Process Biochemistry xxx (2011) xxx–xxx

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Combined use of LC–ESI-MS and antifungal tests for rapid identification of bioactive lipopeptides produced by *Bacillus amyloliquefaciens* CCMI 1051

Ana Teresa Caldeira[a,b,*], J.M. Santos Arteiro[a,b], Ana V. Coelho[a,c], J. Carlos Roseiro[d]

[a] *Universidade de Évora, Departamento de Química, Rua Romão Ramalho no 59, 7000-671 Évora, Portugal*
[b] *Centro de Química de Évora, Rua Romão Ramalho no 59, 7000-671 Évora, Portugal*
[c] *Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. da República, Apt. 127, 2781-901 Oeiras, Portugal*
[d] *Laboratório Nacional de Energia e Geologia, Unidade de Bioenergia, Azinhaga dos Lameiros 22, 1648-038 Lisboa, Portugal*

ARTICLE INFO

Article history:
Received 20 January 2011
Received in revised form 10 April 2011
Accepted 27 May 2011

Keywords:
Antimicrobial activity
Bacillus amyloliquefaciens
Cyclic lipopeptides
Iturin
Biosurfactants

ABSTRACT

The strain *Bacillus amyloliquefaciens CCMI 1051* used in this study has been isolated in our laboratory from healthy *Quercus suber* in the south of Portugal and shows high levels of antagonistic properties against filamentous fungi that attack forest products industry due to the production of bioactive peptides.

A combined use of LC–ESI-MS and antifungal tests allowed a rapid identification of lipopeptides as active compounds produced. Applying autobiographic methods it was possible to obtain active compounds. LC–ESI-MS, a powerful tool for rapid identification, indicates the presence of lipopeptides and MS^2 electrospray ionization showed the partial sequence Tyr–Asn–Pro–Glu in the peptidic portion of some compounds produced. The association of mass spectrometry and chromatography, used in parallel with antifungal tests proved to be an efficient approach for the characterization of active lipopeptides without the need of previous total isolation. This methodology can be employed for screening and optimization the production of antifungal iturinic lipopeptides, showing a great potential for future application.

1. Introduction

Several strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens* have been referred to produce lipopeptides. In response to nutritional stress, a variety of processes are activated by *Bacillus* strains, including sporulation, synthesis of extracellular degradative enzymes and antibiotic production [1,2]. Many strains are known to suppress fungal growth *in vitro* due to the production of antifungal antibiotics [3–6] especially the nonribosomally synthesized cyclic lipopetides surfactin, iturin and fengycin. Bioactive peptides show a great potential for biotechnological, biopharmaceutical and agricultural applications. Among the most promising candidates for bacterial biocontrol agents are several species of the genus Bacillus, their ability to sporulate, assures their prevalence in the environment and guarantees future suitable formulation strategies [7]. The lipopeptides surfactin, fengycin and iturin are amphiphilic membrane-active biosurfactants and peptide antibiotics with potent antifungal activities [8,9], which can be used as biopesticides for plant protection. These compounds, made of amino acids and a fatty acid, are easily biodegradable in the soils [10]. In recent years, there is a considerable interest in using *Bacillus* producing lipopeptide antibiotics like iturin A and surfactin as a biocontrol agent due to its antagonistic and repressive activity over plant pathogens [11]. These amphiphilic cyclic biosurfactants have many advantages over other pesticides: low toxicity, high biodegradability and environmentally friendly characteristics [6,12–15]. In literature, several approaches for lipopeptide identification have been proposed but their biological activity needs to be determined after isolation procedures [16–19]. The *Bacillus* strain, used in this study, Genbank access number AY785773, has been isolated in our laboratory from *Quercus suber* and shows high levels of antagonistic properties against filamentous fungi that attack industrial forest products. The purpose of this study was to access a rapid characterization of the unidentified active metabolites produced by *B. amyloliquefaciens CCMI 1051* using LC–ESI-MS analysis and monitoring, by antifungal tests, active metabolites without previous total isolation. MS/MS was employed separately to establish the partial sequence of each active compound after isolation.

2. Materials and methods

2.1. Microoganisms and inocula preparation

The *B. amyloliquefaciens* CCMI 1051 used throughout this study has been isolated in our laboratory from *Q. suber*. The strain was characterized by the morpholog-

* Corresponding author at: Universidade de Évora, Departamento de Química, Rua Romão Ramalho no 59, 7000-671 Évora, Portugal. Tel.: +351 266 745 313; fax: +351 266 745 303.
E-mail address: atc@uevora.pt (A.T. Caldeira).

1359-5113/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.procbio.2011.05.016

ical, physiological, biochemical characteristics based on the Bergey's manual of systematic bacteriology and the partial sequence of 16S rDNA. The 16S rDNA partial sequence was the GenBank accession number AY785773. *B. amyloliquefaciens CCMI 1051* was maintained on nutrient agar slants and stored at 4 °C. Cells of a new slant were used to inoculate one 500 mL shake flask containing 100 mL of chemical defined medium containing per litre: $(NH_4)_2SO_4$, 1.5 g; KH_2PO_4, 1.7 g; $Na_2HPO_4{\cdot}2H_2O$, 1.7 g; $MgSO_4{\cdot}7H_2O$, 0.2 g; yeast extract (Difco), 0.1 g; glucose 2 g and 2 mL of Vishniac solution sterilised separately [20]. The inoculum culture was incubated for 24 h at 30 °C in an orbital shaker at 200 rpm (Heidolph unimax 1010).

Trichoderma pseudokoningii CCMI 304, *Aspergillus niger* CCMI 296, Rhizopus sp L-122, *Penicillium expansum* CCMI 625, *Trichoderma harzianum* CCMI 783, *Trichoderma koningii* CCMI 868, *Trichoderma harzianum* CCMI 822, *Cladosporium resinae* CCMI 262, *Fusarium oxysporum* CCMI 898, *Fusaruim solani* F4 and *Cephalosporium* sp. F25 were used as test micro-organisms and were obtained from the Culture Collection of Industrial Micro-organisms (Lisbon). The cultures of the test micro-organisms were maintained on malt extract agar slants.

2.2. Growth conditions

The *Bacillus* was grown in batch culture at 30 °C using a bench scale bioreactor (Infors HT CH-4103, Switzerland) with a 1L working volume of chemically defined medium. Control of foam was performed by the addition of 2 mL/L of medium of a sterilised aqueous solution of polypropylene glycol 2000 (2%, v/v).

The bioreactor was inoculated with 100 mL stationary-phase culture. The culture pH was measured by a combined electrode (Ingold) and controlled to 7 ± 0.2 by the automatic addition of base (NaOH 2 M) and acid solutions (H_2SO_4 1 M) through peristaltic pumps fitted in the control unity. Temperature was measured by using a platinum thermocouple (Pt-100) temperature sensor and was controlled at 30 °C by a water thermo circulator, in a double jacket present in the vessel. The dissolved oxygen level was measured by a polarographic electrode (Ingold). The stirring speed was set to 700 rpm and the aeration rate was 2 vvm.

