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BIOSURFACTANT PRODUCTION BY RHIZOBACTERIA ISOLATED FROM AMAZONIAN SOILS

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Abstract

The biosurfactants production by non-pathogenic rhizobacteria to plants and animals can be a desirable microbial characteristic to recommend in biodegradation processes of petroleum hydrocarbons. Laboratory experiments were performed with four rhizobacteria, two classifieds as Rhizobium sp, one as Paenibacillus sp and the other as Bradyrhizobium sp., to evaluate their abilities to produce surfactant metabolites using diesel oil as carbon source. They were used individually and in the form of five consortia, using two culture media, SYM and INPA. All treatments showed significant differences among themselves and the emulsifiers production in the media SYM and INPA. The values of the emulsification index water in oil were high, demonstrating the potential of these rhizobacteria to produce biosurfactants, despite the low and medium values acquired in the emulsifying index of oil in water. Consortium 05, composed by bacteria INPA R332, INPA R555 and INPA R713 was the one that produced the largest quantities of emulsifiers using any one of the two media. These results demonstrate that these rhizobacteria may become a viable and safe alternative for soils bioremediation contaminated with petroleum.

Keywords*: Bioremediation; Microbial metabolism; Emulsifying activity; Paenibacillus sp.; Rhizobium sp.; Bradyrhizobium sp.*

Introduction

Surfactants are amphipathic molecules composed of a hydrophobic portion and a hydrophilic portion. The nonpolar portion is often a hydro carbonate chain, while the polar portion can be ionic (anionic or cationic), non-ionic resins or amphoteric. Some examples of ionic surfactants used commercially include sulfated esters or fatty acids sulphate (anionic) and quaternary ammonium salts (cation). The presences of hydrophilic and hydrophobic groups in the same molecule make the surfactants to distribute in the interfaces among fluid phases with different degrees of polarity (oil/water and water/oil). The formation of a molecular film, ordained in the interfaces, reduces the interfacial and superficial tension, being responsible for the surfactant's unique properties [1].

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The biosurfactants can be produced by a diversity of microorganisms, the majority by bacteria and yeasts, and in smaller quantities, by filamentous fungi [2]. The main classes include glycolipids, lipopeptides and lipoproteins, derived from microbial metabolism [3]. The biosurfactants action can be demonstrated in the degradation process by microorganisms on compounds derived from petroleum, by a significant increase in the hydrocarbon's degradation present in the medium [4-6]. The dynamics of this process is based on the biosurfactants ability to reduce surface tension by molecular rearrangements, through the accumulation of insoluble compounds on surfaces, influencing the hydrogen bonds and hydrophilic-hydrophobic interactions. This increases the surface area, providing a higher bioavailability and consequent increase of the biodegradability [7].

The biosurfactants are more efficient than the existing synthetic surfactants and have properties that they do not have, such as greater selectivity and specific activity under extreme conditions of pH, temperature and salinity. If produced by microorganisms, they can be obtained using relatively simple procedures and inexpensive substrates, through fermentation processes. Sugars and oils are suitable carbon sources to obtain ecologically safe tensioactive substances [8, 9].

Bacteria with potential for the biosurfactants production can reduce the surface tension of the medium (oil in water), increasing the emulsifying activity, contributing to the bioremediation process, removing and degrading the petroleum. Through the surfactant agent production, the possibility of their applications emerges in the degradation processes of hydrophobic contaminants such as petroleum, being a promising alternative in the environment decontamination processes, aiding in the reduction of the concentration and toxicity of various components of spilled petroleum.

Some of the bacteria found in the soil have great potential to be used in remediation of petroleum or its derivatives spills [10], mainly the rhizobacteria found in the plants rhizosphere [11] in higher numbers than in the soil, and species that are not pathogenic to plants and animals must be chosen [12]. Emphasis should be given to the rhizobacteria capable of fixing nitrogen, solubilizing phosphate and producing growth hormones [13, 14], because these characteristics may promote both the petroleum degradation and the vegetation present in the affected area. Some studies have already demonstrated that rhizobacteria producing biosurfactants also showed a capacity to degrade cooking oil, a very common contaminant discarded in the environment by human being after its use in food preparation [15].

