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Identification of a New *Bacillus licheniformis* Strain Producing a Bacteriocin-Like Substance

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The emergence of antibiotic resistance has spurred a great number of studies for development of new antimicrobials in the past decade. The purpose of this study was to screen environmental samples for *Bacillus* strains producing potent antimicrobial agents. A new strain, which showed strong antimicrobial activity against *Staphylococcus aureus* and *Salmonella enterica* ser. Pullorum, was isolated from soil and designated as B116. This new isolate was identified as *Bacillus licheniformis* by morphological, biochemical and genetic analyses. The production of bacteriocin-like substance (BLS) started at early exponential phase and achieved highest level at early stationary phase. The BLS was precipitated by ammonium sulfate and its molecular mass was determined as ~4 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Culture supernatant of the new isolate exhibited antimicrobial activity against both Gram-positive and Gram-negative bacteria, including *Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Micrococcus luteus*, *Escherichia coli*, and *Salmonella* spp. The BLS was resistant to heat, acid and alkaline treatment. Activity of the BLS was totally lost after digestion by pronase and partially lost after digestion by papain and lipase. The new isolate and relevant BLS are potentially useful in food and feed applications.

Keywords: antibiotic-resistance, bacteriocin-like substance, *Bacillus licheniformis*

Introduction

The abuse of antibiotics as therapeutic agents for humans and as prophylactics and growth promoters for animals has conferred antibiotic resistance on pathogens (Barbosa *et al.*, 2005). These antibiotic resistant pathogens pose a serious health challenge and impose a high monetary cost. It was estimated that at least 25,000 patients in Europe die every year due to a lack of effective antibiotics (Cars *et al.*, 2011). Reports from scientific communities have raised concerns about this lack of antibiotics for treatment of drug-resistant pathogens and the urgent need to develop alternatives (Freire-Moran *et al.*, 2011).

Bacillus spp. are Gram-positive, spore forming bacteria that are ubiquitous in the environment. Strains of *Bacillus* have been used in food and agricultural industries for manufacture of enzymes and bioinsecticides (Boer *et al.*, 1994). *Bacillus* spp. have received considerable attention, as a source of antimicrobials, due to the abundance of antimicrobial substance production; these substances include bacteriocin, lipopeptide, polyketide, phospholipid, and aminosugar (Stein, 2005). Bacteriocins are natural antimicrobial peptides that are ribosomally synthesized by bacteria (Cleveland *et al.*, 2001). The most well-known bacteriocin, nisin, which is produced by lactic acid bacteria (LAB), has been applied as a food preservative in more than fifty countries (Bari *et al.*, 2005). Compared to LAB bacteriocins which are only active against Gram-positive bacteria, bacteriocins from *Bacillus* may exhibit a broad antimicrobial spectrum (Abriouel *et al.*, 2011). In addition, mersacidin, a bacteriocin produced by *Bacillus subtilis*, had been reported to be active against methicillin-resistant *Staphylococcus aureus in vivo*, and is potentially useful in medical applications (Kruszewska *et al.*, 2004).

The aim of this study was to screen environment samples for *Bacillus* strains with potent antimicrobial activity. A bacterial strain that showed potent antimicrobial activities against both Gram-positive and Gram-negative bacteria was isolated from a soil sample. The isolate and its associated antimicrobial agent were characterized.

Materials and Methods

Strain screening

Samples were collected, including soil, foods, animal feces and gastrointestinal contents of chickens, and screened for *Bacillus* strains with potent antimicrobial activity. The samples were suspended in sterile phosphate buffer (pH 7.0) and homogenized. The resultant suspensions were placed in a water bath (80°C) for 15 min to inactivate vegetative cells. The heat-treated samples, presumably containing spores, were serially diluted and spread-plated onto dextrose and yeast extract agar (DYE, 10 g Dextrose, 10 g Yeast Extract, 4 g K_2HPO_4, and 15 g agar in 1,000 ml water, pH 7.0). These plates were incubated at 30°C for 24 h and inspected for presence of bacterial colonies. The colonies were marked and subcultured in DYE broth at 30°C, with agitation of

