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SCREENING FOR S-LAYER PRODUCTION BY SOME LACTOBACILLI FROM HOME-MADE FERMENTED FOODS

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Abstract

Production of surface-layer proteins has been described for several species of the genus Lactobacillus. They seem to be responsible for a sum of cell wall functions like protection against physico – chemical agents, adhesion, and aggregation among others. In this study, 15 strains of lactobacilli obtained from different fermented vegetables, cereals, and dairy productswere screened for S-layer production. Five strains were able to produce S-layer proteins, with a molecular mass between 40 and 55 KDa, as shown by sodium dodecyl sulphate polyacrylamide gel electrophoresis .Four of these strains were selected to test the influence of incubation temperature on the bacterial growth and S-layer production. Although the growth at 42°C was slower than at 37°C, similar amounts of S-layer proteins were produced. The proteins were efficiently extracted with 5M LiCl, especially from the cells grown at 42°C.

Key words*:fermented foods, lactobacilli, S-layer, SDS-PAGE.*

INTRODUCTION

Lactobacilli constitute an important group of lactic acid bacteria (LAB). American Food and Drug Administration classifies them as Generally Recognized as Safe (GRAS) microorganisms. Most species are found in natural habitats rich in carbohydrates, but also in the gastrointestinal tract of human and animal organisms (Slover and Danzige, 2008).
Home-made fermented foods have been shown to be rich sources for manylactic acid bacteria, including lactobacilii, with potential biotechnological application (Wouters et al. 2013, Grosu-Tudor et al. 2014). In Romania, such foods are still produced and consumed at a significant extent. Besides the fermented dairy products, Romanians use fermentation as a common method for cereals, vegetables, and fruits conservation, especially for the cold seasons, when fresh vegetables are hard to find.
Lactobacilli are commonly used as probiotics, therefore, they have to withstand different types of stress like high temperatures during product fabrication or harsh conditions inside gastrointestinal tract (low pH and bile salts, Champagne et al., 2002). This is possible because many *Lactobacillus* strains had developed a mechanism of defense making them suitable to be used as probiotics or in biotechnological industry (Tuohy et al., 2003).
Many *Lactobacillus* species possess at their surfaces an array of single, identical proteins, known as S-layer. This is a bi-dimensional crystalline structure of one, two, three, four or six (glycol-) protein subunits with oblique, tetragonal or hexagonal symmetry (Sleytr, 1997).In the case of lactobacilli, these subunits can be easily disintegrated using denaturing agents such as lithium chloride (LiCl), guanidine hydrochloride (GHCl), and re-assembled due to the non-covalent links (Sara and Sleytr, 2000). The S-layers of *Lactobacillus* species are formed by proteins with a molecular weight of 25 – 71 KDa (Åvall-Jääskeläinen and Palva, 2005), the smallest ones when compared to those from other bacteria, which can reach a molecular weight up to 200 KDa (Sleytr and Messner, 1983).

During the past years, S-layer has been characterized for several species of genus *Lactobacillus* and some features and functions have been assigned to it. These include protection role against physico-chemical factors (Engelhardt and Peters, 1998; Chami et al., 1997), aggregation, and adhesion (Åvall-Jääskeläinen and Palva, 2005) among others.
The aim of the present study was to select S-layer producing strains among some lactobacilli isolated from home-made fermented foods and to study the influence of the incubation temperature on the bacterial growth and S-layer production.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The LAB strains used in this study were isolated from Romanian artisan dairy products (9 strains, namely *Lb. brevis*46.5, *Lb. fermentum*419, *Lb. helveticus*34.9, *Lb. plantarum*6.10, 7.5, 16.1, 26.1, 35.1, 44.2; Zamfir et al. 2006,Grosu-Tudor et al. 2013), fermented vegetables (5 strains, namely *Lb. brevis*403, 530, *Lb. parabrevis*196, *Lb. plantarum*198, 619; Wouters et al. 2013), and fermented cereals (one strain, namely *Lb. oris*P49; Grosu-Tudor et al. 2014). All strains were long-termpreserved at -80°C in MRS medium in the presence of 25% (v/v) glycerol as cryo-protectant. Fresh cultures from the freeze-dried stocks were obtained by transferring twice each strain (2% v/v inoculum) in MRS medium followed by 24 h incubation at 37°C.

Screening for S-layer formation by SDS-PAGE analysis

One milliliter of an overnight culture (OD_{600nm}: 1.5) from each of the 15 LAB strains was centrifuged (10 000 x *g*, 10 min, 4°C) and the deposits were washed with distilled water and centrifuged again. The sediments were resuspended in 50 µl Laemli sample buffer and warmed up at 95°C for 5 minutes. After cooling, the suspensions were centrifuged again applying the same parameters and checked for S-layer proteins production using Sodium Dodecyl Sulfate Polyacrylamide Gel electrophoresis(SDS-PAGE). The gels were prepared according to Laemmli (1970) method. Therefore, 10% (w/v) polyacrylamide running gel, and 4% (w/v) polyacrylamide stacking gel were used in a BiometraMinigel Twin (Biometra, Germany). Electrophoresis was conducted at a constant intensity of 40 mA in the stacking gel and 60 mA in the migration gel, respectively. Broad range protein molecular weight (MW) marker (10-225 KDa, Promega, USA) was used as reference. Gels were stained with Coomasie Brilliant BlueR250 (Carl Roth GmbH, Germany) to visualize the bands.

