

Lipases and biosurfactants production by the newly isolated *Burkholderia* sp.

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Abstract. Lipases and biosurfactants are biocompounds produced by microorganisms involved in the metabolism of oily substrates. In this way, our study aimed to evaluate these molecules production by bacteria isolated from contaminated soil with waste vegetable oil and evaluate the optimal culture conditions for lipase production using the response surface methodology. The lipolytic activity was tested on tributyrin agar and rhodamine B agar with olive or soybean oil. All 66 isolates of bacteria were positive on tributyrin medium, while the percentage of lipolytic bacteria on rhodamine B medium varied from 31 (soybean oil, pH 6.0) to 38 (olive oil, pH 7.0 and 8.0; soybean oil, pH 8.0). The oil-spreading technique revealed that all isolates produced biosurfactants and oil emulsification and hemolytic activity tests detected biosurfactants in 60% and 88% of isolates, respectively. Lipolytic activity and biomass value varied de 8.7 to 12.4 U/mL and 2.5 to 4.04 mg/mL, respectively, in nutrient broth with olive oil medium. Six isolates with higher lipase activity were identified as *Burkholderia* sp., according to phylogenetic analysis based 16S rRNA sequences. Only *Burkholderia* sp. O19 strain produced rhamnolipids among bacteria studied. The surface response methodology revealed that the production of lipases by *Burkholderia* sp. O19 occurs in a wide range of pH and temperature with maximum response achieved at pH 8.5 and 65 °C (18.7 U/mL). The results obtained in this study are relevant as they show the simultaneous production of two biocompounds with broad industrial applications.

Keywords: Bacteria; Biosurfactants; Lipase; Soil.

Introduction

A large variety of microorganisms such as bacteria, fungi and yeasts have been reported as potential lipases

producers mostly isolated from the soil enriched with industrial and household waste or decaying food (Shaini and Jayasree, 2016).

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Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) act on the triacylglycerol - water interface and catalyze the hydrolysis of fats and oils to free fatty acids and glycerol (Gopinath et al., 2013). In the area of industrial biocatalysis, these enzymes are the most important, since they can act both as hydrolases or synthetases, besides presenting a huge potential in several biotechnological applications (Bhosale et al., 2016). Microbial lipases are more required in biotechnology processes than those of plant and animal origin because of their stability, high conversion rate of substrate to product, wide range of activities at different temperature and pH, and mostly extracellular production, thus facilitating their acquisition. Moreover, many microorganisms can be genetically manipulated and able to grow in culture media with specific substrates (Gupta et al., 2015).

Biosurfactants are microbial compounds that have the ability to reduce surface and interfacial tension, increase solubility and mobility of hydrophobic compounds (Gudiña et al., 2016). Some of the most important characteristics of biosurfactants are their ecological acceptability, low toxicity and biodegradability (Franzetti et al., 2012), which resulted in increased research interest in their production at a cost compatible to that of surfactants derived from petrochemical products (Geys et al., 2014).

Biosurfactants and lipases can be widely used for industrial, agricultural, food, cosmetics and pharmaceutical application (Lotfabad, et al., 2009), but there are a few studies that focus on lipase and biosurfactants production simultaneously (Colla et al., 2010). The current study aimed to analyze the co-production of lipases and biosurfactants by bacterial isolates from contaminated soil with waste vegetal oil and further evaluate the optimal culture conditions for lipase production using the response surface methodology.

Material and methods

Soil sampling and microorganisms isolation

The soil sample was collected in an experimental area used for the disposal of waste vegetal oil, located at João Pessoa, Paraíba, Brazil (7° 12' 9.5" S - 34° 48' 21.8" W). The soil was contaminated by spreading about 1 L of wasted frying oil on the soil surface (2 m²) every two months during three years. Bacteria were isolated in selective agar medium consisted of nutrient broth, emulsified soybean oil (0.1% v/v), soil extract (10% v/v), urea (0.02%) and mycostatin (50 mg/L) at pH 7.0 (Ko et al., 2005). Culture medium were incubated at 30 °C for 4 days and 66 bacterial isolates were obtained. The biotypes of isolates were determined using the following characteristics: cell morphology, Gram stain reaction and endospore formation. The physical and chemical soil analysis was performed by CEIMIC Environmental Analysis Ltda., São Paulo-SP, Brazil.

