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Selection of Lipid-Producing Fungi Present in Fruits of the Amazon Region

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The objective of the present study was to evaluate lipid production by endophytic microorganisms isolated from three Amazonian fruit species - açaí (*Euterpe oleracea*), pupunha (*Bactris gasipaes* K.) and tucumã (*Astrocaryum aculeatum*). The greatest occurrence of microorganisms was found in tucumã (*A. aculeatum*), with 32 isolates, using Sabouraud agar medium. The most frequent genus found in fruits was *Penicillium* sp., while *Aspergillus flavus* CWA153 was the strain with the largest biomass, 4.016 $g.L^{-1}$. *Gliocladium* sp. CWA537 had the highest total lipid concentration among the microorganisms analyzed, with approximately 20.7%. The *Aspergillus* sp. CWA977 strain presented the best lipid productivity, 0.07 $g.L^{-1}.d^{-1}$. The total percentage of monounsaturated fatty acids varied between 15.0 and 26.8%, and oleic acid (C18:1, cis-9) was the most abundant fatty acid in most species analyzed. Linoleic acid was the polyunsaturated fatty acid with the highest concentrations (8.5% to 17.8%); *Gliocladium* sp. TCU104 was the sole strain in which this type of fatty acid was not present.

1. Introduction

Oleaginous or single cell oil (SCO) micro-organisms are defined as oleaginous species that have the ability to accumulate lipids amounting to 20-25% of the biomass weight, reaching 50% when dry (Meng et al., 2009; Papanikolaou et al., 2007). They are commercially produced as a source of specific lipids for dietary supplements and used for the production of bioactive molecules used in cosmetic, nutritional and pharmaceutical products (Enshaeieh et al., 2013, Huang et al., 2013, Kimura et al., 2004). They can be found within plant species and are considered endophytic; their large biodiversity plays an important role in ecosystems, particularly in tropical climates and in temperate tropical forests widely found in Brazil. Almost 20% of these organisms originate from a biotechnological source (Strobel, 2003; Souza et al., 2004, Molina et al., 2012).

SCOs are regarded as alternatives to agricultural and animal resources for the production of oils and fats because they can be produced under controlled fermentation conditions. Their production bears many advantages compared to vegetable oils, including the short life cycle of microorganism production, abundant and cheap raw materials, and a shorter time required for production, thus contributing to the future by becoming one of the great potential sources of raw material for oil production. However, there is still limited information on the use of these oils in the food industry (Liang and Jiang, 2013).

Despite all the advantages over the production of vegetable oils, cultivation medium costs remain high and may hinder future application. The exploration of agro-industrial waste and by-products as raw materials can

not only greatly reduce these costs but also provide a more sustainable destination for these wastes (Castanha et al., 2013, Huang et al., 2013, Lucena et al., 2016).
Oleaginous fungi belonging to the genera *Mortierella*, *Cunninghamella*, *Fusarium*, *Yarrowia*, *Cryptococcus*, *Lipomyces*, *Rhodosporidium*, *Rhodotorula*, *Trichosporon*, *Yarrowia*, *Aspergillus*, *Thamnidium*, *Candida*, *Zygosaccharomyces*, *Zygorhynchus*, *Mucor*, *Torulopsis* and *Pichia* are employed in the production of polyunsaturated fatty acid (PUFA)-rich lipids such as arachidonic acid (ARA), alpha-linolenic acid and eicosapentaenoic acid (EPA) (Berger et al., 2010; Demir et al., 2013; Enshaeieh et al., 2013; Kang et al., 2011; Liang and Jiang, 2013; Papanikolaou et al., 2007). Thus, considering the richness of the Amazonian fauna and flora, the objective of the present study was to isolate new microbial sources of oils from Amazonian fruits.

2. Materials and Methods

2.1 Raw material

Healthy açaí (*Euterpe oleracea*), pupunha (*Bactris gasipaes* K.) and tucumã (*Astrocaryum aculeatum*) fruits, collected at the Brazilian Agricultural Research Corporation (*Empresa Brasileira de Pesquisa Agrícola* - EMBRAPA) in Western Amazon, located in Manaus/Amazonas (AM), were used. The samples were transported in isothermal boxes containing ice to the Laboratory of Mycology of the National Institute of Amazonian Research (*Instituto Nacional de Pesquisa da Amazônia* - INPA), where they were sanitized according to the method described by De Lima et al. (2011).

2.2 Microorganisms and culture medium

The inoculum was plated on Sabouraud agar (Peptone 10 g/L + Dextrose 40 g/L + Agar 20 g/L) containing an antibiotic (Chloramphenicol 500 mg), followed by incubation at 25 ± 2°C for 3-5 days in a bacteriological oven, according to the methods described in the American Public Health Association's Compendium of Methods for Microbiological Examination of Foods (Salfinger and Tortorello, 2015).

