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Immobilization of *Lactobacillus salivarius* ATCC 11741 on Loofa Sponge Coated with Chitosan for Lactic Acid Fermentation

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Lactic acid (LA) fermentation by *Lactobacillus salivarius* ATCC 11741 immobilized on loofa sponge (LS) was evaluated. To increase the surface area of LS for cell immobilization, H_2O_2 and chitosan were introduced as surface modifying reagents. Four chitosans of different molecular weights were separately coated on LS. All experiments were conducted in shaking flask mode at 100 rpm rotating speed and 37°C with 5% $CaCO_3$ as a pH regulating agent. The effects of initial glucose concentration were investigated in the range of 20–100 g/l on LA fermentation by free cells. The results indicate that the maximum concentration of LA was produced with 50 g/l glucose concentration. The immobilized cell system produced 1.5 times higher concentration than free cells for 24 h of fermentation. Moreover, immobilized cells can shorten the fermentation time by 2-fold compared with free cells at the same level of LA concentration. At 1% (w/v) chitosan in 2% (v/v) acetic acid, the Yp/s and productivities of various molecular weights of chitosans were insignificantly different. Repeated batch fermentations showed 5 effective recycles with Yp/s and productivity in the range of 0.55–0.85 and 0.90–1.20 g/l·h, respectively. It is evident that immobilization of *L. salivarius* onto LS permits reuse of the system under these fermentation conditions. Scanning electron micrographs indicated that there were more intact cells on the chitosan-treated LS than on the untreated LS, thus confirming the effectiveness of the LS–chitosan combination when being utilized as a promising immobilization carrier for LA fermentation.

Keywords: Cell immobilization, lactic acid fermentation, loofa sponge, *Lactobacillus salivarius* ATCC 11741, chitosan

Lactic acid (LA) has become a major chemical commodity with widespread applications, extending across various industries, which include feedstock for polymerization to biodegradable polymers (PLA), oxygenated chemicals, plant growth regulators, green solvents, various specialty chemical intermediates, acidulant, flavoring agents and preservatives in food, pharmaceuticals, and the leather and textile industries [17, 21, 31]. LA is an organic acid that exists as two optical isomers: D(–) and L(+)lactic acids. Both isomeric forms of lactic acid can be polymerized to obtain polymers with different properties depending on the initial composition. Generally, LA can be produced through two production methods: (1) chemical synthesis, and (2) microbial fermentation [8, 17, 32]. Chemical synthesis constitutes 10% of the 80,000 tons of LA produced each year, whereas the remaining 90% are produced through microbial fermentation [8]. The fermentative production process has an advantage over synthetic production, since it can produce optically pure LA when an appropriate strain of microorganism is chosen, which produces only the desired isomer; whereas in chemical synthetic production, the production process always results in a racemic mixture of LA [3, 17, 21, 32]. *Lactobacillus salivarius* subsp. *salivarius* ATCC 11741 is known as a producer of high-yield L(+)LA [3, 29]. Nearly 50% of the world supply of LA is produced through free cell fermentation despite the low productivity levels of conventional processes.

On the other hand, cell immobilization, in comparison with traditional fermentative processes, can maintain higher cell concentrations, enhance fermentative productivity, improve cell stability, as well as lower the costs of recovery, recycling, and downstream processing. [11, 12, 16, 28]. Cell immobilization mimics the natural occurrence of cell growth on surfaces or within natural structures. Many microorganisms have the ability to adhere to various surfaces in nature. Several techniques and supporting materials have been proposed to localize intact cells to a certain

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region of space with preservation of some desired catalytic activity. These techniques can be divided into four major categories based on the physical mechanism employed: (a) attachment or adsorption on solid carrier surfaces, (b) entrapment within a porous matrix, (c) self-aggregation by natural flocculation or with cross-linking agents (artificially induced), and (d) cell containment behind barriers [4, 7, 12, 14, 16, 24, 28], which is the main focus of this research.