Production of lipases by bacteria

Screening for lipase producing bacteria was done initially on the medium with tributyrin (Himedia, Mumbai, India) (1% v/v), peptone (Himedia, Mumbai, India) (0.5%), yeast extract (Himedia, Mumbai, India) (0.3%) and agar (Himedia, Mumbai, India) (1.5%) incubated at 30 °C and pH 7.5 for 4 days. The transparent halo around the colony indicated positive result. The confirmation of lipase production was done on the media consisted of rhodamine B dye (Sigma-Aldrich, USA) (0.001%), nutrient broth (Himedia, Mumbai, India) (0.8%), agar (Himedia, Mumbai, India) (1%), NaCl (Vetec, Brazil) (0.4%) and olive oil (Vale Fertil, Parana, Brazil) (3% v/v) or soybean oil (Soya, São Paulo, Brazil) (3% v/v), at different pH (6.0, 7.0 and 8.0) (Kouker and Jaeger, 1987). The cultures were incubated at

30 °C for 4 days and the isolates that showed formation of a fluorescent orange halo around colonies under UV light (350 nm) were considered lipolytic. Lipolytic activity of isolates that were positive in rhodamine B agar was evaluated by titration method. The isolates were cultured in 10 mL of nutrient broth, for 24 h at 30 °C under shaking (150 rpm). One milliliter of culture was used to inoculate 100 mL of nutrient broth (pH 7.0) with olive oil (1%) or soybean oil (1%). After 48 h of incubation (30 °C, 150 rpm) the cultures were centrifuged (8000 rpm, 15 min) and cell-free supernatant was used for quantification of lipases by measuring the release of fatty acids from olive oil emulsion (50 mL of olive oil, 50 mL of 7% arabic gum (Synth, Brazil) solution w/v) using titrimetric method according to Stuer et al. (1986). One unit of enzyme activity (1 U) was defined as the amount of enzyme that releases 1 µmol of fatty acids per mL of culture supernatant during 1 min under assay conditions. The specific activity of lipase was defined as units per g of dry weight cells. The cell biomass was determined by weighting dry cell pellet (105 °C, 24 h).

Screening of biosurfactants production by bacteria

The biosurfactants production was analyzed by the following tests: oil dispersion, oil emulsification and hemolytic activity. Bacteria were cultured in a nutrient broth (pH 7.0) with olive oil (1%) as mentioned above for measurement of lipase. The oil-spreading test was performed on 140 mm diameter Petri plate with 35 mL of water and 100 µL of diesel oil placed on the water surface. 10 µL of bacterial culture or cell-free supernatant was added to the oil surface and the clear zone that appeared on the surface was measured. The emulsification index of bacteria was determined using 2 mL of diesel oil

without additives and 2 mL of culture (nutrient broth with 1% of olive oil) or cell-free supernatant (Das et al. 1998). The mixture was agitated in a vortex for 2 min and allowed to stand for 5 min and 24 h to measure the height of the emulsion. The emulsification indexes E5 and E24 were calculated by dividing the height of emulsion measured after 5 min and after 24 h, respectively, by the total column height and expressed in percentage (%). The reason between the E24 e E5 indexes was used to calculate the stability of emulsion. The hemolytic activity of isolates was evaluated on the blood agar (Difco, USA) with 5% defibrinated rabbit blood incubated at 30 °C for 48 h. The clear halo around the colony of bacteria was measured. The isolates that showed hemolytic activity were analyzed for the production of rhamnolipids according to the method of Siegmund and Wagner (1991). Rhamnolipids production was detected through formation of a blue halo around the colony after incubation at 30 °C for 21 days.

