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# Biosurfactant Production by *Bacillus* strains isolated from sugar cane mill wastewaters

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**HIGHLIGHTS**

- Interest in biosurfactants has increased in the last two decades.
- Surfactin from *Bacillus* is one of the most effective biosurfactants.
- *Bacillus* species are known as the main producers of extracellular enzymes.
- Isolation of new strains is strategic for several fields of biotechnology.

**Abstract:** Biosurfactants possess diverse chemical properties and provide important characteristics to the producing microorganisms, which can act as surface-actives and emulsifiers of hydrocarbon and others water insoluble substances. Most of them are lipopeptides synthetized by *Bacillus*. This study evaluated the biosurfactant production by strains of *Bacillus* previously obtained from liquid residues of sugar-alcohol industry. The bacterial isolates LBPMA: BSC, BSD, J1, J2 and L1 were cultivated in medium that induces production of biosurfactants (Landy medium). During 48 h of incubation, at intervals of 12 h, the total contents of proteins, reducing carbohydrates and surfactant activity of the filtrated growth media free of cells were evaluated. The results showed that these strains use glucose as a source of carbon, energy and for synthesis of surfactant. In this medium (24 h), the best producer of biosurfactant was the strain LBPMA-J2, molecularly identified

as *Bacillus thuringiensis*. Once the supernatant free of cells of this microorganism disperses the oil phase in the water, this strain has potential for being utilized on bioremediation processes.

**Keywords:** emulsification; oil dispersion; lubricant oil waste; bioremediation.

## INTRODUCTION

Throughout its existence, the human being makes use of microorganisms in many different processes and ways. Interest in surfactants produced by them (biosurfactants), for instance, has increased in the last two decades, because of its high degradability, biocompatibility and tolerance to variations of pH and temperature. These secondary metabolites are amphipathic molecules; therefore, they can reduce surface and interfacial tension of both aqueous and hydrocarbon mixtures, or emulsify non-polar molecules in water, as detergents [1-3].

The first generation of biosurfactants presented high cost of production due to their low recovery in microbial growth media with elevated price of substrates. However, for the second generation, costs were lower after the use of residuary renewable sources, making them more attractive to industries [4].

Many strains of the genus *Bacillus* can produce different surfactants in several carbon and nitrogen sources, as animal fat, industrial olive effluents, burned oil, whey and wastewaters with high contents of starch [5]. Therefore, the use of pollutant substances of liquid and solid wastes, as well as the screening of distinct microorganisms able to produce biosurfactants, as well as the genetic improvement of these strains, can be used to increase such production and reduce its high cost. Others researches investigate forms to increase the contact area of bacterial cells to micelles produced by polycyclic aromatic hydrocarbon (PAHs), polychlorinated biphenyls (PCBs) and petroleum. Such contact area promotes the action of the bacterial enzymes on those compounds, facilitating their biodegradation.

Biosurfactants are molecules from different types, such as glycolipids, lipopeptides/lipoproteins, phospholipids and fatty acids. *Bacillus* species generally produce lipopeptides biosurfactants. In general, mixtures of cyclic lipopeptides are built from variants of heptapeptides and hydroxy fatty acid chains. They are classified in three families: surfactin, iturin and fengycin [6] (Figure 1).

