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A Lipopeptide Biosurfactant Produced by *Bacillus subtilis* C9 Selected through the Oil Film-collapsing Assay

KIM, HEE-SIK[1], CHANG-HO LEE[1], HYUN-HYO SUH[1], KEUG-HYUN AHN[1], HEE-MOCK OH[1], GI-SEOK KWON[2], JI-WON YANG[3], AND BYUNG-DAE YOON[1]*

[1]*Environmental Microbiology R.U., Korea Research Institute of Bioscience and Biotechnology, Taejon 305-600, Korea*
[2]*Natural Science Collage, Andong National University, Andong 760-749, Korea*
[3]*Dept. of Chemical Engineering, Bioprocess Engineering Research Center, KAIST, Taejon 305-701, Korea*

Bacillus subtilis C9 was selected by measuring the oil film-collapsing activity and produced biosurfactant in a medium containing glucose as a sole carbon source. The biosurfactant emulsified hydrocarbons, vegetable oils and crude oil, and lowered the surface tension of culture broth to 28 dyne/cm. A biosurfactant, C9-BS produced by *B. subtilis* C9 was purified by ultrafiltration, extraction with chloroform and methanol, adsorption chromatography, and preparative reversed phase HPLC. Structural analyses, IR spectroscopy, FAB mass spectroscopy, amino acid composition, and NMR analyses, demonstrated that C9-BS was a lipopeptide comprising a fatty acid tail and peptide moiety. The lipophilic part consisting of C_{14} or C_{15} hydroxy fatty acid was linked to the hydrophilic peptide part, which contained seven amino acids (Glu-Leu-Leu-Val-Asp-Leu-Leu) with a lactone linkage.

Surfactants are amphiphilic molecules consisting of hydrophobic and hydrophilic domains. Due to their amphipathic nature surfactants can partition preferentially at the interfaces between different fluid phases such as oil/water or water/air interfaces. Biosurfactants are microbially produced compounds which exhibit surface activity (4, 5). Unlike chemical synthetic surfactants biosurfactants have an easily biodegradable nature, low toxicity and various possible structures. Such variable structures resulted in more potent surface activity than that of chemical synthetic surfactants under extreme temperature conditions, pH or salinity, thus their development may expand the range of applications of biosurfactants (6, 8, 14).

With environmental compatibility becoming an increasingly important factor in the selection of industrial chemicals, the use of biosurfactants in environmental applications, such as bioremediation and the dispersing of oil spills is becoming more common. In addition, biosurfactants have other benefits to the petroleum industry, such as enhanced oil recovery and transportation of crude oil. Other possible applications are in food, cosmetic, and in pharmaceutical industries. In these fields, most biosurfactants are used as emulsifiers (1, 5, 11, 12).

*Corresponding author
Phone: 82-42-860-4320. Fax: 82-42-860-4595.
E-mail: bdyoon@kribb4680.kribb.re.kr.
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However, because of economic and technical limitations, biosurfactant have not yet been employed extensively in industry. The main limiting factor for commercialization is, as for other microbial compounds, the economics of production. To reduce the cost of biosurfactant production it is first necessary to select a microorganism capable of producing the required product in high concentrations.

To select bacteria that produce biosurfactants, they are usually grown individually in a liquid medium. After several days the culture filtrates are tested for surface activity by measuring surface tension or interfacial tension (9). This traditional method is labor-intensive and time consuming.

In this study highly productive bacteria were screened by a sensitive and rapid method of measuring oil film-collapsing activity. The selected strain was identified, and its patterns of biosurfactant production were simply investigated. The biosurfactant was purified from a culture of the selected strain to apparent homogeneity. The structure of this compound was characterized by amino acid analysis and various spectroscopic techniques.

MATERIALS AND METHODS

Medium and Culture Conditions

The isolation medium for biosurfactant-producing bacteria was Cooper's basic mineral salts medium (2) sup-

plemented with yeast extract (Difco, 1.0 g/l), and omitted EDTA. As a carbon source, glucose, *n*-hexadecane, and soybean oil were used to concentrations of 10 g/l in combination or respectively. Incubation was carried out at 30°C and 150 rpm rotary shaking for 3 days. Agar plates were prepared with 1.8% Bacto agar (Difco).

Small scale batch fermentations (1.0 liter) were performed with a 2.0-liter fermentor system (B. Braun). The cultures were grown at 30°C, stirred at 300 rpm, with maintained air volume at a minimum.

