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## Effect of Environmental Stresses on S-Layer Production in *Lactobacillus acidophilus* ATCC 4356

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### 1. Introduction

The gastrointestinal tract (GIT) is the organ with the largest surface area in the human body, having in an adult between 150 and 200 m<sup>2</sup> (Holzapfel et al., 1998). Interesting in this context is the fact that a huge number of microorganisms live and interact with the host in the stomach and gut. The GIT of an adult human is estimated to harbour about 10<sup>13</sup>-10<sup>14</sup> viable bacteria, i.e. 10 times the total number of eukaryotic cells in all tissues of man's body (Holzapfel et al., 1998; Velez et al., 2007). In the gastrointestinal tract, the bacteria are affected both by the physiological conditions (such as low pH, bile salt and enzymes) and by other microorganisms which exist in the GIT. Because of the presence of enzymes, salts and acids in the gastric juice, the environmental conditions in the stomach are destructive to a number of microorganisms (Holzapfel et al., 1998). The microbial community in the gastrointestinal tract is complex and consists of several hundred species, of which lactic acid bacteria constitute a minor proportion. Lactic acid bacteria (LAB) are Gram-positive bacteria which excrete lactic acid as a main fermentation product into the medium. This biochemical definition associates lactic acid bacteria of different phylogenetic branches of bacterial evolution: the "low GC" taxa, e.g. *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*, and the "high GC" genus *Bifidobacterium*. Species of these genera can be found in gastrointestinal tract of man and animal (Klaenhammer et al., 2005; Klein et al., 1998; Mathur & Singh, 2005). They have also been involved since time immemorial in food processing and food preservation, and are applied in particular for the manufacturing of dairy products, fermented meat, vegetables, bread and ensilage (Pouwels et al., 1998). Several lactic acid bacteria have the potential to promote the health of the host or to prevent and treat diseases. Such bacteria are referred to as probiotic, meaning 'for life' (Latin *pro*=for and *biotic*=life) (Herich & Levkut, 2002; Marteau & Rambaud, 2002; Mercenier et al., 2002; Ouwehand et al., 2003). The strains of LAB used as probiotics usually belong to species of the genera *Lactobacillus*, *Enterococcus*, and *Bifidobacterium* (Klein et al., 1998). The effects of probiotic microorganisms on the host have been discussed extensively in the literature. It has been proposed that probiotics possess several advantageous properties, such as antagonistic actions, production of antimicrobial substances, modulation of immune responses, and impact on the metabolic activities of the gut (Marteau & Rambaud, 2002;

O'Toole & Cooney, 2008; Sanders & Klaenhammer, 2001). *In vitro* and animal studies have further shown inhibitory effects of probiotic bacteria to be mediated by their interference with the adhesion of gastrointestinal pathogens or with toxins produced by the pathogenic microorganisms (O'Toole & Cooney, 2008; Sullivan & Nord, 2002). However, beneficial influence of probiotics has been demonstrated in clinical studies (De Roos & Katan, 2000; Kos et al., 2008; Mishra et al., 2008; O'Toole & Cooney, 2008; Park et al., 2008; Resta-Lenert & Barrett, 2003; Sanders & Klaenhammer, 2001). Some possible health effects include immune system stimulation, cholesterol lowering, prevention and treatment of diarrhea, prevention of cancer recurrence, improvement in lactose intolerance, and reduction of allergy (De Roos & Katan, 2000; Mercenier et al., 2002; Reid, 1999; Sanders & Klaenhammer, 2001). The most common probiotic strains belong to two genera, *Lactobacillus* and *Bifidobacterium* (Sanders & Klaenhammer, 2001; Sullivan & Nord, 2002). Some strains of *Lactobacillus* are found as natural commensals of the GIT, the oral cavity and the female uro-genital tract of animals and humans (Pouwels et al., 1998). Also, Lactobacilli are of considerable technological and commercial importance because of their role in the manufacturing and preservation of many fermented food products (Schar-Zammaretti et al., 2005). *Lactobacillus acidophilus* is one of the major species of the genus *Lactobacillus* found in human and animal intestines (Frece et al., 2005). Nowadays, *Lactobacillus acidophilus* strains are commonly used as probiotic (Sanders & Klaenhammer, 2001), and marketed as capsules, powders, enriched yogurts, yogurt-like products, and milk (De Roos & Katan, 2000). Adhesion to intestinal epithelial cells is an important prerequisite for colonization of probiotic strains in the gastrointestinal tract (Altermann et al., 2004; Kos et al., 2003). This is mediated either non-specifically by physico-chemical factors (such as hydrophobicity) or, specifically, by adhesive bacterial surface molecules and epithelial receptor molecules (such as S-layer, fibronectin and mucin-binding proteins) (Holzapfel et al., 1998).

