Research Article

**Identification and natural functions of cyclic lipopeptides from *Bacillus amyloliquefaciens* An6**

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**Keywords:** Antimicrobial activity; Antioxidant activity; *Bacillus amyloliquefaciens*; Lipopeptides; MALDI-ToF-MS

**Practical application**

*B. amyloliquefaciens* An6 produces multiple lipopeptides belonging to surfactin, iturin and fengycin families. An6 lipopeptides exhibited an antioxidant activity and have a protective role against oxidative stress. Furthermore, An6 lipopeptides were active against a variety of Gram-negative and Gram-positive bacteria and some fungi. Consequently, An6 lipopeptides may be useful for cosmetic, therapeutical or pharmaceutical purposes in order to delay or prevent oxidative deterioration of manufactured products. An6 lipopeptides may represent a novel antimicrobial substances with potential applications in food safety, with important implications for the biological control of pathogenic and spoilage microorganisms.
Abstract

Lipopeptides constitute a structurally diverse group of metabolites produced by various bacterial and fungal genera. In the past decades, research on lipopeptides has been fueled by their surfactant activities. However, natural functions of lipopeptides compounds have received considerably less attention. The aim of this study was to isolate and identify the lipopeptides from *Bacillus amyloliquefaciens* An6, and further evaluate their biological activities. An6 lipopeptides were detected by polymerase chain reaction (PCR) using degenerated primers and Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-ToF-MS). An6 strain was found to produce surfactin, fengycin and bacillomycin. Following their purification, the *in vitro* antioxidant activity of An6 lipopeptides was studied through different assays. The scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals at a dosage of 0.75 mg/ml was 81%. Its reducing power was concentration-dependant and reached a maximum of 1.07 at 2.5 mg/ml. Moreover, they showed a strong inhibition of β-carotene bleaching. An6 lipopeptides mixture was also found to display significant antimicrobial activity against several Gram-positive, Gram-negative bacteria, and fungal strains. An6 lipopeptides were insensitive to proteolytic enzymes, stable between pH 4.0 and 12.0 and resistant to high temperature. Our results provided enough evidence proving that An6 lipopeptides could be used as functional-food components.

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1 Introduction
The formation of potentially toxic compounds caused by the oxidative deterioration of lipids in foods is responsible for the decrease in food quality and safety. It is necessary to suppress lipid peroxidation in food in order to preserve its nutritional value [1]. Antioxidant supplementation to foods is the most effective way for delaying the lipid peroxidation. Many synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene, t-butylhydroquinone and propyl gallate have been extensively used in industrial processing. However, there is a growing concern about the use of synthetic antioxidants because they are reported to be carcinogenic and toxic [2]. Therefore recent research has focused on the development and use of antioxidants from natural sources. In recent years, hydrolyzed proteins from many animal and plant sources such as milk casein [3], canola [4], and egg-yolk protein [5] have been found to possess antioxidant activities. In addition, aquatic products and by-products have also proven to be good sources of antioxidant peptides [6].

