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Production of *Bacillus amyloliquefaciens* OG and its metabolites in renewable media: valorisation for biodiesel production and p-xylene decontamination

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Abstract

Biosurfactants are important in many areas; however, costs refrain large-scale production. This work aimed to develop a global sustainable strategy for the production of biosurfactants by a novel strain of *Bacillus amyloliquefaciens*. Initially, *Bacillus* sp. 0G was renamed *B. amyloliquefaciens* subsp. *plantarum* (syn. *Bacillus velezensis*) after analysis of the gyrA/gyrB DNA sequences. Growth in modified Landy’s medium produced three main recoverable metabolites surfactin, fengycin and acetoin, which promote plant growth. Cultivation was studied in the presence of renewable carbon and nitrogen sources, respectively glycerol and arginine. While diverse kinetics of acetoin production was observed in different media, similar yields (6-8 g/l) were obtained after 72 h of growth. Glycerol increased surfactin-specific production, while arginine increased the yield of surfactin and fengycin as well as biomass significantly. The specific production of fengycin increased ~ 10 times, possibly due to a connecting pathway involving arginine and ornithine. Adding value to crude extracts and biomass, these were respectively shown to be useful for the removal of p-xylene from contaminated water, and for biodiesel production, yielding ~ 70 mg/g cells and glycerol, which could be recycled in novel media. This is the first study considering circular bioeconomy to lower the production costs of biosurfactants by valorisation of both microbial cells and their primary and secondary metabolites.

**Keywords:** *Bacillus* sp., sustainability, lipopeptides, biosurfactants, environmental decontamination, circular bioeconomy.
Introduction

The world demand for sustainability requires the use of recycling materials and the valorisation of waste products in a type of circular economy, which is a tendency (Jacquet et al. 2015). As the demand for energy and for food may increase dramatically in the next 50 years, the microbial production of biofuel is an alternative to reduce the usage of crop fields (Hill et al. 2006).

The demand for potable water is another major concern of sustainability. Remediation of contaminated waters can be attained by the use of biosurfactants that have high surface activity and biodegradable properties (Franzetti et al. 2010). A number of microbes produce biosurfactants. For instance, the lipopeptides produced by *Bacillus* spp., represent one of the most promising classes of biosurfactants with applications in many areas including bioremediation. The most promising biosurfactant produced by *B. subtilis* and *B. amyloliquefaciens* is surfactin, which is anionic and has the strongest surface active property yet reported (Jacques 2011). The critical micelle concentration of surfactin (CMC) is in the range of 20 to 40µmol l⁻¹, approximately 200 times less concentrated than a commonly used chemically-derived surfactant, sodium dodecyl sulphate (SDS) that is biodegradable but not promptly oxidized, especially in cold waters (George 2002).

The production of iturins, surfactins and fengycins (three lipopeptide families) has been reported in *B. amyloliquefaciens* as an important feature for plant protection against fungi (Alvarez 2012). Also, *Bacillus* is known to produce the volatile organic compounds acetoin and 2,3-butanediol, which have been proved to elicit induced systemic resistance (ISR) in plants (Rudrappa et al. 2010).

Due to their various applications in industry, the market of surfactant is constantly increasing and microbial biosurfactants trying to make inroads into this market. However, the price remains a major obstacle to their development. The costs of...
growth medium (which can represent more than 30% of the molecule price at industrial scale) and those associated with low yields for the extraction from culture make large-scale production uneconomic. Thus, the use of recyclable substrates (Partovi et al. 2013), or the demonstration of the many applications of biosurfactants are strategies that could lower production costs of such important secondary metabolites (Ongena and Jacques 2008; Jacques 2011). Furthermore, there are a number of cheap substrates accepted by *Bacillus* as a source of carbon/nitrogen such as protein hydrolysates and glycerol, co-products from the industry (Almeida et al. 2012).

This study presents an approach to lower the costs of biosurfactant production. It is based on circular bioeconomy, thus involving the coproduction of microbial biomass, primary metabolites (volatile organic compounds) and secondary metabolites (lipopeptide biosurfactants), using sustainable growth media and potentially recycling glycerol from biomass. First, after molecular biology and analytical analysis we reclassified the strain *Bacillus* sp. 0G used in this study as *B. amyloliquefaciens* 0G (syn. *Bacillus velezensis*) (Dunlap et al. 2016). Then, the biosurfactant production was carried-out using the Landy’s medium as base (Landy et al. 1948) in which the carbon and nitrogen sources were replaced respectively by glycerol and arginine to study the effects on microbial biomass, acetoin, lipopeptide production and yield. The characterization of the lipopeptide extract demonstrates ideal conditions to induce surfactin and fengycin biosynthesis concomitantly. Also, we have shown that the microbial biomass, a waste product from biosurfactant production, can be used for the synthesis of biodiesel. Thus glycerol, which is a waste product from biodiesel synthesis, can be regenerated at this step, and potentially recycled in the growth medium for novel production of biomass and lipopeptides. Finally, the removal of 1,4-dimethyltoluene (p-xylene), from artificially contaminated water has demonstrated a novel environmental application for the crude biosurfactant extract of *B. subtilis* sp. 0G (Figure 1).
Materials and methods