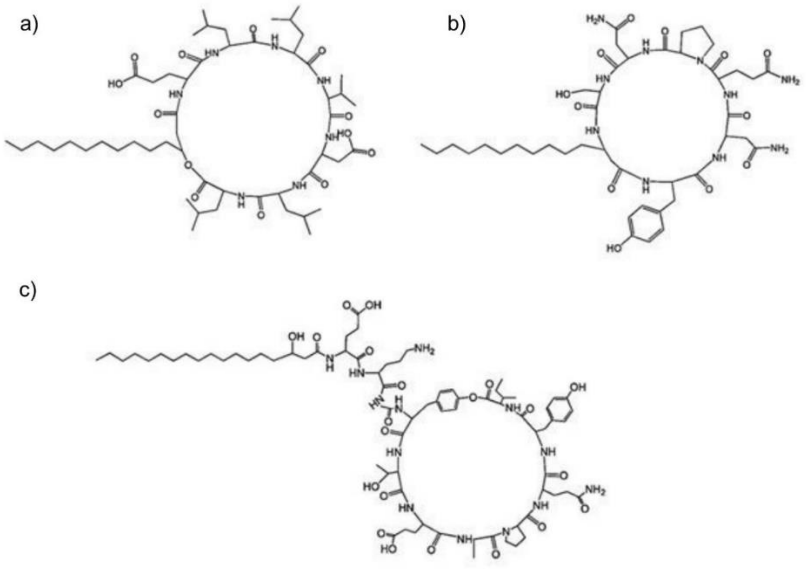


**Figure 1.** Chemical structures of some biosurfactants lipopeptides: (a) surfactin (b) iturin and (c) fengycin.

In *Bacillus*, the production of surfactin is associated with cell growth, occuring especially in the transition from exponential to stationary growth phase. On another hand, the biosynthesis of fengycins and iturins usually occurs later in the stationary phase [7]. Surfactin has been recognized as one of the most effective biosurfactants. Its primary structure is a cyclic lipopeptide consisting of seven amino acids bonded to the carboxyl and hydroxyl groups of a 14-carbon fatty acid [8]. The purpose of the present study was to evaluate the capacity of some *Bacillus* spp., from effluents of a sugar-alcohol industry, in producing biosurfactants after the consumption of proteins and reducing glycids on defined growth medium, providing too their identification (morpho-biochemical and molecular).

## MATERIAL AND METHODS

### Bacteria

Several bacteria were isolated from wastewater samples collected in dry opaque plastic bottles (previously rinsed with boiled distilled water), from the sedimentation pool ("A") of the Effluent Treatment Plant (ETP) in a sugar-alcohol industry ("S.A. Usina Coruripe Açúcar e Álcool"), from the South Coast of the State of Alagoas, Northeast of Brazil. Such bottles were immediately stored in a refrigerated box (≅4 °C) and transported to the LBPMA/IQB/UFAL (Laboratory of Biochemistry of Parasitism and Environmental Microbiology of the Institute of Chemistry and Biotechnology in Federal University of Alagoas, Brazil), before being diluted ($10^{-5}$ to $10^{-1}$) and inoculated in Nutrient-Agar (NA) plates, incubated for 24 h (30 ± 2 °C, at dark). After isolation, the appearance of the colonies was analysed as well as the morphology of the cells under the optical microscope, according to the Gram, Ziehn Nielsen and Malachite Green stains. Finally, the five isolates which showed Gram positive rod shaped and form endospore, were selected for the present study and stored in the LBPMA bacterial collection (- 80°C). Such strains received the internal codes: LBPMA: BSC, BSD, J1, J2 and L1. Their presumptive identification was also based in the investigation of the motility and production of indole, tests of Voges Proskauer and Methyl Red, or for expressions of the Catalase, oxidase, amylase, gelatinase and nitrate reductase, fermentation or oxidation of glycids (with or without the production of gas), use of citrate or urea as the sole carbon source, and growth in 6.5 % NaCl [9-12].

### Biosurfactant production

Flasks containing Landy medium (LM) [13] ($g.L^{-1}$ distilled water): glucose 20.0; glutamate 5.0; $MgSO_4$ 0.5; KCl 0.5; $KH_2PO_4$ 1.0; $FeSO_4.7H_2O$ 0.15; $MnSO_4$ 0.005; $CuSO_4.5H_2O$ 0.00016; yeast extract 10.0 were individually inoculated with 100µL of an aqueous suspension ($7 \times 10^4$ cells) of each isolate (cultures of 24 h in NA, at 30 ± 2 °C, at dark), and incubated for 48 h (at 30 ± 2 °C, 150 rpm, in an orbital shaker).

The cell growth, biosurfactant production, and total proteins and reducing glycids contents were monitored at intervals of 12 h (0, 12, 24, 36 and 48 h). For this, two aliquots (1 mL) of the growth medium were collected. The first one was used to cell counting, with the aid of the Neubauer Chamber. The other was filtered through cellulose membranes (0.22 µm) and the supernatant free of cells recovered to sterilized plastic microtubes stored at ≅4 °C to subsequent evaluation of surfactant activity and contents of total proteins and reducing glycids. All the assays were repeated three times, with three replicates per sample and parameter analyzed.