2.3. Reverse phase column chromatography

Antimicrobial substances in cell-free supernatant cultures were concentrated and subject to chromatographic separation, using as stationary phase reverse phase silica (RP-18, Polygoprep 100-50 C18, Macherey-Nagel, 4 cm × 17 cm) and: water (Merck), methanol (Merck), chloroform (Merck) and hexane (Merck) as eluentes.

2.4. TLC separations

TLC (Thin layer chromatography) analysis of methanolic fractions were performed on precoated plates (0.2 mm, 20 cm × 20 cm, IF254, Merck, Darmstadt, Germany) using the mixture butanol:acetic acid:water (6:1:2) as a mobile phase. UV-active compounds were detected at 254 and 360 nm.

2.5. Preparative chromatography

The active methanolic fraction was applied in plates of preparative chromatography (0.5 mm, 20 cm × 20 cm, IF254, Merck, Darmstadt, Germany), separated by using butanol/acetic acid/water (6:1:2) as eluent and the areas corresponding to the presence of the active compounds were removed.

2.6. Antifungal paper disks diffusion assay

Fungal spore suspension was prepared by adding loopful of hyphae and spores from a Malt Extract Agar (MEA) slant incubated at 25 °C for 7 days, in 5 mL of NaCl 0.85% solution. The suspension was filtered by sterilized cotton or triple gauze (for *Botrytis cinerea*). A 10^8 cfu mL^{-1} spore suspension was obtained through dilutions and fungal suspensions were incorporated in MEA at 45 °C in Petri dishes. Filter paper discs (Macherey-Nagel 827 ATD) impregnated with 20 μL of samples were placed on the agar and the Petri dishes were incubated at 25 °C for 24–48 h. Antifungal activity was indicated by the formation of a inhibition halos around the discs [21].

2.7. Bioautographic TLC methods

2.7.1. Spray fungi assay

The micelium of *Cladosporium cucumerinum* CCMI 206 was harvested from the agar plates in Homans and Fuchs nutrient broth [22], filtered through sterilized gauze and diluted in order to obtain 10^6 cells ml^{-1}.

Developed chromatograms on silica gel precoated TLC plates (0.2 mm 20 cm × 20 cm, IF254, Merck) were dried using a hair-dryer for removing the solvent. UV-active compounds were detected at 254 and 360 nm and marked on the plates. TLC plates were dried overnight and then, a 20 mL of the *C. cucumerinum* cell suspension (10^6 cells mL^{-1}) was sprayed over the TLC plates. Plates were incubated in closed pyrex trays at 25 °C for two–three days, protected from light. Antifungal activity was indicated on the bioautograms by clear spots [23].

Table 1
Antifungal activity of methanolic fraction from *Bacillus amyloliquefaciens* CCMI 1051.

	Antifungal activity
Trichoderma pseudokoningii CCMI304	+
Aspergillus niger CCMI 296	+++
Rhizopus sp.	+++
Penicillium expansum CCMI 625	+
Trichoderma harzianum CCMI 783	++
Trichoderma koningii CCMI 868	++
Trichoderma harzianum CCMI 822	+++
Cladosporium cladosporioides CCMI 680	+
Cladosporium resinae CCMI 262	++
Cladosporium resinae CCMI 667	+
Fusarium oxysporum CCMI 898	+
Botrytis cinerea CCMI 899	++
Fusarium solani F4	+
Cephalosporium sp. F25	++

+ positive test (inhibition halo <15 mm); ++ (inhibition halo 15–20 mm); +++ (inhibition halo >20 mm).

2.7.2. MEA incorporated fungal suspensions assay

Fungal suspensions (10^8 cfu mL^{-1}), were incorporated in MEA at 45 °C. After development, thin layer plates were dried overnight and than placed on the agar in Petri dishes (with MEA incorporated fungal suspensions) being just a fine middle film with fungal suspension on the TLC plate surface. The plates were incubated at 25 °C for 24–72 h. Clear spots (inhibition zones) corresponding to the presence of compounds, indicating antifungal activity [23].

Parallel, for confirmation these inhibition zones, the different compounds, after having cut out from de TLC plate, were also placed on the Petri dishes, as described previously.

2.8. Mass spectrometric analysis

A 100 mL culture sample was spun down (16,000 × *g* for 20 min at 6 °C) and lipopeptides were recovered as described by Mc Keen et al. [24]. The dried material was dissolved in a minimum volume of methanol–water (1:1).

The electrospray mass spectra were recorded on a Bruker, Esquire 3000 plus ion trap mass spectrometer in the positive polarity mode.

Samples were diluted 50-fold in acetonitrile–water (1:1) with formic acid (0.1%) and injected at a rate of 100 $\mu L^{-1}\ h^{-1}$ into the ESI probe. Capillary temperature and voltage were set to 300 °C and 166 V, respectively. All spectra acquisitions were performed by using Esquire Control and analysed with the Data Analysis software.

2.8.1. LC–ESI-MS/MS analysis

LC–ESI–MS/MS analyses were carried out using a Thermo Hypersil Biobasic C18 Column, in a LCQ Advantage ThermoFinnigan mass spectrometer equipped with an electrospray ionization source and using an ion trap mass analyzer. It was controlled by Xcalibur software (ThermoFinnigan). The LC system was equipped with a photodiode array detector (Surveyor ThermoFinnigan) and an auto sampler (Surveyor ThermoFinnigan). The MS conditions were: capillary temperature 250 °C, source voltage 4.0 kV, source current 80.0 μA, and capillary voltage 7.0 V, in positive mode.

3. Results and discussion

3.1. Activity of compounds extracted in the methanolic fraction

The species *B. amiloliquefaciens* has been reported to produce lipopeptides with antifungal proprieties. *B. amyloliquefaciens* CCMI 1051 cultures displayed antifungal activity due to the production of several extracellular compounds [25]. In this work, culture cell-free supernatants were fractionated by using a reverse phase chromatography. Compounds present in the methanolic fraction confirm activity against all tested fungi (Table 1).

A LC–ESI–MS spectral analysis of the methanolic fraction showed a cluster containing molecules that were observed at *m*/*z* 1031, 1045 and 1059 and 1073. These peaks differ by 14 Da, suggesting a series of homologous molecules with different length of fatty acid chain. The spectra of the commercially available iturin A, surfactin and the peak mass exhibited on those experimental conditions was compared. The samples showed no correspondence with the peaks exhibited by the commercial samples subjected to the same conditions. Nevertheless, the commercial iturin A and surfactin samples show the same peak profile (Fig. 1).

