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Development of Antimicrobial Edible Film from Defatted Soybean Meal Fermented by *Bacillus subtilis*

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Abstract In order to extend shelf-life of the packaged or coated foods, an antibacterial edible film was developed. Antimicrobial activities of 9 bacteriocin-like substance (BLS)-producing strains were evaluated after growing them on defatted soybean meal medium (DSMM). *Bacillus subtilis* was selected among those, because it showed the biggest inhibition zone against 6 problem bacteria in food. The antimicrobial edible film, containing 0.32% of BLS, was produced from the fermented soybean meal with *B. subtilis* at the optimum condition of pH 7.0–7.5 and 33°C for 33 h. The antimicrobial activity of the film was over 50% of the maximum activity after film production with heat treatment at 90°C and pH adjustment to 9. When the soy protein film with BLS was applied on the agar media containing *E. coli*, the growth inhibition was much higher than the ordinary soy protein film. These results indicate that the soy protein film with BLS from *B. subtilis* can be used as a new packaging material to extend the shelf-life of foods.

Key words: Bacteriocin-like substance (BLS), functional film, antimicrobial activity, *B. subtilis*, soybean meal

Recently, food safety has become an increasingly important international concern. Besides refrigeration and dehydration, packaging and preservatives have been considered to be the effective ways to extend shelf-lives of foods and to guarantee food safety. However, because of increasing concern over environmental safety of nondegradable synthetic packaging materials, there is a demand for natural degradable products from renewable sources as an alternative to synthetic polymers. Soy protein is a viable renewable resource for producing environmentally safe industrial products. Therefore, a few researches have undertaken to develop and utilize the biodegradable soy protein films as packaging and coating materials [1, 37]. However, expanded utilization of soy protein films to extend the shelf-life and guarantee the safety of food is limited, because the packaging and coating with soy protein film can only prevent the foods from moisture migration and cross-contamination of foods. In order to solve the problem that soy protein film is not able to retard the growth of precontaminated pathogens, chitosan has been used in a biodegradable film [32]. However, the film containing chitosan showed a relatively negligible antimicrobial ability, although enhanced mechanical properties were observed. Therefore, the application of more effective preservatives to the films has been considered in packaging to extend shelf-lives of foods that are highly perishable and are not feasible for direct addition of preservatives. Because most people are presently reluctant to use synthetic preservatives in foods, food industry is searching for substituted preservatives. One of the potential solutions of these problems has been antimicrobial peptides produced by microorganisms. The application of these antimicrobial peptides produced by bacteria has received a great deal of attention due to its low toxic or other adverse effects on foodborne pathogens. Bacteriocins are also the bacteria-originated antibacterial proteins which inhibit the growth of other bacteria. They are different from many other traditional antibiotics which have relatively narrow antimicrobial spectra. They are toxic only to bacteria that are closely related to the producing strains [5, 21, 25, 33, 35]. In the last few years, a number of new bacteriocins from bacteria have been identified and characterized. Because of their strong inhibitory

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effects on the growth of pathogens, the strains and their bacteriocins have potentially been used as natural food preservatives [10, 17, 27, 29, 30, 38]. However, direct application of these strains to commercial food industry still remains questionable due to its economical problem.

In the present study, therefore, we conducted experiments to economically develop an antimicrobial edible film from defatted soybean meal, which is a kind of waste product, by inoculating with bacteriocin-like substance (BLS)-producing bacteria and to verify whether the film could be used as a packaging material in the food industry.

MATERIALS AND METHODS

Materials

Defatted soybean meal used in this study was supplied by Shindongbang Co. (Seoul, Korea).

Bacterial Strains and Culture Conditions

The capabilities for fermenting defatted soybean meal (DSM) and producing bacteriocin-like substance (BLS) were evaluated in nine strains of bacteria: *Bacillus subtilis* KCTC1021, *B. cereus* KCTC1012, *Pseudomonas putida* KCTC1033, *P. methanolica* KCTC2692, *P. aeruginosa* KCTC2742, *Lactobacillus delbrukii* KCTC3635, *L. fermentum* KCTC3112, *L. casei* KCTC3109, and *Paenibacillus macerans* KCTC3723. The antimicrobial activities of BLS-producing bacteria were determined against 6 foodborne problem bacteria: *E. coli* KCTC1039, *Listeria monocytogenes* KCTC3710, *Staphylococcus aureus* KCTC2199, *Salmonella typhimurium* KCTC2515, *Shigella sonnei* KCTC2518, and *Vibrio cholerae* KCTC2715. All 15 strains were purchased from the KCTC (Korean Collection for Type Cultures, Daejeon, Korea). *E. coli* is the index for fecal contamination, and the 5 other foodborne pathogens are main causative bacteria for foodborne diseases in Korea and are related to contamination of food and water, direct contact with farm animals, and person-to-person transmission [11]. Therefore, these 6 strains were selected as indicators in this study.

