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Antimicrobial Edible Film Developed from Defatted Corn Germ Meal Fermented by *Bacillus subtilis*

KIM, HYUNG-WOOK, I-WOO ROH, KYUNG-MI KIM, IN-SUK JANG, SANG-DO HA[1], KYUNG-BIN SONG[2], SANG-KYU PARK[3], WON-YOUNG LEE[4], KWANGSUP YOUN[5], AND DONG-HO BAE*

Division of Bioscience & Biotechnology, Konkuk University, Seoul 143-701, Korea
[1]Department of Food Science & Technology, Chung-Ang University, Anseong 456-756, Korea
[2]Department of Food Science & Technology, Chungnam National University, Daejeon 305-764, Korea
[3]Material Science & Engineering, Kwangju Institute of Science & Technology, Gwangju 500-712, Korea
[4]Department of Food Engineering, Sangju National University, Sangju 742-711, Korea
[5]Department of Food Science & Technology, Catholic University of Daegu, Hayang 712-702, Korea

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Abstract In order to extend the shelf-life of packaged or coated foods, an antibacterial edible film containing 1.8% of BLS was developed from the defatted corn germ meal, which had been fermented with *Bacillus subtilis* under the optimum condition of pH 7.0–7.5 and 33°C for 33 h. Water vapor permeability of the fermented film (88.3 mg/cm^2·h) was higher than those of the normal corn germ films (75.8 mg/cm^2·h). Protein solubility of the fermented film was also higher than ordinary corn germ film at the pH range of 3–10. The fermented corn germ film had higher tensile strength and lower % elongation (elongation rate) than the ordinary corn germ film. The antimicrobial activity of the film was more than 50% of the maximum activity after film production with heat treatment at 90°C and pH adjustment to 9. When the corn germ protein film with bacteriocin-like substance was applied on the mashed sausage media containing *E. coli*, the bacterial growth inhibition was higher than the ordinary corn protein film.

Key words: Bacteriocin, protein film, antimicrobial film, *Bacillus subtilis*, defatted corn germ meal

Corn protein is a viable renewable resource for producing environmentally safe industrial products. Therefore, a few researchers have undertaken to develop and utilize the biodegradable corn protein films as packaging and coating materials [3, 30]. However, expanded utilization of corn protein films to extend the shelf-life and guarantee the safety of food is limited, because the packaging and coating with corn protein film can only prevent the foods from moisture migration and cross-contamination of foods. Because most people are presently reluctant to use synthetic preservatives in foods, the 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 because of its low toxic or other adverse effects on foodborne pathogens. Bacteriocins are also the bacteria-originated antibacterial proteins that inhibit the growth of other bacteria. They are different from many other traditional antibiotics that have relatively narrow antimicrobial spectra: They are toxic only to bacteria that are closely related to the producing strains [18, 24, 28, 29]. In the last few years, a number of new bacteriocins have been identified from bacteria and characterized. Because of their strong inhibitory effects on the growth of pathogens, the strains and their bacteriocins have potentially been used as natural food preservatives [9, 27]. However, direct application of these strains to the commercial food industry still remains questionable owing to its economical problem.

In the present study, we conducted experiments to economically develop an antimicrobial edible film from defatted corn germ meal, which is a by-product of corn oil production, by inoculating with bacteriocin-like substance (BLS)-producing bacteria, to determine the physical and mechanical properties of the developed edible film, and to verify whether the film could be used as a packaging material in the food industry.

*Corresponding author
Phone: 82-2-450-3756; Fax: 82-2-450-7011;
E-mail: donghoya@konkuk.ac.kr

METERIALS AND METHODS

Bacterial Strains and Culture Conditions

Defatted corn germ meal used in this study was supplied by Shindongbang Co. (Seoul, Korea). The capabilities for fermenting defatted corn germ meal (DSM) and producing bacteriocin-like substance (BLS) were evaluated in 9 strains of bacteria, including *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, such as *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 5 other foodborne pathogens are the 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. 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 a single colony prior to experimental uses [24]. 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 a UV-VIS spectrophotometer (TU-1800, General Electric, Seoul) before inoculation for bacteriocin assay [27].