Please cite this article in press as: Caldeira AT, et al. Combined use of LC–ESI-MS and antifungal tests for rapid identification of bioactive lipopeptides produced by *Bacillus amyloliquefaciens* CCMI 1051. Process Biochem (2011), doi:10.1016/j.procbio.2011.05.016

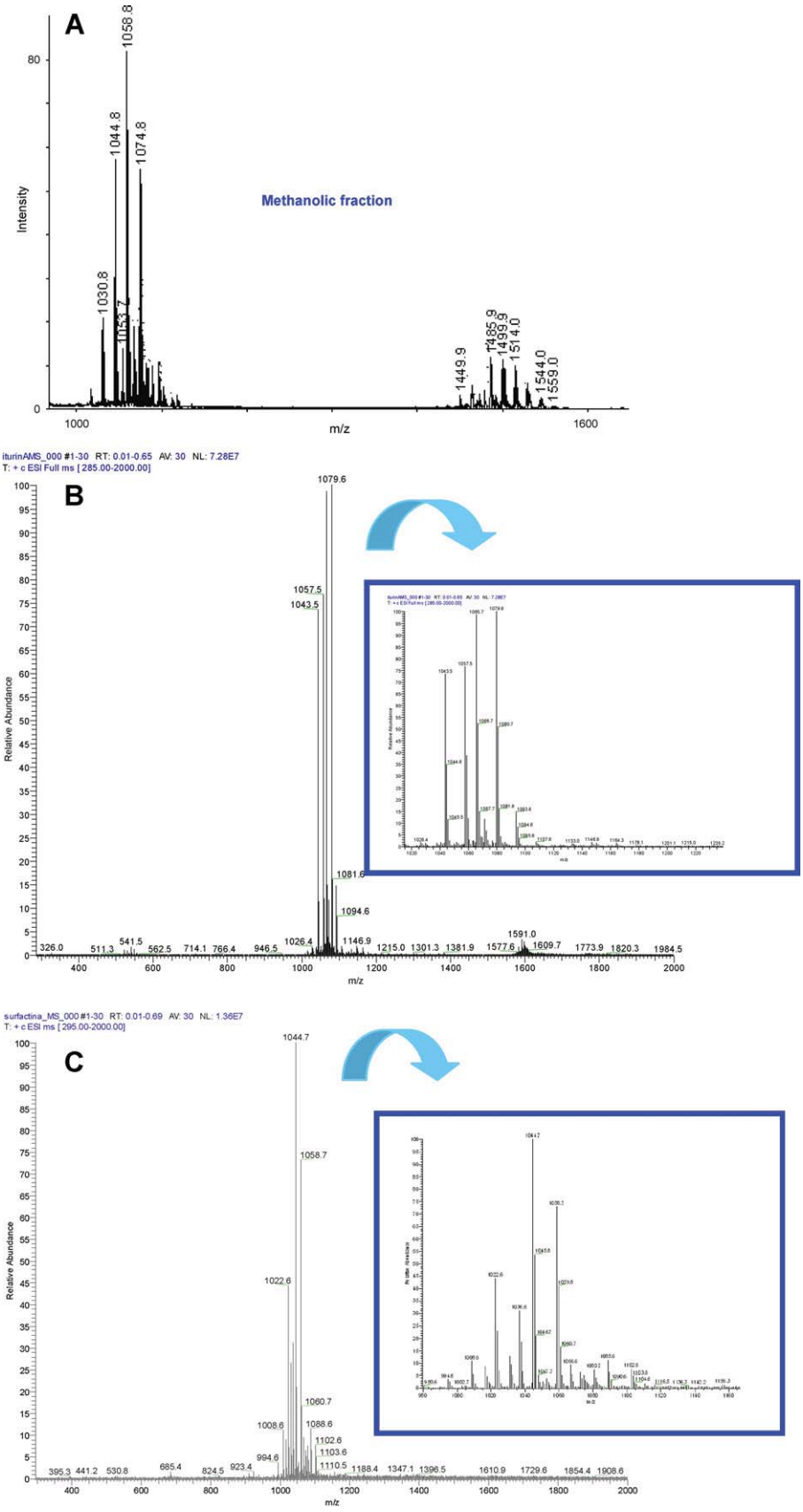

Fig. 1. ESI-MS spectra of the methanolic fraction (A), commercially iturin A (B) and commercially surfactin (C).

Please cite this article in press as: Caldeira AT, et al. Combined use of LC–ESI-MS and antifungal tests for rapid identification of bioactive lipopeptides produced by *Bacillus amyloliquefaciens* CCMI 1051. Process Biochem (2011), doi:10.1016/j.procbio.2011.05.016

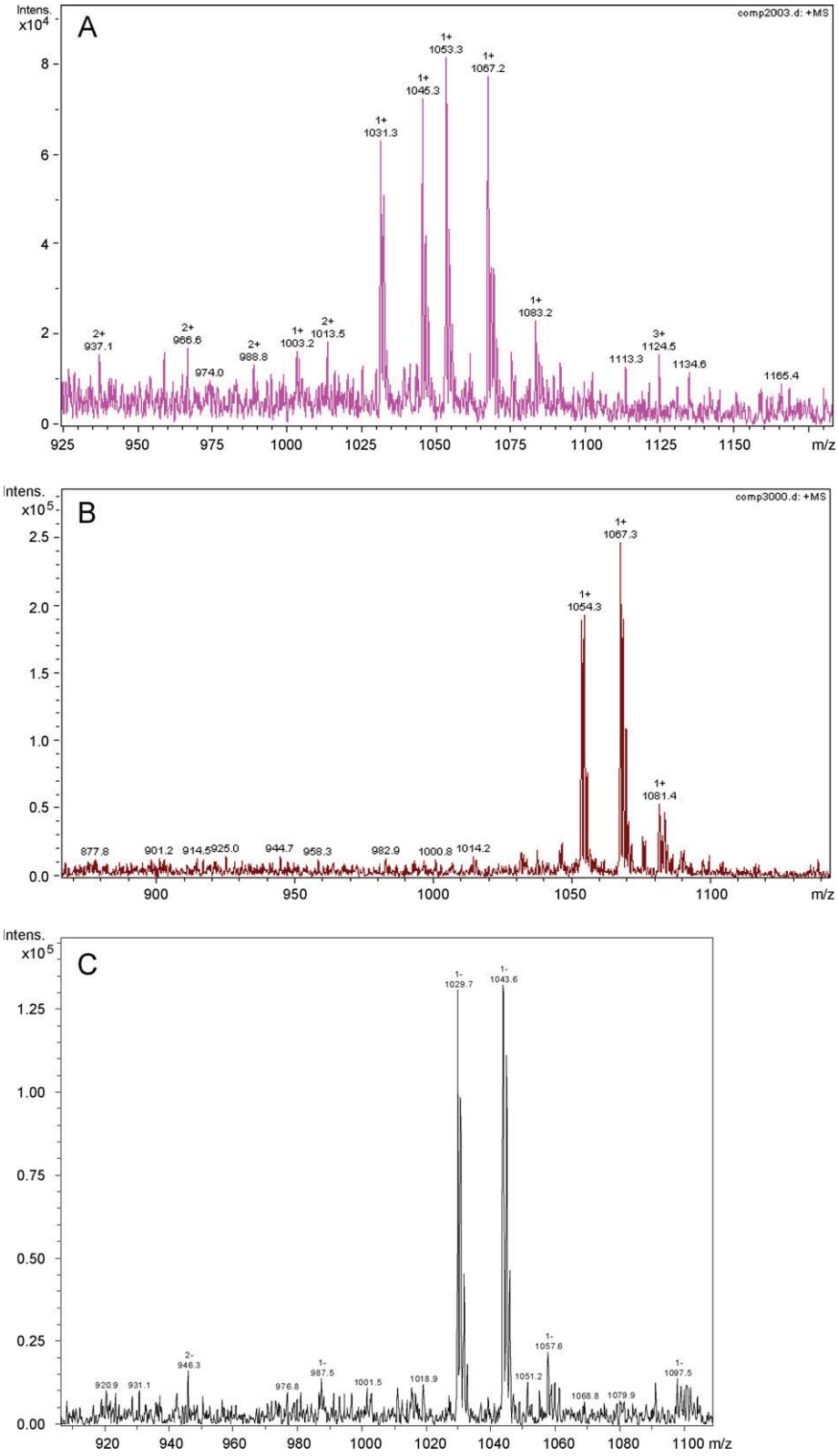

Fig. 2. (A) ESI-MS full scan MS (+) *m*/*z* corresponding to fraction 1. (B) ESI-MS full scan MS (+) *m*/*z* corresponding to fraction 2. (C) ESI-MS full scan MS (−) *m*/*z* corresponding to fraction 2.

