Isolation and structural characterization of Coryxin, a novel cyclic lipopeptide from Corynebacterium xerosis NS5 having emulsifying and anti-biofilm activity

Dina Dalili a, Mohsen Amini b, Mohammad Ali Faramarzi c, Mohammad Reza Fazeli a, Mohammad Reza Khoshayand a, Nasrin Samadi a,∗

a Department of Drug and Food Control, Faculty of Pharmacy and Pharmaceutical Quality Assurance Research Center, Tehran University of Medical Sciences, Tehran, Iran
b Department of Medicinal Chemistry, Faculty of Pharmacy and Drug Design and Development Research Center, Tehran University of Medical Sciences, Tehran, Iran
c Department of Pharmaceutical Biotechnology, Faculty of Pharmacy and Biotechnology Research Center, Tehran University of Medical Sciences, Tehran, Iran

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A B S T R A C T

Herein we reported the structure and several properties of a new biosurfactants produced by Corynebacterium xerosis strain NS5. This strain was capable of producing a novel lipopeptide biosurfactant that we have named coryxin. The biosurfactant structure was characterized by using Fourier transform infrared spectroscopy (FTIR), Nuclear magnetic resonance spectroscopy (NMR), and Liquid chromatography-mass spectrometry (LC–MS). It contained a hydrophobic moiety of 3-hydroxydecanoic acid and a peptide part predicted as a sequence of seven amino acids including Asn–Arg–Asn–Gln–Pro–Asn–Ser. Coryxin lowered the surface tension of water to 31.4 mN/m, with a critical micelle concentration of 25 mg/L. It was a strong emulsifier with an emulsification index of 61% against n-hexane. Coryxin showed antibacterial activity against test organisms belonging to Gram-positive and Gram-negative bacteria and disrupted preformed biofilms of Staphylococcus aureus (82.5%), Streptococcus mutans (80%), Escherichia coli (66%) and Pseudomonas aeruginosa (30%). In conclusion, microbial surfactant from C. xerosis exhibited inhibitory and disruptive activities against biofilm formation that could be of use in biofilm-related menace.

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1. Introduction

Microbial compounds that exhibit pronounced surface and emulsifying activities are classified as biosurfactants. Biosurfactants have unique amphipathic properties derived from their complex structures, including both hydrophilic and hydrophobic moieties. They partition preferentially at the interface between fluid phases that have different degrees of polarity, such as oil and water or air and water interfaces [1]. These traits allow them to form micelles, lower the surface tension, and thereby enhance the solubility of poorly soluble compounds in water [2]. Biosurfactants are considered to be secondary metabolites; some may play essential roles for the survival of biosurfactant-producing microorganisms under hostile environmental conditions through facilitating nutrient transport, antagonistic effects toward other microbes in the environment or by involving in cell adherence [1,3].

Most of biosurfactants are used as emulsifiers, solubilizers, as well as foaming and wetting agents. In recent years they have been found to possess several properties of therapeutic and biomedical importance such as anti-adhesive and anti-microbial activities [4]. Several advantages of these molecules over chemically synthesized surfactants include the absence of toxicity, biodegradability, and possibility of production from renewable feed stocks. Their specific activity at extreme temperatures, pH, and salinity make these microbial products an efficient replacer or possible enhancer of chemically synthesized surface active agents [2,5].

Biosurfactants comprise a wide range of chemical structures including lipopeptides, glycolipids, phospholipids, fatty acids, neutral lipids, and lipopolysaccharides. Among the several categories of biosurfactants, lipopeptides are particularly interesting due to their high anti-microbial activities [1]. Their exceptional surface activity allows for modification of the bacterial surface hydrophobicity, which interfere with microbial adhesion to solid surfaces [6]. Therefore, as a new strategy, these biomolecules may be used as a safe and effective anti-adhesive coating agent for any surface, including medical devices.
Lipopeptides have a hydrophilic part that contains polar amino acids, while the hydrophobic part contains lipophilic fatty acids and non-polar amino acids [4]. Among the lipopeptides, surfactin produced by Bacillus subtilis is the first and the most well-known. Since its discovery, various types of lipopeptides have been isolated and characterized. Fencycin, iturin, bacillomycins, and mycosubtilins produced by B. subtilis, lichenysin produced by Bacillus licheniformis and pumilacin produced by Bacillus pumilus are the other antimicrobial lipopeptides.