Optimization of pH and temperature for lipase production

The determination of the optimal levels of two variables, initial pH (7.0 - 10.0) and temperature (30 °C-70 °C) of lipase production was done by using the response surface methodology (RSM). Briefly, a set of experimental design (central composite design with five coded levels: -1.44, -1, 0, 1, 1.44) was performed. For two factors, the design was made up of a full 2³ factorial design with its eight points augmented with three replications of the center points (both factors at level 0). The set of 11 experiments that consisted of eight unique combinations and three replications in central point (pH 8.0 and temperature 50 °C) were carried out as described in Table 1.

Table 1. Experimental range and levels of independent variables used in Response surface analysis for lipolytic activity of *Burkholderia* sp. O19.

Variables	Levels				
	-1.41	-1	0	1	+1.41
Temperature (°C)	30.0	35.9	50.0	64.1	70.0
pH	7.0	7.4	8.5	9.6	10.0

The culture was grown in nutrient broth with 1% of olive oil at pH and temperature designed for each experimental set for 48 h. The total activity (U/mL/h) of lipases was determined by titrimetric method as described above. Data were analyzed by the Experimental Design Module (Statistica, version 7.0, StatSoft, EUA). The effect of each factor was evaluated by ANOVA (95% and 99%). The model permitted the evaluation of the effects of linear, quadratic and interactive terms of the independent variables on the dependent variable. The response surface plots were drawn to illustrate the main and interactive effects of the independent variables on lipase production. The optimum values of the selected variables were obtained by solving the regression equation and by analyzing the response surface plot.

Identification of lipolytic bacteria by sequencing of the 16S rRNA gene

The 16S rRNA gene was amplified from genomic DNA by polymerase chain reaction (PCR) using the primers: forward 26F (5'- GAG TTT GAT CMT GGC TCA G - 3') and reverse 1492R (5' - ACG GCT ACC TTG TTA CGA CTT - 3'). Amplifications were performed in thermocycler (Primus, Germany) in the following stages: 94 °C for 5 min, 25 cycles (94 °C for 1 min, 57 °C for 2 min, 72 °C for 2 min), and a final extension at 72 °C for 10 min. The partial sequencing of 16S rRNA genes was performed by ACTGene Molecular Analysis Ltda.

(Biotechnology Center, UFRGS, Porto Alegre-RS, Brazil) using the automatic sequencer ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems). The 16S rRNA gene sequences obtained were subjected to BLAST of the National Center for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and analyzed by Sequencher Software, version 5.0. Phylogenetic tree was constructed with Neighbor-Joining Method based on Tamura-Nei Model by Molecular Evolutionary Genetics Analysis (MEGA) Software, version 5.0 (nodal support was assessed by 1000 bootstrap replicates).

Results

Lipase and biosurfactants production

Soil properties (0-20 cm) of the area after enrichment were: pH (H₂O) = 3.87; organic matter = 110.4 g/kg, P = 0.003 g/dm³, K = 0.031 g/dm³, Na⁺ = 0.001 g/kg, Ca²⁺ = 0.26 cmol_c/dm³. All bacterial isolates obtained from the soil contaminated with residual vegetable oil showed lipolytic activity on the tributyrin agar. However, the analysis on rhodamine B agar confirmed the lipase production for 25 isolates out of 66 tested. Among 36 isolates of Gram negative rods 20 were lipolytic, while among 23 strains of Gram-positive spore-forming rods only one isolate showed lipase production. All isolates of non-spore Gram-positive rods tested were lipolytic (Figure 1).