These organisms were maintained in frozen stocks with 20% (w/v) glycerol at -20°C and propagated twice from single colony prior to experimental uses [25]. Each bacterial strain was cultivated in their optimum media and temperatures. Indicating strain cultures were always adjusted to 0.5 of optical density (OD) at 540 nm with UV-VIS spectrophotometery (TU-1800, General Electric, Seoul) before inoculation for bacteriocin assay [6, 29].

Determination of Antimicrobial Activity

Antimicrobial activities of BLS-producing strains against indicator species were established by Spot-On-Lawn assay. BLS was partially purified from the soybean media by Agar well diffusion method (paper disk method), using actively grown cells [4, 14, 22, 29, 33, 35]. A 100 µl of sample cultured in Trypticase Soy Broth (TSB; Difco Laboratories, MD, U.S.A.) was spot-inoculated on the surface of dried soy bean agar plate and grown for 24 h at each temperature. Then, the inoculated plates were overlaid by 10 ml of Trypticase Soy Agar (TSA; Difco Laboratories, MD, U.S.A.) containing 0.75% soft agar with 10^8 cfu/ml of indicator strain. Inhibition zones were observed after 48 h of incubation [10, 33]. Agar well diffusion method (paper disk method) was used for detection of the BLS-producing activity [16, 18, 22, 24, 29, 35]. The culture of BLS-producing strains were grown in a liquefied soybean medium for 24–48 h and inoculated on a rotary shaker fitted at 150 rpm and 33°C aerobically. Cell-free supernatants were obtained by both centrifugation at 8,000 rpm for 20 min of each culture and filtration by using 0.22 µm of Millipore filter (Millipore S.A 67120, France). pH of the concentrated supernatant was adjusted to 7.0 with 1 N NaOH and 1 N HCl. Indicator strains were inoculated into liquefied medium at a final OD_{600} (optical density at 600 nm) of 0.5. The solidified media were spread with 100 µl of the indicator cultures and concentrated to 10^8 cfu/ml prior to use as an inoculum. After the medium was solidified, paper disks of 8 mm in diameter (Adventec, Toyo Roshi Kaisa, Japan), wetted with 100 µl of supernatant, were put on the agar plate. The plates were aerobically incubated at 37°C for 24 h. Sizes of inhibition zones were measured in both directions, and the average diameter of inhibition zone was expressed in mm unit. All experiments were carried out in triplicate.

Optimization of Culture Conditions for BLS

The effects of pH and temperature on the microbial growth and the BLS production were investigated. The growth curve of the selected strain was determined by spread plate culture counting method [9, 25, 26, 30, 34]. The incubation was carried out in a shaking incubator at 33°C and 150 rpm.

Stability of Cell-Free Supernatant (CFS) to Heat and pH

Heat stability was examined by pre-heating CFS at the range from 20 to 100°C for 30 min and the residual inhibition activity of CFS was determined by OD at 540 nm with UV-VIS spectrophotometer (TU-1800, General Electric, Seoul) [8]. In order to investigate the effect of pH on antimicrobial stability, the pH of CFS was adjusted to 3–10 with either 1 N HCl or 1 N NaOH and left in an appropriate pH at room temperature for 12 h. The residual antimicrobial activity was measured by spectrophotometery after the pH of CFS was readjusted to 7.

Fermentation

The fermentation was carried out in a 500-ml flask with culture media, containing 50 g of soybean meal in 250 ml.

The fermenter containing medium was autoclaved at 121°C for 20 min, and the pH was controlled by the addition of 1 N NaOH or 1 N HCl. The media were fermented at 33°C for 33 h after inoculation with *B. subtilis* culture [21].

