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ORIGINAL ARTICLE

Biosurfactant production by AL 1.1, a *Bacillus licheniformis* strain isolated from Antarctica: production, chemical characterization and properties

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Abstract Biosurfactants are of great interest due to the demand for natural products with low toxicity. Nevertheless, their production is not competitive when cost is a limiting factor. Strain AL 1.1, isolated on Deception Island (Antarctica), identified as *Bacillus licheniformis*, produced lipopeptides when grown using a variety of carbohydrates. Biosurfactant production, but not growth, was optimal at 30 °C. The culture conditions and medium composition dictated biosurfactant production. Basic optimization of culture and extraction parameters gave a production yiels of 860 mg/L purified extract in 24 h. The purified biosurfactant yielded a mixture of lipopeptide homologues, with molecular weights between 1006 and 1034. The peptide moiety consists of glutamine as the N-terminal amino acid, two leucines, valine, aspartic, leucine and isoleucine as the C-terminal amino acid. The lipid moiety contains a mixture of β-hydroxy fatty acids ranging in size from C_{14} to C_{16}. These results indicate a similarity with lichenysin groups A, D or G. The organic extract reduced surface tension to 28.5 mN/m and achieved a critical micelle concentration of 15 mg/L. This highly effective and efficient behavior characterized the product as a powerful surfactant. Its stability under a wide pH range, high temperatures and variable concentrations of salt, as well as its emulsifying properties, suggest potential application in cosmetic industrial processes.

Keywords Biosurfactant · Lipopeptide · *Bacillus licheniformis* · Surface tension · Cosmetic

Introduction

Although many bacterial surfactants have been reported in recent decades, and their production, structure and properties have been studied extensively, there is still a demand for new biosurfactants (BS) with improved characteristics. The search for new microorganisms able to produce compounds with industrial or medical application has led to the study of samples collected in unique environments. Strain AL 1.1, isolated from geothermal sediments of Deception Island in the South Shetland archipelago (Antarctica), was studied for its capacity to effectively reduce the surface tension of growth culture media.

BS have distinct advantages over their commercially manufactured counterparts because of their lower toxicity, biodegradability and effectiveness in a wide range of conditions. These favorable features make BS potential alternatives to chemically synthesized surfactants in a variety of applications. In recent decades, BS have become relevant in the environmental field as biocontrol agents or in bioremediation as promoters of biodegradation of hydrocarbon pollutants. Properties of BS, such as soaping, emulsifying, foaming and

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dispersing, make these compounds important in metal extraction, petroleum production and in the textile, paper and chemical industries. Nevertheless, the high cost of production and downstream processing limit their large-scale production and use (Ghribi and Ellouze-Chaabouni 2011). Recently, despite the apparently small market for BS, they have aroused fresh interest due to their unique structures; they are beginning to be appreciated for their potential applications in many demanding and innovative biotechnological aspects of the cosmetics and food industries, in pharmaceuticals (due to their dispersant, emulsifying or anti-adhesive properties) and especially in medicine (antimicrobial, antitumoral, antiviral and anti-inflammatory properties; Soberón-Chavez and Steinbüchel 2011).

Lipopeptides (LP) are amphiphilic compounds in which a cyclic peptide is linked to a long fatty acid chain. Isolated in the 1950s, LPs from *Bacillus* sp. encompass more than 30 different peptides linked to various fatty acid chains and are classified into four families: surfactins, iturins, fengycins or plipastatins, and kurstakins (Jacques 2011). The natural diversity of LPs is associated with the length of the fatty acid chain, giving rise to homologues, as well as with the amino acid composition of the peptidic sequence, giving rise to isoforms (Pecci et al. 2010). Surfactin, the most widely studied LP, is synthesized non-ribosomally by *Bacillus subtilis*, employing a multifactorial enzyme system. The presence of variants can be related to alterations in culture conditions, especially feeding with some specific amino acid residues (Li et al. 2010; Jacques 2011). *Bacillus licheniformis* has the ability to produce many surface-active LP (Li et al. 2008; Madslien et al. 2013). *Bacillus amyloliquefaciens* LP03 and *B. thuringensis* have also been described as LP producers. Among lipopeptides, surfactin is one of the most powerful BS since it lowers the surface tension of water from 72 mN/m to 27 mN/m at a concentration as low as 20 μmol/L (Li et al. 2008). LP exhibit some powerful biological actions due to their exceptional surface activity, which leads to modifications of the bacterial surface.

One method of achieving high BS production is to select appropriate bacteria and media components and optimize culture conditions. In fact, the nature of the carbon substrate, concentration of salts in the medium and operational conditions such as temperature or agitation influence the nature and quantity of BS produced by bacteria enormously (Mnif et al. 2012). This paper describes the physiology of growth for BS production of the previously unexamined *Bacillus licheniformis* strain AL 1.1, the chemical characterization and stability of the BS produced, and their emulsion capacity with oils used in the cosmetics industry.

Materials and methods

Microorganism and culture conditions

Strain AL1.1 was isolated from a non-contaminated sample (sand, Kroner lake) taken from a geothermal area of Deception island, in the South Shetland archipelago (Antarctica), and was identified as *Bacillus licheniformis* by phenotypic characterization and numerical taxonomic and chemotaxonomic analyses (Llarch et al. 1997). The strain was maintained by 2-weekly subculture on Tryticase Soy Agar (Pronadisa, Madrid, Spain) and preserved in cryovials (AES Laboratoire, Comburg, France) at −80 °C. For BS production, TSB (Pronadisa) and mineral salt medium (B-MSM) of the following composition were used (g/L): glucose, 10; NH_4NO_3, 4; KH_2PO_4, 4; Na_2HPO_4 5.7; $FeSO_4 \cdot 7H_2O$, 0.01; $MgSO_4 \cdot 7H_2O$, 0.14; $CaCl_2$ 7×10^{-6}; trace element solution, 0.05 mL; final pH 7 (Marqués et al. 2009). Mineral components and carbon source were autoclaved separately. The inoculum consisted of a 2 % (v/v) cell suspension in saline buffer of strain AL 1.1 grown overnight in TSA, after adjusting turbidity to McFarland scale no. 6. Cultures were incubated at 30 °C in a 500 mL baffled Erlenmeyer flask with 50 mL basal medium for 48 h with agitation at 120 rpm. Samples were collected at time-defined intervals and analyzed for bacterial growth (dry weight), surface and interfacial tension, crude organic extract weight (as BS measurement), pH and consumption of nutrient sources. All experiments were performed in triplicate.

Bacterial identification

Genomic DNA was extracted using the Wizard® genomic DNA purification kit (Promega, Madison, WI). PCR amplification and sequencing of the 16S rRNA gene were performed using universal primers and methods described elsewhere (Martinez-Murcia et al. 1999). The amplified DNA was purified by the MSB® Spin PCRapace kit (Invitek, Westburg, The Netherlands), used according to the manufacturer's instructions. The purified PCR product was sequenced directly on both strands by the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems®, Life Technologies, Foster City, CA) and analyzed on an ABI PRISM® 3700 DNA sequencer (Applied Biosystems®) by the Genomics Unit of the Scientific and Technological Centres of the University of Barcelona (CCiT-UB). The CLUSTAL X program (version 2.1; Larkin et al. 2007) was used for sequence assembly and alignment. After a preliminary BLAST search analysis (Altschul et al. 1990), the 16S rRNA gene sequence of strain AL 1.1 was compared with other related sequences obtained from the GenBank/EMBL/DDBJ databases. Pairwise similarities between 16S rRNA gene sequences were calculated with MEGA 6.0 software (Tamura et al. 2013).