Screening by the Oil Film-collapsing Assay

Water suspensions of soil samples were spread on agar plate with isolation medium as described above, covered with crude oil (sulfur content 0.1%, Yukong Petroleum Chemistry Co., Korea). After 3 days' incubation at 30°C colonies surrounded by a halo on an oil-agar plate were isolated. Isolated colonies were cultured in the isolation liquid medium containing *n*-hexadecane, soybean oil, or glucose as a carbon source, and then the biosurfactant producers were selected by measuring the oil film-collapsing activity of the culture broth. The method, oil film-collapsing assay, is based on the feature of surfactants in that they change the contact angle at the oil/water interface. The surface pressure of the surfactant is pushed over the oil film, and surfactant molecules displace oil molecules. Crude oil of 20 μl was put onto the surface of 5 ml of distilled water in petri dishes (50 mm in diameter). A thin oil film formed immediately. Then, 5 μl of culture broth was gently placed in the center of the oil film. A clear halo was visible under light, and the diameter of this circle was measured.

Identification of Bacterial Strain

The morphological, physiological, and biochemical properties of the selected strain were investigated using the methods of Cowan (3) and Macfaddin (16). The chemical properties, peptidoglycan type (13), fatty acid type (18), quinone composition (23), and DNA G+C molar ratio (22), were also examined. Finally the strain was classified according to the Bergey's manual of systematic bacteriology (10).

Analyses

Cell concentrations were determined by measuring the optical density of samples at 660 nm (OD_{660}). The dry cell mass was determined as a function of the OD_{660}, cell dry weight(g/l)=$0.3 \times OD_{660}$.

Biosurfactant concentration was determined by diluting the culture broth until the critical micelle concentration (CMC) was attained (2, 7, 9, 17). The dilution necessary to reach this point, where the surface tension starts to rise dramatically, was designated the CMD (critical micelle dilution).

The surface tension of the culture broth and surfactant solution was determined at 25°C with a ring tensiometer (K10ST; Krüss, Hamburg, Germany).

Emulsification activity was determined by the modified standard method of Rosenberg (20, 21). Samples to be tested (0.1 to 1.0 ml) were introduced into a 50-ml flask containing distilled water to a final volume of 7.5 ml, next 0.1 ml of a 1 : 1 (by volume) mixture of *n*-hexadecane and 2-methylnaphthalene was added. The samples reacted at 28°C with reciprocal shaking (160 strokes per min) for 1 h. Turbidity was then determined by measuring the optical density of the reaction at 620 nm (OD_{620}). One unit of emulsifying activity per milliliter was defined as the amount of biosurfactant that increased OD_{620} of 0.1 in the assay reaction. Emulsion turbidity was directly proportional to the crude biosurfactant, C9-BS concentration between 0.1 and 3.0 mg/l.

Glucose concentration was determined with a glucose kit using glucose oxidase (Sigma Chemical Co., MO, U.S.A.).

TLC was carried out on Silica gel F_{254} plates (Merck Co.) in chloroform-methanol-water (65 : 25 : 4). Components on the plates were located by spraying them with 30% H_2SO_4 solution or the irradiation of short wavelength UV.

Purification of Biosurfactant

Bacterial cells were removed from the culture broth by centrifugation (12,000×g, 4°C, 10 min), and the cell-free supernatant was concentrated by an Amicon magnetically stirred ultrafiltration cell, containing a YM10 membrane (exclusion molecular size, 10 kDa). The concentrated biosurfactant was extracted three times with an equal volume of chloroform-methanol (2 : 1, by volume). A crude biosurfactant was obtained as a brown-colored powder by removing the solvent under reduced pressure.

Further purification of the biosurfactant was achieved by an adsorption chromatography column (2.5×30 cm) on Silica gel 60 (Merck Co., mesh 230~400) and preparative reversed phase HPLC. The reversed phase HPLC was performed with a Shimadzu SPD-6A system (Shimadzu, Japan) on a Polygosil C_{18}, 10 μm (Metachem Inco., Torrance, 250×10 mm I.D.) column, and eluted with acetonitrile-10% trifluoroacetic acid (70 : 30, by volume) at a rate of 4.0 ml/min. Separation was monitored by UV detection at 214 nm.

Amino Acid Analysis

The amino acid composition of the hydrophilic part was determined on an amino acid analyzer (Pharmacia LKB 415a, Sweden) after total hydrolysis of the lipopeptide in 6 N HCl at 110°C for 18 h.

Amino Acid Sequence Analysis

The biosurfactant was partially hydrolyzed in 30% trifluoroacetic acid (TFA) at 110°C for 2 h. The resulting peptide fragments were separated and isolated by reversed phase HPLC on a Cosmosil C_{18}, 5 μm (150×4.6 mm I.D.) column. Elution was programmed from H_2O containing 0.12% TFA to 50% acetonitrile containing

0.1% TFA in 30 min. Amino acid sequence was determined through the identification and combination of partially hydrolyzed peptide fragments. The hydrolyzed peptide fragments were coupled with phenylisothiocyanate (PITC), and the fragments were identified on a Protein sequencer 476A (ABI). Through the combination of identified fragments, the amino acid sequence was determined.