### Total reducing glycids (TRG) and proteins (TP)

The TRG were determined by using the 3.5 - Dinitrosalicylic acid (DNSA) method [14], using a standard curve with different concentrations of glucose in sterilized distilled water (0-1.0 $mg.mL^{-1}$). On the other hand, the TP were quantified as described by Bradford [15]. The standard curve was prepared using different dilutions of bovine serum albumin (BSA) in 0.15M NaCl (0-50 $mg.mL^{-1}$).

**Biosurfactant activity**

The oil dispersion test was carried out to evaluate the surfactant capacity of supernatants [16]. Diameter of the halo (clear zone) caused by the samples (triplicates) was measured and correlated with the ones caused by different concentrations of the standard synthetic surfactant Sodium Dodecil Sulfate (SDS), it means, (0.01, 0.05, 0.1, 0.25, 0.5 and 1 $mg.mL^{-1}$).

**Molecular identification of most effective strain in surfactant production**

The DNA extraction of the most effective strain regarding the surfactant production was performed using 24h-cultures (30 ± 2°C, 150 rpm) in Nutrient Broth (NB). The Bacterial Genomic DNA Isolation kit (Norgen Biotek®) was used according to the instructions of the manufacturer. The amplification of 16S rRNA gene was carried out using the primers 356F (ACWCCTACGGGWGGCWGC) and 1064R (AYCTCACGRCACGAGCTGAC), designed by WINSLEY *et al.* [17] and synthetized by GenOne.

The mixture for the Polymerase Chain Reaction (PCR) contained the following components (final volume of 25 µL): 100 µg genomic DNA template, 10 ρmol of each forward and reverse primers, 0.8 mM dNTPs, 2.5 mM $MgCl_2$, 10X reaction buffer (25 µL), 5 µg BSA and 1 U Taq polymerase enzyme. The PCR conditions were: initial denaturation for 10 min at 94°C, 2 min at 60°C, 2 min at 72°C, 40 cycles of 20 s at 94°C for denaturation, 45 s at 55.5°C for annealing, 1 min at 72°C for extension and 5 min at 72°C for final extension [18]. The amplified DNA was visualized with the aid of transilluminator (UV emitted directly on agarose gel stained with ethidium bromide). The amplicons were sequenced by the DNA Sequencing Platform from Federal University of Pernambuco (Recife, Brazil). Sequences were compared with others present at NCBI (National Center for Biotechnological Information) and RDP (Ribosomal Database Project). The dendrogram was created using *Tree Dyn software* [19].

**RESULTS**

**Biochemical and morphological aspects of the isolates**

The results of morphological tests (Table 1) of strains LBPMA: BSC, BSD, J1, J2 and L1 indicated they are Gram-positive spore forming rod bacteria. The main biochemical aspects of the studied isolates can be seen in Table 2. In respect to amylase, gelatinase and lipase, all strains were positives to degrade the specific substrates for such enzymes present in the growth medium. On the other hand, only strains LBPMA-L1 and LBPMA-BSD produced urease. In respect to glycids or peptone utilization by fermentation and/or oxidation, in the TSI test the strain LBPMA-J1 was the only able to aerobically desamine the peptone-amino acids, using their carbonic chain to obtain energy and the strain LBPMA-BSD was the only able to utilize citrate as carbon source. Therefore, with such characteristics and the support of identification keys of Skerman and Reva *et al*., the five isolates (strains LBPMA: BSC, BSD, J1, J2 and L1) were identified as *Bacillus* spp [20,21].

**Table 1.** Morphological characteristics of the five strains LBPMA: BSC, BSD, J1, J2 and L1, isolated from the wastewater of a sugar-alcohol industry in Alagoas, Brazil (+ positive result; - negative result).

| Aspects | Strains LBPMA | | | | |
|---|---|---|---|---|---|
| | **BSC** | **BSD** | **J1** | **J2** | **L1** |
| Colony Shape | Circular | Rhizoid | Irregular | Irregular | Irregular |
| Colony Elevation | Convex | Concave | Flat | Flat | Flat |
| Colony Margin | Entire | Filamentous | Lobate | Lobate | Lobate |
| Colony Texture | Smooth | Wrinkled | Filamentous | Wrinkled | Filamentous |
| Colony Brightness | Translucent | Opaque | Opaque | Opaque | Opaque |
| Colony Pigmentation | + | + | + | + | + |
| Colony Aspect | Mucoid | Viscid | Membranous | Membranous | Membranous |
| Gram stain | + | + | + | + | + |
| Malachite green stain | + | + | + | + | + |
| Ziehl –Neelsen stain | - | - | - | - | - |