BS recovery

Bacterial cells were removed from the culture medium by centrifugation at 8,000 *g*, at 4 °C, for 15 min (Avanti^{TM} j-20 centrifuge; Bekman Coulter, Indianapolis IN). The organic extract was recovered from the culture supernatant after three extractions with an equal volume of an ethyl acetate:methanol mixture (8:1, v/v). Organic phases were combined, passed over anhydrous sodium sulfate and concentrated in a rotary vacuum evaporator (Büchi, Flawil, Switzerland). The resulting organic extract was weighed. Other organic solvents [chloroform, ethyl acetate, hexane, diethyl ether, chloroform:methanol (8:1) and ethyl acetate:methanol (8:1)] were assayed for BS recovery and extraction by precipitation was performed as in Li et al. (2010).

Effect of culture conditions on BS production

Stain AL 1.1 was grown in B-SMS and the effects of growth temperature (60–30 °C), agitation (0–150 rpm) and incubation time (24–73 h) on BS production were studied. The effects of various nutrients on BS production and AL 1.1 growth were studied by modifying B-SMS components. For carbon sources (1 %), glycerol, fructose, glucose, saccharose, lactose, C_{12} (9.3 % C_{10}; 32.4 % C_{11}; 31.3 % C_{12}; 26,7 % C_{13}, from Petrepar®120, Petroquímica Española, Madrid, Spain), olive oil (76.1 %, oleic acid ($C_{18:1}$); 11.3 %, palmitic acid ($C_{16:0}$); 9.2 %, linoleic acid ($C_{18:2}$); 1.5 % stearic acid ($C_{18:0}$); 1.3 % linolenic acid ($C_{18:3}$), from Borges® pure olive oil, Spain) and linseed oil (6.3 % palmitic acid ($C_{16:0}$); 2.5 % stearic acid ($C_{18:0}$); 19 % oleic acid ($C_{18:1}$); 24 % linoleic acid ($C_{18:2}$); 47.4 % linolenic acid ($C_{18:3}$), 3.2 % other, from Acofarma, Barcelona, Spain) were used. The following nitrogen sources were evaluated for BS production: NH_4NO_3 (4 g/L), $NaNO_3$ (8.5 g/L), $(NH_4)_2HPO_4$ (6.6 g/L), $(NH_4)_2SO_4$ (4.4 g/L) and urea (3 g/L). The effects of phosphate concentration (Na_2HPO_4-KH_2PO_4, 1.4−1 to 11.4−8), calcium chloride (7×10^{-6}–1×10^{-3} g/L) and magnesium sulfate (0.14–0.28 g/L) were also studied.

Analytical methods

Biomass determination

Samples were taken at different times, centrifuged and washed. The biomass obtained was dried at 105 °C to constant weight.

Surface tension measurements

Equilibrium surface tension (γ_{st}) was measured at 25 °C with a Krüss K9 digital tensiometer (Krüss, Hamburg, Germany), using the ring method. The instrument was calibrated against ultrapure water (γ_{st} 72 mM/m) and pure ethanol (γ_{st} 22.7 mN/m) to ensure accuracy over the entire range of surface tension. Interfacial tension was measured against kerosene.

TLC analysis

Crude extract samples were dissolved in methanol, spotted on silica plates (ALUGRAM® SIL G/UV$_{254}$, Macherey-Nagel, Düren, Germany). Chloroform:methanol:5 M ammonium hydroxide (65:30:5) was used as the mobile phase. Controls were also added. The resulting spots on the TLC were viewed by spraying with ninhydrin (Sigma, St. Louis, MO) for amine groups; Molish solution for sugar observation; and iodine vapors for lipid observation.

Amino acid and fatty acid determination

The purified organic extract (3 mg) was hydrolyzed in 1 mL 6 M HCl at 110 °C overnight in a sealed tube. The fatty acid residue was obtained by extracting the hydrolysate with 1 mL diethyl ether. Extraction was performed three times and the organic phase was rinsed twice with distilled water (2 mL). For ESI-MS analysis, the fatty acid residue was dissolved in methanol and mixed with 0.1 % formic acid, of which 20 μL was used in the LC/MSD-TOF system. The methanol-soluble residue was dried after repeated evaporation and re-dissolved in 20 mM HCl to a final concentration of 0.1 mM. The amino acids were then analyzed by HPLC, using the Waters AccQTag pre-column derivatization method (Cohen and Michaud 1993). Reaction of amino acids with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate yields derivatives that are detected at 254 nm. The analysis was performed on a Nova-Pak C18 column (3.9×150 mm) at a flow rate of 1 mL/min at 37 °C, attached to a Delta 600 chromatographic system with a 2478 Dual Absorbance detector and a 717plus Autosampler (Waters, Bedford, MA).

Mass spectrometry

The molecular weight of the components of the surfactants was determined by negative- and positive-ion mode electrospray ionization (ESI) analyses (LC/MSD-TOF, Agilent Technologies, Palo Alto, CA). The capillary voltage was 4 kV and 3.5 kV for the positive and negative modes, respectively, with nitrogen as the nebulizing and drying gas. Tandem mass spectrometry (4800 Plus MALDI TOF/TOF, ABSciex, Dublin, CA) was used in the experiment. The full-mass spectrum was acquired in the reflector positive-ion mode for the LP, using dihydroxybenzoic acid (DHB) as the matrix. Subsequent fragmentation of the observed ions was obtained by positive MS/MS analysis.

Biosurfactant stability and emulsion capacity of $BS_{AL\ 1.1}$

A BS solution (15 mg/L, pH 7.4) was used for thermal stability studies. BS solution (10 mL) was exposed to 25, 50, 75, 100 and 120 °C for 1 h, cooled at room temperature and the surface tension measured. For stability studies, the pH of the BS solution was adjusted in the range of 2–11, and surface tension was measured. To study the effect of salt addition on surfactant activity, different NaCl concentrations (0–20 %) were added, mixed until complete dissolution, and surface tension was measured. BS emulsion activity was studied with oils used in cosmetics, namely isopropyl palmitate (Acidchem International, Pulau Pinang, Malaysia), octyldodecanol (BASF), cetearyl ethylhexaonate (Industrial Qiuímica Lasem, Barcelona, Spain), caprylic capric triglycerides (DHW Deutsche Hydrierwerke, Rodleben, Germany) and isopropyl myristate (Sigma-Aldrich, St. Louis, MO). The emulsion test was carried out using 3 mL aqueous solution of $BS_{AL\ 1.1}$ (0.1 mg/mL) and 1.5 mL oil in a screw cap tube, and vortexed at high speed for 2 min. As a control, water without BS and isopropyl palmitate was used. Emulsion stability was determined visually after 24 h. The emulsion index (E_{24}) was obtained by dividing the height of the emulsion layer by the total height of the liquid and multiplying by 100.

Results and discussion

Strain selection

Life in the Antarctic is limited by the extreme conditions, and only cold-adapted microorganisms and certain animals survive. About 98 % of Antarctica is covered by an ice sheet, although volcanoes are present, as on Deception Island, where the samples analyzed here were collected. Its isolation as a continent and extreme climatic conditions may have allowed the development in Antarctica of bacteria with unique metabolic profiles. Six strains previously isolated in our laboratory were studied for BS production after growth (48 h, 50 °C, 120 rpm) in B-MSM and TSB. Unlike the other isolates, strain AL 1.1 reduced the surface tension of the culture medium supernatant from 53.1 to 37.1 mN/m after growth in TSB and from 55.2–45.3 to 35.2 mN/m after growth in B-MSM. Due to its capacity to effectively reduce surface tension, the thermophilic *Bacillus licheniformis*, strain AL 1.1, was selected for subsequent study. In a BS production study, factors related to growth and bacterial metabolism need to be understood. Nutritional requirements may contribute considerably to the production cost and bioprocess economics. As TSB is a complex and costly medium for BS production, a simpler and more economical glucose-based minimal medium was used for the present work.