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Table 1. Phenotypic characteristics of *B. licheniformis* B116

Characteristics	Results[a]	Characteristics	Results[a]
Cell diameter >1.0 μm	-	Utilization of citrate	+
Spherical spore	-	Acid from	
Swollen sporangia	-	Glucose	+
Catalase	+	Xylose	+
Oxidase	+	Arabinose	+
Anaerobic growth	+	Mannitol	+
Voges-Proskauer test	+	Growth at/in	
Nitrate reduction	+	50°C	+
Hydrolysis of		pH 5.7	+
Starch	+	7% NaCl	+
Casein	+		

[a] -, negative; +, positive

130 rpm for 24 h. The culture supernatants were tested for antimicrobial activity against five indicators; these were *Staphylococcus aureus* CVCC 1885, *Escherichia coli* CVCC 195, *E. coli* CVCC 245, *Salmonella enterica* ser. Enteritidis CVCC 2181 and *S. enterica* ser. Pullorum CVCC 79301. These indicator microorganisms, which are causative agents of animal diseases (pyogenesis, diarrhea, enteritis, and pullorum respectively), were purchased from the China Veterinary Culture Collection Center. Strains with potent antimicrobial activity were then streaked onto new DYE agar to check purity. A soil sample yielded an isolate that was active against four of these five indicator strains and was given the designation B116.

Cultures and media

DYE agar was used to culture the new isolate and DYE broth was employed for production of antimicrobial agent. The indicator microorganisms and related media are listed in Table 2. For stock preparation, the culture was incubated at 30°C for 3 days, mixed with glycerol at a final concentration of 20%, and stored at -20°C.

Strain identification

Morphological characteristics of the new isolate were examined by Gram staining and spore staining. Phenotypic and biochemical tests (catalase, oxidase, Voges-Proskauer test, acid production from carbohydrates, utilization of citrate, growth at 50°C or pH 5.7, growth in 7% sodium chloride, hydrolysis of casein and starch, and reduction of nitrate), and amplification and sequencing of 16S rDNA were done at the Institute of Microbiology, Chinese Academy of Sciences. A commercial biochemical test kit (API 50 CH strips and CH B/E medium, bioMérieux, Inc., USA) was also applied

Table 2. Antimicrobial activity spectrum of strain B116 culture supernatant

Strains	Broth medium	Antimicrobial activity
Gram-positive bacteria		
B. subtilis CGMCC1.769[a]	DYE	-
B. licheniformis CGMCC1.265[a]	DYE	-
B. licheniformis CGMCC1.807[a]	DYE	-
B. cereus L01[b]	DYE	-
B. cereus CGMCC1.230[a]	DYE	++
E. faecium CVCC1928[d]	MB	-
Listeria monocytogenes CVCC1599[d]	NB	++
Lactobacillus plantarum YJG[b]	MRS	-
Micrococcus luteus CMCC28001[e]	NB	++
S. aureus CMCC26003[a]	NB	++
S. aureus CICC21601[c]	NB	+++
S. aureus CVCC1885[d]	NB	+++
Streptococcus equi subsp. *zooepidemicus* CVCC1903[d]	MB	+++
Gram-negative bacteria		
E. coli CVCC245[d]	LB	+++
E. coli CICC21525[c]	LB	+
E. coli CVCC195[d]	LB	+
E. coli CVCC249[d]	LB	+
Pseudomonas aeruginosa CVCC2087[d]	NB	-
Pasteurella multocida CVCC458[d]	MB	-
Salmonella enterica ser. Enteritidis CVCC2181[d]	BPY	-
S. enterica ser. Pullorum CVCC79301[d]	BPY	+++
S. enterica ser. Typhimurium CVCC541[d]	BPY	++