Many studies related to cell immobilization through cell entrapment have been carried out to produce LA through the use of gels, such as alginate [9, 10, 27, 33] and κ-carrageenan [1, 2]. However, cell immobilization through the use of gel entrapment presents many disadvantages. First, gel beads can be disrupted owing to the build-up of pressure generated during cell division. Second, LA itself cannot easily diffuse through the gel layer, and thus accumulates in the gel beads. Third, the gel beads can solubilize gradually and have low mechanical strength. Moreover, the mass production of gel beads on an industrial scale is uneconomical and very complicated. Immobilization on a solid surface carrier through physical adsorption can overcome all these disadvantages, and has no limitations when being adapted for mass production. Cells are adhered to the solid support surfaces by Van der Waals forces, and ionic or covalent interactions [12]. Another reason why production systems using cell immobilization are popular is due to the relative ease and simplicity of the production process. Keep in mind, however, that despite the simplicity, careful attention must be given in selecting an appropriate type of carrier. The desired carrier should be: nonreactive, non-toxic, inexpensive, available in large quantity, easy to handle, highly stable, and have a high capacity with effective yield and preservation. Examples of solid carriers used in this type of immobilization are cellulosic materials (*e.g.*, DEAE cellulose, wood, sawdust, and delignified sawdust) and inorganic materials (*e.g.*, polygorskite, montmorilonite, hydromica, porous porcelain, and porous glass, *etc*). Solid materials such as glass or cellulose can also be modified with polycations, chitosan, or other chemicals (preformed carriers) to enhance their adsorption ability [12]. Some researchers have employed synthetic or polymer supporting matrices for cell binding in LA fermentation [7, 13, 25, 26]. However, most of these carriers have closed structures and are non-biodegradable. Biodegradability, in and of itself, has become more important over the years in all industries.

Agricultural residue is now more commonly being used as basic immobilization materials. Recycling and reuse help minimize the environmental impact associated with their waste build-up, as well as reduce the consumption of noble materials. This trend has contributed to a shift in focus by the industry to search for better and more environmentally responsible biomaterials. A good biomaterial sample is natural fiber, which is available in great abundance. Cellulose, hemicelluloses, and lignin are three substances that make up the majority of agricultural residues being generated today. Of these three substances, cellulose is the most abundant of all naturally occurring substances, and is the principal structural component of cell walls in all major plants. It is non-toxic, renewable, biodegradable, modifiable, and has great potential in serving as the base material for the industry. Because of all these benefits, it is of no surprise that there have been numerous studies directed toward the search and discovery of alternative, renewable, and biodegradable carriers, which are essentially nonsynthetic, easy to use, inexpensive, and naturally available [8]. Once a carrier is chosen, chitosan, a flocculating agent, is then used to enhance the adhesion of the cells to the solid support [18, 19, 31]. Chitosan acts not only as a flocculating agent but also as polycations in solid support treatment, and assists in the improvement of adsorption of cells to the support surface [13]. The main parameters influencing the characteristics and properties of chitosan are molecular mass (MM), degree of deacetylation (DD), which represents the molar fraction of deacetylated units, crystallinity, and the purity of the product. All of these parameters are determined by the conditions during preparation [20, 22]. In addition, chitosan of different molecular masses possibly have different effects on cell immobilization owing to the presence of differing viscosity levels within the substance.

To the best of our knowledge, there has been no report on the immobilization of the *L. salivarius* strain for LA production. Therefore, this study is the first report on the immobilization of *L. salivarius* ATCC 11741. Additionally, this study appears to be the first integration of immobilization of *L. salivarius* ATCC 11741 on chitosan-coated loofa sponge, which is an intriguing alternative for making a new biocatalyst for lactic acid production. Chitosans with different molecular masses are also applied to investigate their effects on fermentation performance.

MATERIAL AND METHODS

Microorganism
L. salivarius ATCC 11741 was kindly provided by the Thailand Institute of Scientific and Technological Research (TISTR). The stock culture was stored at -20°C in a mixture of MRS broth and glycerol at 1:1 ratio. Each starter culture was obtained by two successive propagations at 37°C in a preculture medium (meat extract 10 g, yeast extract 5 g, peptone from casein 10 g, glucose 20 g, tween-80 1 g, K_2HPO_4 2 g, $MgSO_4$ 0.2 g, and $MnSO_4$ 0.2 g per liter). The cell pellets were obtained by centrifugation (refrigerated centrifuge, Kubota 7820, Kubota, Japan) at 3,000 rpm and 4°C for 10 min. All procedures were done under aseptic condition.