Growth and S-layer production by *Lactobacillus* strains

The effect of incubation temperature on the cell growth and S-layer proteins production was studied in *Lb. parabrevis*196, *Lb. brevis*403, *Lb. brevis*530 and *Lb. helveticus*34.9.
Active cultures of the selected strains were grown at two different temperatures (37°C, and 42°C) for 24h. Cell growth was determined by measuring the optical density at 600 nm(OD_{600nm}), pH and cell count (CFU/ml) using solid MRS medium (MRS supplemented with 1.5% (w/v) agar).

Extraction of S-layer proteins

Cells suspensions were obtained by inoculating 100 ml glass bottles containing 50 ml MRS with each strain(2% inoculation rate). The bottles were incubated at 37°C, and 42°C for 24 hours.The obtained bacterial biomass was separated by centrifugation as described before, washed twice with 10 ml of phosphate – buffered saline (PBS), pH = 7.4. The cell pellets were then resuspended in 5 ml of PBS and an equal volume of 5M LiCl was added in order to extract the S-layer proteins. After 2 hours at 37°C and occasionally shaking, the samples were centrifuged and the supernatants with the putative S-layer proteins were transferred to Vivaspin 6 ultrafiltration modules with a 10-kDa MM cut-off (Sartorius Stedim Biotech, Goettingen, Germany).Milli Q water was added over the retentate to a final volume of 5 ml and centrifuged again. Milli Q water was finally added over the retentate to a volume of 3 ml and transferred to a falcon tube. After a final centrifugation, the sediment representing the presumptive S – layer was resuspended in 60 µl Laemmli buffer(Laemmli,

1970). Both the sediment and supernatant were analyzed by SDS – PAGE.

RESULTS AND DISCUSSIONS

Detection of S-layer proteins

A total of 15 lactobacilli were evaluated for their ability to produce S-layer proteins. SDS-PAGE of whole protein extracts from fivestrains, namely *Lb. brevis* 403, 530 and *Lb. parabrevis* 196, isolated from fermented vegetables, and *Lb. helveticus* 34.9 and *Lb. brevis* 46.5 isolated from fermented dairy products, showed intense protein bands with molecular weightsin range of 40 - 55 kDa(figure 1)corresponding, most likely, to S-layer proteins (according to Åvall-Jääskeläinen and Palva, 2005). These protein bands are in the range of the molecular weights described for S-layer proteins from lactobacilli according to these authors. In addition, we observed variations of the molecular weightsbetween the species, but also between the particular strains of the same species (figure 1).

We observed intense formation of presumptive cell surface proteins in*Lb. brevis* 403 and 530, *Lb. parabrevis* 196, and *Lb. helveticus* 34.9. Consequently, these strains were selected for further studies.

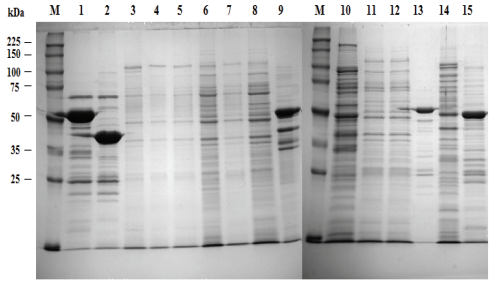

Figure 1. SDS-PAGE profiles of whole cell proteins from 15 LAB strains. Lanes: M: broad range protein molecular weight marker (Promega); 1: *Lb. brevis* 403; 2: *Lb. brevis* 530; 3: *Lb. plantarum* 619; 4: *Lb. plantarum*6.10; 5. *Lb. plantarum*7.5; 6: *Lb. plantarum* 16.1; 7: *Lb. plantarum* 35.1; 8: *Lb. plantarum* 44.2; 9: *Lb. helveticus* 34.9; 10: *Lb. fermentum* 419; 11: *Lb. plantarum* 26.1; 12: *Lb. oris* P49; 13.*Lb. parabrevis* 196; 14: *Lb. plantarum* 198; 15: *Lb. brevis* 46.5.

