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OPTIMIZATION OF BIOFLOCCULANT PRODUCTION BY *Bacillus* spp. FROM SUGARCANE CROP SOIL OR FROM SLUDGE OF THE AGROINDUSTRIAL EFFLUENT

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Abstract - Biological flocculants are advantageous due to their biodegradability and safety to living beings. In this work, four bacterial strains, isolated from sugarcane cropped soil (*Bacillus megaterium* LBPMA-APFSG3Isox and *B. toyonensis* LBPMA-ACOPR1.Isox) and sludge of an agroindustrial effluent (*B. pumilus* LBPMA-BLD07 and *B. thuringiensis* LBPMA-EFIII), were studied. It was found that all of them secreted bioflocculants, at 37 ± 1 °C, with no pH changes over time and the flocculant activity increased during the time course of incubation. These results stimulated the optimization of the culture conditions to improve flocculation rates, such as the pH, nitrogen sources and carbon. For *B. toyonensis* LBPMA-ACOPR1.Isox and *B. thuringiensis* LBPMA-EFIII, the best pH for the bioflocculant production was 5.0. Sucrose and maltose were the best sources of carbon, while urea was the preferred source of nitrogen for two of the tested isolates (*B. pumilus* LBPMA-BLD07 and *B. toyonensis* LBPMA-ACOPR1.Isox), followed by $(NH_4)2SO_4$ (*B. megaterium* LBPMA-APFSG3Isox) and peptone (*B. thuringiensis* LBPMA-EFIII). The FTIR-ATR spectra of each extracted material responsible for the flocculant activities of the strains displayed carboxyl, hydroxyl and methoxy functional groups characteristic of polysaccharides.
Keywords: Bioflocculation; *Bacillus*; Polysaccharides secretion.

INTRODUCTION

Biological diversity is an important source for the development of sustainable tools for ecosystem management and opportunities of substances prospection, especially concerning microorganisms, since this life-group is still unknown compared to others such as animals and plants (Pylro et al., 2014), and they survive and proliferate in distinct environments, from natural to human managed. Hence, their biochemical adaptation makes microbial cells promising tools for biotechnological and ecoefficient utilization. The greatest challenge to rational use of such biodiversity is definitely transforming a high natural genetic heritage into biotechnological richness (Pylro et al., 2014).

Biological processes have conquered a remarkable place in global technological development, exhibiting economical and operational characteristics that confer a benefit regarding classical chemical methods (Barros et al., 2013). The *Bacillus* genus is one of the principal contributors to this issue. It can produce, for example, enzymes utilized to manufacture detergents and paper pulp, besides biosurfactant, polyhydroxyalkanoates (bioplastics) and bioflocculants. Their species are easily cultivated under different laboratorial conditions and can inhibit the growth of other microorganisms. These characteristics

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stimulate screening for strains expressing the synthesis of bioproducts suitable for different environments, especially for certain industrial processes (Martins and Hatti-Kaul, 2002). In this context, researchers have sequenced the complete genomes of bioflocculant-producing strains (Liu et al., 2017).

On another hand, the increase in industrial activities generates a large amount of effluent that can become a serious environmental problem, mainly when untreated wastewater is discharged into water bodies. In Brazil, there is a governamental environmental Council (CONAMA- "Conselho Nacional do Meio Ambiente") that established limits for some parameters for such destination of residues, mainly through legal resolution 430/2011 (CONAMA, 2011). This led to an increase in chemical flocculant utilization for wastewater treatment.

The purpose of the flocculation process is to form aggregates from finely dispersed particles in the presence of natural or synthetic polymeric substances called flocculants. The chemical flocculants are efficient and inexpensive, but have carcinogenic properties (Moussas and Zouboulis, 2009). On the other hand, bioflocculants are secondary metabolites produced during the growth of microorganisms (such as bacteria, fungi and algae), composed predominantly of polysaccharides, proteins, nucleic acids and lipids (Okaiyeto et al., 2015). Among these macromolecules, polysaccharide-based bioflocculant deserve attention due to their biodegradability, high flocculation taxes for metal removal and for use in wastewater treatment (Li et al., 2009; Liu et al., 2010). Few Brazilian studies have been published regarding this significant matter, and the purpose of this work was to screen *Bacillus* strains previously selected for being tolerant to the herbicides isoxaflutole, sulfentrazone and diuron, or producers of polyhydroxyalkanoates/biosurfactants, their capacity to also synthetize bioflocculant.

MATERIALS AND METHODS

Source of bacteria and inoculum preparation

Four bacterial strains maintained in the Biochemistry Laboratory of Parasitism and Environmental Microbial (Chemistry and Biotechnology Institute of University Federal of Alagoas, Brazil) were used. Two microorganisms (*Bacillus megaterium* LBPMA-APFSG3Isox and *B. toyonensis* LBPMA-ACOPR1. Isox) were obtained for the Atlantic Forest whose soil possesses a history of herbicide application, while *B. pumilus* LBPMA-BLD07 and *B. thuringiensis* LBPMA-EFIII were isolated from sludge of a Wastewater Treatment Effluent Station (Coruripe-AL, Brazil). The inoculum was prepared by reactivation of microbial strains on nutrient agar, during 24 h (at 32 ± 1 °C, dark). Then, 9 mL of sterile water were used to suspend each strain from the Petri dishes, and this suspension was submitted to a serial dilution to 10^8 cells.mL^{-1}, using liquid media for bioflocculant production, prepared as described below.

Bioflocculant production

Triplicates of each culture were prepared using aliquots of the particular inoculum suspension in 75 mL of the specific medium in Erlenmeyer flasks (125mL) to reach 10^8 cells.mL^{-1}. The mixture was incubated for 120 h at 37 ± 1°C in the dark and under orbital shaking at 160 rpm (the control was maintained without addition of bacteria). At intervals of 24h, dried biomass, bioflocculant activity and pH were evaluated in samples of the different cultures. The medium for bioflocculant production was composed (g L^{-1}) of: 20 glucose, 0.5 urea, 0.5 yeast extract, 0.3 $(NH_4)_2SO_4$, 2 KH_2PO_4, 5 K_2HPO_4, 0.1 NaCl and 0.2 $MgSO_4 \cdot 7H_2O$. Initial pH was adjusted to 7.0 (Zhang et al., 2007).