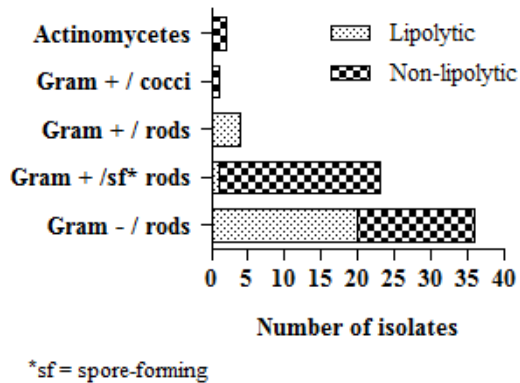


Figure 1. Numbers of lipolytic isolates, in rhodamine B medium, within the biotypes of bacteria isolated from the soil contaminated with vegetal residual oil.

The number of lipolytic bacteria varied slightly on the rhodamine B media with olive oil and soybean oil, and at different pH of media. The highest score of positive isolates (38%) was observed on the rhodamine B agar with olive oil at pH 7.0 and 8.0, and with soybean oil at pH 8.0. The number of positive isolates was lower in the rhodamine B medium with olive oil at pH 6.0 (32%) and with soybean oil at pH 6.0 (31%) and 8.0 (36%). The fluorescent halos around colonies were usually larger (> 30 mm) on the rhodamine B medium containing olive oil, pH 7.0. The lipase activity of bacteria that showed lipase production on olive oil rhodamine B agar was evaluated by titrimetric method in the nutrient broth with olive oil or soybean oil (pH 7.0, 30 °C). Among the isolates tested six (O17, O19, O22, O23, O45, O62) showed high lipolytic activity (8.7-12.4 U/mL; 2.4-4.96 U/mg of cells) and biomass values of 2.5 to 4.04 mg/mL in

nutrient broth with olive oil (Table 2, Figure 2).

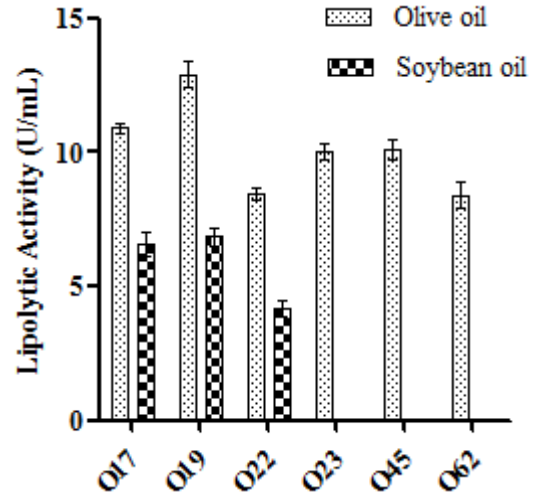


Figure 2. Lipolytic activity of *Burkholderia* sp. isolates in a nutrient broth medium with olive oil or soybean oil.

All these isolates were closely related to *Burkholderia ambifaria*, as revealed by phylogenetic analysis based on nearly full-length 16S rRNA sequences (Figure 3). The highest total activity of lipases was observed for *Burkholderia* sp. O19 strain: 12.4 U/mL and 7.2 U/mL in nutrient broth with olive oil and soybean oil respectively (Figure 2). All lipolytic isolates produced biosurfactants as was shown by the oil-spreading technique. The oil emulsification capacity was observed in cultures and cell-free supernatants of 60% of lipolytic isolates. The emulsification index (E24) of cultures varied from 13.1% to 55.2%,

Table 2. Production of biosurfactants in a nutrient broth medium with olive oil by bacteria isolated from soil.

