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Production of an antimicrobial compound by *Bacillus subtilis* LS 1-2 using a citrus-processing byproduct

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Abstract−The aim of this study was to investigate the antimicrobial activity of *Bacillus subtilis* LS 1-2 grown on citrus juice waste (CJW). Citrus-juice waste (CJW) was obtained from the residue of squeezed citrus fruits. To use CJW as a raw material for the growth of *B. sutilitis*, a citrus-juice medium (CJM) was prepared by treating CJW with $Ca(OH)_2$. No antimicrobial activity was observed either in the culture broth of Luria Broth medium or in CJM itself. The maximum antimicrobial activity was obtained after 24 hr of cultivation (culture) of *Bacillus sutilitis* LS 1-2 in CJM. The culture supernatant exhibited inhibitory activity against *E. coli* O-157 (140 AU/mL), *Staphylococcus aureus* (180 AU/mL), and *Candida albicans* (260 AU/mL), respectively. *Bacillus subtilus* LS 1-2 also produced protease (3,600 U/ml) and amylase (290 U/ml) in CJM. Antimicrobial activity of the culture broth was stable for 1 hr at 100 °C, pH 2-10, and bile acid (concentration needs, 1 mM TDOC and 0.27 mM DOCmM), respectively. These results indicate the potential of CJW as a novel bioresource and the scope of probiotic applications of *Bacillus subtilis* LS 1-2 in various industrial applications.

Key words: Antimicrobial Compound, *Bacillus subtilis*, Citrus Waste Medium, Relative Performance Index

INTRODUCTION

Citrus is the largest fruit crop in the world. Ripe citrus fruits are either used as fresh fruits or processed into juice. In recent years the citrus-juice production processes have been further developed and commercialized. For juice production, citrus fruits are washed, punched without peeling to remove citrus-oil, and directly pressed to extract citrus juice. A byproduct of squeezing the fruit, citrus-juice waste (CJW), is a waste which contains 90% liquid. Typically, this CJW is sent to waste-disposal plants or dumped into the ocean. Various efforts have been launched to find uses for it. Pectin, a product of citrus waste, has long been used as a food additive that thickens and stiffens products such as jams and jellies. Several bioactive compounds including anti-oxidative d-limonene, cancer-inhibiting hesperidin and antimicrobial naringin [1-4] also have been identified in CJW. However, commercialization of valuable bioactive compounds in CJW has made little progress due to the economic and technical difficulties of the separation process. Another approach for utilizing CJW is to develop it as an animal feed stock [5]. CJW can be dried and used as a dry feed additive. However, the presence of pectin and the high water (90%) and sugar contents significantly increase the cost of drying. Another approach is to produce pellet-type feed stock by dewatering the CJW with NaOH or CaO treatment [6]. The solids can be processed into pellet-type feed. However, disposal of eluted water containing high concentration of organics, (i.e., 50,000 ppm B.O.D.), poses a significant burden to the wastewater treatment facility. Dumping of CJW in to the ocean is scheduled to be prohibited by 2012 [7]. This indicates an urgent need to develop an effective strategy for using CJW. Towards this goal, we attempted to produce antimicrobial compounds utilizing the liquid waste of CJW. The growing prevalence of antibiotic resistance due to extreme overuse of antibiotics in animal feed is an emerging public health crisis. Recently, the European Commission prohibited the use of antibiotics in animal feed. Consequently, an urgent need is felt globally to look for alternatives to antibiotics. Probiotics, a microbial culture broth containing antimicrobial compounds, and their producer GRAS strains, are gaining attention because of their safety and their potentials for improving animal health [8]. Several microorganisms under the name of "Probiotics" have been proposed and used in a wide range of field trials, ranging from diarrheal disease treatment to cancer prevention. Due to the numerous applications of probiotics, there is an attempt to screen and to identify new probiotic strains with particular characteristics that would be appropriate for specific applications.

In this study, we isolated a *Bacillus subtilis* from soybean paste that produced antimicrobial compounds using CJW as the growth medium. In addition, the production and characterization of antimicrobial compounds from *Bacillus subtilis* LS 1-2 strain using CJM were carried out.

EXPERIMENTAL SECTION

1. Chemicals and Materials

LB (Luria-Bertani), LBS and TSB were purchased from BactoTM (Becton, Dickinson and Company, USA). Proteinase K, Pronase E, Lipase, Amylase and Catalase were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All chemicals used in this study were of analytical grade.

