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# ANNEX V - MICROBIAL ENHANCED OIL RECOVERY RESEARCH

Summary Annual Report
1991-1992

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# SUMMARY ANNUAL REPORT
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**TITLE:** Annex V - Microbial Enhanced Oil Recovery Research
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**PRINCIPAL INVESTIGATORS:** Mukul M. Sharma and George Georgiou

## OBJECTIVE

The objective of this work is to develop an engineering framework for the exploitation of microorganisms to enhance oil recovery. Specific goals include:

(i) the production, isolation, chemical characterization and study of the physical properties of microbially produced surfactants,
(ii) development of simulators for MEOR and
(iii) model studies in sandstone cores for the characterization of the interactions between growing microbially cultures and oil reservoirs,
(iv) design of operation strategies for the sequential injection of microorganisms and nutrient in reservoirs.

## INTRODUCTION

Three microbial activities play a critical role in microbial enhanced oil recovery.

i) production of surfactants,
ii) production of biopolymers, and
iii) selective plugging of high permeability zones.

The significance of biosurfactants in MEOR depends on several factors including the effectiveness of the biosurfactant in lowering the interfacial tension, the amount necessary to obtain significant surface tension reduction, and the adsorption of the surfactant on the rock surface. Generally, the interfacial tension must be lowered to $10^{-2}$ to $10^{-3}$ mN/m, corresponding to a capillary number $N_{ca} = 10^{-3}$ to $10^{-1}$, before significant oil recovery is achieved. Some biosurfactants are capable of reducing the interfacial tension to such values.

In this summary Annual Report, we briefly review the progress made in the past year. Details of our accomplishments to date will be available in the Final Report that is currently being prepared.

## SUMMARY OF PROGRESS

The surface active lipopeptide produced by *Bacillus licheniformis* JF-2 was isolated to near apparent homogeneity. NMR experiments revealed that this compound consists of a heptapeptide with an amino acid sequence similar to surfactin and a heterogeneous fatty acid consisting of the normal-, anteiso-, and iso- branched isomers. The surface activity of the *B. licheniformis* JF-2 surfactant was shown to depend on the presence of fermentation products and is strongly affected by the pH. Under conditions of optimal salinity and pH the interfacial tension against decane was $6x10^{-3}$ mN/m which is one of the lowest values ever obtained with a microbial surfactant.

Microbial compounds which exhibit particularly high surface activity are classified as biosurfactants. Microbial biosurfactants include a wide variety of surface and interfacially active compounds, such as glycolipids, lipopeptides polysaccharide-protein complexes, phospholipids, fatty acids and neutral lipids (4). Biosurfactants are easily biodegradable and thus are particularly suited for environmental applications such as bioremediation and the dispersion of oil spills (5, 13, 16).

*Bacillus licheniformis* strain JF-2 (8) has been shown to be able to grow and produce a very effective biosurfactant under both aerobic and anaerobic conditions and in the presence of high salt concentrations (7,11). The production of biosurfactants in anaerobic, high salt environments is potentially important for a variety of *in situ* applications such as microbial enhanced oil recovery (12). As a first step towards evaluating the commercial utility of the *B. licheniformis* JF-2 surfactant, we isolated the active compound from the culture supernatant, characterized its chemical structure and investigated its phase behavior. We found that the surface activity of the surfactant is strongly dependent on the pH of the aqueous phase. This may be important for the biological function of the surfactant and is of interest for several applications in surfactancy.

### Surfactant isolation

*Bacillus licheniformis* strain JF-2 ATCC #39307 was grown aerobically in a mineral salt medium (8) supplemented with 1.0 % trace salt solution (3) and 1% glucose in 2-liter Erlenmeyer flasks with a working volume of 1 liter at 42°C for 15 hours. The presence of the surfactant was detected by a decrease in the interfacial tension against decane. The value of the interfacial tension was determined using a spinning drop

interfacial tensiometer (Model 300, University of Texas, Austin, TX). This instrument allows us to very precisely measure ultra low interfacial tensions (down to $10^{-3}$ dyne/cm) by measuring the diameter of a drop of decane in an aqueous phase contained in a rotating horizontal tube (2).