2.3 Identification of microorganisms - classical and molecular

Fungi were identified using the microculture technique, and micromorphological and macromorphological aspects of the vegetative and reproductive structures were observed. The results were compared with taxonomic keys to determine the genus (Barnett and Hunter, 1998). Identification by nucleotide sequencing of the ribosomal ITS1 and ITS4 DNA (rDNA) was performed as described by White (White et al., 1990).

2.4 Bioprocess for selection of fungi with greater lipid production efficiency

A total of 50 mL of culture medium (Sabouraud dextrose agar broth (Oxoid)) was added to 125-mL Erlenmeyer flasks. The medium was autoclaved for 15 minutes at 121°C, 1 atm. The medium was subsequently inoculated with 100 µL of fungi and incubated for 120 hours under controlled temperature and stirring conditions (28°C/100 rpm). Cell growth was quantified by the gravimetric determination of dry biomass collected from culture samples after incubation. Total lipid extraction was performed using the Bligh & Dyer method, modified by Manirakiza et al. (2001).

2.5 Determination of lipid fatty acid profiles of microorganisms

The microorganism oils were trans-esterified according to the IUPAC 2.301 standard method (IUPAC, 1987). Chromatographic profiles were recorded, and the percentage of the compounds was determined against a calibration curve obtained using a gas chromatograph (GC) coupled to a mass detector (Shimadzu® GCMS-QP2010, Kyoto, Japan) equipped with a Durabound DB-23 30 m x 0.25 mm x 0.25 µm column. The injector and detector temperature were set to 230°C, and the column temperature was 90°C. The elution gradient in the column followed three different ramps: 90 to 150°C (10°C/minute), 150 to 200°C (5°C/minute) and 200 to 230°C (3°C/minute), over a total of 34 minutes. Helium was used as carrier gas.

3. Results and Discussion

3.1 Identification of isolates

To characterize the lipid-producing ability of Amazonian fungi, the genera *Acremonium* sp., *Aspergillus* sp., *Colletotrichum* sp., *Cladosporium* sp., *Penicillium* sp., *Rhodotorula* sp., *Coelomycetes* sp., *Fusarium* sp. and *Paecilomyces* sp. and sterile mycelium were isolated from the selected fruits. Table 1 lists the taxonomic identification of isolates obtained from the different fruits investigated. The main isolated genera were *Penicillium* sp. (36 strains), *Aspergillus* sp. (15 strains), and *Cladosporium* sp. (13 strains). The largest number of isolates was obtained from tucumã.

Nascimento and Moraes (2011) examined the incidence of fungal species during the germination of açaí (*Euterpe oleracea*) seeds and found a high prevalence of *Penicillium* sp. and *Aspergillus* sp. in seeds with a moisture content of less than 30% and at temperatures of 15°C.

Table 1. Taxonomic identification of the isolates obtained from different investigated fruits (açaí, tucumã and pupunha)

Fruits	Açaí		Pupunha		Tucumã	
Number of isolates	28		26		32	
Genera/Frequency (%)	*Penicillium* sp.	32	*Penicillium* sp.	31	*Penicillium* sp.	22
	Cladosporium sp.	32	*Cladosporium* sp.	23	*Aspergillus* sp.	8
	Aspergillus sp.	17	*Aspergillus* sp.	19	*Paecilomyces* sp.	3
	Sterile Mycelium	10	Sterile Mycelium	15		
	Acremonium sp.	3	*Acremonium* sp.	3		
	Colletotrichum sp.	3	*Paecilomyces* sp.	3		
	Rhodotorula sp.	3	*Coelomycetes* sp.	3		
			Colletotrichum sp.	3		

3.2 Selection of the most efficient strains for lipid production in culture medium

From the 36 strains isolated, 12 (33%) showed potential for lipid production. Table 2 lists the highest values of microorganism biomass productivities after the bioprocess was completed; *Aspergillus flavus* CWA153, *Aspergillus niger* CWA152, and *Penicillium* sp. CWA177 yielded the highest values - 4.016, 3.194, and 2.428 $g.L^{-1}$, respectively. The lipid contents per species biomass weight varied between 0.921 and 20.69%. The productivity of the filamentous fungi species analyzed varied between 0.001 and 0.07 $g.L^{-1}.d^{-1}$. The *Aspergillus* sp. CWA977 genus presented the highest productivity over 5 days, 0.07 $g.L^{-1}.d^{-1}$. Of the isolated fungi, *Aspergillus* sp. CWA977 presented the greatest oleaginous potential, with greater lipid productivity per biomass production.