Spectroscopic Analyses

Infrared spectroscopy was carried out on a Laser precision analytical (USA) IFX-65S instrument in KBr.

Fast atom bombardment-mass spectroscopy (FAB-MS) analysis was performed on a Kratos (Kratos Analytical Ltd., Manchester, UK) Concept-1S instrument with an NBA (3-nitrobenzyl alcohol) matrix. Mass spectra were collected from 500~1200 AMU. Positive ions were detected.

^{1}H-NMR, ^{13}C-NMR, and HMBC spectra were acquired at 300 MHz and 500 MHz on a Varian UNITY-300 and 500 NMR spectrometer (U.S.A.). The samples were prepared as solutions in 100% dimethylsulphoxide-*d* 6 (DMSO-*d* 6).

RESULTS

Selection of Biosurfactant-producing Bacteria

Three hundred colonies forming halos on agar plates covered with crude oil were isolated from soil samples. Isolated colonies were cultured in the isolation liquid medium in test tubes (15×150 mm) containing *n*-hexadecane, soybean oil, or glucose as a carbon source, and then by measuring the oil film-collapsing activity of the culture broth the twenty strains showing a large halo (more than 2 cm-diameter)were further isolated. These samples were assessed for surface tension and emulsification activity. This isolation program resulted in obtaining 3 bacterial strains (C9, A43, and F6) which were capable of emulsifying hydrocarbons effectively. The strains C9, A43, and F6 showed high emulsification activity only when cultured in glucose-, soybean oil-, and hexadecane-containing medium, respectively (Table 1). Among them, only the strain C9 grown in glucose-containing medium showed any emulsification activity towards crude oil, and reduced the surface tension of culture broth below 30 dyne/cm. Thus, the strain C9 was finally selected as a potential producer of biosurfactant for this study.

Identification of Strain C9

Morphological and biochemical characteristics of strain C9 are shown in Table 2. Strain C9 was gram-positive, motile, spore-forming, rod-shaped bacterium with a length of 1.3 to 2.3 μm and a width of 0.7 to 0.8 μm. Colonies were round and mucoid, and flattened out given a longer incubation time.

This strain grew in the medium with 7% (w/v) NaCl, at a temperature of 40°C , and at pHs of 5.0 to 9.0, but did not grow at 65°C or pH 11.

Strain C9 could use a variety of carbon sources for growth, including arabinose, glucose, mannitol, xylose, fructose, sucrose, and starch but not lactose, ethanol, or methanol. Strain C9 hydrolyzed gelatin, casein, starch; used citrate; and reduced nitrate, but did not produce indole or hydrogen sulfide. Catalase, oxidase, and β-galactosidase activities were observed, but no activities of urease, arginin dihydrolase, or lysine decarboxylase were detected. In addition, strain C9 included *meso*-type diaminopimelic acid in peptidoglycan layer, and branched type (13-methyl tetradecanoic acid and 12-methyl tetradecanoic acid) as a major fatty acid component. Quinone composition and G+C molar ratio of strain C9 were

Table 1. Surface activity and emulsification activity of the selected bacterial strains.

Strain No.	Carbon source	Oil film-collapsing (cm)[a]	Surface tension (dyne/cm)	Emulsification activity (unit/ml)[b]	
				Hydrocarbon	Crude oil
C9	Glucose	4.3	28.5	145.5	+
	Soybean oil	1.3	52.9	135.0	−
	Hexadecane	1.6	47.0	0	−
A43	Glucose	0.5	60.5	8.8	−
	Soybean oil	2.5	41.3	130.0	−
	Hexadecane	1.2	55.8	8.6	−
F6	Glucose	0.6	58.6	3.9	−
	Soybean oil	1.2	56.4	87.2	−
	Hexadecane	3.2	38.3	116.4	−
Control medium		0.4	71.2	0	−

[a]Oil film-collapsing was designated as the diameter of halo on the crude oil film. [b]Emulsification activities were measured against hydrocarbon and crude oil. Hydrocarbon was the equal volune mixture of *n*-hexadecane and 2-methylnaphthalene, and activity against crude oil was designated positive (+) or negative (−).

Table 2. Characterestics of the selected strain C9.

