Full Length Research Paper

Potential biosurfactant producing endophytic and epiphytic fungi, isolated from macrophytes in the Negro River in Manaus, Amazonas, Brazil

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Endophytic and epiphytic fungi isolated from *Eichhornia crassipes* (Mart.) Solms and *Cyperus ligularis* L., macrophytes collected from oil-contaminated waters, were studied to assess their potential for producing biosurfactants; the most promising ones were identified by means of the rDNA region sequencing. In the selection, in the hydrocarbonate biodegradation activity, 2.6-indophenol (DCPIP) in oil-added Bushnell-Haas (BH) medium was the indicator used. The following tests were performed to ascertain the biosurfactant, bioemulsifier activity: emulsification measurement, drop-collapse, surface tension and production slope. Of the twenty fungi isolated, six promoted DCPIP discoloration. The isolate (S31) *Phoma* sp. showed emulsification of diesel (1.5 cm or 52%) and reduction of the surface tension of 51.03 mN/m water identified as *Phoma* sp. The other five fungi were identified as *Rhizopus oryzae* (S24), *Fusarium* sp. (S32, S33, S42, S46), presenting potential for biodegradation of hydrocarbons, as well. New studies on *Phoma* sp. (S31), including its cultivation in different carbon sources will be necessary to improve the production of secondary compounds involved in surface tension bioemulsification and reduction.

Key words: Bioremediation, bioemulsifiers, *Eichhornia*, *Cyperus*, oil, diesel.

INTRODUCTION

Biosurfactants may be of microbial origin and show potential for commercial applications in several fields.

These products are shown to be efficient in processes of microbial enhanced oil recovery and bioremediation in

hydrocarbon-contaminated environments. They also possess potential applications in agriculture, raw materials for cosmetics, pharmaceutical products, detergents, personal hygiene products and food processing, among others (Sourav et al., 2015). They are more advantageous than the synthetic-derived ones when considering their biodegradablity and low toxicity.

Biosurfactants are biologically produced from various substrates, such as waste from tropical agronomic cultures, food processing industries, fruit processing industries, petroleum processing, and coffee processing industries (Bento et al., 2008; Sourav et al., 2015). Given that biosurfactants are produced by bacteria, yeasts and filamentous fungi, they may be produced by endophytic and epiphytic fungi as well. Endophytic microorganisms, according to Azevedo (2008), live inside a plant, at least for one period of their life cycle, as opposed to the epiphytic microorganisms that live on a plant's surface.

Curvularia clavata, *Fusarium proliferatum* and *Phoma* sp. isolated from different hydrocarbon-contaminated environments are the major species of the genera referred to as biosurfactant producers. For the biodegradation of hydrocarbons, genera *Cladophialophora* and *Exophiala* assimilate toluene; *Aspergillus* sp. and *Penicillium* spp. degrade aliphatic hydrocarbons, aliphatic hydrocarbons, chlorophenols, polycyclic aromatic hydrocarbons (PAHs), pesticides, synthetic dyes and 2,4,6-trinitrotoluene (TNT) (Harms and Schlosser, 2011; Bhardwaj et al., 2015; Neoh et al., 2015). Fungi are able to grow under environmental stressed conditions: environments with low pH, poor in nutrients, and low water availability media favor their growth (Mollea et al., 2005). They may still, though more rarely, produce biosurfactants (Luz et al., 2011).

This study is an important contribution to identifying fungal strains collected from similar environments in the Amazon region. These strains can further be exploited commercially, both in the production of new bioemulsifiers or biosurfactants, as well as biodegradation of contaminated oil environments, and open a range of new possibilities for research with these microorganisms, notably with regard to their physiology in producing amphiphilic compounds. The purpose of this work was to assess the potential of endophytic and epiphytic fungi, for the biodegradation of hydrocarbons and production of biosurfactants or bioemulsifiers.