Carrier Preparation
In the beginning, loofa sponge obtained from the factory was washed with tap water to remove dirt and impurities and dried in an oven

(Model 500, Memmert, Germany). Then, it was cut into a square shape with the approximate dimensions 2×2 cm [5]. To ensure good cleaning before use as the immobilization support carrier, the loofa sponge was boiled in deionized water for 30 min, left in deionized water for 24 h, and finally dried in an oven at 70°C [5, 15, 23]. Pretreatment was performed by immersing the fiber in a 0.25% hydrogen peroxide solution at 60°C for 1 h in a water bath [6]. After that, the obtained fiber was washed several times with deionized water and dried at 70°C. Surface modification by chitosan was applied to the H_2O_2-treated fiber. A 1% (w/v) of various molecular mass chitosan solutions in 2% (v/v) acetic acid was prepared as a modifying reagent. The fiber was incubated in the chitosan solution for 30 min [13] and dried at 50°C in an oven.

Adsorption of Cells to the Matrix

The cell suspension containing 16-h-old cells (OD_{600} after 100-fold diluted=0.30–0.35) was inoculated to the fiber in the medium and incubated in a rotary shaker at 100 rpm, 37°C for 24 h. The immobilized biocatalyst was separated from the fermentation broth and washed with sterile 0.85% NaCl solution twice in order to remove free and loosely bound microorganisms.

Lactic Acid Fermentation Using Immobilized *L. salivarius* ATCC 11741

The cells of *L. salivarius* ATCC 11741 immobilized on the treated fiber were inoculated in a preculture medium supplemented with glucose 50 g/l. Other compositions were the same as in the preculture medium. The pH was adjusted with 5% calcium carbonate. The fermentation was propagated in the same conditions as in the above immobilization step. Sampling was done regularly. In a repeated batch mode, immobilized biocatalysts were transferred aseptically to a fresh medium and a new run was carried out in the same configuration as the main run.

Sample Analysis

Glucose and lactic acid concentrations in the fermentation broth were measured by using a YSI 2700 SELECT Biochemistry Analyzer (YSI Incorporated, Yellow Springs, OH U.S.A.) and all samples were carried out in triplicate. During the course of fermentation, samples of carriers were collected for scanning electron microscopy (SEM). The carriers were sputter-coated with gold and examined using SEM (JSM 5410LV, JEOL, Japan). Cell mass was not able to be measured under the application of calcium carbonate as a pH regulating agent because it interfered with the optical density of the cell solution at 600 nm.

RESULTS AND DISCUSSION

Lactic Acid Fermentation by Free *L. salivarius* ATCC 11741

To evaluate the optimum initial glucose concentration for the $CaCO_3$ system, the experiments were conducted under the conditions of a shaking flask at 100 rpm rotating speed, 37°C, and 5% $CaCO_3$. Calcium carbonate was used as the pH regulating reagent because it is relatively inexpensive and can be applied without an expensive pH controller system. The initial glucose concentrations varied from 20 to 100 g/l. The effects of various initial glucose concentrations
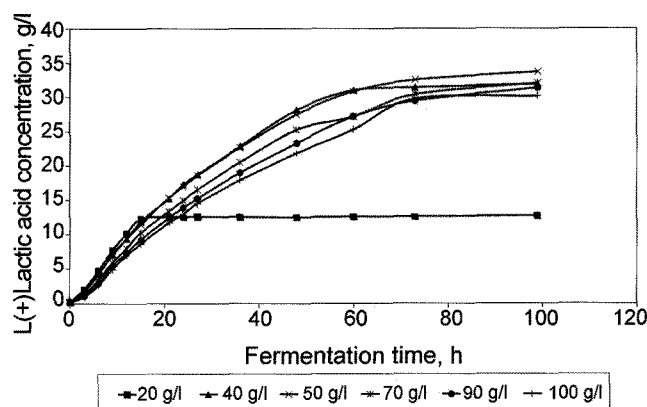

Fig. 1. Time course of glucose consumption and lactic acid production by *L. salivarius* ATCC 11741 at the initial glucose concentration of 50 g/l, with pH controlled by 5% $CaCO_3$.

on the LA production of the immobilized *L. salivarius* ATCC 11741 during the batch fermentation are illustrated in Fig. 1. At the initial glucose concentration of 20 g/l, LA was continuously produced until the 16th hour, after which the production started to level off owing to the depletion of the substrate. LA concentration rose in correlation to the increase in the initial glucose concentration up to 50 g/l, but then declined with further increases in the initial glucose concentration (at 70, 90, and 100 g/l). This decrease in the LA concentration resulted from the substrate inhibition phenomena. Higher substrate concentrations may have increased the osmotic pressure, which in turn affected the cell growth by either removing water from the microbial cells or restricting the normal diffusion process of water into these cells [30]. Hence, the initial glucose concentration of 50 g/l was selected as the optimal concentration for further experiments.

