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Fruit Peel from *Astrocaryum aculeatum* G. Mey. AS Substrate for Lipases Production by *Aspergillus niger* A2B1

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Authors' contributions

This work was carried out in collaboration with all authors. Authors JVBS, RF, WRC and ESS designed the study, managed the literature searches and wrote the first draft of the manuscript. Authors NFLD, RGS, LAO and JGSO performed the laboratorial work and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

The fruit of *Astrocaryum aculeatum* G. Mey. are consumed by population in the Brazil Northern resulting fruit peels. These peels are rich in lignocellulose and fat. The present study investigated peels from the *Astrocaryum aculeatum* G. Mey. as a substrate for lipases production by solid state bioprocess. To reach this objective: 1) we isolated fungi from peels from the fruit of *Astrocaryum aculeatum* G. Mey; 2) we screened the isolates for lipase production (screening in petri dish and

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screening in submerged bioprocess), 3) we investigated the production of lipases by using peels from *Astrocaryum aculeatum* G. Mey as substrate. The isolates belonged to the genera *Aspergillus* (16), *Penicillium* (3) and *Fusarium* (1). These strains were submitted to petri dishes and submerged fermentation for lipases production, these experiments resulted in the selection of five strains belonging to the *Aspergillus genera*. The lipases produced by these five strains performed enzymatic transesterification; however, the lipases from the strain *Aspergillus niger* A2B1 produced the highest ester content. The utilisation of fruit peel from *Astrocaryum aculeatum* G. Mey. as the main substrate, fruit peel from *Astrocaryum aculeatum* G. Mey. oil (13%), moisture (70%) and 75 h of incubation were the optimal conditions identified for the production of lipases by *Aspergillus niger* A2B1(17.42 U/g) in solid state bioprocess (SSB).

Keywords: Tucumã peel; bioprocess; lipases; enzymatic transesterification.

1. INTRODUCTION

The use of on-site natural resources to generate electricity has been considered as a possible alternative for energy production. Biodiesel produced from vegetable oils extracted from seeds native species is one of these possibilities. This source of alternative and renewable energy may contribute to reducing the emission of greenhouse gases (carbon cycling) and toxic compounds containing sulfur, nitrogen or polyaromatic compounds [1,2].

The biodiesel is produced by transesterification of vegetable oils (triacyl glycerides). This reaction involves a short alcohol chain (methanol or ethanol), fatty acid esters (biodiesel) and glycerin which are catalysed by acidic, basic chemicals or enzymatic reactions using lipases [3]. Lipases (triacylglycerol acilhidrolase, EC 3.1.1.3) are carboxyesterases that can catalyze esterification reactions and transesterification of glycerides containing fatty acids with more than 10 carbons [4]. These enzymes are produced by microorganisms, animals and plants. In the past few years, serious efforts have been made in the search for enzymatic catalysis mediated by microbial lipases. The advantages of microbial lipase include the use of non-anhydrous alcohol at room temperature for reactions, easier recovery of glycerol, the absence of soap formation and lack of toxic residues [5–7]. However, low cost lipases are necessary for these reactions and microbial screening for lipases producers and the use of residues as the substrate is one alternative to reduce costs.

Tucumã (*Astrocaryum aculeatum* G. Mey.) from the Amazon stands out among the oleaginous plant species native to the Amazon that can be used for biodiesel production. This palm tree belongs to the Arecacea family and produces a fruit (tucumã). The production of Tucumã biodiesel via acid and base catalysis is well characterised and described in the literature [8]. Since the tucumã mesocarp is edible, the endocarp is an oil source that can be used biodiesel production and aiming for the complete utilisation of the fruit, we would like to use the fruit peel (a sub-product) as a substrate for lipase production, specifically lipases with transesterification activity.

The aim of our study was to carry out a screening of fungal strains for the production of lipases with transesterification activity using fruit peel from *Astrocaryum aculeatum* G. Mey. as substrate. To reach this objective we: a) isolated fungi from Tucumã sub-products; b) screened lipases producers; c) performed the transesterification assays to identify the best lipase producer and d) optimised the lipase production by solid state bioprocess of a selected strain.