Film Preparation

The fermented film solution was filtered through a cotton sieve, cast in a thin layer, dried, and peeled from the surface. In order to overcome film brittleness and to obtain freestanding films, the plasticizer, 3% glycerol, was added. The pH of the solution was adjusted to 9 with 1 N NaOH. After heating at 90°C, the solutions were strained through eight layers of cheesecloth (grade 40) to remove small particles and cast onto a crystal PVC plate (30×30× 0.8 cm). Non-fermented film was also prepared as the control under the same conditions mentioned above. The prepared films were peeled from the plate and stored at 20°C in a desiccator [1, 19, 23, 31, 37].

Quantitative Analysis of BLS Contained in Film by SDS-PAGE

Quantitative analysis of BLS contained in film was performed as follows: 1) the freeze-dried BLS film was gel-electrophoresed, 2) the BLS band on the gel was determined by comparing with the gel of ordinary soy protein film and by assaying inhibitory abilities of each band, 3) the size of BLS band was determined by image analyzer (GeneGenius2, geneSNAP software, Syngene), and 4) the amount of BLS in the film was calculated from the size of BLS band. The approximate protein content in film was previously analyzed by the Kjeldahl method.

The gel-electrophoresis of the freeze-dried BLS film was conducted by SDS-PAGE. Molecular weight of each band was estimated by comparison with the Bio-Rad Kaleidoscope Prestained standards [Myosin (200 kDa), β-galactosidase (116.2 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa)]. After electrophoresis, the gels were stained by the procedure described [7, 10, 12, 13, 20, 22]. At the end of electrophoresis, every band was removed and tested for antimicrobial activity [28]. It was immediately fixed by treating in a solution containing 50% methanol and 10% acetic acid for 3 h. After washing with distilled water, it was aseptically placed in a sterile petri dish, and covered with 20 ml of 0.75% soft agar containing 10^8 cfu/ml of *E. coli* as an indicator strain. Then, the plate was incubated at 37°C for 12 h and examined for sizes of inhibition zone.

Antimicrobial Property of Film

Antimicrobial property of the film was measured by placing the BLS film on a petri dish (5.0×5.0 cm) containing TSA and 10^3 cfu of *E. coli*. *E. coli* was enumerated under a light microscope every 4 h. The same procedures were also used in the non-BLS film as the control [2].

RESULTS AND DISCUSSION

Antimicrobial Spectra of BLS-Producing Strains

As mentioned above, the objective of this study was to produce the antibacterial edible packaging film by inoculation of BLS-producing bacteria to defatted soybean meal. Therefore, selection of the bacteria strains that can grow and produce BLS on the defatted soybean meal was very important. The inhibitory spectra of 9 tested strains were evaluated against 6 indicator strains, *E. coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Shigella sonnei*, and *Vibrio cholerae*, and the results are described in Table 1.

Paenibacillus macerans, *Lactobacillus fermentum*, and *Lactobacillus casei* could grow little and produce BLS on the defatted soybean meal medium. The most superior

Table 1. Inhibition zone of bacteriocin-like substance (BLS) producers against 6 indicator strains in soybean media.

(unit: mm)

BLS-producing strain \ Indicator strain	*E. coli*	*Listeria monocytogenes*	*Staphylococcus aureus*	*Salmonella typhimurium*	*Shigella sonnei*	*Vibrio cholerae*
Bacillus subtilis	6	2	2	2	×	×
Bacillus cereus	1	×	×	×	×	×
Pseudomonas putida	4	2	2	1	×	×
Pseudomonas methanolica	2	1	1	×	×	×
Pseudomonas aeruginosa	3	×	1	1	×	×
Lactobacillus delbruekii	1	1	1	×	×	×
Paenibacillus macerans	1	×	×	×	×	×
Lactobacillus fermentum	×	×	×	×	×	×
Lactobacillus casei	1	1	×	×	×	×

*Measured by Spot-on-lawn method.

1-6 indicates the length of inhibition.

×: smaller than 1 mm.

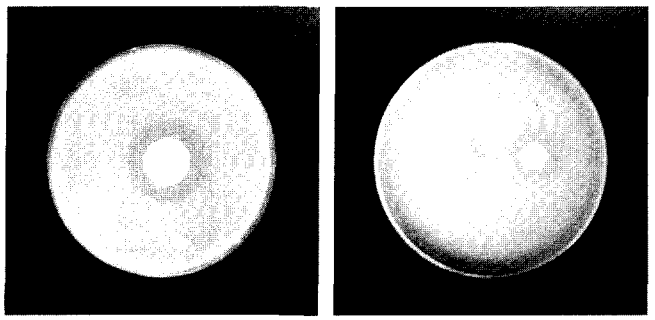

Fig. 1. Inhibitory effect of *Bacillus subtilis* against *E. coli*.
(a) Spot-on-Lawn, (b) Agar Well Diffusion (Paper disk method) Methods.