Strain of *Bacillus* sp. used in this study

The strain of *Bacillus* sp. 0G used in this study was isolated from soil in an environment shared with fungi. DNA from strain 0G was purified using the Wizard Genomic DNA kit from Promega Corp. (Madison, WI, U.S.A.), and amplicons were generated using specific primers for (i) *Bacillus* 16S ribosomal RNA genes, in which the primers were S1: 5’-AGAGTTTGATC(A,C)TGGCTCAG-3’; and S2: 5’-GG(A,C)TACCTTGTTACGA(T,C)TTC-3’), as performed previously (Etchegaray et al. 2008); and (ii) *gyrA* and *gyrB* genes, according to Nihorimbere et al. (Nihorimbere et al. 2012) and Yamamoto and Harayama (Yamamoto and Harayama 1995), respectively. The amplicons were cloned into pGEM-T Easy (Promega Corp.), and transformed in *Escherichia coli* JM109. Sequencing of the amplicons was effected using the pGEM-T Easy primers by either GATC Biotech AG (Konstanz, Germany) or Eurofins MWG Operon (Ebersberg, Germany). Identifications were carried out using Blastn software (NCBI, Bethesda, MD, U.S.A.) and Seqmatch software of the Ribosomal Database Project (Michigan State University, East Lansing, MI, U.S.A.). New partial sequence of *gyrA* and *gyrB* genes from *Bacillus* sp. 0G have been deposited at the NCBI gene bank, respectively under the accession numbers KU321596 (*gyrA*) and KU321597 (*gyrB*).

Culture conditions

A fresh colony of the *Bacillus* sp. 0G was taken to inoculate the pre-culture 1 from fresh Petri dishes containing Luria-Bertani (LB) medium incubated at 30 ºC. The pre-culture 1 was prepared in 100 ml of Landy’s medium containing 0.1 mol l⁻¹ 3-(N-morpholino)-propane sulphonic acid (MOPS), pH 7.0 (medium M1) (11) in 500 ml Erlenmeyer flask and incubated for 8 h at 160 rev min⁻¹ and 30ºC. A second pre-culture was prepared to reach an OD 600 nm close to 3.0 (middle of the exponential phase) in specifically
modified Landy’s medium according to the compositions of the culture media M1, M2, M3 and M4 but under the same incubation conditions. M1 and M2 contained 20 g l⁻¹ glucose, whereas M3 and M4 (20 g l⁻¹ glycerol) as major carbon source. Concurrently, M1 and M3 contained 5 g l⁻¹ glutamic acid, while M2 and M4 (5 g l⁻¹ arginine) as the major organic nitrogen source. Details are presented in Table S1. A volume of pre-culture 2 was withdrawn to inoculate 200 ml of the different culture media M1, M2, M3, and M4 (all at pH 7.0) in a 1 l Erlenmeyer flask to reach a starting OD 600 of 0.2. The growth was carried out for 72 h at 30°C under shaking (160 rev min⁻¹). Every 24 h, a sample of the broth was withdrawn for analysis. Cells and supernatant were separated by centrifugation (10 min at 10,000 g). The optical density OD 600 nm and cell dry weight were determined every 24 h, as described previously (Coutte et al. 2010a). All experiments were prepared in triplicates.

Analytical methods used for primary and secondary metabolites characterization and quantification

Purification and quantification of lipopeptides were carried out by RP-HPLC according to Coutte et al. (2010a). After centrifugation the lipopeptides present in the supernatant were concentrated (~ 25 times) by solid phase extraction, using C-18 cartridges (Bond Elut C18 from Agilent Technologies) before HPLC analysis. Surfactin, fengycin and bacillomycin families were separately analyzed using the solvents acetonitrile/water/trifluoroacetic acid (80:20:0.1, v/v/v) and (45:55:0.1, v/v/v) and (30:70:0.1, v/v/v) respectively. Purified lipopeptides dissolved in methanol 100% were used as references (Lipofabrik, Villeneuve d’Ascq, France). Retention time and second derivatives of UV–visible spectrum (Diode Array Waters PDA 996, Millenium Software) of each peak were used to identify and quantify the eluted molecules.
-Characterization of the lipopeptides was performed by LC-MS analysis on an Accela UHPLC system acquired from Fisher Scientific (Thermo Fisher Scientific, Bremen, Germany) that consisted of an auto-sampler equipped with a column oven, a tray compartment cooler and a quaternary pump with a built-in solvent degasser, all controlled by the software Xcalibur. The chromatographic separation was performed on a Vydac C18 column 5 µm, 250 mm × 3 mm ID (Grace) equipped; furthermore, 10 µl of samples were injected. Analysis were carried out at constant flow rate of 600 µl min⁻¹ using the solvents (A) H₂O/HCOOH (99/1%) and (B) ACN (99/1%) with the following gradients: 0–25 min: isocratic elution system at 40% B; 26 min: 45% B; 31min: 65% B; 32-52 min: isocratic elution 80 % B. A flow divider called "splitter" was used as the output rate of HPLC is higher than that tolerated by the mass spectrometer. The flow was split to give approximately 200 µl min⁻¹ and then directed into the mass spectrometer via the electro-spray interface. The system was equipped with an ESI interface that was used in positive ionization mode with the following conditions: capillary temperature and voltage at 250 °C and 93 V respectively, ion spray voltage at 3.5 kV and tube lens voltage at 180 V. Nitrogen was used as the sheath gas and as auxiliary gas with a flow rate of 10 and 5 arbitrary units. The compounds were identified by comparison with reference compounds from retention times, MS analysis. The spectra were recorded in the range of m/z 100–2000. An external calibration of the equipment for mass accuracy was carried out according to the manufacturer's guidelines.

-MALDI-ToF analysis was performed on the cell pellets using 1 µl of matrix solution (10mg ml⁻¹ cyano-4-hydroxycinnamic acid in 70% water, 30% acetonitrile, and 0.1% TFA). Samples were spotted onto a MALDI-ToF MTP 384 target plate (Bruker Daltonik GmbH, Leipzig, Germany) according to the dried-droplet method. Mass
profile experiments were analyzed with an Ultraflex MALDI-ToF/ToF mass spectrometer (Bruker, Bremen, Germany) as described previously (Hamley 2013).