Production of these anti-microbials by Bacillus probiotics is one of the major mechanisms by which they inhibit the growth of pathogenic microorganisms in the gastrointestinal tract [7]. It has been shown that probiotic organisms, such as Lactobacillus spp., produced multifunctional molecules, known as biosurfactants, which had antagonistic and anti-adhesive properties against microbial pathogens [8]. Velaeds et al. [9,10] showed that the initial adhesion rate of Enterococcus faecalis to glass with the biosurfactant from Lactobacillus acidophilus RC14 or Lactobacillus fermentum B54 was significantly decreased by approximately 70%. They also demonstrated that the biosurfactants from L. acidophilus RC14 and L. fermentum B54 were rich in protein, while those from L. casei subsp. rhamnosus 36 and ATCC 7469 had relatively high polysaccharide and phosphate contents.

Zajic et al. [11] found that Corynebacterium hydrocarboclas- tus produces compounds having both surfactant and emulsifying properties. Panohal and Zajic [12] reported that emulsifying agents which also demonstrate surfactant properties are produced by Corynebacterium sp. PPS-II. Thavasi et al. [13] demonstrated the emulsification capacities of the biosurfactant produced by Corynebacterium kutscheri against hydrocarbons.

Rennie et al. [14] demonstrated that underarm odour (UAO) was almost confined to aerobic coryneforms. It was also demonstrated that bacteria with the ability to generate UAO possess a range of potent enzymes capable of transforming lipids. Based on this, we hypothesized that coryneforms with the ability of metabolizing lipids may be capable of producing biosurfactant. The literature survey also showed that there are several reports on the production of surface active agents by Corynebacterium species [11–13], but there are only a few studies which defined the chemical structure of these compounds. Therefore, the aim of present study was to study the biosurfactant from Corynebacterium xerosis NS5 isolated from the human axillary microbiota. The structure of this new cyclic lipopptide biosurfactant was reported for the first time and some of its physicochemical and biological activities were determined.

2. Materials and methods

2.1. Isolation and identification of bacterial strain

The bacterial strain was obtained from the axilla of a healthy volunteer with sponges wetting in phosphate buffer saline (PBS) containing 1% Tween 80. The sponges were placed onto brain-heart infusion (BHI) agar (Merck Co. Germany) plates and incubated at 37 °C for 24 h. The resulting colonies were transferred to blood agar plates and Gram stained. The suspect Gram-positive bacilli with rusty pink appearance on blood agar were subjected to biochemical tests for identification of Coryneform bacteria, including catalase, indole, motility, urease and sugar fermentation tests [15,16].

Bacterial strain was further identified by 16S rDNA sequence analysis. Genomic DNA was extracted by boiling the cell suspension at 94°C for 10 min and centrifugation at 11000 × g for 5 min. The primer pair of 27F (5′-AGAGTTTGATCCTTGCTCAG-3′) and 1525R (5′-AAGGAGGTGATCCAGGC-3′) were used to amplify a fragment of the 16S rDNA gene [17].

The PCR program comprised an initial template denaturation step of 3 min at 94°C followed by 30 cycles of denaturation for 60 s at 94°C, annealing for 45 s at 60°C, synthesis for 90 s at 72°C, and a final extinction for 90 s at 72°C.

Amplified DNA was then separated on 1% agarose gel and sequenced. The resulting DNA sequence was compared with available sequences of GeneBank using the basic local alignment tool (BLAST) ([http://www.ncbi.nlm.nih.gov/BLAST/]). The 16S rDNA sequence of Corynebacterium xerosis strain NS5 was submitted to the NCBI GenBank with the accession number of KF177174.1.