The described data is in agreement with results in literature. In fact, Leenders et al. [26], using Matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF MS) analysis, refer the secondary metabolites produced by some strains of *B. subtilis* with molecular masses between 1045 and 1123 Da corresponding to surfactin and iturin A. The length of the lipidic chain and the substitution of amino acids in the peptidic ring were dependent of the producing strain as well the nutritional conditions. MALDI-TOF has also been applied by Price et al. [19] to characterize lipopeptide biomarkers from 54 different strains of Bacillis from most

Please cite this article in press as: Caldeira AT, et al. Combined use of LC–ESI-MS and antifungal tests for rapid identification of bioactive lipopeptides produced by *Bacillus amyloliquefaciens* CCMI 1051. Process Biochem (2011), doi:10.1016/j.procbio.2011.05.016

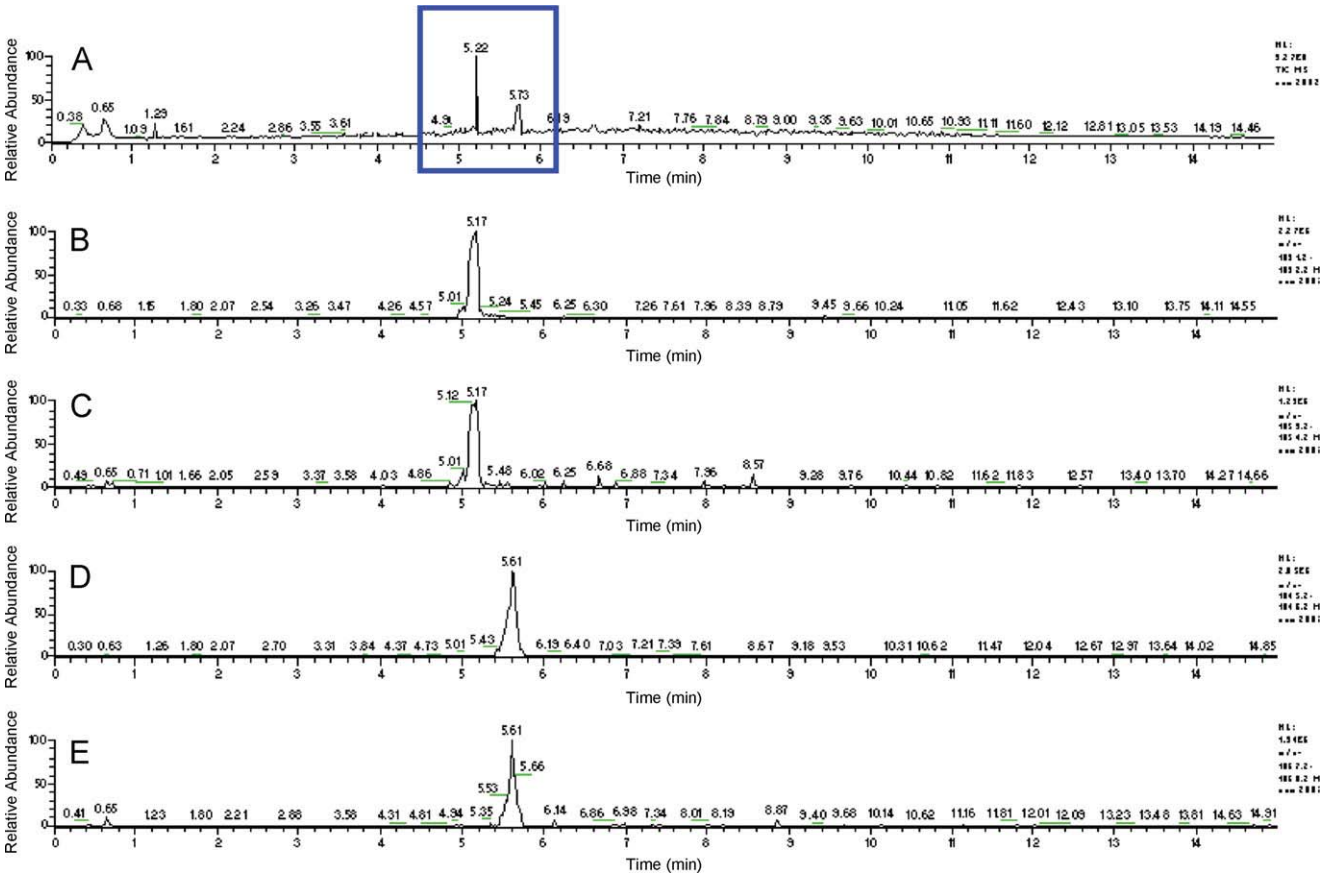

Fig. 3. ESI-LC–MS Chromatogram corresponding to the fraction 1. (A) Total chromatogram. (B) Chromatogram corresponding to *m*/*z* 1031. (C) Chromatogram corresponding to *m*/*z* 1053. (D) Chromatogram corresponding to *m*/*z* 1045. (E) Chromatogram corresponding to *m*/*z* 1067.

taxa within the *B. subtilis–Bacillis licheniformis* clade. Many strains produce previously identified compounds with known antimicrobial properties, whereas other compounds represent novel classes that were hitherto unknown. Mukherjee et al. [27], referred that lipopeptide profile varied according to the producing *B. subtilis* strains.

3.2. Separation and identification of the active compounds

TLC performed to methanolic active fraction showed the presence of two antifungal compounds (Rf = 0.23 and Rf = 0.38) (fractions 1 and 2), detected by using the autobiographical method, after spraying TLC plates with *C. cucumerinum* CCMI 206, it was still observed a much less active band (fraction 3). Parallel, to confirm these inhibition zones, the different compounds, after having been cut out from the TLC plate, were also placed on the Petri dishes, as described previously.

This procedure allows a subsequent separation by preparative chromatography, and the areas corresponding to the presence of the two more active compounds were removed and analyzed by ion-trap MS and LC–MS in order to detect different contributions for the overall biological activity.

The ion-trap MS spectrum obtained by infusion of the preparative chromatography active fractions showed dominant ion peaks at *m*/*z*: 1031.3; 1045.3; 1053.3 and 1067, corresponding to fraction 1 (Fig. 2A) and *m*/*z* 1054, 1067 and 1081 corresponding to fraction 2 (Fig. 2B). A ESI-MS spectrum obtained in negative mode showed two dominant peaks at *m*/*z* 1029.7 and 1043.6 relatively to fraction 1 and three dominant peaks at *m*/*z* 1054, 1067 and 1081 corresponding to the fraction 2 (Fig. 2 C).

