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# Isolation and Characterization of Surfactin Produced by *Bacillus polyfermenticus* KJS-2

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*Bacillus polyfermenticus* KJS-2 (BP-KJS-2) was used to produce a lipopeptide-type surfactin. To accomplish this, a surfactin-producing BP-KJS-2 was fermented by soybeans. The surfactin was then purified by a procedure including ethanol treatment and preparative chromatography. Next, the biochemical structure of the purified surfactin was analyzed by electrospray ionization mass spectrometry (ESI-MS) and high-resolution ESI Q-Tof mass spectrometry (Q-Tof MS). In addition, the masses of the four peaks were determined to be 1007, 1021, 1035, and 1049 *m/z* revealing that the compound was mixture with *quasi*-molecular ions. Taken together, these findings indicated that the lipopeptide had a cyclic structure and amino acid composition of Gln-Leu-Leu-Leu-Val-Asp-Leu-Leu, and that the major lipopeptide product of BP-KJS-2 is the surfactin isoform. In addition, this lipopeptide showed strong antimicrobial activity against bacteria at the level of 0.05 mg/mL.

**Key words:** *Bacillus polyfermenticus* KJS-2, Surfactin, Cyclic lipopeptide, Antimicrobial activity

## INTRODUCTION

Surfactins are amphiphilic molecules that tend to decrease interfacial tension. These compounds are applied in an extremely wide variety of industrial processes involving emulsification, foaming, detergency, wetting, dispersing and solubilization (Gautam and Tyagi, 2006). In addition, surfactins have been widely utilized in the cosmetic, specialty chemical, food, pharmaceutical, and agricultural industries, as well as in cleansers, enhanced oil recovery systems, and the bioremediation of oil-contaminated sites (Yeh et al., 2006). The properties of surfactins are clearly superior to those of its synthetic counterparts; therefore, its commercial potential has increased. Accordingly, there is now interest in the isolation of new microorganisms capable of producing surfactins (Ilori et al., 2005). Several structurally diverse surface-active molecules are produced by a wide spectrum of microorganisms (bacteria, fungi, and yeasts). In addition, there are six major types of surfactins: hydroxylated and cross linked fatty acids (mycolic acids), glycolipids, lipopolysaccharides, lipoproteins-lipopeptides, phospholipids, and complete cells (Dehghan-Noudeh et al., 2005). Natural surfactins are comprised of a mixture of isoforms with physiological properties that differ slightly due to variations in chain length and branching of their hydroxyl fatty acid components, as well as substitutions in the amino acid components of the peptide rings (Lim et al., 2005).

Surfactin production by solid-state fermentation of soybean curd residue (okara), a solid substrate, was performed using a *Bacillus subtilis* (Mizumoto and Shoda, 2007). This process is related to the production of a higher concentration of iturin A and Surfactin in solid–state fermentation, such as occurs in the manufacture of tofu (Lim et al., 2005; Mizumoto and Shoda, 2007).

The production of secondary metabolites such as antibiotics is part of a complex response to conditions of nutritional deprivation. Indeed, surfactins with antibiotic activities are produced as biocontrol agents to protect cells from attacks by other micro-organisms.

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Specifically, cyclic lipopeptide surfactins play an important role in biological activities, such as antibacterial, antifungal or antiviral activity, cytolytic activity, inhibition of fibrin clot formation, and the stimulation of macrophages (Nakano et al., 1988; Kim et al., 2004). In this paper, BP-KJS-2 was identified from 'Bispan' strains, which are described in the Japanese Pharmacopoeia as amylolytic bacilli (Lee et al., 2001). We then conducted preparative separation of a surfactin mixture obtained from BP-KJS-2, which was produced by fermented soybeans. Surfactin homologues were then identified by high resolution reversed phase HPLC and fractionated by preparation liquid chromatography. The isolated surfactins were also characterized by mass spectrometry (electrospray LC/MS and high-resolution Q-Tof mass spectrometry). In addition, we evaluated the potential antimicrobial activity of surfactins produced by BP- KJS-2. Finally, the antimicrobial spectrum and several properties of the surfactin against pathogenic bacteria are described.