The whole experiment was divided into two stages. The first one was the analysis of the bacteria behavior during the time course (120 h), and the second was the selection of the best pH, carbon and nitrogen sources for bioflocculant production among the tested ones, after 24 h of incubation as above mentioned.

After centrifugation (5000 rpm, 20 min, 4 °C), the pH values of the supernatants free of cells were obtained with a pH meter. Precipitated cells were ressuspended in 1 mL of sterile distilled water and transferred to 1.5 mL Eppendorf microtubes, which were submitted to drying in the oven (40 °C during 24 h) until constant mass. Aliquots of 2 mL of the supernatant medium obtained after new centrifugation (5000 rpm, 30 min) were used as the test bioflocculant to determine the flocculating activity as described by Kurane et al. (1994). Briefly, 100 mL of kaolin suspension was added to 3 mL of 1% w/v $CaCl_2$ and supernatant. The mixture was agitated vigorously for 60 s and then poured into a glass volumetric cylinder (100 mL) and allowed to sediment for 5 min at room temperature. The optical density (OD) of the clarifying supernatant was measured at 550 nm with a UV spectrophotometer (Thermo Spectronic, USA) and the flocculating activity determined as follows: Flocculating Activity (%) = [(B - A) / A] x 100, where A and B are optical densities of control and samples, respectively, measured at 550 nm (Pathak et al., 2018).

Effects of initial pH, carbon and nitrogen sources

To determine the best sources of carbon and nitrogen of medium composition for bioflocculant production, each microorganism was inoculated in Falcon tubes (50 mL) containing 40 mL of specific medium for bioflocculant production.

The initial pH value was adjusted to 3.0, 5.0, 7.0 or 9.0 using NaOH 1.0 M or HCl 1.0 M in different

culture media, to identify the best condition. Flocculant activity and dried biomass were analyzed after 24h of incubation.

The carbon sources (20 g L^{-1}) utilized were: sucrose, maltose and glucose. Urea, peptone, ammonium sulfate and a mixed source of nitrogen (yeast extract, ammonium sulfate and urea) were used as nitrogen sources (1.3 $g.L^{-1}$).

Extraction and purification of the bioflocculant

Extraction and purification of bioflocculant produced by the studied bacterial strains were realized as described by Wong et al. (2012) with some modifications. After the selection of the best initial pH, carbon and nitrogen sources, and conditions for bioflocculant production, the culture broth incubated for 72h was centrifuged (4,000 g, 15 min, 4°C) and two volumes of ethanol were added to the supernatant. The mixtures were stirred and left to stand for 24 h at 4 °C. The pellet (bioflocculant) was recovered by centrifugation and dissolved in 25 mL of distilled water with a mixture of chloroform and 1-butanol (5: 2 v/v). After shaking, the mixture was left standing at room temperature for 12 h in a separation-hopper (room temperature). The superior phase was recovered, centrifuged, redissolved in 50 mL distilled water and left to stand at 40 °C. The result was a dry powdered bioflocculant which was characterized as described below.

Bioflocculant characterization

To determine the functional portions of bioflocculant, the infrared spectra of the dried bioflocculants were recorded in the frequency range of 4000-400 cm^{-1} by a Fourier Transform Infrared-Raman Spectrophotometer (Shimadzu, model IRPrestige-21).

Statistical analysis

All the assays were repeated three times, with analysis in triplicate. The results were expressed as mean ± standard deviation, and the analysis of variance (ANOVA) was carried out to verify if there were significant differences ($\rho < 0.05$). Also, the Tukey's test was utilized to verify the significant differences ($\rho < 0.05$). among the means.

RESULTS AND DISCUSSION

Evaluation of bioflocculant activity

Under the assay conditions established, the studied *Bacillus* strains were able to increasingly synthetize bioflocculant in parallel with cell growth. The maximum flocculant activity (MFA) was 57% for *B. pumilus* LBPMA-BLD07, after 72 h of incubation, while *B. thuringiensis* LBPMA-EFIII*, B. toyonensis* LBPMA-ACOPR1.Isox and *B. megaterium* LBPMA-APFSG3Isox presented respectively 33 %, 21 % and 34 % of this activity after 24 h. From this time point, the flocculant activity gradually decreased, proportional to the decrease of the dried biomass in the studied medium (Figure 1). These results agree with observations of Xia et al. (2008), who found maximum flocculating activity (MFA) was reached in the beginning of the stationary phase. This can be explained by the lower number of cells (at that moment, there is depletion of nutrients), so the secretion of products like bioflocculants is also reduces (Nwodo and Okoh, 2013).

Yang et al. (2012) also reported that *Klebsiella* sp N-10 isolated from activated sludge achieved its MFA (86.5 %) at 60 h of cultivation. Likewise, Okaiyeto et al. (2016) registered MFA of 85.8 % after 72 h of cultivation for *Bacillus* sp. AEMREG7. Others studies relate that *B. mojavensis*, *B. licheniformis* X-14 and *Klebsiella* sp. TG-1 achieved their MFA after 24, 20 and 28 h (Li, 2009; Sekelwa et al., 2013). Although fungi and bacterial growth present different tendencies, Aljuboori et al. (2013) also showed that *Aspergillus flavus* had MFA (87.2%) in a medium containing ($g.L^{-1}$) sucrose, 30; peptone, 3.0; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; $FeSO_4$, 0.01; K_2HPO_4, 1.0, at initial pH 6.0, after 60 h of incubation.

In the present study, the temperature of 37 °C favored the growth as well as the bioflocculant production by the studied bacterial strains. According to Salehizadeh and Shojaosadati (2001) and Zhang et al. (2007), the enzymes which are responsible for bioflocculant production are activated in a temperature range between 25 °C and 37 °C.