MATERIALS AND METHODS

Samples and biological material

The isolates studied are from the collection of cultures of the Biodegradation Laboratory of the Agronomic Sciences College, Federal University of Amazonia. The microorganisms were isolated from the macrophytes: *Cyperus ligularis* L. and *Eichhornia crassipes* (Mart.) Solms collected near the effluent output from a Petrobras/Manaus-AM (REMAN) refinery, and stored according to Castellani (1939). Plant species were identified at the herbarium of the National Research Institute of Amazonia – INPA.

Oil

Crude oil was from the Urucu Oil Base, Amazonas, Brazil, and the diesel was acquired at a gas station, and previously filtered using millipore membrane (0.22 mm) in order to sterilize it. Oil and diesel doses were used according to Jaquiche-Matsuura et al. (2014).

Biodegradability test using the redox 2,6-dichlorophenol indophenol (DCPIP) indicator

The biodegradability test was performed through the DCPIP technique (Hanson et al., 1997). The experiment was carried out in a 96-well polystyrene plate and DCPIP concentration was adjusted to 0.010 g/mL. 200 µL of the DCPIP solution, 10 µL of oil from Urucu and hyphaes of fungi grown in BH + oil, inoculation corresponding to 3 mm of the diameter, were added to each well. The plates were kept at room temperature (27±2°C). Medium discoloring-time measurements were taken following 24 and 48-h. DCPIP with oil and without strain was used as a positive control and DCPIP without oil and without strain was used as negative control.

Specific medium for biosurfactant production

Biosurfactant production was undertaken in 50 mL of culture medium composed of $MgSO_4.7H_2O$ (0.5 g/L), KH_2PO_4 (1 g/L), $NaNO_3$ (3 g/L), yeast (1 g/L) and peptone (0.3 g/L) extract, with pH adjusted at 5 for filamentous fungi, modified by Rapp and Backhaus (1992). Diesel oil or oil at 1.0% v/v was used as carbon source. The diesel was used in biosurfactant production or bioemulsifier because their carbon chains are less complex than the oil initially used.

Microbial culture of isolate (S31) was carried out in 125-mL Erlenmeyer flasks at 30°C in an orbital incubator (New Brunswick Scientific) with 150 rpm steady stirring for 20 days. Erlenmeyer flasks with 50 mL of culture medium and 1% diesel oil (v/v), with no inoculation, was used as control. Every microorganism was cultured in triplicate. Following the incubation period, the culture media were filtered in filtering membrane (TPP, Europe/Switzerland) with 0.45 mm porosity coupled with a 20-mL sterilized syringe.

Oil drop-collapse qualitative test

The test was conducted in 60 x 12 mm Petri dishes containing 3.5 mL of filtered cell-free extract. To carry out the test, an oil drop was added to the cell-free extract in triplicate and observed for 0, 1, 5, 30 min, 1 and 72 h. The result was regarded positive when the oil drop dispersed. A total of 3.5 mL fungus-free extract and 3.5 mL 1 M dodecyl sulfate sodium (DSS) surfactant solution were used as negative and positive control, respectively.

Emulsification activity assessment

The specific medium for the production of biosurfactants was filtered and assessed as the water in oil (W/O) type, emulsification. The test was performed in triplicate. 3 mL of the cell-free culture extracts and 2 mL of diesel were added to test tubes. These were vortexed for 2 min at 70 rpm and kept at rest for 24 h. Following this period, the emulsified oil height (cm) was compared with the total. The emulsification was calculated according to Equation 1:

$$E_{24} = \frac{He}{Ht} \times 100$$

Table 1. Molecular identification of endophytic and epiphytic fungi isolated from macrophytes in the Negro River-Amazonas/Brazil, with the deposition number, National Center for Biotechnology Information (NCBI) data as reference.

Iso*	Microorganisms	NCBI Sequences	Hosts	Endo.*	Epi.*	Id.*(%)
S24	*Rhizopus oryzae*	KU948381	*Cyperus ligularis*		X	99
S31	*Phoma* sp.	KU948382	*C. ligularis*		X	100
S32	*Fusarium* sp.	KU948385	*Eichhornia crassipes*		X	99
S33	*Fusarium* sp.	KU948386	*C. ligularis*		X	98
S42	*Fusarium* sp.	KU948384	*C. ligularis*		X	99
S46	*Fusarium* sp.	KU948383	*C. ligularis*	X		99

*Iso = Isolate; Endo. = endophytic; Epi. = epiphytic; Id. = identity.