In addition, this work had as objective to test the rhizobacteria capacity, as well as five of their consortia, regarding the biosurfactants production, since the reduction of surface tension and increased emulsifying activity are leading factors to assess the ability of these microorganisms to degrade the petroleum.

Experimental

Materials

Previous tests performed at the Laboratory of Ecology and Biotechnology of Microorganisms of the Amazon, at the National Institute for Amazonian Studies (INPA) with 169 rhizobacteria, using petroleum hydrocarbons as carbon sources, indicated four strains that showed degradation capacity of all the tested derivatives (naphtha, gasoline, diesel oil, lubricating oil and kerosene) [16-19]. These four rhizobacteria and five consortia with their presences were used in this study.

Methods

Molecular identification

For taxonomic identifications, their genomic DNA was extracted using the extraction kit Pure link Invitrogen. The region 16S rRNA [20] was amplified using the oligonucleotide primers: 530F (5' - TGA CTG ACT GAG TGC CAG CMG CCG CGG - 3`) and 1492R (5' -

TGA CTG ACT GAG AGC TCT ACC TTG TTA CGM YTT - 3`). As a control test, the bacterial DNA of *Escherichia coli* ATCC 25922 was used. The amplification system was performed in the thermocycler Biocycle, and the thermal profile of the CRP was: an initial cycle at 95°C/2 minutes, 35 cycles at 95°C/40 seconds, 60°C/40 seconds and 72°C/2 seconds and finally an extension cycle at 72°C/5 minutes. The amplified products were subjected to electrophoresis in agarose gel 0.8% for the fragment's verification. The PCR products were purified using polyethylene glycol 20% (NaCl 2.5M, PEG 20%) and quantified in agarose gel 0.8%. The samples were sequenced (ABI 3130 - Applied Byosistems DNA sequence) and the sequences obtained were edited and evaluated using the program PHRED available at the address (http://helix.biomol.unb.br/phph/index.html). After obtaining the sequences F (forward) and R (reverse), comparative alignment were performed in the database of bacterial genomes deposited in the GeneBank" using the tool BLAST (Basic Local Alignment Searching Tool) [21] of "National Center for biotechnology Information (NCBI) <www.ncbi.nlm.nih.gov> (Access in: July 2017) and were also compared with the database "Ribosomal Data base Project"(RDP).

Biosurfactants production by rhizobacteria and their consortia

The bacteria INPA R302, R332, INPA R555 and INPA R713 were used individually or as five consortia (Table 1) for the biosurfactants production. For each formed bacterium and consortium, 100 mL of the mineral medium salts SYM [22] and liquid mineral medium INPA [23] (Table 2), plus 0.1mL of Urucu's crude oil (extracted from the Base of Operations Geólogo Pedro de Moura, on the banks of Urucu river, municipality of Coari, Amazonas) in 250-mL Erlenmeyer flasks. Flasks were used as control containing only the culture media (SYM or INPA) and petroleum. The flasks were incubated at 30ºC in rotary shaker at 65rpm for nine days. The initial and the final pH of each sample were measured to check if they changed.

Table 1. Bacteria consortia tested for biosurfactants production

Consortium	Bacteria
C01	INPA R302, R332, R555 e R713
C02	INPA R302, R332 e R555
C03	INPA R302, R332 e R713
C04	INPA R302, R555 e R713
C05	INPA R332, R555 e R713

Table 2. Salts constitution of the media SYM and INPA

Medium SYM [22]		Medium INPA [23]	
Components	**Quantity**	**Components**	**Quantity**
K_2HPO_4	0.5 g	$MgSO_4 \cdot 7H_2O$	0.4 g
$MgSO_4 \cdot 7H_2O$	0.2 g	$Ca(CH_3COO)_2 \cdot H_2O$	0.02 g
NaCl	0.1 g	KH_2PO_4	1.5 g
Yeast extract	0.5 g	K_2HPO_4	0.5 g
Distilled water	1000 mL	$(NH_4)_2SO_4 \cdot 7H_2O$	2.5 g
		$FeCl_3 \cdot 6H_2O$	0.05 g
		$FeSO_4 \cdot 7H_2O$	0.05 g
		$ZnSO_4 \cdot 7H_2O$	0.01 g
		$CuSO_4 \cdot 5H_2O$	0.01 g
		$MnSO_4 \cdot 7H_2O$	0.01 g
		Na_4SeO_3	0.01 g
		Distilled water	1000 mL