Microorganisms are also a rich source of bioactive compounds. Bacillus strains are the most important producers of bioactive secondary metabolites with great potential in different fields. Some of these peptides are produced by the non-ribosomal biosynthetic pathway, using large enzymes, referred to as nonribosomal peptide synthetases (NRPS), which use the multiple-carrier thiotemplate mechanism. Many species of Bacillus such as Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus pumuli, Bacillus cereus, Bacillus thuringiensis, and...
Bacillus licheniformis are able to produce these nonribosomal peptides, particularly lipopeptides [7]. Lipopeptides are valuable microbial amphiphilic molecules with efficient surface-active and biological properties. Fengycin, surfactin and iturin are the main lipopeptides families produced by Bacillus strains [8]. They are made up of seven (surfactins and iturins) or 10 α-amino acids (fengycins) linked to one unique β-amino (iturins) or β-hydroxy (surfactins and fengycins) fatty acid [9]. Lipopeptides have gained a great scientific interest thanks to their attractive properties like high biodegradability, reduced toxicity, better environmental compatibility and enhanced specific activity at extreme conditions of temperatures, pH levels and salinity [10]. Lipopeptide properties may lead to applications in diverse areas of industry. In the pharmaceutical industry, for instance, lipopeptides were given a great deal of attention when classic antibiotics were no longer working against pathogenic bacteria or fungi. Due to their structure, lipopeptides exert their toxicity on the cell membrane permeability bearing the similitude of a detergent like effect. Ongena and Jacques [8] demonstrated that surfactin presents an antagonistic activity against bacteria whereas iturin and fengycin show important antifungal spectra. Lipopeptides were also active against several enveloped viruses, including herpes viruses and retroviruses. Several studies revealed that lipopeptides act directly on the lipid envelope of the virus. Hydrophobicity is another important factor due to its nonspecific detergent activity which easily disintegrates the virus particles [11]. Owing to their low cytotoxicity against human cells and their biological properties, lipopeptides received a great interest in cosmetics. Indeed, they are used in dermatological products as anti-aging, cleansers and moisturizing agents [12]. Lipopeptides are also applied for environmental applications such as bioremediation of petroleum hydrocarbons [13] and in agriculture as biocontrol agents of plant diseases.
Finally, in food production, lipopeptides are used specially as emulsifiers, foaming, wetting, solubilizers, antiadhesives and antimicrobial agents [14]. In addition, lipopeptides are characterized by their antioxidant activity [15]. They exhibit a scavenging activity towards free radicals [12] and they are effective in delaying lipid peroxidation. Thus, they could serve as a food preservative and might be a good alternative to synthetic antioxidants that have some side effects such as carcinogenicity [16].

Therefore, the objectives of this study were to detect the presence of NRPS genes by PCR and characterize the produced lipopeptides by MALDI–TOF-MS. The antioxidant and antimicrobial activities of partially purified lipopeptides were investigated.

2 Materials and Methods

2.1 Bacterial strain

The microorganism used in this study was isolated from the soil of a detergent industry in Tunisia. It was identified as *B. amyloliquefaciens* An6 according to the methods described in Bergey’s Manual of Determinative Bacteriology and on the basis of the 16S rDNA sequence analysis. It was assigned the accession number FJ517583 [17].

2.2 Detection of NRPS genes by PCR and DNA sequencing

The primer pairs (Af2-F/Tf1-R, As1-F/Ts2-R and Abl1-F/Tbl1-R) were used to detect, by PCR, the nonribosomal genes coding for lipopeptide synthetases in the Genomic DNA of *B. amyloliquefaciens* An6. These primer pairs were determined based on the conserved motifs obtained after the alignment of adenylation or thiolation domains that
compose lipopeptide synthetases [18] (Table 1). The DNA was prepared using the Wizard Genomic DNA Purification Kit and protocol (Promega Corp., Madison, USA).

The PCR conditions included an initial denaturation step at 94°C for 3 min, followed by 30 cycles, with denaturation at 94°C for 1 min, annealing for 30 s at 43°C with As1-F/Ts2-R and at 45°C with Af2-F/Tf1-R and Abl1-F/Tbl1-R, respectively, and extension at 72°C for 45 s. Final extension was performed at 72°C for 10 min. The Taq polymerase used was “Master Mix” (Thermo Scientific Fermentas, Illkirch, France) with a final primer concentration of 1.2 μM. The desired bands were purified with QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and then ligated into pGEM-T Easy Vector (Promega Corp.). Recombinant plasmids were transferred into E. coli JM109 cells by a heat shock, according to the manufacturer’s protocol (Promega). Plasmids were extracted from the transformed cells using the QIAprep Spin Miniprep kit (Qiagen, Germany). Cloned PCR products were sequenced using the universal primers pUC-M13-R/F (Eurofins MWG Operon, Ebersberg, Germany). DNA sequences were analysed with the GenBank databases using the BLAST (Basic Local Alignment Search Tools) software provided online by the National Center for Biotechnology Information (Bethesda, MD, USA).

2.3 Lipopeptides identification by Mass spectrometry analysis

Matrix-assisted laser desorption ionization-time of flight (MALDI-ToF) mass spectrometry was used to screen lipopeptides from whole bacterial cells on solid media as previously reported [19].

2.4 Lipopeptides quantification by HPLC analysis
For lipopeptides production, the isolated organism was grown aerobically in Landy medium [20]. Culture was carried out for 72 h at 30 °C with shaking at 160 rpm. Culture was then centrifuged at 15,000 × g for 15 min.