Determination of Antimicrobial Activity

Antimicrobial activities of BLS-producing strains against indicator species were established by the spot-on-lawn assay. BLS was partially purified from the corn germ media by the agar well diffusion method (paper disc method), using actively grown cells [8, 10, 15, 20, 27–29]. A 100 μl sample cultured in Trypticase Soy Broth (TSB; Difco Laboratories, MD, U.S.A.) was spot-inoculated on the surface of dried corn germ agar medium 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 [9]. The agar well diffusion method (paper disc method) was used for detection of the BLS-producing activity. The cultures of BLS-producing strains were aerobically grown in a liquefied corn germ medium for 24–48 h and inoculated on a rotary shaker fitted at 150 rpm and 33°C. 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 Millipore filters (Millipore S.A 67120, France). The 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 discs 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 the spread-plate culture counting method [24, 25]. 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 a UV-VIS spectrophotometer (TU-1800, General Electric, Seoul). 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 re-adjusted to 7.

Fermentation

The fermentation was carried out in a 500-ml flask with culture media, containing 30 g of corn germ 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 [18].

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, 2% glycerol, the plasticizer 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). Nonfermented 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 [3, 14, 21, 32].

Quantitative Analysis of BLS Contained in Film by SDS-PAGE

Quantitative analysis of BLS contained in film was performed as follows: 1) freeze-dried BLS film was gel-electrophoresed, 2) the BLS band on the gel was determined by comparing with the gel of ordinary corn protein film and by assaying inhibitory abilities of each band, 3) the size of the 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 the BLS band. The approximate protein content in the film was previously analyzed by the Kjeldahl method. The gel electrophoresis of the freeze-dried BLS film was conducted by SDS-PAGE. The molecular weight of each band was estimated by comparison with Bio-Rad Kaleidoscope Prestained standards. After electrophoresis, the gels were stained by the procedure described by Kim *et al.* [12]. At the end of electrophoresis, every band was removed and tested for antimicrobial activity [26]. 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.

Measurements of Physical and Mechanical Properties

Film thickness was measured using a 0–25-mm micrometer screw gauge (Mitutoyo, Tokyo, Japan). The mean thickness of films was determined from the average of measurements at 10 locations [7]. The water vapor permeability of the films was determined as described in ASTM [2]. After equilibration, films were trimmed to an 8.0-cm diameter and sealed with lubricant grease over test cups (diameter= 7.0 cm). Each cup contained distilled water that created internal humidity. All films were oriented with the side that faced the petri dish during drying, facing the lowest humidity. The whole device was weighed and then placed in a climatically controlled chamber (25°C and 50±5% of relative humidity). The weight changes of the cups were periodically recorded and described as the % of moisture evaporated. Forced air movement was not provided and performed by the ASTM method [2, 19]. The protein solubility of films was also determined by the ASTM method [2]. Film samples (2 cm×2 cm) were weighed and transferred into test tubes containing 10 ml of distilled water. The tubes were mildly shaken for 12 h at ambient temperature (25°C). After centrifugation, protein concentrations of the supernatant were measured using Lowry's method. Protein solubility was expressed in percentage of dissolved protein [6, 31].

Tensile strengths and % elongation of the films were measured by the ASTM method [1] using a texture analyzer (TA plus, Lloyd Instruments, England). The films were cut into strips of 20 mm width and 50 mm length. The film samples were clamped into the metal grips of the tensile geometry and stretched at an overhead crosshead speed (120 mm/min). Tensile strength at the maximum and % elongation at break were calculated [1].

Antimicrobial Properties of Fermented Film on Foodstuffs

The antimicrobial activities of BLS-producing bacteria were determined against *E. coli*. Antimicrobial property of the fermented film on foodstuff was measured by placing the films on mashed sausage media, containing 10^2–10^3 CFU/plate 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 [4, 23].

RESULTS AND DISCUSSION

Antimicrobial Spectra of BLS-Producing Strains

The inhibitory spectra of 9 tested strains were evaluated against 6 indicator strains including *E. coli*, *L. monocytogenes*, *St. aureus*, *S. typhimurium*, *Sh. sonnei*, and *V. cholerae*, and

Table 1. Inhibition zone of Bacteriocin-like Substance (BLS) producers against 6 indicator strains in corn germ media.

