal pH) or pH 4.0 (acidic pH) and incubated at 37 ℃ until the culture reached late exponential-phase growth.

2.2. Morphological tests using electron-microscopy

Bacterial cells from acidic or optimum pH were fixed with 3% glutaraldehyde (ProSciTech) fixative in 0.2 M Na-citrate phosphate buffer pH 6.5. After fixation, cells were washed and postfixed in osmium tetroside, followed by uranyl acetate. The cells were dehydrated in increasing concentrations of ethanol and acetone and subsequently embedded in Spurr's epoxy resin. Ultrathin sections were prepared and collected onto copper grids, contrasted 1% uranyl acetate and Reynolds lead citrate, then it was examined and photographed using a JEOL 1200EX transmission electron microscope. Sections were photographed onto Kodac 4489 electron image film.

2.3. Cell fractionation procedure

Cultures were harvested at 5,000 *g*, 4 ℃ for 15 min and washed with 40 mM Tris-HCl buffer, pH 7.0. Cell lysis was performed in 40 mM Tris-HCl buffer, pH 7.0 by bead beating in a Mini Bead Beater-8 (Biospecs Products Inc) six burst of 1 min at maximum speed with 2-min intervals on ice. The tubes were kept in -20 ℃ until further analysis as whole cells or followed by brief centrifugation (5,000 *g*, 15 min, 4 ℃) to settle the beads and unbroken cells as the first pellet. The cytosolic fraction was obtained by removal the second pellet at 22,000 *g* for 30 min at 4 ℃.

2.4. Extraction of S-layer proteins

S-layer protein was extracted using LiCl method, developed by Lortal (1992) [4], for that bacterial cells were grown on MRS at normal and low pH and incubated at 37 ℃ anaerobically for 18 .

Cells were harvested at exponential phase and washed twice with deionized water, then resuspended in 0.1-0.15 w/v 1 M LiCl (30 min at 4 ℃). After removing the LiCl extract by centrifugation, 10-15 mg of the moist pellet was followed by the second extraction with 5 M LiCl (1h, 4 ℃). Subsequently bacterial cell suspensions were pelleted at 30,000 *g* for 30 min. Supernatant was filtered through 0.2 μm filters and extensively dialyzed against deionized water (4 ℃) for at least 24 h, to remove any LiCl from the liquid. The liquid was then concentrated by laying the dialysis tubes on spectra/gel™ absorbent (Spectrum Laboratories, Inc., rancho Dominguez, CA, USA). This crude extract designated as concentrated S layer envelopes was analyzed and chemically characterized by SDS-PAGE, 2D electrophoresis and MALDI-TOF-TOF.

3. Results and DISCUSSION

Analysis of microscopic changes and examination of the cell morphology following growth at low pH together using electron microscopic examinations with the proteomic observations of *Lactobacillus casei* showed evidence of re-structure of cell surface at acidic conditions and indicated that the cell surface alterations are important for adapting to conditions that are normally faced during manufacturing and environmental conditions.

Growth at low pH (acidic pH 4) caused induction of several cell-surface proteins, where the pattern of proteins induced varied between strains. In some, a surface antigen (MW 42 kDa) was strongly expressed throughout growth, and this was likely to be a membrane protein-from hydrophobicity analysis and removal on centrifugation with cell debris after lysis (Fig. 1).

Nterminal sequence analysis and DNA sequencing of the cloned gene, showed that one of the proteins was a surface protein of unknown function. However, a conserved domain belonging to the NPLC_P60 superfamily, which occurs in bacterial lipoproteins, was detected: this domain occurs in amidases associated with cell wall and peptidoglycan metabolism. Other proteins were extracted into 5M LiCl, demonstrating an extracellular location in the S-layer.