-Quantification of substrates and primary metabolites (glucose, glycerol, lactate, acetate and acetoin) was carried out after collection of growth medium aliquots, which were filtered onto 0.2 µm membranes and analyzed by HPLC Spectra SYSTEM P1000 XR (Thermo Fisher Scientific Inc., Waltham, MA, USA) using a Fast Fruit Juice column (150 × 7.8 mol l-1, Waters Corp, Milford, MA, U.S.A.) according to Coutte et al. (2010b).

Sample treatment for p-xylene extraction and analysis
The analytical curve was constructed adding increasing volumes of p-xylene to water in a final volume of 500 µl (Table S2). The mixture was then treated with 500 µl of hexane in order to recover p-xylene in the organic phase for further analysis by gas chromatography. GC analysis was carried out on a HP chromatographer 5860 after recovering 400 µl of the organic phase from the previous mixture (water: p-xylene/hexane). Thus 400 µl of the hexane layer was treated with anhydrous sodium sulfate to remove water that could be carried through. The mixture was centrifuged and 1 µl was taken for GC analysis using the column HP Wax - Crosslinked Polyethylene Glycol 30 m x 0.25 mm id, x 0.50 µm, coupled to the Hewlett Packard 5890 Series II gas chromatographer with flame ionization detector. The results were plotted in a graph of peak area against p-xylene concentration.

Cloud point extraction of p-xylene
Extracts of *Bacillus* 0G that were obtained from cultures in exponential growth, thus harvested by centrifugation, and solutions prepared using purified lipopeptides (surfactin and/or fengycin - Lipofabrik, Villeneuve d’Ascq, France) were used to try and
remove the contaminating p-xylene from water. Initially a mixture of artificially contaminated water was prepared using 50 µl of p-xylene and 450 µl of lipopeptide extract to form a two-phase system containing water saturated with p-xylene (Table S3). In the presence of crude biosurfactant extracts, a layer of denatured micelles is obtained (cloud point), allowing the recovery of water from underneath (water layer). This was made possible by carefully leaning the polypropylene tube and laterally inserting a 200 µl pipet tip to remove a total of 400 µl of the water phase in two steps (Figure S1).

- *Extractions using purified surfactin and fengycin* were carried out using separate or mixed solutions - with and without - a protein hydrolysate from bovine collagen (Sanavita, Piracicaba, Brazil), which was added in order to lower the biosurfactant solubility by ion-pairing (Vanhoute et al. 2009). In addition, a control experiment was set up using the anionic surfactant sodium dodecyl sulphate (SDS). The stock solutions of surfactin, fengycin and collagen (hydrolysed) were prepared at the following concentrations: (i) lipopeptides were prepared in water at 1 g l⁻¹, respectively 1 mmol l⁻¹ surfactin and 0.7 mmol l⁻¹ fengycin; (ii) collagen hydrolysate at 10 g l⁻¹; 20 mmol l⁻¹ sodium dodecyl sulfate (SDS). Several proportions of lipopeptides were prepared in water artificially contaminated with p-xylene as shown in Table S3.

**Transesterification of fatty acids from microbial biomass and GC analysis**

After 72 h of growth, a 100 ml aliquot of culture was centrifuged and the pellet was kept. The cell pellet was re-suspended in 8 ml of methanol (100%) using a vortex. To the suspension a volume of 1.4 ml of concentrated sulfuric acid, 2 ml of hexane and a few glass beads were added and the solution was placed in a water bath at 80°C for 30 min according to the work of Johnson and Wen (2009). All extractions were performed in hexane. For quantification, the hexane was transferred to a clean and pre-weighed tube for solvent evaporation and quantification of the synthesized biodiesel.
GC–MS analysis of total lipid constituents

One µl was injected with PTV inlet used in splitless mode into a gas chromatography coupled with mass spectrometry TRACE DSQ (Thermofinigan, USA). The GC column was a Zebron ZB-FFAP (Phenomenex, Lane Cove, NSW, Australia), length 30 m, internal diameter 0.25 mol l-1, film thickness 0.25 µm. Injector temperature is at 200°C. The gas flow rate through the column was 1 ml/min. The column initial temperature was kept at 70 °C for 5 min. Then temperature was increased from 70 to 250 °C at a rate of 4 °C/min, held on for 1 min. Transfer line temperature was 270 °C. Ionization was achieved at EI mode (ionization energy: 70 eV) at temperature of 200°C, gas: Helium at 1 Bar. Masses were acquired in TIC between m/z 50 at 600 when the acceleration voltage was turned on after a solvent delay of 3min. All data were processed by Xcalibur (Thermofinigan, USA). All compounds were identified by comparing both the MS spectra and retention index with those available in libraries, i.e. NIST, Wiley.