2. Screening and Isolation

Korean traditional soybean paste and Korean hot pepper paste were homogenized with 0.85% saline and serially diluted (10^{-1} to

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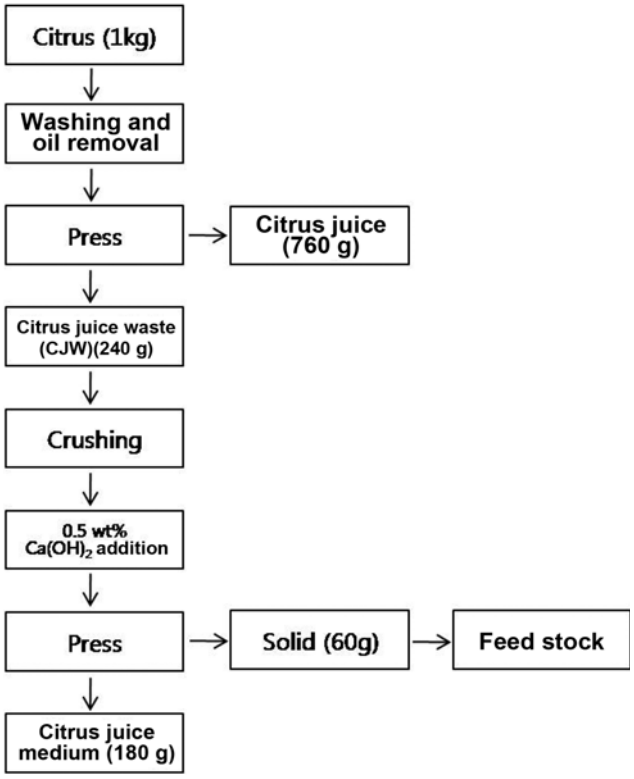

Fig. 1. Production process of citrus-juice medium (CJM) from citrus-juice waste (CJW); CPP-citrus pulp pellet.

10^{-8}). One hundred microliters of appropriately diluted samples was spread on an LB and nutrient agar plate. Single colonies were isolated on agar plates after incubation at 37 ºC for 12 hr. Following isolation, individual colonies were tested for the ability to grow on citrus-juice medium (CJM) (5% solid phase, 2% agar, pH 7) at 30 ºC for 24 hr. CJM was prepared from CJW by treating it with $Ca(OH)_2$ (Fig. 1) [9].

All colonies were stained by the basic Gram method selective for *Bacilli* strains. Bacilli obtained from the resulting colonies were microscopically confirmed. Cellular catalase activity levels of the supernatant and the cell extract were determined at room temperature, according to the method of Beer and Sizer [10] which monitored the degradation of hydrogen peroxide at 240 nm.

The screening of prospective antimicrobial activity was performed based on the compound's ability to inhibit pathogens measured by the relative performance index (RPI) [11]. For gram-negative and gram-positive bacterium, RPI was calculated as follows:

1) RPI anti-*S.aureus* for anti- *Staphylococcus aureus* ($p<0.9544$)
$-100<[\{(X1-X1a)/\sigma\}-2]\times25<0$

2) RPI anti-*E. coli* for anti- *Escherichia coil* O-157 ($p<0.9544$)
$0<[\{(X1-X1a)/\sigma\}-2]\times25<100$

3) RPI overall

RPI anti-*S.aureus*+RPI anti-*E. coli*=RPI Overall

X: Single observation value of a strain

Xa : Average of all observations from all strains being ranked

σ: Standard deviation of all observations from all strains being ranked

3. Identification of the Strain

We performed biochemical testing on cultures using the API 50 CHB kit (bioMerieux Co. France). Genotypically, the 16S bacterial rRNA gene (16S rDNA) was amplified by the polymerase chain reaction (PCR) and sequenced. The sequence of the PCR product was compared with known 16S rRNA gene sequences at the National Center for Biotechnological Information (NCBI) 13.16 (Weisbug 1991).

4. Growth and Production of Anti-microbial Substance from *Bacillus subtilis* LS 1-2

Bacillus subtilis LS 1-2 was cultured with 2% culture of CJM in 50 mL CJM in 250 mL flasks for 24 hr at 30 ºC. In a 2.5 L jar fermenter (Kobiotech, Korea), culture conditions were as follows: inoculum 10% (v/v); working volume, 1 L; temperature 30 ºC, pH, 6.5; agitation, 550 rpm; aeration rate, 1 vvm and cultivation time; 3 days. Samples were collected from cultures at regular intervals and tested for antimicrobial activity by the agar well diffusion method. After growth, culture broth was centrifuged at 13,000 rpm for 5 min at 4 ºC for removing cells, and the supernatants were filtered through 0.22 μm membranes (Sartorius, USA).