(unit: mm)

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

*Measured by spot-on-lawn method. 1–4 indicates the length of inhibition.
×: smaller than 1 mm.

the results are presented in Table 1. *Paenibacillus macerans*, *L. fermentum*, and *L. casei* could grow little and produce BLS on the defatted corn germ meal medium. The most superior BLS activities in the other 6 producers were observed against *E. coli*. Specifically, *B. subtilis* among the 9 tested strains showed the highest antibacterial activity against *E. coli*. The strongest inhibition of BLS against *E. coli* was produced by *B. subtilis* and 3 *Pseudomonas* species. *Listeria monocytogenes* and *Salmonella typhimurium* were only partially inhibited by BLS from the above 4 strains, but *Sh. sonnei* and *V. cholerae* 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 often used as a probiotic agent for biocontrol [5, 13, 28].

Growth and BLS Production of *B. subtilis*

In order to examine the antimicrobial activity of *B. subtilis* during the growth, cell-free samples were collected at various time intervals [5]. The relationship between the growth of *B. subtilis* and antimicrobial activity against *E. coli* is shown in Fig. 1. 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 the death phase, in accordance with the report of Park *et al.* [28]. There was a remarkable difference between cell growth and BLS production in corn germ broth medium: At the beginning, bacterial growths and BLS activity on corn germ media were negligible; however, after 24 h of incubation, a fast bacterial growth was found on corn germ medium until 30 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 from corn germ particles, in addition to the inhibitory activity of secondary metabolites.

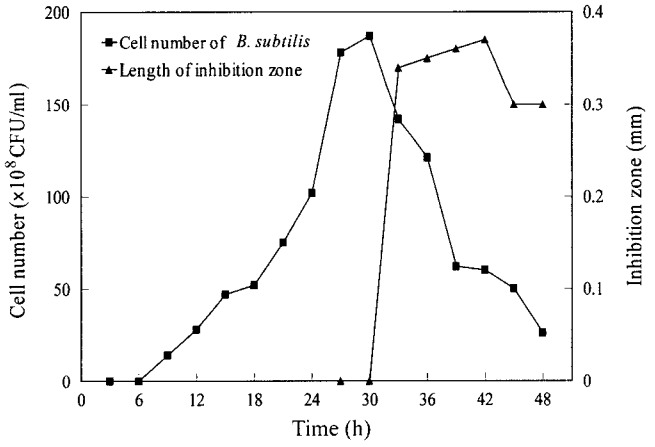

Fig. 1. Relationship between the growth of the *B. subtilis* and the antimicrobial activity against *E. coli*.

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 [9, 20], especially in commercial production [14, 24, 27]. Therefore, the stabilities of BLS against heat and pH were examined, and the results are presented in Fig. 2. 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 90°C for 20 min, but reduced by heating at 121°C for 20 min. Although a substantial loss of the activity occurred under excessive acidic (pH<4) and alkaline (pH>10) conditions, it was also stable at room temperature and wide range of pHs. These results are in accordance with other reports [3], demonstrating that the

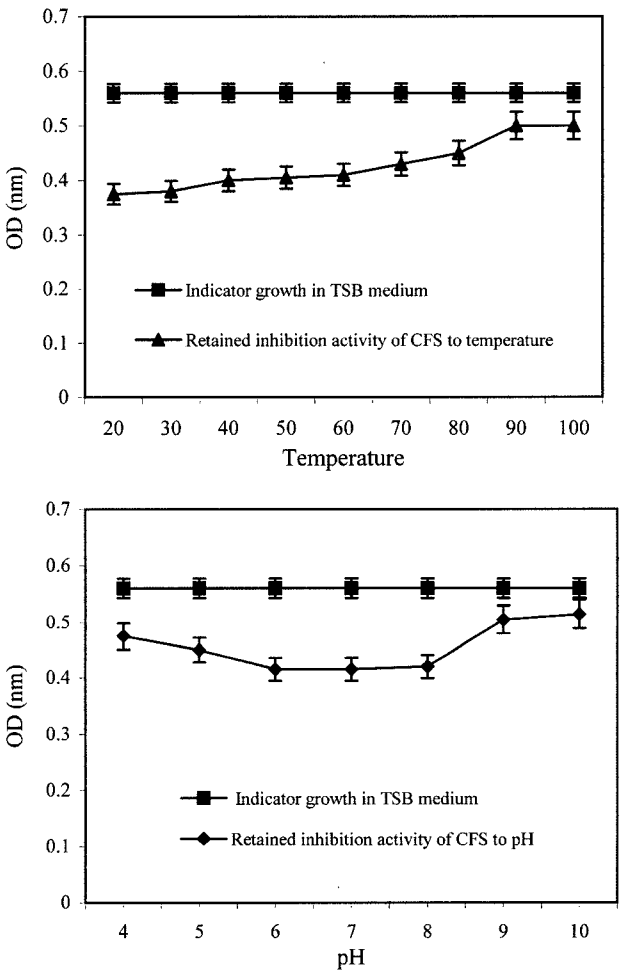

Fig. 2. Stability of cell-free supernatant (CFS) to heat and pH.
*Within errors of less than 5%.