## MATERIALS AND METHODS

### Chemicals

Standard surfactin was purchased from Sigma (St. Louis, MO, USA). HPLC grade ethanol was purchased from Burdick and Jackson (Muskeson, MI, USA). All other analytical grade chemicals were purchased from Sigma (St. Louis, MO, USA).

### Microorganisms

BP-KJS-2 was isolated from *B. polyfermenticus* n. sp, which is comprised of several strains of *B. polyfermenticus* (Kim et al., 2008). The basal medium used for the growth of bacteria was tryptone soy broth (TSB). The microorganisms used to evaluate the antimicrobial activity were *Enterococcus faecalis* ATCC 2921, *Streptococcus parauberis* DSM 6631, *Streptococcus iniae* ATCC 29178, *Lactococcus garviae* KCCM 40698, *Staphylococcus aureus subsp. aureus* ATCC 29213, *Staphylococcus aureus subsp. aureus* ATCC 25923, *Staphylococcus epidermidis* KCTC 1917, *Flexibacter tractuosus* ATCC 23168, *Flexibacter* sp. KCTC 2670 *Vibrio harveyi* KCCM 40866*, Vibrio vulnificus* ATCC 27562, *Vibrio ordalii* KCCM 41669, *Vibrio harveyi* ATCC 14126, *Escherichia coli* ATCC 25922, *Salmonella enteritidis* KCCM 12021, and *Edwardsiella tarda* KCTC 12267. All microorganisms used in this study were obtained from the American Type Culture Collection (ATCC), the Korea Culture Center of Microorganisms (KCCM) or the Korean collection for type cultures (KCTC).

### Preparation of seeding culture

Each bacterium was cultured in 3 mL of TSB medium (Becton and Dickinson, USA). One milliliter of preculture was then used to inoculate 100 mL of TSB medium, after which the samples were incubated in a rotary shaker at 200 rpm and 37°C for 16 h. Bacterial concentrations were determined by measuring the optical density at 600 nm and then used as a seed for solid-state fermentation when a value of 1.2 was attained.

### Solid-state fermentation

Soybeans (Hamyang, Korea) were obtained and wrapped in a sheet of commercial wrapping film until use. Prior to use, 100 g of soybeans were placed in a 1 L Erlenmeyer flask and autoclaved twice at 120°C for 20 min at an interval of 5 h to kill any spore-forming microorganisms inhabiting the material. Next, the samples were cooled to room temperature, after which 30 mL of the seeding culture described above were added and the samples were mixed with a stainless steel spatula. The flasks were then incubated statically at 40°C for 48 h.

### Extraction and partial purification of surfactins

Two equivalent volumes of ethanol were added to the fermented soybeans, after which the mixture was shaken for 10 min to extract the surfactins. The crude extract was then centrifuged at 18,000×g for 10 min at 4°C, filtered through a 0.2-μm pore size polytetrafluoroethylene (PTFE) membrane (JP020, Advantec, Tokyo) and then concentrated in 4 mL of ethanol. The surfactins were then analyzed using a modified version of the method described by Mukherjee et al., 2005. The optimal conditions of detection were achieved using an HPLC (Agilent Technologies, 1100 series, USA) column (Agilent Zorbax SB-C18, 250 mm × 4.6 mm, 5 μm) and detected at 205 nm with a UV monitor (Agilent Technologies, 1100 series, Photo-Diode Array UV/Vis detector, USA). The column temperature was held at room temperature and the mobile phase consisted of 60:40 (*v*/*v* %) ethanol and 10 mM phosphate buffer (pH 6.0). Chromatography was performed isocratically at a flow rate of 0.6 mL/min. Each fraction was then applied to a preparative chromatography (Buchi, Hwashin Co. Ltd. Seoul, Korea) column (Buchi, C18, 75 mm × 12 mm) and evaluated at 205 nm using a UV monitor (Buchi, UV photometer C-635, Korea). The same mobile phase described above was used. Next, ESI-MS analysis was performed on an Agilent Technologies G2708DA electrospray mass spectrometer equipped with an Agilent Technologies Atmospheric Pressure Ionization (API) interface fitted with

a hexapole ion guide. The optimal conditions for the analysis of surfactin employed pneumatic nebulization with nitrogen (45 p.s.i.) and a counter flow of nitrogen (12 L/min) heated to 350°C for the nebulization and desolvation of the introduced liquid. The molecular mass of the purified biosurfactant was determined by Q-Tof MS.