2.2. Biosurfactant production

The mineral salt medium (MSM) was made according to Lindhardt et al. [18]. Seed culture was prepared by transferring a loopful from a fresh culture grown on case agar (Merck Co. Germany) plate into 50 ml of sterile MSM contained in 250 ml Erlenmeyer flask. The flask was incubated in a rotary shaker at 150 rpm and 30°C for 24 h. Biosurfactant production was carried out in 500 ml Erlenmeyer flasks containing 100 ml sterile MSM. The flasks were inoculated with the seed culture at 5%v/v and incubated at 150 rpm and 30°C for 24 h [19].

The time course study of biosurfactant production was carried out for 48 h, as described above. Samples were taken at regular interval during the incubation period and variation in surface tension, as an indication of biosurfactant production, was recorded by du Nouy ring method using a K20 tensiometer (Krüss GmbH, Hamburg, Germany).

2.3. Extraction and purification of biosurfactant

The bacterial cells were removed by centrifugation at 10,000 × g for 20 min at 4°C. Biosurfactant was precipitated from the culture supernatant by adjusting the pH to about 1.0–2.0 and standing overnight at 4°C. Biosurfactant was extracted with a mixture of CHCl3 and CH3OH (2:1 v/v) and concentrated under reduced pressure using a rotary evaporator (Buchi, Switzerland). This yielded a viscous honey-colored biosurfactant product. About 1.0 g of the crude biosurfactant was extracted per liter of culture medium. The crude biosurfactant was further purified by column chromatography (2.5 × 50 cm; silicagel 60; minus 230 mesh; Macherey Nagel Co., Germany). The column was first eluted with chloroform in order to separate fatty acids. It was then eluted with increasing amounts of methanol in chloroform to liberate the absorbed product. The separation of biosurfactant was monitored by TLC using chloroform/methanol/water (85:15:2, v/v/v) as mobile phase. For the detection of peptides, the TLCs were visualized using ninhydrin solution (1.5%) in acidified butanol and heating at 100–110°C.

The purified fraction was analyzed by reverse-phase high-pressure liquid chromatography (HPLC) system (Waters Chromatography Division, Milford, MA, USA) which consists of Waters 510 pump and solvent delivery system. The analysis was operated at a flow rate of 1.0 ml/min with a solvent of 0.1% trifluoroacetic acid (TFA)/acetonitrile (4:6, v/v) as mobile phase. Instrument control and data acquisition were performed with a personal computer running the software Autochro-2000 (Chromatograph Data System). Separation was achieved using a column Intersil ODS-3 V (5 μm, 4.6 × 250 mm, GL Sciences Inc, Japan) and detection was performed using a Waters 486 UV Detector at 220 nm.

The surface activity of the isolated compound was determined by measurement of surface tension, critical micelle concentration (CMC) and emulsification index.
2.4. Determination of surfactant activity

2.4.1. Measurement of surface tension and critical micelle concentration (CMC)

The surface tension was determined by a K20 tensiometer (Krüss GmbH, Hamburg, Germany) at 25 ± 1 °C, according to the du Noý ring method. Surface tension measurement was performed on aqueous solution of purified biosurfactant in the concentration range of 100 to 1.5 mg/l. Each measurement was repeated 5–10 times with the experimental deviation of ±0.1 mN/m. The accuracy of the measurements is controlled by the surface tension measurements of water before each set of measurements. The critical micelle concentration (CMC) is the concentration of a surface-active compound at which the surface becomes fully loaded with surfactant molecules and above which the formation of micelles in the solution is initiated.

The CMC and the surface tension at the CMC were estimated from the breakpoint in the surface tension and the surfactant concentration plot [19].