2. METHODOLOGY

Fungi isolation identification: Mature fruits obtained commercially (Manaus open markets) were washed and submitted to decomposition for 30 days in a shady place, 25 ^{o}C and humidity of 70%. The fungi were isolated as described by Souza et al. [9]. Peel and endosperm, with a diameter of 5 mm, decayed for 30 days under environmental conditions, were quantified (1g), subjected to serial dilutions (10^{-1}, 10^{-2} and 10^{-3}) and inoculated onto plates containing culture medium containing Tucumã oil as the main carbon source (4% Tucumã oil, 2% peptone, 0.07% $MgSO_4.7H_2O$, 0.5% NaCl, 0.1% $CaCl_2.2H_2O$, 2% Agar, 1.2% Tween 80). These cultures were incubated at room temperature for 72 hours and following this twenty isolates were transferred to tubes containing the culture medium described above.

Fungi identification: The genera of isolated fungi were identified using their macro and microscopic morphological characteristics [9] and the Specifically, fungal DNA was extracted from mycelium using the QIAamp Tissue and Blood (Qiagen®, Hilden, Germany) extraction kit according to the manufacturer's recommendations. The Internal Transcribed Spacer (ITS) was amplified using the primers ITS1/ITS4 [10]. PCR products were purified with polyethylene glycol, based on the protocol described by Lis and Schleif [11]. The sequencing reaction was performed with the Big-Dye terminator cycle sequencing reagents (BigDye®, AppliedBiosystems, Foster City, CA, USA) kit, and sequencing was carried out in the ABI Prism® Seq 3130 Genetic Analyzer (Applied Biosystems). The sequences obtained were compared to those in the GenBank database (database incorporating DNA sequences of all publicly available sources).

Screening for lipase producers: The screening of the lipase producer was carried out using two different methods: Screening Assay in Petri Dishes (SAPD) and Screening Assay in Submerged Bioprocess (SASB).

The SAPD was carried out as previously described by Souza et al. [9]. The isolates were inoculated with a platinum loop into three equidistant points in culture medium (yeast extract 0.5%, 1.5% Tween 80 and 1% Tucumã oil, 2% Agar) contained in Petri dishes (90 mm diameter). The plates were incubated for 72 hours and visible halos of hydrolysis of lipids were quantified.

The SASB was performed as described by Gutarra et al. [12]. Into Erlenmeyer flasks (125 mL), 25 ml of culture medium was added (yeast extract 0.5%, 1.5% Tween 80 and 1% oil Tucumã). This medium was sterilized and inoculated (10^5 cells / ml medium). Flasks were kept for 5 days at room temperature under agitation (100 rpm). The medium was filtered (Whatman n.4) and lipases present in the culture medium were quantified.

Lipases quantification: The determination of lipase activity was performed by titrimetric method as previously described by Gutarra et al. [12]. A reaction mixture containing 5 mL of emulsion (50 mL water, 50 mL of olive oil and 7 g of Arabic gum), 4 ml of sodium phosphate buffer (200 mM, pH 7.0) and 1 ml of the enzyme extract was incubated for 15 min at 35°C. The reaction was carried out adding 10 mL of a 1:1:1 solution of acetone/ ethanol/ water. The solution was titrated with 0.05 N NaOH to pH 11. One unit of lipase was defined as the amount sufficient to produce 1 mmol of fatty acid per minute under the experimental conditions.

Transesterification assays: It was performed as described by Wu et al. [13] using the enzymes produced in submerged fermentation by five major producers of lipases. In micro tubes (2 ml) refined soybean oil (1 ml), ethanol or methanol (130 µL) and the culture filtrate (120 µL) containing the enzyme were added. They were incubated for 5 days at 37°C under agitation (70 rpm). The commercial lipase Lipozyme TL-IM (kindly provided by Novozymes Latin Americas LTD) was used as a control (20 mg/mL). The reaction products were analysed by High Performance Thin Layer Chromatography (HPTLC) in sheets of AL Silica gel 60 F254 (Merck Darmstadt, Germany), using a silica adsorbent phase and a mobile phase composed of 95:5 petroleum ether/ethyl acetate. The plates, after chromatographic elution, were stained with iodine vapour or 5% phosphomolybdic acid. Biodiesel, fat acid, glycerol and triglycerides were used as standards (obtained chemically). The conversion percentage was calculated from the corresponding peak areas of the glycerides and biodiesel product by densitometry using imaging software (ImageJ 1.45).