The procedure employed for the isolation and purification of the biosurfactant is outlined in Figure 1. The cells were removed from the culture by centrifugation at 10,000xg for 15 minutes. Surface-active compounds were isolated from the culture supernatant either by acid-precipitation by adjusting the pH to 2.0 using concentrated HCl (1) or adsorption chromatography on an XAD-2 resin (Sigma Chemical Co.). For the former, the acidified culture supernatant was incubated at 4°C overnight and the precipitate was collected by centrifugation at 15,000xg for 15 minutes. Following separation of the precipitate and adjustment of the pH to 6.0, the interfacial tension increased from 0.085 mN/m for the cell-free culture fluid to more than 15 mN/m. The surfactant-containing precipitate was resuspended in 15 ml of water, pH 6.0, lyophilized and extracted with 5 ml of a mixture of chloroform and methanol (v:v = 1:2). A yield of about 250 mg of lyophilized acid-precipitate per liter of culture was obtained. Following extraction, the organic soluble material obtained after evaporation of the solvent was about 110 mg per liter of culture. For XAD-2 adsorption chromatography, the fermentation broth supernatant was loaded onto a 16x500 mm column at a flow rate of 1 ml/min. The column was eluted with 1.5 bed volumes of methanol following 1.5 bed volumes of water wash. Non-volatile material in the effluent was concentrated by evaporation in a Müchi rotary evaporator (Fisher Scientific) at 45°C. Upon evaporation, approximately 65 mg per liter of culture was obtained from the XAD-2 column.

The crude biosurfactant preparation obtained after either XAD-2 chromatography or acid precipitation was dissolved in 3 ml of a mixture of chloroform and methanol (1:1) and further separated into five fractions by silica gel chromatography on a silica gel (Mallinckrodt, Grade 62) column (28 x 500 mm). The column was eluted with 5% methanol in chloroform at a flow rate of 1 ml/min. Interfacial tension measurements showed that only one fraction, eluted after 1.8 bed volumes, contained surface active material. Analytical reverse phase $C_{18}$ HPLC, using a Waters HPLC system equipped with a Waters 7.8X300 mm μBondapak $C_{18}$ column, indicated that there were at least three major compounds in this fraction (Figure 2). The solvent system consisted of mobile phase A : 10 mM $KH_2PO_4$ buffer at pH6.0 and mobile phase B : 20 % tetrahydrofuran in acetonitrile (HPLC grade, Fisher Scientific). The column was eluted

with 53% B isocratically at a flow rate of 1 ml/min and the absorbance was monitored at 210 nm.

The active compound was eventually purified by preparative reverse phase liquid chromatography with a Waters $C_{18}$ μBondapak column (19X300 mm). Fractions collected were lyophilized and extracted with methanol to remove inorganic material which can affect the surface activity. Interfacial tension measurements indicated that fraction C contained the most surface active compound. At a concentration of 100 mg/l in freshly prepared medium with 0.5% NaCl at pH 6.0, the material obtained from fraction A, B, and C gave interfacial tensions against decane of 1.317, 1.873, and 0.060 mN/m, respectively. Overall, after the final preparative HPLC step approximately 35 mg of pure biosurfactant was obtained per liter of culture.

**Structure Determination.**

A series of experiments, including amino acid analysis, infrared spectroscopy, FAB/MS spectroscopy and NMR analysis, were employed to elucidate the chemical structure of the purified surfactant. FAB/MS (fast atom bombardment mass spectroscopy), analysis was performed on a Finnigan TSQ 70 mass spectrometer with a NBA matrix. The FABS/MS ion fragmentation pattern indicated that the molecular weight of the surfactant is 1035. FTIR spectra were obtained on a Nicolet 60SXR FT-IR spectrometer. Bands characteristic of peptides and aliphatic chains were observed in the IR spectrum (data not shown), indicating that the compound is a lipopeptide. Also observed was a band at wavenumber 1733 corresponding to an ester carbonyl group. To determine the amino acid composition of the lipopeptide, a sample was hydrolyzed in 6 M HCl at 105°C for 24 hours and injected to an Applied Biosystem 420H Derivatizer/Analyzer (Foster City, CA). Amino acid analysis indicated the presence of glutamic acid, aspartic acid, valine and leucine at a ratio of 1 : 1 : 1 : 4 respectively.