Table 2. Concentration of lipid-based biomass ($g.L^{-1}$) produced by different fungi, the biomass fraction of lipids (%) and productivity of lipids ($g.L.d^{-1}$) over 5 days

Genera	Biomass ($g.L^{-1}$)	Lipid (%)	Productivity ($g.L^{-1}.d^{-1}$)
Aspergillus sp. CWA977	1.8886	19.185	0.07
Aspergillus flavus CWA153	4.016	7.918	0.06
Penicillium sp. CWA177	2.428	10.296	0.05
Gliocladium sp. TCU 104	1.208	20.695	0.05
Aspergillus niger CWA152	3.194	8.202	0.05
Cladosporium sp. CWA537	1.492	13.404	0.04
Aspergillus sp. CWA151	3.054	3.405	0.02
Penicillium sp. CWA251	0.56	2.857	0.003
Black fungus CWPA27	0.434	0.921	0.001

Venkata Subhash and Venkata Mohan (2011) showed the potential of the filamentous fungi *Aspergillus* sp. as a whole-cell biocatalyst for the production of biodiesel using corn residue as a substrate. The use of Sabouraud dextrose broth resulted in improved biomass production (13.6 g dry weight/1000 mL) and lipid yield (23.3%). André et al. (2010) employed crude glycerol (discarded after the biodiesel production process) as a suitable substrate for the growth of two strains of *A. niger*, which they used to produce lipids and biomass in considerable amounts and with satisfactory conversion yields (3.1-3.5 g.L-^{1} SCO, biomass 5.4 $g.L^{-1}$, over 96 hours). In the Netherlands and in New Zealand, Ratledge (1991) investigated approximately 125 species, including filamentous fungi and yeasts, to analyze their lipid contents, obtaining 49%, 53%, 51%, 48%, 57%, and 57% SCOs for *Cladosporium herbarium*, *Aspergillus fischeri*, *A. nidulans*, *A. ochraceus*, *A. oryzae* and *A. terreus*, respectively. For *Penicillium javanicum*, *P. lilacinum*, *P. soppi* and *P. spinulosum*, SCO values were 39%, 56%, 40% and 64%, respectively.

The growth kinetics of the five most productive fungi were monitored over 5 days. The highest amount of biomass (4.8 $g.L^{-1}$) was obtained for the strain *Aspergillus* sp. CWA 977 after 120 hours of fermentation (Figure 1). The five kinetic profiles show great reproducibility among the triplicate experiments performed for each kinetic profile studied, with small, almost imperceptible, mean standard deviations.

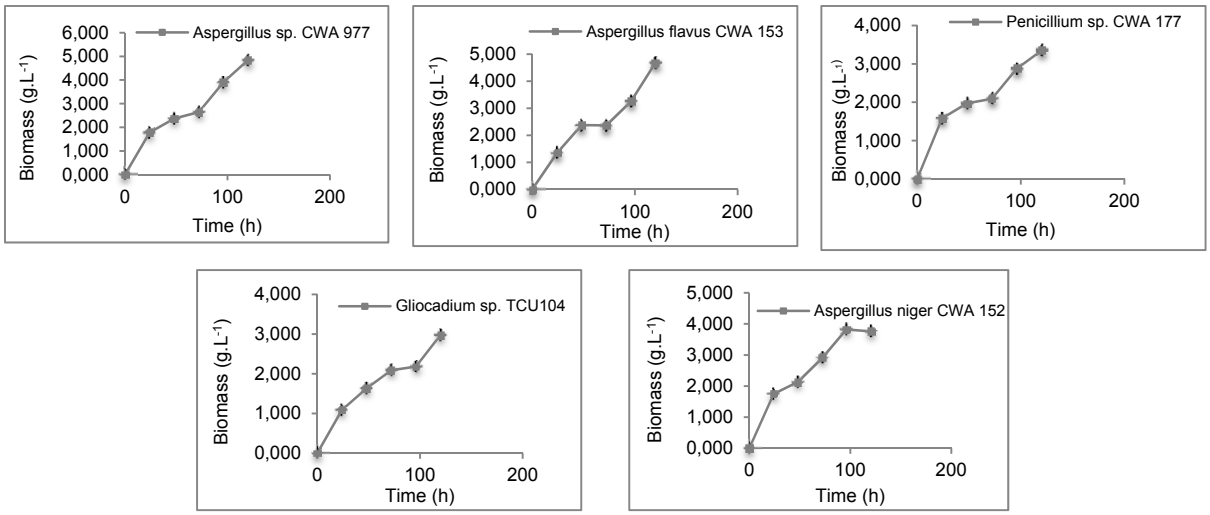

Figure 1. Kinetics of biomass productivity over 120 hours of Amazon isolates from Amazon fruits that were subjected to bioprocessing for the purpose of lipid production