Evaluation of the emulsification activity

Suspensions of consortia of rhizobacteria in mineral media SYM and INPA, added of petroleum were used for this experiment. For each 3.5mL of bacteria suspension, 3.5mL of petroleum were added in test tubes that were agitated during 2 minutes in vortex and left for 48 hours at rest. After this time, with the aid of a pachymeter, measurements were carried out at the

height of the emulsion layer (EL), water in oil (WO) and Total Height (TH) of liquids in the tubes, expressed in cm. The emulsifying index was obtained using the formula: EI (%) = (EL/TH)*100 [24]. The emulsifying activity of oil in water was measured by optical density in a spectrophotometer at 610 nm absorbance [25] (Fig.1) and the qualification of the emulsifying activity was performed [26], as shown in Table 3. Two evaluations were made of emulsifying layers, one initial, with five minutes of incubation and a final, with 48 hours of incubation.

Table 3. Adopted convention to qualify the emulsificant activity [26]

Emulsificant activity	Height of emulification (water in oil)	Optic density 610 nm (oil in water)
High	≥2 cm	≥1.2 U
Moderate	1.0 - 1.9 cm	0.7 – 1.1 U
Low	‹ 1.0 cm	0.1 – 0.6 U

The experiments followed a completely randomized design, with 3 repetitions. The data were subjected to analysis of variance (ANOVA) by F test. When significant, comparisons of means were performed by the Scott-Knott test at 5% probability, using the program ASSISTAT 7.0.

Results and discussion

Rhizobacteria molecular identification

From the sequencing of the 16S rRNA gene, the genera of four rhizobacteria were identified, two of which classified as *Rhizobium* sp, one as *Paenibacillus* sp and the other one as *Bradyrhizobium* sp. (Table 4).

Table 4. Bacteria identification using comparison of 16s rDNA sequences from GenBank (analise BLASTn)

Bacteria	Results of BLASTn	Identity (%)	Acess number
INPA R302	*Paenibacillus* sp.	99	DQ3424561
INPA R332	*Rhizobium* sp.	99	KM4337361
INPA R555	*Bradyrhizobium* sp.	100	KJ1254011
INPA R713	*Rhizobium* sp.	100	TJ167789789

Biosurfactants production by the formed consortia

Figure 1 shows the potential for the biosurfactants production in test tubes, being possible to visualize the emulsifying activity in the water in oil layer (W/O) by the formation of foam with bubbles appearance between the carbon source and the supernatant soon after the first minutes of the test (Table 5).

It was clearly observed, with some exceptions, that the treatments where the bacteria were used individually produced thicker layers in the SYM medium, but upon being used in mixture in the form of consortia, the largest layers were in the INPA medium.

All the bacterial treatments produced significant layers of emulsifiers, as comparing them with the control treatment (Table 5).

The water/oil layer represents the water dispersed inside the oil, where the liposoluble emulsifiers are found (nonionic, hydrophobic) [27]. The thicker the emulsifying layer the greater the amount of hydrophobic biosurfactants produced will be, which can contribute to the bioremediation process and demonstrates abilities of these microorganisms to remove and degrade petroleum hydrocarbons.

Upon comparing the constitutions of the SYM and INPA media (Table 2), one must consider that the yeast extract present in the SYM medium is an organic component that has nitrogen quantities of essential vitamins for better growth and microbial biosynthesis [28].

