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Research Article

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

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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 PCR using degenerated primers and 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 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.

Keywords: Antimicrobial activity / Antioxidant activity / *Bacillus amyloliquefaciens* / Lipopeptides / MALDI-TOF-MS

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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 nonribosomal 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 pumilus*, *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus licheniformis* are able to produce these nonribosomal peptides, particularly

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Abbreviations: BHA, butylated hydroxyanisole; DPPH, 1,1-diphenyl-2-picrylhydrazyl; NRPS, nonribosomal peptide synthetases

Table 1. List and characteristics of primers used in this study

Primers	Sequences ^{a)}	Expected fragment size (bp)	HyC ^{b)}	Lipopeptides
As1-F	CGCGGMTACCGVATYGAGC	419, 422, 425, 431	12	Surfactin
Ts2-R	ATBCCTTTBTWDGAATGTCCGCC		36	
Af2-F	GAATAYMTCGGMCGTMTKGA	443, 452, 455	34	Fengycin
Tf1-R	GCTTTWADKGAATSBCCGCC		72	
Abl1-F	GATSAWCARGTGAAAATYCG	428, 431, 434	16	Bacillomycin
Tbl1-R	ATCGAATSKCCGCCRARATCRAA		32	

^{a)} Using IUPAC DNA code: Y = C or T; M = A or C; K = G or T; W = A or T, D = G, A, or T; S = G or C; B = G, T, or C; R = A or G.

^{b)} Coefficient of hybridization calculated as described by Tapi et al. [18].

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 such as 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 that 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 antiaging, 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 toward 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, WI, 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 μ M. 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 *Escherichia 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 analyzed 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 MS analysis

MALDI-TOF-MS 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 $15\,000 \times g$ for 15 min.

For their quantification, lipopeptides were purified from culture supernatant by SPE 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 HPLC (600 s, Waters, USA) using a C18 column (5 μ m, 250 mm $\times$ 4.6 mm, 218 TP, VYDAC). Each family of lipopeptides was separately analyzed with the ACN–water–TFA solvent system (40:60:0.5 v/v/v for iturin, 80:20:0.5 v/v/v 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. Samples (20 μ L) 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 Da). The separation was carried out in two steps: (i) in the first step, 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%) that disrupted the micelles. Finally, the permeate was collected, concentrated, and lyophilized, resulting in partially purified lipopeptides mixture that was used for subsequent analysis.

2.6 Evaluation of antioxidant activity of An6 lipopeptides

2.6.1 1,1-Diphenyl-2-picrylhydrazyl free radical-scavenging assay

The 1,1-diphenyl-2-picrylhydrazyl (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]. Five hundred microliters of An6 lipopeptides mixture and standard BHA (used as a positive control) at different concentrations (0.25–5 mg/mL) was mixed with 375 μ L of 99% ethanol and 125 μ L 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]. One microliters of An6 lipopeptides at different concentrations (0.25–5 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. In total, 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 μ L 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 pCRIITMTOPO by the method of Yanagimoto et al. [24] with slight modifications. A mixture of 10 μ L of An6 lipopeptides at the concentration of 2 and 5 mg/mL and plasmid DNA (0.5 μ g/well) were incubated for 10 min at room temperature followed by the addition of 10 μ L of Fenton's reagent (30 mM H_2O_2 , 50 μ M L-ascorbic acid, and 80 μ M $FeCl_3$). After incubation for 5 min at 37°C, the mixtures were subjected to electrophoresis (agarose gel: 1% w/v) using Tris-acetate-EDTA (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), *B. cereus* (ATCC 11778), *Enterococcus faecalis* (ATCC 29212), and *Micrococcus luteus* (ATCC 4698), and Gram-negative bacteria: *Salmonella typhimurium* (ATCC 19430), *E. coli* (ATCC 25922), *Pseudomonas*,

Table 2. BLAST results of the sequenced PCR products with degenerated primers

Primers	Product size (bp)	Probable proteins	Identity (%)
As1-F/Ts2-R	441	Surfactin synthetase of <i>Bacillus subtilis</i>	93
Af2-F/Tf1-R	421	Fengycin synthetase of <i>B. subtilis</i>	96
Abl1F/Tbl1-R	419	Bacillomycin B synthetase of <i>Bacillus amyloliquefaciens</i> FZB42	95

aeruginosa (ATCC 27853), and *Klebsiella pneumoniae* (ATCC 13883). Antifungal activities were tested against *Rhizoctonia bataticola*, *Pythum ultimum*, *Rhizoctonia solani*, *Fusarium 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 μ L) of the indicated strains (about 10^6 colony forming units/mL for bacterial cells and 5×10^4 spores/mL for fungal strains) were spread over the Luria-Bertani or Sabouraud dextrose agar, respectively. Then, wells (7 mm depth, 6 mm diameter) were cut in the agar. Sixty microliters of An6 lipopeptides mixture at a concentration of 2 mg/mL was 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 lipopeptides 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 that 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 nonconventional processes. The most interesting finding in this study was the self-assembly of three lipopeptides obtained from the same strain. In the same context, Koumoutsis 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 MS

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-MS. 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 and 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 isoforms. 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 antifungal properties, while surfactins are mostly antiviral and antibacterial.