5. Detection of Antimicrobial Activity

To test the antimicrobial activity of the supernatant, the agar well diffusion method using 8-mm (outer diameter) stainless steel cylinders, was used [12]. *E. coli* O-157, *Staphylococcus aureus* and *Candida albicans* were used as test organisms. Ten ml of soft agar (0.8%) containing 10^6-10^7 CFU/mL of test organisms was overlaid on a previously poured 24-hr-old sterile trypticase soy broth agar (1.5%) plate. Sterilized culture supernatant (100 μL) was placed in the cup on the agar plate. The plates were incubated at 37 ºC for 24 hr. Antimicrobial activity was evaluated by measuring the diameter of inhibition zones without bacterial growth in mm (8 mm of the cylinder diameter was included). Activity was defined as the reciprocal of the dilution after the last serial dilution that produced a zone of growth inhibition, and it is expressed as activity units (AU) per milliliter (e.g., 10 mm of inhibition zone=100 AU/ml).

6. Enzyme Activity and Bile Tolerance Test

The protease activity of the culture broth was assayed according to the method of Shimizu et al. [13]. One unit of enzyme was defined as the amount of the enzyme that solubilized a 1 μg equivalent of tyrosine in 1 min. Amylase activity was assayed according to Bernfeld [14] using soluble starch as the substrate. Briefly, an aliquot of enzyme was incubated with a reaction mixture, containing 50 mM phosphate buffer at pH of, 7.0 and 1% soluble starch, at 37 ºC for 30 min. The reaction was stopped and the liberated maltose was measured by the addition of DNS (3,5-dinitrosalicylic acid) reagent. One enzyme unit is defined as the amount required to liberate 1 μmol of reducing sugar under the specified conditions.

Bile tolerance was determined by minimum inhibitory concentration (MIC) of the conjugated bile salt, taurodeoxycholic acid sodium salt (TDOC 25 mM=1.2% w/v), and the nonconjugated salt deoxycholic acid (DOC 25 mM=1% w/v). The MICs for bile tolerance were evaluated [15] by comparing the MICs against TDOC and DOC of *Bacillus subtilis* ATCC21332, *B. licheniformins*, *B. coagulans*, *B. cereus* (Bactisubtil) and *B. clausii* (Enterogermina), *in vitro*. *Bacillus subtilis* ATCC 21332, *Bacillus coagulans*, *Bacillus licheniformis*, *Bacillus cereus* and, *Bacillus clausii* were used as the positive controls for bile tolerance.

7. Stability Against Enzymes, Heat and pH

The chemical nature of the antimicrobial substance produced by

the strain *Bacillus subtilis* LS 1-2 in CJM was investigated by determining its sensitivity with four enzymes (Proteinase K, Pronase E, Lipase and α-amylase) for 2.5 hr at 37 ºC incubator. One milliliter of the media supernatant was mixed with equal volumes of each enzyme (4 mg/mL) and incubated for 2.5 hr at 37 ºC. Before antimicrobial activity testing, the reaction samples were boiled at 100 ºC for 5 min to inactivate the enzymes, thereby stopping the reaction.

To determine the heat stability, the supernatants were exposed to temperatures ranging from −70 to 100 ºC for 24 hr and transferred to an autoclave at 121 ºC for 15 min. Incubated samples at different temperatures are taken at regular intervals and tested for antimicrobial activity. The pH stability of the antimicrobial compound was evaluated by the agar diffusion method at a variety of pH values (pH 2 to pH 10) for 26 hr. The supernatant was neutralized to pH 7.0 before testing for antimicrobial activity.

RESULTS AND DISCUSSION

1. Screening and Isolation

Bacilli are widely used as probiotics or feed and food additives, and they are generally regarded as safe strains. They are well known for their ability to produce pectinase, by using pectin in citrus peel as the sole carbon source [16-18]. Although commercial pectin is obtained from citrus peel, soy hull is a potentially inexpensive commercial source of pectin. Since citrus and soy pectin have a structural similarity, it was anticipated that bacilli strains isolated from soybean paste would be able to use the pectin in citrus peel as a sole carbon source. We isolated 124 bacilli strains from soybean food pastes and tested isolated strains for their antimicrobial activities against gram-positive and gram-negative strains. From the screening, we obtained 63 strains showing antimicrobial activity against both gram-positive and gram-negative microbes: five strains showing antimicrobial activity against gram-positive bacteria (*E. coli* O-157) and three strains showing activity against gram-negative bacteria (*S. aureus*) (data not shown). Based on these results, 71 strains that produced antimicrobial substances on citrus juice medium were chosen as potential candidates for probiotics and were compared with the commercial counterparts.