Regarding the pH, since no significative alterations from the initial value (7.0) happened, it is probable that the composition of the medium has a good buffer composition and it is efficient for the bioflocculant synthesis by the studied strains. Prasertsan et al. (2006) found that *Enterobacter cloacae* WD7 bioflocculant activity was optimal at pH 6.0. On the other hand, Li et al. (2010) verified that *Agrobacterium* sp. M-503 produced bioflocculant with high activity at neutral to alkaline pH (7-12). Wang et al. (2011) observed that the MAF of the CBF-F26 bioflocculant (mixed culture of *Rhizobium radiobacter* F2 and *B. sphaericus* F6) occurred in neutral and weak alkaline conditions, while Xiong et al. (2010) detected MFA of *B. licheniformis* CGMCC 2876 at pH 7.5.

With respect to non-bacterial organisms, Deng et al. (2005) reported that the bioflocculant was secreted by the fungus *A. parasiticus* in a pH range of 5.0-6.0, and the increase in pH decreased the biomass and bioflocculant activity production, while the lower pH favored the synthesis, secretion and accumulation of the bioflocculant in the medium. It was observed that the flocculation efficiency of the biopolymer produced by dinoflagellate *Gyrodinium impudicum* KG03 was

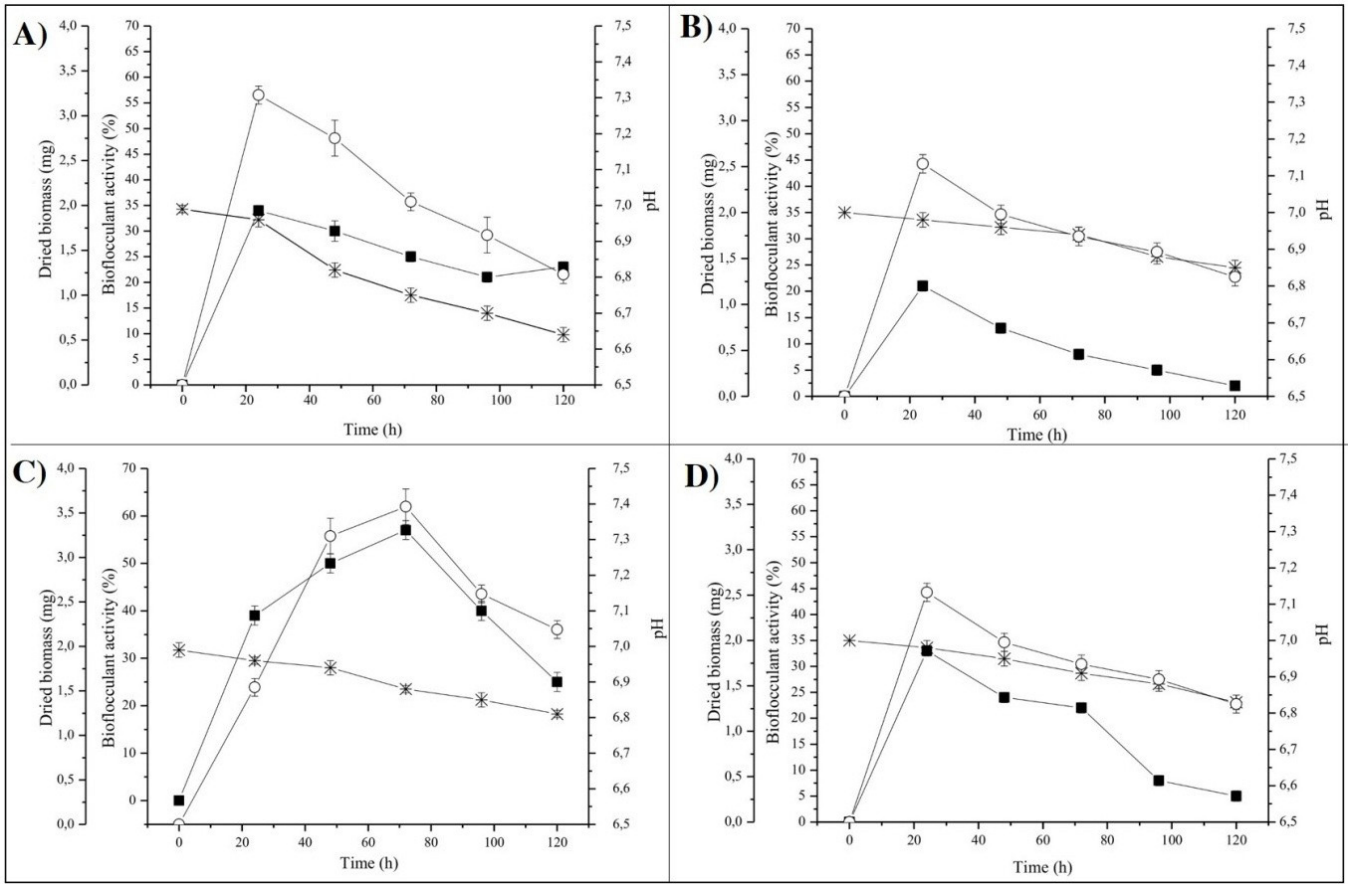

Figure 1. Percentage of bioflocculant activity using kaolin suspension (■), dried biomass (O) and pH (*) of the cultures (glucose as carbon source) of *Bacillus megaterium* LBPMA-APFSG3Isox (A), *B. toyonensis* LBPMA-ACOPR1.Isox (B), *B. pumilus* LBPMA-BLD07 (C) and *B. thuringiensis* LBPMA-EFIII (D), during 120 h of incubation (at 37ºC, dark, 160 rpm of orbital shaking).

optimal within a pH range of 3.0-6.0 (MFA at pH 4.0) (Yim et al., 2007).

