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NMR-based metabolic profiling to follow the production of anti-phytopathogenic compounds in the culture of the marine strain *Streptomyces* sp. PNM-9

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ABSTRACT

Actinobacteria are the major source of bioactive secondary metabolites and are featured in the search for antimicrobial compounds. We used nuclear magnetic resonance (RMN)-metabolic profiling and multivariate data analysis (MVDA) to correlate the metabolites' production of *Streptomyces* sp. PNM-9 from the algae *Dictyota* sp. and their biological activity against the rice phytopathogenic bacteria *Burkholderia* spp. The compounds 2-methyl-N-(2′-phenylethyl)-butanamide (**1**) and 3-methyl-N-(2′-phenylethyl)-butanamide (**2**) were identified through MVDA and 2D NMR experiments in the organic extract of a 15-days LB media culture of *Streptomyces* sp. PNM-9. Compounds **1** and **2** were isolated and their structures confirmed by one- and two-dimensional NMR and mass spectrometry (MS) data. Compounds **1** and **2** were active against the rice pathogenic bacteria *Burkholderia glumae* (ATCC 33,617) displaying minimal inhibitory concentration (MIC) values of 2.43 mM and 1.21 mM, respectively. The metabolomics-guided approach employing NMR-metabolic profiling was useful for marine microbial bioprospecting and suggested *Streptomyces* sp. PNM-9 strain and its compounds as a potential control against phytopathogenic bacteria.

1. Introduction

Rice (*Oryza sativa* Linneo) is one of the most important crops for the world economy with an estimated global production of 759 million tons (FAO Rice Market Monitor, 2018) in 2017 being a fundamental part of the human diet. However, about 75 % of the world's production of this cereal is severely affected by the diseases known as bacterial panicle blight (BPB) and sheath rot (Nandakumar et al., 2009). The main etiological bacterial agents of these diseases are the phytopathogenic bacteria *Burkholderia glumae* and *Burkholderia gladioli* (Sheath rot and less frequently BPB) (Ham et al., 2011; Nandakumar et al., 2009; Naughton et al., 2016), which causes drastic reductions in crop yields. BPB is an emerging disease characterized by spikelet sterility, emergent grain discoloration, grain size decrease, inhibition of seed germination and flower sterility (Jeong et al., 2003).

Countries, such as Panama (Nandakumar et al. (2007)), Ecuador (Riera-Ruiz et al., 2014), Costa Rica (Quesada-González and García-Santamaría, 2014), South Africa (Zhou, 2014), Korea (Jeong et al., 2003), India (Mondal et al. (2015)), China (Luo et al., 2007) and Colombia (Florez-Zapata and Velez, 2011), among others, have reported the occurrence of BPB. Despite its high incidence, few management strategies are available to control these pathogens efficiently (Melanson et al., 2016). The chemical treatment for *B. glumae* is based on quinolone derivative compounds such as oxolinic acid (Hikici et al., 2001). However, the rapid emergence of resistance in *B. glumae* strains in Japan – less than ten years since the oxolinic acid was introduced in rice crops – has diminished the interest in applying these compounds for disease control in other countries (Hikichi et al., 1998; Stockwell and Duffy, 2012).

Given this scenario, microbial inoculants as disease-suppressive agents are an alternative to the use of chemical pesticides. Microorganisms have been recognized as a sustainable source for

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producing antibiotics and may have potential use for the control of phytopathogens (O'Callaghan, 2016). Currently, terrestrial microorganisms have been studied and used as a source of these compounds. However, in the last decade, marine microorganisms have gained relevance as an important source of secondary metabolites from marine environment (Carroll et al., 2019). In recent years, some of these metabolites have shown their potential for the control of phytopathogens (Kong, 2018; Rashad et al., 2015).

Actinobacteria are one of the class with the highest biotechnological potential for the production of antibiotics and have been isolated from terrestrial and marine environments (Manivasagan et al., 2013) (Newman, 2016). About two-thirds of all biologically active compounds are produced by Actinobacteria, most of them by strains belonging to the genus *Streptomyces* (Newman and Cragg, 2007). In solid culture, the production of metabolites by *Streptomyces* spp. is associated with its pre-sporulation stage (Bibb, 2005; van Wezel and McDowall, 2011; Yagüe et al., 2012). The production of compounds by Actinobacteria is also dependent on other factors such as pH, available nutrients, oxygen levels, and temperature. Together, they affect metabolic expression and thus the productivity in the fermentation process (Wu et al., 2016; Yoon and Nodwell, 2014).

Recently, we described 24 Actinobacteria strains belonging to the genera *Streptomyces*, *Micromonospora* and *Gordonia* (Betancur et al., 2017) recovered from different marine samples. Among them, 11 *Streptomyces* exhibited the ability to inhibit the *in vitro* growth of phytopathogens *B. glumae* and *B. gladioli*.

When the goal is monitoring metabolite production for biotechnological applications, nuclear magnetic resonance (NMR) is an analytical technique that offers advantages such as the possibility of relative and absolute quantification, complete identification of the produced metabolites, automation of sample preparation and high reproducibility of acquired data (Li and Hu, 2016; Schripsema, 2010). Many applications have been reported, such as investigating the phytochemical variations in plants (Marchev et al., 2016), quantifying metabolites in human fluids (Li and Hu, 2016), agricultural researches (Tian et al., 2016), among others.