In order to compare the immobilization results, an experiment using free cells of *L. salivarius* ATCC 11741 was also conducted under the same conditions mentioned above. The experimental data shown in Fig. 2 indicate
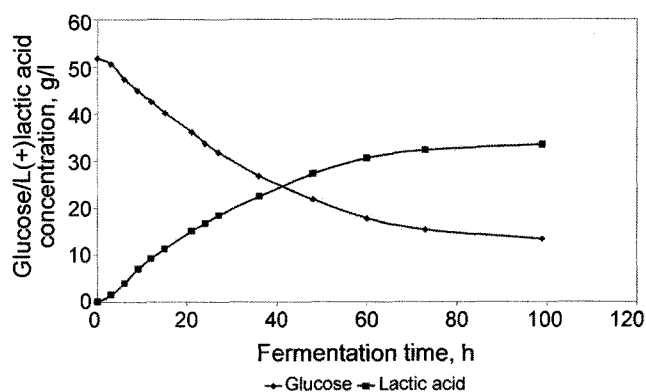

Fig. 2. Effect of initial glucose concentration on lactic acid production.

Table 1. Characteristics of chitosan used in this research.

Molecular mass, (kDa)	DD (%)	Viscosity[a] (cp)	Zeta potential (mV)
83	90	10.2	45.9
185	90	18.6	55.5
380	91	36.6	55.8
800	92	50.4	55.0

[a]Viscosity of 1% (w/v) chitosan in 2% (v/v) acetic acid.

that normal glucose consumption and LA production took place, and a slight lag phase occurred during the first 2 h. At 48 h of fermentation, the LA concentration was 27.52 g/l with $Y_{p/s}$ of 0.91 and productivity of 0.57 g/l.h.

Lactic Acid Fermentation by Immobilized *L. salivarius* ATCC 11741

For the purpose of improving cell immobilization on the LS, chitosan was applied as a surface modifier of the natural fiber. The chitosan structure was composed of an amine group in every repeating unit that resulted in the cations on the surface of the LS, enhancing the binding of cells to the fiber. Therefore, chitosan with a high molecular mass should contain high levels of amine groups that can be protonated in water with hydrogen ions to create a cationic surface [34]. Consequently, the chitosan molecule should possess a high power of static attraction and adsorption to anionic molecules, including cells. Molecular mass was one of the important factors that affected the characteristics and properties of chitosan [20, 22]. Characteristics of the chitosan used in this experiment are tabulated in Table 1.

In this experiment, four cultures using chitosan of different molecular weights for cell immobilization on LS were evaluated. Each of the cultures were labeled and are tabulated in Table 2. The initial glucose concentration was approximately 50 g/l. This is the level that is considered ideal for maximized LA production. The reusability of the biocatalyst in LA fermentation was also determined in the repeated batch mode after the main batch.

In looking at Fig. 3, as expected, the immobilized cell system produced a higher LA concentration than the free

Table 2. List of samples and labels for loofa sponge fermentation.

Immobilized carrier	Label
H_2O_2-treated loofa sponge coated with chitosan MM 83 kDa[a]	L1
H_2O_2-treated loofa sponge coated with chitosan MM 185 kDa[a]	L2
H_2O_2-treated loofa sponge coated with chitosan MM 380 kDa[a]	L3
H_2O_2-treated loofa sponge coated with chitosan MM 800 kDa[a]	L4

[a]1% (w/v) chitosan in 2% (v/v) acetic acid.
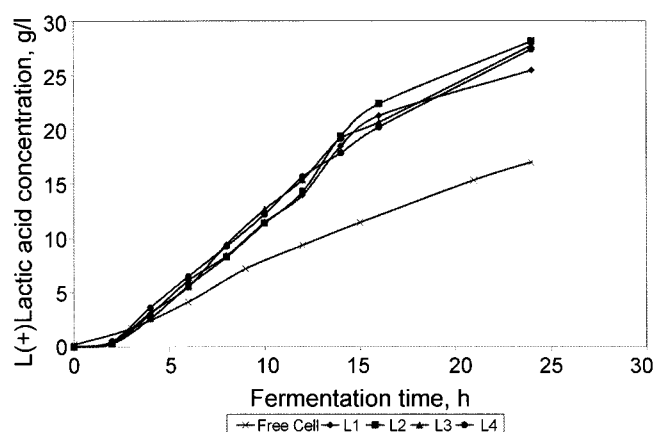
Fig. 3. Lactic acid concentration of free cell and immobilized cell during the first 24 h of fermentation.