	Selected strain C9	*Bacillus subtilis*
Morphological		
Gram stain	+	+
Shape	rod	rod
Width of rod	0.7~0.8 μm	0.7~0.8 μm
Length of rod	1.3~2.3 μm	2.0~3.0 μm
Spore	+	+
Motility	+	+
Acid fast	−	−
Cultural		
Colony color	yellowish-white	
Growth in air	+	+
Growth anaerobically	−	−
Growth at 40°C	+	+
Growth at 65°C	−	−
Growth at pH 5.7	+	+
Growth at pH 11.0	−	−
Growth in 7% NaCl	+	+
Growth in alcohols	−	−
Physiological		
Catalase	+	+
Oxidase	+	d[a]
Glucose (acid)	+	+
O/F (Oxidation/Fermentation)	F	d
Carbohydrates, acid from		
glucose	+	+
arabinose	+	+
mannitol	+	+
xylose	+	+
fructose	+	+
sucrose	+	+
lactose	−	d
Hydrolysis of		
gelatin	+	+
starch	+	+
casein	+	+
Utilization of citrate	+	+
VP (Voges-Proskauer reaction)	+	+
Nitrate reduction	+	+
Indole	−	−
H_2S production	−	
Urease	−	d
β-galactosidase	+	d
Arginine dihydrolase	−	−
Lysine decarboxylase	−	−
Chemical		
DAP (Diaminopimelic acid)	*meso* type	*meso* type
Fatty acid	branched C_{15}	branched C_{12-17}
Quinone	MK-7 (menaquinone)	MK-7
G+C molar ratio	43.30%	41.8~46.3%

[a]d: diffrent reactions in different strains.

menaquinone (MK-7) and 43.3%, respectively. These properties of strain C9 indicated a close similarity to *Bacillus subtilis* (13, 16, 18, 22, 23), and the strain was named as *B. subtilis* C9.

Production of Biosurfactant by *B. subtilis* C9

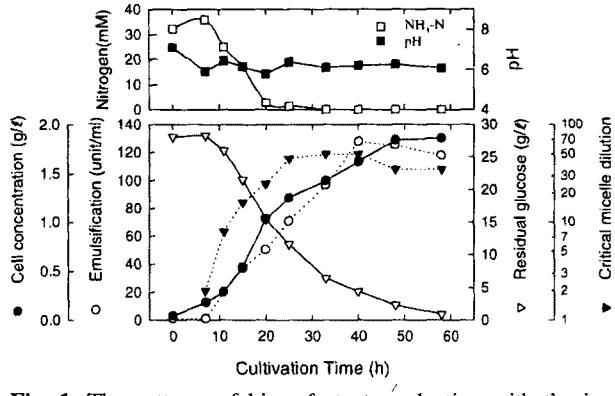

Fig. 1. The patterns of biosurfactant production with the jar-fermentor by *Bacillus subtilis* C9.
Cultivation was done with the basal medium containing 30 g/l of glucose, 1.0 l of working volume, 0.2~0.1 vvm of aeration rate, 300 rpm of agitation, and at 30°C.

Fig. 1 shows the biosurfactant production pattern in the culture medium containing glucose as a carbon source in the jar fermentor. The patterns of surface tension, CMD, emulsification activity, residual substrates, and cell concentration were examined. The biosurfactant production by *B. subtilis* C9 showed a growth-associated pattern. Nitrogen sources were all consumed at 25-h culture, and further increases in biosurfactant concentrations was not observed in nitrogen limited conditions. However, some other strains can produce a biosurfactant only in growth-limiting conditions. For example, *Pseudomonas aeruginosa* can overproduce rhamnolipid in nitrogen limiting conditions when the culture reaches the stationary phase (24).

The minimum surface tension of 28.1 dyne/cm was obtained during the early exponential growth phase (12 h). The maximum surfactant concentration was 50×CMC of the biosurfactant formed at the culture time of 25 h. Emulsification activity reached a maximum (127.7 unit/ml) after 40-h culture. The maximum biomass was 1.9 g/l. Glucose was almost consumed after 60-h of culture. During fermentation the culture medium changed to a dark brown color, and the foam was evident from the beginning the end of fermentation.

Purification of Biosurfactant

From the fermented broth, a compound capable of reducing surface tension and emulsifying hydrophobic material was isolated by ultrafiltration and solvent (chloroform : methanol=2 : 1, by volume) extraction. The ability of surfactant molecules to form micelles at concentrations above CMC allows these aggregates to be retained by relatively high molecular weight cut-off membranes (19). Lower molecular weight impurities such as salts, free amino acids, peptides and small proteins were easily removed by ultrafiltration. This isolation scheme

resulted in obtaining the major compound contributing to surface tension reduction and the emulsification of hydrocarbons and crude oil. The isolated powder was yellow-colored and we obtained 1.2 g from 1 l of culture broth.