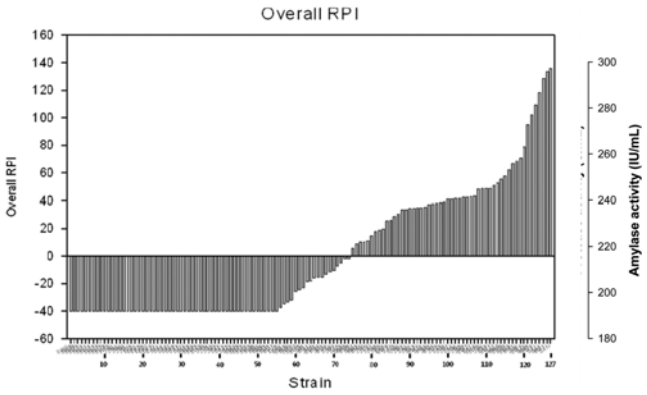

Fig. 2. Overall RPI values of total screened strains.

The RPI statistical method was used to identify the strains with the best potential and was applied in antimicrobial activity tests against *Escherichia coli* O-157 and *Staphylococcus aureus* (50%: 50% inhibition). The antimicrobial activity was determined as described earlier. Therefore, in this study, isolated strains were ranked in terms of their antimicrobial activity based on RPI. Fig. 2 shows the RPI values of all the isolated strains. Based on the results, *Bacillus subtilis* LS 1-2 was ultimately selected as the potential candidate for further study.

2. Strain Identification

Physiological and biochemical analysis of *Bacillus subtilis* LS 1-2 showed that the strain was gram positive and catalase positive. Biochemical tests using API 50 CHB Kit (bioMerieux Co. France)

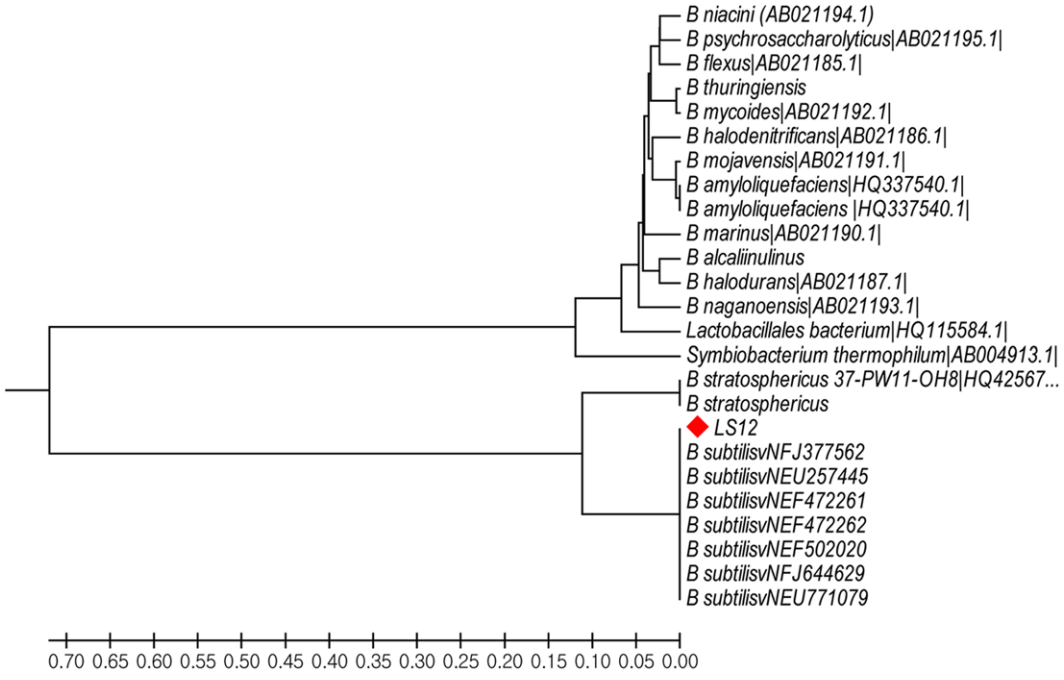

Fig. 3. Phylogenetic tree of *Bacillus subtilis* LS 1-2 and related *Bacillus* sp.