**Table 2.** Biochemical characteristics of the five Gram-positive spore forming rods LBPMA: BSC, BSD, J1, J2 and L1, isolated from the wastewater of a sugar-alcohol industry in Alagoas, Brazil (TSI= triple sugar-iron; OF- oxidation/fermentation; + positive result; - negative result).

| Aspects | Strains LBPMA | | | | |
|---|---|---|---|---|---|
| | **BSC** | **BSD** | **J1** | **J2** | **L1** |
| TSI – agar medium | Glucose + lactose and/or saccharose | Glucose + lactose and/or saccharose | Glucose and peptone | Glucose + lactose and/or saccharose | Glucose + lactose and/or saccharose |
| $H_2S$ (TSI/SIM) | - | - | - | - | - |
| Motility | + | + | + | + | + |
| Indol | - | - | - | - | - |
| OF medium | Facultatively anaerobic | Facultatively anaerobic | Facultatively anaerobic | Facultatively anaerobic | Facultatively anaerobic |
| Oxidase | - | - | - | - | - |
| Catalase | + | + | + | + | + |
| Citrate | - | - | - | - | - |
| Voges-Proskauer | + | + | + | + | + |
| MethylRed | + | + | + | + | + |
| Urease | + | - | - | - | + |
| Gelatinase | + | + | + | + | + |
| Nitrate-reductase | + | + | + | + | + |
| Amylase | + | + | + | + | + |
| Lipase | + | + | + | + | + |
| Growth in 6,5% NaCl | + | + | + | + | + |

**Capacity of biosurfactant production by the studied *Bacillus* strains**

The Figure 2 shows the growth of the studied bacterial strains during 48 h of incubation in LM (indicated to stimulate the biosurfactant synthesis), while the Figures 3-5 express respectively the TRG, TP and oil dispersion capacity of their supernatants.The growth of the isolates LBPMA: BSC, J1, J2 and L1 at the studied conditions was exponential during the initial 24 h (Figure 2), with a stationary phase between 24 and 48 h. Only for LBPMA-BSD the stationary phase was initiated 12 h after inoculation. On the other hand, the population of the strain BSC declined after 24 h with a second phase of growth from 36 h.

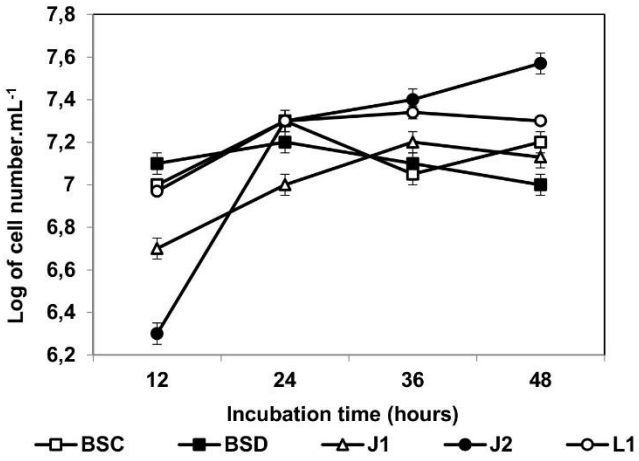


**Figure 2.** Growth of the bacterial strains LBPMA: BSC, BSD, J1, J2 and L1, in Landy medium (LM) during 48 h (at 30 ± 2°C under the dark and 150 rpm). The results are expressed as media of triplicate ± standard deviation.

In general, there was a consume of TRG by most of the studied strains along the time (Figure 3), and an increase in the TP (Figure 4), which can be explained respectively by the utilization of glucose as the initial carbon source for the cells growing and secretion of extracellular enzymes contributing for the amino acids and other compounds absorption. The cultures of LBPMA-BSC and LBPMA-BSD, which had a declination in their growth (Figure 2) between 24-36 h (more accentuated to the strain LBPMA-BSC), showed an increase in the TRG in such interval (Figure 3), being possible that some of their died cells have released this type of molecules to the medium. At the same time, it was seen a reduction in the content of TP in cultures of LBPMA: BSC, BSD and L1 (Figure 4), whilst for the others, at this interval, the TP content stabilized.