### Antimicrobial activity of surfactins via the spot-on-lawn method

The antimicrobial activity of the surfactins was assayed by the spot-on-lawn method (Kim et al., 2004). Briefly, $10^7$ cells of each of the indicated strains per overlay were plated on TSA plate. 50 mL of surfactin (0.1 g and 0.05 mg suspended in 1 mL of 50% acetone) were spotted onto the plate. The antimicrobial activity of the surfactin was then evaluated based on the diameter of the cleared zone that developed after incubation (Table I).

## RESULTS AND DISCUSSION

Cyclic lipopeptides including surfactin, iturin, fengycin, and lichenysin are the major classes of biosurfactants produced by *Bacillus* species (Mukherjee et al., 2005). lipopeptides were obtained by solid-state fermentation using soybeans. Although BP-KJS-2 is comprised of several strains of *Bacillus subtilis*, each of the strains produce lipopeptide molecules that are qualitatively and quantitatively similar. When standard surfactins (Sigma, USA) were subjected to RP-HPLC on a C18 column, they resolved into four major peaks (Fig. 1). Similarly, crude surfactins obtained from the cell-free supernatants of BP-KJS-2 that were subjected to further purification by preparative chromatography with an isocratic comprised of 60:40 (v/v %) ethanol and 10 mM phosphate buffer (pH 6.0) the partial peak was resolved into four fractions (Fig. 1). The molecular mass and identities of each fraction revealed that the sample contained *quasi*-molecular ions at $m/z$ = 1008, 1022, 1036, and 1050 ($[M+H]^+$), and $m/z$ = 1030, 1044, 1058, and 1072 ($[M+Na]^+$). Standard surfactin, which was originally isolated from a soil strain of *Bacillus subtilis*, is comprised of a macrolide containing the heptapeptide sequenced Glu-Leu-Leu-Val-Asp-Leu-Leu and a lipid portion comprised of a mixture of several a-hydroxy-fatty acids with a chain length of 13-15 carbon atoms (Lim et al., 2005). The products were further investigated by evaluating the surfactins produced by BP-KJS-2 by Q-Tof MS. Fig. 2 shows the product patterns determined by Q-Tof MS for surfactins produced by BP-KJS-2. The amino acid sequence of BP-KJS-2 represents Gln-Leu-Leu-Leu-Val-Asp-Leu-Leu. These results indicate that the amino acids present in the surfactins produced by BP-KJS-2 are similar to those present in sigma surfactin. There were also well-resolved groups of peaks with $m/z$ values between 1000 and 1060. These groups of peaks could be attributed to the isoform ensembles of surfactins, iturins, and fengycins, which represent the well-known surfactin families produced by *Bacillus* strains (Vater et al., 2002). Fig. 2 shows

**Table I.** Antimicrobial activity of surfactin isoforms produced by BP-KJS-2 using the spot-on-lawn method. TSA, tryptic soy agar; ME, malt extract

| Pathogenic Microoganisms | Culture Medium | Incubation Temperature (°C) | Inhibition (mm) |
|---|---|---|---|
| | | | Crude surfactin isolated from *B. polyfermenticus* KJS-2 |
| Gram-Positive bacterial | | | |
| *Streptococcus parauberis* DSM 6631 | TSA | 37 | 12 |
| *Streptococcus iniae* ATCC 29178 | TSA | 25 | 12 |
| *Lactococcus garviae* KCCM 40698 | TSA | 25 | 14 |
| *Enterococcus faecalis* ATCC 2921 | TSA | 25 | 14 |
| *Staphylococcus aureus subsp. aureus* ATCC 29213 | TSA | 25 | 12 |
| *Staphylococcus aureus subsp. aureus* ATCC 25923 | TSA | 25 | 12 |
| *Staphylococcus epidermidis* KCTC 1917 | TSA | 25 | 15 |
| Gram-negative bacteria | | | |
| *Flexibacter tractuosus* ATCC 23168 | TSA | 25 | 12 |
| *Flexibacter* sp. KCTC 2670 | TSA | 25 | 12 |
| *Vibrio harveyi* KCCM 40866 | TSA | 25 | 12 |
| *Vibrio harveyi* ATCC 14126 | TSA | 25 | 12 |
| *Vibrio vulnificus* ATCC 27562 | TSA | 25 | 10 |
| *Vibrio ordalii* KCCM 41669 | TSA | 25 | 12 |
| *Edwardsiella tarda* KCTC 12267 | TSA | 25 | 10 |
| *Escherichia coli* ATCC 25922 | TSA | 25 | 10 |
| *Salmonella enteritidis* KCCM 12021 | TSA | 25 | 9 |