The isolated biosurfactant was dissolved in chloroform and then placed on a column of silica gel. The surface-active compound was eluted with 500 ml of methanol-chloroform (1 : 3). Eluted compounds were finally separated by reversed phase HPLC, and then named C9-BS. Surfactin (Sigma, St. Louis, MO, U.S.A.) and C9-BS samples from each of the purification steps were analyzed by TLC. The purified biosurfactant, C9-BS had the same R_f value (0.63) to surfactin.

Structural Analysis of Biosurfactant C9-BS

IR spectroscopy. The IR spectrum of the purified C 9-BS in KBr (Fig. 2) showed strong bands, indicating the presence of peptide components, at 3282 cm^{-1}, 1648 cm^{-1}, and 1541 cm^{-1} resulting from the N-H stretching mode, CO-N stretching mode, and the deformation mode of the N-H bond combined with the C-N stretching mode, respectively. This spectrum also suggested the presence of an aliphatic chain (2950~2920 cm^{-1} and 1400-1380 cm^{-1}, $-CH_3$ and $-CH_2-$), and an ester carbonyl group (1737 cm^{-1}, ester C=O), suggesting a lactone ring. These results indicated that C9-BS was a lipopeptide containing a peptide moiety and aliphatic hydrocarbons.

FAB mass spectroscopy. The molecular weight of C9-BS was determined by FAB mass spectrometry (Fig. 3). The $[M+H]^+$ peaks of C9-BS were found at m/z 1022 and 1036 accompanied by $[M+Na]^+$ peaks at m/z 1044 and 1058. This analysis revealed the presence of molecular ion species of 1021 and 1035 Da. The difference of 14 Da in molecular weight may represent the difference of a methylene group in the fatty acid chain. Assuming that C9-BS contained 7 amino acid residues like those of surfactin, it could be supposed that C9-BS was bound to a C_{15} or C_{14} hydroxy fatty acid.

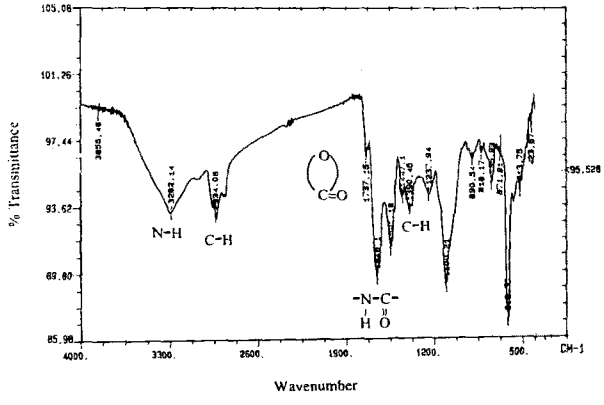

Fig. 2. Infrared spectrum of C9-BS surfactant in KBr.

Amino acid analysis. Ninhydrin assays performed on native biosurfactant C9-BS were negative. Following acid hydrolysis with 6 N HCl at 110°C for 18 h, C9-BS was positive to ninhydrin, indicating the presence of a peptide with a blocked N-terminus. Thus, it was conceivable that the native C9-BS had a cyclic closed structure for the peptide part. The amino acids present in acid-hydrolyzed C9-BS were identified by an amino acid analyzer. The following amino acid composition was obtained; Glx : Asx : Val : Leu=1 : 1 : 1 : 4. Glx and Asx can be in both the amine form (glutamine and asparagine) and the acid form (glutamic acid and aspartic acid). Acid hydrolysis would prevent detection of glutamine and asparagine due to conversion to glutamic acid and aspartic acid, respectively.

Amino acid sequence analysis. For amino acid sequence determination, C9-BS was partially hydrolyzed in 30% TFA, and subsequent fragments were separated by reversed phase HPLC. The selected ninhydrin positive fragments were identified yielding the sequences shown in Table 3. Glutamic acid was not detected in any of the fragments but was in the direct linkage of glutamic acid to the fatty acid part. The amino acid sequence, determined from the amino acid composition ra-

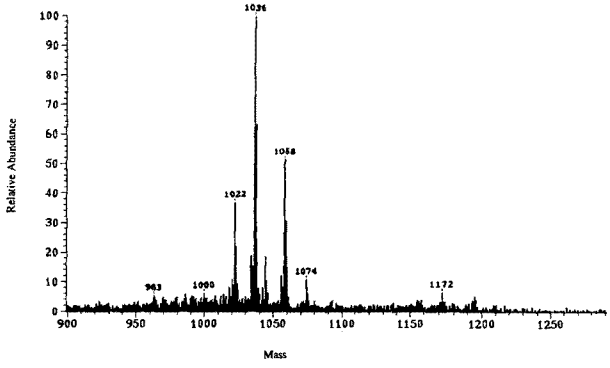

Fig. 3. FAB-MS spectrum of C9-BS surfactant.