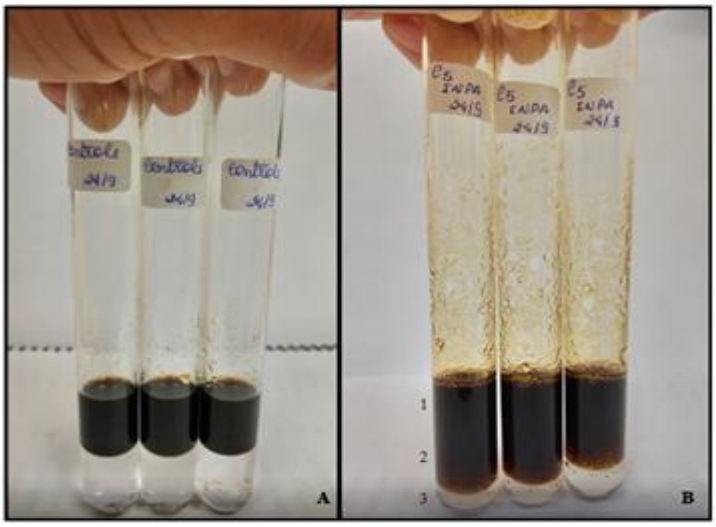

Fig. 1. Biossurfactants production. A: control tubes petroleum + INPA medium. B: petroleum + meio INPA + bacteria: 1 = petroleum; 2 = emulsificated layer; 3 = médium with bacteria. Tests with triplicates.

Tablel 5. Emulsificant layer thickness (EL) from rhizobacteria using two different media and petroleum as carbon source.

Treatments	EL			
	---------- cm ----------			
	SYM		INPA	
	Initial (5 minutes)			
Control	0.00 gX	B	0.00 hX	L
INPA R302	1.07 fX	M	0.78 gX	L
INPA R332	1.45 fX	M	0.88 g Y	L
INPA R555	4.55 bX	A	1.92 e Y	M
INPA R713	3.38 cX	A	1.17 f Y	M
Consortium 01	2.07 eY	A	2.65 dX	S
Consortium 02	2.81 dY	A	3.59 cX	S
Consortium 03	2.09 eY	A	3.29 cX	S
Consortium 04	2.83 dY	A	4.56 bX	S
Consortium 05	9.36 aX	A	9.14 aX	S
Means *	3.29 X		3.11 X	
	Final (48 hours)			
Control	0.00 eX	B	0.00 fX	L
INPA R302	5.84 cX	A	3.66 eY	S
INPA R332	6.32 cX	A	5.21 dY	S
INPA R555	6.50 cX	A	4.17 eY	S
INPA R713	7.37 bX	A	5.82 dY	S
Consortium 01	4.97 dY	A	7.96 bX	S
Consortium 02	6.22 cX	A	6.23 dX	S
Consortium 03	5.16 dY	A	6.93 cX	S
Consortium 04	5.96 cX	A	5.72 dX	S
Consortium 05	12.23 aX	A	11.15 aY	S
Means *	6.73 X		6.32 Y	

Emulsificant activity: S (Strong), M (Moderate) and L (Low) [26].
Means with the same lowercase letters in the columns and capital letters in the lines do not differs statistically by the Scott-Knott test at 5% of probability. * Means without the control.

The presence of the yeast extract in the SYM medium favored the achievement of better percentage of petroleum degradation in the treatments where the rhizobacteria were uses individually, indicated by the largest thicknesses of the emulsifier layer. Studies indicate that the use of more easily assimilated nutritional sources are beneficial to the biodegradation of recalcitrant compounds, because some favor the increase of biomass and energy gain, resulting in better removals of these compounds [29]. However, this fact did not occur in the tests containing the consortia in the same medium (SYM), because different microorganisms together tend to compete among themselves for resources [27].

The INPA medium, on the other hand, contains Se^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, considered enzymatic cofactors essential for catalytic activity of several enzymes [30, 31]. These metallic components may act as triggers that accelerate the rate of enzymatic reaction to promote an active state of enzymes involved with the biosurfactant production.

In general, the presence of the yeast extract of the SYM medium may have favored the bacteria action when placed individually, while for an integrated and more efficient action (consortia), the enzymatic cofactors included in the INPA medium may have acted with more intensity.

However, despite the microbial growth not being high in the early hours, at the end of the test all bacteria and their consortia presented emulsifying layers considered high [26]. The addition of nutrients that encourage the growth of degrading microorganisms of environmental contaminants is well known [32], because a higher microbial activity leads to greater action against these undesirable organic components.

