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Improvement of biomass and cyclic lipopeptides production in *Bacillus amyloliquefaciens* MEP$_2$18 by modifying carbon and nitrogen sources and ratios of the culture media

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Abstract

Bacillus amyloliquefaciens MEP_218 is a native isolate with a broad spectrum of antifungal activity against plant pathogenic fungi. The ability of strain MEP_218 to antagonize phytopathogens is due to the production of cyclic lipopeptides (CLPs). In this work, different carbon (C) and nitrogen (N) sources and C to N ratios were evaluated in order to improve both, biomass and CLPs production by strain MEP_218. Among the C and N sources and C to N ratios tested, glucose and NH_4NO_3 at the C to N ratio of 10:1 enhanced significantly biomass and CLPs yield. Moreover, CLPs produced in this medium exhibited higher antibacterial activity against *Xanthomonas axonopodis* pv. *vesicatoria* (*Xav*) than those obtained in the recommended medium for CLPs production. Interestingly, CLPs addition influenced the development of *Xav* biofilm on biotic and abiotic surfaces. A comparison of HPLC-chromatograms of CLPs obtained in the optimized medium versus the ones obtained in the recommended medium showed a notable increase of surfactin in the CLPs obtained in the optimized medium. Furthermore, two peaks with antibacterial activity against *Xav*, identified by mass spectrometry analysis as fengycins A and B, were detected in the CLPs from the strain MEP_218, grown in the optimized culture medium. The results obtained in this work suggest that changes in C and N sources and C to N ratios affect the yield and type of CLPs produced by *B. amyloliquefaciens* MEP_218. To the best of our knowledge, this is the first study to report the finding of fengycins with antibacterial activity. CLPs produced by the strain MEP_218 are potential candidates for controlling bacterial spot disease in tomato and pepper.

Keywords: Cyclic lipopeptides, *Bacillus*, culture media, fengycin, *Xanthomonas*, biofilm

1. Introduction

Nowadays, pest management is expected to be agro-economically, socially and environmentally sustainable. Some species of the genus *Bacillus* fulfill all the above-mentioned requirements; therefore, the U.S. Food & Drug Administration (FDA) and the United States Environmental Protection Agency (EPA) have recommended its use for controlling plant diseases caused by phytopathogenic microorganisms (Borriss, 2011; Calvo et al., 2017). Particularly, *B. amyloliquefaciens* and *B. subtilis* have been developed into commercial products for controlling a wide range of diseases caused by fungi and oomycetes (Borriss, 2011; Pérez-García et al., 2011). Despite the high diversity of antibacterial metabolites produced by *Bacillus* sp., the major fraction of the pathogens-suppressing antibiotics, are nonribosomally synthesized peptide derivatives, mainly cyclic lipopeptides (CLPs). A combination of different types and number of ring amino acids and the length and composition of fatty acid side chains provides notable structural diversity that influences the physicochemical properties and antimicrobial activity of CLPs.

CLPs play a crucial role in the antagonism towards phytopathogens and confer superior properties than antibiotics, such as low toxicity, high biodegradability, wide antimicrobial spectrum and biosurfactant activity. These features qualify CLPs as competitive candidates in cosmetics, food and the pharmaceutical industry (Georgiou et al., 1992; Varvaresou and Iakovou, 2015). Based on their structure, CLPs can be generally classified into three families or groups: surfactin, iturin and fengycin (Ongena and Jacques, 2008; Stein, 2005). In addition to their antimicrobial properties, CLPs are also involved in root colonization and in the systemic stimulation of host plant immune system (Ongena and Jacques, 2008).

In previous studies, we showed that *B. amyloliquefaciens* MEP_218 (hereafter referred to as MEP_218), a plant growth-promoting rhizobacterium (PGPR) was able to inhibit *in vitro* the growth of *Fusarium* spp. and *Sclerotinia* spp. (Príncipe et al., 2007). Moreover, foliar application of MEP_218 suppressed sclerotinia stem rot, caused by *Sclerotinia sclerotiorum*, in soybean. The main mechanism associated with the antifungal activity exerted by MEP_218 was antagonism through the production of CLPs. The major CLP produced by MEP_218, was iturin A C15 (Alvarez et al., 2012).

In general, CLPs from *Bacillus* spp. are frequently described by their antifungal activity, but few reports have demonstrated their effects on bacteria. Antibacterial activities of CLPs are mainly attributed to surfactins instead of iturins or fengycins (Ongena and Jacques, 2008). Nevertheless, some reports have addressed the antibacterial effects of iturins and fengycins on pathogenic bacteria (Cochrane and Vederas, 2016; Mora et al., 2015; Zeriouh et al. 2011).

Culture conditions and media composition affect CLPs production since several reports described that both, carbon (C) and nitrogen (N) sources in the culture media, and growth conditions such as temperature, pH, and oxygen rate, can influence the type and yield of the CLPs produced (Singh et al., 2014; Volpon et al., 2000). Particularly, the carbon source is an important factor influencing microbial growth and biosurfactant production (Sen, 1997).

Bacterial adhesion and biofilm formation are early and crucial events in plant pathogenesis. Biofilm formation by plant pathogens allows them to resist the most commonly used antibacterial agents since bacteria within a biofilm are up to 1000 times more resistant to the effect of antibacterial agents than when they are in a planktonic state (Bogino et al., 2013; Stewart, 2015). Few antibiotics have been demonstrated to be effective for the prevention and disruption of biofilms (Parra-Ruiz

et al., 2010; Raad et al., 2007), therefore biosurfactants become an excellent strategy since they can interfere the bacterial adhesion to surfaces.

Management of plant diseases caused by phytopathogenic microorganisms includes mainly the application of chemical pesticides (Mueller et al., 2002), but since these compounds cause damage to the environment and human health, alternative and more sustainable strategies are required. Therefore, the finding of new and innovative antimicrobial products became a priority. In this sense, CLPs produced by native isolates of *Bacillus* could represent a good strategy for the development of new antimicrobial agents for fighting plant diseases.

In this work, we describe the effect of C and N sources and C to N ratios on the yield of the culture, CLPs production, and biofilm formation on abiotic surfaces of $MEP_2$18. By HPLC and mass spectrometry (MALDI TOF) we identified new metabolites as well as those whose production was increased as a result of the new formulation.

2. Materials and methods

2.1. Microorganisms and growth conditions

Bacillus amyloliquefaciens $MEP_2$18 was selected because of its biocontrol properties from a bacterial collection obtained in our lab from a saline soil of the south of Cordoba province, Argentina (Alvarez et al., 2012; Príncipe et al., 2007).

The *Bacillus subtilis* laboratory strain JH642 (Δ*trpC2, pheA1*), a derivative from *B. subtilis* strain 168, was kindly provided by Mansilla and de Mendoza (1997). This

strain, a nonlipopeptide producer (genotypically *sfp0*), was used as a negative control.

Bacillus strains were grown in Lysogeny broth (LB) or in **M**edium **O**ptimal for **L**ipopeptide **P**roduction (MOLP) (Gu et al., 2005) at 30 ºC and 150 rev min^{-1}. *Xav,* the causative agent of bacterial spot disease on pepper (*Capsicum* spp.) and tomato (*Lycopersicon* spp.) plants (Thieme et al., 2005), was routinely grown at 30 ºC in nutrient agar (NA) plates (or nutrient broth), and in minimal medium MMX (Daniels et al., 1984). All strains were stored at -80 ºC in saline buffer plus 20 % glycerol.