Table 3. Amino acid seguences of peptide fragments.

Fragment	Rt (min)	Amino acid sequences
1	11.6	Val-Asp
2	13.2	Asp-Leu
3	14.7	Leu-Val
4	16.5	Leu-Leu-Val
5	18.4	Leu-Leu
6	20.2	Leu-Val-Asp-Leu
7	22.6	Asp-Leu-Leu
Total sequence		Leu-Leu-Val-Asp-Leu-Leu

The partially hydrolyzed peptide fragments were separated and isolated by reversed phase HPLC on Cosmosil C_{18} column. Elution was programmed from H_2O containing 0.12% TFA to 50% acetonitrile containing 0.1% TFA in 30 min.

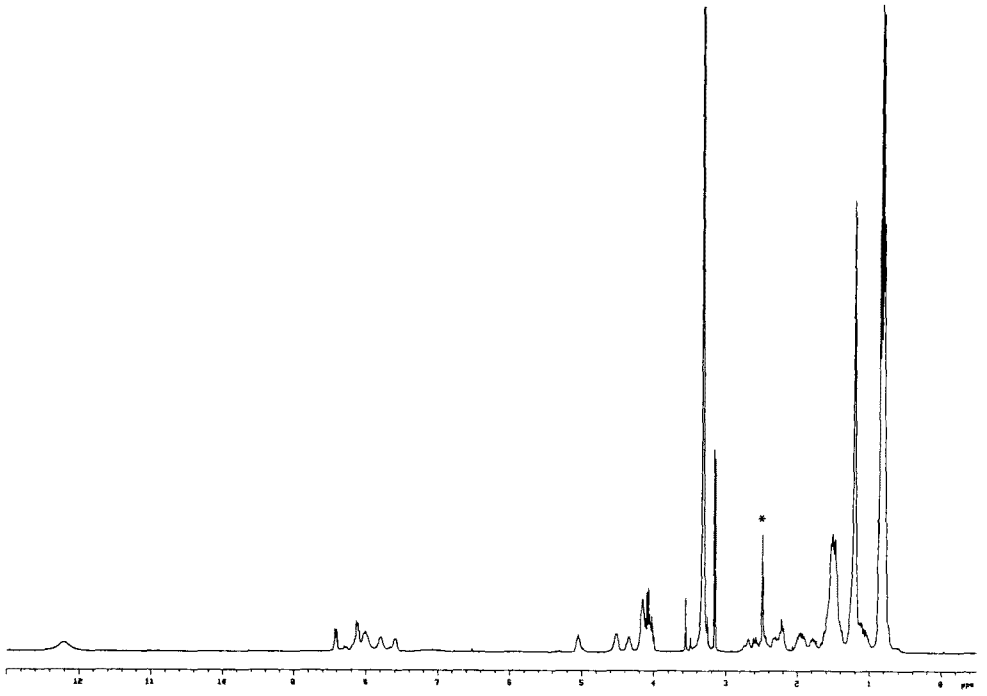

Fig. 4. ^{1}H-NMR spectrum of C9-BS surfactant in DMSO-*d6* at 25°C (300 MHz).
Chemical shifts are referenced to residual ^{1}H resonance of DMSO-*d6*(*) arising at 2.49 ppm.