Growth of the selected strains

After 24 hours of incubation, lactobacilli cells belonging to the four selectedstrains grew well at 37°C reachingcell counts between $4.7x10^8$ and $5.6x10^9$ CFU/ml. The pH values dropped from 6.2 to 3.88 - 4.35 (Table 1). In the case of incubation at 42°C lactobacilli cultures obtained in the experimental conditions displayed a poor development comparing to incubation at 37°C. Cultures belonging to the strains*Lb. helveticus* 34.9 and *Lb. brevis* 530 displayed a mildlyreduced development, with a difference in cell counts of only 1 log CFU/ml comparing to the cell counts obtained at 37°C. These results show good tolerance to higher temperature for the strains *Lb. helveticus* 34.9 and *Lb. brevis* 530. Poor tolerance to higher temperature was marked for *Lb. brevis*403 with a significant decrease of cell counts (3 logs CFU/ml lower comparing to the cell counts obtained at 37°C). The poorest growth was observed for *Lb. parabrevis* 196 with a difference in cell counts of 5 logs CFU/ml comparing to the culture obtained at 37°C. These results indicate important sensibility of thisstrainto temperatures above 37°C.

Table1. Growth parameters of the selected bacterial strains

Temperature	*Strain*	*pH*	*CFU/ml*
37°C	FV 196	4.35	5.6×10^9
	FV 403	4.29	2.9×10^9
	FV 530	4.33	4.7×10^8
	RFF 34.9	3.88	1.7×10^9
42°C	FV 196	5.37	2.2×10^4
	FV 403	4.80	3.1×10^6
	FV 530	4.75	3.6×10^7
	RFF 34.9	4.11	3.3×10^8

Extraction of S-layer proteins

An intense band corresponding to about 40-55KDa, most likely the S-layer proteins, was observed for all tested strains, both grown at 37°C and 42°C (figure 2). Furthermore, after incubation of lactobacilli at 42°C, the bands corresponding to S-layer are similar to those obtained after incubation at 37°C. This points out that these four strains have the capability to produce S-layer both under the optimal cultivation temperature and under temperature stress conditions.

After the 5M LiCl treatment, the putative S-layer proteins were efficiently extracted from most of the strains. With a few exceptions (especially for strains grown at 37°C), in the cells subjected to LiCl extraction (for 2h), the band corresponding to the S-layer proteins was

not detected on SDS-PAGE. The corresponding band was, on the other hand, the major band inthe sediment recovered from the LiCl treatment and ultrafiltration. In some cases, the same bandwas also detected as the major band in the supernatant recovered from the treatment.

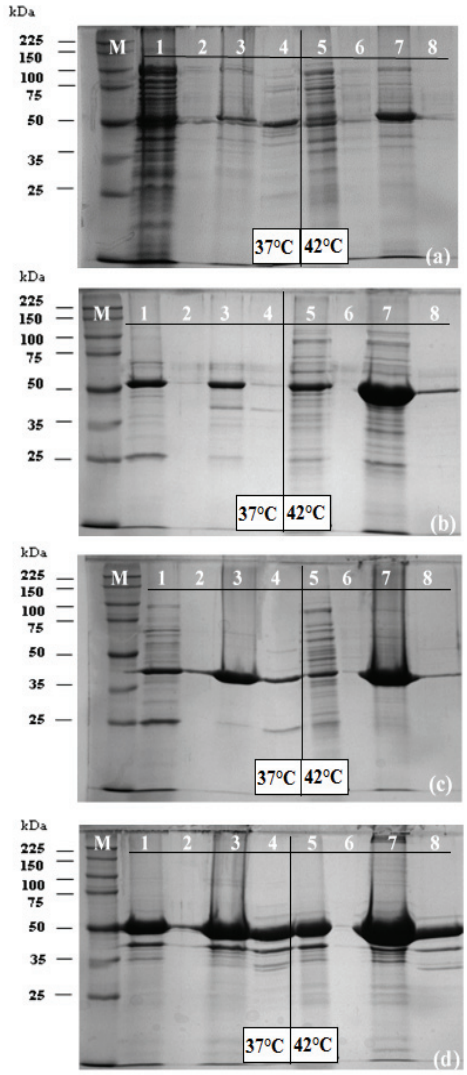

Figure 2. SDS-PAGE analysis of S-layer proteins of (a) *Lb. parabrevis* 196; (b) *Lb. brevis* 403; (c) *Lb. brevis* 530; (d) *Lb. helveticus* 34.9.
Lanes: M: Molecular weight marker; 1 and 5: cells before 5M LiCl treatment; 2 and 6: cells after 5M LiCl treatment (2h); 3 and 7: S-layer proteins extracted with 5M LiCl; 4 and 8: supernatant recovered after 5M LiCl extraction.

Overall, a better extraction was obtained for the strains cultivated at 42°C than 37°C. A possible explanation can be the ratio between the volume of 5M LiCl and the cell density in the suspensions used for extraction (significantly lower for the cultures obtained at 42°C compared with the ones at 37°C), but other processes might be responsible, too. Further studies will be made.

CONCLUSIONS

All four strains selected in our study showed the ability to produce cell surface-associated proteins with molecular weights in the range of 40-55 KDa. The proteins can be efficiently extracted using 5M LiCl. These proteins are produced in similar amounts when cells were grown at 37°C and 42°C and are, most likely, S-layer proteins, offering potential bio(nano)-technological applications to the producing strains.

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