2.2. Experimental design

To determine the influence of C and N sources on the culture yield, CLPs production, and biofilm formation, MEP_218 was grown in MOLP or in modified MOLP media. MOLP was modified by removing yeast extract and replacing sucrose and NH_4Cl by glucose or fructose and KNO_3 or NH_4NO_3, respectively. The ratios of C to N assayed were 10 to 1, 5 to 1 and 2 to 1. The concentrations corresponding to different C to N ratios are given in Table 1.

2.3. Quantification of cyclic lipopeptides

A turbidometric method according to Mukherjee et al. (2009) with slight modification (Meng et al., 2016) was used to quantify the CLPs production. Two hundred µL of cell-free supernatants from MEP_218 cultures, and 100 µL 10 % trichloroacetic acid were added into 96 multi-well plates. After 30 min at room temperature, turbidity was measured by using an Epoch Microplate

Spectrophotometer (BioTek) at an optical density of 600 nm (OD_{600}). Distilled water of the same volume replaced 10 % trichloroacetic acid to mix with cell-free supernatant served as blank control.

2.4. Isolation of cyclic lipopeptides

CLPs were isolated by acid precipitation with concentrated HCl (Kim et al., 2004; Vater et al., 2002). Briefly, $MEP_2$18 was grown in MOLP or modified MOLP media until the stationary phase of growth. Bacterial cells were removed by centrifugation at 12000 g for 15 min. CLPs were precipitated from the remaining supernatants by adding 3 N HCl to a final pH=2.0 and stored for 30 min at 4 ºC. The precipitates were collected by centrifugation at 10 000 g for 20 min, suspended in 100 % methanol, concentrated twenty times (20X) and stored at 4 ºC.

2.5. Analysis of antibacterial activity of cyclic lipopeptides

The antibacterial activity of CLPs against phytopathogenic *Xav* was tested on nutrient agar (NA) plates by using the disc diffusion method. 100 µL of a log culture of *Xav* were spread evenly on a NA plate by using a glass spreader. Sterile paper discs were imbibed with 10 µL of 20X CLPs. Once methanol was evaporated, the discs were deposited onto the plate and incubated at 30 ºC for 24 hours. After incubation, the zones of inhibition were visualized as the diameter of the zone formed around each paper disc. Paper discs imbibed with 10 µL of methanol and acidic precipitate from cell-free supernatant of strain JH642 were used as controls. The analysis was done in triplicate to check the reproducibility.

2.6. Determination of Minimal Inhibitory Concentrations (MICs)

A microtiter plate dilution assay (Baindara et al., 2013) was performed for determining the minimum inhibitory concentration (MIC) of CLPs on *Xav*. *Xav* was grown until the logarithmic phase of growth in nutritive broth (OD_{600} between 0.3-0.4). CLPs were used at the following concentrations (µg ml^{-1}): 0, 3, 6, 7.5, 15, 30, 60, 90, 120, 300 and 600. The lowest concentration of CLPs that inhibited the growth of *Xav* and did not show any increase in the OD_{600} after 48 h of incubation was considered as the MIC.

2.7. Biofilm Assays

To quantify the biofilm formation on plastic surfaces, overnight cultures of *Xav* grown in MMX medium or in LB medium were centrifuged and the cells washed with 10 mM $MgCl_2$ and suspended in fresh medium to an OD_{600} of 0.5. Then, 100 µl of each bacterial suspension were dispensed into each well of 96-well polyvinylchloride (PVC) and incubated for 12 h at 28 ºC. After that, 5 µL of 20X CLPs (the final concentration in the bacterial culture was 1X) or 5 µL of methanol (control without CLPs) were added. At 6 h of incubation (Gottig et al., 2009), bacterial biofilm was measured after repetitive washing of the plates to remove non-adherent cells and staining with 0.3 % crystal violet (CV) for 15 min at room temperature. Excess stain was removed by washing under running tap water, and the CV stain was solubilized by the addition of 150 µl of 95 % ethanol added to each well. CV was quantified with a microplate reader at 570 nm (OD_{570nm}) (O'Toole and Kolter, 1998).

To determine the biofilm formation by $MEP_2$18 on PVC, cultures were grown until the stationary-phase growth in MOLP and media with different C and N sources and C to N ratios. Then, the same procedure described above was used to quantify the bacterial biofilm.

To determine the biofilm of *Xav* on biotic surfaces (pepper and tomato leaves), the bacterium was grown in the same conditions described for the microplate colorimetric assay. For pre-treatment with CLPs, healthy leaflets were mounted onto petri dishes, adaxial side down and 1 mL of 1X CLPs were added to abaxial side. After 6 h incubation at room temperature (Gottig et al., 2009), the leaf was inoculated with 3 ml of the bacterial suspension of *Xav* at a concentration of 10^8 CFU ml^{-1} and incubated for 24 h at 28°C in a humidified chamber. In post-treatment, bacterial suspension was first added and, after 6 h incubation, 1 mL of 1X CLPs were added and incubated in the same conditions as pre-treatment. The surface-attached biofilm was determined by staining with 3 ml of 0.30 % crystal violet for 15 min at room temperature. The unbound crystal violet stain was removed and the leaves were washed gently three times with distilled water. The stained cells attached to the leaf surface were analyzed macroscopically by the intensity of the blue dye (Rigano et al., 2007) and quantified by using ImageJ software (Schneider et al., 2012).

2.8. RP-HPLC analysis

Precipitated CLPs, obtained as described earlier, were suspended in 100 % methanol, filtered through 0.22 µm nylon membrane, and injected into a high-performance liquid chromatography (HPLC) (Infinity LC Grad, Agilent) system

equipped with a reverse-phase Analytical Zorbax C18 column (4.6 mm x 150 mm, Agilent) and a UV detector (Agilent).

CLPs were eluted using a two component solvent system of which solvent A is acetonitrile and solvent B is water acidified with 0.1 % formic acid. For each run, 20 µl of sample were injected onto a column and eluted using the following gradient (% A:B *v*/*v*): injection start (10:90), 10 min isocratic (10:90), then (35:65) with an increasing gradient of solvent A to 65 % through 25 min, then 10 min isocratic (65:35), followed by 10 min isocratic (80:20) and finally an increasing gradient of solvent A to 100 % through 25 min. The elution pattern was monitored by determining absorbance at 210 nm. Elution program used a flow rate of 1 mL min^{-1}.

Eluted fractions were collected, concentrated in a centrifugal vacuum concentrator (SC110 Savant), suspended in 20 µl of 100 % methanol and challenged against *Xav*. Negative controls consisted of 100 % methanol and precipitates obtained from cell-free supernatants of *B. subtilis* JH642 (a strain deficient in CLPs biosynthesis) suspended in 100 % methanol.