Table 1. Comparison of antimicrobial activity in different culture media

Medium	Antimicrobial activity
LB	-
LBS	-
TSB	-
MRS	-
Pectin	-
LB/LBS/TSB/MRS+Pectin	-
CJM	+
CJM (No cells)	-

+: Antimicrobial activity
−: No Antimicrobial activity

Table 2. Tolerance of *Bacillus subtilis* LS 1-2 against bile acids

Indicator strains	MIC of TDOC (mM)	MIC of DOC (mM)
Bacillus subtilis LS 1-2	≥1.0	0.27
Bacillus subtilis ATCC 21332	≥1.0	0.20
Bacillus licheniformis	≥1.0	0.15
Bacillus coagulans	≥1.0	0.1
Bacillus cereus	0.2	0.2
Bacillus clausii	0.05	0.2

TDOC: bile acid form existing in liver
DOC: bile acid form existing in intestine

showed 98% similarity to the established species *Basillus subtilis*. Phylogenetic analysis using the 16S rRNA sequence showed that strain LS 1-2 was closest to *Bacillus subtilis* FJ377562 strain (alignment score is 99.9%). Phylogenetic tree of *Bacillus subtilis* LS 1-2 and related *Bacillus* sp. is shown in Fig. 3. Therefore, the candidate strain LS 1-2 was identified as a new strain of *Bacillus subtilis* and was designated *Bacillus subtilis* LS 1-2. Since *Bacillus subtilis* has been given generally regarded as safe (GRAS) status and reported in probiotics use [19], *Bacillus subtilis* LS 1-2 is a potential candidate for probiotics use.

3. Production of Antimicrobial Substance by *Bacillus subtilis* LS 1-2

Antimicrobial production by the selected strain, *Bacillus subtilis* LS 1-2, was greatly influenced by growth medium. When supernatants of different culture broths were tested for antimicrobial activity, we noted that antimicrobial activity was obtained in CJM and not in any other commercial medium (LB/LBS/TSB/MRS) (Table 1). This suggests the possibility that some compounds in the CJM may have induced the antimicrobial production in *Bacillus* LS 1-2. Identification of the compound is under progress in the lab and synthetic pathway will be elucidated based on this result.

4. Growth Characteristics and Antimicrobial Activity of *B. subtilis* LS 1-2 in CJM

As shown in Fig. 4, the antimicrobial compound was produced after 12 hr of cultivation (culture) and reached its maximum at 24 hr. In contrast, growth of *B. subtilis* LS 1-2 reached the stationary phase at 12 hr. Uncoupled production of antimicrobial compound and cell growth indicate the characteristic production of secondary metabolites, including antibiotics [20]. Production of protease and amylase was correlated with microbial growth patterns. Both enzymes in the animal digestion tract are beneficial for the digestion of feed. When a viable spore count was made, it was found that almost all the vegetative cells were transformed into spores. Spores are dormant cells that are relatively tolerant to heat, pH, and humidity and are critical for the long-term preservation of cells in microbial pesticides. The high concentrations of hydrolytic enzymes and the effective conversion of vegetative cells to dormant spores in CJM indicate the potential of *B. subtilis* LS 1-2 to function as an effective probiotic.

5. Bile Tolerance

Bile tolerance is an important characteristic of probiotics used as dietary adjuncts because it enables the probiotic strains to survive,