2.4.2. Emulsification index

The emulsifying capacity of the biosurfactant was evaluated by determination of emulsification index (E24). Emulsification index was measured according to the method of Cooper and Goldenberg [20] with some modifications. The purified lipopeptide dissolved in 5.0 ml distilled water (0.5% w/v) was mixed with 5 ml of n-hexane (as a hydrophobic substrate), and then vortexed at high speed for 2 min. The mixture was allowed to stand for 24 h at 25 °C prior to measurement. Synthetic surfactant, Tween 80 (Merck Co. Germany), was used for comparison.

The emulsification index (E24) is defined as the height of the emulsified layer (cm) divided by the total height of the liquid column (cm) and expressed as percentage. The higher the emulsification index, the higher the emulsification activity of tested surfactant.

2.5. Structural characterization of the biosurfactant

2.5.1. Fourier transform infrared spectroscopy (FTIR)

FTIR spectroscopy was used to detect the functional groups of purified biosurfactant. The FTIR spectra, with a resolution of 4/cm, were collected from 400 to 4000 wave numbers (cm⁻¹), with an average of 128 scans.

2.5.2. Nuclear magnetic resonance spectroscopy (NMR)

Chemical composition of the purified biosurfactant was characterized by ¹H NMR and ¹³C NMR. The purified compound was dissolved in CDCl₃ and the ¹H NMR and ¹³C NMR spectra were recorded at room temperature using a Bruker DRX (Germany) spectrometer 500 MHz and 125 MHz respectively. Chemical shifts (δ) are given on the ppm scale relative to tetramethylsilane (TMS).

2.5.3. Liquid chromatography-mass spectrometry (LC-MS)

LC-MS analysis was conducted on Agilent 6410 Triple Quadrupole mass spectrometer coupled to an Agilent 1200 series liquid chromatography equipped with an auto sampler (1200 series). 20 μl of the purified sample, (10 μg/ml in methanol) was introduced into the instrument. Methanol-water (90:10) was used as the mobile phase. The HPLC flow rate was 0.35 ml/min and directly introduced into the mass spectrometer. Mass spectrometric conditions were as follows: Drying gas (nitrogen) flow rate: 6.0 l/min; Nebulizing gas adjusted at 10 psi, Desolvation gas temperature at 300 °C, Capillary voltage adjusted at 4 kV. Electro spray ionization (ESI+) was used as the method of ionization and Mass spectrometer was operating on positive mode, over the mass range of 100–2000 m/z. Mass Hunter software was used for data processing.

2.6. Anti-bacterial activity

Anti-bacterial activity of the lipopeptide biosurfactant against Streptococcus mutans ATCC 35668, Staphylococcus aureus ATCC 6538, Pseudomonas aeruginosa ATCC 9027, and Escherichia coli ATCC 8739 was evaluated by microdilution method [21] in 96-well U shaped plastic tissue culture plates. A 200 μl aliquot of stock solution (100 mg/ml) of the biosurfactant in Mueller–Hinton broth (MHB) (Merck Co. Germany) was transferred into the first well in each row and serially diluted by mixing with 100 μl of MHB in subsequent wells. Then, 100 μl of bacterial suspension (1 × 10⁵ CFU/ml) was added to each well (except for the negative control column) to reach the final inoculum size of about 5 × 10⁵ CFU/well. After 24 h incubation at 35 °C, the microdilution trays were tested for the presence of visible growth in comparison with that of the growth in biosurfactant-free control wells. The endpoint MIC is the lowest concentration of the compound at which the test strain does not demonstrate visible growth.

2.7. Biosurfactant-mediated disruption of pre-formed biofilms

Inhibition of biofilm production by the lipopeptide biosurfactant was quantified according to the procedure described by O’Toole [22]. Four known biofilm forming strains, P. aeruginosa ATCC 9027, S. mutans ATCC 35668, S. aureus ATCC 6538, and E. coli ATCC 8739 were used for comparative purposes.