2.9. Mass spectrometry analysis

Ultraviolet matrix assisted laser desorption-ionization mass spectrometry (UV-MALDI MS) performed on the Bruker Daltonics Ultraflex Time-of-Flight/ Time-of-Flight (TOF/TOF) mass spectrometer (Bremen, Germany) was used to identify RP-HPLC purified fractions corresponding to CLPs. Desorption/ionization was obtained by using the frequency-tripled Nd:YAG laser (355 nm). For CLPs analysis, portions of 1- to 2-µL of fractions obtained after RP-HPLC were each mixed with an equal volume of matrix medium (a saturated solution of α-cyano-4-hydroxycinnamic acid in 70 %

aqueous acetonitrile containing 2,5 % [vol/vol] TFA). Sample solutions (1 µL) were spotted on a MTP 384 target plate polished steel (Bruker Daltonics). Mass spectra were acquired in linear positive ion modes in a range of 700 to 3500 Da. External mass calibration was made using trypsin-digested BSA standard with HCCA as matrix in positive ion mode. The laser power was adjusted to obtain high signal-to-noise ratio (S/N) while ensuring minimal fragmentation of the parent ions and each mass spectrum was generated by averaging 100 lasers pulses per spot. Spectra were obtained and analyzed with the programs FlexControl and FlexAnalysis, respectively (Torres et al., 2015).

2.10. Statistical analysis

All experiments were performed at least three times with three biological replicates per treatment. Data are expressed as the mean ± SEM. The data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test or Tukey test (considering significant at $P < 0.05$). The statistics software SPSS v23.0 (SPSS Inc., Chicago, USA) was employed.

3. Results

3.1. Effect of different carbon and nitrogen sources and ratios on the growth, CLPs production and biofilm formation by B. amyloliquefaciens *MEP$_2$18*

The maximal biomass achieved by growing MEP$_2$18 in MOLP was in the order of ten to eight CFU mL^{-1} (Table 2). With the aim of improving the culture yield as well

as the production of CLPs, we assayed different C and N sources and different C to N ratios.

The pairs glucose-KNO_3 and fructose-KNO_3 at the C to N ratio of 10 to 1 did not result in a better growth compared to MOLP, which is composed of sucrose-NH_4Cl at the C to N ratio of 10 to 1. The growth rates under these conditions were reduced by half, compared with that observed in MOLP (Figure 1, Table 2). In contrast, the pairs fructose-NH_4NO_3 and glucose-NH_4NO_3 at the C to N ratio of 10 to 1 enhanced bacterial growth reaching ten to nine and ten to ten CFU mL^{-1}, respectively. In fact, glucose and NH_4NO_3 10 to 1 produced the highest culture yield. In all cases, however, the growth rate in MOLP was higher, possibly due to the fact that MOLP contains traces of yeast extract.

When the C to N ratios of 5 to 1 and 2 to1 were tested, no substantial increases in bacterial growth were found compared to MOLP. In these conditions, the culture yields were in the range of ten to eight CFU mL^{-1}. Additionally, the growth rates were lower than the ones observed in MOLP.

The production of CLPs improved when NH_4NO_3 was the source of N, exceeding in all cases the production obtained in MOLP. The lowest growth rate and CLPs production were obtained in glucose and KNO_3 at the C to N ratios of 2 to 1.

In general, bacterial biofilm on PVC was enhanced by increasing the C to N ratio (Table 2). The biofilm formed by $MEP_2$18 on PVC improved when NH_4NO_3 was the N source, obtaining the maximum biofilm production at the C to N ratio of 10 to 1. Therefore, independently of the C source, the most appropriate N source and C to N ratio for the growth of $MEP_2$18 were NH_4NO_3 and 10 to 1, respectively. Although the growth rate obtained in MOLP was not improved in any condition, glucose and NH_4NO_3 at the C to N ratio of 10 to 1 were by far the most suitable combination in

terms of culture yield (Table 2). Additionally, this C and N combination also resulted in the highest CLPs production and improved the biofilm formed by $MEP_2$18 (Table 2).

Altogether, these results indicate that the modified medium containing glucose and NH_4NO_3 at the C to N ratio of 10 to 1 is more appropriate than traditional MOLP for biomass and CLPs production by $MEP_2$18. For this reason, this medium, called hereafter as modified MOLP (MMOLP) was chosen to continue the experiments.

3.2. Antibacterial activity of cyclic lipopeptides from B. amyloliquefaciens *$MEP_2$18 on the phytopathogenic* Xanthomonas axonopodis pv. vesicatoria

Considering the fact that the growth of $MEP_2$18 in the MMOLP resulted in an enhanced production of CLPs, we wondered whether the new formulation could also be effective for controlling the bacterial spot disease caused by *Xav.* Thus, we first determined, using the disc diffusion method and the MIC determination, the antibacterial activity against *Xav* of CLPs produced by $MEP_2$18 in the MMOLP in comparison with that from CLPs produced in MOLP.

Purified CLPs obtained from cell-free supernatants of cultures grown in MOLP or MMOLP exhibited antibacterial activity against *Xav* (Figure 2A). The diameters of the inhibition zones were higher for the CLPs produced in MMOLP (halo diameter=3.27±0.31 cm) than for those produced by CLPs produced in traditional MOLP (halo diameter=2.57±0.16 cm). Accordingly, the MIC values of purified CLPs, for *Xav*, obtained from MMOLP or MOLP showed clear differences being the MIC obtained from MMOLP (MIC=15 µg ml^{-1}) 4 times lower than that obtained from MOLP (MIC=60 µg ml^{-1}). *B. subtilis* JH642, a strain deficient in CLPs biosynthesis,

failed to show an antibacterial effect against *Xav*. Methanol, used for CLPs suspensions, did not show any inhibitory effect against *Xav*.

Our results clearly show that an antibacterial compound present in the CLPs fraction obtained from culture supernatants from $MEP_2$18 is responsible for the antibacterial activity against *Xav*.

3.3. Influence of cyclic lipopeptides on the stability of Xanthomonas axonopodis pv. *vesicatoria biofilms*

Since the target organ of the bacterial spot disease caused by *Xav* is the leaf of tomato and pepper plants and taking into account that the biofilm formed by *Xav* on leaf surfaces (phyllosphere) contributes hugely to disease progress, we investigated whether the exogenous application of CLPs can reduce the biofilm of *Xav* on both, biotic (plant leaf) and abiotic (PVC) surfaces. The bacterial biofilm on PVC was evaluated by incubating cultures of *Xav*, grown in MMX or LB media, in PVC wells.

The data obtained show that the biofilm formation on PVC by *Xav* was more efficient in MMX medium than in LB (Figure 3A). When CLPs were added, a statistically significant decrease in biofilm formation was observed for bacteria grown in MMX (Figure 3A). Methanol had no effect on biofilm formation by *Xav*.

The biofilm formation of *Xav* on biotic surfaces was determined on the abaxial surface of tomato and pepper leaves as described in Materials and methods. Representative leaves of each treatment are shown in Figures 3B and 3C respectively.

CLPs were applied on the leaves at two different moments: six hours before adding *Xav* (named pre-treatment) and six hours after adding *Xav* (named post-treatment).

When tomato or pepper leaves were treated only with *Xav*, the leaf area stained with the CV dye was 75 % or 55 %, respectively. In contrast, the application of CLPs to leaves in the pre-treatment (CLP+*Xav* in Figure 3B and 3C), resulted in almost no staining of leaves with CV, indicating that CLPs inhibit the phytopathogen ability to form the biofilm on biotic surfaces, possibly due to the antibacterial activity of CLPs.