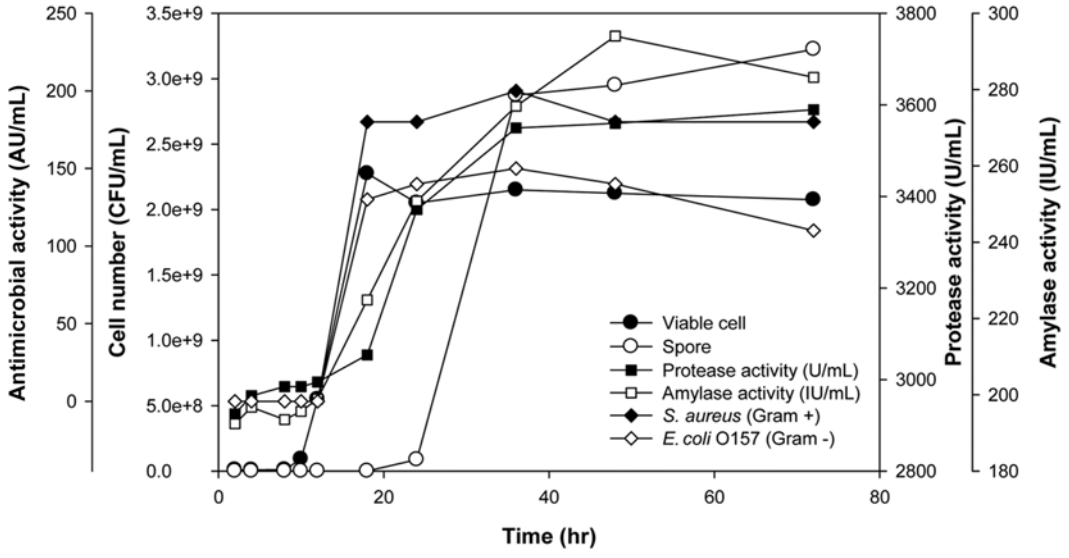

Fig. 4. Time-course cultivation (culture) of *B. subtilis* LS 1-2 using citrus juice medium (CJM) in 2.5 L fermentor.

Table 3. Effect of enzymatic digestion by *Bacillus subtilis* LS 1-2 on antimicrobial activity

Enzyme	Antimicrobial activity (%)	
	Anti-*S. aureus*	Anti-*E. coli* O-157
No treatment	100	100
Proteinase K	100	100
Pronase E	0	0
Lipase	100	100
Amylase	100	100
Catalase	100	100

grow, and exert their beneficial action in the host [21]. Table 2 shows the comparative tolerance of the selected strain and four commercial strains against liver and intestinal bile acid salts. The lowest concentration of bile acids that totally inhibited the growth of *Bacillus subtilis* LS 1-2 at 1 mM of TDOC and 0.27 mM of DOC. The ability of *B. subtilis* LS 1-2 to survive at low pH in the presence of bile salts was similar to that of commercial probiotic strains. This is an important observation indicating that the selected strain *B. subtilis* LS 1-2 will not only survive the low pH of the stomach but also may able to grow in the high bile environment of the intestine.

6. Physical-chemical Characterization of Antimicrobial Compound

To discover the chemical nature of the inhibitory substance produced by *Bacillus subtilis* LS 1-2, a stability test of the culture media supernatant from CJM was conducted in the presence of proteolytic enzymes and a variety of temperature and pH conditions.

It was found that the antimicrobial compound did not lose activity after treatment with Proteinase K, Lipase, α-Amylase, or Catalase but lost activity on treatment with Pronase E (Table 3). The proteinaceous nature of this antibacterial compound was confirmed by loss of activity upon exposure to Pronase E. No activity loss upon treatment with Lipase or α-Amylase indicates that the bacterial peptide does not require a glucosidic or a lipidic moiety for its biological activity. Our results are consistent with those reported for other bacteriocins [22-24].

The ability of the compound to retain activity under elevated temperatures was tested by incubating the culture broth at various temperatures ranging from −70 to 121 ºC, for 0-24 hr. Fig. 5(a) shows the heat stability of the antimicrobial compounds at different temperatures. Results show that the treatment of the culture supernatants exposed to temperatures from −70 to 4 ºC for 24 hr did not show any significant difference from the control. This indicates that cold temperature does not significantly decrease the potential of these active compounds; hence, the substance can be applied to foods and therapeutics that must be kept under cold conditions for a considerable length of time. It was found that antimicrobial activity is stable up to 100 ºC for 1 hr. When exposed to high temperatures (~121 ºC), antimicrobial activity significantly decreased after 15 min followed by the complete loss of activity at 1 hr. The interesting feature of heat stability at 100 ºC for 1 hr supports the view that it might be advantageous via potential use as a food additive in processes like pasteurization and drying. The loss of the antimicrobial activity at autoclaving conditions was probably due to the prolonged period of exposure to heat and pressure. The antimicrobial activities of the