NMR spectra were obtained ($^1H$, $^{13}C$, and 2-D NMR) in 1, 2-dideuteriotetrachloroethane ($CDCl_2CDCl_2$) (Norell) at 348°K using a Bruker high-field (11.9T) nuclear magnetic resonance spectrometer. The proton NMR spectrum showed seven N**H** signals (δ 7.0 - 7.7) and seven corresponding C**H** signals (δ 3.9 - 4.9) for the α-amino acids of the peptide part (data not shown). These were readily correlated with one another as well as with the signals of the corresponding alkyl residues via 2-D COSY and TOCSY spectra. Since there were no signals for free $CONH_2$, eliminating asparagine and glutamine as possibilities, the identities of the amino acids were confirmed as aspartic acid, glutamic acid, leucine, and valine. The observation of an

additional low field signal at δ 5.3 confirmed the presence of lactone as indicated in the IR spectrum.

Attempts to obtain the sequence of the amino acids using 2-D NOE (ROESY) (Figure 3) in combination with 2-D TOCSY were only moderately successful because of signal overlap in both $CDCl_2CDCl_2$ and $90\%H_2O/10\%D_2O$. The following partial sequences were obtained (not in particular order) :

fatty acid-Glu-Leu, Asp-Leu, Leu-Leu

Carbon-13 NMR (90% $H_20$/10% $D_2O$) confirmed all the above proton NMR assignments (data not shown). Peak multiplicity was determined using the DEPT sequence and inverse CH correlation gave direct relationships between peaks in the proton and carbon spectra, aiding the assignment of both. The fatty acid component of the lipopeptide consisted of a mixture of normal, anteiso, and iso branched forms ($CH_3$ at δ 13.4, 10.7 and 18.7 and ca. 22, respectively), with the latter predominating as seen in the proton NMR spectra. Furthermore, the presence of only one major molecular ion species in the MS spectra indicated that the compound consists of a $C_{15}$ chain with little if any size heterogeneity. The chemical structure of biosurfactant produced by *B. licheniformis* JF-2 is shown in Figure 4. The presence of a family of lipopeptides with the same peptide domains but different chain lengths of lipid tails, which has been frequently observed for lipopeptides produced by other microorganisms (6, 9), was not observed in the *B. licheniformis* JF-2 compound.

The partial amino acid sequences in the *B. licheniformis* JF-2 lipopeptide are the same as those found in surfactin (10) and other related lipopeptides from *Bacillus* sp. such as esperin from *Bacillus mesentericus* (15) and BL-86 from *Bacillus licheniformis* strain 86 (6). Recent studies have indicated that *Bacillus* lipopeptides exhibit some heterogeneity in the composition of the peptide, particularly at the ultimate position (6,14). For example, Peypoux and Michel (14) reported that surfactin, the best studied biosurfactant, consists of two groups of homologous lipopeptides differing by the presence of valine instead of leucine at the seventh amino acid position. The relative ratio of $Val^7$ to $Leu^7$ was found to depend on the strain of *B. subtilis* and the growth conditions. However, in contrast to the results with surfactin no heterogeneity in the amino acid composition of the peptide could be detected in the compound from *B. licheniformis* JF-2.

**Interfacial Behavior.**

Interfacial tension measurements of the biosurfactant diluted in freshly prepared medium indicated that the critical micelle concentration (CMC) is 10 mg/l. The surface activity of the lipopeptide is strongly affected by the pH, microbial metabolites, and NaCl concentration. Interfacial tensions of various solutions containing 50 mg/l of pure biosurfactant were measured at a wide range of pH values, Figure 5. In all solutions the interfacial tension of the biosurfactant was significantly lower at pH 6.0 than at pH 7.0. For a biosurfactant solution consisting of fresh medium with 0.5% NaCl, the interfacial tension at pH 6.0 was 0.043 mN/m, while that at pH 7.0 was 1.571 mN/m. The same behavior was observed in solutions consisting of the supernatant of acid-precipitation with 5% or 0.5% of NaCl. Microbial metabolites, such as organic acids and alcohols in the supernatant of acid-precipitation, also positively affect the interfacial activity of the biosurfactant. At pH 6.0 , the interfacial tension of the biosurfactant solution consisting of the supernatant of acid-precipitation with 0.5% NaCl was two times lower compared to purified biosurfactant dissolved in fresh medium with 0.5% NaCl. The interfacial activity of the biosurfactant is favorably affected by the NaCl concentration. The interfacial tensions in the presence of 5% and 0.5% NaCl at pH 6.0 were 0.006 and 0.023 mN/m, respectively. We observed that NaCl and microbial metabolites act synergistically with the biosurfactant to obtain the maximum interfacial activity. The interfacial activity of biosurfactant in the supernatant of acid-precipitation, which contained microbial metabolites such as organic acids and alcohols, with 5% NaCl (0.006 mN/m) was about 10 times higher than that of the acid precipitate dissolved in freshly prepared medium with 0.5% NaCl (0.043 mN/m).