A drastic reduction of the preformed biofilm of *Xav* on leaves was observed in the post-treatment with CLPs (*Xav*+CLP in Figure 3B and 3C). Altogether, these results indicate that the foliar application of CLPs could be helpful for reducing the incidence and severity of the bacterial spot by altering both the establishment of new biofilm and the preformed *Xav* biofilm on leaves of tomato and pepper.

3.4. Identification of antibacterial metabolites present in the CLPs fraction

With the aim of identifying the compound/s present in the CLPs fraction and responsible for the antibacterial activity, this fraction was subjected to reverse phase HPLC on a C18 reverse phase column. The elution strategy used for HPLC analysis allowed the separation of the three major CLPs families. Iturins eluted first followed by fengycins and finally surfactins. Eluted sub-fractions were collected and assayed against *Xav*, as described above.

Comparing the HPLC profiles from CLPs obtained from bacterial cultures grown in MOLP or MMOLP, it can be observed that both are basically similar in terms of the types of CLPs produced (Figure 4A). Nevertheless, some peaks were modified in the CLPs fraction obtained in MMOLP. For example, peaks with retention times of 13 and 30.2 min appeared, while peaks with retention times of 28, 32 and 37 min

disappeared. Other peaks were increased, such as those corresponding to retention times of 12, 22, 25.5, 27.4, 49, 54, 56 and 61 min.

According to the range of acetonitrile concentration where the three CLPs families elute, the HPLC profiles obtained for CLPs from $MEP_2$18 are in agreement with iturin, fengycin and surfactin production (Yang et al., 2015). Two active fractions, which showed strong antibacterial activity, were found in the CLPs extract from $MEP_2$18 grown in MMOLP. These peaks eluted in the range of acetonitrile concentration where fengycins elute (55-60% acetonitrile) (Malfanova et al., 2012; Nihorimbere et al., 2012). Peaks corresponding to surfactin were also recognized in the HPLC profiles by comparison with purified standard of surfactin but the antibacterial activity against *Xav* was absent in these peaks (data not shown). As expected, HPLC analysis of acidic precipitates from cell-free supernatants from *B. subtilis* JH642 did not show any active fraction (data not shown).

A detailed view of the chromatogram region that includes the peaks with antibacterial activity is shown in Figure 4B. The peak with retention time of 27.4 min showed an increased area (167.78 %) in comparison to that observed in the CLPs fraction obtained in traditional MOLP. Moreover, a new peak with retention time of 30.2 min appeared in the CLPs fraction of $MEP_2$18 grown in MMOLP. Both peaks were active against *Xav* growth *in vitro* showing extremely large inhibition zones (Figure 2B).

Figure 4C shows a closer view of the chromatogram region where surfactin eluted. Surfactin peaks were remarkably enhanced when $MEP_2$18 grew in MMOLP in comparison to the traditional MOLP. Quantification using a commercial standard of surfactin (Surfactin from *Bacillus subtilis*, Sigma) showed that the surfactin production increased from 8 to 16 mg mL^{-1} of purified CLPs when $MEP_2$18 grew in the MMOLP.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was applied to identify the compounds responsible for *in vitro* antibacterial activity present in the peaks with retention times of 27.4 min and 30.2 min (Figure 4D). The mass-spectra analysis of purified fractions displayed peaks that corresponded to different isoforms of fengycins A and B in both fractions, majority C_{17} fengycin B (for peak 27.4 minutes) and C_{16} / C_{17} fengycin A (for peak 30.2 minutes). Peaks 1435.9 and 1489.9 m/z, coincident with C_{14} fengycin A and C_{16} fengycin B, respectively, are exclusive for the CLP fraction of 30.2 min eluted from $MEP_2$18 grown in MMOLP. Moreover, some peaks can be recognized as sodium adducts (Table 3).

The results presented in this study show that the culture medium influences both the amount and the isoforms of CLPs produced by the same *Bacillus* strain, suggesting that it is possible to modulate the production of certain metabolites as CLPs through modifications of the sources and ratios of C and N of the culture media.

4. Discussion

CLPs produced by *Bacillus* species are promising and interesting antimicrobial compounds due to their agronomic, pharmaceutical and biotechnological applications (Jacques, 2011). CLPs are produced as a combination of isoforms and homologs (Roongsawang et al., 2011). Variations in CLPs are highly dependent on strain, culture conditions and growth media composition and these variations influence biological activities of CLPs (Abdel-Mawgoud et al., 2008; Akpa et al., 2001; Kilani-Feki et al., 2016; Singh et al., 2014). In the present study, with the aim of improving

the culture yield and CLPs production by the native isolate *B. amyloliquefaciens* $MEP_2$18, an iturin, fengycin and surfactin-producing strain, we assayed different C and N sources and different C to N ratios. A direct influence on the growth of $MEP_2$18 was observed by modifying C and N sources and C to N ratios and this in turn altered culture yield and CLPs production. Although sucrose is commonly used for CLPs production (Gu et al., 2005; Zhao et al., 2014), glucose is the preferential C source for all living organisms. In *Bacillus circulans* (Das et al., 2009), *Bacillus amyloliquefaciens* BZ-6 (Liu et al., 2012), *Bacillus amyloliquefaciens* fmb-60 (Lu et al., 2016) and *Bacillus subtilis* strains (Makkar et al., 2011), a direct correlation between CLPs production and biomass yield was reported. $MEP_2$18 exhibited optimal growth and enhanced CLPs production with glucose as the C source. Similarly, Roychoudhury et al. (1989) reported that glucose supports a faster growth and biomass accumulation in *B. amyloliquefaciens*. Moreover, this C source has been used for optimizing the production of surfactin by *B. subtilis* DSM 3256 (Sen and Swaminathan, 2004) and lichenysin by *B. licheniformis* (Coronel-León et al., 2015; Joshi et al., 2008). Bacillomycin production has been shown to be dependent on biomass concentration (Qian et al., 2015). Recently, Lu et al. (2016) suggested that biomass yield could be an important parameter for fengycin production and that glucose had a positive effect on fengycin biosynthesis in *Bacillus amyloliquefaciens* fmb-60.

Nitrate availability is another variable influencing bacterial growth and CLPs production. Although $MEP_2$18 was able to utilize all of the nitrogen sources tested, the optimal nitrogen source was NH_4NO_3. Here, we suggest that the C to N ratio could be an important factor to consider for improving CLPs production since significant differences in biomass and CLPs yield were observed in $MEP_2$18 by using

the same C and N sources but varying the C to N ratio. For all C and N sources assayed, the optimal C to N ratio for $MEP_2$18 was 10 to 1, suggesting that CLPs production is enhanced when low amounts of nitrate are provided. In *B. subtilis* ATCC 202152, nitrate addition affected negatively the production of antimicrobial compounds (Pryor et al., 2007).

Gu et al. (2005) reported on the optimization of medium constituents for CLPs production by *Bacillus subtilis*, in this study those constituents (named MOLP medium) were used as a starting point to improve the biomass and CLPs production by $MEP_2$18. The production of these secondary metabolites by $MEP_2$18 was increased by modifying the C and N sources of the culture medium, even though the most appropriate C to N ratio was the same used in the MOLP medium.