Lipases production optimisation in SSB using fruit peel from *Astrocaryum aculeatum* G. Mey. as a substrate: The strain selected in the previous test was subjected to optimisation using a full factorial design (2^3 + axial points) and the response surface methodology [14]. The factors that were chosen to study were: moisture (%), oil concentration (%) and bioprocess time (h). We carried out 17 assay and three replicates in the centre point. The dependent variable was the lipase activity (U/g). The SSB assay was performed as described by Damaso et al. [15], Erlenmeyer flasks (125 ml) containing 20 g of dried fruit peel particle size of approximately 5 mm, moisture corrected, inoculated (10^5 cells / g) and incubated for 168 hours at room temperature. The lipases were extracted with water (1:10), under orbital shaking (100 rpm, 15 min), filtered by (Whatman n.4) for separation of biomass and submitted to quantification.

3. RESULTS

3.1 Isolation and Identification of Fungi

The fungal isolates were identified to genera level by micromorphology. We found that the isolates belonged to Ascomycota Phylum, specifically to the genus *Aspergillus* (16), *Penicillium* (3) and *Fusarium* (1) (Table 1).

3.2 Screening of Lipases Producers

Aiming to find a good lipase producer, two screening assays were carried out a SAPD and a SASB (Table 1). In SAPD, it was observed that 16 cultures produced halos of triglycerides hydrolysis. In the SASB the strains *Aspergillus* A1C3, *Aspergillus* A1P, *A. niger* A2B1, *Aspergillus* C4V2 and *Aspergillus* A2V1 produced the highest lipase levels 1.58, 1.41, 1.83, 1.33 and 1.08 U/mL, respectively. These five strains were selected for the transesterification assay.

The culture filtrate (SASB) from *Aspergillus* A1C3, *Aspergillus* A1P, *A. niger* A2B1, *Aspergillus* A2V1 and *Aspergillus* C4V2 were submitted to the transesterification assay by mixing culture filtrate, methanol or ethanol and triacylglycerol. It was observed that lipases of *A. niger* A2B1 promoted the highest ester production (5,5%, w/w) under the experimental conditions. This strain was selected for the solid state bioprocess by using the fruit peel from *Astrocaryum aculeatum* G. Mey.as substrate. The strain *Aspergillus* A2B1 was identified to specie level by sequencing the ITS region of rDNA demonstrating 97 % of homology with the specie *Aspergillus niger* being nominated was *A. niger* A2B1.

3.3 Fruit Peel from *Astrocaryum aculeatum* G. Mey. as Substrate for Lipase Production in Solid State Bioprocess

In order to evaluate the utilization of fruit peel from *Astrocaryum aculeatum* G. Mey. as substrate for lipases production, it was carried out an experimental design (2^3+ axial points). This design intended to investigate the influence of moisture (%), Tucumã oil (%) and bioprocess time (h) in the lipase production (U/g). Table 2 shows the levels and the factors and the lipase production studied in variable experimental settings. The lipase levels ranged from 0 to 15 U/g.

Table 1. Lipase production in screening assay in Petri dishes and screening assay in submerged bioprocess microorganism isolated from the Tucuman fruit in decomposition and their results obtained in the screening assays for lipase production

Microrganism	Screening assay in petri dishes (mm)*	Screening assay in submerged bioprocess (UI/mL)*
Aspergillus niger A_2B_1	4	1.83
Aspergillus A_1C_3	3	1.58
Aspergillus A_1P	4	1.41
Aspergillus A_2V_1	3	1.33
AspergillusC_2B_1	3	1.08
Aspergillus A_1Act	3	0.83
Aspergillus A_4C_2	3	0.83
Aspergillus A_2P	2	0.66
Aspergillus A_1B	2	0.58
Aspergillus A_2B_2	2	0.58
Aspergillus A_2V_2	3	0.5
Fusarium A_1C_1	2	0.25
Aspergillus A_4C_3	1	0.16
Aspergillus C_4V_1	2	0.16
Penicillium A_1V_3	0	0.08
Aspergillus C_4V_2	2	0.08
Penicillium A_1V	0	0
Penicillium A_2G	0	0
Aspergillus A_3G	0	0
Aspergillus A_4C_1	0	0