For the evaluation of the emulsifying activity (Table 6), the absorbance values of oil in water - O/A were measured (Figure 1), defined as the amount of biosurfactants needed to increase the absorbance in 1.0 unit at 610nm and the treatments did not show high quantities of emulsifiers in the analyzed media. After 48 hours of testing, the consortia 03 and 04 showed moderate activity with 0.74 and 0.70 Us in the SYM medium and the Consortia 02 and 04 with 0.73 and 1.04 Us in the INPA medium (Table 6). It was observed that the bacteria showed higher emulsifying activity when in the SYM medium, except for the consortia 02, 04 and 05 that showed greater activity in the INPA medium.

Table 6. Optic density – Emulsification activity from the bacteria using two different media solutions and petroleum as carbon source

Treatments	Oil in water			
	SYM		INPA	
	---------- U ----------			
Control	0.03 eX	B	0.03 fX	L
INPA R302	0.19 dX	B	0.07 eY	L
INPA R332	0.33 bX	B	0.02 fY	L
INPA R555	0.20 dX	B	0.03 fY	L
INPA R713	0.32 bX	B	0.07 eY	L
Consortium 01	0.27 cX	B	0.10 eY	L
Consortium 02	0.34 bY	B	0.73 bX	M
Consortium 03	0.74 aX	M	0.51 dY	L
Consortium 04	0.70 aY	M	1.04 aX	M
Consortium 05	0.20 dY	B	0.55 cX	L
Means *	0.37 X		0.35 X	

Evaluation: S (Strong), M (Moderate) and L (Low) [26].
Means with the same lowercase letters in the columns and capital letters in the lines do not differs statistically by the Scott-Knott test at 5% of probability. * Means without the control.

It was observed that there was also a significant difference between the media for the emulsifying index (Table 7), reflecting statistically in the indices of emulsification and stability of hydrophobic emulsifiers. In general, as observed with the layers of emulsifiers (Table 5), the

bacteria individually also produced more emulsifiers in the SYM medium, but when mixed as Consortia, they showed higher production of emulsifiers, with few exceptions, in the INPA medium.

Table 7. Emulsification Index (EI) of the bacteria.

Treatments	SYM		INPA	
	% Initial			
Control	0.00	fA	0.00	hA
INPA R302	2.24	eA	1.50	gA
INPA R332	2.71	eA	1.78	gA
INPA R555	8.41	bA	3.66	eB
INPA R713	6.97	cA	2.33	fB
Consortium 01	4.78	dB	5.93	dA
Consortium 02	6.28	cB	8.13	cA
Consortium 03	4.70	dB	7.45	cA
Consortium 04	6.59	cB	10.37	bA
Consortium 05	21.54	aA	21.01	aA
Means *	7.14	A	6.91	A
	% Final			
Control	0.00	eA	0,00	iA
INPA R302	13.20	dA	7.21	hB
INPA R332	12.81	dA	11.18	fB
INPA R555	13.20	dA	8.72	gB
INPA R713	16.69	bA	12.29	fB
Consortium 01	12.33	dB	19.20	bA
Consortium 02	14.07	dA	15.06	dA
Consortium 03	12.61	dB	16.97	cA
Consortium 04	14.94	cA	13.79	eA
Consortium 05	29.31	aA	27.46	aB
Means *	15.46	A	14.64	B

The EI % formed in the SYM medium ranged from 2.24 to 21.54% (initial) and 12.33 to 29.31% (final), while in the INPA medium, 1.50 to 21.01% (initial) and 7.21 to 27.46% (final), with the Consortium 05 being the best biosurfactants producer using petroleum.

The emulsifying index (EI) and the emulsion stability are important for the assessment of the emulsifying power [33]. The emulsifying layers formed by all these isolates, classified as high (very thick), have a direct influence on the emulsifying index. As the emulsion and complexation processes production is affected by a number of factors, including pH, temperature and the loads present in the bio emulsifiers and bio sorbents [34], the additional presence of these salts in the INPA medium can be related to better emulsification indexes produced by bacteria in this medium.

These results demonstrate that these bacteria produce surfactants like the ones observed by 15 rhizobia isolated from the rhizosphere of leguminous plants [15]. These authors reported that three strains, UNIP R19, UNIP R21 and UNIP R22 stood out by presenting emulsifying indices respectively of 1.58, 1.51 and 1.56. Many organisms have enzymatic capacity to degrade the hydrocarbons existing in the petroleum [15-19, 25].