CLPs are usually known by its broad-spectrum of antifungal properties (see reviews: Chowdhury et al., 2015; Cochrane and Vederas, 2016; Meena and Kanwar, 2015; Ongena and Jacques, 2008). However, some studies have also highlighted the inhibitory activity of CLPs produced by *Bacillus* against phytopathogenic bacteria. For example, in *Bacillus* spp. isolated from plant environments there was a direct correlation between the presence of the CLPs biosynthetic genes *ituC* (iturin), *bmyB* (bacillomycin), *fenD* (fengycin) and *srfAA* (surfactin), and the antibacterial activity against the plant pathogens *Erwinia amylovora* PMV6076, *Pseudomonas syringae* pv*. syringae* EPS94, *X. arboricola* pv*. fragariae* CFBP3549, *X. axonopodis* pv*. vesicatoria* CFBP3275, *Rhizobium radiobacter* CECT472 (syn. *Agrobacterium tumefaciens*), *Ralstonia solanacearum* CECT125,*Clavibacter michiganensis* sbsp*. michiganensis* CECT790 and *Pectobacterium carotovorum* sbsp*. carotovorum* CECT225 (Mora et al., 2015). In rhizospheric *Bacillus* spp., bioactive compounds, like CLPs, were proposed as responsible for the inhibition of *Xanthomonas* strains

(Issazadeh et al., 2012; Mishra and Arora, 2012; Monteiro et al., 2005; Wulff et al., 2002). Moreover, Zeriouh et al. (2011) reported that the iturin-like lipopeptides are essential components in the biological control of *B. subtilis* against *X. campestris* pv. *cucurbitae.* Accordingly, iturins A produced by *B. amyloliquefaciens* S20 were reported as the main antagonistic compounds against *Ralstonia solanacearum* (Chen et al., 2014).

The *Xanthomonas* spp. are widely distributed in different geographical regions resulting in severe problems for agriculture (Kebede et al., 2014). Bacterial spot disease produced by xanthomonads group causes yield loss and impairs fruit quality representing a worldwide risk to production of Solanaceae crops, especially tomato (*Lycopersicon esculentum*), sweet pepper (*Capsicum annuum*), and chili pepper (*Capsicum frutescens*). Upon immigration onto the leaf surface, *Xanthomonas* colonizes the phyllosphere, (Potnis et al., 2015) and the formation of bacterial biofilms on the leaf surface has been implicated in the epiphytic survival of this bacterial pathogen (Castiblanco and Sundin, 2016).

For many years bacterial spot disease has been managed by foliar sprays of copper-based compounds, and attempts have been made to increase the effectiveness of copper bactericides by increasing the availability of free copper ions in combination with fungicides, such as maneb or mancozeb. A research effort is being made on biocontrol strategies for bacterial spot caused by *Xav* because resistant cultivars to *Xanthomonas* are unusual, and bacterial resistance to antimicrobial compounds, such as copper, is often reported (Abbasi et al., 2015).

In this study, an enhanced antibacterial activity against *Xav* was observed in CLPs fractions from $MEP_2$18 grown in the MMOLP. Interestingly, the application of CLPs, produced in this modified medium, significantly prevented the formation of

biofilm by *Xav* on biotic and abiotic surfaces. More striking was the finding that foliar application of these CLPs resulted in the disruption of pre-formed biofilm of *Xav* on tomato and pepper leaves. These findings suggest that foliar application of CLPs could be a more effective strategy than copper treatment, for reducing incidence and severity of bacterial spot caused by *Xav* in tomato and pepper plants.

Previously, Alvarez et al. (2012) reported that the major CLP with antifungal activity produced by $MEP_2$18 grown in LB broth, was iturin A C_{15}. Surprisingly, mass spectrometry analyses of HPLC-purified CLPs fractions from $MEP_2$18 grown in MMOLP, with antibacterial activity against *Xav*, allowed the identification of fengycins A and B. In *B. amyloliquefaciens* FZB42, one of the most extensive studied *Bacillus* strains, fengycins were exclusively associated with antifungal effects (Wu et al., 2015). Roy et al. (2013) reported that fengycins, produced by *B. thuringiensis* became moderately active against bacteria when they were artificially modified by self-assembly.

Because fengycins were the major CLPs from $MEP_2$18 detected in active HPLC fractions against *Xav*, we suggest that this CLP could have a protective and effective role in avoiding biofilm formation and/or disrupting the preformed *Xav* biofilm. Fengycin as biofilm disruptor has been investigated in some pathogenic bacteria. For example, Rivardo et al. (2009) reported that fengycins from *B. subtilis* V19T21 and *B. licheniformis* V9T14 inhibited selectively biofilm formation of *Staphylococcus aureus* and *Escherichia coli*, respectively.

Surfactins produced by *Bacillus* are known to perform a protective role against phytopathogens through biofilm formation by *Bacillus* in roots and leaves (Aleti et al., 2016; Zeriouh et al., 2014). Moreover, surfactin can trigger root colonization by *Bacillus* and plant immune response mediated by induced systemic resistance (ISR)

(Aleti et al., 2016). Although the HPLC peaks containing surfactins did not show a direct antibacterial activity against *Xav,* the production of this CLP by $MEP_2$18 was increased by 100 % in the optimized medium. Therefore, we cannot exclude the possibility that surfactin produced by $MEP_2$18 can exert a synergistic effect with fengycins for the inhibition or disruption of *Xav* biofilm. Future work using mutants affected in surfactin production will address this issue.

Overall, results from this work demonstrated that changes in C and N sources and C to N ratios affected quali- and quantitatively the production of CLPs in $MEP_2$18. The growth of $MEP_2$18 in the optimized culture medium resulted in an enhanced biomass and CLPs yield. Our results also suggest that application of CLPs produced by $MEP_2$18 could be an efficient and ecological alternative for controlling the development of biofilms and pathogenesis caused by *Xav* in tomato and pepper plants. We have demonstrated here, that the increased antibacterial activity of CLPs produced by $MEP_2$18 was due to the production of fengycins A and B. This work provides new evidence about the poor characterized role of fengycins produced by *Bacillus* as antibacterial compounds and raise up the possibility of using this cyclic lipopeptide for controlling the bacterial spot of tomato and pepper caused by *Xanthomonas axonopodis* pv. *vesicatoria*.

Future work will attempt to identify C and N sources derived from agronomical and/or industrial activities for an improved low-cost production of CLPs by *B. amyloliquefaciens* $MEP_2$18.

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Figure captions

Figure 1. Time course of growth in different culture media by *Bacillus amyloliquefaciens* $MEP_2$18. Growth curve obtained with C to N ratios of 10 to 1**(A)**; 5 to 1 **(B)**; and 2 to 1 **(C)**. Values are the mean ± SD of three independent experiments with three biological replicates per treatment.