Despite the increased interest in biosurfactants in recent years, surprisingly little is known about their interfacial properties. For other lipopeptides, such as surfactin, the lowest interfacial tensions that have been reported are in the order of 0.1 to 1.0 mN/m (4). These values are too high for many applications such as microbial enhanced oil recovery. In contrast, we found that the *B. licheniformis* JF-2 lipopeptide is highly surface active. The lowest interfacial tension values we have measured using the fermentation broth supernatant are as low as $2.5 \times 10^{-3}$ mN/m (M. Powalla, unpublished observations). Given the structural similarity between the JF-2 surfactant and surfactin, we believe that the much higher interfacial tensions that have been obtained for the latter may not be accurate. The values may have been caused by an incomplete understanding of the effects of NaCl and pH as well as the measurement of interfacial tensions using low sensitivity techniques. In any case, we believe that the interesting interfacial behavior of

the *B. licheniformis* JF-2 lipopeptide we have observed call for a more detailed examination of the commercial potential of microbial surfactants for industrial and environmental applications.

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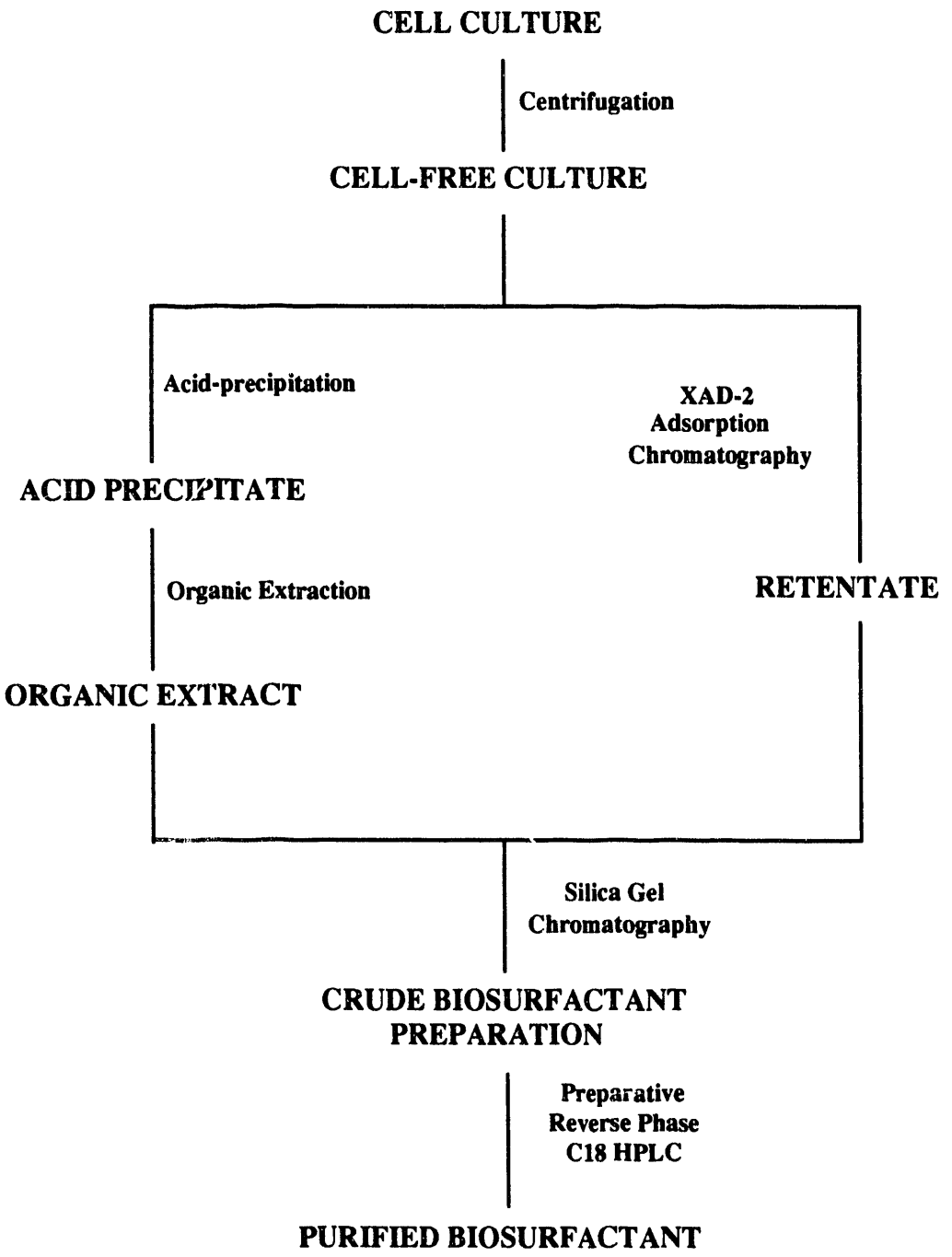