NMR-based metabolomics methods are a tool for screening diverse biological sources of potentially novel antibiotics. These methods can be applied for the identification and optimization of the production of active secondary metabolites. Also, NMR-based metabolomics can efficiently help to establish the optimum culture conditions for compounds production at a small scale (Kim et al., 2011; Verpoorte et al., 2008). The metabolic differences can be identified using statistical analysis with the NMR data, such as chemometric methods, principal component analysis (PCA), and partial least squares regression (PLS-DA). (Wu et al., 2016).

We aimed to evaluate the metabolic production of *Streptomyces* sp. PNM-9, a strain isolated from the *Dictyota* sp. algae, cultured in TSB, TSBm, LB, and LBm media for 15 days. Its NMR-metabolic profiling analysis and the biological assays were performed to correlate the metabolic production upon different growth conditions and the bioactivity against rice phytopathogenic bacteria *B. glumae* and *B. gladioli* by using multivariate data analysis (MVDA).

2. Materials and methods

^{1}H- and ^{13}C-NMR (1D and 2D) spectra were recorded on a Bruker®-DRX500-Ultra Shield® (^{1}H: 500.13 MHz, ^{13}C: 125.77 MHz). ^{1}H-NOESY 1D spectra were recorded on a Bruker® Advance 400 spectrometer (400.13 MHz for ^{1}H and 100 MHz for ^{13}C). $CDCl_3$ and CD_3OD were used as solvents and residual solvent signals were used as internal standards. High-resolution mass data were collected on an Accurate-Mass quadrupole Time-of-Flight (q-TOF) (Agilent Technologies) mass spectrometer, using electrospray ionization (ESI) and detecting in positive mode under the following analysis conditions: Nebulizer 50 (psi); Gas Flow 10 L/min; Gas Temp. 350 °C. Fragmentor 175 V, Skimmer 75 V, Vpp 750 V.

HPLC-DAD-ELSD was performed on a Thermo Dionex ultimate 3000 system, coupled to a DAD detector and an LT-ELSD Sedex 85 (Sedere, France) detector, with a gain set at 10 and a temperature of 80 °C. Optical rotations were measured on a Polartronic E, Schmidt + Haensch polarimeter in 1 mL 5 cm cells. An AccuReader Metertech (Taipei, Taiwan) was used at 600 nm for reading the antibacterial bioassay in 96-well plates. All solvents were HPLC grade.

2.1. Streptomyces sp. PNM-9 and growing conditions

Streptomyces sp. PNM-9 was obtained from our in house marine strain collection (Betancur et al., 2017). In order to conduct this research, the ANLA (Autoridad Nacional de Licencias Ambientales) and the Ministerio de Ambiente y Desarrollo Sostenible granted permission to collect samples and study the recovered bacteria (Permission No. 4 of 10/02/2010, Anexo 2, Contrato de Acceso a Recurso Genético No 108) (Betancur et al., 2017). The Trypticase Soy Broth (TSB), Trypticase Soy Broth minimum (TSBm), Luria-Bertani (LB) and Luria-Bertani minimum (LBm) media were used for liquid fermentation. Fully supplemented LB contained 5 g yeast extract, 10 g sodium chloride, and 10 g tryptone per liter of water and the pH adjusted to 7.3 with NaOH. TSB contained 17 g tryptone, 3 g Bacto™ Soytone, 2.5 g glucose, 5 g sodium chloride, and 2.5 g dipotassium hydrogen phosphate per liter of water and adjusted pH 7.3. Both, LBm and TSBm, are minimum media ten times diluted constituted by the same components of the LB and TSB media, respectively. 5 plugs of *Streptomyces* sp. PNM-9 previously grown on ISP2 solid medium were inoculated into 100 mL of LB, LBm, TSB, and TSBm in flasks of 500 mL; each flask of 500 mL was incubated at 25 °C with continuous shaking at 130 rpm.

2.2. Culture extraction

The *Streptomyces* sp. PNM-9 cultures in all the different four culture media and their media controls were sampled (in a destructive assay) at the days 1, 2, 3, 4, 5, 10 and 15. At each day, one flask from the four culture media was extracted along with their corresponding blank. Mycelia were removed by centrifugation at 5000 rpm for 20 min. The culture supernatant and the media culture blanks were extracted three times with 30 mL of ethyl acetate. The water in the organic phase was removed with anhydrous sodium sulfate and filtrated. Then, the ethyl acetate solvent was evaporated under vacuum at 40 °C. The obtained extracts were cryopreserved at −80 °C pending NMR analysis and bioassays evaluation.