cell system. The maximum LA concentration that resulted from the immobilized cell was in the range of 25.47 to 28.18 g/l at hour 24, whereas the free cell provided the maximum LA concentration of 16.96 g/l. The productivities of the immobilized cell, 1.06–1.17 g/l.h, were higher than the free cell system, 0.70 g/l.h, as shown in Table 3. Focusing on the LA concentration, the free cell system production took about 48 h of fermentation to produce 27.52 g/l of LA, whereas the immobilized cell system used only 24 h to produce the same level. Thus, a conclusion can be made that immobilized cell shortened fermentation time and results in the higher productivity.

Focusing on the variation in the molecular mass of chitosan, there were no marked differences in glucose consumption and LA formation. The reason for this might be due to an unsuitable chitosan concentration [1% (w/v)] being applied, or the slightly different zeta potential values of chitosan, which prevented the variation in molecular masses from affecting the efficiency of the coated chitosan molecule on the LS surface. Analysis also indicated that the productivities and the product yields of different molecular masses of chitosan were not significantly different.

Since production by means of cell immobilization was faster than the production *via* the free cell system, further analyses were made into the amounts of time that cells immobilized on LS can be reused. Thus, additional fermentation was carried out in the repeated batch mode.

Table 3. Lactic acid concentration and productivity of free cell and immobilized cell after 24 h of culture.

Culture	Lactic acid concentration g/l	Productivity g/l·h
Free cell	16.96	0.70
L1	25.47	1.06
L2	28.18	1.17
L3	27.75	1.16
L4	27.41	1.14
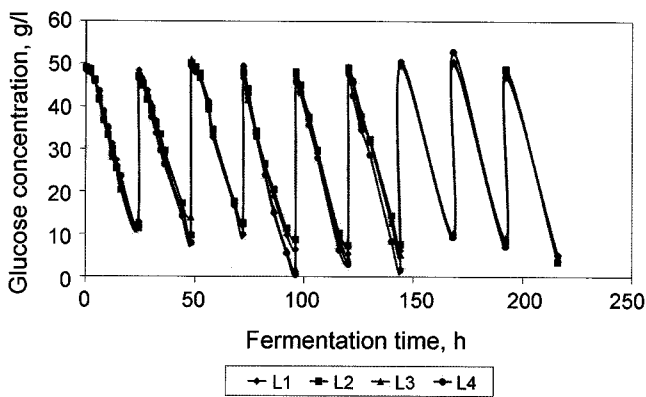
Fig. 4. Glucose concentration profile of cell-immobilized loofa sponge for all cycles.

Fig. 6. Total Yp/s of cell-immobilized loofa sponge for all cycles (1 cycle=24 h).