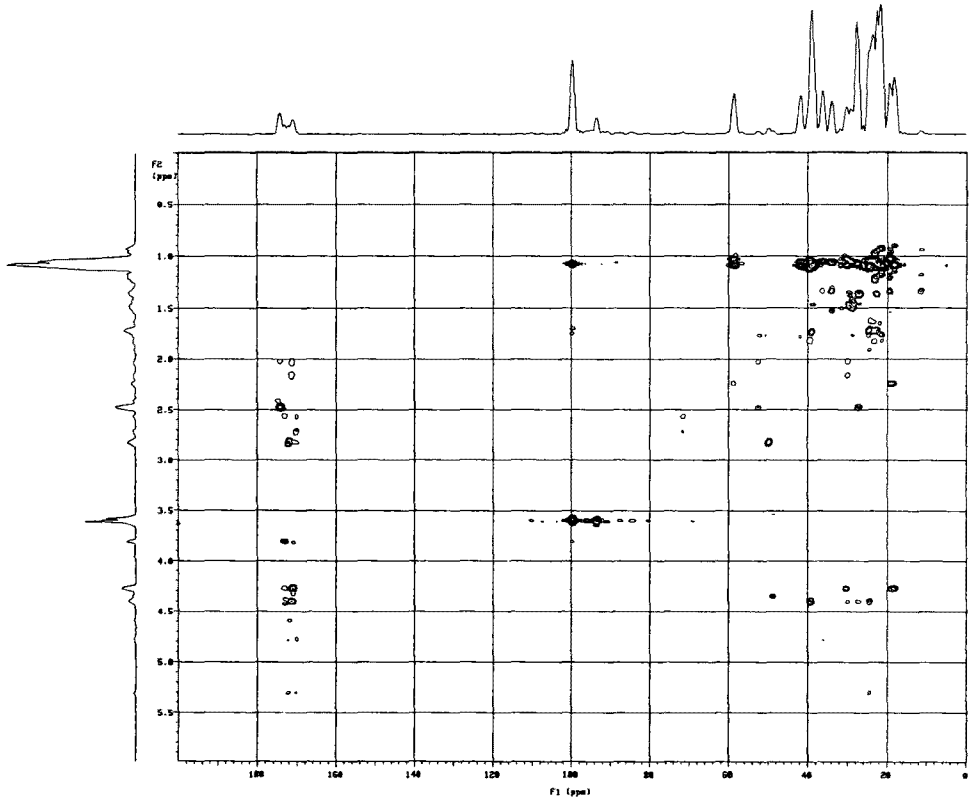

Fig. 5. HMBC spectrum of C9-BS surfactant in DMSO-*d6* at 25°C (500 MHz).

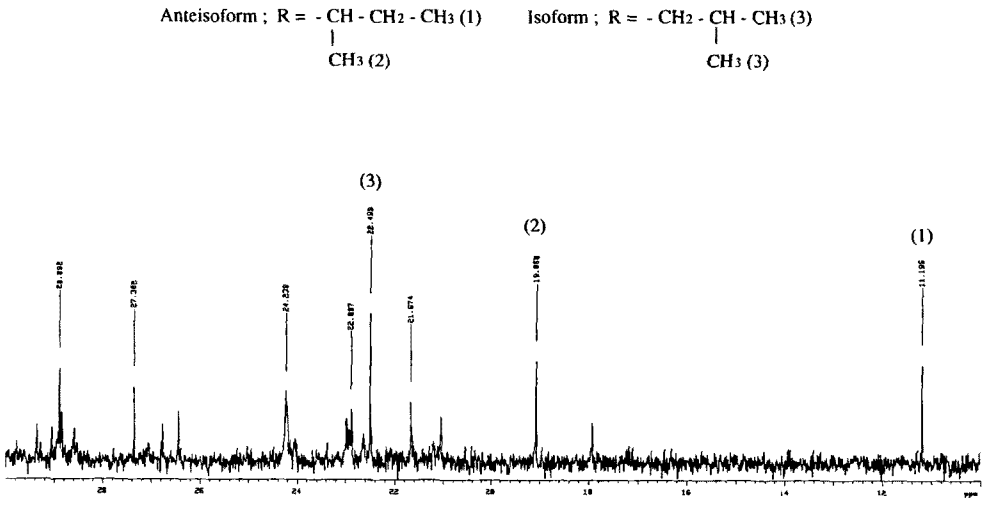

Fig. 6. ^{13}C-NMR spectrum of C9-BS surfactant in DMSO-*d*6 at 25°C (75 MHz).
Chemical shifts are referenced to residual ^{13}C resonance of DMSO-*d*6(*) arising at 39.5 ppm.

tio and the identification of partially hydrolyzed ninhydrin positive fragments, was as follows: Glu-Leu-Leu-Val-Asp-Leu-Leu.

NMR spectroscopy. The proton NMR spectrum of C9-BS was presented in Fig. 4. The spectrum confirmed the presence of a long aliphatic chain (CH_2 at 1.6~1.0 ppm) and a peptide backbone (NH at 8.6~7.4 ppm and CH at 4.6~4.0 ppm). The spectrum indicated the presence of the following amino acids: valine with peptide CH at 4.3, CH at 1.5, $2CH_3$ at 0.84 ppm; leucine with peptide CH at 4.1, CH_2 at 1.5, CH at 1.2, $2CH_3$ at 0.84 ppm; aspartic acid or asparagine with peptide CH at 4.5, CH_2 at 2.7 ppm; and glutamic acid or glutamine with peptide CH at 4.0, internal CH_2 at 1.7, and CH_2 α to the carbonyl at 1.9 ppm. Due to the complexity and overlap of the NMR spectrum most of these assignments are the result of detailed analyses of the 2-dimensional HMBC spectrum (Fig. 5). A resonance at 5.1 ppm indicated the presence of an ester group which may be a part of a lactone ring. Integration of the spectrum (assuming a value of 1.00 to the H of the resonance at 5.1 ppm, which is believed to be part of the lactone group) suggests the presence of 7 amino acids, based on the number of peptide CHs. Twelve terminal CH_3 groups were calculated (1.0~0.75 ppm region). The lipid chain seems to consist of mostly 8~9 CH_2 groups (1.3~1.0 ppm region). ^{13}C NMR analysis (Fig. 6) indicated that the lipid moiety of C9-BS is present in two different configurations, iso- and anteisoforms. It was observed that a mixture of anteiso- (11.2 and 19.1 ppm) and iso-branched forms (22.5 ppm) were present.