2.3. NMR measurement and data analysis

Two mg of each extract was dissolved in 500 µL of CD_3OD in a 5 mm diameter NMR tube. A mixture of the TSB organic extracts was used as the quality control pool sample (QC pool), which was analyzed under the same conditions as other samples (Alonso-herranz et al., 2015). Each sample was analyzed on a 400 MHz Bruker Ultrashield Avance spectrometer (Bruker ®). Samples were measured at 298 K in a fully automatic mode. After each sample was inserted, a long delay (180 s) before acquisition was used to ensure that the temperature of the sample inside the magnet was equilibrated. Automated tuning, matching (ATMA), locking (LOCK) and shimming (TOPSHIM) were achieved using standard Bruker routines. Deuterated methanol was used as the internal lock.

The ^{1}H-NOESY 1D (noesygppr1d) experiment consists in a 1D experiment with suppression of the water signal during relaxation delay, 4 dummy scans and 64 scans were measured, operating at a proton NMR frequency of 400.13 MHz, using the following parameters: 0.12 Hz/point, pulse width (PW) = 12.3 µs and relaxation delay (RD) = 2.0 s. Free induction decays (FIDs) were Fourier transformed with a line broadening (LB) = 0.30 Hz. With a spectral window of 10 ppm. The

resulting spectra were manually phased, baseline corrected, and calibrated to residual methanol-d_4 at 3.31 ppm, using TopSpin (version 3.5 pl6, Bruker). The ^{1}H-NOESY 1D were processed as described by Kim and Verpoorte (Kim and Verpoorte, 2010). The MestreNova software was used to convert the ^{1}H-NOESY 1D spectra to an ASCII file, with total intensity scaling. Binning was performed to the spectral data, reducing the region between 0.5–10.0 ppm into little portions (bins) of equal width (0.04 ppm). The region δ 3.25–3.35 was removed in the analysis due to the presence of the methanol-d_4 residual signal. Principal component analysis (PCA), hierarchical cluster analysis (HCA) and orthogonal projection to latent structures (OPLS) based on Pareto scaling were performed with the SIMCA-P + software (version 14.1, Umetrics, Umea, Sweden).

2.4. Isolation of bioactive compounds

Streptomyces sp. PNM-9 was cultured in ten liters of TSB medium under the same incubation conditions described previously. Mycelia were removed by centrifugation at 5000 rpm for 20 min and culture supernatant was extracted three times with ethyl acetate (1:1). The water of the organic phase was dried with anhydrous sodium sulfate and the ethyl acetate solvent was evaporated under vacuum at 40 °C. Bio-guided fractionation of 1000 mg of organic extract was done using a solid-phase extraction cartridge (SPE) (HyperSep Thermo Scientific C-18) in a discontinuous gradient of methanol-water mixtures. Five fractions were obtained (F1- F5) from elution with 10, 30, 50, 70 and 100 % of methanol, respectively. The fraction F4 (25.0 mg) resulted active and was separated into six fractions (F4 − 1 to F4 − 6) by HPLC using a Phenomenex Kinetex C8 column (100 Å 2.6 µm 100 × 4.6 mm) and a gradient of acetonitrile:water (10 % of acetonitrile for 3 min, from 10 % to 40 % in 17 min, from 40 % to 100 % in 15 min, 100 % for 10 min and from 100 % to 10 % in 2 min). Fraction F4 − 4 (12.8 mg) was further fractionated by HPLC using a C6-Phenyl, Gemini 110A column (250 × 10 mm, 5 µm) and a gradient of acetonitrile:water (10 % of acetonitrile for 3 min, from 10 % to 100 % in 20 min. 100 % for 7 min, from 100 % to 10 % in 2 min and 10 % for 3 min), obtaining six fractions (F4 − 6-1 to F4 − 6-6). Finally, fraction F4 − 6-5 (6 mg) was separated by HPLC on a Phenomenex Kinetex C18 column (100 Å, 2.6 µm, 100 × 4.6 mm) using a gradient of acetonitrile:water (10 % of acetonitrile for 3 min, from 10 % to 25 % in 10 min and from 25 % to 95 % in 5 min) at 0.5 mL/min flow rate, to yield compounds **1** (t_R = 23.0 min, 0.6 mg) and **2** (t_R = 24.0 min, 1.0 mg), which were analyzed by 1D and 2D NMR in order to establish their structures.

2.5. Antimicrobial activity

To evaluate the antibacterial activity of extracts and purified compounds obtained from *Streptomyces* sp. PNM-9, antimicrobial broth dilution tests against bacterial phytopathogens *B. glumae* (ATCC 33,617) and *B. gladioli (3704 − 1-FEDEARROZ)* were performed (Balouiri et al., 2016). For this purpose, it was used a solution of 2 mg/mL of the obtained extracts during the 15 days of incubation from those four evaluated media. Two hundred microliters of each extract were added to 190 µL of KB (10 g peptone, 1.5 g anhydrous K_2HPO_4, 15 g glycerol, $MgSO_4$ sterile; per liter adjusting pH to 7.0 with HCl) medium and then 10 µL of *B. glumae* (Absorbance $\lambda_{600\ nm}$ = 0.25) were inoculated in the first vertical line of a 96 well-plate. Four serial dilutions were done using the first wells as the concentrated solution to obtain five different concentrations in each well: 500, 250, 125, 62.5 and 32.25 µg/mL. Twenty µL of ethyl acetate extract of blank growth media and 20 µL of sterile water were used as negative controls. In the same way, 10 µL of gentamicin (final concentration of 20 µg/mL- 42 µM) was used as positive control. The plates were stationary incubated at 37 °C for 24 h (Sussulini, 2017) and the differences in growth patterns were measured by absorbance at λ 600 nm and confirmed by visual checking of macroscopic growth in the 96 well-plate (Wiegand et al., 2008). In all cases, absorbance obtained from negative control (sterile water) was used as 100 % value of growth and served to determine the percentage of bacterial survival as follows:

$$\text{Percent survival} = (OD_{sample} - OD_{pos}) / (OD_{neg} - OD_{pos}) \text{ X } 100$$