For their quantification, lipopeptides were purified from culture supernatant by solid phase extraction using C18 Maxi clean cartridges (Extract – Clean SPE 500 mg, Alltech Deerfield, IL) used according to the recommendations of the supplier. Lipopeptides were eluted with methanol (100%). The extract was then characterized by high-performance liquid chromatography (600 s, Waters, USA) using a C18 column (5 µm, 250 mm × 4.6 mm, 218 TP, VYDAC). Each family of lipopeptides was separately analyzed with the acetonitrile-water-trifluoroacetic acid (TFA) solvent system (40:60:0.5 [vol/vol/vol] for iturin, 80:20:0.5 [vol/vol/vol] for surfactin, and a gradient from 45/55/0.1 to 55/45/0.1 for fengycin) and a flow rate of 0.6 ml min⁻¹. Samples (20 µl) were injected and compared to the appropriate standards (purified iturins, fengycins and surfactins provided by ProBioGEM laboratory). The retention time and second derivatives of UV-visible spectra (Waters PDA 996 photodiode array detector; Millenium Software) of each peak were used to identify the eluted molecules.

2.5 Lipopeptides purification

An6 lipopeptides were recovered from fermentation cultures by ultrafiltration (molecular weight cut off of 10,000 Daltons). The separation was carried out in two steps: (i) in step 1, lipopeptides were effectively rejected in the micellar form and (ii) in the second step, they were recovered in the permeate upon addition of methanol (75%) which disrupted
the micelles. Finally, the permeate was collected, concentrated and lyophilized, resulting in partially purified lipopeptides mixture which was used for subsequent analysis.

2.6 Evaluation of antioxidant activity of An6 lipopeptides

2.6.1 DPPH free radical-scavenging assay

The DPPH free radical scavenging potential of the An6 lipopeptides was determined by measuring the decrease of the absorbance of the reaction mixture as described by Bersuder et al. [21]. 500 µl of An6 lipopeptides mixture and standard BHA (used as a positive control) at different concentrations (0.25 to 5 mg/ml) was mixed with 375 µl of 99% ethanol and 125 µl of DPPH solution (0.02% in ethanol) as a free radical source. After incubation at room temperature in the absence of light for 60 min, the absorbance of the mixture solution was measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the equation of DPPH radical scavenging activity.

\[
DPPH\% = \frac{A_{control} - A_{sample}}{A_{blank}} \times 100
\]

where \(A_{control}\) is the absorbance of the control sample containing distilled water instead of the sample, and \(A_{sample}\) is the absorbance of An6 lipopeptides/standard BHA samples. The experiment was carried out in triplicate and the results were the mean values.

2.6.2 Reducing power assay

Ferric reducing antioxidant power assay of An6 lipopeptides was carried out as described by Oyaizu [22]. 1 ml of An6 lipopeptides at different concentrations (0.25 to 5 mg/ml) was mixed with 2.5 ml phosphate buffer saline (pH 6.6, 0.2 M), and 2.5 ml of potassium ferricyanide \((K_3Fe(CN)_6)\) solution (1%, w/v). After incubation for 30 min at 50 °C.
2.5 ml of trichloroacetic acid (10%, w/v) was added to the mixture, and then centrifuged at 10,000 rpm for 10 min. A volume of 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 2.5 ml of ferric chloride (0.1%, w/v) and then the absorbance at 700 nm was measured.

2.6.3 β-carotene bleaching by linoleic acid assay

The ability of the An6 lipopeptides to prevent bleaching of β-carotene was assessed as described by Koleva et al. [23]. Aliquots (2.5 ml) of the β-carotene/linoleic acid emulsion were transferred to test tubes containing 500 µl of An6 lipopeptides samples at different concentrations. The emulsion system was incubated for 2 h at 50 °C, and the absorbance of each sample was measured at 470 nm. BHA was used as a positive standard. The control tube contained no sample. Tests were carried out in duplicate.

2.6.4 DNA nicking assay

DNA nicking assay was performed using plasmid pCRII™TOPO by the method of Yanagimoto et al. [24] with slight modifications. A mixture of 10 µl of An6 lipopeptides at the concentration of 2 and 5 mg/ml and plasmid DNA (0.5 µg/well) were incubated for 10 min at room temperature followed by the addition of 10 µl of Fenton's reagent (30 mM H₂O₂, 50 μM L-ascorbic acid and 80 μM FeCl₃). After incubation for 5 min at 37 °C, the mixtures were subjected to electrophoresis (agarose gel: 1% w/v) using Tris-Acetate-EDTA (TAE) (40 mM Tris-acetate and 1 mM EDTA, pH 7.4) as running buffer. The properly developed gel was immediately visualized under a UV transilluminator.