Isolate	Emulsification index (E24) (%)		Oil-spreading zone (mm)		Hemolysis (mm)
	Culture	Cell-free supernatant	Culture	Cell-free supernatante	
02*	0	0	140	140	18
04*	44.7 ^a	47.3 ^b	140	130	21
05*	53.3 ^a	36.6 ^b	140	110	27
06*	0	0	140	110	21
07*	30.0 ^a	23.3 ^c	140	90	22
08*	21.0 ^a	7.8 ^c	45	35	23
013**	44.7 ^a	28.9 ^a	90	90	17
015*	46.6 ^c	43.3 ^b	140	120	21
017 ^{*,B}	0	0	140	120	0
019 ^{*,B}	42.1 ^a	44.7 ^a	50	30	12
021*	44.7 ^a	36.8 ^c	140	95	17
022 ^{*,B}	0	0	140	40	17
023 ^{*,B}	52.6 ^b	50.0	140	140	17
030**	0	0	140	140	20
045 ^{*,B}	47.3 ^b	42.1 ^b	140	140	14
047*	39.4 ^a	31.4 ^a	110	100	24
050*	55.2 ^c	60.5 ^a	90	55	24
052*	43.3 ^b	46.6 ^a	120	120	28
054*	44.7 ^a	26.6 ^b	50	35	14
056*	0	0	140	120	23
057*	0	0	140	149	20
058*	0	0	140	140	0
062 ^{*,B}	13.1 ^a	15.7 ^c	90	90	30 ^R
064**	0	0	140	140	0
064**	0	0	40	40	39

*Gram negative rods; B - *Burkholderia* sp.; **Gram positive rods; Emulsion stability: a - 1.0; b - 0.9; c - 0.8; ^R - Rhamnolipids production.

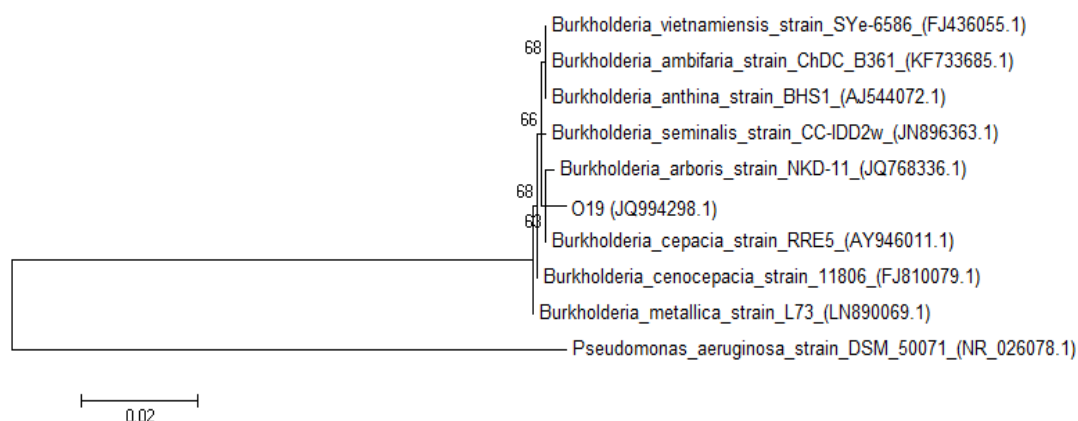


Figure 3. Phylogenetic positions of *Burkholderia* sp. O19 based on its 16S rRNA gene sequence. *Pseudomonas aeruginosa* DSM 50071 was used as an outgroup for this analysis. The isolates O17, O19, O22, O23, O45 and O62 showed the same 16S rRNA sequence. GenBank accession numbers of the sequences are given in parentheses.

and of cell-free supernatant from 7.8% to 60.5% (emulsion stability: 0.8-1.0). Only one bacteria (O62, *Burkholderia* sp.) produced rhamnolipids (Table 2).

Optimization of lipase production by response surface methodology

The *Burkholderia* sp. O19 that showed the highest total and specific

lipolytic activity was submitted to the response surface methodology to determine the optimal conditions of pH and temperature for lipases production (Table 3).