Several other factors, besides the pH (Salehizadeh and Yan, 2014), temperature (Nakata and Kurane, 1999), cell concentration and macro and micronutrients, can affect MAF in a given environment. The agitation speed, for example, determines the concentration of dissolved oxygen available, which interferes with the absorption of nutrients and enzymatic reactions of bioflocculant production. Once the nutrients deplete in the culture, the level of oxygen available to the microorganisms becomes reduced, as well as the products of toxic residues of the metabolic activity, such as the presence of some deflocculating enzyme among them, which increases drastically the number of cells still viable and the accumulation of secreted flocculants (Zheng et al., 2008). According to Li et al. (2009), the optimum shaking speed for bioflocculant production by the strain of *B. licheniformis* X14 was 140-160 rpm.

Optimization of culture medium for bioflocculant production

Several carbon sources can stimulate specific microorganisms to produce bioflocculants. In the present study, different carbon sources (sucrose, glucose and maltose), at four pH values (3.0; 5.0; 7.0 and 9.0) for production of bioflocculants by different strains of *Bacillus* spp. were evaluated. Similarly, Guo et al. (2018) investigated bioflocculant production of *Pseudomonas boreopolis* G22 under different pHs (5 - 10). In the present study, sucrose was the most favorable carbon source for bioflocculant production by *B. megaterium* LBPMA-APFSG3Isox, and *B. toyonensis* LBPMA-ACOPR1.Isox, respectively, with 41% and 39% bioflocculating activity after 24 h. For *B. pumilus* LBPMA-BLD07 and *B. thuringiensis* LBPMA-EFIII, maltose was the best source of carbon to stimulate this bioflocculant activity (13% and 25%, respectively) (Figures 2 and 3).

Similar findings were reported by Makapela et al. (2016) with a strain of *B. pumilus* from Thyume River in Alice, Eastern Cape (South Africa). For this strain, maltose and sucrose were the favorable carbon sources for the production of bioflocculants (respectively 71.7% and 69.8% of activities), after 120 h of incubation in a production medium composed of ($g.L^{-1}$): maltose, 20; K_2HPO_4, 5; KH_2PO_4, 2, urea 0.5; yeast extract, 0.5; $(NH_4)_2SO_4$, 0.2 and $MgSO_4.7H_2O$,

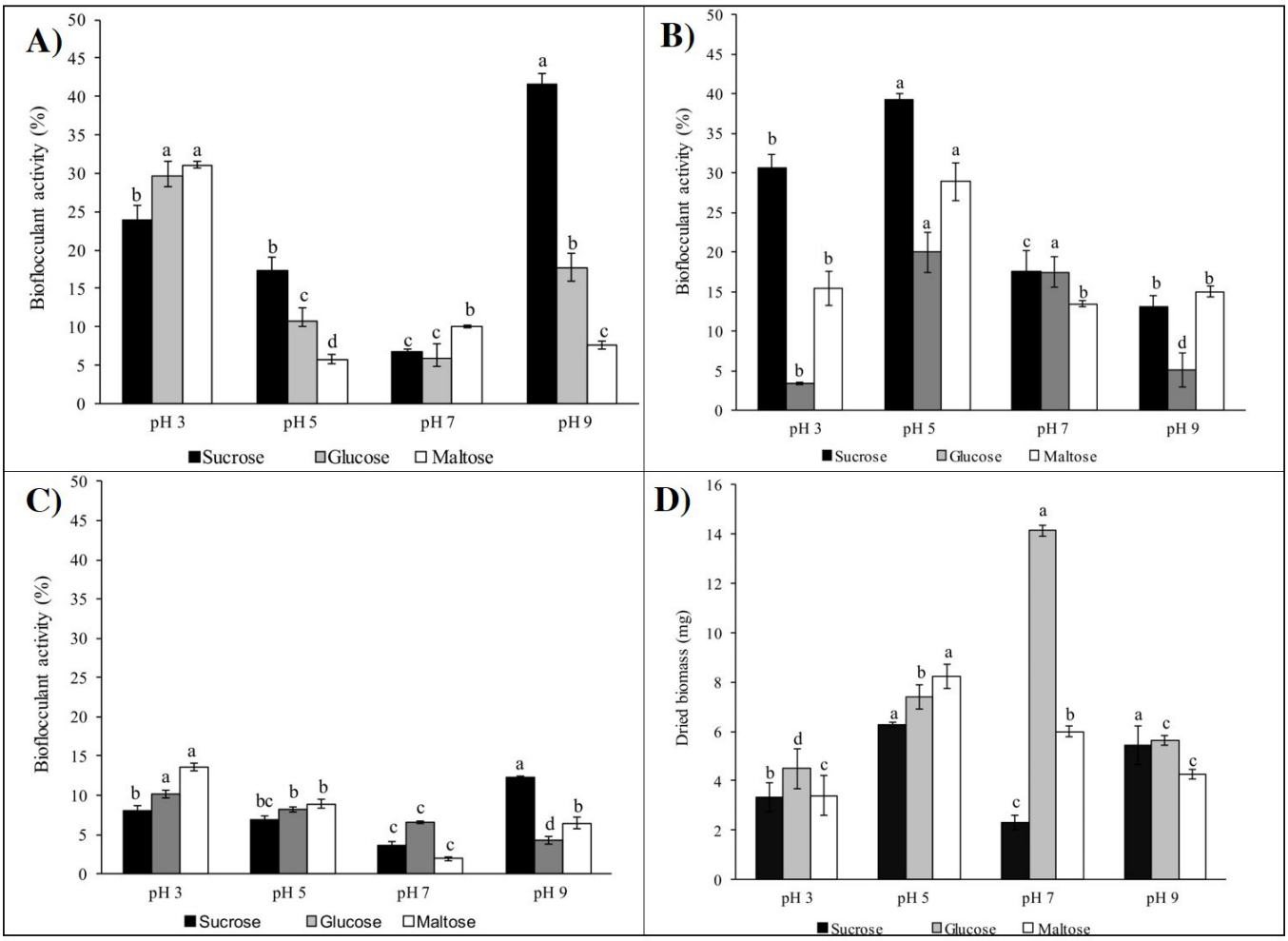

Figure 2. Effect of carbon sources and pH on the bioflocculant production by *Bacillus megaterium* LBPMA-APFSG3Isox (A), *B. toyonensis* LBPMA-ACOPR1.Isox (B), *B. pumilus* LBPMA-BLD07 (C) and *B. thuringiensis* LBPMA-EFIII (D), after 24 h of incubation (at 37ºC, dark, 160 rpm of orbital shaking). For each strain, bars of the same color followed by different letters indicate subgroups with statistically significant differences, according to Tukey test ($\rho < 0.05$).