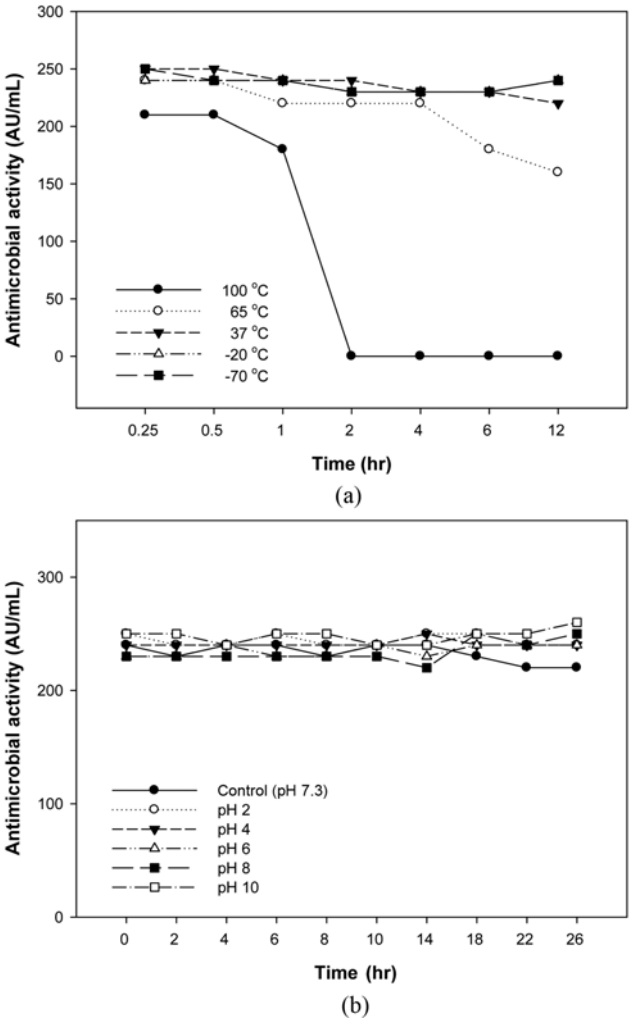

Fig. 5. Effect of temperature (a) and pH (b) on antibacterial activity of *B. subtilis* LS 1-2.

culture supernatants after treatment at different tested pHs are shown in Fig. 5(b). The results demonstrated that the antimicrobial substance was highly stable from pH 2 to 10. Such a wide range of pH tolerance is an extremely important feature, because the isolates have the ability to survive, grow, and produce their antimicrobials under both acidic and alkaline conditions. This pH stability is in agreement with the previous reports on bacteriocins [25].

7. Antimicrobial Spectrum of the Compound Produced by *Bacillus subtilis* LS1-2

The antimicrobial spectrum of the compound from *Bacillus subtilis* LS 1-2 was compared, by using agar well diffusion method, with those of the commercially available bacteria. As shown in Fig. 6, the compound from *Bacillus subtilis* LS 1-2 was effective against gram-positive and gram-negative strains. Since Vancomycin and Streptomycin are ineffective against *E. coli*, these results demonstrate that the antimicrobial compound from *B. subtilis* LS 1-2 has a broad spectrum of action.

CONCLUSIONS

The manipulation of citrus juice waste is becoming a very seri-

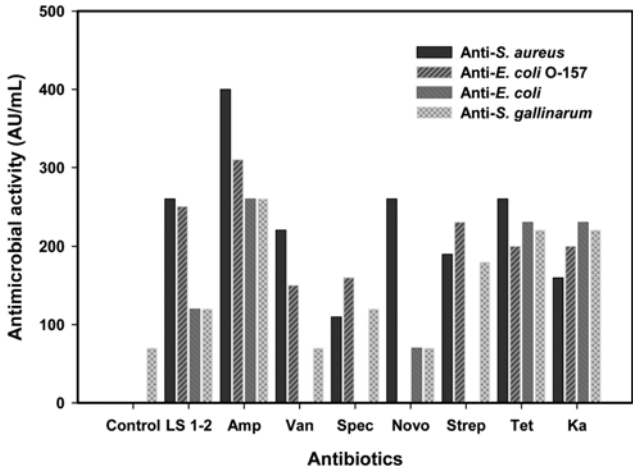

Fig. 6. Antimicrobial spectrum of the antimicrobial substance produced by LS 1-2 v/s commercial antibiotics; Blank: Citrus Juice Medium (No cells), Control: cultured *B. subtilis* LS 1-2 strain supernatant (100 μL), Amp: Ampicillin (1 mg/mL), Van: Vancomycin (1 mg/mL), Spec: Spectinomycin (1 mg/mL), Novo: Novobiocin (1 mg/mL), Strep: Streptomycin (1 mg/mL), Tetra: Tetracycline (3 mg/mL), Kana: Kanamycin (3 mg/mL).

ous environmental issue. We successfully utilized CJW for the production of an antimicrobial compound from *Bacillus subtilis* LS 1-2 that could be a potential candidate for use as a probiotic. Apart from the antibacterial activity against *E. coli* O-157 (140 AU/mL) and *Staphylococcus aureus* (180 AU/mL), the strain also produced protease (3,600 U/ml) and amylase (290 U/ml) in CJM. The antimicrobial compound was stable up to 100 ºC for 1 hr and over a wide range of pH, from 2 to 10. Bile tolerance, which is an important property of functional probiotics, also was exhibited by *Bacillus subtilis* LS 1-2. Based on these results we suggest a cost-effective utilization of citrus juice waste and the use of *Bacillus subtilis* LS 1-2 as a probiotic in various industrial applications.