Strain AL 1.1 characterization

Strain AL 1.1 was identified as *Bacillus licheniformis* in a numerical taxonomic study using morphological and biochemical data (Llarch et al. 1997). The phenotypical identification was confirmed by 16S rRNA gene sequence analysis in the current study. An almost complete 16S rRNA gene sequence (1,287 bp) of strain AL 1.1 was determined (GenBank accession number: KJ872525). The sequence obtained was compared with those in the GenBank nucleotide database by a BLAST search analysis and showed high identity scores with members of the genus *Bacillus*. Strain AL 1.1 had the highest 16S rRNA sequence similarity value (100 %) with the type strain of *B. licheniformis* ATCC 14580 (GenBank accession number: CP000002), confirming its previous biochemical identification. The number of newly described species of *Bacillus* has grown considerably in the last 20 years. At the time of writing, the genus *Bacillus* comprises 282 recognised species (Euzéby 1997), 80 % of them described since the description of strain AL 1.1 in 1997. At present, the nature of species description has changed to the extent that morphological, physiological and biochemical properties, complemented by genotype data, especially the 16S rRNA gene sequences, are being used to support the identity of new strains at the species level. This, together with the extensive reclassification of species of the *Bacillus* genus in recent decades, points to the importance of confirming the initial phenotypic characterization of strain AL 1.1 with the 16S rRNA gene sequence analysis. This study confirmed the total identity of AL 1.1 belonging to the species *B. licheniformis*—a saprophytic bacterium widespread in nature.

Effect of temperature and agitation on BS production and growth

Although BS are very active and stable, the greatest limitation to their commercialization is their high production cost. To obtain powerful LP and maximise their application, appropriate methods for their cultivation and characterization are needed. To increase BS production, culture conditions and nutritional requirements of *B. licheniformis* AL1.1 were studied. As *B. licheniformis* AL 1.1 is a thermophile bacteria with optimum growth at 65 °C and a wide growth temperature range (17–68 °C), growth and production were first studied at different temperatures. At 50 °C, AL 1.1 grows very rapidly, showing visible colonies in TSA after 3–4 h incubation. BS production was studied at different temperatures to find a possible relationship with bacterial growth (Fig. 1a). After 24 h incubation, cell dry weight increased as the temperature increased. BS production, measured as surface and interfacial tension and especially as a crude organic extract, worsened as temperature increased. The yield of the crude organic extract was highest at 30° (208 mg/L) and 37 °C (106 mg/L),

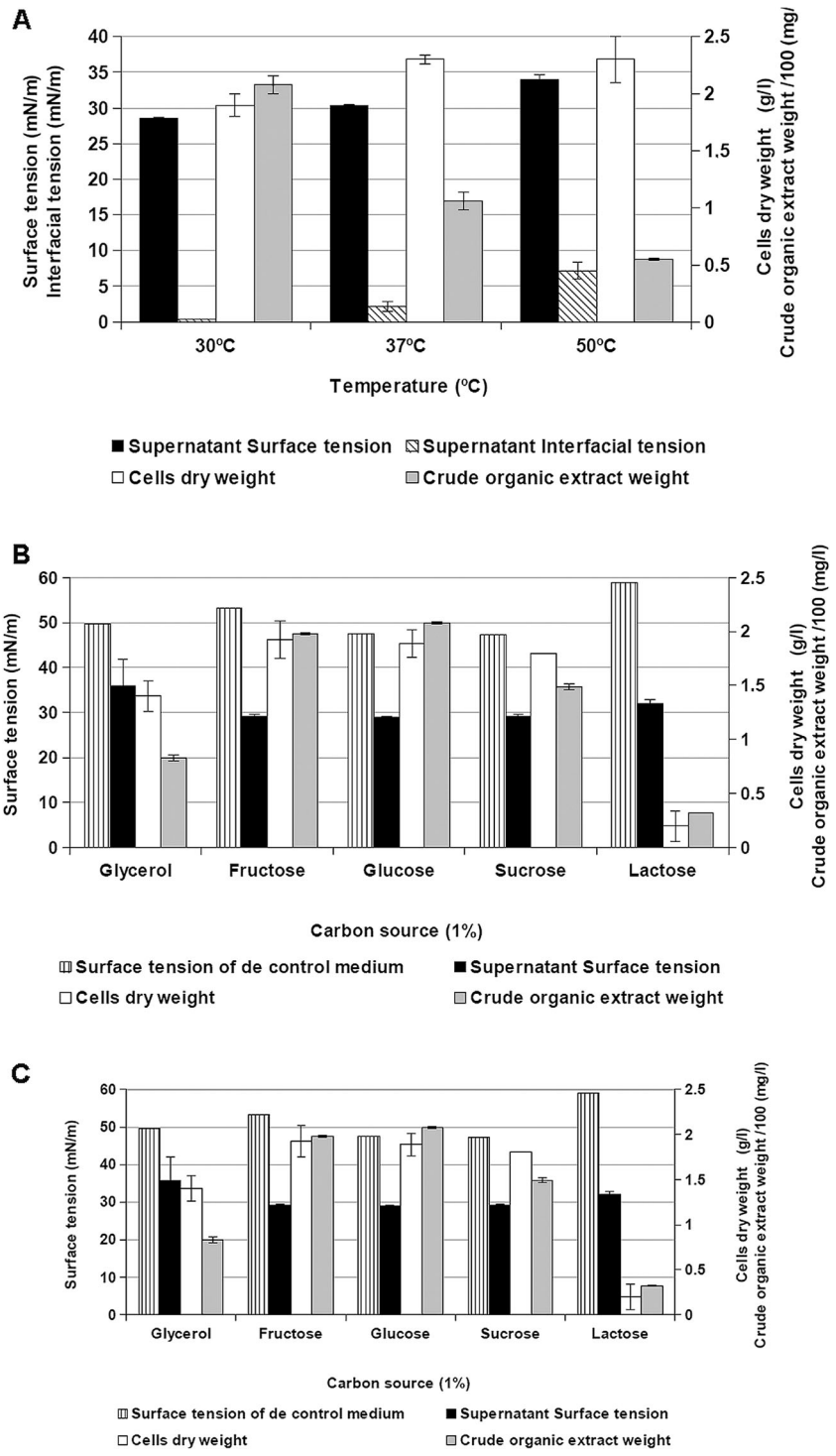

Fig. 1 Influence of **a** growth temperature and **b** agitation on surface and interfacial tension, cell dry weight and crude organic extract weight obtained after 24 h culture. **c** Influence on decrease in surface tension, cell dry weight and crude organic extract weight of carbon source (1 %) in culture medium, after 24-h culture at 30 °C and 120 rpm of AL 1.1. Data are presented as mean±SE

indicating that a high temperature (50 °C) inhibits BS production and metabolic energy may be spent for the extremely rapid cellular growth. The same optimum temperature was selected by Gogotov and Miroshnikov (2009) for BS production by *B. licheniformis* VKM B-511, while Joshi et al. (2007) observed maximum BS production by *B. licheniformis* at 45 °C. In *B. licheniformis* AL 1.1, optimum temperatures for growth and BS production were clearly different. Being more economical and productive, 30 °C was chosen for bacterial culture.