Results

Strain characterization and identification of recoverable metabolites

- Genetic characterization

*B. subtilis* and *B. amyloliquefaciens* are important lipopeptide-producing bacterial species, which are closely related. Considering the morphological and biochemical similarities amongst the *Bacillus* genus and especially in relation to these two members (Blackwood et al. 2004), the classification of these organisms is troublesome. Moreover, a high proximity amongst these two species has been reported at the molecular level (Xu, and Coté 2003). Characterization of the strain *Bacillus* 0G used in
this study was carried out by sequencing the 16S rRNA and gyrase sub-unit genes. Three *Escherichia coli* JM109 clones, harbouring pGEM-T Easy with a 16S rRNA gene amplicon were sequenced, but only one insert could be sequenced from both pGEM-T Easy primers M13-FP and M13-RP. After analyses of the whole and partial sequences (from the other two clones) by both NCBI BLASTn and Ribosomal Database Project softwares, it was concluded that this strain unambiguously belongs to the *Bacillus subtilis/Bacillus amyloliquefaciens* group (identical scores; 99% identity), which is in agreement with our previous analyses (Etchegaray et al. 2008). However, the identification of strain 0G was more accurately determined using its *gyrA* and *gyrB* genes (both inserted in pGEM-T Easy) sequences. Analyses by the NCBI software showed that this strain is closely related to *Bacillus amyloliquefaciens* subsp. *plantarum* (99% identity for both *gyrA*/ two clones and *gyrB*/ two clones). Considering the novel DNA sequence data, based on *gyrA*/*gyrB* DNA sequencing, we propose a novel classification for strain 0G, thus *Bacillus amyloliquefaciens* 0G. Moreover, the LC-MS chromatogram in TIC mode (Figure 2C) has strengthened this identification as it confirms the production of the three lipopeptide families (Hao et al. 2012). The current analysis and the recent data available in literature indicates that this strain could also be named *Bacillus velezensis* (Dunlap et al. 2016).

- **Growth, primary and secondary metabolites production in Landy’s medium**

Initial studies were carried out to analyze the yields of interesting recoverable primary metabolites, lipopeptides and biomass using the original conditions of Landy et al. (1948), which uses glucose and glutamic acid, respectively as the major carbon and nitrogen sources (M1). Figure 2A shows the results of biomass, surfactin and fengycin production observed every 24 hours. Glucose consumption and the consequent production of the primary metabolites lactate, acetate and acetoin are shown in Figure
2B. Figure 2C presents the pattern of lipopeptides observed by LC-MS analysis of concentrated supernatant.

Figure 2A shows that within the first 24 h, the culture presented fast growth and reached a maximum of 1.48 g DW l⁻¹. The biomass did not change significantly, but decreased slowly until 48 h of cultivation. An interesting observation is that specific metabolites from primary metabolism, \textit{i.e.}, lactate, acetate and acetoin, normally produced under low oxygen levels, can be detected in high levels in the extracts of M1 (glucose-containing media). Lactate production reached 3.67 g L⁻¹ after 24 h of culture. Acetoin production in M1 reached its maximum level between 24 and 48 h of growth (9 g l⁻¹), and then showed a slight decrease (Figure 2B). Moreover, a high increase of acetate production was observed between 48 and 72 h of growth. This phenomenon can be explained in terms of acetoin degradation, the concentration of which being reduced to about 1 g l⁻¹ during this period (Figure 2B).

Strain 0G produced very low amounts of lipopeptides in M1. Surfactin and fengycin specific production reached their maximum after 24 h of growth at 2 mg g DW⁻¹ and around 5.5 mg g DW⁻¹, respectively. Surprisingly, the surfactin concentration and specific production decrease until the end of culture. LC–MS analysis was carried out on concentrated supernatant revealing primarily the presence of fengycin (between 25 and 40 min of elution), small quantities of surfactin (between 40 and 48 min of elution) and bacillomycin (between 4 and 11 min of elution). The use of mass spectrometry (TIC mode) as detector in LC-MS analysis reveals the presence of bacillomycin F, which was not detectable by RP-HPLC with UV detection, for certainly below the detection threshold.

\textbf{Growth, primary and secondary metabolite production using renewable media}

- \textit{Growth analysis}
Figure 3 presents the results of biomass, primary and secondary metabolite production in modified Landy’s medium (M2-M4). In order to facilitate the interpretation, the results obtained in M1 were included. Figure 3A shows that within the first 24 h, all cultures presented fast growth for the four different media (M1-M4). After 24 h, the cell weight in the M2 media reached 1.91 g DW l⁻¹. These biomasses did not change significantly, but decreased slowly until 48 h of cultivation. In M3 and M4, cell weight increased during 72 h, yielding 3.06 and 2.15 g DW l⁻¹ respectively. Media containing glucose as major carbon source showed higher growth rate up to the first 24 h, and then the growth rate changed sharply. In contrast, the growth rate presented in media containing glycerol as the major carbon source remained constant up to 48 h of growth. This pattern changed into a slower increasing rate up to 72 h of growth (Figure 3A). Comparative results were also obtained by optical density measurements (data not shown).

- **Production of lipopeptides using renewable media**

Two different patterns for specific surfactin production were obtained as presented in Figure 3B. Considering the first 24 hours of cultivation, the specific production of surfactin was considerably lower in M1 and M2, which presented only approximately 4% of the yields of M3 that uses glycerol as a substitute for glucose. Also, in the first 48 hours of growth, the specific production of surfactin in M2 reached 20% of its corresponding production in M4. After 72 h of cultivation, the specific production of surfactin increased in each media; which could be explained by a decrease in biomass yields during the last 24 h of growth (Figure 3A).