## 2. Probiotics and stress

In the intestinal tracts of mammals and avians, species of the genera *Lactobacillus*, *Enterococcus*, *Streptococcus* and *Bifidobacterium* are the dominant indigenous lactic microbiota. Commonly recovered *Lactobacillus* isolates from the human gastrointestinal tract include *L. acidophilus*, *L. salivarius*, *L. casei*, *L. plantarum*, *L. fermentum*, *L. brevis* and *L. reuteri*. The findings that colonization by lactobacilli and other lactic acid bacteria improves infection resistance of the host have led to the production and consumption of probiotics (Kosin & Rakshit, 2006). The probiotics must resist multiple stresses including the GIT conditions and food processing. An important attribute for certain probiotic bacteria functions are survival and growth in the intestinal tract (Sanders & Klaenhammer, 2001). The gastric juice contains hydrochloric acid, which induces an extremely low pH. The fasting pH in the stomach is approximately 1.5, while the pH increases to between 3.0 and 5.0 when food is eaten (Cotter & Hill, 2003). In the intestine, the conditions are less extreme because of in the intestine pH is higher than the stomach, but the bacteria still have to endure bile and pancreatic juices (intestinal fluids). Other factors affecting microbial life in this environment are immunoglobulins, defensins, a continuously regenerating epithelium, peristaltic movement of intestinal content, and a viscous mucus layer (Dunne et al., 2001; Tannock et al., 1999). Moreover, probiotics used in food technology are exposed to various adverse conditions during processing, such as temperature changes, acidity, osmotic and oxidative stress (Kosin & Rakshit, 2006). Such stresses may reduce the physiological activity

of the cells and readily kill the cells. Once the cells have survived the stresses, they can colonize and grow to adequate numbers to provide the beneficial effect to the host. Survival mechanisms exhibited by bacteria when confronted with stress are generally referred to as the stress response (De Angelis & Gobbetti, 2004; Jan et al., 2001; Kim et al., 2001). However, the ability to survive passage through the intestinal tract and potentially establish residence there is considered as an important feature. The degree of retention is likely dependent on the ability of the bacteria to interact with eukaryotic cell surfaces or with the mucosal layer surrounding these cells (Altermann et al., 2004). Several factors contribute to the interaction of Lactobacilli with the host tissues, such as cell surface hydrophobicity (Vadillo-Rodriguez et al., 2005; Van der Mei et al., 2003), autoaggregation (Kos et al., 2003), lipoteichoic acids (Granato et al., 1999) and external surface proteins (such as S-layer, fibronectin and mucin-binding proteins) (Altermann et al., 2004; Avall-Jaaskelainen & Palva, 2005; Frece et al., 2005; Kos et al., 2003; Kosin & Rakshit, 2006; Pouwels et al., 1998; Velez et al., 2007; Ventura et al., 2002). Surface layer (S-layer) has been identified as the outermost structure of cell envelope in numerous organisms from the domains Bacteria (in both Gram-positive and Gram-negative Eubacteria) and Archaea (Debabov, 2004; Sara & Sleytr, 2000). S-layer proteins are non-covalently bound to the cell wall and assemble into surface layers with high degrees of positional order often completely covering the cell wall, and can be disintegrated into monomers by denaturing agents such as urea or guanidine hydrochloride (Avall-Jaaskelainen & Palva, 2005; Engelhardt & Peters, 1998; Lortal et al., 1992; Sleytr et al., 2001). The S-layer has been detected in a few species of the genus *Lactobacillus* (Avall-Jaaskelainen & Palva, 2005). Lactobacilli surface layer proteins (S-layers) are generally monomolecular crystalline arrays exhibiting a morphologically similar, oblique lattice structure and representing 10-15% of the total protein content of the bacterial cell wall (Avall-Jaaskelainen & Palva, 2005; Jakava-Viljanen et al., 2002). Several reports have appeared in which functions of S-layer are described or assumed (Boot et al., 1993; Jakava-Viljanen et al., 2002; Sara & Sleytr, 2000). However, no general function has been identified for S-layer proteins, but several lactobacillar S-layers have been identified as putative adhesions with affinity for intestinal epithelial cells, extracellular matrices and/ or to lipoteichoic acid (LTA) of other bacterial species (Avall-Jaaskelainen & Palva, 2005; Buck et al., 2005; Frece et al., 2005; Garrote et al., 2004; Hynonen et al., 2002; Kos et al., 2003; Velez et al., 2007; Vidgren et al., 1992). Of the various roles proposed for the bacterial S-layer, it is a protective sheath against hostile environment (Avall-Jaaskelainen & Palva, 2005; Frece et al., 2005; Khaleghi et al., 2010, 2011; Kos et al., 2003; Schar-Zammaretti et al., 2005). Adhesive S-layers have a role in inhibition of adhesiveness of pathogenic bacteria and thus can contribute to probiotic effects of lactobacilli. To date, several lactobacilli S-layer protein-encoding genes have been cloned, sequenced and deposited in GenBank (Avall-Jaaskelainen & Palva, 2005; Velez et al., 2007). The presence of multiple S-layer protein genes seems to be quite common for bacteria (Avall-Jaaskelainen & Palva, 2005; Ben-Jacob et al., 2000; Boot & Pouwels, 1996; Jakava-Viljanen et al., 2002). Multiple S-layer genes have been identified in the genomes of *L. acidophilus*, *L. amylovorus*, *L. gallinarum*, *L. crispatus*, *L. brevis*, *L. gasseri* and *L. johnsonii* (Avall-Jaaskelainen & Palva, 2005; Boot & Pouwels, 1996; Jakava-Viljanen et al., 2002; Ventura et al., 2002). There is also increasing evidence that S-layer-carrying bacteria may use S-layer variation, by expressing alternative S-layer protein genes, for adaptation to different stress factors such as the immune response of the host for pathogens and drastic changes in the environmental conditions for nonpathogens (Boot & Pouwels, 1996; Frece et al., 2005; Jakava-Viljanen et al., 2002; Pouwels et al., 1998; Sara & Sleytr, 2000). According to Pouwels'

study (1998), phase variation or antigenic variation, as a result of inversion of the *slp* segment, might enable *Lactobacillus acidophilus* bacteria to better adhere to specific regions of the mucosa. Variation in S-layer gene expression as a response to environmental changes has also been described in *Geobacillus stearothermophilus*, *Bacillus anthracis*, and *Campylobacter fetus* (Boot & Pouwels, 1996; Mignot et al., 2002).