Table 2. Levels, factors and lipase production in the 2^3 experimental design for production of lipases by *A. niger* A2B1 in solid state

Run	Moisture (%)	Oil (%)	Time (h)	U/g
1	70	10	120	15
2	50	10	120	15
3	82	16	63	15
4	70	10	120	13.3
5	70	10	120	14.0
6	70	20	120	8.3
7	82	4	63	13.3
8	82	4	177	1.7
9	90	10	120	13.3
10	82	16	177	0
11	70	0	120	13.3
12	70	10	216	0
13	70	10	24	0
14	58	16	177	3.3
15	58	4	177	12.0
16	58	16	63	3.3
17	58	4	63	8.3

The variables effects and their interactions were calculated and are presented in Table 3. The standard errors of the effects (σ) were estimated through the authentic triplicate in the centre point. In Table 3 only the significant effects are marked (*), at 95% confidence.

Table 3. Estimated effects of which variables and interactions affecting the lipase production revealed by 2^3 experimental

Variables	Estimated effects ± standard
Average	14.2±0.5*
A: Moisture	0.03±0.45
B:Oil	-3.2±0.5*
C:Time	-3.4±0.5*
AA	-0.3±0.5
AB	3.4±0.6*
AC	-7.8±0.6*
BB	-2.7±0.5*
BC	-1.8±0.6
CC	-10.3±0.5*

Standard error estimated from pure error with 2 f.d.
**Significant effects at the 5% level (t = 4.3025)*

Considering only statistically significant effects, a quadratic model was fitted to the observed data presented in Table 3. Equation 1: Lipase (U/g) = -36.1887 + [0.4291 * Moisture (%)]–[1.22906 * Oil (%)] + [0.7387 * Time (h)] + [0.0242065 * Moisture (%)* Oil (%)]–[0.00558068 * Moisture (%)*Time (h)]–[0.0368738 * Oil (%)]^2 – [0.00157262*Time (h)]^2.

An ANOVA test was used to evaluate the regression and the lack-of-fit of the model (Table 4). It may be noted that there was no evidence of lack of fit in the model. These results, added to the high percentage of variance explained (93%), indicate that the quadratic model proposed in Equation 1 was appropriated to describe the experimental region analysed.

In order to present graphically the estimation of lipase production, it was elaborated surface response that is shown in Fig. 1. Fig. 2 is the thin layer chromatography demonstrating the biodiesel obtained from enzymatic transesterification with lipase produced by *Aspergillus* A2B1 in SSF.

4. DISCUSSION

The new energy paradigm requires regional and multiple sources of energy and lipases are important for the transesterification reactions for biodiesel production [3,4]. We found that: a) fungi from Ascomycota phylum were isolated from fruit samples, b) most part of microorganisms involved in the fruit decay are able to produce lipase and some of them produce lipases with transesterification activity and c) fruit peel from *Astrocaryum aculeatum* G. Mey. was used as substrate for producing high levels of lipases using the selected strain *Aspergillus niger*A2B1.

The fungi isolated from endosperm and fruit peel belonged to the genera *Aspergillus, Penicillium* and *Fusarium*. These genera are often isolated

from air and environmental substrates in decomposition and several commercially important enzymes of industrial interest [amylases, lipases and proteases] are produced by these genera [4,16].

Most part of the strains presented lipase production, this result was expected since, during the isolation, these strains were cultivated in a culture medium containing triglycerides as the sole carbon source. In experimental conditions, the screening assays SAPD and SASB presented linear correlation (R^2= 0.73). The isolates selected presented production of levels of lipases in both methods.

The SSB optimisation tests performed with *Aspergillus niger* A2B1 resulted in lipases activities ranging from 0 to 15 U/g. This demonstrates the strong influence of the factors investigated, specifically inducer concentration (oil) and bioprocess time [1,17,18]. The lipases levels are high and similar in comparison with previous works that used similar methods of lipases quantification. Cavalcanti E d'Avila et al. [19] investigated packed bed bioreactors the aim of increasing productivity and scaling up of lipase production using *Penicillium simplicissimum* in solid-state fermentation. The maximum lipase activity obtained 26.4 U/g. Griebeler et al. [20] investigated the isolation and screening of microorganisms with the potential to produce lipases. The levels of the best lipases producers were between 10-21 U/g. Recently, Oliveira et al. [21] demonstrated the fungi *Aspergillus niger* was able to grow in Palm kernel cake and palm pressed fibre for lipase production by SSF. *A. niger* showed the highest lipase activity (20.7 U g−1) at 72 h.