0.2. Similarly, Wan et al. (2013) reported that maltose was the preferred carbon source for the production of bioflocculants by *Solibacillus silvestris*, isolated from activated sludge, showing 88.7% flocculant activity after 72 h of incubation in a medium containing ($g.L^{-1}$): yeast extract, 10; KH_2PO_4, 1 and $MgSO_4 \cdot 7H_2O$, 1 g; at pH 7.0. In addition, Luo et al. (2016), using a strain of *B. megaterium* (isolated from biofloc in pond water) and sucrose as carbon source, obtained bioflocculant activity higher than 80% after 72 h of incubation.

These findings support the affirmation that preferences for carbon sources to produce bioflocculants vary among microorganisms (Salehizadeh and Yan, 2014), but also that the ability of most of them to use sucrose as a carbon source for such synthesis led to the option of using molasses for the production on an industrial scale (Okaiyeto et al., 2016).

Regarding the medium-pH, the best bioflocculant production was obtained at pH 5.0 for *B. toyonensis* LBPMA-ACOPR1.Isox and *B. thuringiensis* LBPMA-EFIII, and at pH 3.0 for *B. pumilus* LBPMA-BLD07 and pH 9.0 for *B. megaterium* LBPMA-APFSG3Isox. Choi et al. (1998) and Zheng et al. (2008), reported that the activities of the polysaccharide flocculants of *Anabaena* sp. PC-1 and *Bacillus* sp. F19 decrease as the pH increases, probable because of the interference of the hydroxyl ion (OH^-) during the formation of the complex bioflocculant-clay, mediated by the metallic ions, finally leading to its suspension.

On the other hand, Zheng et al. (2008) found a maximum flocculation activity for *B. megaterium* strain from soil when it was grown in medium with pH ranging from acid to very alkaline, at initial pH 9.0. He et al. (2010), in turn, studying the activity of a bioflocculant produced by *Halomonas* sp. V3a, found that it was higher than 80% in the pH range of 3-11, with the highest flocculation (97%) at pH 7.0. The optimum initial pH of the growth medium aiming at the best bioflocculant