Oxygen availability may affect both growth and metabolite production. As culture agitation increased (0–150 rpm), both cell dry weight and crude extract weight increased, with the best performance at 120 rpm, with 208 mg/L crude organic extract (Fig. 1b). Crude organic extract production was also high at 100–150 rpm. Surface and interfacial tensions were

low under all conditions, indicating the production of a BS with high surface activity. These results suggest that aeration is another important factor to consider in BS production. Overall, the best physical conditions for AL 1.1 BS production were 30°, 120 rpm and 24 h. *B. licheniformis* is a facultative anaerobic bacterium and produces active BS under both anaerobic and, even more so, aerobic conditions (Yakimov et al. 1995). The study of medium optimization for *B. subtilis* SPB1 lipopeptide production showed that aeration of the medium affects BS yields and that oxygen transfer is one of the key parameters for process optimization (Ghribi and Ellouze-Chaabouni 2011). Aeration was also an important factor for BS production by *B. licheniformis* strain AL 1.1. Adequate aeration promotes production with a shorter incubation time, and a more rapidly obtained product is more economically profitable. Rapid BS production is a notable characteristic of AL 1.1, as only 24 h were needed for each production cycle. Moreover, long incubation phases may induce product degradation.

BS production with different carbon sources

To select a suitable carbon source, various carbohydrates (glucose, fructose, sucrose, lactose), oils ($C_{11–13}$, olive oil, linseed oil) and alcohol (glycerol) were tested for BS production. No growth or BS production was obtained when oils were used as the carbon source. The high yield of crude extract and degree of emulsion observed were related to carbon source excess. Strain AL 1.1 grew well with glycerol (1.4 g/L dry weight), while the surface tension of the supernatant was reduced to 35.8±6 mN/m and the crude extract obtained (83±2 mg/L) reduced surface tension to 33.4 mN/m. Good results were obtained with carbohydrates and, to a lesser extent, lactose. Bacterial growth was high (1.8–2.0 g/L) and the crude extract obtained (149–208 mg/L) effectively reduced surface tension to 29.1±1 mN/m (Fig. 1c). The best crude organic extract production was obtained with glucose, utilizing this substrate in subsequent studies. Carbon and nitrogen sources are usually the primary factors in lipopeptide production. Most microorganisms require a hydrophobic substrate (hydrocarbon or oil) as a carbon source for BS production. This includes *Rhodococcus erythropolis* and the phylogenetically related bacteria (Marqués et al. 2009), *Pseudomonas aeruginosa*, other *Pseudomonas* sp., *Alcaligenes calcoaceticus* and *Brevibacterium luteolum* (Vilela et al. 2014). The use of *n*-alkanes by *Bacillus* sp. as a carbon source is probably related with contamination in the isolation environment. In fact, some BS-producing *B. licheniformis* bacteria have been isolated from oily media such as oily waste, petroleum-polluted soil and sludge (Yakimov et al. 1995; Li et al. 2008; Biria et al. 2010). BS increase the availability of oily carbon sources, making bacterial survival possible in such harsh media (Biria et al. 2010). The *B. licheniformis* strain AL 1.1 does not grow in the presence of *n*-alkane mixtures or oils. AL 1.1 isolation from non-contaminated soil may explain its lack of adaptation to these carbon sources. Other *Bacillus* sp. can grow and produce BS on an oily substrate, but the growth and BS production rate in a carbohydrate medium is higher. *B. licheniformis* VKM B-511 produces BS with saccharose (750 mg/L), glucose (730 mg/L) and fructose (680 mg/L) (Gogotov and Miroshnikov 2009). The results we obtained with glycerol are interesting, especially if the substrate can be obtained cheaply as residue from biodiesel production and be transformed into a value-added product.

Effect of glucose concentration on BS production and bacterial growth

Different concentrations of glucose were examined for the best BS yield from strain AL 1.1. Glucose was added to the production medium in concentrations from 0.5 to 2 % (w/v). The results obtained are shown in Fig. 2a. Bacterial growth increased as the glucose concentration increased, up to a concentration of 1.5 %. With an initial glucose concentration of 1.5 and 2 %, residual glucose was present in the medium supernatant at the end of culture (24 h). The crude extract weight increased markedly from 70 to 254 mg/L as glucose concentration increased. Thereafter, 2 % glucose was supplied to the culture medium. Surface and interfacial tensions were low, remaining similar at different glucose concentrations. The presence of residual glucose in the medium from an initial concentration of 1.5 % suggests that other substrates should be optimized for total glucose consumption and improved BS production. Ghribi and Ellouze-Chaabouni (2011) described maximum BS production by *B. subtilis* with 4 % glucose, which is in line with the findings of Saimmai et al. (2011), who described a maximum BS production with a medium of 4 % molasses, a more complex sugar-based substrate.

Effect of different nitrogen sources and phosphate concentration on BS production and bacterial growth

Apart from the carbon source, the production of BS by *B. licheniformis* is also known to be influenced by the nature of the nitrogen source. Different organic or inorganic nitrogen sources (equimolar in nitrogen, 0.1 M) were added to B-MSM to study BS production. *B. licheniformis* grew with all nitrogen sources, but urea was the least favorable substrate for AL 1.1 strain growth and BS production (Fig. 2b). Its nitrate reductase activity could explain bacterial growth with all nitrogen salts studied. The best results were obtained with ammonium salts (364 mg/L with ammonium phosphate and 261 mg/L with ammonium sulfate). Thaniyavarn et al. (2003) also described