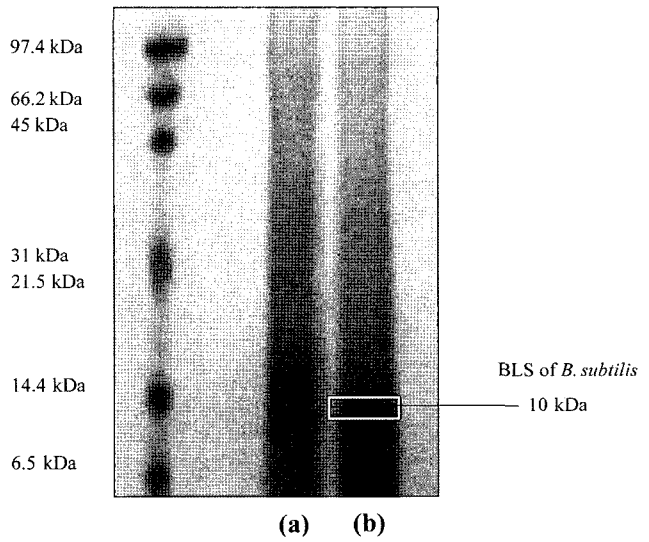

Fig. 3. SDS-PAGE of edible films; (a) ordinary corn germ film, (b) fermented corn germ film with BLS.
The side lane contained protein molecular weight standards (Sigma).

inhibitory compounds from *B. subtilis* are stable proteinaceous compounds [13]. These results suggest that heat treatment at lower than 90°C and pH between 4 and 10 would be allowable for film production.

Production of Fermented Film Containing BLS

In order to verify the presence of BLS in the edible film produced, SDS-PAGE of the film was conducted. The gel-electrophoretic pattern of the film containing BLS revealed a unique band at the region of approximately 10 kDa that was not present on the gel of the ordinary corn protein film (Fig. 3), in concordance with the sizes of inhibition zone determined by the direct detection methods. Consequently, the edible film 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 [28]. However, the BLS produced in this study was estimated to have a 10 kDa molecular size. According to the result of the image analyzer, the BLS fraction in the gel-electrophoretic pattern of the film was quantitatively 10% of the total bands. Considering an approximately 18% protein content in the film, the amount of BLS in the resulting film was 1.8%.

Physical and Mechanical Properties of Films

For the production of fermented corn germ film, the film-forming solution was heated at 75°C for 20 min to unfold protein structure and pasteurization. Heating of film-forming solutions cleaves disulfide bonds and exposes sulfhydryl and hydrophobic groups of corn germ proteins [14, 16, 17]. Formation of new disulfide, hydrophobic, and hydrogen bonds during heat treatment and drying of the film-forming solution are considered to be important in the formation of corn germ film structure [17]. Moreover, proteins undergoing structural changes in the presence of plasticizers affect the mechanical properties of films. Plasteins, the proteins in the plasticizers-containing solution, have higher surface hydrophobicity than the original peptide mixture and tend to aggregate at relatively low temperature [16, 26]. When the film-forming solution is cast, the reformed disulfide bonds link the polypeptide chains together to produce the film structure with aids of noncovalent interactions, resulting in the formation of corn germ film. The antimicrobial activity of the fermented film is still retained after heat treatment. In the present study, the film thicknesses were measured to determine the mechanical properties and water vapor permeability. The central part of the films was thinner than the edges because of the drying behavior, in accordance with other reports [17, 31]. Water vapor permeability is a barrier property, which has most commonly been investigated to assess the ability of edible films to protect foods from the environment and adjacent food components with different water activity. As shown in Fig. 4, the water vapor permeability of the fermented film was higher than those of the control corn germ films. The control and fermented corn germ films permeated 75.8 and 88.3 mg/cm^2·h of moisture, respectively. Considering the amount of moisture evaporated from the unsealed cup (133.8 mg/cm^2·h), the control and fermented corn germ films permeated 57 and 66% of evaporated