Figure 1. MALDI-TOF mass spectrometric analysis of intact whole cells of *Bacillus amyloliquefaciens* An6 grown on agar plates. (A) Peaks at m/z between 1045.6 and 1109.73 and (B) peaks at m/z between 1436.19 and 1516.26.

Table 3. Assignments of all mass peaks obtained by MALDI-TOF MS of whole cells of *Bacillus amyloliquefaciens* An6

Mass peak (m/z)	Assignment
1045.69	C13 surfactin [MM + K] ⁺
1060.79	C14 surfactin [MM + K] ⁺
1069.69	C14 bacillomycin D [MM + K] ⁺
1075.56	C15 surfactin [MM + K] ⁺
1083.74	C15 bacillomycin D [MM + K] ⁺
1097.78	C16 bacillomycin D [MM + K] ⁺
1109.73	C16 bacillomycin F [MM + K] ⁺
1436.19	C14 fengycin [MM + H] ⁺
1466.25	NI
1478.27	C17 fengycin A [MM + H] ⁺
1502.23	C16 fengycin A [MM + K] ⁺
1516.26	C17 fengycin A [MM + K] ⁺

NI = not identified.

When different families are coproduced, 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 SPE cartridges and then lipopeptides were desorbed with methanol (100%). The resulting sample was analyzed by reversed-phase HPLC. The chromatographic profile confirms its high content in surfactin, bacillomycin, and fengycin families, identified on the basis of their typical retention times, compared to those of prepurified 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. *Bacillus amyloliquefaciens* CCMI 1051 has also been reported to produce

Figure 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 $\pm$ standard deviation ($n = 3$). (D) Gel electrophoresis pattern of the plasmid pCRIITMTOPO incubated with Fenton's reagent in the presence and absence of An6 lipopeptides. Lane 1: untreated control: native pCRIITMTOPO DNA (0.5 μ g); 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.

lipopeptides belonging to iturin and surfactin families [31]. Yu et al. [32] demonstrated that the antifungal compounds produced by *B. amyololiquefaciens* 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 cutoff 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 toward 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 μ 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 β -Carotene bleaching by linoleic acid assay

In this method, β -carotene undergoes a rapid discoloration in the absence of an antioxidant. β -Carotene bleaching method is based on the loss of the yellow color of β -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

Table 4. Antimicrobial activity spectrum of An6 lipopeptides mixture

Indicator organism	Inhibition (mm)
Gram-positive	
<i>Staphylococcus aureus</i> (ATCC 25923)	18 ± 1.0 ^b
<i>Bacillus cereus</i> (ATCC 11778)	20 ± 1.0 ^a
<i>Enterococcus faecalis</i> (ATCC 29212)	13 ± 2.0 ^c
<i>Micrococcus luteus</i> (ATCC 4698)	15 ± 1.0 ^c
Gram-negative	
<i>Pseudomonas aeruginosa</i> (ATCC 27853)	11 ± 1.0 ^c
<i>Klebsiella pneumoniae</i> (ATCC 13883)	8 ± 1.0 ^d
<i>Escherichia coli</i> (ATCC 29212)	17 ± 1.0 ^b
<i>Salmonella typhimurium</i> (ATCC 19430)	11 ± 0.0 ^c
Fungi	
<i>Rhizoctonia bataticola</i>	17 ± 2.0 ^b
<i>Pythium ultimum</i>	25 ± 2.0 ^a
<i>Rhizoctonia solani</i>	25 ± 1.0 ^a
<i>Fusarium oxysporum</i>	17 ± 1.0 ^b
<i>Mucor rouxii</i>	12 ± 1.0 ^c

Values are given as mean ± SD from triplicate determinations ($n = 3$), a, b, and c in the same column indicated significant differences ($p < 0.05$).

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 counteract the oxidative stress produced by Fenton reaction on susceptible biomolecule such as DNA.

3.5 Antimicrobial 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-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. *Bacillus 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,

Table 5. Effects of heat, pH, and proteolytic enzymes on antimicrobial activity of An6 lipopeptides

Treatment	Inhibition zone (mm)
Temperature (°C)	
Control	17 ± 1.0 ^a
60	17 ± 1.0 ^a
70	17 ± 1.0 ^a
80	17 ± 1.0 ^a
90	16 ± 1.0 ^{ab}
100	15 ± 1.0 ^{ab}
pH	
4	15 ± 1.0 ^b
6	17 ± 2.0 ^a
8	17 ± 1.0 ^a
10	16 ± 1.0 ^{ab}
12	14 ± 1.0 ^b
Enzyme (1 mg/mL)	
Control	17 ± 1.0 ^a
Trypsin	17 ± 1.0 ^a
Pepsin	17 ± 1.0 ^a
Chymotrypsin	16 ± 1.0 ^a
Alcalase	16 ± 1.0 ^a

Values are given as mean ± SD from triplicate determinations ($n = 3$). a, b, and c in the same column indicated significant differences ($p < 0.05$).

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 preincubation at different pH values was also determined. An6 lipopeptides mixture was highly stable over a pH range of 6.0–10.0. Nevertheless, the activity was slightly reduced 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 3.0–11.0. 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 nontreated 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].

4 Concluding remarks

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, therapeutic, 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.

Practical application

Bacillus 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, therapeutic, 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.

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