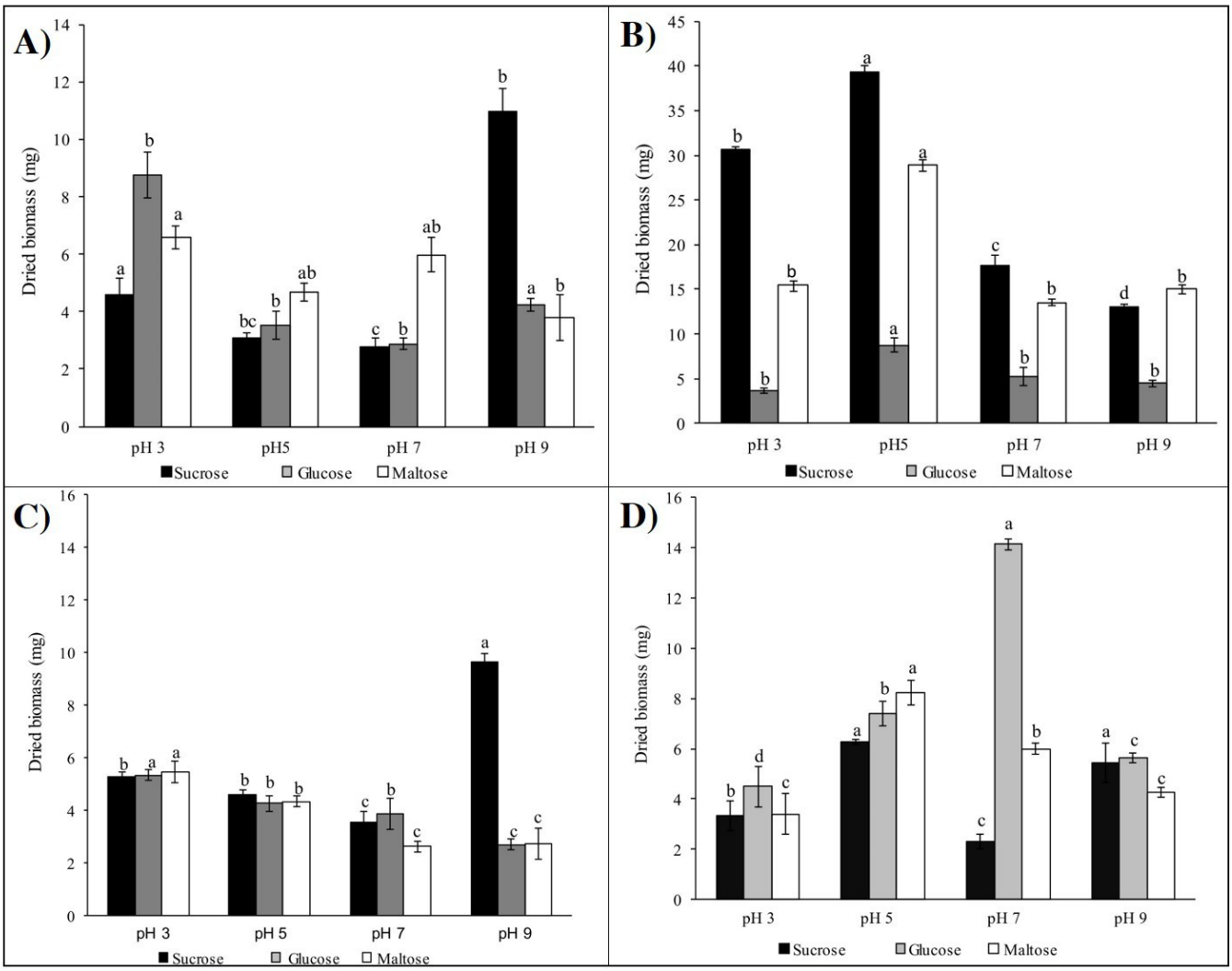

Figure 3. Effect of carbon sources and pH on the dried biomass of *Bacillus megaterium* LBPMA-APFSG3Isox (A), *B. toyonensis* LBPMA-ACOPR1.Isox (B), *B. pumilus* LBPMA-BLD07 (C) and *B. thuringiensis* LBPMA-EFIII (D), after 24 h of incubation (at 37ºC, dark, 160 rpm of orbital shaking). For each strain, bars of the same color followed by different letters indicate subgroups with statistically significant differences, according to Tukey test ($\rho < 0.05$).

activities of *Bacillus* xn12 and *Streptomyces* xn17 (97% for both) was observed at pH 5.0 (Dongchen et al., 2013). On the other hand, Okaiyeto et al. (2015), using a strain of *B. toyonensis* from sediment of Algoa Bay of the Eastern Cape Province (South Africa), found a best bioflocculant production under acidic conditions, especially at pH 5.0.

To evaluate the effect of nitrogen sources, the best combination carbon source and pH value of the initial culture medium for bioflocculant production was selected, using the ratio of flocculation activity to dried-cell biomass (A/m). Therefore, these best combinations, as described in Table 1 for *B. megaterium* LBPMA-APFSG3Isox, *B. thurigiensis* LBPMA-EFIII and *B. toyonensis* LBPMA-ACOPR1.Isox were sucrose (respectively ratios of 5.66 in pH 3.0 and 3.73 and 7.92 in pH 5.0) and maltose for *B. pumilus* LBPMA-BLD07 (highest ratio of 2.49 in pH 3.0).

As can be seen in Figure 4, among the nitrogen sources analyzed, $(NH_4)_2SO_4$ provided the best flocculating activity (36%) for *B. megaterium* LBPMA-APFSG3Isox, whilst urea made the same for *B. pumilus* LBPMA-BLD07 (36%) and *B. toyonensis* LBPMA-ACOPR1.Isox (22%), and peptone for *B. thuringiensis* LBPMA-EFIII (26%) (Figure 4).

According to Okaiyeto et al. (2016), a strain of *Bacillus* from sediment of Algoa Bay of the Eastern Cape Province (South Africa), incubated by 72 h in medium with $(NH_4)_2SO_4$ as source of nitrogen showed a bioflocculant activity of 79.89%. Sheng et al. (2006), studying the production of bioflocculant by a strain of *Klebsiella* sp., also found that ammonium sulphate was the best nitrogen source to stimulate its synthesis. On the other hand, Li et al. (2013) found that peptone was the best source for flocculant production by *Paenibacillus elgii* B69, and the same was seen for

Table 1. The ratio of bioflocculant activity/dried cell growth of four *Bacillus* strains tested after 24 h of incubation (at 37 °C, dark, 160 rpm orbital shaking).

Strains	Parameters			
	pH	Carbon sources		
		Glucose	Maltose	Sucrose
B. megaterium LBPMA-APFSG3Isox	3.0	3.40	4.71	5.66
	5.0	3.03	1.24	5.24
	7.0	2.06	1.69	2.42
	9.0	4.18	2.01	3.79
B. toyonensis LBPMA-ACOPR1.Isox	3.0	0.92	4.72	7.30
	5.0	2.28	5.17	7.92
	7.0	3.33	2.86	6.96
	9.0	1.14	3.49	6.66
B. pumilus LBPMA-BLD07	3.0	1.91	2.49	1.52
	5.0	1.93	2.06	1.50
	7.0	1.70	0.73	1.03
	9.0	1.60	2.36	1.26
B. thuringiensis LBPMA-EFIII	3.0	0.55	3.65	3.40
	5.0	1.39	3.11	3.73
	7.0	1.55	2.37	2.88
	9.0	1.29	0.62	2.93

the filamentous fungus *Aspergillus flavus* studied by Aljuboori et al. (2013).

Comparing the flocculation activities of the tested microorganisms for the different nitrogen sources studied (Table 2), it was verified that these were individually less efficient after 24 h of cultivation of *Bacillus* spp. than the standard combined source [urea, yeast extract and $(NH_4)_2SO_4$].

Bacterial strains can use combined nitrogen sources to produce bioflocculants (Deng et al., 2005; Gong et al., 2008; Xia et al., 2008). For example, Gong et al. (2008) indicated that a mixture of urea and beef extract showed substantial improvement in the production of bioflocculants by *Serratia*. Xia et al. (2008) showed that the combination of peptone, yeast and beef extract as source of nitrogen was quite effective, but only peptone (organic nitrogen source) was the most profitable, with high bioflocculant production by *Proteus mirabilis* strain TJ-1. Also, Sekelwa et al. (2013) reported that a complex nitrogen source consisting of urea, yeast extract and $(NH_4)_2SO_4$ supported optimum bioflocculant production by *Virgibacillus* sp. On another hand, Deng et al. (2005) reported that peptone combined with sodium nitrate is the most suitable source of nitrogen for bioflocculant production by the fungus *Aspergillus parasiticus*, but when it was combined with $(NH_4)_2SO_4$, no bioflocculant was produced.