Where, E_{24} = Emulsification index following 24 h (%); $E_{mulsion}$ = emulsion height; H_t = total height.

Diesel emulsion production slope

For the production slope, the specific medium for the production of biosurfactants and diesel was used at 1% (v/v) as carbon source, by maintaining pH 5. Into each 50-mL Erlenmeyer flask, was added, as inoculation, five 5-mm disks of the fungus culture through 150 rpm steady stirring in an orbital incubator (New Brunswick Scientific) at 30°C. The emulsification index was measured by using Equation 1 every 48 h for a total of 14 triplicate measurements. The statistical analysis was performed through standard deviation means calculated with the *software* BioEstat 5.3 (Ayres et al., 2007).

Surface tension assessment

Surface tension is a common metric and direct method for monitoring the production of biosurfactants. As the microorganism grows, it synthesizes the biosurfactant and this metabolite is excreted to the metabolic broth, reducing the surface tension. The surface tension was measured in the Kruss model tensiometer (K-6, Germany), by the ring method (Du Noüy). The analyses were performed with the supernatant obtained after the raw sample centrifugation at 25°C. Every time, the analyses began, the ring was sterilized by gas burner and calibrated by checking the distilled water surface tension whose value is about 72.8 mN/m. Three measurements of surface tension were made, considering the arithmetic mean of the results (Jaquiche-Matsuura et al., 2014).

Molecular identification of filamentous fungi

Molecular identification was performed only on samples that presented a positive result in the indicator test with redox 2,6-dichlorophenol indophenol (DCPIP) containing oil and on those with a result above 1 cm for emulsification index. For the extraction of the DNA, the plant/fungi DNA isolation kit (Norgen Biotek Corp) was used according to the manufacturer's instructions.

Primers ITS1 (5' – TCCGTAGGTGAACCTGCG G – 3') and ITS4 (5' – TCCTCCGCTTATTGATAT GC – 3') were used for the amplification of the region kept in specific positions of the 18S and 28S of the rDNA gene. The amplicons were purified with polyethylene glycol 8000 and sequenced in an ABI 3500xL genetic analyzer (Applied Biosystems®). Sequences were aligned and edited in a MEGA program with grouping by neighbor-joining method and, employed for identifying isolates by comparing themselves with the type sequences based on the results found in BLASTn.

Toxicity test using *Artemia salina*

The aqueous extract of *Phoma* sp. selected as the producer of bio emulsifier was used to evaluate the toxicity front larvae *Artemia salina*. Toxicity assay was performed with *A. salina* according to Meyer et al. (1982) by preparing a solution with sea-salt in the concentration of 30 g.L^{-1}. The pH was adjusted to 8 with NaOH 0.1 mol/L^{-1} solution. This solution was used in the preparation of the remaining dilutions. The eggs were placed to hatch in a saline solution for 48 h, with steady aeration at 25°C.

The test was conducted in multi-welled plates, by using the cell-free extract concentrations: 100, 50, 25, 13, 7 and 3 µL; ten *A. salina* larvae, with 2000 µL being the final value. After 24 h, mortality was determined with the aid of a stereoscopic binocular microscope (Leica El 224). The LC$_{50}$ was calculated according to Finney (1947).

RESULTS

Isolate molecular identification

Eight fungi selected by growth in oil-added BH medium were identified through sequencing part of the rDNA gene. Its fragments containing the ITS1-5,8S-ITS2 region were presented between 531 and 562 bp. Epiphytic fungi S31 and S36 isolated from *C. ligularis* were identified as *Phoma* sp.; S24 *Rhizopus oryzae*; S32 *Fusarium* sp.; S33 *Fusarium* sp.; and S42 *Fusarium* sp. Among endophytic fungi, S42 was identified as *Fusarium* sp., both originating from *E. crassipes*. The sequences were deposited in the National Center database for Biotechnology Information and are shown (Table 1).