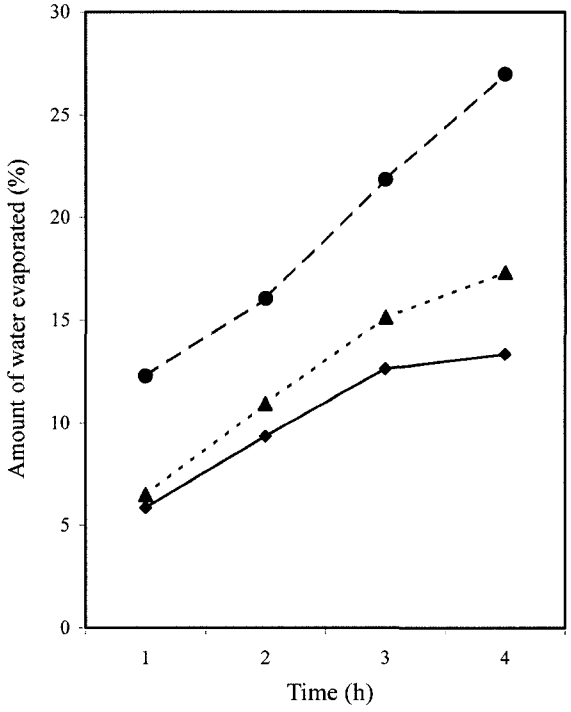

Fig. 4. Amounts of evaporated water in the cup covered with the fermented corn germ film (-▲-) and the control corn germ film (-◆-), and without cover (-●-).

moisture, respectively. The higher water vapor permeability of the fermented film was probably due to a large amount of glycerol added and fragmentation of corn germ proteins during the fermentation. The increases in water vapor permeability of protein films with increasing amounts of plasticizers have earlier been observed by others [19, 32], due to an increase in the free volume between the protein chains. Since most of the plasticizers are hydrophilic, an increase in their concentration favors the absorption of water by the network, thus resulting in a higher level of vapor transfer. The hydrolysis of peptide bonds by the hydrolytic enzymes produced by *B. subtilis* during the fermentation might also cause the changes in water vapor permeability of the film. Changes in water vapor permeability of the film due to hydrolysis of peptide bonds at high pHs have also been noted in other reports [6, 19, 31, 32]. The hydrolysis of peptide chain increases the solubility of corn germ protein, the affinity to water, and consequently, water vapor permeability of the fermented film. Protein solubility profiles of the films are shown in Fig. 5. Protein solubility of the fermented film was higher than the control corn germ film at the pH range of 3–10. The increase in protein solubility of the fermented film resulted from hydrolytic enzymes produced by the BLS-producing strain during the fermentation. Monti and Jost [22] investigated the ability of trypsin, papain, and a neutral protease of *B. subtilis* to break down whey protein aggregates formed during film production. Small protein fragments were observed on SDS-PAGE of the fermented film (Fig. 3) [11]. Hydrolysis of 2.0 and 6.7% of casein with *St. aureus* protease increased the solubility by 25 and 50%, respectively [7]. Therefore, partial hydrolysis of globular proteins by the enzyme increased the protein solubility of the fermented films at a wide pH range, in accordance with other reports [14].

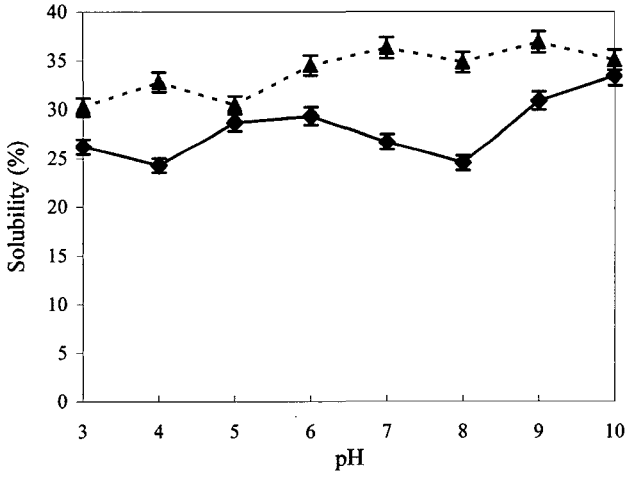

Fig. 5. Protein solubility profiles of the fermented corn germ film (-▲-) and the control corn germ film (-◆-).
* Within errors of less than 5%.

Table 2. Tensile strengths and % elongations of the control corn germ film and the fermented corn germ films at 25°C and 50±5% of RH.