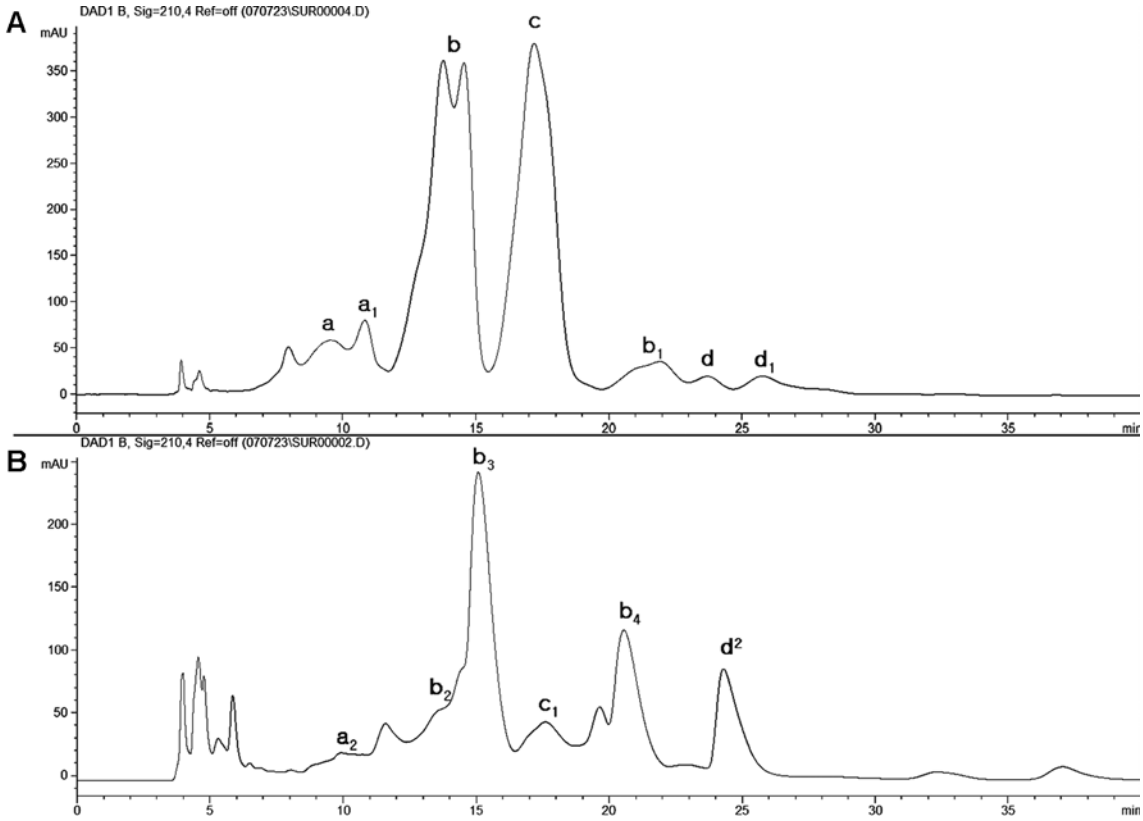


**Fig. 1.** Standard surfactin provided by Sigma Co. Ltd. **A** and Separation of surfactin isoforms isolated from BP-KJS-2, **B** using a mobile phase solution consisting of 60:40 (v/v %) ethanol and 10 mM phosphate buffer (pH 6.0). The compositions of the surfactin isoforms showed a significant similarity. a, $a_1$, $a_2$, M.W. 1007; b, $b_1$, $b_2$, $b_3$, $b_4$, M.W. 1021; c, $c_1$, M.W. 1035; d, $d_1$, $d_2$, M.W. 1049.