There is some evidence that the surface properties of microorganisms are dependent on the growth conditions and the composition of the fermentation medium (Schar-Zammaretti et al., 2005; Waar et al., 2002; Dufrene & Rouxhet, 1996; Millsap et al., 1997). Schar-Zammaretti (2005) suggested that S-layer protein is preferentially expressed under different fermentation media. Furthermore, it has been shown that the S-layer production is changed with the change in medium (such as bile salt, penicillin G) (Khaleghi et al., 2010, 2011).

The aim of this study was to gain more knowledge about S-layer production and *slpA* gene expression in different growth conditions (pH and temperature) in *Lactobacillus acidophilus* ATCC 4356. Moreover, the reassembly of S-layer subunits was studied under these stresses.

## 2.1 S-layer protein, *slpA* expression and stresses

The S-layer proteins of Lactobacilli are relatively small, 25 kDa to 71 kDa in size (Avall-Jaaskelainen & Palva, 2005), whereas the molecular masses of S-layers in other bacterial species range up to 200 kDa (Sara & Sleytr, 2000). The Lactobacillar S-layers are highly basic proteins with calculated isoelectric point values ranging from 9.35 to 10.4. Yet, all the other S-layer proteins characterized are weakly acidic (Avall-Jaaskelainen & Palva, 2005). Evidence shows that the S-layer protein is important for *Lactobacillus acidophilus* (Frece et al., 2005; Khaleghi et al., 2010, 2011; Kim et al., 2001; Toba et al., 1995).

*Lactobacillus acidophilus* strains isolated from humans and animals, which belong to DNA homology groups A, are reported to possess a *slpA* gene, while the strains which belong to the DNA homology groups B appear not to have an *slpA* gene (Boot et al., 1993). According to Boots' study (1995, 1996, 1996c), there are two S-layer protein encoding genes, *slpA* and *slpB*, in *Lactobacillus acidophilus* ATCC 4356; of the two, *slpA* is active and *slpB* is silent in normal growth conditions. The two S-protein genes are located 6-kb apart on the chromosome, in a reverse orientation relative to each other. The *slpA* gene is interchanged with the *slpB* gene through inversion of a chromosomal fragment in a fraction of a culture (0.3% of the cell growth under laboratory conditions). Thus, it seems that S-layer variation of non-pathogenic lactobacilli has the same function as S-layer variation for pathogenic organisms such as *Campylobacter fetus*, namely to circumvent an immune response of the infected host (Boot & Pouwels, 1996; Boot et al., 1995, 1996b, 1996c).

Therefore, the present study investigated the effects of some stresses on the S-layer production, reassembly of S-layer subunits, and *slpA* gene expression.

### 2.1.1 Materials and methods

#### 2.1.1.1 Stress conditions

To study the effect of heat and pH stresses on S-layer production and *slpA* gene expression, the *Lactobacillus acidophilus* ATCC 4356 was cultivated in MRS broth (Merck) for heat stress (30, 45, 50 and 55 °C) (Kim et al., 2001); for pH stress, MRS broth was adjusted to pH 3-7 (adjusted with HCl and NaOH) (Jan et al., 2001; Lorca et al., 1998). The pH and temperature



of the control culture (MRS broth) were 6.5 and 37 °C, respectively, according to manufacturer's recommendation and as described previously (Boot et al., 1993; Silva et al., 2005; Smit et al., 2001).

### 2.1.1.2 Isolation of S-layer

*Lactobacillus acidophilus* ATCC 4356 was obtained from the Germany Type Culture Collection and was cultivated anaerobically (in jar with Anaerocult A-strip, Merck) in MRS broth (Merck) at 37 °C (Boot et al., 1993; Smit et al., 2001). *Lactobacillus casei* ATCC 393 was used as negative control for isolation of S-layer.

For isolation of S-layer and total RNA, the recommended optical density is 0.7 at 695 nm (the end of log phase) (Boot et al., 1993; Smit et al., 2001) and 0.2-0.4 at 600 nm (mid-log phase), respectively (Boot et al., 1995). But in this study, we compared *slpA* gene expression and S-layer production at the same time. In addition, the S-layer production was compared in  $OD_{600} = 0.4$  and  $OD_{600} = 0.7$ . Therefore, S-layer protein and total RNA were isolated in exponentially growing cells ( $OD_{600} \approx 0.4$ ). Also, S-layer protein was extracted at  $OD_{600} = 0.7$ . For extraction of S-layer, *Lactobacillus acidophilus* ATCC 4356 was cultivated anaerobically in MRS broth at 37 °C. In general, 100 ml of pre-warmed MRS medium (under stress conditions and control) was inoculated 1: 100 (v/v) with an overnight culture and cultivated until the optical density at 600 nm reached 0.4 and 0.7. Cells were harvested by centrifugation at  $15000 \times g$  for 15 min at 4 °C. The cells were washed twice with 100 ml of ice-cold water. The cell pellet was extracted with 0.1 volume of 4 M guanidine hydrochloride (pH 7) for one hour at 37 °C and centrifuged at  $18000 \times g$  for 15 min. The supernatant, containing S-layer protein monomers, was dialyzed against water at 4 °C for 16-24 h (Boot et al., 1993). The dialyzed extracts were analyzed by SDS-PAGE (Smit et al., 2001). SDS-PAGE of protein samples was carried out using Precision Plus Protein Standard [low molecular weight marker (10-250 kDa) - Biorad]. The samples were run on 12% polyacrylamide gel at 100 V. Protein bands were visualized by Coomassie blue staining. Protein concentration was determined according to Bradford's method (Bradford, 1976). For normalization of the measured absorption values, BSA (Merck) was used.