[a] Strains obtained from China General Microbiological Culture Collection.
[b] Strains obtained from the culture collection of China Agriculture University feed biotechnology laboratory.
[c] Strains obtained from China Center of Industrial Culture Collection.
[d] Strains obtained from China Veterinary Culture Collection Center.
[e] Strains obtained from China Medical Culture Collection Center.
-, not detectable; +, diameter of inhibitory zone smaller than 15 mm; ++, diameter of inhibitory zone between 15–20 mm; +++, diameter of inhibitory zone larger than 20 mm.
DYE, Dextrose and Yeast Extract Broth; NB, Nutrient Broth; MRS, deMan Rogosa Sharpe; MB, Martin Broth; LB, Luria Bertani; BPY, Beef Extract, Peptone and Yeast Extract; PDA, Potato Dextrose Agar.

to determine the carbohydrate fermentation profile of the new isolate. Results were recorded after incubation of the inoculated wells at 30°C for 24 h and 48 h. Identification was done by referring the profile to the database provided by the manufacturer.

Determination of antimicrobial activity

An agar diffusion assay was employed for both qualitative and quantitative antimicrobial activity tests. For the qualitative test, indicator strains were diluted to 10^7 CFU/ml and aliquots of 200 µl were spread onto corresponding agar. Sterile Oxford cups were placed on these agar plates and 100 µl of antimicrobial agent was added into each Oxford cup. These plates were incubated at 37°C overnight to manifest antimicrobial activity. For the quantitative test, the antimicrobial agent was two-fold serially diluted and 100 µl of each dilution was dispensed into Oxford cups on the agar plate, which had been spread with *S. aureus* CVCC 1885. The plate was incubated at 37°C overnight then examined for inhibitory zones. The antimicrobial activity was expressed as an arbitrary unit per ml (AU/ml), the reciprocal of the highest dilution displaying inhibition that corresponds to 1 ml of the non-diluted antimicrobial agent.

Production of bacteriocin-like substance during growth

A single colony of B116 was inoculated into 10 ml DYE broth and incubated at 30°C overnight. The overnight culture was then subcultured in flasks containing DYE broth and incubated at 30°C with agitation at 200 rpm. Optical density and antimicrobial activity of the culture were examined at different time intervals. Results are presented as the average value from three independent experiments. *S. aureus* CVCC 1885 was used as indicator strain for the antimicrobial activity test.

Protease activity and inhibition tests

The protease activity was tested as described previously (Dischinger *et al.*, 2009) with modifications. Briefly, plates were prepared using 25 g/L skim milk and 15 g/L agar. Strain B116 was incubated in 10 ml DYE broth at 30°C for 48h and the culture supernatant was collected by centrifugation. The culture supernatant was filtrated (0.22 µm, Millipore, Ireland) and an aliquot of 200 µl was dispensed into a well in the skim milk agar. The plate was incubated at 37°C for 48 h before it was inspected for signs of protease activity.

The protease inhibition experiment was performed to investigate the effect of protease produced by B116 on antimicrobial activity of the BLS during fermentation. Briefly, strain B116 was inoculated into several 250 ml flasks, each containing 50 ml DYE broth. The inoculated cultures were incubated at 30°C for 16 h before they were supplemented with ethylenediaminetetraacetic acid (EDTA, 0.1 mM) or phenylmethanesulfonyl fluoride (PMSF, 0.1 mM) (Ivanova *et al.*, 1998). The culture without supplementation of protease inhibitor (EDTA or PMSF) served as control. After a subsequent cultivation of 48 h, these cultures were tested for antimicrobial activity against *S. aureus* CVCC 1885. Meanwhile, samples were collected from the culture, which had been incubated for 16 h and not supplemented with protease inhibitor. These samples were centrifuged and the culture supernatants were mixed with EDTA or PMSF to a final concentration of 10 mM (Kouakou *et al.*, 2008). The mixtures of culture supernatant and protease inhibitor were incubated at 37°C for 48 h before the antimicrobial activity test. Culture supernatant that did not contain any protease inhibitor was incubated at 37°C for 48 h for antimicrobial activity comparison.