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REFERENCES

1. F. Belshaw, *Food. Prod. Devel*., **12**, 36 (1978).
2. R. J. Braddock, *Food. Technol.*, **12**, 85 (1983).
3. J. V. Formica and W. Regelson, *Food. Chem. Toxicol*., **33**, 1061 (1995).
4. H. E. Swisher and D. E. Pritchett, *Baker's Digest*, **43**, 53 (1969).
5. C. Gladine, C. Morand, E. Rock, D. Bauchart and D. Durand, *Anim. Feed Sci. Technol.*, **136**, 281 (2007).
6. K. Manderson, M. Pinart, K. M. Tuohy, W. E. Grace, A. T. Hotchkiss, W. Widmer, M. P. Yadhav, G. R. Gibson and R. A. Rastall, *Appl. Environ. Microbiol.*, **71**, 8383 (2005).
7. E. J. Yan, S. S. Kim, T. H. Oh, J. S. Baik, N. M. Lee and C. G. Hyun, *Int. J. Agr. Biol.*, **9**, 791 (2009).
8. R. Fuller, *A rev. J. Appl. Bacteriol.*, **66**, 365 (1989).
9. S. I. Kim, J. Y. Chang, I. C. Kim and H. C. Chang, *Korean J. Appl. Microbiol. Biotechnol.*, **29**, 50 (2001).
10. R. F. Beer and I. W. Sizer, *J. Biol. Chem.*, **195**, 133 (1952).
11. B. S. Lee, H. B. Lee, S. W. Choi, H. S. Yun and E. K. Kim, *J. Microbiol. Biotechnol.*, **15**, 701 (2005).
12. A. W. Bauer, W. M. Kirby, J. C. Sherris and M. Turck, *Am. J. Clin. Pathol*., **45**, 493 (1966).
13. Y. Shimizu, T. Nishino and S. Murao, *Agric. Biol. Chem.*, **48**, 3109 (1984).
14. P. Bernfeld, *Meth. Enzymol.*, **1**, 149 (1955).
15. R. J. W. Lambert, P. N. Skandamis, P. J. Coote and G. J. E. Nychas, *J. Appl. Microbiol.*, **91**, 453 (2001).
16. A. U. Mahmood, G. J. Reenman and A. H. Scragg, *Enzym. Microb. Technol.*, **22**, 130 (1998).
17. T. Matsumoto, T. Sugiura, A. Kondo and H. Fukuda, *Biochem. Eng. J.*, **6**, 81 (2000).
18. O. Stabnikova, J. Y. Wang, H. B. Ding and J. H. Tay, *Bioresour. Technol*., **96**, 747 (2004).
19. A. Anadon, M. R. Martínez-Larrañaga and M. A. Martínez, *Regul. Toxicol. Pharm*., **45**, 91 (2006).
20. A. L. Demain and A. Fang, *Eng. Biotechnol*., **69**, 1 (2000).
21. M. Begley, C. G. Gahan and C. Hill, *FEMS Microbiol. Rev.*, **29**, 625 (2005).
22. E. J. Gray, K. D. Lee, A. M. Souleimanove, M. R. Falco, X. Di Zhou, A. Ly, T. C. Charles, B. T. Driscoll and D. L. Smith, *J. Appl. Microbiol.*, **100**, 545 (2006).
23. B. Hyronimus, C. L. Marrec and M. C. Urdaci, *J. Appl. Microbiol*., **85**, 42 (1998).
24. M. L. Teixeira, F. Cladera-Olivera, J. dos Santos and A. Brandelli, *Food Res. Int.*, **42**, 63 (2009).
25. M. V. Leal-Sanchez, R. Jimenez-Diaz, A. Maldonado-Barragan, A. Garrido-Fernandez and J. L. Ruiz-Barba, *Appl. Environ. Microbiol.*, **68**, 4465 (2002).