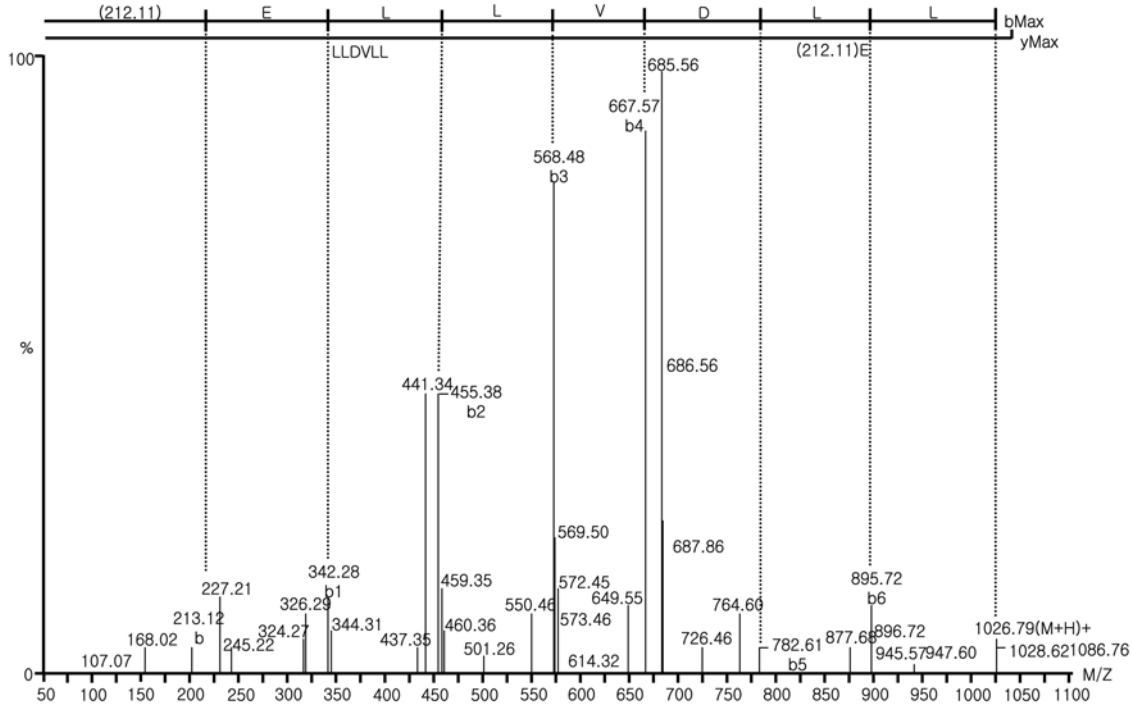


**Fig. 2.** ESI Q-TOF MS of purified surfactins produced by BP-KJS-2

that Q-Tof MS can be used to detect lipopeptide surfactins with high sensitivity and precision. The antimicrobial activity of lipopeptides produced by BP-KJS-2 against pathogenic bacteria are shown in Table I. The lipopeptides showed higher activity against Gram-positive cocci than against Gram-negative bacilli. These results are similar to previously reported antimicrobial activities of biosurfactants against pathogenic bacteria (Fernandes et al., 2007). In this study, the compounds were found to exert antimicrobial activity against most of the *Staphylococcus, Streptococcus,* and *Enterococcus,* as well as against *Flexibacter, Vibrio, Edwardsiella, Escherichia*, and *Salmonella.* Specifically, the mean size of the zones of inhibitions produced against the gram positive and gram negative bacteria were 13 and 11 mm in the 0.05 mg/mL of surfactin, respectively. It is important to note that some of these strains are resistant to at least two β-lactams (Fernandes et al., 2007). The activity against Gram-negative bacteria was lower than the activity against Gram-positive bacteria. Recently, interest in the potential industrial application of surfactins in medicine has increased. This is partially because their anti-adhesive activity against a variety of pathogens indicates their potential usefulness for the protection of biomaterials and functional food composition (Walencka et al., 2008). The results of the present study indicate that the lipopeptide surfactants produced by the *Bacillus* genus present a great potential for biotechnological and biopharmaceutical applications due to their biological properties. Future studies should be conducted to investigate the chemical structure and cellular toxicity of these compounds.