2.7 Evaluation of antimicrobial activity of An6 lipopeptides
Antibacterial activities were tested against several Gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 11778), *Enterococcus faecalis* (ATCC 29212) and *Micrococcus luteus* (ATCC 4698), and Gram-negative bacteria: *Salmonella typhimurium* (ATCC 19430), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Klepsiella pneumoniae* (ATCC 13883). Antifungal activities were tested against *Rhizoctonia bataticola*, *Pythium ultimum*, *Rhizoctonia solani*, *Fusarum oxysporum* and *Mucor rouxii*.

Antimicrobial activity of An6 lipopeptides was assessed according to the agar-well diffusion method as described by Millette et al. [25]. Culture suspension (100 µl) of the indicated strains (about 10^6 colony forming units (Cfu)/ml for bacterial cells and 5 x 10^4 spores/ml for fungal strains) were spread over the Luria-Bertani (LB) agar or Sabouraud dextrose agar, respectively. Then, wells (7 mm depth, 6 mm diameter) were cut in the agar. 60 µl of An6 lipopeptides mixture at a concentration of 2 mg/ml were delivered into them. After incubation overnight at 37 °C for bacteria and 72 h at 30 °C for fungal strains, inhibition zones were measured and recorded as a mean diameter (mm).

2.7. 1 Effect of temperature, pH and proteolytic enzymes on the antimicrobial activity of An6 lipopeptides

The thermal stability was carried out by incubating the crude lipopeptide mixture for 15 min in water bath at different temperatures (60, 70, 80, 90 and 100 °C). The residual antimicrobial activity was then determined.
The effect of pH on lipopeptides activity was examined by assaying antimicrobial activity using *S. aureus* after incubation for 2 h at 4 °C in the pH range of 4.0–12.0. Samples were neutralized to pH 7.0 before measurement of the antimicrobial activity.

In order to evaluate stability to proteolytic enzymes, An6 lipopeptides were incubated for 2 h at 37 °C with 1 mg/ml (final concentration) of the following enzymes: trypsin, chymotrypsin and alcalase in 0.05 M Tris-HCl buffer (pH 8.0). Pepsin in 0.05 M glycine-HCl buffer (pH 2.0). Enzymes were purchased from Sigma (Sigma-Aldrich, St Louis, MO, USA). All of these tests were evaluated by the agar-well diffusion assay against *S. aureus* as an indicator strain.

### 3 Results and discussion

#### 3.1 Detection of lipopeptide genes by PCR

As1-F/Ts2-R, Af2-F/Tf1-R and Abl1-F/Tbl1-R primer pairs were used to detect nonribosomal lipopeptide synthetase genes involved in the biosynthesis of surfactin, fengycin and bacillomycin, respectively. The three degenerated primers gave amplicons with the expected sizes (data not shown). The PCR products were then sequenced after cloning in pGEM-T Easy Vector. As shown in Table 2, the sequence amplified by As1-F/Ts2-R showed a high homology with the surfactin synthetase operon of *B. subtilis* 168 (93%). As expected Af2-F/Tf1-R primers amplify fragment which showed high homology (96%) with the fengycin synthetase gene from *B. subtilis*. Furthermore, Abl1-F/Tbl1-R primers amplified a fragment exhibiting a high homology (95%) with the bacillomycin B synthetase of *B. amyloliquefaciens* FZB42.
In summary, we have an evidence for the presence in An6 strain of three gene clusters directing biosynthesis of chemically different lipopeptides, fengycin, surfactin and bacillomycin. This highlighted the potential of this strain to produce an array of bioactive compounds through non conventional processes. The most interesting finding in this study was the self-assembly of three lipopeptides obtained from the same strain. In the same context, Koumoutsi et al. [26] reported that both bmyD and fenA genes encoding bacillomycin and fengycin synthetase, respectively, were detected in the genome of B. amyloliquefaciens SQR9.