Figure 1 The procedures for the isolation and purification of biosurfactant produced by *Bacillus licheniformis* JF-2.

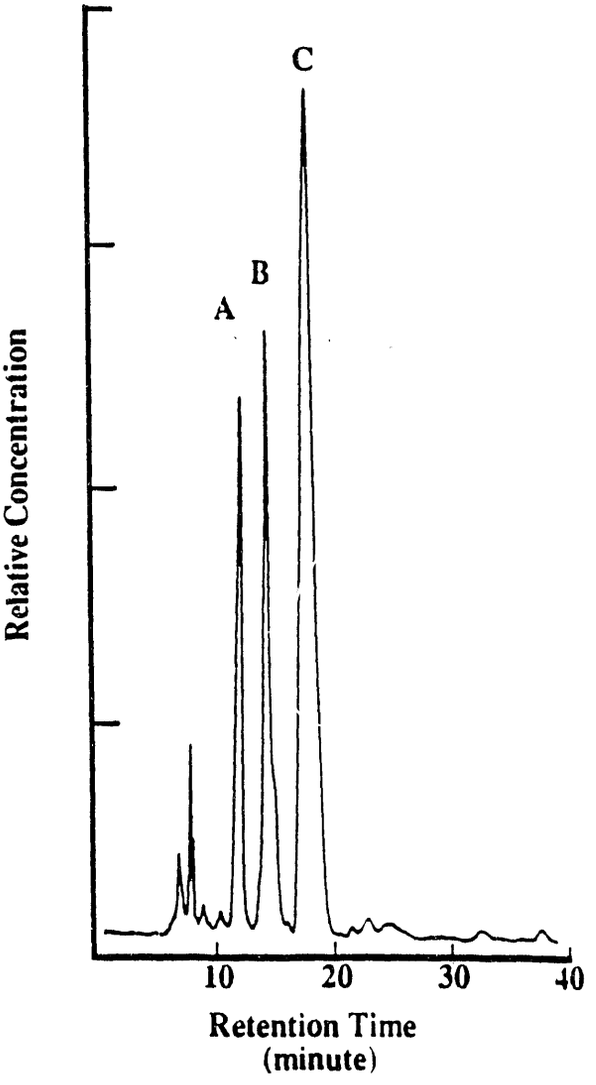



Figure 2 Reverse phase $C_{18}$ HPLC chromatogram of surface active material from silica gel liquid chromatography. Fraction C was identified as the most active compound.