From the experimental data shown in Table 3 the following model equation for the effects of variables (pH - $X_{(pH)}$, temperature - $X_{(Temp)}$) on lipase activity (LA) was generated:

$$LA = -134.0973 + 34.5596 X_{(pH)} - 2.3919 X_{(pH)}^2 + 0.1715 X_{(Temp)} - 0.0091 X_{(Temp)}^2 + 0.1071 X_{(Temp)} X_{(pH)}$$

Table 3. Two-level factorial design for lipase production by *Burkholderia* sp. O19 showing observed and predicted responses.

Experiment	Independent variables		Lipase activity (U/mL)	
	pH	T (°C)	Observed	Predicted
1	7.4	35.9	10.83	13.54
2	7.4	64.1	10.80	15.06
3	9.6	35.9	6.68	8.57
4	9.6	64.1	13.30	16.74
5	7.0	50.0	13.62	13.92
6	10.0	50.0	8.25	11.68
7	8.5	30.0	7.65	10.40
8	8.5	70.0	16.40	17.98
9	8.5	50.0	15.70	18.19
10	8.5	50.0	15.15	18.19
11	8.5	50.0	15.20	18.19

As shown in Table 4 the experimental model was significant with coefficient of determination (R^2) of 0.912, indicating a good agreement between the experimental and predicted

values of the enzyme activity. As indicated by *P-values*, temperature and pH showed significant quadratic and linear effects on the lipolytic activity ($p < 0.05$) (Table 4).

Table 4. Analysis of variance (ANOVA) of second order polynomial model for optimization of lipase activity of *Burkholderia* sp. O19.

Source of variation	Sum of squares	Degrees of freedom	Mean Square	F ratio (model significance)	p-value
pH (Quadratic)	10.0153	1	10.01532	108.2737	0.009110*
pH (Linear)	36.2952	1	36.29519	392.3804	0.002539*
Temperature (Quadratic)	44.9559	1	44.95591	486.0098	0.002051*
Temperature (Linear)	19.2010	1	19.20096	207.5779	0.004783*
1L x 2L	11.0556	1	11.05563	119.5203	0.008263*
Lack of fit	9.9371	3	3.31238	35.8095	0.027290*
Pure error	0.1850	2	0.09250	-	-

*Significant factors ($p < 0.05$).

The Figure 4 indicates that the region with high lipolytic activity of *Burkholderia* sp. O19 was located at pH range from 7.5 to 9.0 and at temperature

from 45 °C to 70 °C. The model predicted a maximum response for lipolytic activity of *Burkholderia* sp. O19 isolate at pH 8.5 and temperature 65 °C (18.71 U/mL).