Production of the fengycin lipopeptide family was also evaluated during the growth of *Bacillus 0G* in these different media. It reached its maximum after 24 h in M4 (40 mg l⁻¹), and did not change with time, remaining almost constant until the end of cultivation.
Peaks of fengycin production were observed, for instance, 86 mg l⁻¹ and 25 mg l⁻¹, respectively after 24h of cultivation in M2 and 48 h in M3 (data not shown). It is interesting to note that the sharp decrease in fengycin levels observed in M2, correlated with glucose consumption after 24 hours, suggesting that possibly the produced fengycin that accumulated in the broth could have been degraded by the cells due to a decrease in available carbon source. Such starving period thus starts after 24 hours of growth as shown in Figures 2 and 4. This rational would explain the steep decrease in the recently produced fengycin. On the other hand, the positive effect of arginine in the production medium is highlighted when the yields of fengycin biosynthesis are correlated to biomass production (Figure 3C). Thus, for fengycin biosynthesis, there were two patterns of substrate induction, one based on the use of arginine as the major nitrogen source for both medium combinations glycerol/arginine (M4), and glucose/arginine (M2), which yielded approximately 5 to 9 times more lipopeptide than in the media combinations using glutamic acid as the major nitrogen source, respectively M3 and M1, especially considering the initial 24 h of growth. The highest specific production of fengycin occurred in M2 and M4, yielding more than 40 mg g DW⁻¹. In order to confirm that this effect of arginine is not only an additional nitrogen input due to its concentration in M2 and M4, which is equivalent to glutamate (e.g: 5 g l⁻¹), we have carried out an experiment in which the molar ratio of nitrogen was exactly the same used in medium M1 by providing exactly the same amount of nitrogen that is present when you add 5 g l⁻¹ of glutamic acid. Since one mol of arginine has 4 mols of nitrogen atoms and 1 mol of glutamic acid has only 1 mol of nitrogen, the amount of arginine was reduced 4 times, i.e., we added only 1.75 g l⁻¹ of arginine instead of 5 g l⁻¹. In this latter medium, the specific production of fengycin was 3 times higher than in M1 (data not shown). This result shows clearly that arginine induces the production of fengycin and, more importantly, it also shows that an input of nitrogen in M2 and M4.
(in which the amount of nitrogen is 4 times higher than in M1) also contributed to the production of fengycin. Moreover, the use of glycerol instead of glucose also appears to be beneficial for fengycin production. The specific production replacing glucose by glycerol was two times higher in M3 than in M1 up to 48 h of growth (Figure 3C). This additional effect was not observed if arginine was the major nitrogen source. Production of bacillomycin was also detected by RP-HPLC in the 25 times concentrated supernatant, but no significant peaks could be quantified.

MALDI-ToF analysis of the cell pellet (Figure 5) demonstrates the presence of surfactin A (C13: m/z 1030.9 and 1046.9, C14: m/z 1060 and C15: m/z 1074), bacillomycin F (C13: m/z 1081, C14: m/z 1095, C15: m/z 1109, C16: m/z 1123 and C17: m/z 1137.9) and fengycin A and B (A C15 or MB C13: m/z 1487, A C16 or B C14: m/z 1501, A C17 or B C15: m/z 1515.78, A C18 or B C16: m/z 1529.85 and B C17: m/z 1543.9). In view of the analysis it is tentatively assumed that M4 is the best medium for the coproduction of bacillomycin, fengycin and surfactin.

- **Glucose consumption and primary metabolite production**

Considering glucose, glycerol and amino-acid as the major carbon sources, all cultures started with approximately the same quantity of carbon. For a pattern of substrate consumption during the growth of strain 0G in different media, Figure 4A shows that a similar pattern was observed for growth media M2 as observed previously in M1, demonstrating that after 24 h of growth, glucose was exhausted. This result explains the lower levels of biomass production observed in media containing glucose (Figure 2B and 4A), indicating that strain 0G reached the end of growth. On the other hand, the glycerol-containing media (M3 and M4), showed a constant rate of glycerol degradation, as demonstrated by the decreasing curve, that reaches ~ zero substrate level after 72 h of growth (Figure 4A). For glucose containing media (M1 and M2), the
pattern of primary metabolites production is fairly similar. Lactate production reached 2.38 g L\(^{-1}\) after 24 h of cultivation in M2. However, it remained very low in the glycerol-containing media (M3 and M4). This feature can be observed in Figure 4B. Additionally, lactate concentration started a steep decreasing rate within the following hours, almost reaching zero at harvesting time.

In Figure 4C, we can identify two distinct patterns for acetoin production. These behaviors are in consistency with the different substrates that were used. Acetoin production in M2 is similar to the observed in M1, it reached a maximum level of 9 g l\(^{-1}\) after 48h of growth), and then slightly decreasing (Figure 4C). In media containing glycerol (M3 and M4) acetoin production was constantly increasing and reached a maximum value of respectively 6.33 g l\(^{-1}\) and 7.65 g l\(^{-1}\) after 72h. Acetoin production appears directly related to the consumption of carbon source characterized by an inverted pattern. Rather similar patterns are observed for acetate production in each media until the first 48h of growth (Figure 4D). In M3 and M4, the acetate production increased gently until 24 h of growth and remained stable up to the end of cultivation. Interestingly, a high increase was also observed in M2 between 48 and 72 h of growth (similar to M1). However, the highest values are once more detected in the glucose-containing media (Figure 4D).

**Sustainable growth and applications of biomass to produce biodiesel**

In our experiments, a direct transesterification reaction was designed that allowed production of biodiesel just after harvesting the cells by centrifugation to promptly separate the most important products (biosurfactants), which are segregated in the broth. The experiments revealed that the best source of lipids for biodiesel production corresponded to the biomass produced in M1 and M2, which yielded respectively 75.94 ± 10.83 mg g of DW\(^{-1}\) and 75.37 ± 9.78 mg g DW\(^{-1}\) of biodiesel. The yields for M3 and
M4 were respectively 66.88 ± 3.35 mg g DW⁻¹ and 69.48 ± 7.83 mg g DW⁻¹ (data not shown). The pattern of the biodiesel produced from the biomass of M1, which gave the best yields is presented in Figure S2 (Supporting Information). When compared to fatty acid standards, the results indicated that C14, C15 and C16 are the major fatty acids present in the cells, a pattern that was observed in all growth media (M1-M4).