Among the eight analyzed fungi, only *Phoma* sp. (S31), was efficient in the tests undertaken to select biosurfactant producers. Though, they had dispersed an oil drop at different times, the remaining isolates formed no emulsion above 1 cm, according to the method

Table 2. Selection of endophytic and epiphytic fungi isolated from macrophytes in the Negro River– Amazonas/Brazil for the production of biosurfactants.

Isolates		Oil drop-collapse						Emulsification index	Surface tension (mN/m)
		0	1 min	5 min	30 min	1 h	72 h	H_u/E_{24}	
(S31)	*Phoma* sp.	+	+	+	+	+	+	1.5 cm/52%	51.03
(S24)	*Rhizopus oryzae*	-	-	±	±	±	±	0	-
(S46)	*Fusarium* sp.	-	-	-	-	-	-	0	-
(S42)	*Fusarium* sp.	-	+	+	+	+	+	0	-
(S32)	*Fusarium* sp.	-	-	±	±	±	±	0	-
(S33)	*Fusarium* sp.	-	±	±	±	±	±	0	-
	Control								
	DSS	+	+	+	+	+	+	100%	
	BH+FFO	-	-	-	-	-	-	0	53.03 mN/m
	Water								71.26 mN/m

*DSS = Dodecyl sodium sulfate was used as positive control ; BH+FFO = fundus free oil medium used as negative control; H_u = emulsified height; E_{24} = emulsification index in 24 h. ± = low collapse, + = high collapse.
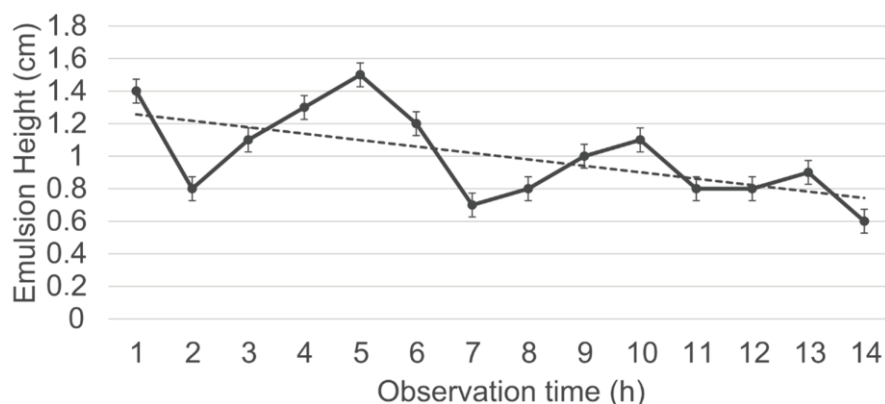

Figure 1. Bioemulsifier production slope of the endophytic fungus *Phoma* sp. (S31) isolated from *Cyperus ligularis* L. occurring in an oil-contaminated area in the Negro River Amazonas State, Brazil.

adopted (Table 2).

Only *Phoma* sp. (S31) was selected for surface tension test, since it was positive in the oil drop-collapse test sooner and its bioemulsifier was above 1 cm. The surface tension initially observed was 51.03 mN/m. The difference between fungus extract and control surface tensions was 2 mN/m, yet when it was added to water, it was 18.23 mN/m, indicating it would be able to break surface tension. To ascertain the fungus extract-promoted diesel emulsification, a production slope was performed, taking emulsified height-fungus growth relativeness into account (Figure 1). The slope shows bioemulsifier production occurred up to the 14th measurement, yet it peaked at the 5th or 10th day following its growth, forming 1.5 cm high emulsion. This variation continued until the very end of the experiment. The cell-free aqueous extract of *Phoma* sp. (S31), showed no toxicity with *A. salina* in the concentrations used (Figure 2).

DISCUSSION

Oil with BH medium-grown isolate behavior may be analyzed according to Jaques et al. (2007), Maciel et al. (2013) and Cruz et al. (2014), wherein, both fungi and bacteria may be hydrocarbon biodecomposers. Microorganisms shown to be successful in degrading these compounds should produce enzymes able to use complex oil molecules in their metabolic pathways' intermediate products. In this case, fungi had non- and ligneous metabolic pathways, like the fungi *Cunninghamella elegans*, *Pleurotus ostreatus*, *Aspergillus fumigatos* and *C. lunata*, which have been already studied as effective decomposers of oil and its derivatives (Bhatt et al., 2010).