A standard biofilm assay medium for P. aeruginosa and E. coli was M63 minimal medium, one-quarter strength (25%) of brain–heart infusion broth supplemented with 2% sucrose (BHIS) for S. mutans, and caso broth (CB) supplemented with an additional 0.25% (W/V) glucose for S. aureus [23]. Briefly, the bacteria were grown over night in lysogeny broth (LB), after which they were diluted 1:100 into biofilm assay medium and dispersed in sub-100 μl volumes into the wells of a flat bottom 96-well microtiter plate. For quantitative assay, 4–8 replicate wells were used for each treatment. Plates were incubated for 24 h at 37 °C. After incubation period, unattached cells were removed and the medium was replaced with CB containing different concentrations of biosurfactant (0.05–100 mg/ml). These plates were further incubated at 30 °C for 24 h. At the end of incubation, the planktonic cells were discarded by turning the plate over, shaking out the liquid and washing the wells three times with PBS. Adhesion of cells and biofilm formation were quantified by using the crystal violet assay [24]. The adherent microorganisms were fixed with 200 μl of methanol (99% purity) per well, and after 15 min, the plates were emptied and left to dry. Then the wells were stained with 200 μl of 2% solution of crystal violet in water for 5 min at room temperature.

Excess stain was rinsed out by placing the plate under running tap water. Subsequently, the plates were air-dried, and the dye bound to the adherent microorganisms was resolubilized with 200 μl of 30% acetic acid in water per well. The absorbance of each well was measured in a microplate reader at 600 nm using 30% acetic acid in water as the blank. The medium without the biosurfactant was used as non-treated control.

The percentage of inhibition at different biosurfactant concentrations for each microorganism was calculated as:

Percentage inhibition = \[1 - \frac{(A_{\text{sample}})}{(A_{\text{control}})}\] × 100

2.8. Anti-adhesive assay

The anti-adhesive activity of lipopeptide biosurfactant toward four bacterial strains was determined by using 96-well microtiter plates [25]. A 2-fold dilution of biosurfactant stock solution (100 mg/ml) was prepared in the first horizontal row. Then 100 μl...
of the bacterial suspension (10^6 CFU/ml) was transferred into the each well to yield the final volume of 200 μl. Negative control contained sterile medium instead of bacterial suspension. After 24 h incubation at 37°C, the planktonic cells were discarded and the crystal violet assay was performed as mentioned earlier. The results were expressed in terms of percentage of cell adhesion compared to biosurfactant-untreated wells.

2.9. Scanning electron microscopy (SEM)

Cells of S. aureus were grown overnight in LB at 30°C. Aliquots of 200 μl of the culture (~10^6 cells/ml) were added to sterile Petri dishes containing 20ml of LB medium and pre-sterilized microscopic glass slides as surfaces for biofilm formation. The plates were incubated at 30°C for 24 h. After incubation, the glass slides were removed and placed in LB medium containing biosurfactant (1.6 mM). The Petri dishes were further incubated at 30°C for 24 h to determine the effect of biosurfactant on the pre-formed biofilm. Then, the glass slides were removed, rinsed twice with PBS, and air dried. Dehydration procedure was carried out by transferring the samples to ethanol/water solutions of increasing concentrations (10, 25, 40, 50, 70, 80, 90, and 95%) for 15 min in each solution. The samples were maintained desiccated until gold sputtering and visualized by scanning electron microscope (Hitachi S4160) operating at 20 kV. Biofilms without biosurfactant treatment served as controls.

3. Results

3.1. Identification of the bacterial isolate

An effective biosurfactant-producing strain, NS5, was isolated from the underarm microbiota.

Cells of the isolated strain were Gram-positive non spore-forming bacilli with positive catalase, glucose, fructose, mannose, galactose and sucrose fermentation, producing acid but no gas and negative indole, motility and urease reactions. These biochemical results classified it as belonging to Corynebacterium genus. Comparison of 16S rDNA nucleotide sequence from strain NS5 with sequences in the GenBank database, NCBI, USA, revealed 99% similarity with the corresponding sequences from C. xerosis.