BLS activities in the other 6 producers were observed against *E. coli*. In particular, *Bacillus subtilis* showed the highest antibacterial activity against *E. coli* among the 9 tested strains. Figure 1 shows the inhibitory activity of *B. subtilis* against *E. coli*. The strongest inhibition of BLS against *E. coli* was produced by *B. subtilis* and 3 *Pseudomonas* sp. *Staphylococcus aureus* and *Salmonella typhimurium* were only partially inhibited by BLS from the above 4 strains, but *Shigella sonnei* and *Vibrio cholera* were almost insensitive. Among the BLS producers, *B. subtilis* was considered to be the most effective isolate with the broadest antimicrobial spectrum against the indicators. *B. subtilis* is well known to possess antagonistic activities against many bacterial as well as fungal pathogens, and is often used as a probiotic agent for biocontrol [3, 15, 33, 35].

Growth and BLS Production of *Bacillus subtilis*

In order to examine the antimicrobial activity of *B. subtilis* during the growth, cell-free samples were collected at various time intervals [3, 33]. The relationship between the growth of *B. subtilis* and antimicrobial activity against *E. coli* is shown in Fig. 2.

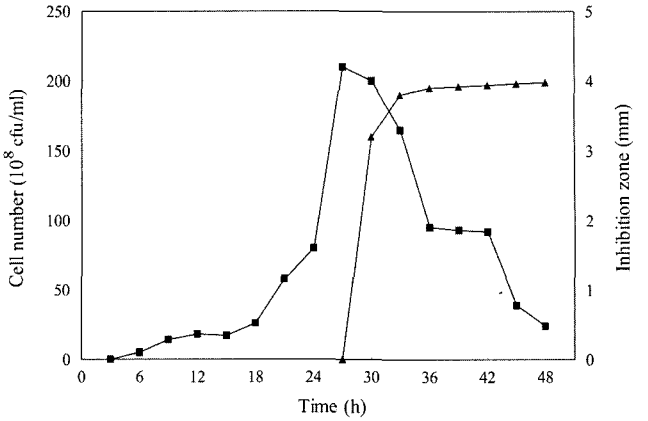

Fig. 2. Relationship between the growth of the *B. subtilis* and the antimicrobial activity against *E. coli*.
-■-: cell number of *B. subtilis*; -▲-: length of inhibition zone.

The BLS activity was detected early in the death phase and persistently remained during this phase until its end. BLS activity showed a maximum level at the end of death phase, in accordance with the report of Park *et al.* [33]. There was a remarkable difference between cell growth and BLS production in the soybean broth medium: At the beginning, bacterial growths and BLS activity on soybean media were negligible until 24 h of incubation. After 24 h of incubation, a fast bacterial growth was found on soybean medium until 27 h, at which BLS started to be produced. Maximum antibacterial activity was observed after 33 h of incubation. It is highly likely that this result was due to the slow elution of carbon and nitrogen sources