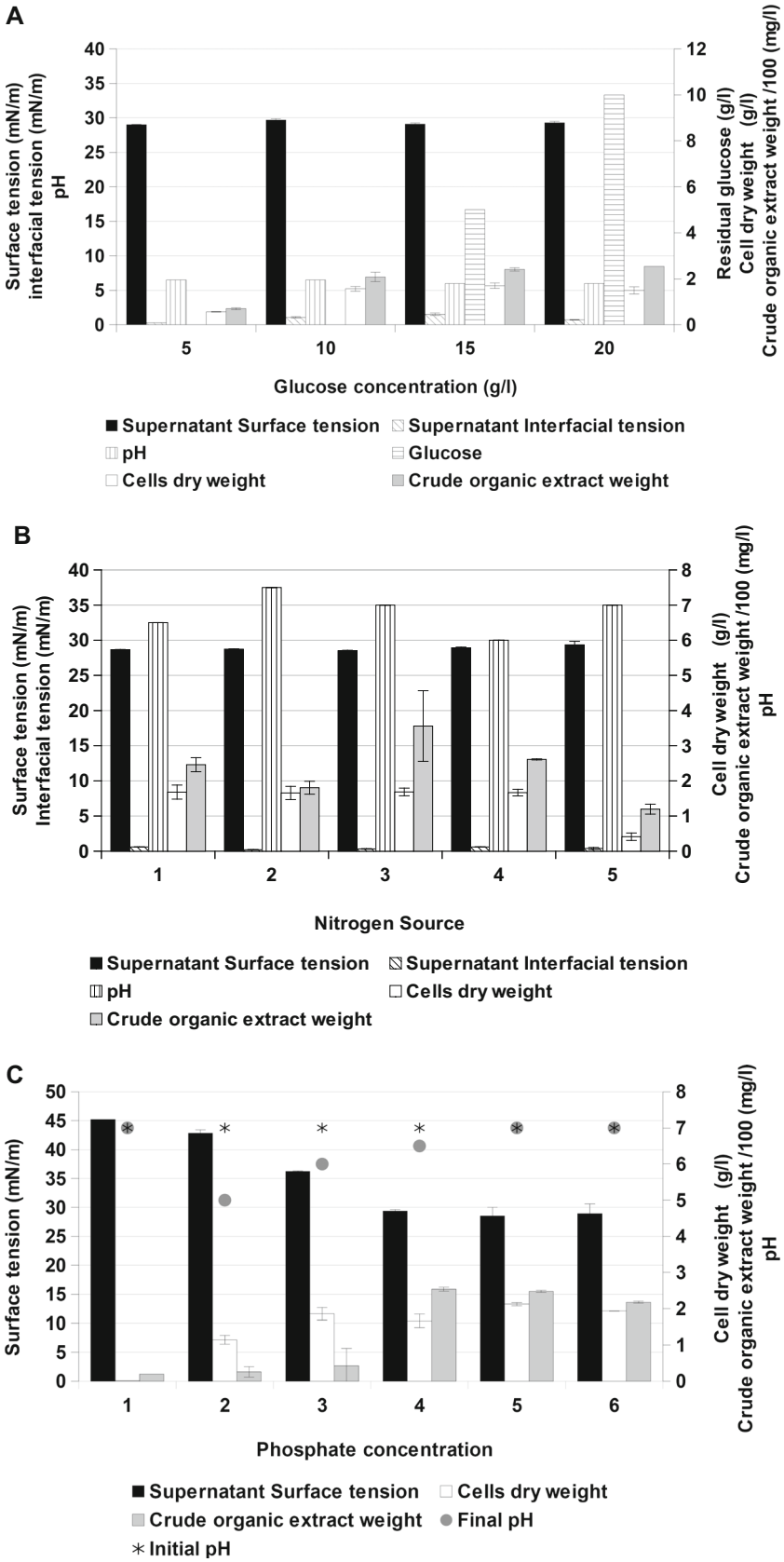

Fig. 2 Effect of **a** glucose concentration, **b** nitrogen source [*1* NH_4NO_3, *2* $NaNO_3$, *3* $(NH_4)_2PO_4$, *4* $(NH_4)_2SO_4$, *5* urea] and **c** phosphate concentration [*1* Medium control, *2* Na_2HPO_4+ KH_2PO_4 (1.4+1 g/L), *3* (2.9+ 2 g/L), *4* (5.7+4 g/L), *5* (8.6+6 g/L), *6* (11.4+8 g/L)] on growth and BS production by strain AL 1.1 at 30°C and 120 rpm. Data are presented as mean±SE

an improved BS production by *B. licheniformis* F2.2 with ammonium nitrate salt.

Phosphates are an important component of the culture medium as a source of phosphorus for growth, as well as acting as a buffer. The best results were obtained with the initial concentration of phosphates used, or a slightly higher one [Na_2HPO_4 (5.7–8.6 g/L)+KH_2PO_4 (4–6 g/L); Fig. 2c]. At this concentration, the final pH of the medium was kept at 7–6.5 and surface activity was maintained. With lower phosphate concentrations, the final pH dropped to 6 or 5 and surface activity fell markedly, despite the presence of crude organic extract. At pH 6 or lower, the organic extract formed a turbid solution associated with BS precipitation. Crude extract precipitation was a reversible process when the pH was basic. According to the results obtained, a high phosphate concentration is good for the bacterial growth, BS production and surface activity of the BS produced by the AL 1.1 strain. Many media combinations are possible, but similar phosphate concentrations have been used for *B. licheniformis* culture by other authors (Yakimov et al. 1995; Gogotov and Miroshnikov 2009). As a cheap synthetic medium is preferred for the large-scale production of BS in industry (Thaniyavarn et al. 2003), the lower phosphate concentration was chosen for BS production.

Effect of calcium, iron and magnesium concentration on BS production and bacterial growth.

Finally, the effects of calcium, iron and magnesium salts were also studied. The initial concentration of calcium and iron used in B-MSM was optimum, but an increase in magnesium concentration (0.21 g/L) achieved an increase in crude extract production of 551 mg/L after 24 h culture. Metal ions including magnesium and iron were shown to have an important impact on the yield of surfactin by a *B. subtilis* strain (Li et al. 2008). According to these results, the new medium G-MSM has the following composition (g/L): glucose, 20; $(NH_4)_2HPO_4$, 6,6; KH_2PO_4, 4; Na_2HPO_4 5.7; $FeSO_4{\cdot}7H_2O$, 0.01; $MgSO_4{\cdot}7H_2O$, 0.21; $CaCl_2$ 7×10^{-6}; trace element solution, 0.05 mL; final pH 7, 24h culture at 30 °C and 120 rpm. This change increased crude extract production from 210 to 550 mg/L (2.6 times) and improved the surface activity measured in the culture supernatant from 37.7 to 28.5 mN/m. This decrease in surface tension may be due to higher BS production and a better control of medium pH and BS solubility

Recovery of BS

The crude extract of BS was recovered from the culture supernatant of *B. licheniformis* AL 1.1 by organic extraction with several organic solvents: chloroform, ethyl acetate, hexane, diethyl ether, chloroform:methanol (8:1) and ethyl acetate:methanol (8:1). Ethyl acetate:methanol gave the highest crude extract dry weight (480–550 mg/L) after growth in G-MSM. TLC indicated the presence of a noteworthy spot, identified as a lipopeptide with a R_f. of 0.50, and another product with higher polarity. However, because the most frequent extraction method for *Bacillus* surfactants is overnight acid precipitation (Li et al. 2010; Pecci et al. 2010; Sivapathasekaran et al. 2010), the organic extraction method was compared with acid precipitation and subsequent organic extraction. The extract obtained after precipitation and solvent evaporation was weighed and clearly showed better BS production (731 mg/L). Organic extract TLC indicated a high-purity product, probably due to the double extraction. The surface tension response with different concentrations of purified organic extract revealed a critical micelle concentration (CMC) of 15 mg/L. Lichenysins are among the most efficient lipopeptides. $BS_{AL\ 1.1}$ has a CMC similar to that of lichenysin A (12 mg/L), arthrofactin (13.5 mg/L) and surfactant BL86 (10 mg/L), but lower than surfactin (25 mg/L) and lichenysin B (20 mg/L) (Yakimov et al. 1995).

Downstream processing is an important step in major biomolecule production processes. It has been considered an obstacle to a reasonably economical production, since it accounts for 50–80 % of total production costs (Mnif et al. 2013). BS organic extraction from the culture supernatant is a frequent practice for obtaining hydrophobic products. However, the anionic nature of lichenysins (Nerurkar 2010) causes BS precipitation at acidic pH, facilitating its extraction. Additionally, subsequent organic extraction from precipitate gave a very pure extract. When studying BS production by *B. licheniformis* TR7, Saimmai et al. (2011) described the chloroform:methanol mixture (2:1) as the most efficient in BS extraction, with a very high recovery of 3.26 g/L. However, it is important to know the BS composition in the extract after organic extraction.