Inhibitory spectrum test

A qualitative agar diffusion assay was used for the antimicrobial spectrum test. Indicator microorganisms included Gram-positive and Gram-negative, pathogenic, and non-pathogenic bacteria (Table 2).

Stability tests

Culture supernatant was tested for sensitivity to enzymes, heat and pH changes. For enzyme digestion, a volume of culture supernatant was mixed with an equal volume of enzyme solution. Enzymes (trypsin, pepsin, papain, pronase, α-amylase, and lipase) were purchased from Sigma and dissolved in phosphate buffer (50 mM, pH 7.0), except pepsin which was dissolved in 10 mM HCl, at a concentration of 2 mg/ml. The mixtures of culture supernatant and enzyme were incubated at 37°C for 2 h and tested for residual antimicrobial activity. The solution containing culture supernatant and pepsin was neutralized before the antimicrobial activity test. Untreated sample was mixed with phosphate buffer and incubated, to serve as control. For the thermal sensitivity test, the BLS was exposed to various temperatures (50, 60, 70, 80, 90, and 100°C) in a water bath for 15 min. Samples were cooled to room temperature and measured for antimicrobial activity. For the pH stability test, culture supernatants were adjusted to different pHs (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0), followed by incubation for 2 h. These samples were then brought to pH 7.0 and tested for antimicrobial activity. Each experiment was performed in triplicate and diameters of inhibitory zones were recorded. Residual activity was compared with the control and expressed as a percentage.

Preparation of crude extract

The new isolate was fermented in 500 ml DYE broth at 30°C with agitation for 16 h. Cells were removed by centrifugation of the culture at 8,000×g for 20 min and ammonium sulfate was added into the cell free supernatant to 70% saturation. The resultant solution was stirred overnight and was centrifuged to collect the precipitate. The precipitate was dissolved in 10 ml phosphate buffer (25 mM, pH 7.0) and dialyzed in the same buffer for 10 h using a dialysis membrane with 3,000 Da molecular weight cut-off (MWCO). The buffer was changed three times during dialysis and the solution containing BLS was freeze dried. The resultant powder was reconstituted in 10 ml phosphate buffer (25 mM, pH 7.0), centrifuged and passed through a 0.22 µm filter (Millipore). This solution, referred to as crude extract, showed an activity of 2560 AU/ml and was stored at 4°C until use.

Table 3. Effect of selective enzymes, heat, and pH changes on antimicrobial activity of the bacteriocin-like substance

Treatment	Residual activity[a]
Enzymes	
Trypsin	91.7%
Papain	68.0%
Pepsin	79.0%
pronase	0
α-amylase	92.8%
Lipase	62.9%
Heat	
60°C	99.1%
70°C	98.6%
80°C	88.1%
90°C	85.0%
100°C	78.7%
pH	
3	85.9%
4	82.6%
5	88.0%
6	97.0%
8	92.3%
9	76.5%
10	59.0%

[a] Experiments were performed in triplication. Residual activity was compared to control and expressed as a percentage.

SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was conducted to estimate the molecular weight of the BLS. The preparation of gel and parameters of electrophoresis were described previously (Schagger, 2006). After electrophoresis, the gel was vertically cut into two parts and one half of the gel was stained with Coomassie blue G-250 (Sigma, USA). The other part was washed with sterile distilled water for 1 h, placed in a petri dish, and overlaid with 10 ml of soft agar that had been seeded with an overnight culture of *S. aureus* CVCC 1885 (Barboza-Corona *et al.*, 2007). This plate was then incubated at 30°C overnight and inspected for presence of an inhibitory zone.