### 2.1.1.3 Reassembly of S-layer monomers and transmission electron microscopy (TEM)

S-layer self-assembly subunits were studied by the negative staining technique. To prepare the TEM samples, several droplets of the dialyzed protein were pipetted onto the carbon-coated grids and left for 1-16 h to immobilize the proteinaceous structures. Samples were washed once with distilled water and then stained with 2% uranyl acetate for two minutes (Avall-Jaaskelainen et al., 2002; Smit et al., 2001). The grids were dried by nitrogen flow and studied by Zeiss/CEM 902 a transmission electron microscopy (TEM) at 60 kV.

### 2.1.1.4 Isolation of total RNA

For isolation of total RNA, *L. acidophilus* ATCC 4356 cells were grown in MRS broth (under stress conditions and control) until they reached an optical density of approximately  $OD_{600} = 0.4$ . The cells were subsequently harvested by centrifugation ( $5000 \times g$  for 10 min at 4°C) and washed with an ice-cold TE buffer (Boot et al., 1995). The total RNA was isolated using a protective RNeasy Minikit (Qiagen) according to the manufacturer's recommendations, and then treated with deoxyribonuclease I (DNase I, RNase-free; Fermentas) at 37°C for 30 min according to manufacturer's recommendations.

### 2.1.1.5 RT-PCR

The reverse transcription (RT) of the RNA samples was performed with 150 ng of total RNA and 0.5  $\mu$ g of Oligo dT primer using a First Strand cDNA Synthesis kit (Fermentas) at 42°C for 60 min, as recommended by the manufacturer. Forward and reverse primers were designed for the *slpA* gene of *Lactobacillus acidophilus* ATCC 4356 as follows: *slpA* forward (5'-TGG CCG TTC TTG AAT GTG TA-3') and *slpA* reverse (5'-ACA TCA ACG CTG CAA ACA TC-3'). These primers generated a 154 bp PCR product in the PCR reaction.

16S rRNA was used as the internal control gene based on previously reported primers (Trotha et al., 2001) that generate a 370 bp PCR product.

The final volume of the PCR reaction was 25  $\mu$ l with the following components: 1  $\mu$ l cDNA ( $\approx$  7.5 ng), 1  $\mu$ l (100 pmol/ $\mu$ l) from each primer, 0.5  $\mu$ l dNTPs mix, 0.5  $\mu$ l MgCl<sub>2</sub>, and 0.25  $\mu$ l (5 U/ $\mu$ l) *Taq* DNA polymerase (Fermentas). The Mastercycler (Eppendorf) was programmed as follows: initial denaturation for 5 min at 94°C; 30 cycles at 94°C for 45 sec, 54°C for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 8 min. The PCR products (and 50 bp DNA ladder, Fermentas) were separated on a 1% agarose gel and visualized by ethidium bromide staining.

### 2.1.1.6 Statistical assessment

All the experiments and measurements were repeated at least three times. All the statistical analyses were performed using SPSS and Excel 2003 software. All the experimental results were analyzed using mean descriptive statistics, the correlation coefficient, and a single-factorial analysis of variance. A value of  $P < 0.05$  was regarded as statistically significant.

## 2.1.2 Results

The growth curve of *Lactobacillus acidophilus* ATCC 4356 showed that it took approximately 8 h to reach OD<sub>600</sub> = 0.4 and approximately 14 h to reach OD<sub>600</sub> = 0.7 (Data not shown). It is important to know that *Lactobacillus acidophilus* ATCC 4356 was live and grew after 14 h under stress conditions. However, the results indicated that *Lactobacillus acidophilus* was not live in 50 °C, 55 °C, pH 3 and 4 after 14 h (Table 1). In this study, pH 5, 6, and 7, as well as temperatures 30 °C and 45 °C were chosen as the stress conditions.

Culture condition	Cell count (CFU/ml)
Control*	$9.6 \times 10^9$
pH 3	No growth
pH 4	No growth
pH 5	$8.38 \times 10^6$
pH 6	$7.24 \times 10^8$
pH 7	$9.03 \times 10^9$
30 °C	$8.98 \times 10^8$
45 °C	$3.63 \times 10^7$
50 °C	No growth
55 °C	No growth

\*pH 6.5 & 37 °C.

Table 1. Viable cell counts of *Lactobacillus acidophilus* ATCC 4356 under stress conditions and the control after 14 h of inoculation.