LC/ESI-MS was used to confirm these results and to analyze the composition of the total methanolic active fraction (Figs. 3–5).

Fig. 3 shows that the peaks corresponding to the masses 1031 and 1053 Da have the same retention time, as well as the peaks 1045 and 1067 Da. The difference of masses between these peaks is 22 Da, corresponding probably to sodium adducts (M+Na). The peak corresponding to a *m*/*z* 1083.2, visible just in positive mode, can be correspondent to a potassium adduct (M+K) of the peak 1045.3 Da (difference of 38 Da).

Fig. 4 illustrates the fraction 2 chromatogram, obtained by LC–MS. The peaks *m*/*z* 1031 and *m*/*z* 1053 have the same retention time and the peaks *m*/*z* 1045 and *m*/*z* 1067 also present the same time of retention, as it had already been observed in the fraction 2. A third pair of peaks was observed, *m*/*z* 1059 and 1081, with identical time of retention. On the other hand, ESI-MS (−) *m*/*z* corresponding to this fraction revealed a peak at *m*/*z* 1057.6. Probably, the dominant ion peaks corresponding to Fig. 2B correspond to the sodium adducts of ion peaks (*m*/*z*) 1031.3; 1045.3 and 1059. This fraction is made of the same compounds as the previous fraction and a third compound with *m*/*z* 1059.

Bacillus species are well known producers of metabolites with antimicrobial properties. Usually, three different classes of bioactive peptides can be distinguished: antifungal peptides, such as bacilysin and rhizocticin; antifungal lipopeptides, such as surfactins, iturins and fengycins; and antimicrobial polypeptides such as subtilin. The antifungal activity of *Bacillus* spp. seems to be an indicator of the presence of lipopeptides belonging to the class of the iturin and surfactin [28]. The peak mass exhibited by active fractions on those experimental conditions was compared with commercial sample of iturin A and surfactin. The commercial iturin A and surfactin samples show

Please cite this article in press as: Caldeira AT, et al. Combined use of LC–ESI-MS and antifungal tests for rapid identification of bioactive lipopeptides produced by *Bacillus amyloliquefaciens* CCMI 1051. Process Biochem (2011), doi:10.1016/j.procbio.2011.05.016

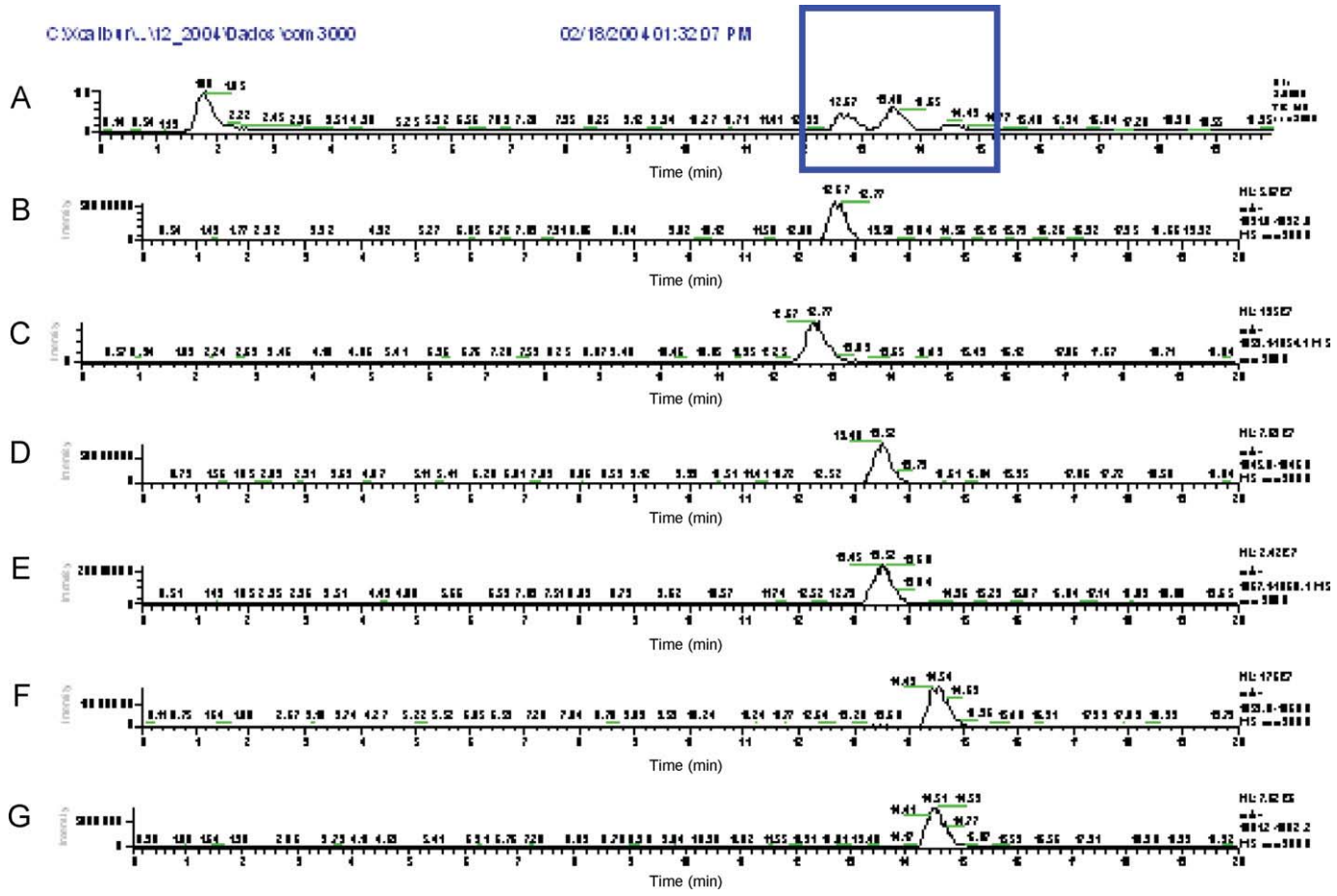

Fig. 4. ESI-LC–MS Chromatogram corresponding to the fraction 2. (A) Total chromatogram. (B) Chromatogram corresponding to *m*/*z* 1031. (C) Chromatogram corresponding to *m*/*z* 1053. (D) Chromatogram corresponding to *m*/*z* 1045. (E) Chromatogram corresponding to *m*/*z* 1067. (F) Chromatogram corresponding to *m*/*z* 1059. (G) Chromatogram corresponding to *m*/*z* 1081.

the same peak profile and the presence of adducts (data not shown).

In the spectra of ESI of organic molecules the most intense adducts are, usually, Li+ > Na+ >K+ [29]. On MS analyses of iturin, obtained by electrospray ionization, they prevail the adducts of metallic salts (iturin A + metal). The adducts of sodium, potassium and calcium are usually the most intense and, per times, they present a larger relative intensity than the iturin [30,31].

The compounds produced by *B. amyloliquefaciens* CCMI1051, in these culture conditions, presented a difference of masses among the peaks of *m*/*z* 1031.3; 1045.3 and 1059, of 14 Da, which corresponds to the molecular weight of one CH_2 group. Different isoforms exist for each lipopeptide, which vary in the chain length of their fatty acid components and amino acid residues in their peptide rings.