Figure 2. a. Antibacterial activity of CLPs from *Bacillus amyloliquefaciens* $MEP_2$18 against *Xanthomonas axonopodis* pv. *vesicatoria*

Paper discs were embedded with: 10 µL of 20X CLPs obtained from $MEP_2$18 grown in MOLP **(A)** or in MMOLP (glucose/NH_4NO_3, C to N ratio of 10 to 1) **(B)**; 10 µL of CLPs obtained from *Bacillus subtilis* strain JH642 (negative control) **(JH642)**; 10 µL of methanol (**MeOH)**.

b. Antibacterial activity of high-performance liquid chromatography (HPLC)-purified peaks from CLPs fractions of *Bacillus amyloliquefaciens* $MEP_2$18 against *Xanthomonas axonopodis* pv*. vesicatoria.*

Paper discs were embedded with: 10 µL of CLPs obtained from $MEP_2$18 grown in MMOLP (glucose/NH_4NO_3, C to N ratio of 10 to 1) (**A)**; 10 µL from peak with retention time of 27.4 min (C17 -fengycin B) (**B)**; 10 µL from peak with retention time of 30.2 min (C16/C17-fengycin A) (**C)**; 10 µL of extract obtained from *Bacillus subtilis* strain JH642 (negative control) (**JH642)**; 10 µL of methanol (**MeOH)**.

Figure 3. Effect of CLPs from *B.amyloliquefaciens* $MEP_2$18 on the biofilm formation by *Xanthomonas axonopodis* pv. *vesicatoria* (*Xav*) on abiotic and biotic surfaces. *Xav* biofilm on PVC **(A)** was measured at 570 nm after crystal violet staining. 5 µL of 20X CLPs (the final concentration in the bacterial culture was 1X) were added to log cultures of *Xav* and biofilm was evaluated 6 h post-treatment with CLPs. Values are expressed as the mean ± standard deviation from three independent experiments (n = 3). Different letters represent significant differences ($P < 0.05$) per Duncan's multiple range test.

Xav biofilm on biotic surfaces: **(B)** tomato leaflets and **(C)** pepper leaves. In the pre-treatment (CLP+*Xav*), leaves were previously treated with 1 mL of 1X CLPs and 6 h later infected with *Xav* and incubated for 12 h prior to CV staining. Post-treatment (*Xav*+CLP) includes CLPs addition in leaves previously infected with *Xav*.

The stained area of each leaf was analyzed macroscopically by the intensity of the CV dye and quantified by using ImageJ (Schneider et al., 2012). Twenty leaves of each treatment were analyzed from three independent experiments.

Figure 4. Characterization and identification of CLPs produced by *Bacillus amyloliquefaciens* MEP_218. **(A)** Analytical HPLC chromatograms of CLPs obtained from strain MEP_218 grown in MOLP (red line) or in MMOLP (glucose/NH_4NO_3, C to N ratio of 10 to 1) (blue line), **(B)** detailed view of the chromatogram region containing the peaks with antibacterial activity against *Xav*. Increased active fractions are marked with *****, **(C)** detailed view of the chromatogram region corresponding to surfactin retention times (green line correspond to Surfactin from *Bacillus subtilis*, Sigma), and **(D)** mass spectra obtained by MALDI-TOF from the antibacterial fractions against *Xav***.**

Tables

Table 1. Growth media composition used in this study [a]

C to N ratio	**C** source	**N** source	
	Glucose/Fructose (g L^{-1})	NO_3K (g L^{-1})	NH_4NO_3 (g L^{-1})
10:1	20	6	2.4
5:1	20	12	4.8
2:1	20	30	12

[a]Mineral supplements were added: Na_2HPO_4 5 g L^{-1}, KH_2PO_4 3.5 g L^{-1}, $FeSO_4.7H_2O$ 8.5 µM; $ZnSO_4.7H_2O$ 0.04 mM; $MgSO_4.7H_2O$ 0.2 mM; $MnSO_4.H_2O$ 0.02 mM.

Table 2. Culture yield (CFUx10^8 mL^{-1}), CLPs and biofilm production by *Bacillus amyloliquefaciens* $MEP_2$18 grown in MOLP and modified MOLP media

C and N sources	C to N ratio	Culture yield * CFUx10^8 mL^{-1}	Growth rate (h^{-1})	CLPs quantification (mg mL^{-1}) *	Biofilm on PVC surface *,§
Glucose/KNO_3	10:1	0.97 ± 0.03^a	0.0495	0.74 ±0.17^a	2.1688± 0.3623^b
	5:1	1.30 ± 0.35^a	0.0580	0.68 ± 0.14^a	1.7488± 0.1967^b
	2:1	8.67 ± 1.33^b	0.0257	0.58 ± 0.11^a	1.3370± 0.1957^a
Glucose/NH_4NO_3	10:1	91.67 ± 4.41^c	0.0715	1.70 ± 0.21^c	3.7648± 0.3019^c
	5:1	3.23 ± 0.39^b	0.0553	1.11 ± 0.24^{c,d}	3.7419± 0.6416^c
	2:1	1.33 ± 0.33^a	0.0470	1.21 ± 0.21^c	1.8751± 0.2846^b
Fructose/KNO_3	10:1	1.07 ± 0.15^a	0.0582	0.84 ± 0.11^b	2.2966± 0.2035^b
	5:1	1.33 ± 0.67^a	0.0628	0.74 ± 0.13^b	2.0574± 0.2654^b

	2:1	2.00 ± 0.58[a]	0.0461	0.61 ± 0.11[a]	1.1348± 0.2567[a]
	10:1	23.00 ± 1.15[c]	0.0667	1.04 ± 0.15[b]	3.2865± 0.4185[c]
Fructose/NH_4NO_3	5:1	0.93 ± 0.07[a]	0.0570	1.03 ± 0.16[b]	2.6162± 0.2057[b]
	2:1	2.00 ± 0.58[a]	0.0504	1.26 ± 0.28[c]	1.8996± 0.3442[b]
Sucrose/NH_4Cl (MOLP)	10:1	6.33 ± 0.67[b]	0.0942	0.93 ± 0.09[b]	2.5290± 0.3027[b]

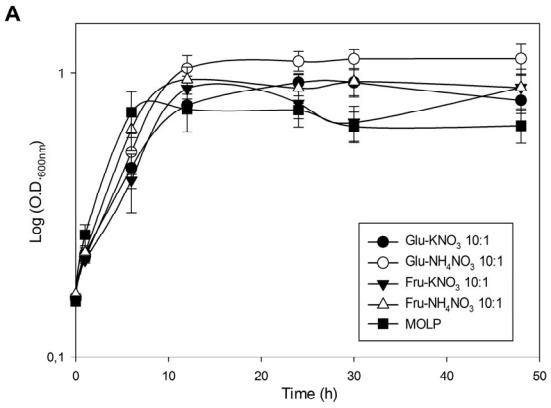
* Values are the mean ± SEM from three independent experiments (n=3). Different letters represent significant differences ($P < 0.05$) according to the Tukey test.

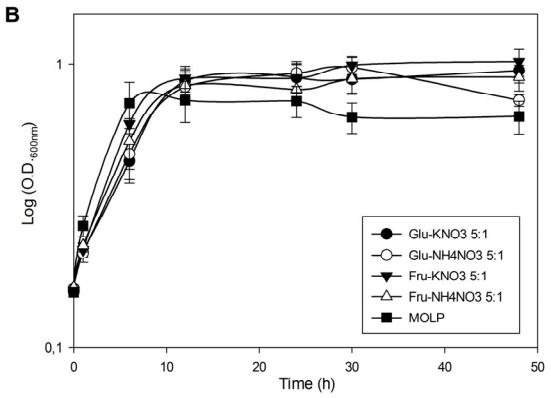
§ Values represent the mean of crystal violet stain measured spectrophotometrically (OD_{570nm}) ± SEM from three independent experiments (n=3).