DISCUSSION

The isolation program, using oil film-collapsing assay, resulted in our obtaining 3 strains (C9, A43, and F6) capable of emulsifying hydrocarbons. *B. subtilis* C9 degraded glucose as a substrate, and synthesized fatty acids as lipophilic part through lipogenesis like other *Bacillus* sp. producing lipopepetide biosurfactant (7), whereas the strains A43 and F6 degraded fatty acids, and synthesized sugars the gluconeogenesis, the reverse glycolytic pathway (6) like *Pseudomonas* sp. producing glycolipid (24).

However, only *B. subtilis* C9 grown in glucose-containing medium effectively emulsified crude oil, and reduced the surface tension below 30 dyne/cm.

A biosurfactant, C9-BS was purified by ultrafiltration, solvent extraction, silica gel chromatography, and preparative-scale C_{18} reversed phase HPLC.

The structure and composition of C9-BS was studied by several analytical techniques. IR spectroscopy indicated that C9-BS was a lipopeptide containing a peptide moiety and aliphatic hydrocarbons, and FAB mass indicated that the molecular weight of the main peak was 1035.

Firstly the peptide portion of C9-BS was analyzed. The presence of amino acids, Leu, Val, Asx, and Glx were confirmed by amino acid analysis and NMR. Glx and Asx can be in either the amine form (glutamine and asparagine) or the acid form (glutamic acid and aspartic acid). Acid hydrolysis would prevent detection of glutamine and asparagine due to conversion to glutamic acid and aspartic acid, respectively. The ratio of these amino acids was determined to be 1 : 1 : 1 : 4 for Glx : Asx : Val : Leu. The amino acid analysis data, in which the sum of the amino acid molar ratio was 7, predicted the presence of a total of 7 amino acids per molecule. This prediction was in agreement with the NMR results. Amino acid sequences determined from the amino acid

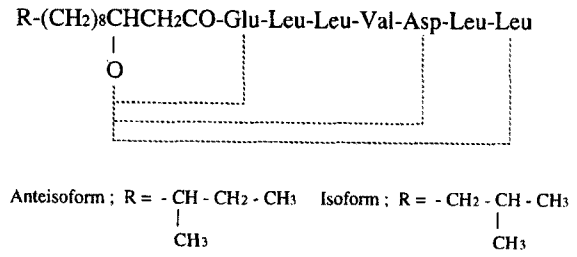

Fig. 7. The proposed structure of C9-BS surfactant.
The location of the lactone ring in the peptide was not determined. It could be either to the C-terminal leucine carboxy group or to side chain carboxy groups of aspartic acid or glutamic acid (·····).

molar ratio and the identification of partially hydrolyzed ninhydrin positive fragments were as follows; Glu-Leu-Leu-Val-Asp-Leu-Leu.

The presence of a lactone ring in the C9-BS was revealed by the IR and NMR spectra, which detected an ester carbonyl group, and was supported by the fact that the peptide has a blocked N terminus, and a negative ninhydrin result on intact biosurfactant. The location of the lactone ring in the peptide was not determined. It could be either to the C-terminal amino acid carboxy group or to the side chain carboxy groups of aspartic acid or glutamic acid within the peptide sequence.

The presence of an aliphatic hydrocarbon chain was detected by IR and NMR spectra. FAB mass spectrum revealed the presence of molecular ion species of 1021 and 1035 Da. The difference of 14 Da in molecular weight may represent the difference of one methylene group in the fatty acid chain. From the above results we can see that C9-BS contains 7 amino acid residues, and that the molecular weight of C9-BS was 1021 or 1035 Da. It also revealed that C9-BS was bound to a C_{15} or C_{14} hydroxy fatty acid. The NMR integration indicated 12 terminal methyl groups. The peptide portion could contribute 10 of the terminal methyl groups (i.e. methyl groups of Leu and Val). The remaining 2 terminal methyl groups were in the fatty acid chain. In addition, the carbon NMR analysis indicated that the lipid moiety of C9-BS is present in two different configurations, namely, iso- and anteisoforms. The anteisoform of fatty acids has been observed in a lipopeptide biosurfactant produced by *Bacillus licheniformis* (15).

Based on the analytical data obtained for C9-BS and previous literature concerning lipopeptides produced by the genus *Bacillus*, the following description of C9-BS could be proposed (Fig. 7).

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