Junior et al. (2012) identified *Phoma* genus fungi as good producers of laccase, a phenol group enzyme able to biodegrade phenolic compounds and hydrocarbons.
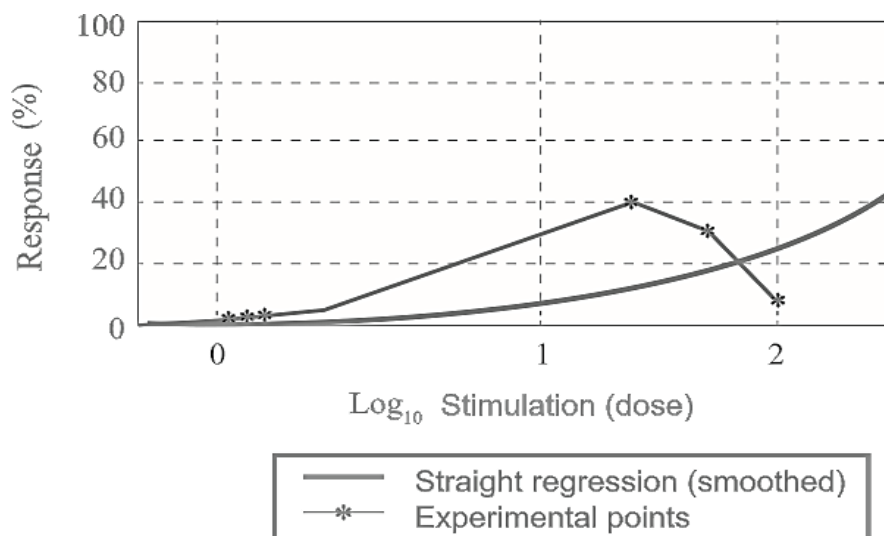
Figure 2. Probit analysis of the six cell-free *Phoma* sp. (S31) fungal extract concentrations: 100, 50, 25, 13, 7 and 3 µL.

Carneiro and Lucas (2010) confirmed this view in an important study on bioremediation, using microorganisms in the metabolization of several compounds, including oil.

Harms and Schlosser (2011) reported *Rhizopus* genus cultures to be potential HPA decomposers; this, in some regard, may account for this genus species presence in selecting tests using oil-added BH medium (Table 1). Balaji et al. (2014), in a study done in India, cited nine genera fungi such as, *Aspergillus*, *Curvularia*, *Drechslera*, *Fusarium*, *Lasiodiplodia, Mucor, Penicillium, Rhizopus* and *Trichoderma*, isolated from oil-contaminated soil.

The filamentous fungus *C. clavata* was cited by Neoh et al. (2015) to bioremediate palm industry effluents, produce ligneous enzymes, in addition to dramatically reduce polyphenolic compounds. Detoxification of effluent indicates C. *clavata* suitability in the bioremediation of organic effluents, which is important, since it can direct future studies using fungi, identified in the present research in similar processes. With regards to the DCPIP oxidation time, the identified Amazonian fungi findings are consistent with those of Maciel et al. (2013), who found that it takes from 14 to 25 h for these fungi to promote indophenol discoloration. Isolates from macrophytes promoted it within 24 h. In a study by Luz et al. 2011), it took 96 h.

Maciel et al. (2013) pointed out the isolates responsible for degradation to be *Penicillium aurantiogriseum, Penicillium corylophilum* and *Penicillium griseofulvum.* Other species from this genus were identified amongst the isolates from macrophytes as well (Table 2). According to Silva and Esposito (2004), the degradation of pollutants is performed by the intracellular enzyme cytochrome P450 monooxygenase system, which makes water-soluble products less toxic and leads to a detoxification process. These enzymes are likely involved

in this process. These authors reported genera *Trichoderma* and *Fusarium* in oil-contaminated soils; *Fusarium* was isolated from macrophytes in an oil-contaminated environment.