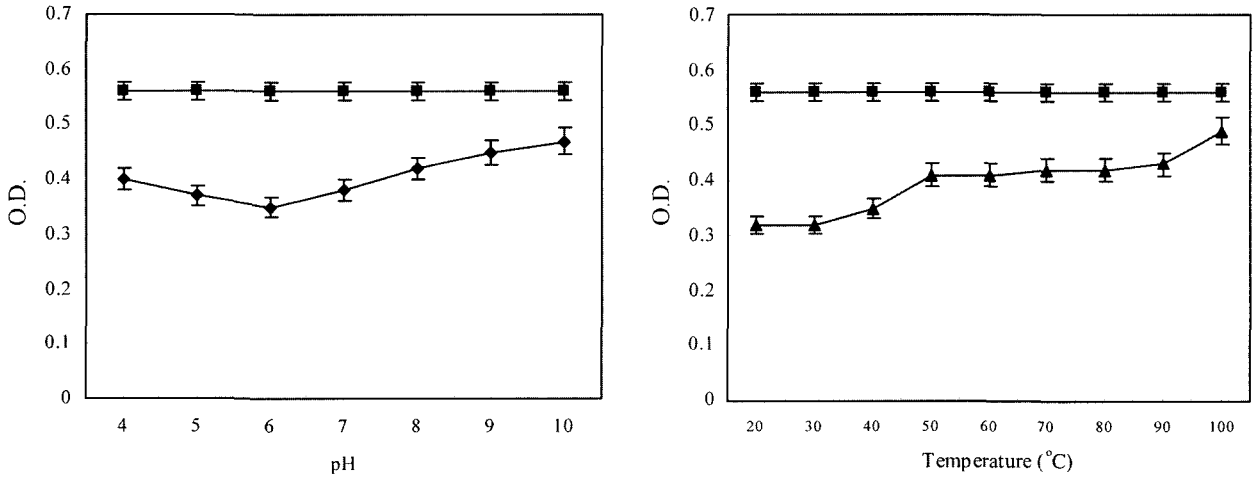

Fig. 3. Stability of cell-free supernatant (CFS) to heat and pH.
-■-: indicator growth in TSB medium; -◆-: retained inhibition activity of CFS to pH; -▲-: retained inhibition activity of CFS to temperature. *Within errors of less than 5%.

from soybean particles, in addition to the inhibitory activity of secondary metabolites.

Heat and pH Stabilities of BLS

The stability of BLS is influenced by many factors. Among those, the most important factors are pH and temperature [10, 22, 33], especially in commercial production [19, 25, 29]. Therefore, the stabilities of BLS against heat and pH were examined, and the results are described in Fig. 3. The antimicrobial activity of *B. subtilis* against *E. coli* was maintained over a wide range of pH and even after heat treatment, which was attributed to the production of antimicrobial substance resistant to high temperature and acidity. Antimicrobial activity of CFS was not reduced after heat treatment at 100°C for 20 min, but reduced by heating at 121°C for 20 min. Although a substantial loss of the activity occurred under an excessive acidic (pH<3) and alkaline (pH>10) conditions, it was also stable at room temperature and wide range of pHs. These results were in accordance with other reports [1, 15, 19, 33], demonstrating that the inhibitory compounds from *B. subtilis* are stable proteinaceous compounds [14]. These results suggest that heat treatment at lower than 100°C and pH between 3 and 10 would be allowable for film production.

Production of the Film Containing BLS

In the production of fermented soybean film, the film-forming solution has to be heated to unfold protein chains and pasteurize. Since proteins undergo structural changes in the presence of plasticizers, the extent of denaturation and exposure of functional groups may affect the mechanical properties of films. When the film-forming solution is cast, reformed disulfide bonds link the polypeptide chains together to produce the film structure with the aid of hydrogen and hydrophobic bonding [1, 19, 36], resulting in the production of BLS-containing soy protein film.

In order to verify the presence of BLS in the edible film produced, SDS-PAGE of the film was conducted. Gel-electrophoretic pattern of the film containing BLS revealed a unique band at the region of approximately 12 kDa which was not present on the gel of the ordinary soy protein film (Fig. 4), in concordance sizes of inhibition zone determined by the direct detection methods. Consequently, the edible films produced by the presently developed method was verified to contain BLS. Some of the bacteriocins produced by *Bacillus* sp. showed different molecular weights: For example, cerein from *B. cereus* was 8.2 kDa and subtilin from *B. subtilis* was 9.5 kDa [33]. However, the BLS produced in this study was estimated to have 12 kDa molecular weight. According to the result of Image analyzer, the BLS fraction in the gel-electrophoretic pattern of the film was quantitatively 13% of the total bands. Considering an approximately 27% protein content in the film, the amount of BLS in the resulting film was 0.32%.