The presence of sodium and potassium adducts also favour differences of 22 Da (1031 *m*/*z* and 1053 *m*/*z*; 1045 *m*/*z* and 1067 *m*/*z*) or 38 Da (1045 *m*/*z* and 1083 *m*/*z*) in the peaks.

The fragmentation pattern corresponding to MS^2 fragmentation of the peaks 1053 and 1067 show fragments that can correspond to differences among some amino acids in the molecules. The sequence of amino acids Tyr–Asn–Pro–Glu found in some fragments (data not shown) can be attributed to part of the sequence of bacilomicina D, a lipopeptide belonging to the iturinic group which sequence of amino acids in the cyclical part of the molecule is: Asn–Tyr–Asn–Pro–Glu–be–Thr. The bacilomicina D exhibits strong antifungal activity against several pathogenic fungi and is produced by some strains of *Bacillus*, namely *B. subtilis* AU195 [32] and some strains of *B. amyloliquefaciens* [33].

LC–ESI-MS of total methanolic fraction was used to confirm this study and to analyze the composition of the total active fraction. Fig. 5 shows the current total ion chromatogram (A) and the chromatograms obtained at different corresponding molecular weights (B–H). Seven compounds designated as 1, 2, 3, 4, 5, 6 and 7 were visualized at 12.7 min (M = 1031.5 Da), 13.61 min (M = 1045.5 Da), 14.81 min (M = 1059.5 Da), 15.83 min (M = 1435.7 Da), 17.18 min (M = 1449.8 Da), 17.90 min (M = 1463.8 Da) and 18.46 min (M = 1477.9 Da) of analysis. Some of those picks correspond to the compounds present on fractions 1 and 2 (*m*/*z* 1031; *m*/*z* 1045 and *m*/*z* 1059). Compounds corresponding to a mass 1436–1478 Da was also detected, forming a cluster, in LC–ESI-MS spectra (Fig. 1A), and probably it can be related to fraction 3, a band much less active observed on bioautographic TLC.

The species *B. amiloliquefaciens* has been reported to produce lipopeptides with antifungal proprieties. Yu et al. [6] demonstrated that the antifungal compounds produced by the strain B94 of *B. amiloliquefaciens* (*m*/*z* values 1044.3; 1047.9 and 1069.5) are isomers of iturin A. Hiradate et al. [3] using the strain *B. amiloliquefaciens* RC-2 attributed the compounds bioactivity to the production of iturin A2–A8 (*m*/*z* values 1043- iturin A2; 1057- Iturin A3–A5; 1071- irurin A6 and A7; 1085- iturin A8). The iturinic composition seems to differ among strains of *B. amiloliquefaciens*. The results presented in this work show that several compounds between 1000 and 1100 Da, comparable to that of iturin and surfactin compounds, are produced by *B. amyloliquefaciens* CCMI 1051 suggesting

Please cite this article in press as: Caldeira AT, et al. Combined use of LC–ESI-MS and antifungal tests for rapid identification of bioactive lipopeptides produced by *Bacillus amyloliquefaciens* CCMI 1051. Process Biochem (2011), doi:10.1016/j.procbio.2011.05.016

the productin of different strong antifungal compounds. This strain also produces compounds between 1436 and 1478 Da less actives against tested fungi. *B. amyloliquefaciens* CCMI 1051 have a function as biocontrol agent, which may contribute to the alleviation of the excessive use of chemical pesticides, and at the end, the reduction of environmental pollution, in fact these compounds made of amino acids and fatty acid, are easily biodegradable in the soils.

The splitting of the samples by using a reverse phase chromatography, monitored by antifungal tests and bioautographic methods, associated to preparative chromatography allowed to obtain relevant amounts of the more active lipopeptides, already quite purified. The association of mass spectrometry and chromatography, used in parallel with antifungal tests proved to be an efficient approach for the character-