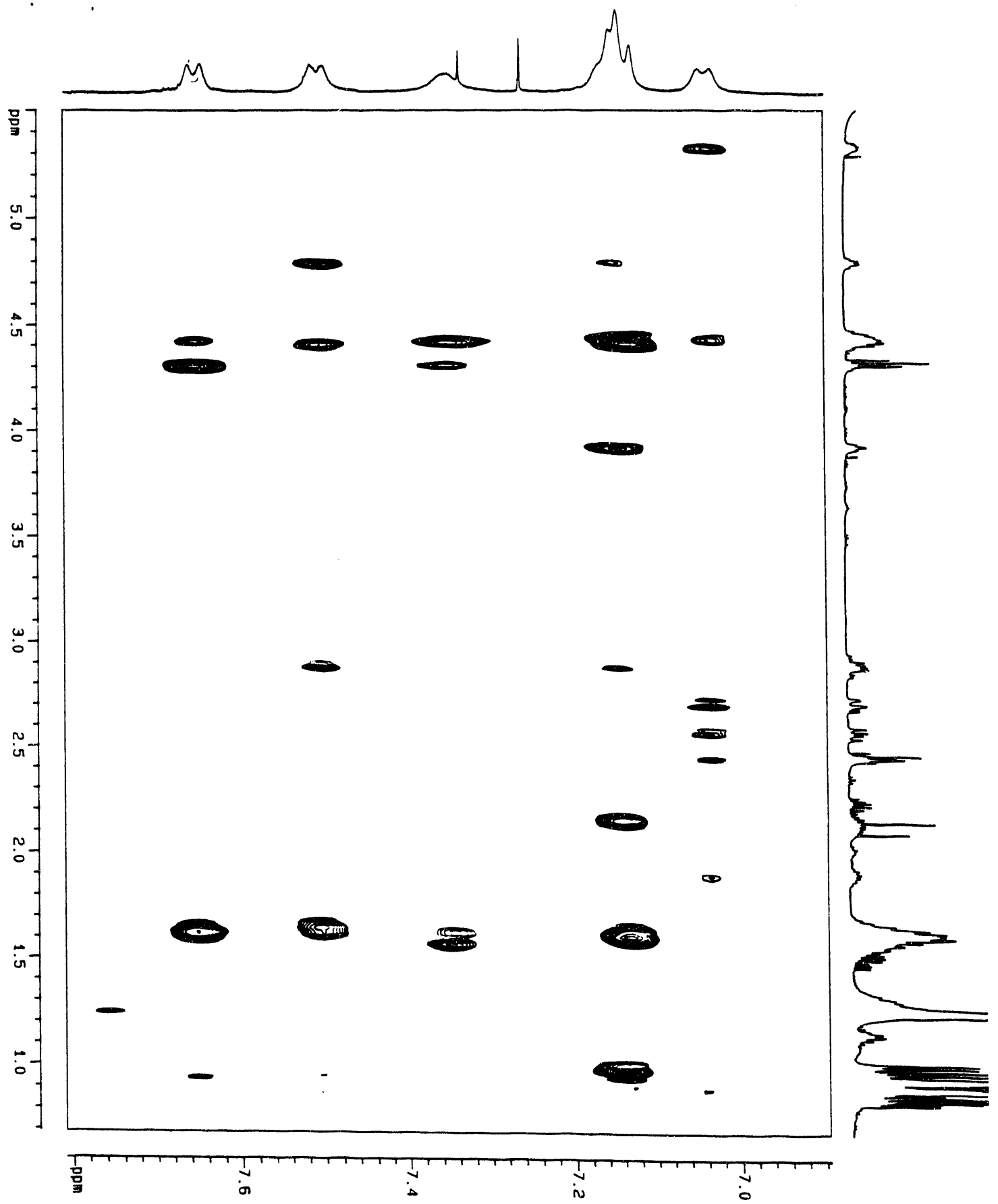
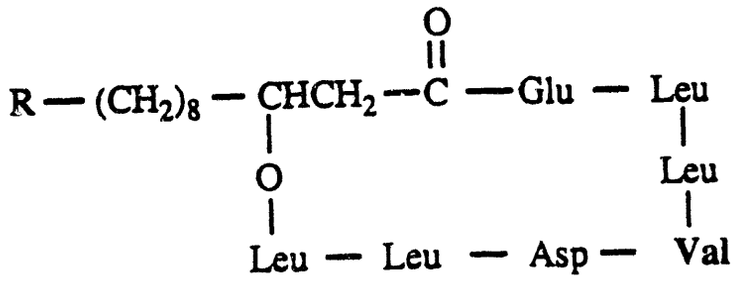
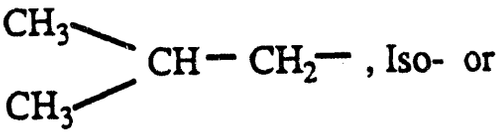



Figure 3 Two dimensional ROESY NMR spectrum of the purified biosurfactant obtained in $CDCl_2CDCl_2$ at 348 °K. Both direct and indirect C-H connectivities were observed.