Time course of growth and BS production

A growth curve of strain AL 1.1 in G-MSM was obtained to study BS production dynamics. Figure 3 shows the growth profile of Al 1.1 when cultured at 30 °C, 120 rpm, for 48 h. After a lag phase of 12 h, exponential growth began until 35 h of incubation, when the initial glucose and nitrogen sources were consumed. The pH was maintained with a later tendency to alkalinity. Crude extract production was associated mainly with exponential growth, with decreased recovery after 24 h. Surface tension decreased markedly in the initial 12 h of culture. This result indicated, as mentioned above, the production of a highly efficient BS, because a very small quantity of crude extract was enough to reduce surface tension to a value of 32 mN/m. In fact, BS produced by *Bacillus* species is

characterized by a lipopeptide nature and exceptional surface activity (Nerurkar 2010). According to our results, BS production was growth-associated, with an almost parallel relationship between BS production, cell growth and substrate utilization. A cultivation time of 24 h gave the highest BS production, which was therefore selected as optimum for BS production by the strain Al 1.1. With these growth and BS extraction conditions, 860 mg/L purified extract was obtained, showing a 4-fold increase from the initial production. The growth-associated production of BS has been reported in several other microorganisms (Sivapathasekaran et al. 2010; Ghribi and Ellouze-Chaabouni 2011; Burgos-Díaz et al. 2013; Chooklin et al. 2014). Like *Bacillus circulans* (Sivapathasekaran et al. 2010), with *B. licheniformis* AL 1.1, a decrease in BS concentration was measured in the stationary growth phase, indicating possible surfactant degradation. Saimmai et al. (2011) described BS production by *B. licheniformis* TR7 with molasses as the carbon source as a growth-associated process, obtaining the highest BS yield as the cultivation time approached 48 h, which corresponded with the beginning of the stationary phase. This slower process may be related to the carbon source used, as assimilation and metabolism of molasses is slower and more difficult than glucose—a simple and readily available sugar. The rapid BS production characteristic of AL 1.1 is notable, as only 24 h are required to achieve a good BS concentration, which has important economical advantages when compared with a production process lasting several days (Vilela et al., 2014). Crude oil was not required for growth or production, although members of the *B. subtilis/B. licheniformis* group have been isolated repeatedly from oil reservoirs and may be presumed to tolerate and produce BS under reservoir conditions (Nerurkar 2010; Jacques 2011; Simpson et al. 2011).

Chemical structure characterization

The amino acid content of the product was determined by analysis of a hydrolyzed sample followed by derivatization using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate reagent (AQC). The HPLC analysis of the hydrolyzed sample showed that the peptide moiety contained five different amino acids: Asx, Glx, Val, Ile and Leu. The corresponding peak areas showed a ratio of 1:1:1:1:3, respectively (Fig. 4).

The molecular weight of the components of the surfactants was determined by negative- and positive-ion mode ESI analyses. In the positive-mode ESI, the mass spectra showed a series of ions $[M+H]^+$ at *m/z* 1007, 1021 and 1035 plus the corresponding ions $[M+Na]^+$ at *m/z* 1029, 1043 and 1057 (Fig. 5a). The negative ionization showed deprotonated molecular ions $[M-H]^-$ at *m/z* 991, 1005, 1019, 1033 and 1047, with *m/z* 1005, 1019 and 1033 as the major ions (Fig. 5b). This 1 Da difference might be due to the presence of a single amide side chain corresponding to a glutamine or an asparagine instead of the glutamic or aspartic acids present in lichenysins B or C. The pattern of structural analogs with mass shifts of 14 Da is due to a mixture of closely related molecules varying in their fatty acid residues (Yakimov et al. 1995; Yang et al. 2006; Pecci et al. 2010).

The tandem mass analysis of the sodium-ionized molecular ion, with a mass of 1043.7, is given in Fig. 5c. To determine the connection of the amino acid residues in a peptide chain, the mass difference of the peaks can be used. A series of peaks, 1043.7—930.6—817.6—702.6 was detected, which suggested a Leu–Leu–Asp sequence, and a second series of peaks, 594.4—481.3—382.2—267.2 suggested a Leu–Val–Asp sequence. Furthermore, the difference between fragment ions, 1043.7 and 799.5, agrees well with an even mass loss

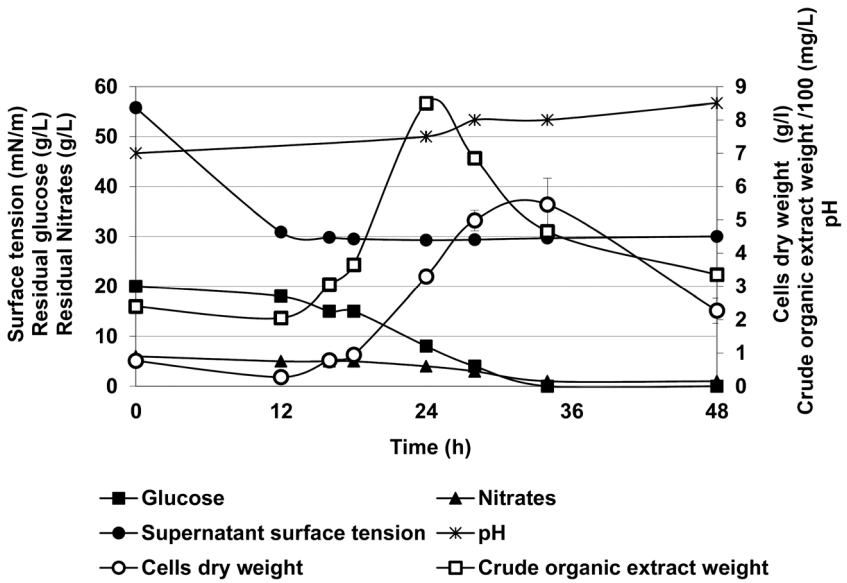

Fig. 3 Time course profiles of residual glucose (g/L), residual nitrates (g/L), supernatant surface tension (mN/m), pH, cell dry weight (g/L), crude organic extract weight (mg/L) during *Bacillus licheniformis* AL 1.1 growth in G-MSM. The points enclose the error bars (±standard deviation) of three measurements

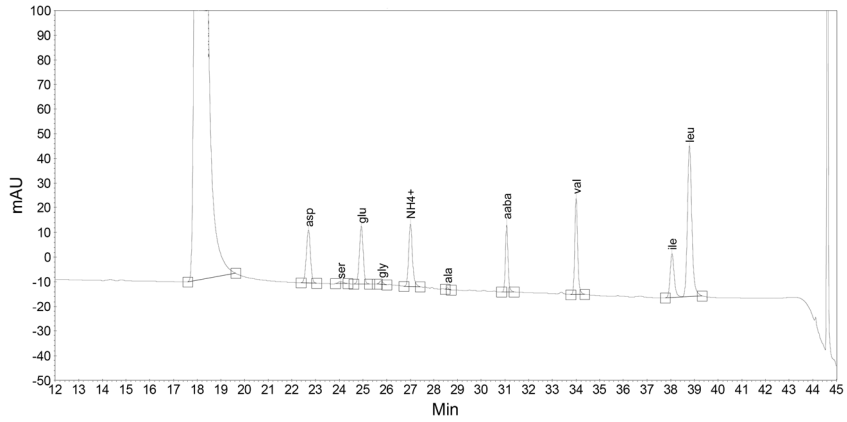

Fig. 4 Amino acid analysis by HPLC of the lipopeptide hydrolyzed sample

corresponding to a fragment Leu–Ile+H_2O (m/z 244.2). This fragment can be explained by a McLafferty rearrangement at the alkoxy group of the ester and confirms that the carboxy group of Ile^7 residue forms the ester bond of the lactone. In addition, the 18-unit increase of the McLafferty rearrangement indicates that the fatty acid in the lipopeptide is a β-hydroxy fatty acid. The presence of a small peak at m/z 267'2 also confirms the Leu–Ile sequence as it accounts for the sodium-ionized fragment (Leu–Ile–OH_2+Na^+). In this regard, it is important to note that it is not possible to distinguish between Leu and Ile residues by ESI mass spectrometry. However, we will hypothesize that the single Ile detected by amino acid analysis is located in position 7, forming the ester bond with the β-hydroxy fatty acid, as occurs with all Ile-containing lichenysins reported so far in the literature (i.e., lichenysins A, D, and G, see Nerurkar 2010). Altogether, these fragmentation patterns allow the establishment of the hexapeptide sequence Leu–Leu–Val–Asp–Leu–Ile in the cyclic lipopeptide (Fig. 5d). The last residue determined, Glx, could only be connected to the N-terminal Leu; this fact is supported by the series of ions 702'5 (Na^+ hydroxyacyl–Gln–Leu–Leu–Val), 817'8 (Na^+ hydroxyacyl–Gln–Leu–Leu–Val–Asp), 930'6 (Na^+ hydroxyacyl–Gln–Leu–Leu–Val–Asp–Leu), and, finally, 1043'7 (Na^+ hydroxyacyl–Gln–Leu–Leu–Val–Asp–Leu–Ile). The mass of the fragments indicates that Glx has a side chain amide, i.e., a Gln, also in agreement with the even molar mass of the compound (it has an even number of nitrogen atoms, eight to be precise, as Asx is aspartic acid, as explained above). The sequence of amino acid residues in the peptide is therefore Gln-Leu-Leu-Val-Asp-Leu-Ile (Pecci et al. 2010).