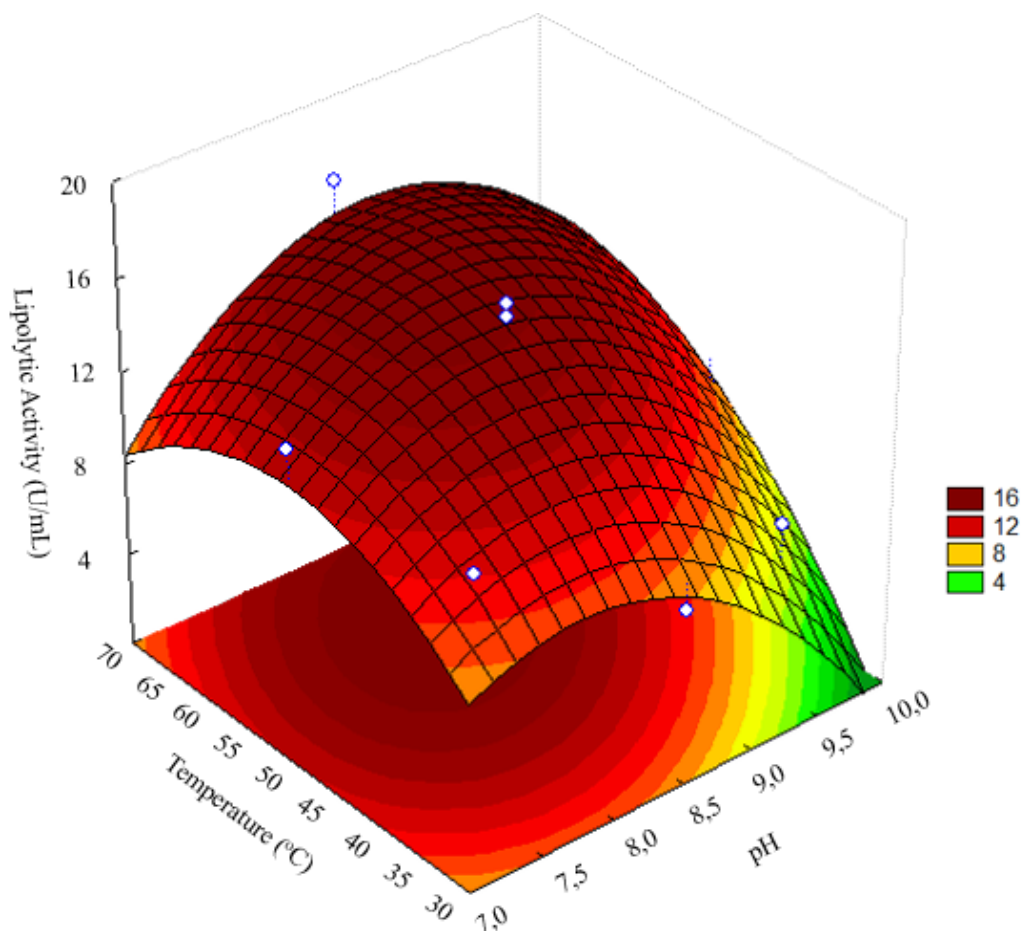


Figure 4. Response surface curve and contour diagram of the effect of pH and temperature on the lipase production by *Burkholderia* sp. O19.

Discussion

Among the substrates used to select the lipase and biosurfactants producing microorganisms vegetal oils have been increasingly prominent. As was shown in the present and other studies, the soil, once contaminated with residual oil, provides favorable conditions for the development and selection of lipolytic microorganisms capable of using oil as carbon source and

adapted to specific environmental characteristics, such as low pH, alterations of macro and microelements contents, and reduced humidity (Peil et al., 2016; Zafar et al., 2016). In this study all bacterial isolates from the soil contaminated with waste vegetable oil showed lipolytic activity on tributyrin agar, however, only 32%-38% of isolates produced lipases on the rhodamine B nutrient agar media with olive oil or soybean oil. It is known that tributyrin

can be hydrolysed by lipases and esterases, and therefore the rhodamine B agar is more suitable for the detection of lipases than tributyrin medium (Kim et al., 2001). The rhodamine B agar with olive oil at pH 7.0 gave the highest numbers of positive isolates, all with oil hydrolysis zones above 30 mm. According to Kouker and Jaeger (1987) the size of hydrolysis zone on rhodamine B agar shows positive correlation with lipolytic activity. Olive oil is considered the best inducer for lipase production due to the presence of high amount of oleic acid (Ciafardini et al., 2006).

Over all bacteria studied in this work, six isolates stood out by the rest exhibiting significant lipolytic activity. All these isolates belonged to the *Burkholderia cepacia* complex and were closely related to *B. ambifaria*. The *Burkholderia* genus is one of the most important group of bacterial, since they are good producers of lipases and consists of about 35 species, most of which are soil saprophytes and phytopathogens occupying a wide range of environmental niches. *B. ambifaria* is generally found as the dominant *Burkholderia cepacia* complex species in natural environments and is often associated with plant roots showing biocontrol effects on other microorganisms (Ciccillo et al., 2002; Ramette et al., 2005).