**p-Xylene extraction from artificially contaminated water**

When p-xylene was added to the crude extract of lipopeptides, a top layer of denatured micelles mixed with p-xylene was formed at the water interface (cloud point). Upon leaning the microcentrifuge tube (Figure S1), we recovered water that contained only minor amounts of p-xylene. Table 1 presents the best results for p-xylene extraction, which were basically given by the crude biosurfactant extract and by the mixtures of purified lipopeptides prepared in the presence of collagen hydrolysate, in order to promote instability in the colloidal suspension by ion-pairing (Vanhoute et al. 2009).

**Discussion**

Gunaseelan and Ramkrishna (2014) made a prediction for a global consumption of about half a million tons of biosurfactants in about two years. However, the production of biosurfactants is not yet economically viable. On the other hand, it is possible to lower the production costs, either using cheaper substrates and/or adding value to microbial secondary products such as biomass and other biotechnological products present in the cell-free extracts. One of the most important applications for the biosurfactants is in the pharmaceutical industry, thus requiring the use of chemically defined media to facilitate their isolation (Rangarajan and Clarke 2015). Despite the use of a chemically defined medium such as the Landy’s medium, in order to lower the production costs, the composition of the medium can be modified. Therefore, in the present work, we have developed alternative media based on recyclable substrates, for
instance, glycerol, which is a co-product of biodiesel production, corresponding to 10% of the weight of the product after transesterification reactions (Yang et al., 2012). Another alteration in the original medium was the exchange of glutamic acid for arginine, which is an extra source of organic nitrogen. After the growth and the production of biosurfactant in these renewable media, we developed an original and sustainable approach based on circular bioeconomy to take an advantage of the biosurfactant and biomass produced. Firstly, the biomass was used for biodiesel production, and a crude biosurfactant extract was successfully used for water decontamination (Figure 1).

**Production of biomass, acetoin and lipopeptides**

Using *B. amyloliquefaciens* 0G we showed that the coproduction of biosurfactants can be attained in modified Landy’s media containing glycerol and arginine. The results showed that during the growth of this strain in the four different media, fengycin specific production was higher than surfactin specific production (Figure 3B and 3C). However, the use of glycerol as carbon source induces the production of surfactin in comparison with the results obtained for glucose (Figure 3B). Alternatively, lower production levels of fengycin under glucose starvation suggest that its biosynthesis is down-regulated under specific conditions as in the case of surfactin (Mueller et al. 1992; Koumoutsi 2006). For example, under carbon starvation, *Aspergillus niger* has been shown to express a number of hydrolases and to modify its metabolism in order to survive (Nitsche et al. 2012).

In addition, there are studies on the availability of nitrogen. Davis et al. (1999) showed that inorganic nitrogen is an important factor for surfactin synthesis. The authors studied the influence of carbon and nitrogen limitations on surfactin production in aerobic and in oxygen-limited conditions, concluding that the evaluated strain (*B. subtilis* ATCC
21332) also may require a source of organic nitrogen for growth and lipopeptide production. An alternative is the use of basic amino acids, which have been reported previously to be an important organic source of carbon and nitrogen (Poirier and Demain 1981). More recently, Liu et al. (2012) reported on the importance of medium composition for the specific production of the surfactin isoform (C15), in which the fatty acid moiety has 15 carbons and presents novel synergistic antifungal activity. Here, the addition of arginine in the medium was shown to induce the growth and production of fengycin. The lipopeptide production profile presented is relevant information since it provides the best harvesting stages for the different biosurfactants, produced under different growth conditions. It also demonstrates that the best carbon/nitrogen sources for fengycin production at early stages of growth (up to 24 h) are respectively glucose/arginine and glycerol/arginine. This latter result suggests that arginine induces production in both combinations of carbon and nitrogen sources; possibly, given that ornithine, which is incorporated on the structure of fengycin (Ongena and Jacques 2008), has a connecting biosynthetic pathway involving arginine degradation (Belitsky and Sonenshein 1998). Poirier and Demain (1981) also reported that arginine induces growth and gramicidin S biosynthesis by Bacillus brevis, presumably because it contributes indirectly by supplying a substrate for ornithine biosynthesis. Production of bacillomycin was not detectable by RP-HPLC analysis, but it was by LC-MS and MALDI-ToF in the concentrated supernatant as well as in the cell pellet. The hypothesis that bacillomycin was mostly adsorbed on the cell is plausible as demonstrated previously on other strain of B. amyloliquefaciens (Yuan et al. 2014).

Selectivity is an important factor due to coproduction of lipopeptide families and isoforms (Rangarajan and Clarke 2015). This study indicates that the use of arginine in chemically defined medium can select for fengycin production and this, in connection with aeration could be studied to further increase specific yields.
The use of glycerol as carbon source was also important as it contributes for microbial growth and for the carbon balance. Indeed, the amount of biomass obtained using glycerol is almost 2-fold higher than with glucose. Moreover, this correlates with the amount of primary metabolite secreted in the different media. For instance, using glucose, large amounts of lactate and acetoin are quickly produced, which represent a divergent use of carbon for biomass production. These distinct primary metabolite-producing profiles could be explained by an excess of carbon source uptake in the case of glucose. This primary metabolite production has been demonstrated previously with other strains of *Bacillus* using glucose as the main source of carbon (Nakano et al. 1997) and specially using Landy’s medium during growth under oxygen limitation (Coutte et al. 2010b; Coutte et al. 2013).