Amplification of putative bacteriocin gene

Several sets of primers were employed to detect the potential bacteriocin gene (Table 4). Primers Licfwd and Licrev were designed to amplify a fragment spanning 480 bp upstream and 435 bp downstream of the lichenicidin structural gene (Begley *et al.*, 2009). Degenerate primers (SB5, SB6, LanBfwd, LanBrev, LanCfwd, and LanCrev) were specific to conserved motifs of bacteriocin modification proteins and had led to the discoveries of genes responsible for production of ericin and salivaricin 9 (Stein *et al.*, 2002; Wescombe *et al.*, 2011). Primers osboP1 and osboP2 had been used to amplify a DNA fragment containing the subtilosin gene. Polymerase chain reactions (PCR) were conducted in a thermal cycler (MJ Mini Gradient, Bio-Rad, USA). For primers LanBfwd, LanBrev, LanCfwd and LanCrev, PCR was carried out using a HotStarTaq DNA polymerase (QIAGEN, USA) as follows: after an activation of 15 min at 95°C the solutions were subjected to 30 cycles, each consisting of 1 min at 94°C, 1 min at 40°C and 2 min at 72°C; a final extension was performed for 10 min at 72°C (Wescombe, 2011). For other primers, Taq DNA polymerase (QIAGEN) was used for amplification. The amplification included three steps: an initial 3 min incubation at 94°C; 30 cycles of denaturation (1 min) at 94°C, annealing (1 min) at 55°C and elongation (2 min) at 72°C; and the final extension at 72°C for 10 min (Stein, 2002). PCR products were examined by agarose gel electrophoresis.

Results

Identification of a new *B. licheniformis* strain, B116

Several hundreds of *Bacillus* strains were isolated from diverse environmental samples and screened for antimicrobial activity. A soil sample yielded an isolate with potent antimicrobial activity against *S. aureus* CVCC 1885 and *Salmonella* Pollorum CVCC 79301. The new isolate is a Gram-positive, rod-shaped, and spore-forming bacterium. The phenotypic characteristics of the new strain are listed in Table 1. Genetic analysis showed that the 16S rDNA of the new isolate shares 99% similarity with that of *B. licheniformis*. Carbohydrate fermentation analysis (API 50 CH kit) of the new strain also showed 94.1% similarity with *B. licheniformis*. Through the combination of phenotypic and genotypic properties, the isolate was identified as *B. licheniformis* and was given the designation B116.

Table 4. Oligonucleotide primers used in this study

Primer	Sequence	Source
Licfwd	CGGGCTACATTCAGCTCTGT	this study
Licrev	CAAGCCTTAAAATGTTTAGTTCCAG	
SB5	GAWKNACWCCWTWTGG	Stein *et al.* (2002)
SB6	CCRCCATATCSWTMTRYYTC	
LanBfwd	TATGATCGAGAARYAKAWAGATATGG	Wescombe *et al.* (2011)
LanBrev	TTATTAIRCAIATGIAYDAWACT	
LanCfwd	TAATTTAGGATWISYIMAYGG	Wescombe *et al.* (2011)
LanCrev	ACCWGKIIIICCRTRRCACCA	
osboP1	CCTCATGACCAGGACTTCGCCTTCGCTTACTTT	Zheng *et al.* (1999)
osboP2	CGGTGCCGAGCGCTTCAGGTAAGCTTTCCAAA	

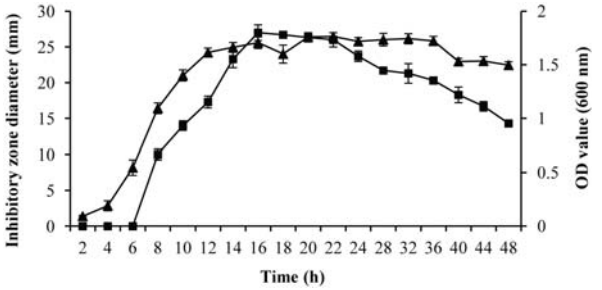

Fig. 1. The relationship of bacteriocin-like substance production and cell growth. Triangle, optical density of B116 culture. Square, antimicrobial activity of the culture supernatant.

B. licheniformis B116 produces a bacteriocin-like substance during growth

The production of BLS was determined by the antimicrobial activity of the culture supernatant. The relationship between cell growth and antimicrobial production is shown in Fig. 1. Antimicrobial activity of strain B116 was detected at early exponential phase and reached a maximum at early stationary phase. This indicated that the BLS is synthesized along with cell propagation. Since *B. licheniformis* strains are known to produce protease (Hadj-Ali *et al.*, 2007), the reduction of antimicrobial activity in late stationary phase could be due to proteolytic degradation of the BLS.