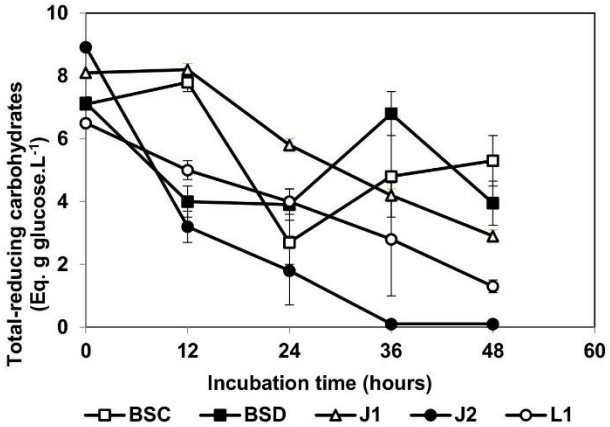


**Figure 3.** Concentration of total reducing-glycids (equivalent mg.mL$^{-1}$ of glucose) in the supernatant free of cells of cultures in Landy medium (LM) of the bacterial strains LBPMA: BSC, BSD, J1, J2 and L1, during 48 h (at 30 ± 2°C under the dark and 150 rpm). The results are expressed as media of triplicate ± standard deviation.

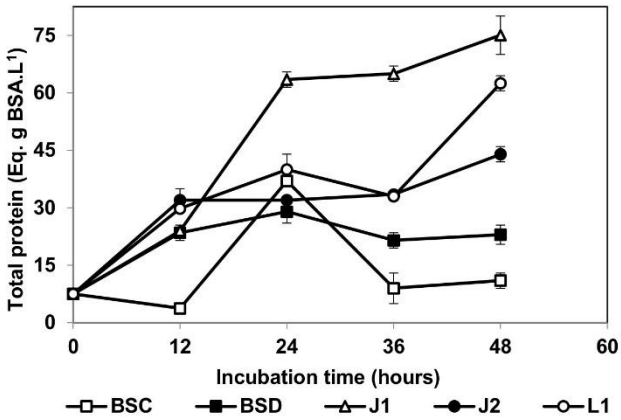


**Figure 4.** Concentration of total proteins (equivalent mg.mL$^{-1}$of bovin serum albumin) in the supernatant free of cells of cultures in Landy medium (LM) of the bacterial strains LBPMA: BSC, BSD, J1, J2 and L1, during 48 h (at 30 ± 2°C under the dark and 150 rpm). The results are expressed as media of triplicate ± standard deviation.

On the other hand, 12 h after the incubation, the strains LBPMA-J1 and LBPMA-L1 secreted more proteins than the others (Figure 4), and showed lower surfactant activity than the strain LPBMA-J2 (Figure 5). So, the strain LBPMA-J2 was selected for its best capacity to produce biosurfactant (Figure 5) after 48h of incubation in LM.

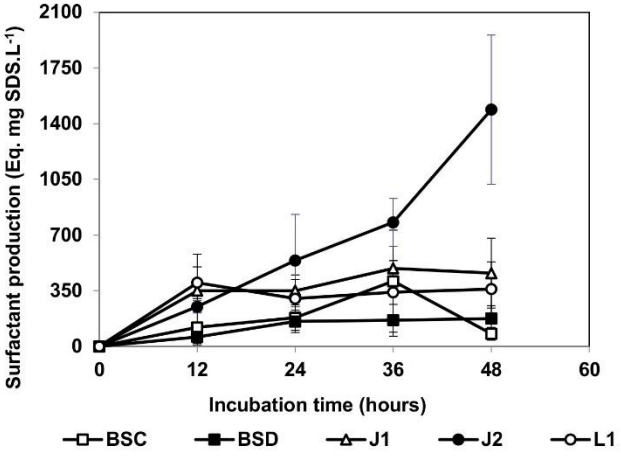


**Figure 5.** Concentration of biosurfactant (equivalent µg.mL$^{-1}$ of SDS) in the supernatant free of cells of cultures in Landy medium (LM) of the bacterial strains LBPMA: BSC, BSD, J1, J2 and L1, during 48 h (at 30 ± 2°C under the dark and 150 rpm). The results are expressed as media of triplicate ± standard deviation.