Regarding biosurfactant production, though filamentous fungi had grown in oil-added BH medium, as the sole carbon source, most of them were unable to produce it through selective tests such as drop-collapse, emulsification index and surface tension. *Phoma* sp. (S31) was the only one able to produce oil drop-collapse in less than 1 min, showing 1.5 cm or a 52% emulsification index, as compared to oil and diesel; this index is regarded to be moderate. It also decreased surface tension by 51.03 mN/m. The emulsification index of *Phoma* sp. is comparable to the emulsification rate of the bacterium, *Gordonia amicalis* (51%) and *Bacillus licheniformis* (70%) (Dewaliya and Jasodani, 2013). This shows that the crude extract of the selected fungus has potential as an emulsifier.

This fungus has emulsifying properties, yet little ability to reduce surface tension (51.03 mN/m) (Table 2). The low surface tension might be due to the difficulty of using the hydrocarbonate present in diesel oil for the synthesis of biosurfactants (Decesaro et al., 2013). On the other hand, it is likely that, through the tests performed, the biosurfactant present in fungal extract are of high molecular weight, which would indeed account for the diesel oil's low surface tension and stabilizing property (Bento et al., 2008).

The emulsion formed was of the A/O type, suggesting organic compounds present in this emulsifier, possess hydrophilic characteristics due to emulsion formation always occurring between water and diesel oil or oil. This characteristic is paramount in pollutant bioremediation, since it can aid in the bio stimulation or bio augmentation

process, facilitating other microorganisms' physiological growth in the oil hydrocarbonates-contaminated medium (Deon et al., 2012).

Jackisch-Matsuura et al. (2014) cited the difficulty in finding filamentous fungi that are good producers of biosurfactants and have the ability to reduce surface tension, a fact observed in this study. On the other hand, bio emulsifier production by fungi and bacteria should be thoroughly studied, since they may be used in several applications, such as food processing and paint manufacturing industries, among others (Bezerra et al., 2012).

The best bioemulsifier production activity by *Phoma* sp. (S31) occurred on the 10th day. The emulsification index observed in the slope ranged from 0.6 to 1.5 cm. Such emulsification measurements are comparable to those presented by extracts from *Lactobacillus pentosus* used in the bio emulsification of kerosene, gasoline and octane (Moldes et al., 2013) and *F. proliferatum* for n-dodecane compounds (Bhardwaj et al., 2015). From tropical *Phoma* sp., a cyclic-structured lipopeptide compound was identified and classified as *Phomafungy*, which was shown to be efficient as an antifungal in several tests. Apart from the work by Herath et al. (2009), it was not possible to find or cite any other studies of the genus *Phoma* sp. as a biosurfactant or bio emulsifier producer. Consequently, further studies on the *Phoma* sp. (S31) isolate are needed. Through the toxicity test, the aqueous extract from bio emulsifier producing species, showed no toxicity in the different concentrations used. Such findings were also observed by Decesaro et al. (2013), who found no toxic activity for bio emulsifiers from fungi and bacteria tested in their work.

Further studies involving *Phoma* genus species addressing biosurfactant production mainly related to cultivation in different carbon sources are needed to better understand the production of the involved secondary compounds, both on emulsification and reduction of surface tension. The sequence of studies involving *Phoma* sp. (S31) might be promising to different fields of the food, pharmacological and oil industries. The selection of filamentous fungi continues to be a great challenge due to their low biosurfactant yield.

Conclusion

Every identified fungus is shown to be promising in oil and diesel degradation. Thus, further study pertaining to these fungi enzyme yields is necessary, particularly for enzymes of commercial interest, which is related to oil and its derivative biodegradation, as well as on production of biosurfactants by the selected *Phoma* sp. Microorganisms originating from areas with a history of oil contamination and contamination of its derivatives, which possess physiological mechanisms that actually assist them in capturing hydrocarbons used as a carbon source was found in the test carried out in this study.

Conflict of interest

The authors declare that they have no conflict of interest.

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