3.2. Production and purification of biosurfactant

Time course analysis of the production of biosurfactant showed that the surface tension started to decrease by surfactant production, reaching to its lowest value after about 22–25 h of incubation. The partially purified biosurfactant that was obtained after acidification of the cell-free broth and solvent extraction was subjected to column chromatography. The surface-active fraction migrated as a single ninhydrin positive spot on the TLC (Rf of 0.60). It was detected by reversed-phase HPLC and one pure pick with retention time of 3.18 min revealed (data not shown).

3.3. CMC and Emulsification index of the biosurfactant

The surface tension of the isolated biosurfactant was measured by du Noüy ring method. Fig. 1 shows the plot of surface tensions versus concentrations of the biosurfactant. The extracted lipopeptide showed high-surface activity, as it could lower the surface tension of water from 72 to 31.4 mN/m. CMC was determined with the aid of a series of concentrations and was around 25 mg/l. The results suggested that the crude biosurfactant provided excellent properties in terms of the reduction of surface tension and a low value of the CMC.

![Graph showing surface tension versus concentration of the biosurfactant produced by Corynebacterium xerosis NS5.](image)

The emulsifying properties of the biosurfactant from C. xerosis strain NS5 was studied with n-hexane as a hydrophobic substrate and compared with commercial surfactant Tween 80. This biosurfactant had 61% emulsification ability in comparison to 55% for Tween 80.

3.4. Structural characterization of the purified biosurfactant

3.4.1. IR Spectrometric analysis

Direct characterization of the purified biosurfactant was performed by using FTIR analysis (Fig. 2). The characteristic absorbance bands of peptides at 3391/cm (NH-stretching), and 1644/cm resulting from the stretching mode of the amide bond could be clearly observed. In addition, the presence of aliphatic chains (–CH3; –CH2–) is also clear, which is represented by the bonds between 2957 and 2855/cm, and 1437–1377/cm. The bond observed at 1734/cm is attributed to the lactone carbonyl absorption. Peaks at 1233 and 1193/cm are referred to C–O–C vibrations in esters. FTIR analysis indicated the presence of aliphatic hydrocarbons combined with a peptide moiety which confirmed the lipopeptide nature of the isolated biosurfactant.

3.4.2. NMR analysis

The 13C and 1H NMR analysis of the purified biosurfactant are shown in Fig. 3A, B. The 1H NMR spectrum confirmed presence of an aliphatic chain (CH2 at 1.25–1.55 ppm), and a peptide backbone (NH at 7.00–7.60 ppm and CH at 4.8–4.2 ppm). The 13C NMR spectrum indicated the presence of 10 amide and ester carbonyl groups (C=O at 160–170 ppm). Furthermore, the peaks at 10–48 ppm indicated the presence of an aliphatic side chain in the lipophilic moiety and some CH2 groups in the side chain of the amino acids. The peaks at 50–76 ppm could be attributed to –NH–CH–CO– of amino acids. R

3.4.3. LC–MS analysis

The structure of purified biosurfactant was further elucidated by means of LC–MS analysis. The mass spectrum is shown in Fig. 4. In this spectrum, specific fragment ions were recognizable, and sequential losses of the C-terminal amino acid were observed. By referring to the mass unit of the amino acids, the sequence of Asn–Arg–Asn–Gln–Pro–Asn–Ser seemed to be the most probable amino acid sequence (Table 1). Thus, the lipopeptide surfactant produced by C. xerosis NS5, most likely composed of seven amino acids coupled to one molecule of 3-hydroxydecanoic acid.

Since this surface active compound was first isolated from C. xerosis strain NS5, it was named “coryxin”. The proposed complete structure of coryxin is shown in Fig. 4.
3.5. Anti-bacterial activity

The antimicrobial activity of the lipopeptide biosurfactant isolated from C. xerosis strain NS5 was measured by its MICs against two Gram-positive and two Gram-negative bacteria. The isolated biosurfactant presented anti-microbial activity against all test microorganisms, although depending on the microorganism the biosurfactant presented different effective concentrations. It showed the MIC value of 0.19 mg/ml against S. aureus and S. mutans and the MIC values of 3.12 mg/ml and 100 mg/ml against E. coli and P. aeruginosa, respectively.