3.2 Structure analysis of An6 lipopeptides by MALDI-TOF mass spectrometry

MALDI-TOF-MS analysis, performed on whole cells of B. amyloliquefaciens An6, was used to characterize the structure of the different lipopeptides produced by this strain. Figure 1 shows the product patterns determined by MALDI-TOF mass spectrometry. Two well-resolved groups of peaks at m/z values between 1046-1110 and 1436-1516 are detected. By comparing the mass data with the mass reported for the lipopeptide complexes from other Bacillus strains [27], the group of peaks at m/z values between 1046-1110 could be attributed to the isoform sets of surfactins, and iturins, and that of peaks at m/z values could be attributed to the fengycin isoformes. The mass numbers for the different lipopeptide families observed in the mass spectra shown in Fig. 1 are summarized in Table 3. Our results show that lipopeptide molecules are detected, in their protonated form or as potassium adducts. In fact, the ions at m/z 1045.69, 1060.79 and 1075.56 could be assigned as potassium adduct of surfactin C13, C14 and C15, respectively. The peak at m/z 1069.69 1083.74, 1097.78 and 1109.73 observed...
in spectrum could be attributed to [MM+K]$^+$ forms of bacillomycin DC14, DC15, DC16 and FC16, respectively. On other hand, four peaks at m/z 1436.19, 1478.27, 1502.23 and 1516.26 were also detected which were putatively assigned as potassium adducts (m/z 1436.19 and 1478.27) and protonated form (m/z 1502.23 and 1516.26) of fengycin AC14, AC17, AC16 and AC17, respectively.

On the basis of the above results, An6 strain can be added to the limited number of *Bacillus* strains, reported to produce three families of lipopeptides simultaneously [28]. As reported by Razafindralambo et al. [29], this coproduction could improve the biological properties of the produced mixture. Indeed, both of iturins and fengycins are mainly known for their anti-fungal properties, while surfactins are mostly anti-viral and anti-bacterial. When different families are co-produced, their interaction can become synergistic and enhances each of their respective activities [30].

3.3 An6 lipopeptides quantification by HPLC analysis

Because MALDI-TOF-MS is not suitable for determining the exact concentration of lipopeptide produced, the various lipopeptide compounds were quantified from broth cultures by HPLC analysis. To this end, the An6 strain was cultivated in Landy medium for 72 h at 160 rpm in a shaker incubator. Supernatant was loaded on C18 solid-phase extraction cartridges and then lipopeptides were desorbed with methanol (100%). The resulting sample was analyzed by reverse-phase HPLC. The chromatographic profile confirms it high content in surfactin, bacillomycin and fengycin families, identified on the basis of their typical retention times, compared to those of pre-purified standards.
The total amounts of fengycins, bacillomycins and surfactins produced by the strain An6 were found to be 120.37, 80.5 and 130.09 mg/l, respectively. In literature, the B. amyloliquefaciens species have been reported to produce lipopeptides. Sun et al. [12] reported that B. amyloliquefaciens ES-2, screened from a medicinal plant, has been discovered to produce antibiotic substance, whose main components were identified as surfactins and fengycins at levels of 294 and 81.2 mg/l, respectively. B. amyloliquefaciens CCMI 1051 has also been reported to produce lipopeptides belonging to iturin and surfactin families [31]. Yu et al. [32] demonstrated that the antifungal compounds produced by B. amyloliquefaciens B94 are isomers of iturin A.

3.4 In vitro antioxidant activities of An6 lipopeptides

An6 lipopeptides were isolated from culture supernatant through a different step of ultrafiltration (using a 10 kDa cut-off membrane) and then lyophilized. The in vitro antioxidant activity was determined through three assays: DPPH scavenging activity, reducing power and β-carotene bleaching by linoleic acid.

3.4.1 DPPH radical-scavenging activity

The DPPH radical scavenging activity is based on the ability of antioxidants to donate a hydrogen atom or an electron to stabilize free radicals and convert them to a more stable product.