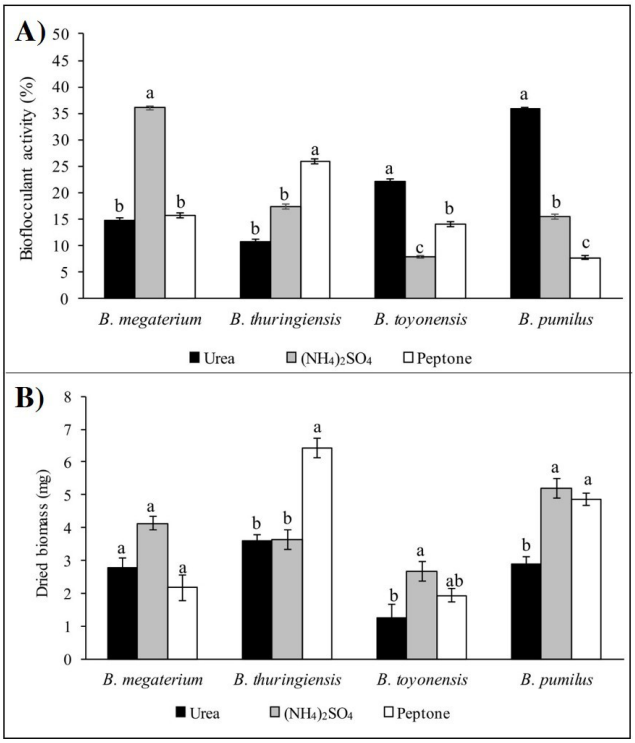

Figure 4. Effect of nitrogen sources on the bioflocculant production (A) and dried biomass (B), after 24 h of incubation (at 37ºC, dark, 160 rpm of orbital shaking) of *B. megaterium* LBPMA-APFSG3Isox, *B. toyonensis* LBPMA-ACOPR1.Isox, *B. pumilus* LBPMA-BLD07and *B. thuringiensis* LBPMA-EFIII, on media with the best carbon sources and initial pH values for such production. Bars of the same color followed by different letters indicate subgroups with statistically significant differences, according to Tukey test ($\rho < 0.05$).

Therefore, according to the composition and initial pH of the culture media, different bacteria produce different chemical classes of the flocculants.

Characterization of produced biosurfactant

The infrared absorption spectrum (FTIR) of a compound is probably its most original physical property, so the spectrum is often called the fingerprint

Table 2. Bioflocculant activity (%) of the cultures of *B. megaterium* LBPMA-APFSG3Isox, *B. toyonensis* LBPMA-ACOPR1.Isox, *B. pumilus* LBPMA-BLD07 and *B. thuringiensis* LBPMA-EFIII with different nitrogen sources during 24 h of incubation (37 ºC, dark, 160 rpm of orbital shaking).

Strains	Nitrogen source			
	Urea, yeast extract and $(NH_4)_2SO_4$	Peptone	Urea	$(NH_4)_2SO_4$
B. megaterium LBPMA-APFSG3Isox	34	16	15	**36**
B. toyonensis LBPMA-ACOPR1.Isox	21	8	**22**	14
B. pumilus LBPMA-BLD07	39	8	**36**	15
B. thuringiensis LBPMA-EFIII	33	**26**	11	17

of a molecule. Then, FTIR analysis of the bioflocculant produced by each studied *Bacillus* strain was performed to detect the presence of any functional groups that could contribute to its activity. In the achieved spectra (Figure 5), an intense broad-stretching peak was found at 3261 cm^{-1} for the bioflocculant of *B. megaterium* LBPMA-APFSG3Isox, 3269 cm^{-1} for that of *B. toyonensis* LBPMA-ACOPR1.Isox, 3263 cm^{-1} for that of *B. pumilus* LBPMA-BLD07 and 3259 cm^{-1} for that of *B. thuringiensis* LBPMA-EFIII, all characteristic of the hydroxyl groups (-OH).

In addition, bands of 1309 cm^{-1}, 1377 cm^{-1} and 1402 cm^{-1}, characteristic of symmetric stretch (C = O) of carboxylate (Deng et al., 2003), are representative of the carboxyl group, and they were found for the flocculants, respectively, of *B. toyonensis* LBPMA-ACOPR1. Isox, *B. thuringiensis* LBPMA-EFIII and *B. pumilus* LBPMA-BLD07. Ranges of a weak stretching (C-H) vibration were observed at 2960 cm^{-1}, 2916 cm^{-1}, 2960 cm^{-1} and 2958 cm^{-1}, respectively, in the spectra of the flocculants of *B. megaterium* LBPMA-APFSG3Isox, *B. toyonensis* LBPMA-ACOPR1.Isox, *B. pumilus* LBPMA-BLD07 and *B. thuringiensis* LBPMA-EFIII. Bands at 1037 cm^{-1}, 1051 cm^{-1}, 1020 cm^{-1} and 1022 cm^{-1}, were respectively found for the biofloculants of *B. megaterium* LBPMA-APFSG3Isox, *B. toyonensis* LBPMA-ACOPR1.Isox, *B. pumilus* LBPMA-BLD07and *B. thuringiensis* LBPMA-EFIII, and indicate the presence of the methoxy group (Zheng et al., 2008). On another hand, a peak of absorption at 1261 cm^{-1} was found in the spectra of biofloculants of *B. megaterium* LBPMA-APFSG3Isox, *B. toyonensis* LBPMA-ACOPR1.Isox and *B. thuringiensis* LBPMA-EFIII, while for *B. pumilus* LBPMA-BLD07 this band appeared at 1228 cm^{-1}, and both indicate the presence of CO stretch in an ether or alcohol (Abd-El-Haleem et al., 2008). Hydroxyl and carboxyl groups play a key role in the flocculation of suspended particles because they provide adsorption sites where the suspended particles can be attached (Okaiyeto et al., 2016). Absorption peaks around 1000-1100 cm^{-1} are known to be characteristic of all sugar derivatives (Zheng et al., 2008).