3.6. Disruption of pre-formed biofilm

In the present study, coryxin disrupted the one-day developed biofilms of all test cultures, albeit to different degrees. The anti-biofilm activity was dependent on the biosurfactant concentration and the microorganism tested (Fig. 5). Treatment of preformed biofilms (24 h grown) of S. aureus, S. mutans, E. coli and P. aeruginosa in polystyrene microtiter plates with coryxin (100 mg/ml) resulted in up to 82.5%, 80%, 66% and 30% biofilm dislodgement, respectively.

The preformed biofilms were effectively removed at concentrations greater than the MIC values in a dose-dependent manner. At lower concentrations (0.4 mg/ml or less), biofilm disruption by the coryxin was not quite evident. At higher concentrations, there was a dose-dependent increase in biofilm disruption caused by the coryxin.

3.7. Anti-adhesive effect

Lipopeptides, a class of biosurfactants, are known to decrease adhesive interactions [1]. To test the anti-adhesive activity of the isolated biosurfactant, we measured the adherence of four different bacteria to 96-well microtiter plates at different concentrations (0.05–100 mg/ml) of coryxin. As shown in Fig. 6, the highest anti-adhesive activity was exhibited against S. aureus with inhibition percentage of up to 77.4%. The biosurfactant also inhibited adhesion of S. mutans (72%), E. coli (65%) and P. aeruginosa (52%).

3.8. Scanning electron microscopy (SEM) analysis

Disruption of preformed biofilm promoted by conditioning the surface with isolated biosurfactant was visualized by SEM (Fig. 7). The untreated S. aureus cells grew as biofilms (Fig. 7A) and showed the presence of cells tightly bound to the surface. Treatment of the biofilms with biosurfactant resulted in destruction of microcolonies (Fig. 7B).

4. Discussion

The present study reported the potential of C. xerosis for the production of a new lipopeptide biosurfactant that was named "coryxin". The predicted structure of this lipopeptide was different from other lipopeptides reported so far. The structure of coryxin was consisting of a heptapeptide and a β-hydroxy fatty acid with 11 carbon atoms. The amino acid sequence of coryxin was Asn–Arg–Asn–Gln–Pro–Asn–Ser. A lactone bridge between the β-
Fig. 3. $^1$H (A) and $^{13}$C (B) nuclear magnetic resonance (NMR) spectrum of Corynebacterium xerosis NS5 biosurfactant.

Fig. 4. Electrospray ionization mass spectrum and structure of the lipopeptide biosurfactant (coryxin) produced by Corynebacterium xerosis NS5.
hydroxyl group of the acid and the carboxyl terminal group of the peptide confers a cyclic structure to the molecule.

Microbial surfactants are commonly differentiated on the basis of their biochemical nature, functional properties, and microbial species producing them [26]. There are numerous reports on the isolation of biosurfactant-producing bacteria, of which only a few have well-defined chemical structures. Lipopeptide biosurfactants are cyclic structures generally produced by Bacillus and Pseudomonas species. They mainly consist of hydrophilic peptides, usually between 7 and 10 amino acids long, linked to a hydrophobic fatty acid structure. Bacillus cyclic lipopeptides consist of three major groups known as the surfactin, iturin and fengycin families. Surfactin consists of a 7 amino acid cyclic sequence connected to a C13–C16 fatty acid. Iturin also consists of 7 amino acids, though different to surfactin, linked to C14–C17, while fengycin is composed of ten amino acids with fatty acid chain length of C14–C18 [27]. Pseudomonas lipopeptides consist of a fatty acid attached to a peptide, which is cyclized to form a lactone ring between two amino acids in the peptide chain. Both iturin and coryxin have similar amino acid residues except in position 2 of peptide moieties (Arg in coryxin, and Tyr in iturin). The fatty acid moiety is straight in coryxin while it is branched in iturin. It is a general tendency that a straight-chain fatty acid has more surface activity than a branched-chain fatty acid. In comparison with surfactin (27 mN m\(^{-1}\)) the relatively low surface activity of coryxin suggest that Asp and Glu (in surfactin) are more effective hydrophilic residues than Asn, Gln or Ser (in coryxin) in exhibiting high surface activity. CMC is one of the main characteristics of surfactants as above this concentration no further effect is expected in the surface activity. For practical purposes, it is important to distinguish between an effective biosurfactant and an efficient biosurfactant. Effectiveness is measured by the minimum value to which the surface tension can be reduced, whereas efficiency is measured by the biosurfactant concentration required to produce a significant reduction in the surface tension of water. The latter can be determined from the CMC of the biosurfactant [28]. In this study, coryxin effectively lowered the surface tension of water to 31.4 mN/m. Coryxin also showed to be highly efficient as well, since its CMC value was found to be 25 mg/l. Coryxin has higher surface activity than iturin (37.5 mN/m). The various isoforms of the surfactin produced by different strains of B. subtilis have been reported to lower the surface tension of water to 25–40 mN/m [4].