The scavenging effect on DPPH radicals of An6 lipopeptides at various concentrations was measured and compared to that of BHA. As shown in Fig. 2A, An6 lipopeptides up to 2.5 mg/ml exhibited a scavenging activity towards DPPH free radicals in a concentration-dependent manner. However, no significant change in the radical scavenging activity was
observed when An6 lipopeptides concentration exceeds 2.5 mg/ml. This statement is also supported by the EC$_{50}$ value, which was found to be 370 $\mu$g/ml. In line to our study, Tabbene et al. [33] reported also the scavenging activity of C14, C15 and C16 bacillomycin D-like lipopeptides produced by B. subtilis B38. This activity was believed to be due to their hydrophobic and aromatic side-chain groups of their amino acids as well as to the aliphatic chain of their beta amino fatty acids.

3.4.2 Reducing power assay

The reducing powers of An6 lipopeptides as well as BHA as a function of their concentrations are shown in Fig. 2B. The reducing capacity of An6 lipopeptides (absorbance at 700 nm) increased with the increase of the sample concentration and reached a maximum of 1.07±0.02 at a dose of 2.5 mg/ml. The obtained values were lower than those of BHA at the same concentrations.

Similar findings have been reported for a lipopeptide produced by B. subtilis RW-I [34]. It was demonstrated that the reducing power increased with the increase of the lipopeptide concentrations. This indicates the existence of some functional groups in their structure which were electron donors and could react with free radicals to convert them into more stable products. In this context, Ren et al. [35] reported that hydrophobic amino acids are at the basis of the reducing power of antioxidant compounds.

3.4.3 $\beta$-carotene bleaching by linoleic acid assay

In this method, $\beta$-carotene undergoes a rapid discoloration in the absence of an antioxidant. $\beta$-carotene bleaching method is based on the loss of the yellow color of $\beta$-carotene, which is monitored spectrophotometrically, due to its reaction with free radicals.
formed by linoleic acid oxidation in the emulsion system. The β-carotene bleaching rate slowed down in the presence of antioxidants. The antioxidant activities of An6 lipopeptides, as well as the positive control (BHA), as measured by the bleaching of β-carotene, are presented in Fig. 2C. An6 lipopeptides mixture was found to inhibit the oxidation of β-carotene in a dose-dependent manner. At 5 mg/ml, the inhibition value was 53 %, while it was only 17.15% at 1 mg/ml. It is believed that lipid peroxidation is one of the causes of the occurrence of cardiovascular diseases and cancer [36]. The high inhibition rate of An6 lipopeptides might therefore contribute to their therapeutical potential.

3.4.4 DNA nicking assay

To investigate the scavenging ability of An6 lipopeptides against hydroxyl radical, the DNA nicking assay was used and results are reported in Fig. 2D. Line 1 represents the untreated plasmid (native DNA) with its two forms: supercoiled (S) and open circular (O). Incubation of plasmid with Fenton’s reagent in the absence of An6 lipopeptides resulted in the complete degradation of the two DNA bands. Nevertheless, it is interesting to note that addition of An6 lipopeptides to the reaction mixture resulted in a protection against hydroxyl radical induced DNA breakage. These results indicate that An6 lipopeptides counteracts the oxidative stress produced by Fenton reaction on susceptible biomolecule such as DNA.

3.5 Antibacterial activity of An6 lipopeptides

3.5.1 Spectrum of antibacterial activity

The results of the antimicrobial activity of the lipopeptides produced by B. amyloliquefaciens An6 against pathogenic bacteria are shown in Table 4. An6 lipopeptides mixture showed important antimicrobial activity against microorganisms with multidrug-

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resistant profiles. The activity against Gram-negative bacteria was lower when compared to
Gram-positive bacteria. Indeed, the inhibition zones were in the range of 8-17 mm and 13-20
mm with Gram-negative and Gram-positive bacteria, respectively. *B. cereus, S. aureus* and
*E.coli* were the most sensitive with a halo diameter of 20, 18 and 17 mm, respectively. It is
important to note that some of these strains are resistant to the conventional antibiotics. An6
lipopeptides mixture was also characterized by an important antifungal activity, especially,
against *P. ultimum, R. bataticola* and *R. solani* and lower activity against *M. rouxii*.

Bacteria of the genus *Bacillus* are known for being producers of a broad spectrum of
bioactive lipopeptides with potent antibiotic activity against bacteria, fungi and yeast. Wang
et al. [37] isolated a *B. amyloliquefaciens* strain ES-2 from a medicinal plant, which produces
an antibiotic substances, whose main components were identified as surfactin and fengycin,
which inhibited the growth of *E. coli, B. cereus* and *S. typhimurium*. Singh and Cameotra [15]
have also observed that lipopeptide N1, produced by *B. subtilis* C1, was active against several
microorganisms, especially *S. aureus* and *Mycobacterium* sp. Similar results were also
reported for the antimicrobial activity of lipopeptide produced by *B. cereus* NK1 [38] and *B.
natto* TK-1 [39] against several pathogens.