Polysaccharides contain a significant number of hydroxyl groups, which have a broad absorption band

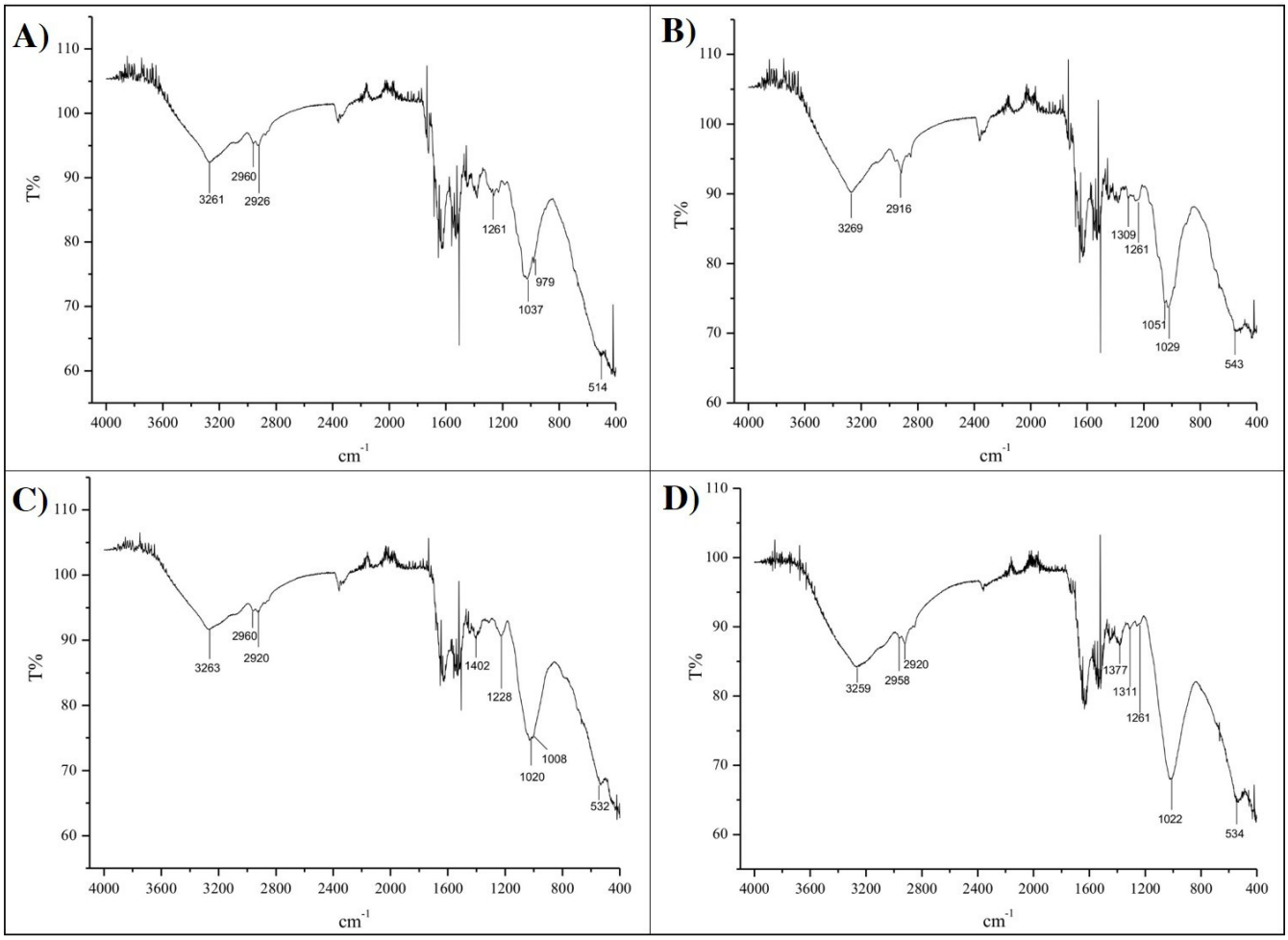

Figure 5. Fourier-transform infrared (FTIR) spectroscopy of bioflocculant produced by *Bacillus megaterium* LBPMA-APFSG3Isox (A), *B. toyonensis* LBPMA-ACOPR1.Isox (B), *B. pumilus* LBPMA-BLD07 (C) and *B. thuringiensis* LBPMA-EFIII (D) after 72 h of incubation (at 37ºC, dark, 160 rpm of orbital shaking).

above 3000 cm^{-1}, as in the studied bioflocculants, suggesting they are polysaccharides. Deng et al. (2003) documented that *B. mucilaginosus* secreted a bioflocculant named MBFA9, which, from infrared analysis, revealed the presence of carboxyl and hydroxyl groups as the major functional groups in this molecule - a polysaccharide composed mainly of amino-sugars, uronic acids and neutral sugars. Feng and Xu (2008) observed that the acid flocculant produced by *Bacillus* sp. BF3-3 is composed by polysaccharide and protein, while Gao et al. (2006) found that the flocculant produced by *Vagococcus* sp. W31 is a heteropolysaccharide formed mainly by neutral glycol and uronic acid. Gomaa (2012), studying the bioflocculant produced by *Pseudomonas aeruginosa,* observed the presence of carboxyl, hydroxyl, amino and sugar groups, characteristics of exopolymers. Dongchen et al. (2013) and Kavita et al. (2013), investigating the FTIR spectra of flocculants produced by isolates of the fungus *Phanerochaete chrysosporium* and the bacteria *Vibrio campbellii*, observed groups characteristic of polysaccharides.

Therefore, the FTIR spectra of the bioflocculants produced by the studied strains showed the presence of carboxyl, hydroxyl and methoxy groups (Figures 7 and 8), which are characteristic of polysaccharides and preferred for flocculation (Ntsangani et al., 2017).

CONCLUSION

In the present study, we investigated the flocculating efficiency and chemical properties of the bioflocculants produced by four strains of *Bacillus* (*B. megaterium* LBPMA-APFSG3Isox, *B. toyonensis* LBPMA-ACOPR1.Isox, *B. pumilus* LBPMA-BLD07 and *B. thuringiensis* LBPMA-EFIII). Maximum flocculating activity measurements were established and the results showed the maximum value of 57% for *B. pumilus* LBPMA-BLD07 after 72h. The presence of hydroxyl, *carboxyl* and methoxy groups in the *bioflocculant of all strains indicate they are* polysaccharides.

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