In this work we have found that strain 0G can also produce large amounts of acetoin (~9 g l⁻¹) regardless of the source of carbon used, but with different kinetics. This observation adds a very interesting product to be used in crop protection, and thus add value to the extracts that also contain the lipopeptides that have recognized biopesticide properties (Ongena and Jacques 2008; Deravel et al. 2014). Indeed, it is now proven that acetoin is able to induce systemic resistance (ISR) in plants. In addition, its recovery from a fermentation broth can easily be carried out following the procedure described previously (Li et al. 2010). The steps of this method could also be easily integrated into the continuous production and purification process of the lipopeptides using a multistage membrane bioreactor developed by Coutte et al. (2013).

**Biodiesel production**

Biodiesel is a biofuel prepared from vegetable oils and animal fat that has been produced worldwide in order to contribute with sustainability. In principle, biofuels such as bioethanol and biodiesel are sustainable because they originate from crops.
Therefore, the CO₂ produced from their burning can be recycled. However, the major source of lipids used in biodiesel production is from edible oils, therefore the production of biofuel from crops adds a pressure on the price of food in addition to cause deforestation in some regions (Martínez et al. 2015). An alternative is the microbial production of biodiesel, which has been proven successful from oleaginous microorganisms such as microalgae, yeast and fungi (Meng et al. 2009). When compared to plant oils, microbial lipids have a number of advantages as quick production, which is not affected by season and climate and it is easy to harvest. The costs of biodiesel production using vegetable oil is about US$ 1.00 per liter, while the price using waste cooking oil is approximately 1/3 of that (Skarlis et al. 2012). The microbial production has been evaluated primarily for microalgae (Gao et al. 2012). If other microorganisms are to be used, in order to reduce costs, it is necessary to evaluate the expenses with growth media. In this sense, microalgae are less expensive as they are photosynthetic organisms and can also be used to clean the water. However, in the case of microbes used at biotechnology industrial units, the costs associated with biomass production are null, since the profit is based on the products rather than on biomass, which is in fact residue. Therefore, the use of biomass as a novel source of lipids, in this case, is considered profitable as this revenue can lower the costs of the actual biotechnological process. Though the biomass from Bacillus is not considered a good source of lipids (only 20% of the weight), it is for sure characterized as a source of branched chain fatty acids, which are important biodiesel additives to ameliorate the flow of biodiesel in cold weather (Kaneda, 1977; Tao et al. 2015). Besides the potential applications as additives, some fatty acids identified in the biomass of B. amyloliquefaciens 0G potentially correspond to hydroxy-fatty acids, which are also important to use as diesel additives and for the chemical synthesis of surfactins, or yet as antifungal compounds (Black et al. 2013).
Here, considering circular economy, we evaluated the synthesis of biodiesel from the biomass of *B. amyloliquefaciens* 0G, which is a source of important lipopeptides. The results indicated that it would be appropriate to explore this possibility considering that large quantities of microbial biomass would be generated every 48/72 h at industrial level. Since biodiesel production generates glycerol as a co-product, indicated in Figure 1, this could be recycled in novel growth media, thus making a full cycle for glycerol.

The production of biosurfactants using large fermenters (~ 300 m$^3$), would produce ~ 1 ton of biomass, based on an average production of 3g l$^{-1}$ using Landy’s medium (Coutte et al. 2010b). Considering that *Bacillus* biomass has ~ 16% of lipids (Kaneda, 1977), if fully converted into biodiesel a single 72 h growth in a 300 m$^3$ fermenter would produce ~ 160 kg of biodiesel containing branched-chain and hydroxylated fatty acids. Also, it would generate approximately 16 kg of glycerol. GC analysis (Figure S2) reveals that fatty acids present in the cell were mostly C14, C15 and C16. In addition, MALDI-ToF analysis of the produced lipopeptides shows that the isoforms of lipopeptides contain mostly C14, C15 and C16 fatty acids in surfactin and fengycin structures, whereas C16 and C17 fatty acids were found in bacillomycin. Hence, there is a potential correlation between the fatty acids in the cells and those that are incorporated into the lipopeptide. This is an important finding because it shows that, at the end of lipopeptide production, the unused microbial biomass is still a source of valuable material. Branched chain and hydroxylated fatty acids are good additives for the operation of biodiesel in cold temperatures. At low temperatures, hydroxylated fatty acids have been shown to contribute to increase lubricity of diesel (Goodrum and Geller 2005).

Water decontamination
Applications of lipopeptides (biosurfactants) for environmental decontamination have been previously described (Franzetti et al. 2010). Here, we show for the first time the application of a biosurfactant crude extract from *B. amyloliquefaciens* 0G to remove p-xylene from artificially contaminated water. The crude lipopeptide extract from strain 0G grown in M4 was able to remove approximately 96% of p-xylene. This result adds another environmental application for biosurfactants from *Bacillus* spp., demonstrating their importance in other fields besides the known plant protection (biocontrol), food industry, cosmetics and medical fields. The main purpose of lipopeptide production in chemically defined growth media is easier recovery, for instance for pharmaceutical applications (Rangarajan and Clarke 2015). However, the above results indicate that during isolation of the coproduced lipopeptides, some impure fractions mixed with proteins and other peptides originated from the broth, would find usefulness in water decontamination. These results demonstrate other applications for the biossurfactants and add value to crude extracts.