The mass spectrum of the fatty acid residue obtained from the hydrolyzed organic extract is shown in Fig. 6. The molecular masses of 229, 243 and 257, with 14 units difference between peaks, indicate the presence of fatty acids of different lengths. These results suggest that peaks correspond with β-hydroxyl fatty acid chains that are C_{14}, C_{15} and C_{16} long. These results indicate a structural relationship between $BS_{AL\ 1.1}$ and lichenisyn A, D and G (Nerurkar 2010).

The structural determination of the isolated LP mixture was studied by a variety of analytical techniques. First, an amino acid analysis of a hydrolyzed sample proved the presence of a peptide moiety and showed a composition of amino acids containing Asx, Glx, Val, Ile and Leu in a 1:1:1:1:3 ratio (Fig. 4). The pairs Glu/Gln and Asp/Asn cannot be determined by this technique as hydrolysis of the peptide converts these amino acids into Glu and Asp. The molecular weight of the surfactant components was determined by negative- and positive-ion mode ESI mass spectrometry. The molecular weights of the main components were 1006, 1020 and 1034, deduced from the series of ions $[M+H]^+$ and $[M+Na]^+$ obtained by positive ionization and $[M-H]^-$ in the ESI negative-mode. The difference in m/z 14 mass units was found to correspond to the different number of methylene units of the lipid moieties, as explained below. The LP mixture had the most abundant structural analogues ranged in even-numbered molecular weight, unlike the previously studied LP, surfactin and lichenysins B and C, which have odd-numbered molecular weights of 1007 to 1035 (Arima et al. 1968; Jenny 1991). This is indicative of an even number of nitrogen atoms in the sequence and hence of an additional amide present in a side chain, corresponding to a glutamine or an asparagine instead of the Glu or Asp present in these lipopeptides. The tandem mass analysis of the sodium-ionized molecular ion m/z 1043.7 permitted the determination of the sequence of amino acids. In this regard, it is important to note that ESI mass spectrometry does not allow the L or D configuration of amino acids to be assigned or the Leu and Ile residues to be distinguished. Nevertheless, consistent series of ions resulting from fragmentation at each peptide bond was recognized and, by referring to the mass unit of the respective amino acid residues, the amino acid sequence Gln–Leu–Leu–Val–Asp–Leu–Ile was deduced. The presence of Gln supported the hypothesis that the LP was closely related to lichenysins A, D, or G. Hence, the single Ile detected by amino acid analysis could similarly be located at

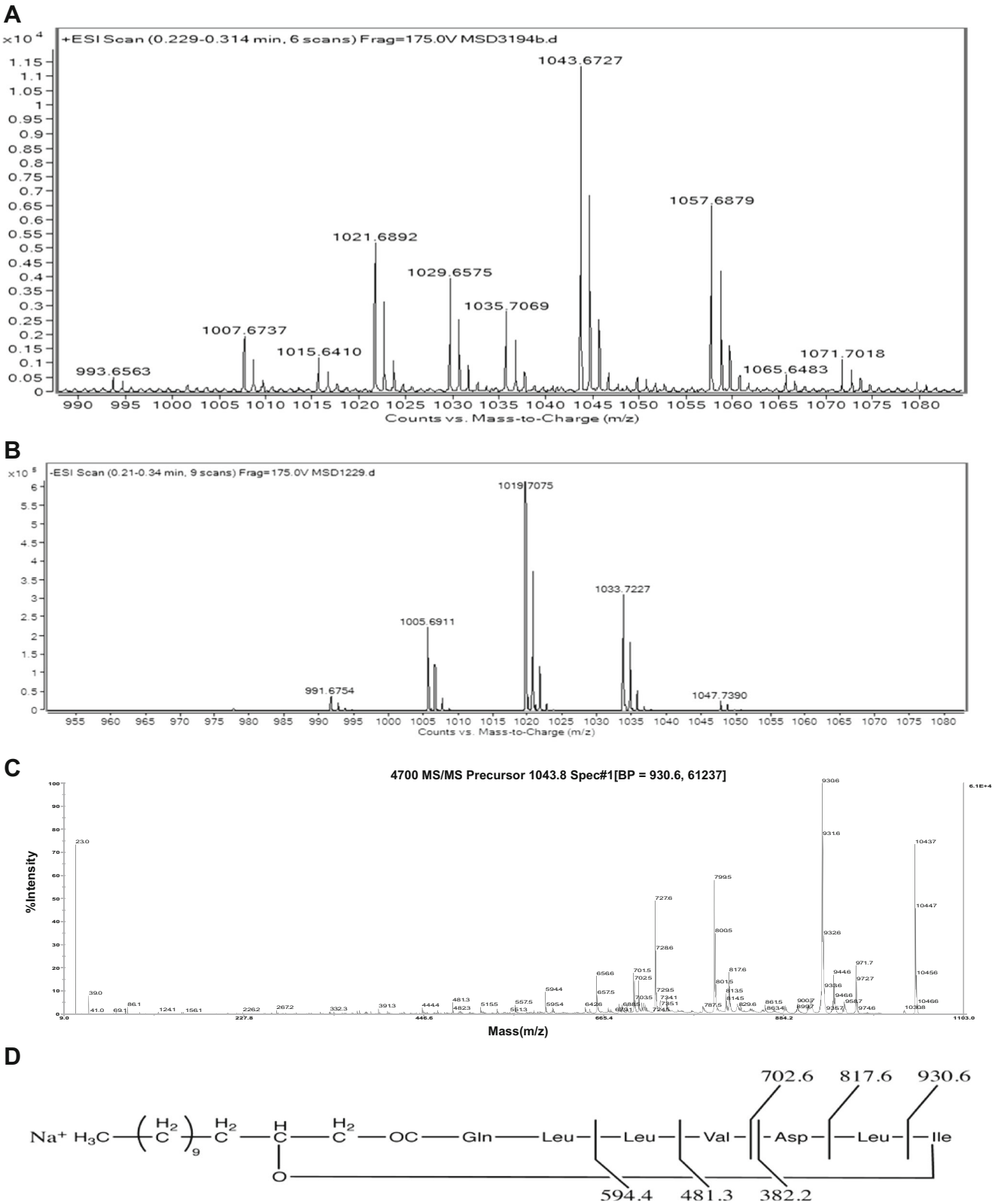

Fig. 5 **a** Partial electrospray mass spectrum in the positive-ion mode. **b** Partial electrospray mass spectrum in the negative-ion mode. **c** MS/MS of the sodium-ionized peak of the lipopeptide with m/z 1043'7. **d** Structure of the cyclic lipopeptide and fragmentations obtained by MS/MS

position 7, forming the ester bond with the β-hydroxy fatty acid, as occurs with all Ile-containing lichenysins reported so far in the literature (Nerurkar 2010). Furthermore, the detection of fragments arising from an even mass loss (Leu-Ile+