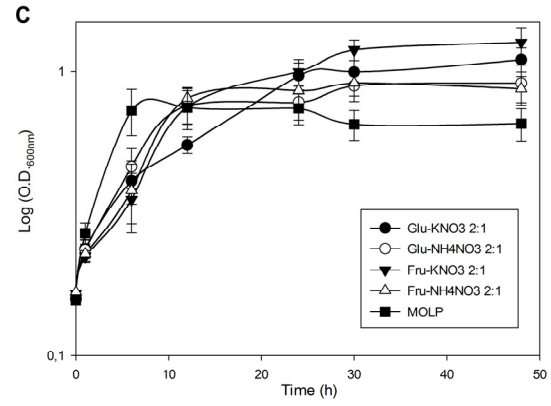
Table 3. Assignment of CLPs to mass peaks obtained by MALDI-TOF mass spectrometry analysis of HPLC peaks with antibacterial activity from CLPs produced by *Bacillus amyloliquefaciens* $MEP_2$18

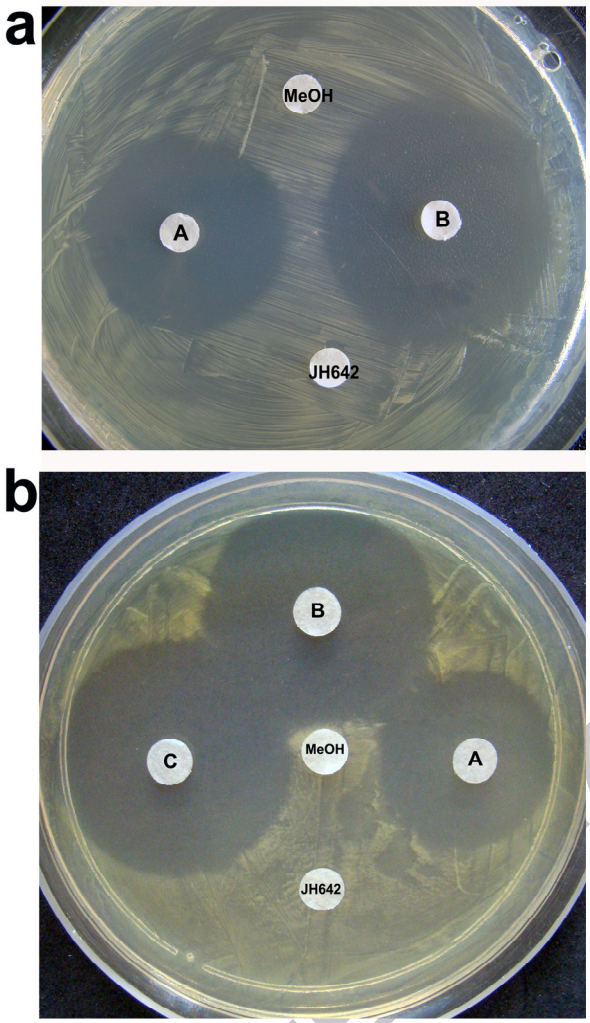
HPLC fraction (minutes)	Mass peak	Assignment
27.4	1447.9, 1469.9	C_{15} fengycin $[M+H]^+$ Ala-6, C_{15} fengycin $[M+Na]^+$ Ala-6
	1461.9, 1483.9	C_{16} fengycin, $[M+H]^+$ Ala-6, C_{16} fengycin, $[M+Na]^+$ Ala-6
	1505.9, 1527.9	C_{17} fengycin, $[M+H]^+$ Val-6, C_{17} fengycin, $[M+Na]^+$ Val-6
	1519.9, 1541.9	C_{18} fengycin, $[M+H]^+$ Val-6, C_{18} fengycin, $[M+Na]^+$ Val-6
30.2	1435.9	C_{14} fengycin, $[M+H]^+$ Ala-6
	1461.9, 1483.9	C_{16} fengycin, $[M+H]^+$ Ala-6, C_{16} fengycin, $[M+Na]^+$ Ala-6
	1489.9	C_{16} fengycin, $[M+Na]^+$ Val-6
	1475.9, 1497.9	C_{17} fengycin, $[M+H]^+$ Ala-6, C_{17} fengycin, $[M+Na]^+$ Ala-6
		C_{17} fengycin, $[M+H]^+$ Val-6, C_{17} fengycin,

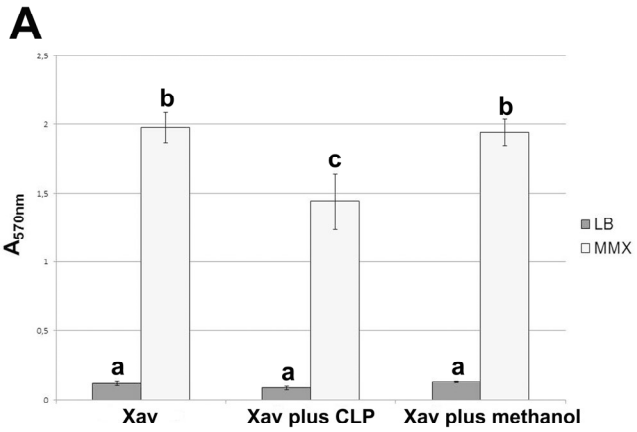
	1505.9, 1527.9	$[M+Na]^+$ Val-6
	1519.9, 1541.9	C_{18} fengycin, $[M+H]^+$ Val-6, C_{18} fengycin, $[M+Na]^+$ Val-6