Where OD_{sample}, OD_{neg}, and OD_{pos} corresponds to the mean absorbance of each concentration tested, negative control and positive control, respectively (Campbell, 2011).

Purified compounds were tested following a similar approach. Briefly, six serial dilutions were prepared using the first wells as the concentrated solution, obtaining seven different concentrations in each well: 500, 250, 125, 62.5, 32.25, 16.12 and 8.1 µg/mL. 20 µL of methanol 10 % and 10 µL of gentamicin (20 µg/mL) were used as negative and positive controls, respectively. The plates were stationary incubated at 37 °C for 24 h and the growth of *B. glumae* was determined by absorbance as previously described. In this case, the minimal inhibitory concentration (MIC) of the purified compounds was defined as the lowest concentration (in mM) of the antimicrobial compound that generates a reduction of at least 90 % in the growth of the phytopathogenic bacteria in comparison with the negative control (Balouiri et al., 2016).

3. Results

In our previous search for marine Actinobacteria as a source of compounds for the control of phytopathogens, *Streptomyces* sp. PNM-9 was selected based on the activity of its organic extract against the rice phytopathogens *B. glumae* and *B. gladioli* (Betancur et al., 2017). To investigate the effect of growth media in the production of bioactive compounds, *Streptomyces* sp. PNM-9 was grown in four liquid media (TSB, TSBm, LB, LBm) during 15 days, and samples were collected at days 1, 2, 3, 4, 5, 10 and 15.

The obtained extracts were tested against *B. glumae* in a broth dilution test. The strongest antibacterial activity was displayed by TSB and LB extract obtained from 2- and 15-days cultures, (Fig. 1A). Interestingly, the reduction of more than 50 % of bacterial survival was achieved in TSB and TSBm medium since day 2. However, the TSB extract of days 3, 4 and 5 and the TSBm extract of day 3 showed an unexpected inflection point, indicating an increase in bacterial survival. After day 5, it was observed another inflection point due to a decreased bacterial survival. These results suggest an early production of antimicrobial compounds during days 1 and 2, followed by a second production peak from day 5 up to 15. A similar pattern was seen on LB medium, where the reduction of bacterial survival below 50 % was achieved on day 4, followed by an increase on bacterial survival on day 5 and another reduction of bacterial growth on day 10 and 15 (Fig. 1A).

3.1. NMR-based metabolic profiling of Streptomyces sp. PNM-9 and its correlation with bioactivity

NMR-based metabolic fingerprinting was applied to observe metabolite production across the 15 days of culture assay and to find a correlation with the described antimicrobial activity. The metabolite concentrations within samples are usually quite small (50 µM to 50 mM) compared to proton concentration in water (approximately 110 M); therefore, water suppression is required to remove the otherwise dominant signal of water-related protons within the NMR spectrum (Sussulini, 2017). ^{1}H-NOESY-1D experiments were selected for the NMR analysis. In these experiments, the excitation pulse is replaced by three consecutive pulses where a short (typically 10 ms) mixing time is introduced between the second and the third pulse. The three pulses effectively replace the single excitation pulse in the pre-saturation experiment and result in a more uniform excitation of the NMR sample, which leads to enhanced water suppression better than the common pre-saturation experiments (Mckay, 2011; Zheng and Price, 2010).