The surface proteins of *Lactobacillus acidophilus* ATCC 4356 were extracted by treatment of whole cells with 4M guanidine hydrochloride, and analyzed by SDS-PAGE. One dominant band of 43-46 kDa, which is known as the S-protein (Boot et al., 1993; Smit et al., 2001) and a few faint bands were visible on gel (Fig. 1b). In the mid-log-phase ( $OD_{600}=0.4$ ), S-protein production was low in control group, so the 43-46 kDa band was not seen on gel clearly (Fig. 1a, c; lane 1). However, in the control group with  $OD_{600}=0.7$ , a 43-46 kDa band was visible on SDS-PAGE gel (Fig. 1b, d; lane 1).

Under stress conditions ( $OD_{600}=0.4$  & 0.7), S-protein band was visible and the band became sharper in pH 5 and 45 °C (Fig. 1). It seemed that S-protein bands were not different in pH 6, 7 and the control (Fig. 1b).

No protein bands (43-46 kDa) were visible on SDS-PAGE gel from isolated protein of *Lactobacillus casei* ATCC 393.

To determine the total proteins, the Bradford method was used. The total proteins were compared between the control and the group under stress conditions. In the case of pH 5 and 45 °C, total protein content was higher than others (Fig. 2). Moreover, the total protein production level was lowest at 30 °C ( $p < 0.001$ ). In pH 6, 7 and control, the protein content was almost similar. After comparing the results of total protein analysis under stress and control conditions, the range of difference in protein content was similar in  $OD_{600} = 0.4$  and 0.7.

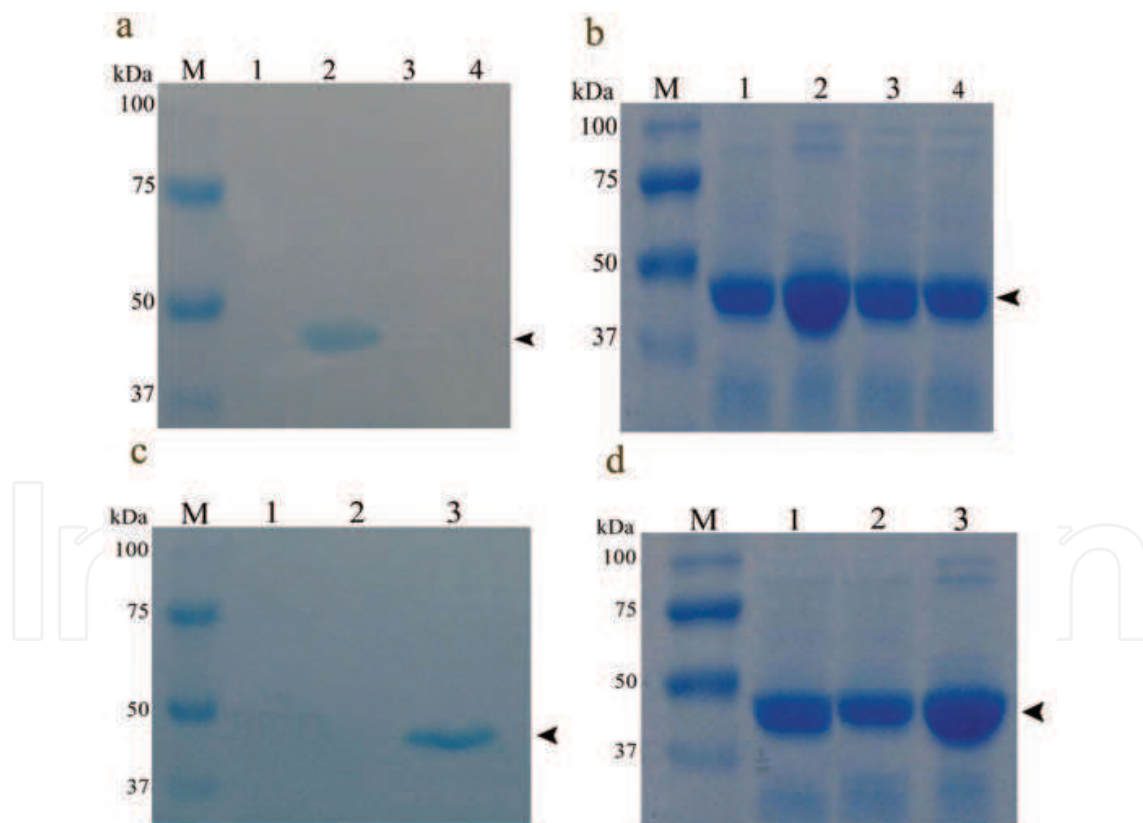


Fig. 1. SDS-PAGE gel (12% polyacrylamide) analysis of isolated surface proteins of *Lactobacillus acidophilus* ATCC 4356 at: **a)** mid-log phase ( $OD_{600}= 0.4$ ) and **b)** exponential growth phase ( $OD_{600}= 0.7$ ) under pH stress (lane 1, control; lane 2, pH 5; lane 3, pH 6; lane 4, pH 7). **c)** mid-log phase ( $OD_{600}= 0.4$ ) and **d)** exponential growth phase ( $OD_{600}= 0.7$ ) under heat stress (lane 1, control; lane 2, 30 °C; lane 3, 45 °C). M, Protein marker.