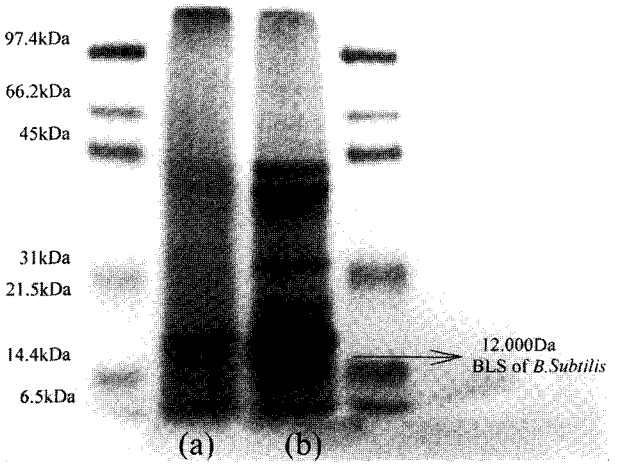

Fig. 4. SDS-PAGE of edible films.
(a) Ordinary soybean film, (b) soybean film with BLS. Each of side lanes contained molecular weight protein standard (Sigma).

Resistance of BLS Film to the Indicator Strain

Figure 5 shows the growth of *E. coli* in the TSA which was covered by either ordinary soy protein film or soy protein film with BLS. The inhibitory effect of cell growth was observed in the soy protein film with BLS. The time required to reach 10^6 cfu/ml of *E. coli* was about 14 h, when covered by ordinary soy protein film, and 24 h when covered by soy protein film with BLS. These results indicate that packaging foods with the BLS film would extend the shelf-life of foods. The maximum cell number in the samples covered by the BLS-containing film was also lower than those covered by ordinary film. The differences between the two kinds of wraps on the surfaces of the samples are shown in Fig. 6, and were visually significantly different after 24 h of storage. The death rate after reaching

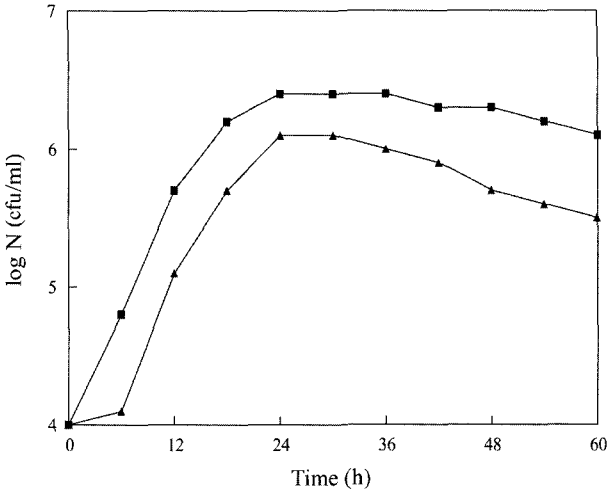

Fig. 5. Growth of *E. coli* on the surface of medium wrapped with two types of films. The initial cell number was all 10^4 cfu/ml.
-■-: ordinary soybean film; -▲-: soybean film with BLS.

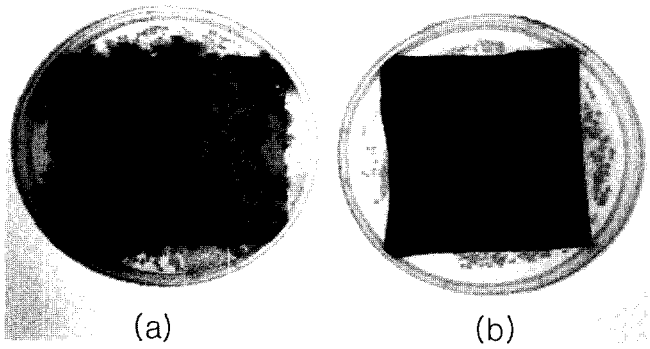

Fig. 6. Microscopic pictures of *E. coli* grown on the surface of media wrapped with the films after 24 h.
(a) Ordinary soybean film, (b) soybean film with BLS.

the peak was also faster in the sample covered with the BLS-containing film than the ordinary film. This might be due to gradual diffusion of BLS from the film. The advantage of the antimicrobial edible film is that the antimicrobial agents in this film can specifically be targeted to post-processing contaminants on the food surface. The diffusion rate of antimicrobials into the product is incorporated into the film and the film properties [1, 19]: Diffusion of antimicrobials through an edible film is influenced by the raw material, procedure for production, hydrophilic characteristics, storage temperature, and duration.

As mentioned, the application of the antimicrobial agent to film material in this study will be useful to prevent the growth of microorganisms on the food surface. It may extend the shelf-life, improve microbial safety of the product, and provide economic advantages for consumers and food industries. Moreover, since byproduct or waste of soybean oil is used for the production of this film, it is also highly attractive from the viewpoint of economics for commercialization.

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