Crystallographic studies have shown that the lipases have two different conformations: open state and closed state. When the active site is not accessible to the solvent, the enzyme surface is mainly hydrophilic and its lid is closed. On a hydrophobic surface environment, the active site becomes accessible by opening the lid and the lipase becomes active (Rehm et al., 2010).

In this work, lipases produced by *Burkholderia* sp. O19 were alkaline with optimal values of pH and temperature of 8.5 and 65 °C, respectively, according to the model obtained by the response surface methodology. The high lipase

production by this isolate was observed in a wide range of pH and temperature. Other studies show that, in generally, bacterial lipases have neutral or alkaline pH optima and are stable over a wide pH range (Gupta et al., 2004; Hasan et al., 2006). The diverse optimal reaction temperature for lipase activity of different *Burkholderia* strains were reported: 37 °C (Ma et al., 2010), 55 °C (Liu et al., 2006; Yang et al., 2007), 60 °C (Liu et al., 2006) and 70 °C (Lu et al., 2009).

Interestingly, all isolates that showed lipolytic activity produced also biosurfactants detected by the oil-spreading technique. This technique was more efficient in the biosurfactants detection than the emulsification and hemolysis tests. Other works also indicate the oil-spreading method as a sensitive and convenient assay able to detect small quantities of biosurfactants (Morikawa et al., 2000). A high numbers of biosurfactants producers was also observed in hemolytic activity test. However, according to Siegmund and Wagner (1991), hemolysis test may lead to false positive results in biosurfactant detection because hemolytic bacteria can secrete not only biosurfactants, such as surfactin or rhamnolipids (Moran et al., 2002), but also other hemolytic factors, e.g. proteases. In this study only one hemolytic isolate, highly lipolytic and closely related to *Burkholderia ambifaria*, produced rhamnolipids. Other isolates may produce surfactin or other hemolytic agents such as proteases, since all lipolytic isolates analyzed in this study were proteolytic (data not shown). The production of rhamnolipids is extensively studied in *Pseudomonas aeruginosa* and there is a little information on their production by other bacteria. The bacteria of *Burkholderia* genus produce rhamnolipids that differ in structure from *P. aeruginosa* rhamnolipids, as was shown by Hörmann et al. (2010) for *B. plantarii* strain. The rhamnolipids produced by non-pathogenic bacteria exhibit essential

advantage in comparison to *P. aeruginosa* biosurfactant. The production of rhamnolipids is considered of great importance, since they increase the availability of lipid compounds for bacteria as also by promoting the acceleration of the degradation of such compounds (Irorere et al., 2017).

The simultaneous synthesis of lipases and biosurfactants by microorganisms can help to metabolize insoluble compounds in water (Zarinviarsagh et al., 2017). However, the relationship between the production of lipases and biosurfactants is not well established. It is known that esterification of fatty acids and sugars catalyzed by lipases leads to the synthesis of biosurfactants (Paula et al., 2005). Colla et al. (2010) studied the production of lipases and biosurfactants by *Aspergillus* sp. and observed that both compounds can be produced simultaneously or not, depending on the bioprocess type. The fact, that all lipolytic isolates studied here produced biosurfactants indicate that such property may give advantage for bacteria in the environment rich of waste vegetal oil, increasing biodegradation capacity of oily substrate, since oil pollution is a problem that affects very terrestrial environments.

Conclusions

The soil used for the discard of vegetable oil proved to be favorable for the prospection of lipolytic microorganisms and biosurfactant producers. The fact that all lipolytic isolates studied here produced biosurfactants indicate that such property may give advantage for bacteria in the environment rich of residual vegetal oil. Moreover, the results obtained in this study are relevant as they showed the simultaneous production of two biocompounds like lipases and biosurfactants with broad industrial applications. Although, further research are required to evaluate the

thermostability analysis of these biocompounds as also to increase their production of these biocompounds by *Burkholderia* sp. O19 at the industrial scale.

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Conflict of interests

The authors declare that there are no conflicts of interest to disclosure.

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