Corn germ film	Film thickness (mm)	Tensile strength (Mpa)	% Elongation
Control film	0.186±0.01	1.2±0.25	119±5
Fermented film	0.206±0.01	1.0±0.25	138±5

Reported values are means of 5 replicates±standard deviation.

The mechanical properties of the films were measured after 48 h of equilibration in controlled environment at 50± 5% relative humidity and temperature of 23±2°C. The control corn germ film had higher tensile strength than the fermented film containing partially hydrolyzed proteins (Table 2). The molecular weight of protein mainly affected the mechanical properties of corn germ films [6]. Aging time taken for film formation has also to be taken into consideration prior to characterization of the mechanical properties of the film [21]. Formation of ordinary corn germ film took only 30 h, while the fermented film formation took 45 h because the high content of glycerol in the fermented film. On the contrary, % elongation of the fermented film was higher than the control corn germ film. The negative correlation between tensile strength and % elongation of films has generally been observed by many researchers [31, 32]. Increasing amount of plasticizer in the fermented film also leads to a decrease in tensile strength and an increase in % elongation of the film. Generally, the mechanical properties of protein films are inferior to those of synthetic films. Nevertheless, because of increasing concern over the environmental safety of nondegradable synthetic packaging materials, there is a demand for natural degradable products from renewable sources as an alternative to synthetic polymers [17]. The fermented film has an adequate durability to be utilized in small food pouches or coatings, although not enough.

Resistance of BLS Film to Indicator Strain on Foodstuff

The fermented film could effectively inhibit both pathogenic and spoilage organisms on a wide variety of foods, which might provide a viable microbial reduction strategy for reducing the incidence of pathogens on foods, even when these packaged products had been opened and contaminated by the consumer [23]. Figure 6 shows the growth of *E. coli* in the mashed sausage media, which was covered by either the ordinary corn germ film or the fermented corn germ film with BLS. The inhibitory effect of cell growth was observed in the fermented film. The time required to reach 10^2 CFU/plate of *E. coli* was about 18 h, when covered by ordinary corn germ film, and 30 h when covered by the fermented film. 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 fermented film was also lower than those covered by

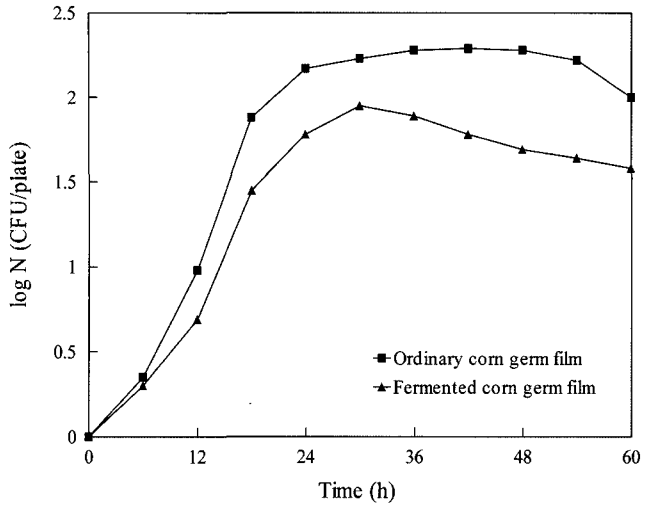

Fig. 6. Growths of *E. coli* on the surface of mashed sausage media covered with the fermented corn germ film and the control corn germ film.
The initial cell numbers were 10^2–10^3 CFU/plate.

ordinary film. The differences between two kinds of wraps on the surfaces of the samples were visually significantly different after 24 h of storage. The death rate after reaching the peak was also faster in the sample covered with the fermented film than the ordinary film. This might be due to gradual diffusion of BLS from the film. The advantage of 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 [3, 14]. Diffusion of antimicrobials through an edible film is influenced by the raw material, procedure for production, hydrophilic characteristics, storage temperature, and duration.

In the present study, we successfully developed an antimicrobial edible film from defatted corn germ meal by inoculating with bacteriocin-like substance (BLS)-producing bacteria and verified the antimicrobial property of the film. The application of the antimicrobial agent to film material in this study will be useful to prevent the growth of microorganisms on food surfaces. It may extend the shelf-life, improve microbial safety of the product, and provide economic advantages for consumers and food industries. Moreover, since by-product or waste of corn germ is used for the production of this film, it is also highly attractive for commercialization from the viewpoint of economics.

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