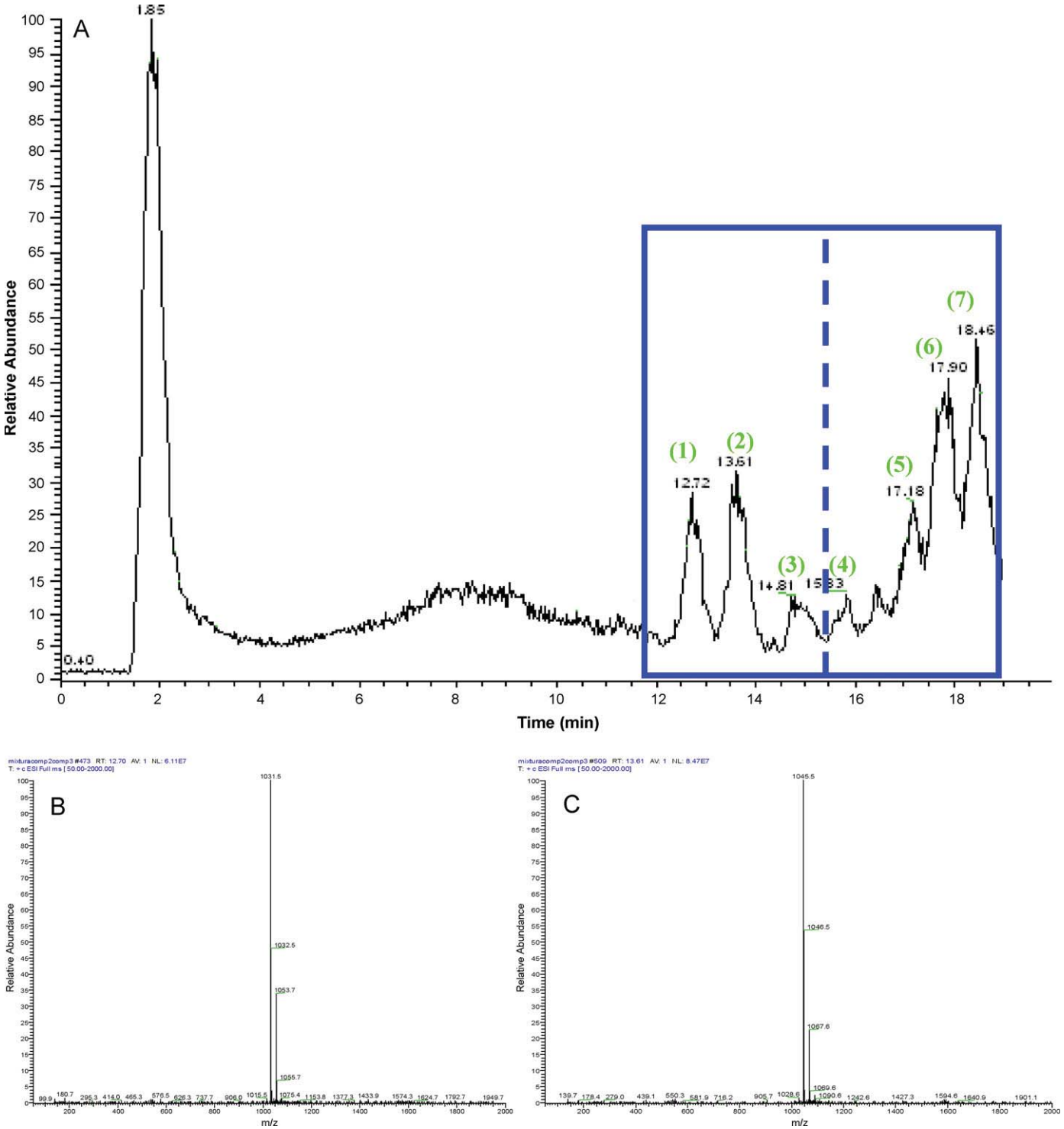

Fig. 5. Chromatogram corresponding to LC–MS of the methanolic fraction (A) and mass spectra of the compounds (B–H). (B) Mass spectra corresponding to the peak (1) *m*/*z* 1031.5. (C) Mass spectra corresponding to the peak (2) *m*/*z* 1045.5. (D) Mass spectra corresponding to the peak (3) *m*/*z* 1059.5. (E) Mass spectra corresponding to the peak (4) *m*/*z* 1435.7. (F) Mass spectra corresponding to the peak (5) *m*/*z* 1049.8. (G) Mass spectra corresponding to the peak (6) *m*/*z* 10463.8. (H) Mass spectra corresponding to the peak (7) *m*/*z* 1477.9.

Please cite this article in press as: Caldeira AT, et al. Combined use of LC–ESI-MS and antifungal tests for rapid identification of bioactive lipopeptides produced by *Bacillus amyloliquefaciens* CCMI 1051. Process Biochem (2011), doi:10.1016/j.procbio.2011.05.016

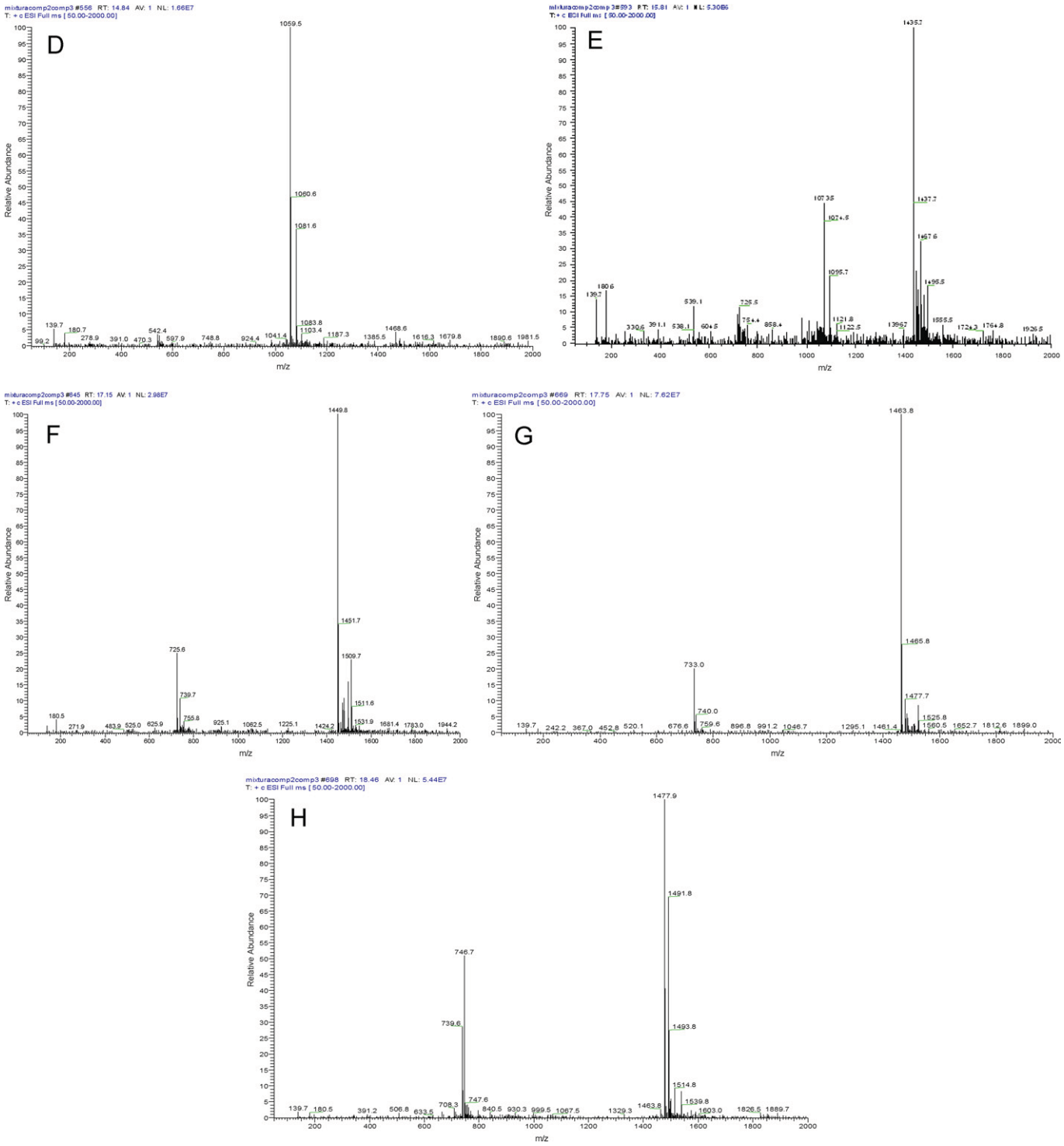

Fig. 5. (*Continued*).

ization of active lipopeptides without the need of previous total isolation. This approach can be employed for screening and optimization the production of antifungal iturinic lipopeptides.

4. Conclusions

A combined use of LC–ESI-MS and antifungal tests allowed a rapid identification of lipopeptides produced by *B. amyloliquefaciens* CCMI 1051 as new active compounds produced, without need of previous total isolation. Compounds of masses between 1000 and 1100 Da, comparable to that of iturin and surfactin compounds, are produced by *B. amyloliquefaciens* CCMI 1051 and active against phytopathogenic fungi. This strain also produces compounds between 1436 and 1478 Da less active against tested fungi. This methodology can be used as a viable alternative to the total isolation approach and also can be employed for screening and optimization the production of antifungal

Please cite this article in press as: Caldeira AT, et al. Combined use of LC–ESI-MS and antifungal tests for rapid identification of bioactive lipopeptides produced by *Bacillus amyloliquefaciens* CCMI 1051. Process Biochem (2011), doi:10.1016/j.procbio.2011.05.016

iturinic lipopeptides, showing a great potential for future application.