Where R = $CH_3$-- $(CH_2)_3$-- , Normal- or



, Iso- or

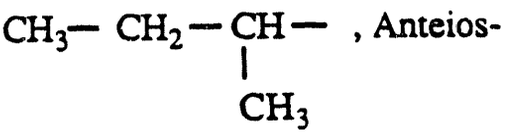


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Figure 4 Proposed chemical structure of the biosurfactant produced by *Bacillus licheniformis* JF-2 based on NMR experiments and the structure of the peptide part of surfactin. The position of valine (**Val**) can not be unambiguously identified because of signal overlap in the ROESY spectrum.

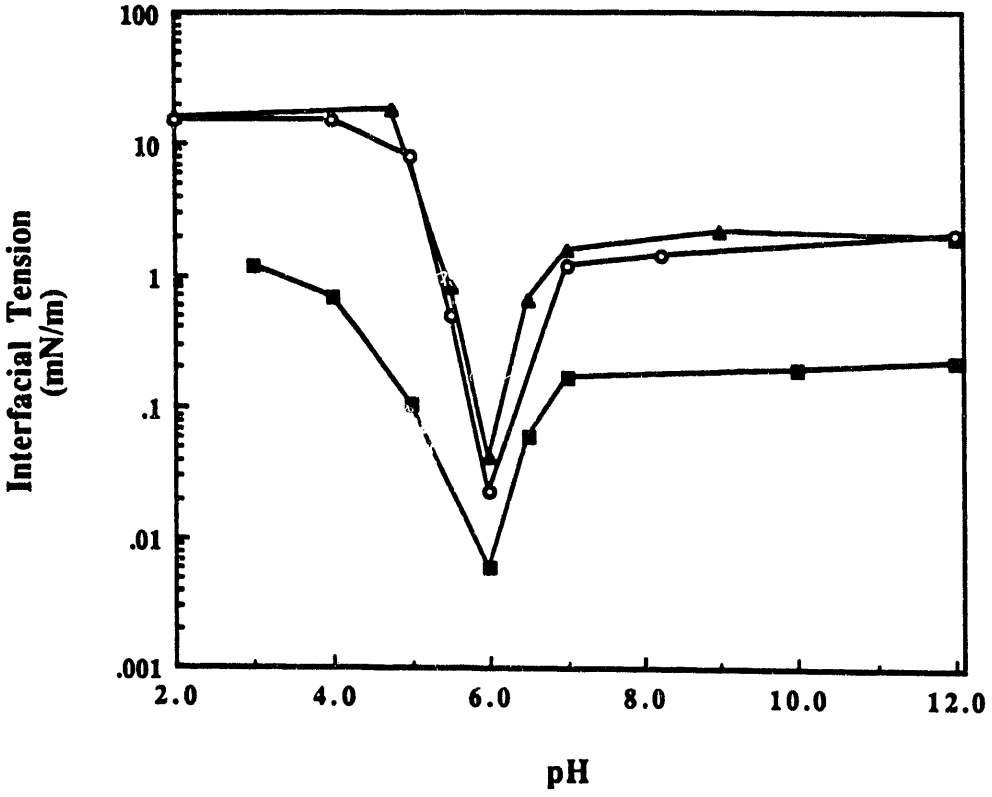


Figure 5 The effects of pH, microbial metabolites, and NaCl concentration on the interfacial activity of the biosurfactant produced by *Bacillus licheniformis* JF-2. The interfacial tension against decane for solutions of purified biosurfactant resuspended at a concentration of 50 mg/l in : fresh medium with 0.5% NaCl (s), the supernatant of acid-precipitation with 0.5% NaCl (m), and the supernatant of acid-precipitation with 5% NaCl (n) were measured as a function of pH.

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