One of the criteria to determine the level of emulsion stability of an emulsifier is its ability to maintain its EI at least 50% after 24h of its formation [35]. EI of 58.1% was obtained with *Lactobacillus* sp. when gasoline was used as substrate [36]. Once that *Lactobacillus* are known for their importance in maintaining human health, mainly to produce antimicrobial substances and adhesion inhibitors, such as biosurfactants, it is expected that this type of bacterium possesses a metabolism directed to the production of emulsifying substances fairly stable, because these play a crucial role on the inhibition of pathogens adhesion. Emphasis should be given to the EI% obtained in the results of rhizobacteria, both in the SYM and INPA media, because these organisms are part of the microbial population in the soil and can be used

in environments contaminated with petroleum hydrocarbons in case they are not pathogenic to plants and animals. The results of IE% found in the present study corroborate with the ones obtained by [37] using microorganisms isolated from food in decomposition, in Nigeria. These authors reported EI equal to 27.14% when using kerosene as a carbon source, and EI equal to 28.12% when the carbon source was the plant oil.

Table 8. pH values during the test for the emulsification potential of the bacteria.

Treatments	pH			
	SYM		INPA	
	Initial	Final	Initial	Final
Control	7.26 c	7.58 c	6.59 b	6.57 c
INPA R302	7.36 b	7.70 b	6.67 a	6.72 b
INPA R332	7.26 c	7.96 a	6.53 b	6.87 a
INPA R555	7.36 b	7.79 b	6.65 a	6.77 b
INPA R713	7.37 b	7.90 a	6.66 a	6.75 b
Consortium 01	7.49 a	8.06 a	6.66 a	6.61 c
Consortium 02	7.44 a	7.93 a	6.67 a	6.64 c
Consortium 03	7.38 b	7.96 a	6.65 a	6.56 c
Consortium 04	7.32 b	8.00 a	6.62 a	6.58 c
Consortium 05	7.45 a	8.02 a	6.67 a	6.74 b
Means	7.37	7.89	6.64	6.68

Obs.: Means with the same letters in the columns do not differs statistically by the Scott-Knott test at 5% of probability.

In addition to the differences of IE%, it was observed that during the emulsification, the pHs of the treatments were different and dependent on these media (Table 8). It is noticed that the pH, when using the salts of SYM medium, was above 7.0, between 7.26 and 8.06, slightly alkaline, while using the salts of the INPA medium, the pH was slightly acid, ranging between 6.56 and 6.87.

pH is a chemical factor that directly affects the microbial activity, due to the effects of H^+ ions in cellular permeability and enzymatic activity, and, indirectly, by the influence on the availability of macro and micronutrients and the aluminum and other heavy metals solubility, which can be toxic to the microorganisms [38].

On the other hand, the phosphate buffers (KH_2PO_4 and K_2HPO_4) present in the INPA medium provided greater stability to the solution, preventing large pH fluctuations. Studies suggest that when the ratio of quantities of phosphates KH_2PO_4 and K_2HPO_4 buffers is three to one, the solution tends to stabilize at pH 6.4 [39]. These results are like those found in other studies, which showed a pH between 7.0 and 8.0 as conducive to the microbial activity in the petroleum degradation [40, 41].

Some characteristics have advantages over conventional surfactants as tolerance to temperature, pH and ionic strength. Some biosurfactants have high thermal stability and pH and can be used in environments with more drastic conditions. The lipopeptide of *Bacillus licheniformis* JF-2 is stable at temperatures around 75 °C for up to 140 h and pH between 5 and 12 [1]. According to these parameters, even with variations in pH between 4 and 7 in the samples with the saline media (INPA and SYM) and with the carbon source (petroleum), the obtained values are within the range of ideal pH (Table 7).

Conclusions

Significant differences occurred among the treatments and the emulsifiers production in the media SYM and INPA.

The values of the emulsification index water in oil were high, demonstrating the potential of these bacteria to produce biosurfactants, despite the low and medium values acquired in the emulsifying index of oil in water.

Consortium 05, composed by bacteria INPA R332, INPA R555 and INPA R713, was the one that produced the largest quantities of emulsifiers using any one of the two media.

These results demonstrate that these rhizobacteria may become a viable and safe alternative for soils bioremediation contaminated with petroleum hydrocarbons.

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