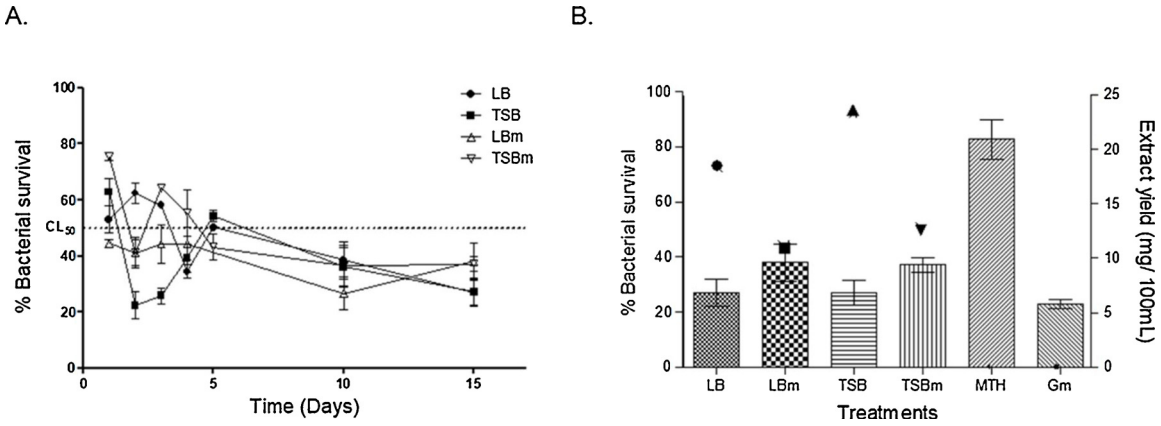

Fig. 1. Antibacterial activity of *Streptomyces* PMN-9 organic extracts. (**A**) The graphic shows the antibacterial activity at 250 μg/mL expressed as bacterial survival percentage; (**B**) the bacterial survival percentages of the different organic extracts on day-15 growth are represented in bars, whereas the extract yield (mg of extract per 100 mL of culture) of each evaluated medium are represented by symbols. Gentamicin at 20 μg/mL was used as a positive control and a solution of 10 % methanol in water was the negative control. The figure shows the media ± standard deviation of three independent replicates.

Additionally, a statistical analysis was carried out to determine the NMR data variability and the found coefficient of variation less than 5% confirmed the ^{1}H-NOESY-1D robustness.

MVDA was performed to correlate the antibacterial activity with accountable signals in the ^{1}H-NOESY-1D spectra. Metabolic differences were identified using PCA. The formation of a well-defined cluster for the QC samples allows assuring the variation observed in the subsequent analyzes for the fermentation samples is due mainly to the intrinsic characteristics of the fermentation time and the culture media.

It was not observed clustering either between the culture media (TSB or LB) or the culture time (in days) samples, indicating a low variability in the metabolic profiles, which are mainly dominated by the signals of the medium components.

As a next step, to correlate the metabolic profiles obtained by the ^{1}H-NOESY-1D NMR spectra with the bioactivity, an orthogonal projection to latent structures discriminant analysis (OPLS-DA) was applied to the data. The antibacterial activity (concentration lower than 125 μg/mL) was used as the Y-variable (Fig. 2A). The quality and robustness of the OPLS-DA model was validated by a permutation test (n = 100). The Q^2 intercept value was -0.481 (below 0.05), showing that the original model is statistically effective (Fig. 2B) (Triba et al., 2015; Westerhuis et al., 2008). Moreover, the model was validated by calculating the area under the receiver operating characteristic (ROC) curve. The value of the area under the curve (AUC) was 0.85, which gives more confidence for the obtained model (Fig. 2C) (Triba et al., 2015).

Separation of the active groups was observed in the OPLS score plot, with the active groups (black circles) on the positive side along the OPLS1 axis and the inactive groups (white circles) on the negative side along the OPLS2 axis. (Fig. 3 A). The two most active samples can be identified in the negative side of the OPLS1 axis (triangular shape).

The S-plot (Fig. 3 D), which shows the most relevant variables in the discriminant function, indicating the metabolites chemical shifts that could be responsible for the antibacterial activity. The S-plot analysis shows signals at $_{H}$ 0.80 (aliphatic proton), 2.77 (benzylic proton), 5.62 (amide NH proton), and others at $_{H}$ 7.42, 7.47 and 8.07 (aromatic protons).

3.2. Isolation and identification of the antimicrobial compounds from *Streptomyces sp. PNM-9*

Streptomyces sp. PNM-9 was cultured for 15 days in TSB media. Compounds **1** and **2** were isolated from the ethyl acetate extract and identified using 1D and 2D NMR.

Compound **1** was isolated as an amorphous solid $[\alpha]_{D}^{20} = +43.15$ (c = 0.059; $CHCl_3$). It showed a protonated molecular ion peak at *m/z* 206.1560 $[M+H]^+$ by HR-ESI-MS (calcd. for $C_{13}H_{20}NO$ *m/z* = 206.1540, error 9.7 ppm). The ^{1}H NMR spectrum showed the following signals: δ_H 7.31 (t, *J* = 7.4 Hz, 2 H), 7.24 (m, 1 H), 7.20 (d, *J* = 7.4 Hz, 2 H), 5.41 (brs, 1 H), 3.54 (tt, *J* = 13.5, 6.8 Hz, 2 H), 2.82 (m, 2 H), 2.01 (m, 1 H), 1.63 (m, 1 H), 1.39 (m, 1 H), 1.07 (d, *J* = 6.9 Hz, 3 H), 0.86 (t, *J* = 7.4 Hz, 3 H) (Supp. Information). Through dereplication by Antimarin® and Antibase databases (Blunt (2012); Laatsch (2012)), compound **1** was identified as 2-methyl-N-(2′-phenylethyl)-butanamide (Fig. 4).