Table 4. Analysis of variance for the evaluation of model (Equation 1)

Source	Sum of squares	Df	Mean square	F-Ratio	P-value
A:Moisture	0.00425128	1	0.00425128	0.01	0.9461
B:Oil	35.7919	1	35.7919	49.03	0.0198
C:Time	38.3985	1	38.3985	52.60	0.0185
AB	23.4613	1	23.4613	32.14	0.0297
AC	114.761	1	114.761	157.21	0.0063
BB	21.0484	1	21.0484	28.83	0.0330
CC	324.263	1	324.263	444.20	0.0022
Lack-of-fit	38.9304	7	5.56149	7.62	0.1209
Pure error	1.46	2	0.73		
Total (corr.)	577.315	16			

R^2 = 93,00%

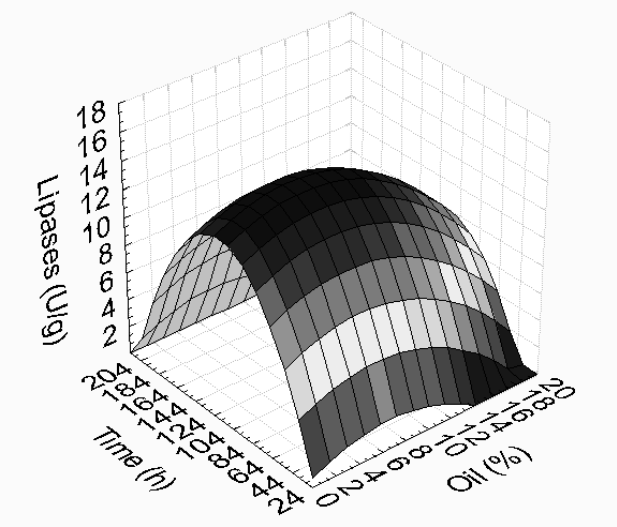

Fig. 1. Response surface presenting the production of lipases relative to oil concentration and the time of the bioprocess in accordance with Equation 1

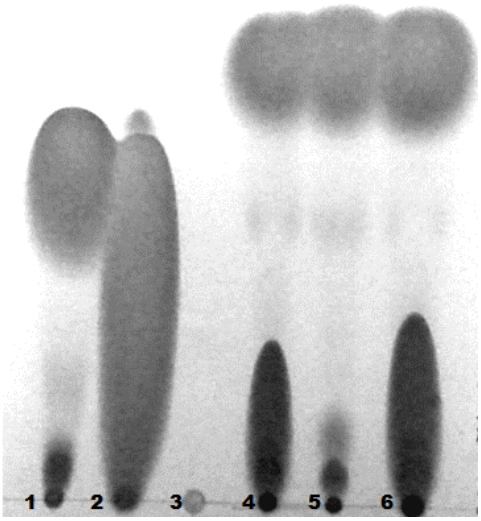

Fig. 2. Production ethyl ester using the lipase produced by A2B1 *Aspergillus*
1- Triglyceride soybean oil; Sample 2- Fatty acid; Sample 3- Glycerin; Sample 4- Transesterification products using Lipozyme TL – IM; Sample 5- Biodiesel and Sample 6- Transesterification products using enzymes produced by Aspergillus A2B1

The present work complements studies for integral use of the fruit of *A. aculeatum* G. Mey. The epicarp has been used as food, endocarp is a source of vegetable oil [8] and fruit peel can be used to produce lipases.

5. CONCLUSION

Brazilian Amazon presents interesting microorganisms and substrates for biofuel industry. We found that fruit peel from *Astrocaryum aculeatum* G. Mey. can be used as a substrate for producing lipases from *Aspergillus niger* A2B1. These lipases were able to transesterification reactions involved in biodiesel production.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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