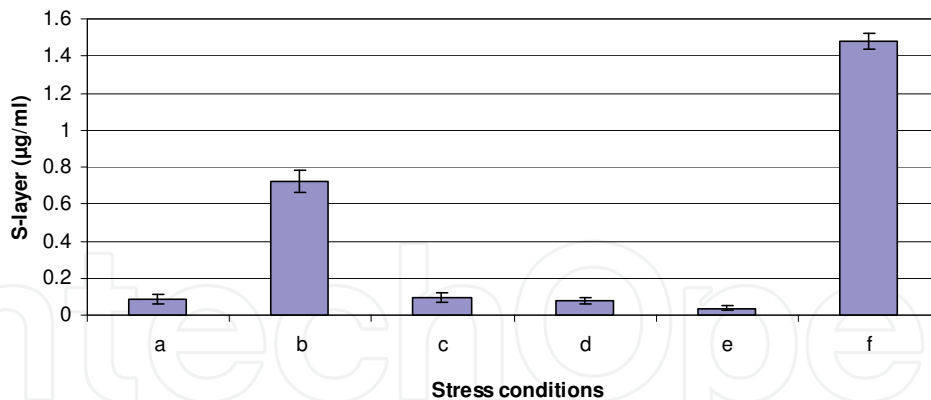


Fig. 2. Extracted surface proteins (extracted in mid-log phase) were compared in control and the group under stress conditions by Bradford method. (a) Control (pH 6.5 & 37 °C); (b) pH 5; (c) pH 6; (d) pH 7; (e) 30 °C; (f) 45 °C. Error bars represent standard deviations of the mean values of results from three independent experiments.

To assess the change in S-layer protein content of the cell wall under stress conditions by transmission electron microscopy (TEM), we chose 45 °C in which S-layer production was highest (Fig. 2). In the electron microscopy study, the presence of the S-layer on the outer surface of *Lactobacillus acidophilus* ATCC 4356 was clearly demonstrated (Fig. 3). In particular, the bacterial surface was completely covered with an S-layer in the control (Fig. 3a, b), but an excess of S-layer protein was found at the both ends of the bacterial cell under stress condition (Fig. 3c, d).

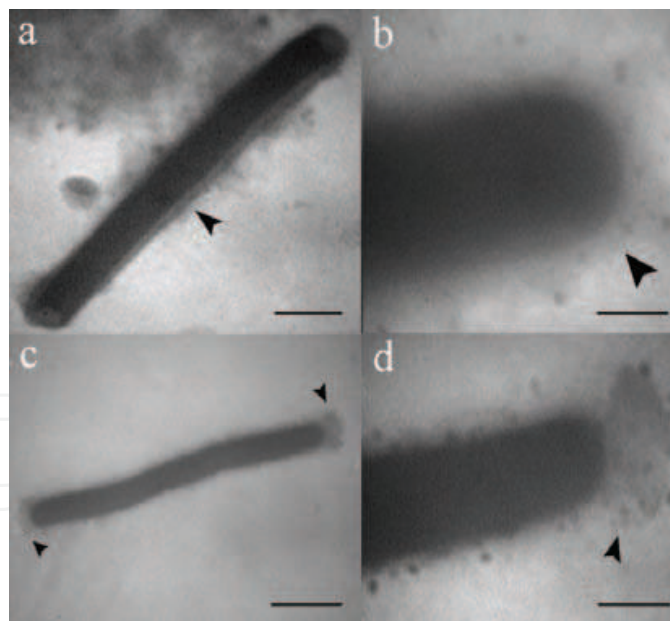


Fig. 3. Electron microscopic images of *Lactobacillus acidophilus* ATCC 4356. **a, b:** *Lactobacillus acidophilus* ATCC 4356 was completely covered by the S-layer in the control. **c, d:** an excess of S-layer was found at the both ends of the bacterial cell at 45°C. S-layer protein was indicated by black arrow (scale bar (a, c) = 2.5 µm and scale bar (b, d) = 0.6 µm).

The crystallization of S-layer was investigated by TEM. S-protein, which was isolated by guanidine hydrochloride, aggregated readily upon removal of the salt by dialysis, and formed a white precipitate. Analysis of these precipitates by TEM showed that they were

composed exclusively of crystalline lattice (Fig. 4). It seems that the reassembly of S-layer subunits was similar in the group under stress condition (45°C) and control. The results indicated that S-layer has two-fold (p2) symmetry with a periodicity of 11.3 and 5.5 nm in the control. After comparing the lattice parameters, we found that they were similar (under stress condition and control).

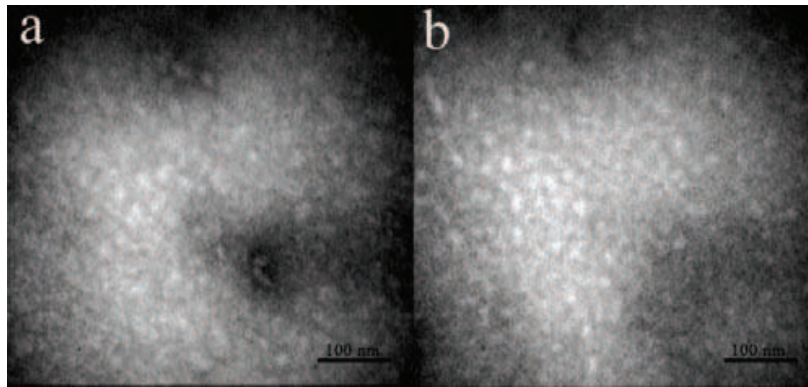


Fig. 4. Negatively stained TEM image of isolated S-layer from *Lactobacillus acidophilus* ATCC 4356. **a)** The control (37 °C); **b)** 45 °C (scale bar = 100nm).