**Concluding remarks**

An integrated design for lipopeptide production, including synthesis of biodiesel/biodiesel additives and recycling of substrates (circular bioeconomy) was proposed. These findings may be relevant on large-scale biotechnological processes of *Bacillus* sp., given the volumes and yields of crude biosurfactant and biomass that are generated. Considering the balance between costs and productivity, the integrated approach of lipopeptide production in modified Landy’s medium, including biodiesel production, substrate recycling and remediation (using crude lipopeptides) is an alternative to reduce production costs.

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Yuan, J., Zhang, F., Wu, Y., Zhang, J., Raza, W., Shen, Q. and Huang, Q. 2014. Recovery of several cell pellet-associated antibiotics produced by *Bacillus*
### Tables

**Table 1.** Results of p-xylene removal from artificially contaminated water by the isotropic-phase separation method.

<table>
<thead>
<tr>
<th>Test</th>
<th>Surfactant</th>
<th>Collagen hydrolysate (10 mg ml(^{-1}))</th>
<th>Water (µl)</th>
<th>Ammonium sulfate (mg)(^{b})</th>
<th>HCl (1 mol l(^{-1}))(^{c})</th>
<th>p-xylene mol l(^{-1})</th>
<th>Removal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Water control(^{a})</td>
<td>-</td>
<td>450</td>
<td>-</td>
<td>-</td>
<td>0.81</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Extract from <em>Bacillus</em> sp. 0G M4 (450 µl)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.81</td>
<td>93.4 ± 2</td>
</tr>
<tr>
<td>3</td>
<td>Extract from <em>Bacillus</em> sp. 0G M4 (450 µl)</td>
<td>-</td>
<td>-</td>
<td>150</td>
<td>-</td>
<td>0.81</td>
<td>96.5 ± 1</td>
</tr>
<tr>
<td>4</td>
<td>Extract from <em>Bacillus</em> sp. 0G M4 (350 µl)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100 µl</td>
<td>0.81</td>
<td>97.0 ± 1</td>
</tr>
<tr>
<td>5</td>
<td>(20 mmol l(^{-1})) SDS (50 µl)</td>
<td>100 µl</td>
<td>250</td>
<td>-</td>
<td>-</td>
<td>1.98</td>
<td>97.4 ± 0.3</td>
</tr>
<tr>
<td>6</td>
<td>(1 mmol l(^{-1})) Surfactin (100 µl)</td>
<td>100 µl</td>
<td>250</td>
<td>-</td>
<td>-</td>
<td>1.76</td>
<td>83.7 ± 0.2</td>
</tr>
<tr>
<td>7</td>
<td>(0.7 mmol l(^{-1})) Fengycin (100 µl)</td>
<td>100 µl</td>
<td>250</td>
<td>-</td>
<td>-</td>
<td>1.76</td>
<td>93.0 ± 2</td>
</tr>
<tr>
<td>8</td>
<td>50 µl of Surfactin (1 mmol l(^{-1})) and 50 µl of Fengycin (0.7 mmol l(^{-1}))</td>
<td>100 µl</td>
<td>250</td>
<td>-</td>
<td>-</td>
<td>1.76</td>
<td>95.6 ± 0.9</td>
</tr>
</tbody>
</table>

\(^{a}\) A control with water only was tentatively prepared, however, it was not applicable because the system (water/xylene) only forms cloud point above 40°C (Pryor and Jentoft Jr, 1961). As demonstrated in the present work, at room temperature the isotopic-phase separation is only obtained in the presence of surfactants and proteins.  
\(^{b}\) In the presence of the lipopeptide extract and at saturating concentrations of p-xylene the system spontaneously becomes cloudy (thus it undergoes isotopic-phase separation) as shown in test 2. However, to induce cloud point the mixture was also treated with solid ammonium sulfate.  
\(^{c}\) Also, considering that surfactin and surfactin micelles lose solubility in acid pH, HCl 1 mol l\(^{-1}\) was added to facilitate the process of micelle denaturation at room temperature.
Figure legends

Figure 1: Gait of the circular bioprocess followed in this study.

Figure 2: Productivity of strain 0G cultivated in M1 for 72 h and the corresponding standard deviations from triplicate samples. (A) Biomass production and yields of fengycin and surfactin (mg of peptide/g of dry cell). (B) Substrate consumption and primary metabolite production: ♦ Glucose; ■ Lactate; ▲ Acetoin; × Acetate. (C) LC-MS analysis of the concentrated cell-free medium extracts showing the major isoforms of surfactin, bacillomycin and fengicyn.

Figure 3: Comparative analysis of dry cell and lipopeptides produced during 72 h of growth using (M2-M4). (A) Biomass; (B) Surfactin; (C) Fengycin. Values are in mg of peptide/g dry cell. The results obtained using M1 were also presented for comparison.

Figure 4: Substrate consumption and primary metabolite production throughout 72 h of growth in modified Landy’s media (M2-M4) and the corresponding standard deviations from triplicate samples. (A) Glucose or glycerol consumption; (B) Lactate production; (C) Acetoin production; (D) Acetate production; ■ M2 using glucose; ▲ M3 using glycerol; × M4 using glycerol.

Figure 5: MALDI-ToF analysis of the cell pellet after growth in different media: (A) M1; (B) M2; (C) M3; (D) M4. Inset shows details of the fengycin isoforms present in the extract.
Microbial culture using renewable source of carbon and nitrogen

Production of Biomass

Production of Biosurfactant and Acetoin

Production of Biodiesel

Recycling of Glycerol

Recycling of Arginine and Glutamate

Protein from food waste products

Crop protection

Removal of organic pollutants
Glucose or Glycerol consumption (g l⁻¹)

Lactate production (g l⁻¹)

Acetoin production (g l⁻¹)

Acetate production (g l⁻¹)