Protease activity and inhibition tests

The decrease of antimicrobial activity gave rise to the hypothesis that BLS was inactivated by the producer's protease in late stationary phase. Therefore, strain B116 was first tested for protease production. The production of protease was confirmed by observation of a clear zone in the skim milk plate which had been dispensed with B116 culture supernatant (data not shown). Second, the effect of protease on the antimicrobial activity of BLS was investigated, by supplementation of protease inhibitors during fermentation

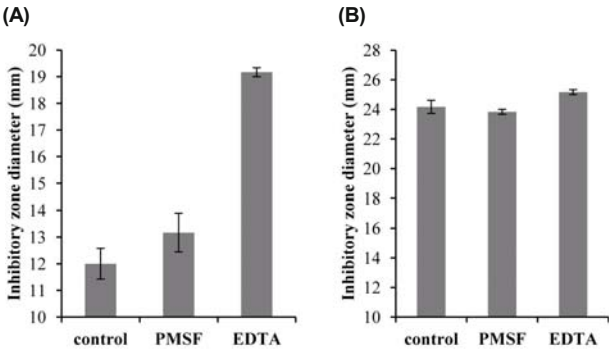

Fig. 2. Effect of protease inhibitors, phenylmethanesulfonyl fluoride (PMSF) and ethylenediaminetetraacetic acid (EDTA), on antimicrobial activity of B116 during fermentation. (A) Strain B116 was supplemented with PMSF or EDTA in early stationary phase during fermentation, cultivated for another 48 h and tested for antimicrobial activity. (B) Culture supernatant of B116 was mixed with PMSF or EDTA and incubated for 48 h before antimicrobial activity test.

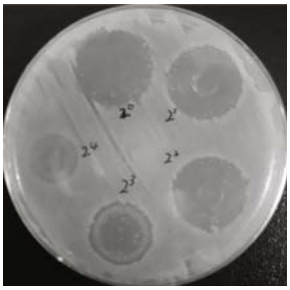

Fig. 3. Antimicrobial activity of B116 culture supernatant (160 AU/ml) against *S. aureus* CVCC 1885. The culture supernatant was 2-fold serially diluted and each dilution (from 2^0 to 2^4) was tested for antimicrobial activity. Result was expressed as arbitrary units (AU).

and co-incubation of culture supernatant with protease inhibitors, respectively. When EDTA was added into the broth culture in early stationary phase and incubated for 48 h, the sample retained most of its activity (Fig. 2A). Meanwhile, the antimicrobial activity of the control decreased dramatically (Fig. 2A). On the other hand, culture supernatant collected from early stationary phase did not lose activity after 48 h incubation, with or without protease inhibitors (Fig. 2B). The results implied that a metalloproteinase was produced by strain B116 in stationary phase. The addition of EDTA, a metal chelator, depleted the metal ions essential for catalytic activity of the metalloproteinase and protected the BLS from degradation. Compared to EDTA, supplementation of PMSF, a serine protease inhibitor, did not inhibit the proteinase activity and led to the reduction of antimicrobial activity.

Inhibitory spectrum

When tested against a panel of Gram-positive and Gram-negative bacterial strains, B116 culture supernatant showed broad antimicrobial activity (Table 2). Microorganisms susceptible to B116 included *B. cereus*, *Listeria monocytogenes*, *Micrococcus leutes*, *S. aureus*, *Streptococcus equi*, *E. coli*, and *Salmonella enterica*.