**Fig. 5.** The effect of Corynebacterium xerosis NS5 biosurfactant at different concentrations (0.2–100 mg/ml) on 1 day developed biofilm of different bacteria. Data represent the mean and SD of three independent experiments each in triplicate format.

**Fig. 6.** The anti-adhesive activity of Corynebacterium xerosis NS5 biosurfactant at different concentrations (0.2–100 mg/ml) toward a variety of bacteria to 96-well microtiter plates. Data represent the mean and SD of three independent experiments each in triplicate format.
The emulsifying power is another important character of any surfactant. Therefore, it was assessed for the test biosurfactant using the emulsification index (E24) against n-hexane. The emulsifying activity was measured using an oil/water ratio of 1:1, which means that the oil phase constitute 50% of the total volume. The E24 values greater than or equal to 50 entails a complete emulsification of the oil phase [28]. Coryxin showed the emulsification index of 61% which was higher than that of Tween 80. This was similar to the range of 59–66% exhibited by *B. subtilis* surfactin against hydrophobic substrates, as reported by Abdel-Mawgoud et al. [28].

The interest for application of biosurfactants in the medical field has been increased during the past decade due to their potential anti-microbial and anti-biofilm properties [29]. The results of this study indicated that test Gram-positive bacteria were more sensitive to the isolated biosurfactant than Gram-negative bacteria. This result is quite comparing to earlier reports on antimicrobial actions of the biosurfactants where the lipopeptide biosurfactants have been reported to be active mostly against Gram-positive bacteria [7]. The SEM images obtained in this study showed that before treatment of biosurfactant, the *S. aureus* cells showed normal smooth surfaces. Meanwhile, clustering of cells was observed and they were tightly bound to the glass surface. In contrast, the outer membrane of the cells treated with biosurfactant was rough. Cells were scattered and few clustering were seen. Our results suggest that the new biosurfactant produced by *C. xerosis* NS5 is a promising compound for inhibition/disruption of biofilms. However, its potential for application in industrial and medical fields needs to be studied in detail.

5. Conclusions

In this study, a new cyclic lipopeptide biosurfactant was isolated from *C. xerosis* NS5. The structure of this biosurfactant consisted of a heptapeptide and a β-hydroxy fatty acid with 11 carbon atoms. The amino acid sequence of coryxin was Asn-Ile-Arg-Glu–Gin–Pro–Asn–Ser. Coryxin lowered the surface tension of water to 31.4 mN/m, with a critical micelle concentration of 25 mg/l and a high emulsification index with n-hexane. Coryxin showed anti-bacterial activity against test organisms belonging to Gram-positive and Gram-negative bacteria and showed anti-adhesive and anti-biofilm activities against *S. aureus, S. mutans, E. coli*, and *P. aeruginosa*. In conclusion, microbial surfactant from *C. xerosis* exhibited inhibitory and disruptive activities against biofilm formation that could be of use in biofilm-related menace.

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