Fig. 4 shows that glucose concentration profiles were similar for all systems, indicating a similar behavior of cells in term of glucose consumption. The same limiting factors might have affected all systems so that the glucose concentration reached a similar level of not higher than 10 g/l by the end of every batch. The lactic acid concentration profile shown in Fig. 5 indicates the opposite trend of the glucose consumption. The concentration of LA produced was in the range of 25 to 30 g/l for every treatment, from the main batch to the repeated batch 5. Then, it rapidly decreased until the lowest value of approximately 10 g/l was reached in the repeated batches 6–8. The chitosan-treated LS might have possibly lost its ability to immobilize the microbial cells in repeated batch 6 and above. The LA concentrations were consequently reduced. One explanation can be that more cells were lost when the fermentation medium was drained in repeated batches 6–8, in comparison with batches 1–5, resulting in smaller amounts of microorganisms remaining attached to the LA support in repeated batches 6–8. A lag phase was noticeable in every other cycle, beginning with the main batch, as shown in Figs. 4 and 5.
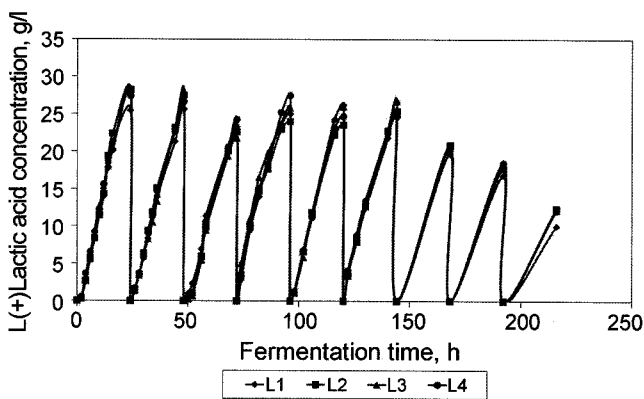
Marginal differences in glucose consumption and LA production in each treatment indicated that the difference in molecular masses of chitosan has insignificance influence on cell behaviors. Fig. 6 indicates that there was a gradual decrease of Yp/s from the main batch (0.85) to the repeated batch 5 (0.55), followed by a rapid drop until the lowest value of approximately 0.25 was reached in repeated batches 6–8. One explanation for this can be that the efficiency of the immobilized cell decreased after every passing cycle. Unsurprisingly, according to Fig. 7, the productivity level also followed the same trend as the Yp/s. The level of productivities in the main batch to the repeated batch 5 was rather constant, ranging from 0.90 to 1.20 g/l.h. Thereafter, it decreased rapidly from repeated batches 6–8, reaching the lowest value of approximately 0.40 g/l.h. The results clearly indicate that cells, when immobilized and used in a repeated batch system, had more reusability than free cells did in a nonrepeated batch system.

SEM micrographs of cell-immobilized LS are shown in Fig. 8. The chitosan-treated LS allowed cells to better adhere throughout the narrow channel embedded in the surface, as well as the broad surfaces of the LS itself,

Fig. 5. L(+)Lactic acid concentration profile of cell-immobilized loofa sponge for all cycles.

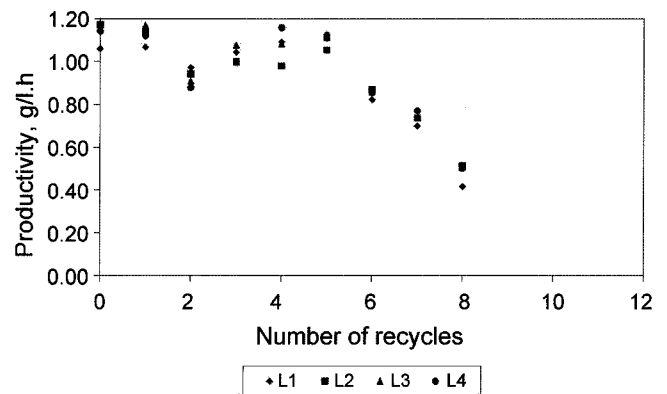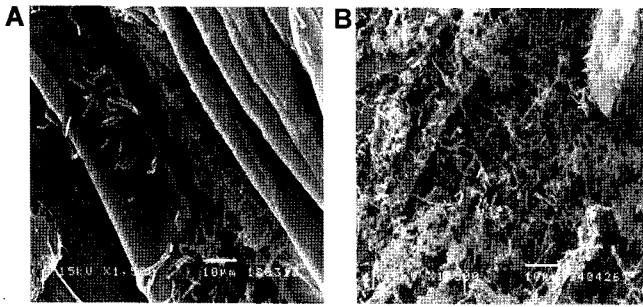Fig. 7. Productivity of cell-immobilized loofa sponge for all cycles (1 cycle=24 h).

Fig. 8. Cell-immobilized loofa sponge (A) untreated and (B) chitosan-treated.

whereas in the untreated LS, cells could only adhere in the narrow channels of the surface.

In conclusion, to develop an LS for *L. salivarius* ATCC 11741 immobilization, chitosan was applied to enhance the cell adhesion ability of the LS. The results showed that immobilized cells provided a higher LA concentration than did free cells. However, the molecular mass of chitosan did not play an important role in cell immobilization and LA production. The cells immobilized on chitosan-treated LS could be reused up to 5 cycles. Consequently, chitosan-treated LS may be of great interest for cell immobilization of other microorganisms. To the best of our knowledge, this is the first report on LA production by *L. salivarius* ATCC 11741 on chitosan-treated LS as a support carrier.

Acknowledgments

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