Table 3. Gas chromatography spectra and their fragmentations on mass spectrometry of lipid fractions in the biomass of five fungal isolates obtained from Amazonian fruit

	Aspergillus sp. CWA977	*Aspergillus flavus* CWA153	*Penicillium* sp. CWA177	*Gliocladium* sp. TCU104	*Aspergillus niger* CWA152
Fatty Acids (%)	3.3	3.4	3.5		3.6
Saturated	3.7	3.8	3.9	3.10	3.11
< C16:0	29.0	12.7	7.0	10.7	10.8
C16:0	18.0	20.7	39.3	33.1	23.1
C17:0	nd	nd	0.6	nd	1.2
C18:0	23.7	14.0	16.5	32.7	15.6
C20:1	nd	nd	nd	nd	1.7
C22:0	nd	18.1	nd	nd	nd
Unsaturated	3.12	3.13	3.14	3.15	3.16
Monounsaturated	3.17	3.18	3.19	3.20	3.21
C16:1, cis-9	nd	nd	0.7	nd	nd
C18:1, cis-9	16.9	nd	25.6	15.5	24.6
C18:1, trans-9	nd	15.0	0.5	1.8	1.8
Polyunsaturated	3.22	3.23	3.24	3.25	3.26
C18:2, cis-9,12	12.4	16.0	8.5	nd	17.8
C18:3, cis-6,9,12	nd	3.5	1.3	8.0	3.4
	3.27	3.28	3.29	3.30	3.31
Saturated (%)	70.7	65.5	63.4	76.5	52.4
Unsaturated (%)	29.3	34.5	36.6	23.5	47.6
Monounsaturated	16.9	15.0	26.8	15.5	26.4
Polyunsaturated	12.4	19.5	9.8	8.0	21.2

nd: not determined

3.3 Fatty acid profile of microorganism-produced lipids

The total fatty acid compositions were determined by gas chromatography coupled to mass spectrometry and are shown in Table 3. The chromatographic analysis revealed an array of saturated and unsaturated fatty

acids. The data indicated the presence of 13 fatty acid compounds with different chain lengths, ranging from 12 to 22 carbons. The saturated and unsaturated fractions represented 52.4 to 76.5% and 23.5 to 47.6% of total fatty acids, respectively. Although PUFAs were not the most dominant fraction, their content varied between 8.0% and 21.12% for *Gliocladium sp.* TCU104 and *Aspergillus niger* CWA152. Among the monounsaturated fatty acids (MUFAs), oleic acid (C18:1, *cis*-9) was the most abundant in almost all species analyzed, varying from 15.5 to 25.6%. The total MUFA content varied between 15.0 and 26.8%. Despite its low lipid productivity, *Penicillium* sp. CWA177 presented the greatest production of MUFAs, with 26.8%, followed by *Aspergillus niger* CWA152, with 26.4%. *Aspergillus* sp. CWA977 showed good lipid productivity and was the second best producer of saturated lipids, with 70.7% total content. Among the filamentous fungi species analyzed, palmitic and stearic acid were the most abundant fatty acids. The C:16 content was highest in *Gliocladium* sp. TCU104 (39.3%) and lowest in *Aspergillus* sp. CWA977. The stearic acid content ranged between 14% and 32.7%. For PUFAs, linoleic acid presented the best results among the analyzed strains, varying between 8.5% and 17.8%; *Gliocladium* sp. TCU104 was the sole strain in which PUFAs were not found. Studies of fatty acids in filamentous fungi have been mainly focused on the production of gamma-linolenic acid (GLNA), EPA, docosahexaenoic acid (DHA) and ARA due to their role in improving health (Fakas et al., 2008; Akpinar-Bayizit et al., 2014).

4. Conclusions

Eighty-six fungal species were isolated from the Amazonian fruits açaí, tucumã, and pupunha. Most belonged to the genera *Penicillium* sp., *Aspergillus* sp., and *Cladosporium* sp., with 36, 15 and 13 strains, respectively. Tucumã (*Astrocaryum aculeatum*) exhibited the highest occurrence of microorganisms, with 32 isolates. *Penicillium* sp. was the most frequent microorganism genus among Amazonian fruits. The strain that presented the largest amount of biomass was *Aspergillus flavus* CWA153; the *Gliocladium* sp. CWA537 strain yielded the highest total lipid concentration among the microorganisms analyzed. The greatest lipid yield was produced by *Aspergillus* sp. CWA977. *Gliocladium* sp. TCU104 yielded the greatest saturated fat production, with 76.5%. *Aspergillus niger* CWA152 presented the highest percentages of unsaturated fatty acids, followed by *Penicillium* sp. CWA177, with 47.6% and 36.6%, respectively.

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