Compound **2** was isolated as an amorphous solid and showed a molecular ion peak at *m/z* 206.1562 for the pseudomolecular ion $[M+H]^+$ by HR-ESI-MS, consistent with the molecular formula $C_{13}H_{20}NO$ (calcd for $C_{13}H_{20}NO$ *m/z* = 206.1540, error 10.6 ppm). The ^{1}H NMR spectrum showed signals: $_{H}$ 7.31 (t, *J* = 7.4 Hz, 2 H), 7.24 (d, *J* = 7.4 Hz, 1 H), 7.20 (d, *J* = 7.4 Hz, 2 H), 5.38 (brs, 1 H), 3.54 (dd, *J* = 13.1 Hz; 6.6 Hz, 2 H), 2.82 (t, *J* = 6.9 Hz, 2 H), 2.06 (m, 2 H), 1.98 (d, *J* = 6.9 Hz, 2 H), 0.92 (d, *J* = 6.6 Hz, 6 H) (Supp. Information). By derreplication using Antimarin® and Antibase data bases (Blunt (2012); Laatsch (2012)), compound **2** was identified as 3-methyl-N-(2′-phenylethyl)-butanamide (**2**) (Fig. 3).

Evaluation of the antimicrobial activity of **1** and **2** showed both were active against bacterial phytopathogen *B. glumae* (ATCC 33,617) with a MIC value of 2.43 mM and 1.21 mM, respectively, indicating that these compounds are the accountable for the antibacterial activity exerted by the crude extract.

4. Discussion

Streptomyces's life cycle is usually described for solid cultures. After spore germination, rapid development of compartmentalized hyphae into the medium occurs (mycelium MI) (Manteca et al., 2008). Then, a differentiation process occurs to generate multinucleated (MII) antibiotic-producing mycelium. The mycelium starts to grow into the air forming the aerial mycelium (late MII). Next, the aerial hyphae undergo massive septation to create a series of uninucleoid compartments. Finally, these compartments differentiate to create spore chains (Yagüe et al., 2012).

It was previously assumed that in liquid cultures there was no differentiation since most *Streptomyces* do not sporulate in these conditions. Nevertheless, industrial antibiotic production is mostly performed in liquid cultures. Now, it is known that in liquid cultures differentiation is comparable to that observed in solid cultures (Manteca and Yagüe, 2018).

The differentiation and therefore secondary metabolite production

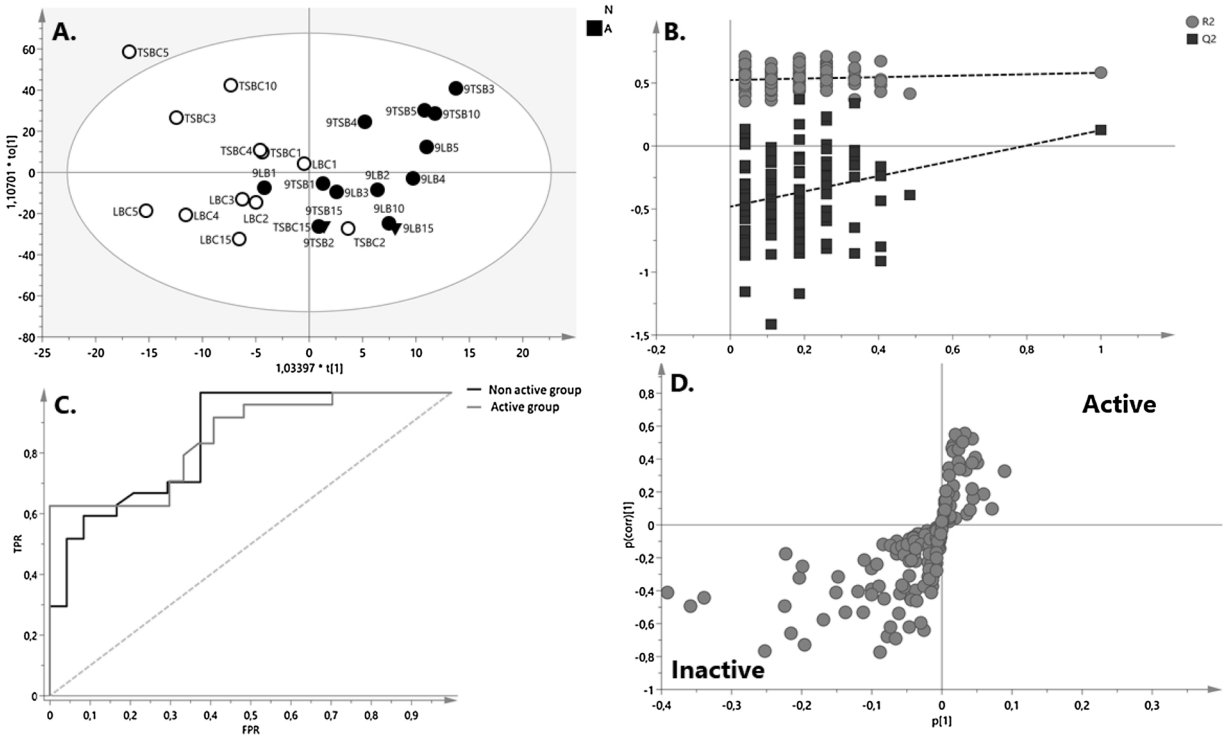

Fig. 2. Multivariate analysis of antibacterial activity against *B. glumae*. (**A**) Score Scatter Plot of twenty-eight extracts of TSB and LB inoculated with *Streptomyces* sp. PNM-9 with their respective blank of media. Active extract (black color) *vs*. non-active extract (white color). (**B**) The permutation test result of the OPLS-DA model. (**C**) ROC curve to affirm the validity of prediction OPLS-DA model of inactive group *vs* active group, the area under the receiver operating characteristic (ROC) curve was calculated. The area under the curve was 0.85 (an ideal model would have an AUC of 1) which clearly states that the prediction model was robust. (**D**) S-Plot generated from the OPLS-DA model shows the endpoint signals for compounds that are the predicted metabolites responsible for the bioactivity.