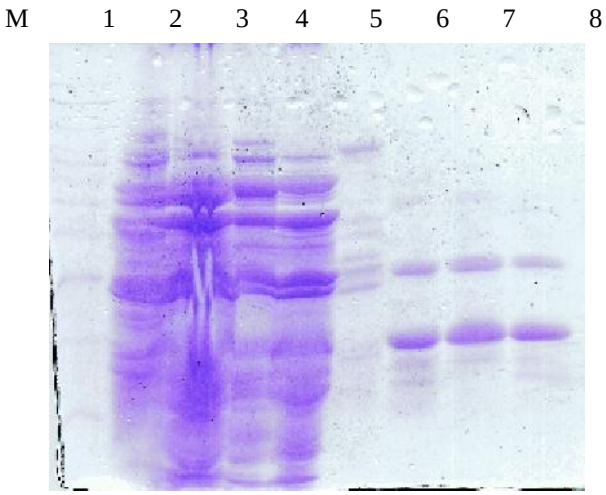

Fig. 1: Preliminary SDS-PAGE profile of protein samples from bead beaten cells of strains Bb 12, *Lb. casei* strain 46, *Lb. casei* 163 and *Lb. paracasei* 292.pellets (lines 1-4) and supernatants (lines 5-9).

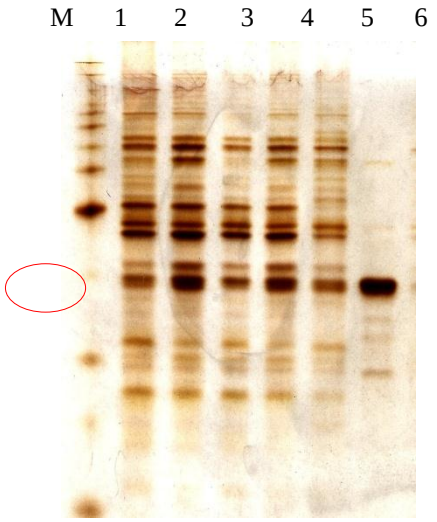

Fig. 2: SDS-PAGE profile of protein samples from whole cells and cytosolic fractions (Lines 1-4) and LiCl extracts (Lines 5-6) of *Lb. casei* exposed to normal pH (1, 3, 5) and acidic growth conditions (2, 4, 6).

Fig. 2 shows a strong band of 42 kDa over-expressed at acidic conditions in LiCl extract which is assumed to be S-layer.

2D electrophoresis analysis of LiCl extracts showed strong up-regulation of enolase- and GAPDH-like proteins, both known to bind fibronectin and contribute to immune-modulation in probiotic LAB. Heat shock failed to modulate expression of these proteins, demonstrating a specific cellular response to growth at low pH that favoured synthesis of surface proteins. Other proteins involved in pyruvate metabolism (Ldh) and GroEL also increased at the cell surface [5].

EM examination displayed some alternations in the cell surface structure under acid stress (Fig. 3).

There was a graded increase in electron density from the inside to the outside with a distinctly different cell wall interface with the environment compared to non-stressed cells, which might contribute to the protection of the bacteria.

These results show that growth conditions can significantly modify the relative expression of cell surface S-layer proteins by *Lb. casei*, and this has implications for improving probiotic activity in different strains and in dairy products.

Native S layer proteins can directly be used or they can be genetically modified as recombinant proteins especially in nanotechnology where they must be tolerant to extreme temperature, pressure and acidic conditions. Fragments of S-layer, layered on microfiltration membrane can be used in ultra filtration as they have uniform pore size. Because of small size (less than 100 nm) they are having greater surface area as compared to larger molecules hence are more reactive species, so they can be having vast applications in medicines and chemistry [6], [7]. Our research demonstrated over-expression of these high potential nanoparticles from probiotic bacterial cultures.