3.5.2 Effects of heat, pH and proteolytic enzymes on antibacterial activity of An6
lipopeptides

In general, lipopeptides were reported to be highly thermostable and resistant to
proteolytic enzymes. Therefore, in order to check their resistance against proteases and
extreme conditions, including pH and temperature, An6 lipopeptides were subjected to
different conditions and their residual antimicrobial activity was evaluated using *S. aureus* as a strain indicator.

As shown in Table 5, thermal treatment below 80 °C did not affect the antibacterial activity. Indeed, no activity loss was detected after 15 min incubation at temperatures from 60 to 80 °C. Nevertheless, An6 lipopeptides activity was slightly affected after incubation for 15 min at 90°C and 100 °C.

An6 lipopeptides activity after pre-incubation at different pH values was also determined. An6 lipopeptides mixture was highly stable over a pH range of 6.0 to 10.0. Nevertheless, the activity was slightly reduced and at pH 4.0 and 12.0 (Table 4). Results are similar to those obtained by Sabaté and Audisio. [40] who showed that the antimicrobial activity of surfactin lipopeptide from *B. subtilis* remained highly stable after boiling (96°C) for 15 min and was active over a pH range of 3.0 to 11.0. Similar properties of stability at high temperatures and over a wide pH range have been also reported, in our previous work, for a lipopeptide mixture produced by *B. mojavensis* A21 [19].

The effect of the proteolytic enzymes on the antimicrobial activity was evaluated by measuring the residual activity against *S. aureus* in the agar-disk diffusion assay. Antimicrobial activity was found to be resistant to all the tested proteases, since there was no remarkable difference in the inhibition zone when compared to a non-treated control. Similarly, a novel lipopeptides from *Streptomyces amritsarensis* was found to be resistant to trypsin and proteinase K [41]. In our previous study, we also reported the stability of lipopeptides mixture produced by *B. mojavensis* A21 against several proteolytic enzymes [19].

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4 Conclusion

In conclusion, *B. amyloliquefaciens* An6 was found to produce multiple lipopeptides. MALDI-ToF-MS analysis of An6 lipopeptides revealed that they belong to surfactin, iturin and fengycin families. An6 lipopeptides exhibited an antioxidant activity and have a protective role against oxidative stress. Furthermore, they were active against a variety of Gram-negative and Gram-positive bacteria and some fungi. Further, they were found to retain their activity during exposure to elevated temperatures (up to 100 °C) and to proteolytic enzymes. Consequently, An6 lipopeptides may be useful for cosmetic, therapeutical or pharmaceutical purposes in order to delay or prevent oxidative deterioration of manufactured products. An6 products may represent a novel antimicrobial substance with potential applications in food safety, with important implications for the biological control of pathogenic and spoilage microorganisms. Further research should be achieved to separate and purify the different lipopeptide families and characterize the family type responsible for the antibacterial and antioxidant activities.

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Conflict of interest: *The authors declare that they have no conflicts of interest.*

5 References


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Fig. 1: MALDI-TOF mass spectrometric analysis of intact whole cells of *B. amyloliquefaciens* An6 grown on agar plates. (A) peaks at m/z between 1045.6-1109.73 and (B) peaks at m/z between 1436.19-1516.26.
Fig. 2. **A:** DPPH radical-scavenging activity of An6 lipopeptides at different concentrations.; **B:** Reducing power assay of An6 lipopeptides at different concentrations.; **C:** β-carotene bleaching activity of An6 lipopeptides at different concentrations. Each value represents mean ± standard deviation (n=3); **D:** Gel electrophoresis pattern of the plasmid pCRII™TOPO incubated with Fenton's reagent in the presence and absence of An6 lipopeptides. Lane 1: untreated control: native pCRII™TOPO DNA (0.5μg); lane 2: DNA sample incubated with Fenton's reagent; lanes 3 and 4: Fenton's reagent +DNA+2 mg An6 lipopeptides and 5 mg An6 lipopeptides, respectively.