Stability tests

The BLS was sensitive to pronase (Table 3), which implied its proteinaceous nature. The antimicrobial activity was partially lost after digestion with papain, pepsin and lipase (Table 3). Inactivation by lipase indicated that the BLS may also contain a lipid moiety. When held at various temperatures (60, 70, 80, 90, and 100°C) for 15 min or exposed to different pHs ranging from 3 to 8, the BLS retained most of its activity. The stability at high temperature and in neutral

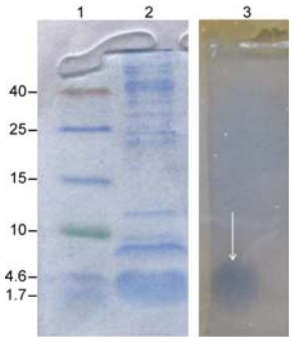

Fig. 4. SDS-PAGE of crude extract of strain B116 culture supernatant and determination of molecular mass of the bacteriocin-like substance produced by B116. Lanes: 1, molecular mass marker (in kilodaltons); 2, crude extract stained with Coomassie blue G-250; 3, the gel overlaid with *S. aureus* CVCC 1885; the arrow indicated the inhibitory zone.

pH is an ideal characteristic of the BLS produced by B116 for applications in food and feed.

The bacteriocin-like substance is a 4 kDa protein

The BLS in culture supernatant was precipitated by ammonium sulphate. After dialysis and reconstitution in phosphate buffer (25 mM, pH 7.0), the crude extract showed antimicrobial activity of 2560 AU/ml against *S. aureus* CVCC 1885. This activity increased more than 15 fold when compared to culture supernatant (160 AU/ml, Fig. 3). The crude extract was then subjected to SDS-PAGE to estimate the molecular mass of BLS. An inhibitory zone was shown on the half of the gel that had been overlaid with indicator strain, and corresponded to a band in the stained half of the gel (~4 kDa, Fig. 4). The SDS-PAGE experiment supported the proteinaceous nature of the BLS produced by strain B116. However, polymerase chain reaction using primers specific to conserved motifs of bacteriocin proteins failed to detect any putative bacteriocin gene in B116 (data not shown).

Discussion

A new bacterial strain, B116, was isolated from a soil sample during a screening for *Bacillus* strains with potent antimicrobial activity. The new isolate was identified as *B. licheniformis* by phenotypic and genetic analyses. This strain produces a bacteriocin-like substance and is active against both Gram-positive and Gram-negative pathogenic bacteria.

The biosynthesis of BLS by B116 exhibits a primary metabolite kinetic, with the peak activity at early stationary phase. A similar kinetic property has been reported for other bacteriocins, e.g., amylovorin L471 and lactococcin 140 (Parente *et al.*, 1994; De Vuyst *et al.*, 1996). The decrease of antimicrobial activity in late stationary phase was proposed to result from adsorption of the bacteriocins to the bacterial cells in these studies. In the case of strain B116, the reduction of antimicrobial activity was due to proteolytic degradation of BLS by a metalloproteinase. Besides protease, the BLS is moderately sensitive to lipase. It has been reported that bacteriocins may conjugate with carbohydrates or lipids, such as colicin, which existed in liquid culture as low molecular weight proteins and high molecular weight lipoprotein-carbohydrate complexes (Reeves, 1972). West and Warner (1988) found that the antimicrobial activity of lactacin 27 was reduced after treatment with lipase or α-amylase and they assumed that the bacteriocin was possibly produced initially as low molecular weight molecules, and subsequently formed complexes with carbohydrates or lipids. Alternatively, the lipid moiety may not be essential for antimicrobial activity but contribute to stabilization of bacteriocin (Lee *et al.*, 1999).

Many BLS have been reported to be produced by *B. licheniformis* strains. However, none of these, except lichenicidin, has structural and genetic information (Dischinger *et al.*, 2009). Polymerase chain reaction provided rapid identification of well characterized bacteriocins (Rodriguez *et al.*, 1995; Wieckowicz *et al.*, 2010). The failure of amplification of the putative bacteriocin gene using primers specific to known bacteriocins suggested that the BLS produced by strain B116 may be a novel protein. Mass spectrometry and nuclear magnetic resonance techniques have been extensively employed for bacteriocin sequencing and are needed for characterization of the compound (He *et al.*, 2007); however, a pure sample is required for these analyses. The identity of the BLS is currently under investigation.

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