is trigged by programmed cell death (PCD) of the vegetative hyphae (MI) (Manteca et al., 2008; Manteca and Yagüe, 2018), which, in liquid cultures, depends on the growth rate of the strain and hypha aggregation (Manteca et al., 2008; Manteca and Yagüe, 2018; Marín et al., 2017; Treppiccione et al., 2017; Yagüe et al., 2014; Zhang et al., 2012).

We evaluated the effect of culture media in the production of bioactive compounds by *Streptomyces* sp. PNM-9 using NMR-metabolic profiling. The profiles obtained showed two points of strong antimicrobial activity at days 2 and 15 of growth in the submerged cultures. A comparison of the ^{1}H NMR spectra shows the production of differential metabolites at the day 2, 5 and 15, showing a good correlation with the observed bioactivity (Fig. 4). This behavior is in accordance with previous reports for the metabolite production by other *Streptomyces* strains, where the metabolites are produced since the early stages (during the logarithmic phase of growth), followed by a decrease in the production rate during the stationary phase, and finally an increase in that active metabolite production at the end of the life cycle can be observed (Marín et al., 2017; Treppiccione et al., 2017; Turło et al., 2012; Zhang et al., 2012). This can be explained because in liquid culture some mycelia suffer PCD since early growth stages, triggering metabolite production (Manteca et al., 2019; Manteca and Yagüe, 2018).

At the same time, NMR-based metabolic profiling was employed to observe metabolite production over the fifteen days culture assay and to find correlations with the above described antimicrobial activity, through MVDA analysis. To do this, the same extracts previously obtained at a five teen-days sampling period were analyzed by NMR spectroscopy. This technique was selected due to its reproducibility and suitability for high-throughput sample analysis (Dona et al., 2014). This analysis leads to the identification of some chemical shifts from the metabolites potentially responsible for the antimicrobial activity.

After bio-guided isolation of compounds **1** and **2**, the presence of these signals was checked in the S-plot and both compounds were located on the active side of the plot, suggesting that they are the main responsible for the antibacterial activity (Fig. 5).

These two compounds were previously isolated from cultures of the symbiotic bacteria *Xenorhabdus nematophilus* recovered from the entomopathogenic nematode *Steinernema glaseri* from a soil sample collected at several places of the South Korea. Their cytotoxic activity against gastric adenocarcinoma (SNU668), colon adenocarcinoma (HT-29) and lung adenocarcinoma (NCIH1703) was reported in those studies (Paik et al., 2001).

Additionally, the isolated compounds have been tested as antifoulants (Yang et al., 2007) and as quorum sensing (QS) inhibitors against the biosensors *Chromobacterium violaceum* CV026, *Vibrio fischeri* and the mutant *Escherichia coli* JB525 (Kumar et al., 2017; Teasdale et al., 2011, 2009a). Like *N*-acylhomoserine lactones (AHL), the phenethylamides possess a ring system with a side chain connected *via* an amide bond. The similarity in the molecular sizes and structures between phenethylamides and AHL-autoinducers suggested that the

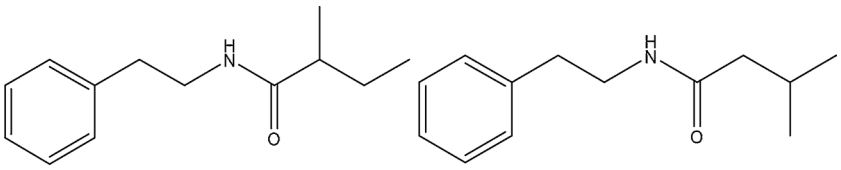

Fig. 3. Structures of the compounds **1** and **2** obtained from the culture of *Streptomyces* sp. PNM-9.