The results indicated that the stress influenced *slpA* gene expression. Interestingly, the *slpA* gene expression increased in pH 5 and 45 °C. Under pH stress, comparison of the *slpA* gene expression showed that in the pH 6 and 7, the *slpA* gene expression was lower than that in the control (Fig. 5a) ( $p < 0.001$ ). In addition, the *slpA* gene expression decreased at 30 °C, and the *slpA* gene expression was highest at 45 °C (Fig. 5b) ( $p < 0.001$ ). However, major differences in the *slpA* gene expression were observed between the control and the group under stress conditions (Fig. 5).

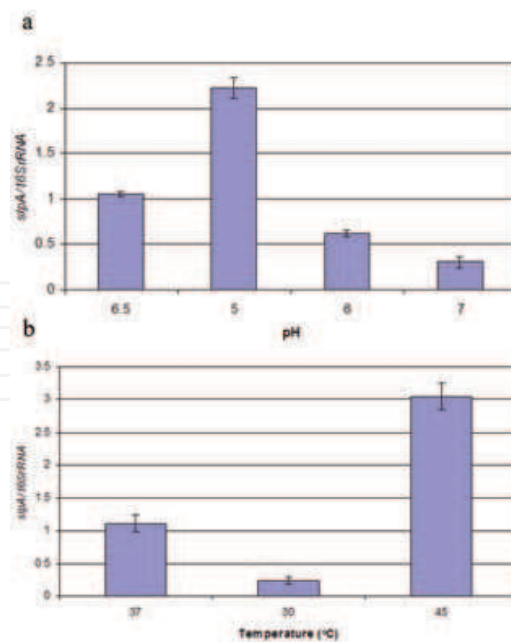


Fig. 5. Comparison of *slpA* gene expression under control and stress conditions (a: pH stress; b: heat stress) as compared to expression of housekeeping gene (*16S RNA*) in the same reaction to normalize the data. Error bars represent standard deviations of the mean values of results from three independent experiments.

### 3. Discussion

To investigate the effects of pH and heat stresses on S-layer production and *slpA* gene expression ( $OD_{600} = 0.4$  and  $0.7$ ) in *Lactobacillus acidophilus* ATCC 4356, we studied the survival of bacteria under stress conditions for 14 h. It was found that in pH 3, 4 and the temperatures 50-55 °C, *Lactobacillus acidophilus* ATCC 4356 could not survive. Then, pH 5, 6, and 7, as well as temperatures 30 °C and 45 °C were chosen as the stress conditions. SDS-PAGE gel (12% polyacrylamide) of S-layer protein showed that a single dominant band (43-46 kDa) was visible (Fig. 1). According to the previous studies, *Lactobacillus acidophilus* ATCC 4356 has S-layer protein with 43 kDa molecular weight (Boot et al., 1993; Smit et al., 2001). Also, *Lactobacillus acidophilus* ATCC 4356 has been used as positive control for S-layer protein in other studies (Fitzsimons et al., 2003; Frece et al., 2005). In our study, S-layer extraction was carried out according to Boot's method (Boot et al., 1993) in *Lactobacillus acidophilus* ATCC 4356 and *Lactobacillus casei* ATCC 393 (as negative control), and the results were compared together. As S-layer is the outermost structure of cell envelope in *Lactobacillus acidophilus* ATCC 4356, the extracted protein was only from cell wall without lysis of bacterial cells. In SDS-PAGE gel, there was one dominant band of 43-46 kDa and a few faint bands which were not 43 kDa. But there was no protein band in negative control. Therefore, we confirmed that these proteins are S-layer proteins. The S-layer protein was isolated with 4 M guanidine hydrochloride. During the mid-log phase ( $OD_{600} = 0.4$ ), the S-layer protein production was low in the control group, but it was clearly visible in  $OD_{600} = 0.7$  (Fig. 1, lane 1). Under stress conditions, the production of S-layer protein increased at pH 5 and 45°C. However, the increase in S-protein production was found in  $OD_{600} = 0.4$  and  $0.7$  (Fig. 1, 2). Evidence showed that S-layer production was increased under stress conditions (Khaleghi et al., 2010, 2011) and medium components (Schar-Zammaretti et al., 2005). It was found that S-layer proteins were present during all growth phases of *Lactobacillus acidophilus* M92 under heat stress (Frece et al., 2005). This suggested that the S-layer protein is preferentially expressed under conditions which are not optimal for bacterial growth. It has been proposed that S-layer plays a role as a protective sheath in *Lactobacillus acidophilus* ATCC 4356. In addition, some studies identified that the S-layer proteins of Lactobacilli were important for hydrophobicity, autoaggregation and adherence of this bacteria to different host surfaces (Frece et al., 2005; Greene & Klaenhammer, 1994; Hynonen et al., 2002; Kos et al., 2003; Pelletier et al., 1997; Sillanpaa et al., 2000; Toba et al., 1995; Vadillo-Rodriguez et al., 2004, 2005; Van der Mei et al., 2003).