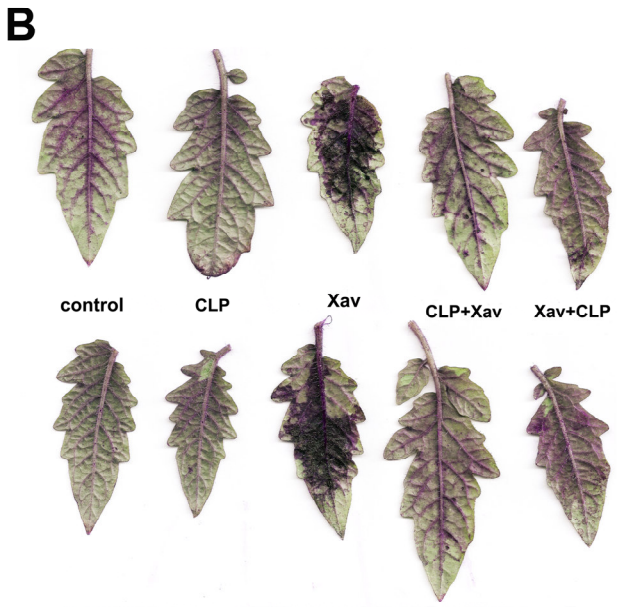


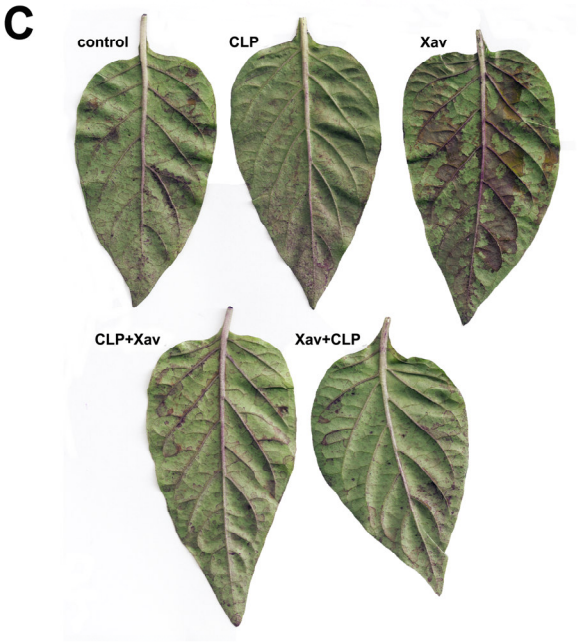












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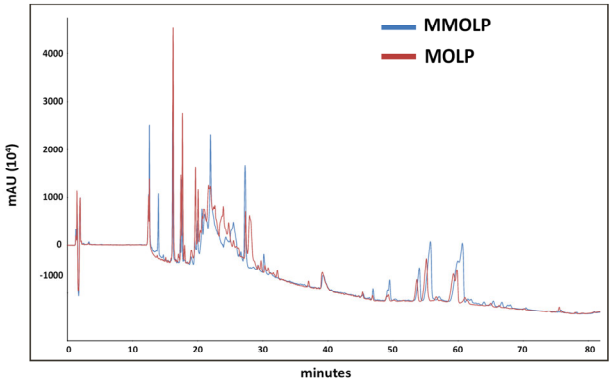

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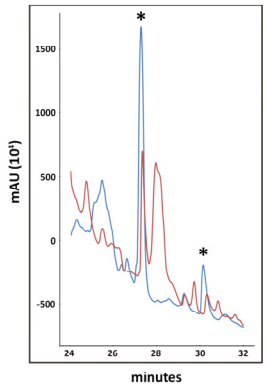

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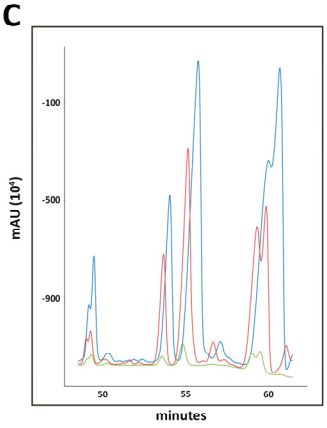

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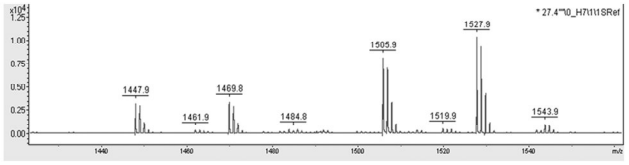

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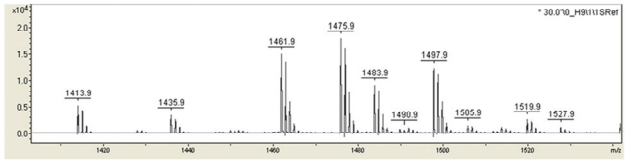

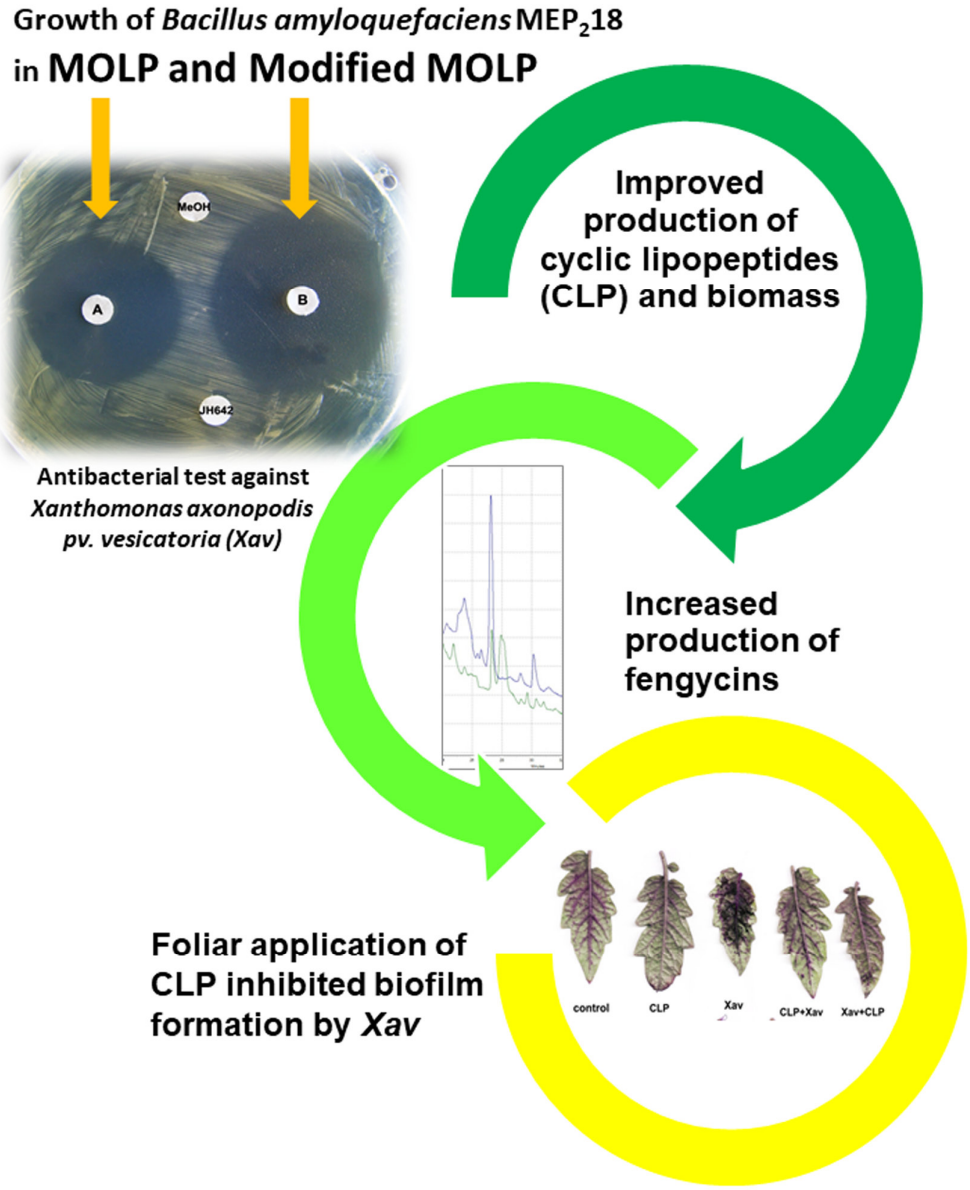

Highlights

- Changes in C and N sources and ratios modify biomass and cyclic lipopeptides (CLP) production in *B. amyloliquefaciens* $MEP_2$18
- CLP produced by strain $MEP_2$18 exhibited strong antibacterial activity against *Xanthomonas*
- Foliar application of CLP inhibited biofilm formation by *Xanthomonas* on tomato and pepper leaves
- Fengycins A and B were identified as the metabolites implicated in the antibacterial activity