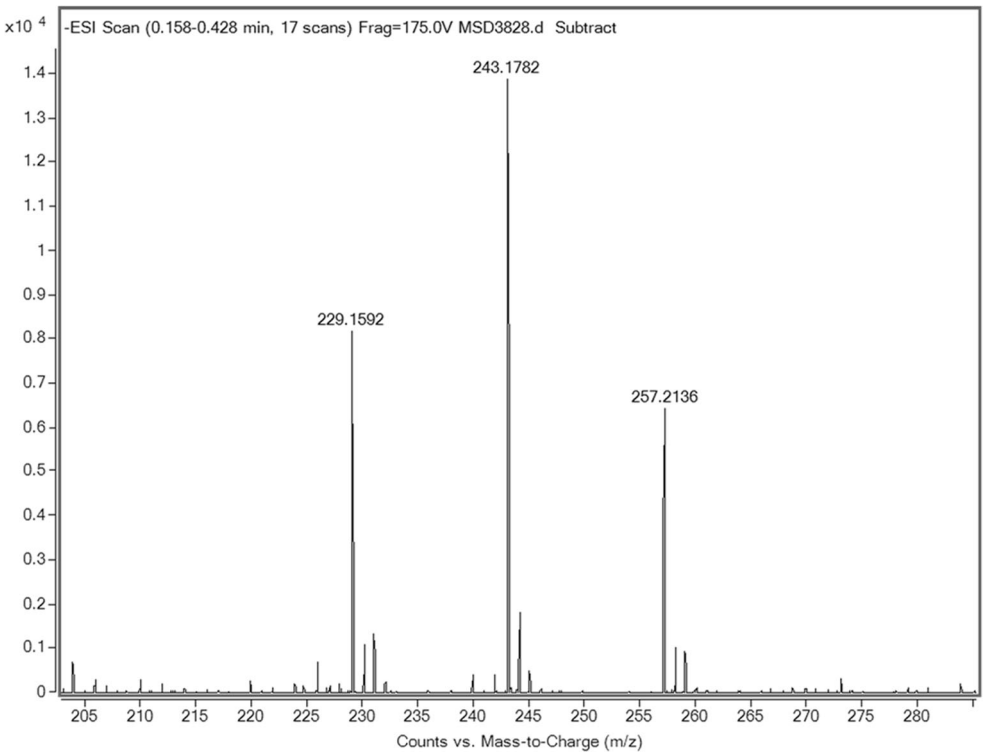

Fig. 6 Electrospray mass spectrometry of the fatty acid residue from hydrolyzed organic extract in the negative-ion mode

H_2O, m/z 244.2) could be attributed to a McLafferty rearrangement at the alkoxy group of the ester, confirming that the carboxy group of Ile[7] residue was the one forming the ester bond with the β-hydroxy fatty acid of the lactone (Yakimov et al. 1995; Yang et al. 2006; Pecci et al. 2010). Finally, the mass spectrum of the fatty acid moiety indicated that the β-hydroxy fatty acid chains were C_{14}, C_{15} and C_{16} long. These results confirmed a structural relationship between $BS_{AL\ 1.1}$ and lichenisyn A, D and G (Nerurkar 2010).

Biosurfactant stability and emulsion capacity of $BS_{AL\ 1.1}$

To assess the potential application of $BS_{AL\ 1.1}$ in the cosmetics industry, properties such as thermal, pH, salt stability and emulsion capacity were studied. After heat treatment, $BS_{AL1.1}$ solution maintained its surface activity, with surface tension decreasing to 29.5–30.7 mN/m, and after sterilization (120 °C) surface tension reached 37.1 mN/m. These results indicate a high stability under high temperatures. The surface activity of BS remained stable at a pH range of 6–11. At a pH below 6, a cloudy suspension was formed, the consequence of BS precipitation, an observation also described by other authors (Vilela et al. 2014). Few changes in surface tension were observed with an increased concentration of NaCl up to 20 %. Emulsion formation was observed with various oils. A translucent emulsion was observed in the aqueous phase (E_{24}= 66.7 %) in the presence of isopropyl palmitate and isopropyl myristate. With octyldodecanol, cetearyl ethylhexaonate and caprylic capric triglycerides, an emulsion was observed in both the aqueous and, even more so, the oily phase (Fig. S1).

The study of BS properties is essential for their application in different fields. Thermic resistance indicates possible applicability in industrial fabrication processes involving heat treatment prior to commercialization. Emulsion capacity is another valuable property in many areas. The results obtained suggest that emulsifier activity depends on the affinity of BS for oily substrates and oil polarity. With hydrocarbon substrates bearing longer carbon chains, isopropyl palmitate (C_{16}) and isopropyl myristate (C_{17}), an O/W emulsion was formed, but with the other studied oils (C_{12}–C_8) an emulsion was formed in both phases, showing a higher concentration in the W/O phase. Abu-Ruwaida et al. (1991) studied the emulsion capacity of a BS produced by *Rhodococcus* sp. ST-5 with crude oil and other hydrocarbon substrates and described a higher emulsion capacity as the hydrocarbon carbon chain length increased. Nano-emulsions (droplet size range 20–299 nm) appear transparent or translucent to the naked eye and possess stability against sedimentation. These properties make nano-emulsions of interest for basic studies and for practical applications in the pharmaceutical or cosmetic fields (Solans et al. 2005). A microemulsion system usually presents a wide range of nano-structures with different forms (liquid crystalline, worm like, hexagonal, bicontinuous sponge-like, spherical swollen micelles) involving the formation of one, two or three phases (Gudiña et al. 2013). The consumption of BS-containing cosmetics is increasing as a result of the remarkable surface and biological activities of LP, which facilitate

applications in this industry. In addition, the broad spectrum of BS properties (anti-adhesive, antimicrobial, anti-inflammatory) has revealed applications as multifactorial cosmetic ingredients (Kanlayavattanakul and Lourith 2010).

The screening of Antarctic BS-producing microorganisms offers an excellent opportunity to discover new molecules with novel properties. Generally, *Bacillus* species co-produce various families of lipopeptides with different homologues and isoforms (Biria et al. 2010; Pecci et al. 2010). Medium components are believed to be important factors affecting the type of lipopeptide obtained. Lichenysins are produced as complex mixtures except for Lichenysin B (Nerurkar 2010). Lichenysins can be added to the group of lipopeptides that have promising applications in high added value products because of their high surface activity. $BS_{Al\ 1.1}$production, stability and emulsion capacity with oils used in cosmetics suggests its suitability for this kind of industry. Growing social concerns about environment pollution and demand for natural products has focused attention on BS as a green alternative to synthetic surfactants (Vilela et al. 2014). However, before an optimal formulation can guarantee safety and efficacy, further research is needed on the optimal concentration of the different components for emulsion production, as well as cytotoxicity studies.

Conclusions

Bacillus licheniformis AL 1.1 produces extracellular BS with carbohydrates as the carbon source. BS production was found to be growth-associated. A four-fold increase in production was obtained by adjusting media composition and physical conditions. A purified organic extract showed a BS mixture with molecular weights of the main components ranging between 1006 and 1034 Da. $BS_{AL\ 1.1}$ was characterised as a lipopeptide with seven amino acids (Gln-Leu-Leu-Val-Asp-Leu-Ile) and three isoforms (C_{14}, C_{15}, C_{16}). This chemical structure identified it as a lipopeptide of the lichenysin group A, D or G. $Lichenysin_{AL\ 1.1}$ decreases water surface tension to 28.5 mN/m and achieves the critical micelle concentration of 15 mg/L, indicating high efficiency and effectiveness. Its heat resistance and capacity to emulsify oils used in cosmetics indicate a possible application in this industrial field.

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