4. Conclusion

The cell envelope of bacteria is a shield against environmental stresses, such as acidic environment; therefore, to increase the acidic responses, bacteria can change the composition of the cell membrane or cell wall in order to decrease the permeability of protons [8]. This article has highlighted the evidence of over-producing S-layer proteins under acid stress condition. This shows the crystalline surface proteins of lactic acid bacterium *Lb. casei* can be modified by culturing at low pH. The tested strain of *L. casei* group appeared to change the composition of the cell surface in order to cope with the growth environment. Considering the regeneration property of this nano-layer at the cell surface, the probiotic beneficial bacteria can be taken into account as a high potential source of S-layer production at a large scale. The unique structural patterning of S-layers is the basis of its broad application potential in nano-biotechnology. Biochemical characterization and detailed studies of the molecular mechanism and cellular roles of these proteins are required to elucidate their role in bacterial response to acidic environments.

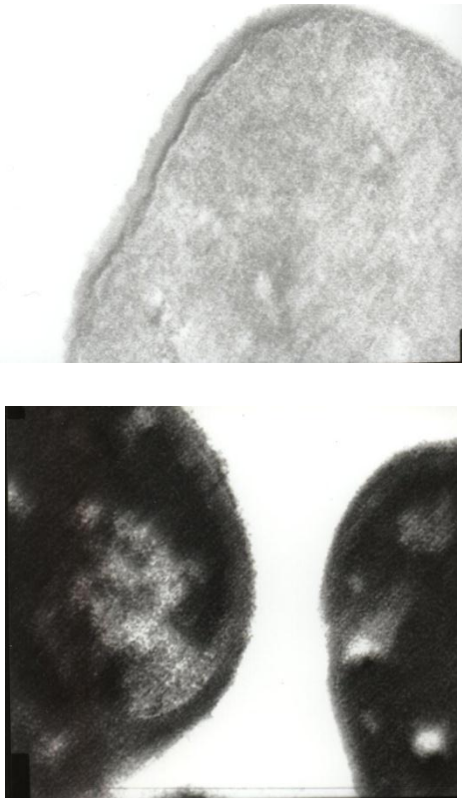

Fig. 3: Cell envelope changes as a function of pH in cells of *Lb. casei* grown in sodium citrate phosphate buffered MRS set on optimum pH 6.5 (A) or acidic pH 4.0 (B).

5. References

[1] M. S. Mulani and D. R. Majumder. "S- layer protein: tailor - made nanoparticles". *International Journal of Innovative Research in Science, Engineering and Technology*. 2 (2013) 4693-4706.

[2] V. G. Debabov. "Bacterial and Archaeal S- layer as subject of Nanobiotechnology". *Molecular Biology*, 38 (4) 482-493. Translated from Molekulyarnaya Biologiya, (2004) 578 – 591.

[3] M. P. Taranto, G. Perez-Martinez, G. f. X de Valdez. "Effect of bile acid on the cell membrane functionality of lactic acid bacteria for oral administration". *Reearch in Microbiol0gy*. (2006); 157 (8): 720-725.

[4] S. Lortal, J. van Heijenoort, K. Gruber and U. B. Sleytr. "S-layer of *Lactobacillus helveticus* ATCC 12046: Isolation, chemical characterization and re-formation after extraction with lithium chloride". *Microbiology* 1992; 138 (3): 611-618.

[5] M. Hosseini Nezhad, M. Knight and M. Britz. "Evidence of changes at membrane bound proteins during growth of *Lactobacillus casei* under acidic conditions". *Food Science and Biotechnology*. 21(2012): 253-260.

[6] U. B. Sleytr, P. Messner, D. Pum, and M. Sara. "Crystalline bacterial cell surface layers (S-layers): rom supramolecular cell structure to biomimetics and nanotechnology. *Angew. Chem. Int. Ed.* 38 (1999) 1034 – 1054.

[7] U. B. Sleytr, C. Huber, N. Ilk, D. Pum, B. Schuster and E. M. Egelseer. "S-layers as a tool kit for nanobiotechnological applications". *FEMS Microbiology Letters* 267 (2007) 131–144.

[8] M. Hosseini Nezhad, M. Hussain, and M. Britz. "Acid stress response in probiotic *Lactobacillus casei.*" *Critical Reviews in Food Science and Nutrition*. 2013. in press.