References

[1] Caldeira AT, Savluchinske-Feio S, Arteiro Santos JM, Roseiro JC. Antimicrobial activity of steady-state cultures of *Bacillus* sp. CCMI 1051 against wood contaminant fungi. Biochem Eng J 2006;30:231–6.
[2] Dieckmann R, Pavela-Vrancic M, Döhren H. Synthesis of (di)adenosine polyphosphates by non-ribosomal peptide synthetases (NRPS). Biochim Biophys Acta 2001;1546:234–41.
[3] Hiradate S, Yoshida S, Sugie H, Yada H, Fujii Y. Mulberry anthracnose antagonists (iturins) produced by *Bacillus amyloliquefaciens* RC-2. Phytochemistry 2002;61:693–8.
[4] Leifert C, Li H, Chidburee S, Hampson S, Workman S, Sigee D, et al. Antibiotic production and biocontrol activity by *Bacillus subtilis* CL27 and *Bacillus pumilus* CL45. J Appl Bacteriol 1995;78:97–108.
[5] Seifert KA, Hamilton WE, Breuil C, Best M. Evaluation of *Bacillus subtilis* C186 as a potencial biological control of sapstain and mould on unseasoned lumber. Can J Microbiol 1987;33:1102–7.
[6] Yu GY, Sinclair JB, Hartman GL, Bertagnolli BL. Production of iturine A by *Bacillus amylolequefaciens* suppressing *Rhizoctonia solani*. Soil Biol Biochem 2002;34:955–63.
[7] Schallmey M, Singh A, Ward OP. Developments in the use of Bacillus species for industrial production. Can J Microbiol 2004;50:1–17.
[8] Moyne AL, Cleveland TE, Tuzun S. Molecular characterization and analysis of the operon encoding the antifungal lipopeptide bacillomycin D. FEMS Microbiol Lett 2004;234:43–9.
[9] Tsuge K, Ano T, Shoda M. Isolation of a gene essential for biosynthesis of the lipopeptide antibiotics plipastatin B1 and surfactin in *Bacillus subtilis* YB8. Arch Microbiol 1996;165:243–51.
[10] Cho SJ, Lee SK, Cha BJ, Kim YH, Shin KS. Detection and characterization of the *Gloeosporium gloeosporioides* growth inhibitory compound iturin A from *Bacillus subtilis* strain KS03. FEMS Microbiol Lett 2003; 223:47–51.
[11] Bais HP, Fall R, Vivanco JM. Biocontrol of *Bacillus subtilis* against infection of *Arabidopsis* roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. Plant Physiol 2004;134: 307–19.
[12] Kim PI, Bai H, Bai D, Chae H, Chung S, Kim Y, et al. Purification and characterization of a lipopeptide produced by *Bacillus thuringiensis* CMB26. J Appl Microbiol 2004;97:942–9.
[13] Magnet-Dana R, Peypoux F. Iturins, a special class of poreforming lipopeptides: biological and physiological properties. Toxicology 1994;87:151–74.
[14] Stein T. *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Mol Microbiol 2005;56:845–57.
[15] Yoshida S, Hiradate S, Tsukamoto T, Hatakeda K, Shirata A. Antimicrobial activity of culture filtrate of *Bacillus amyloliquefaciens* RC-2 isolated from mulberry leaves. Phytopathology 2001;91:181–7.
[16] Chenikher S, Guez JS, Coutte F, Pekpe M, Jacques P, Cassar JP. Control of the specific growth rate of *Bacillus subtilis* for the production of biosurfactant lipopeptides in bioreactors with foam overflow. Process Biochem 2010, doi:10.1016/j.procbio.2010.06.001.
[17] Thasana N, Prapagdee B, Rangkadilok N, Sallabhan R, Aye SL, Ruchirawat S, et al. *Bacillus subtilis* SSE4 produces subtulene A, a new lipopeptide antibiotic possessing an unusual C15 unsaturated β-amino acid. FEBS Lett 2010;584(14):3209–14.
[18] Liu X-Y, Yang S-Z, Mu B-Z. Production and characterization of a C15-surfactin-O-methyl ester by a lipopeptide producing strain *Bacillus subtilis* HSO121. Process Biochem 2009;44:1144–51.
[19] Price NPJ, Rooney AP, Swezey JL, Perry E, Cohan FM. Mass spectrometric analysis of lipopeptides from *Bacillus* strains isolated from diverse geographical locations. FEMS Microbio Lett 2007;271(1):83–9.
[20] Vishniac W, Santer M. The Thiobacilli. Bacteriol Rev 1957;21:195–213.
[21] Caldeira AT, Vicente H, Arteiro JM, Roseiro JC, e Neves J. An artificial intelligence approach to *Bacillus amyloliquefaciens* CCMI 1051 cultures: application to the production of antifungal compounds. Bioresour Technol 2011;102: 1496–502.
[22] Homans A, Fuchs A. Direct bioautography on thin layer chromatography as a method for detecting fungitoxic substances. J Chomatogr 1970;51: 327–9.
[23] Caldeira AT, Savluchinske-Feio S, Arteiro Santos JM, Roseiro JC. *Bacillus amyloliquefaciens* CCMI 1051 in vitro activity against wood contaminant fungi. Ann Microbiol 2007;57(1):29–34.
[24] McKeen C, Reilly C, Pusey P. Production and partial characterization of antifungal substances antagonistic to *Monilinia fruticola* from *Bacillus subtilis*. Phytopathology 1986;76:136–9.
[25] Caldeira AT, Feio S, Arteiro JM, Coelho AMV, Roseiro JC. Environmental dynamics of *Bacillus amyloliquefaciens* CCMI 1051 antifungal activity under different nitrogen patterns. J Appl Microbiol 2008;104:808–16.
[26] Leenders F, Stein T, Kablitz B, Franke P, Vater J. Rapid typing of *Bacillus subtilis* strains by their secondary metabolits using matrix-assisted laser desorption/ionization mass spectrometry in intact cells. Rapid Commun Mass Spectrom 1999;13:943–9.
[27] Mukherjee AK, Das K. Correlation between diverse cyclic lipopeptides production and regulation of growth and substrate utilization by *Bacillus subtilis* strains in a particular habitat. FEMS Microbiol Ecol 2005;54:479–89.
[28] Pabel C, Vater J, Wilde C, Franke P, Hofemeisrer J, Adler B, et al. Antimicrobial activities and matrix-assisted laser desorption/ionization mass spectrometry of Bacillus isolates from the marine sponge *Aplysina aerophoba*. Mar Biotechnol 2003;5:424–34.
[29] Xiang Y, Abliz Z. Clevage reactions of the complex ions derived from self-complementary deoxydinucleotides and alkali-metal ions using positive ion electrospray ionization with tandem mass spectrometry. J Am Soc Mass Spectrom 2004;15:689–96.
[30] Wang W, Liu Z, Ma L, Hao C, Liu S, Voinov V, et al. Electrospray ionization multiple-stage tandem mass spectrometric analysis of diglicosyldiacylglycerol glycolipids from the bacteria *Bacillus pumillus*. Rapid Commun Mass Spectrom 1999;13:1189–96.
[31] Williams S, Brodbelt J. MS^n characterization of protonated cyclic peptides and metal complexes. J Am Soc Mass Spectrom 2004;15:1039–54.
[32] Moyne A, Shelby R, Cleveland T, Tuzun S, Bacillomycin D. an iturin with antifungal activity against *Aspergillus flavus*. J Appl Microbiol 2001;90:622–9.
[33] Koumoutsi A, Chen X, Henne A, Liesegang H, Hitzeroth G, Franke P, et al. Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. J Bacteriol 2004;186:1084–96.

Please cite this article in press as: Caldeira AT, et al. Combined use of LC–ESI-MS and antifungal tests for rapid identification of bioactive lipopeptides produced by *Bacillus amyloliquefaciens* CCMI 1051. Process Biochem (2011), doi:10.1016/j.procbio.2011.05.016