## Molecular identification of the best biosurfactant producer strain

Based on morphological and biochemical tests, all strains were found to be closely related to species of the *Bacillus* genus. As above mentioned, the strain LBPMA-J2 was selected to molecular identification due to its best performance in the dispersion oil test. The 16S rDNA of this strain showed 99% homology with *B. thuringiensis* – a member of the *B. cereus sensu lato* group – strains in the in the blast search analysis performed at Gene bank of the NCBI (Nucleotide database of National Centre for Biotechnological Information). The phylogenetic tree comprising this strain and some nearest bacterial species is present in Figure 6. The nucleotide sequence of the strain LBPMA J2, identified by its high homology with the ones of *Bacillus thuringiensis*, has been deposited in the NCBI and GenBank, and received the accession number MF580970.

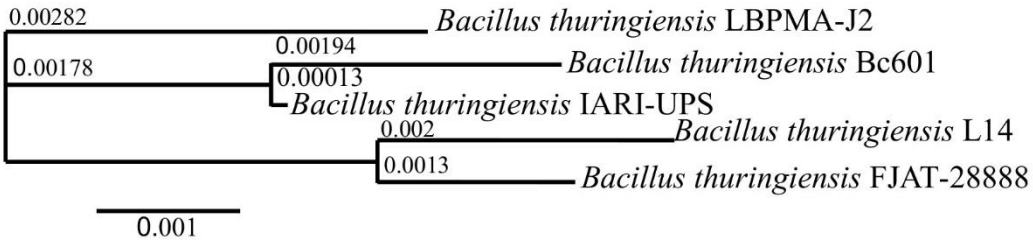


**Figure 6.** Phylogenetic tree inferred for the Bacillus isolate with the best biosurfactant production performed in this study, the strain LBPMA-J2, and some other species of *Bacilus* with 99 % identity (obtained from GenBank). Support values are calculated from 100 bootstrap replicates.

## DISCUSSION

*Bacillus* genus is characterized by peculiar colonies that are big, irregular and rhizoid, with ability to express catalase and grow under aerobiosis or facultative anaerobiosis [22]. Such sporeforming bacterial group exhibit a higher degree of resistance to inactivation by various physical insults, including wet and dry heat, UV and gamma radiation, extreme desiccation and oxidizing agents [23].

In terms of biotechnological uses, microbial enzymes are preferred to those from both plant and animal sources because they are cheaper to produce, and their enzyme contents are more predictable, controllable and reliable [24]. The urease, for example, is the enzyme responsible to split the urea molecule into carbon dioxide and ammonia, which may be a virulence marker of some pathogenic bacteria. However, in the present paper, the detection of this enzyme on strains LBPMA-L1 and LBPMA-BSD indicate they can participate in the recycling of environmental nitrogen [25], being rapidly degraded in soil by other proteolytic enzymes.

*Bacillus* species are known as the main producers of extracellular proteases, such as the ones that hydrolyses gelatin - a protein derived from the collagen that is a structurally more complex animal protein. Due to the excellent catalytic activity, in different conditions, of Bacillus proteases, they are largely used by industries [26]. This genus is also extensively used to produce amylase, an enzyme with application in industries of food, starch liquefaction, saccharification, detergent, brewing, paper, textile and distilling. In this study, the most specific substrate to the screening of amylases-producers, the starch, was used, although others could be utilized, such as starchy with high content of starch [27].

Lipases also have great potential in various industrial applications, such as chemical, pharmaceutical, paper manufacture, biosurfactant and agrochemicals. The ones from microbial sources receive great attention due to their rapid production and versatility. They acts on ester bonds of the hydrophobic triacylglycerols, so that the polysorbate Tween® - a surfactant used as substrate to lipases and esterases production, can be incorporated into the growth medium as substrate to monitoring such activity [28].

Various researchers have investigated the relation among extracellular biosurfactant, cell growth and the carbon source used as substrate. Rane *et al.* [29], for example, evaluated the effect of incubation time on the growth and biosurfactant production by *B. subtilis* ANR 88. They found 75% of the biosurfactant production during the first 24 h of incubation, whilst the bacterial growth ceased 12 h after the inoculation but the utilization of sugar and production of biosurfactant continued. Studies performed by Batista *et al.* and Zhou et al. showed that bacteria can grow and produce biosurfactant in medium with 2% glucose as carbon source [30,31], as it was seen in the present study. Al-Ajlani *et al.* [32] also observed high concentration of surfactin in cultures of *B. subtilis* MZ-7, after incubation of 24-48 h, but specially during the stationary phase (44 h). Studying the strain *B. subtilis* LAMI005, Souza *et al.* [33], found the highest surfactant production (263.64 $\mu g.mL^{-1}$) 30 h after the incubation in a medium using only glycerol as carbon source. de Sousa *et al*. [34] also used glycerol

obtained from the biodiesel production process and verified that strain ATCC 6633 from B*. subtilis* was able to produce surfactin.