## REFERENCES


Dehghan-Noudeh, G., Housaindokht, M., and Bazzaz, B. S. F., Isolation, characterization, and investigation of surface and hemolytic of a lipopeptide biosurfactant produced by *Bacillus subtilis* ATCC 6633. *J. Microbiol.*, 43, 272-276 (2005).

Fernandes, P. A. V., Arruda, I. R. D., Santo, A. F. A. B. D., Araújo, A. A. D., Maior, A. M. S., and Ximenes, E. A., Antimicrobial activity of surfactants produced by *Bacillus subtilis* R14 against multidrug-resistant bacteria. *Braz. J. Microbiol.*, 38, 704-709 (2007).

Gautam, K. K. and Tyagi V. K., Microbial surfactants: A review. *J. Oleo. Sci.*, 55, 155-166 (2006).

Ilori, M. O., Amobi C. J., and Odocha A. C., Factors affecting biosurfactant production by oil degrading *Aeromonas* spp. Isolated from a tropical environment. *Chemosphere*, 61, 985-992 (2005).

Kim, P. I., Bai, H., Bai, D., Chae, H., Chung, S., Kim, Y., Park, R., and Chi, Y. T., Purification and characterization of a lipopeptide produced by *Bacillus thringiensis* CMB26. *J. Appl. Microbiol.*, 97, 942-949 (2004).

Kim, S. M., Lee, K. H., Lee, N. K., Kim, C. J., Kim, C. H., and Paik, H. D., Antagonistic activity of polyfermenticin SCD against *Helicobacter pylori* KCTC 2948. *J. Microbiol. Biotechnol.*, 14, 148-152 (2004).

Kim, K. M., Lee, J. Y., Hong, Y. G., Lee, S. K., and Kang, J. S., The stabilization of lactic acid bacteria, *Bacillus polyfermenticus* KJS-2. *J. Korean Soc. Food Sci. Nutr.*, 37, 1044-1048 (2008).

Lee, K. H., Jun, K. D., Kim, W. S., and Paik, H. D., Partial characterization of polyfermenticin SCD, a newly identified bacteriocin of *Bacillus polyfermenticus*. *Lett. Appl. Microbiol.,* 32, 146–151 (2001).

Lim, J. H., Park, B. K., Kim, M. S., Hwang, M. H., Rhee, M. H., Park, S. C., and Yun, H. I., The anti-thrombotic activity of surfactins. *J. Vet. Sci.*, 6, 353-355 (2005).

Mizumoto, S. and Shoda, M., Medium optimization of antifungal lipopeptide, iturin A, production by Bacillus subtilis in solid-state fermentation by response surface methodology. *Appl. Microbiol. Biotechnol.*, 76, 101-108 (2007).

Mukherjee, A.k. and DAS, K., Correlation between diverse cyclic lipopeptides production and regulation of growth and substrate utilization by *Bacillus subtilis* strains in a particular habitat. *FEMS Microbiol. Ecolo.,* 54, 479-489 (2005).

Nakano, M. M., Marahiel, M. A. and Zuber, P., Identification of a genetic locus required for biosynthesis of the lipopeptide antibiotic surfactin in *Bacillus subtilis*. *J. Bacteriol.*, 170, 5662-5668 (1988).

Vater, J., Kablitz, B., Wilde, C., Franke, P., Mehta, N., and Cameotra, S. S., Matrix-assisted laser desorption ionization-time of flight mass spectrometry of lipopeptide biosurfactant in whole cells and culture filtrates of *Bacillus subtilis* C-1 isolated from petroleum sludge. *Appl. Environ. Microb.*, 68, 6210-6219 (2002).

Walencka, E., Różalska, S., Sadowska, B. and Różalska, B., The influence of Lactobacillus acidophilus-derived surfactants on staphylococcal adhesion and biofilm formation. *Folia Microbiol.* 53, 61-66 (2008)

Yeh, M. S., Wei, Y. H. and Chang, J. S., Bioreactor design for enhanced carrier-assisted surfactin production with *Bacillus subtilis*. *Process Biochem.*, 41, 1799-1805 (2006).