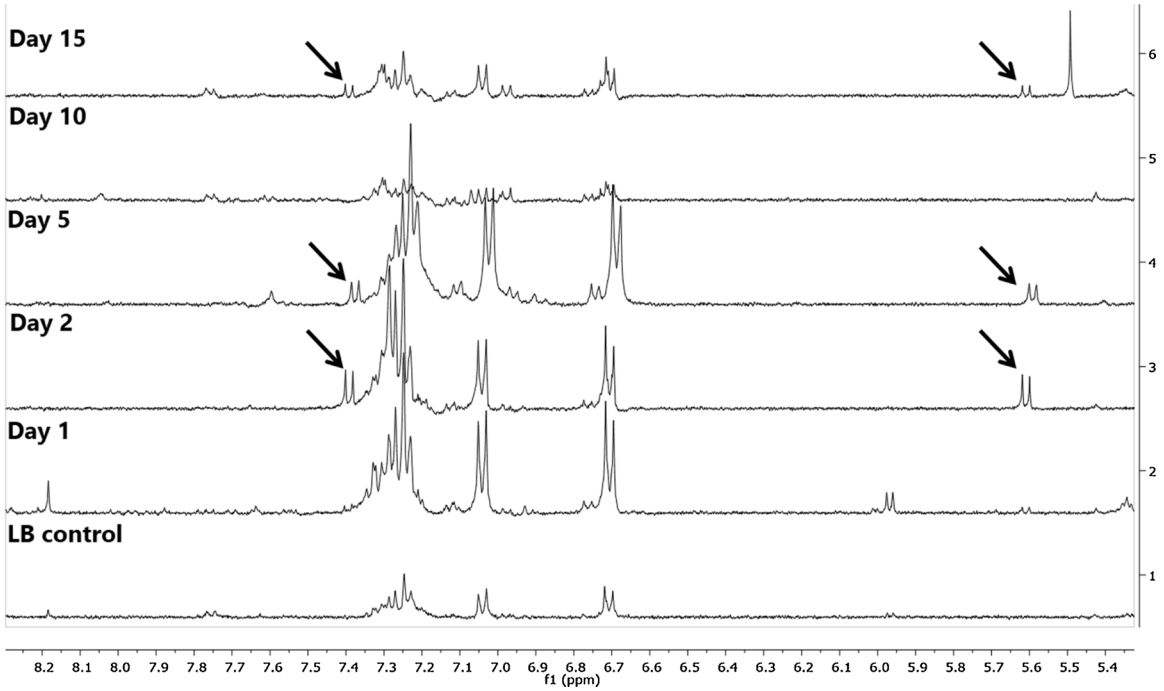

Fig. 4. ^{1}H NMR spectra comparison of the extracts at the days 1, 2, 5, 10, 15 and the media culture control. Arrows shows the observed differential signals trough the fermentation.

phenethylamides might be AHL structural mimics and compete for receptor binding (Teasdale et al., 2009b).

This activity is especially important because QS signals elicited responses in microorganisms that contribute directly to pathogenesis through the production of virulence features, such as toxins, proteases, and other immune-evasive factors (Kumar et al., 2017). Therefore, if the QS signal is blocked, it is possible that bacteria lose their ability to attack the host and thus would be less able to form an organized community resistant to antimicrobial agents (Fuqua and Greenberg, 2002; K. Bhardwaj et al., 2013; Kumar et al., 2017; Rutherford and Bassler, 2012). Thus, molecules that inhibit QS have emerged as an alternative to bacterial resistance observed in different fields, including agriculture (Torres et al., 2019).

5. Conclusions

The comparison of metabolic profiles obtained by NMR using MVDA allowed to select LB and TSB culture media for metabolite production and isolation of bioactive compounds from *Streptomyces sp*. PNM-9. Additionally, the correlation between activity and metabolic production evidenced, before isolation, the structural features of active compounds. In this way, this study contributes to buildup NMR-based tools as starting points for the development of novel biotechnological products with possible applications in agriculture and environmental preservation fields. This study also contributed to investigate the biodiversity in the southwest Caribbean Sea as a source of underexploited microbial diversity, which has enormous potential to provide chemical compounds to develop biotechnological products.

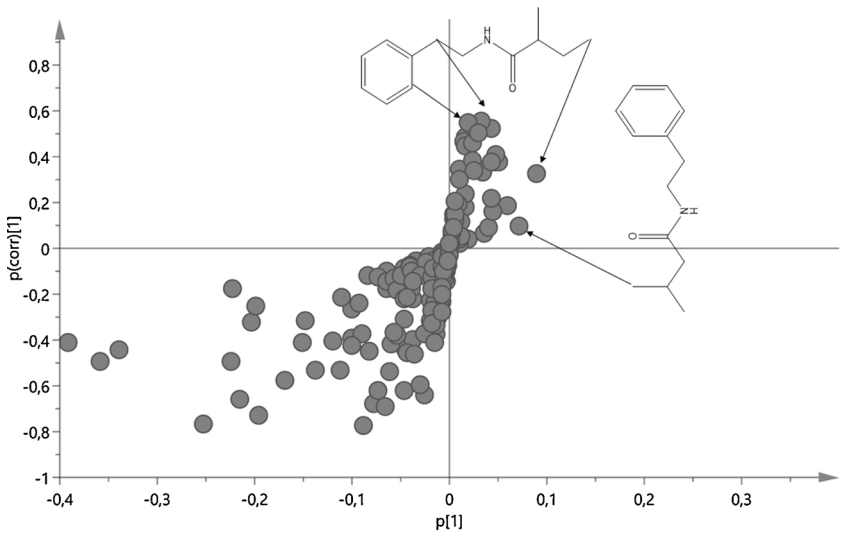

Fig. 5. Two isolated compounds from *Streptomyces* sp. PNM-9 labeled in the S-plot. Both are on the active side, suggesting a most antibacterial activity.

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