Transmission electron microscopic analysis showed that *Lactobacillus acidophilus* ATCC 4356 was completely covered by S-layer at 37 °C. Also, S-layer covered the bacterial cells at 45 °C, but an excess of S-protein was found at the both ends of the bacterial cell (Fig. 3). It seems that the S-layer protein has a protective role for *Lactobacillus acidophilus* ATCC 4356. Previously, the excess of S-layer was found at the site of separation of the two daughter cells in *Clostridium thermosaccharolyticum*, which prevented the exposure of the newly synthesized parts of the cell wall to the environment. It has been proposed that several bacteria produce an excess of S-protein to ensure complete coverage of the cell wall during cell division, and either store excess S-layer protein in the peptidoglycan layer or secrete it into the environment (Boot & Pouwels, 1996).

Because of the adhesion role of S-layer to the epithelial cells in Lactobacilli, it is important to investigate of the self-assembly ability of S-protein monomers under stress conditions. One of these stresses is heat stress that *Lactobacillus* is encountered it during food processing. According to the investigation, the TEM images showed that dialyzed S-protein monomers were able to recrystallized at 45 °C as same as 37 °C. The S-layer structure was an oblique

lattice with p2 symmetry, and its parameters were respectively 11.3 and 5.5 nm. This finding was corresponded to the lattice parameters in *Lactobacillus acidophilus* ATCC 4356 (Smit et al., 2001), *Lactobacillus helveticus* ATCC 12046 (Lortal et al., 1992) and *Lactobacillus brevis* ATCC 14869 (Jakava-Viljanen et al., 2002).

According to the investigation of *slpA* gene expression, it was found that the *slpA* gene expression increased at 45 °C and pH 5. It proves that S-layer is very important for *Lactobacillus acidophilus* ATCC 4356, and is a protective sheath for the bacteria. In the pH 6, 7 and 37 °C, the *slpA* gene expression was lower than that in the control. It is not known why the *slpA* gene expression was different from the S-protein production in pH 6 and 7. It has been suggested that, either a little inversion has happened on *slpA* and *slpB* in unfavorable growth conditions or the presence of HCl and NaOH, used for adjusting of pH, can influence or block *slpA* gene expression. Another explanation could be that the S-layer mRNA has a relatively long half-life of 15 min and it can be repeatedly translated. As S-layer proteins represent 10-15% of the total amount of proteins in *Lactobacillus* cells, their transcription and secretion mechanisms must be efficient and tightly regulated. Multiple promoters precede several S-layer genes (Boot & Pouwels, 1996) including S-layer genes of *Lactobacillus acidophilus* (Boot et al., 1996a) and *Lactobacillus brevis* (Vidgren et al., 1992) and are likely to ensure efficient transcription of these genes. Also, the half-lives of mRNA-encoding lactobacillar S-layer proteins are relatively high, approximately 15 min, which enables efficient protein translation (Boot et al., 1996a). In addition to the actively transcribed S-layer protein gene (*slpA*), *Lactobacillus acidophilus* has also the silent *slpB* gene. The inversion of the *slp* segment causes an interchange of the active and the silent S-layer genes, which resembles a mechanism of phase variation in expression of bacterial surface antigen (Avall-Jaaskelainen & Palva, 2005; Boot & Pouwels, 1996; Boot et al., 1996b; Pouwels et al., 1998). It was found that the frequency of inversion was high (1/300), yet all attempts to demonstrate expression of the *slpB* gene have so far been unsuccessful (Boot et al., 1996b; Pouwels et al., 1998). Phase variation or antigenic variation, as a result of inversion of the *slp* segment, might enable *Lactobacillus acidophilus* bacteria to better adhere to specific regions of mucosa (Pouwels et al., 1998).

#### 4. Conclusion

In conclusion, we found that environmental conditions influenced the S-layer protein and *slpA* gene expression. Nevertheless, it seems that high temperature (45 °C) did not influence the self-assembly of S-layer monomers.

For future investigations, the *slpB* gene expression and adhesion of *Lactobacillus acidophilus* to the epithelial cells should be studied under stress and control conditions.

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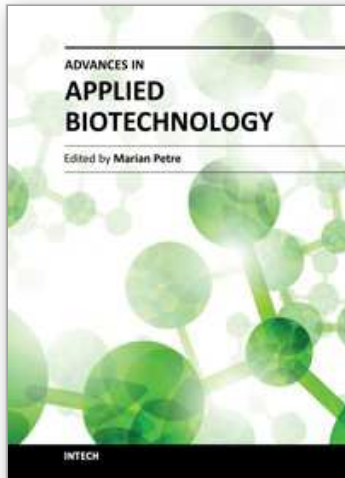
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Biotechnology is the scientific field of studying and applying the most efficient methods and techniques to get useful end-products for the human society by using viable micro-organisms, cells, and tissues of plants or animals, or even certain functional components of their organisms, that are grown in fully controlled conditions to maximize their specific metabolism inside fully automatic bioreactors. It is very important to make the specific difference between biotechnology as a distinct science of getting valuable products from molecules, cells or tissues of viable organisms, and any other applications of bioprocesses that are based on using the whole living plants or animals in different fields of human activities such as bioremediation, environmental protection, organic agriculture, or industrial exploitation of natural resources. The volume *Advances in Applied Biotechnology* is a scientific book containing recent advances of selected research works that are ongoing in certain biotechnological applications. Fourteen chapters divided in four sections related to the newest biotechnological achievements in environmental protection, medicine and health care, biopharmaceutical producing, molecular genetics, and tissue engineering are presented.

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