According to Desai and Banat [35], the kinetic parameters to biosurfactant activity, quantity and quality, can be classified into four major types: growth-associated, production under growth-limiting conditions (carbon and nitrogen sources, minerals such as iron, magnesium, manganese, etc.), by resting-immobilized cells or dependent of precursors supplementation. Yakimov *et al.* emphasizes that glucose and saccharose stimulates more the biosurfactant production than others sugar sources [36].

Haddad *et al.* [37] found that biosurfactat production by *B. subtilis* HOB2 was growth-associated, since the strain produced surfactant within 24 h of incubation. However, in the study of Oliveira and Garcia-Cruz [38] with *B. pumilus*, the biosurfactant biosynthesis in medium containing vinasse or frying oil as carbon source, was not growth-associated.

Defining bacterial species is an ongoing debate and it is no different for Bacillus genus, in which about 70 species from distinct environments are of great technological interest, making the isolation of new strains strategic for several fields, from the pharmacology to the bioremediation of pollutants. *Bacillus* strains have been isolated, for instance, from cassava fermented products [39] as well as from petroleum refinery effluent [40]. Sidkey and Al hadry [41] demonstrated the ability of *B. cereus* B7, for instance, to synthesize biosurfactant with antimicrobial activity against *Candida albicans*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, whilst Tuleva *et al.* [42] were the first to register a B. cereus strain able to produce a ramnolipid biosurfactant under aerobiosis using naftalene (2%), n-alkanes or crude and vegetable oils as only sources of carbon.

The *B. cereus* sensu lato, also known as the *B. cereus* group, is known to contain several divergent pathogenic or not bacteria, and some are producers of biosurfactants. This group has a significant impact on human health, agriculture, and food industry, being formed by species very closely related, such as *B. anthracis*, *B. cereus*, *B. cytotoxicus*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis*, *B. toyonensis* and *B. weihenstephanensis* [43].

Once identification and phylogenetic relationships of bacteria within the *B. cereus* group are controversial, microbiologists have been working on alternative methods, besides the 16S rDNA analysis (i.e. its amplification and sequencing), like a novel variant of multi-locus sequence analysis (nMLSA) and screening of virulence genes, to better identify them [44,45].

Plaza *et al*. [46] screened between 13 environmental isolates of *Bacillus* obtained from diverse habitats to detect surfactant producers. They found that strains of *B. cereus*, *B. subtilis*, *B. pumilus* and *B. thuringiensis* produce in common the biosurfactant surfactin. *B. thuringiensis*, is commonly found in soil, phylloplanes, fresh water, marine sediments, as well as in activated sludges of a sewage treatment plant [47,48] and agro-industrial residues [49]. Ferreira *et al.* [40] found a strain of *B. thuringiensis* in contaminated marine sediments able to degrade pollutants such as phenanthrene and imidacloprid. The entomopathogenic and antifungal properties of *B. thuringiensis* are very well known, and the lipopeptide biosurfactant produced by its strain pak2310, like the commercial surfactin obtained from other *Bacillus*, albeit with a lower molecular weight, inhibits the fungus *Fusarium oxysporum* - an emerging human pathogen.

## CONCLUSION

The five strains of *Bacillus* studied in this research secrete amylase, gelatinase and lipase, and in the selective Landy medium (LM) they use glucose as the only carbon source for their growth and biosurfactant synthesis. Strains LBPMA-J1 and LBPMA-J2 produced 50% of their biosurfactant during the first 24 h of incubation, and the best production was reached by the isolate LBPMA-J2, a spore-forming rod-bacterium molecularly identified as *Bacillus thuringiensis*. Albeit *B. thruringiensis* has been often isolated from samples of soil, in this study it was found in sugar cane washing effluent, which generally has soil particles

and fragments of leaves in its composition. Further field experiments could prove the usefulness of this isolate in bioremediation of contaminated environments.

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