Sugar cane vinasse as a renewable and sustainable feedstock for lipid production from*Aspergillus Niger* and *Aspergillus Flavus*

.

ABSTRACT

Background and Aims: In the search for sustainability and the use of renewable energies, biodiesel has stood out as a notable alternative to the growing energy demand of modern society. Microbial fat synthesis for lipids has been a possibility to produce this biofuel.In this context,the research work aimed to cultivate *Aspergillus niger* and *Aspergillus flavus* fungi in commercial and homemade broth, plus variable concentrations of sugarcane vinasse as a substrate to extract lipids produced using different extraction techniques and solvents.
Methodology: The fungi were grown in a commercial medium and a homemade one made with potato extract. After cultivation, they were submitted to two pre-treatments: the Bead Mill technique and the Ultrasound technique. The extraction of lipids produced in a commercial medium was performed via Soxhlet with a mixture of solvents. Therefore, the lipids produced in a homemade medium were extracted with the adapted Bligh-Dyer method.
Results: The species grew to the different culture media submitted, but the home culture medium increased the reproductive.The industrial residue proves to be a good alternative as a supplement to the culture medium, helping to reduce costs and preserve the environment. The extraction by Soxhlet showed satisfactory results when the fungi were grown in a commercial medium, with *Aspergillusniger* being extracted from the ethyl acetate solvent, obtaining a yield of 18.78%, and for A. flavus extracted with dichloromethane/methanol (9:1) and chloroform, bringing yield of 32.98% and 32%, respectively. Although extraction by Soxhlet showed some excellent results, extraction with the Bligh-Dyer method demonstrated better lipid fractions ranging from 25% to 67% and reduced energy expenditure, indicating a promising use, cost, and benefit.
Conclusion: Filamentous fungi of the genus *Aspergillus* can be used to produce biodiesel as they proliferate and can produce the same amount of lipids as a vegetable, which requires a much larger area of soil, for example.

Keywords: Aspergillus Genus, Vinasse, Lipids, Fatty Acids.

1. INTRODUCTION

With the advancement of global warming and natural disasters, efficiency is increasingly demanded and sought in chemical processes to generate as little waste as possible in industries. Specifically, in the sugar-ethanol industry, vinasse is one of the residues formed in the most significant volume[1]. Depending on the distillery equipment, 10 to 15 liters of vinasse are generated for each liter of ethanol produced[2].

Brazil is the world's largest producer of sugarcane, and in the 2022/23 harvest, it was responsible for produncing 610.8 million tons destined for 36.8 million tons of sugar and 26.5 billion liters of ethanol[3]. Looking at the ethanol process, the vinasse produced was more than 30 billion liters.

Industrial waste and by-products have become the target of interest and research due to their nutritional value and low cost as a cultivation medium. A sugarcane vinasse is an agricultural waste that can be used to develop biotechnological products of industrial interest, generated by many microorganisms such as yeast, algae, fungi, and bacteria [4]. Different authors present the composition of sugar cane vinasse in Table 1.

In the case of fungi, their biochemical activity is influenced by the species' characteristics and the growth medium's conditions. In general, most work is based on a substrate composed of glucose and nutrients. However, many carbohydrates in the culture medium are necessary for more remarkable lipid synthesis; fungi grow as long as there are specific nutrients for this, and after they are depleted, glucose stimulates the formation of lipids [5]. These lipids are synthesized throughout the growth process as part of the fungus' metabolic process and as a carbon reserve [6]. They are considered oleaginous microorganisms within their classes that produce lipids, as they can produce up to 40% of their biomass in lipids [4].

Among the fungi used to produce lipids, the *Aspergillus* genus is well known for its versatility in fermentation and enzymatic reactions. *Aspergillus carbonarius* can produce the laccase enzyme[7], while *Aspergillus nigar* is used in fermentation to produce biodiesel[8], in the production of citric acid[9], and malic acid from *Aspergillus oryzae*[10]. Still, many other works use this genus to produce lipids, as seen in Table 2.

Despite countries' incessant search for safe energy sources, biofuels currently require many lipids (oils and fats) destined for the food industry, making their production more expensive and unfeasible [11–13]. In this context, using lipids obtained from the biomass in sugar cane vinasse in biodiesel production becomes highly advantageous.

In search of sustainability and the use of renewable energy, this work aimed to study the cultivation and applicability of sugar cane vinasse as a substrate in the production of lipids of microbial origin from the fungi *Aspergillus niger* and *Aspergillus flavus* and the extraction of these lipids using different techniques and different solvents, which can be used as raw material in the production of biodiesel.

Table 1. Compositional characterization of sugarcane vinasse.

Parameter	Concentration (mg/L)								
	[14]	[15]	[16]	[17]	[18]	[19]	[20]	[21]	[22]
COD*	17,850	27,000 – 42,000	29,000 – 99,100	67,300	36,000 – 49,000	31,723	81,600	35,750 – 41,316	20,400
Carbohydrates	4,300	400 – 3,350	4,500 – 6,100	–	–	–	19,982	7,672 – 8,097	–
Glycerol	2,598	90 – 5,440	1,500 – 1,600	–	–	–	3,295	2,399 – 3,798	–
Acetic acid	153	60 – 3800	4,300 – 5,600	–	–	–	409	210 – 357	–
Citric acid	–	10 – 550	–	–	–	–	–	–	–
Aconitic acid	–	210 – 2,290	–	–	–	–	–	–	–
Succinic acid	–	90 – 3,440	–	–	–	–	–	–	–
Propionic acid	0	400 – 2,550	–	–	–	–	38	14 – 46	–
Lactic acid	917	610 – 5,360	1,600 – 3,500	–	–	–	1,803	–	–
Butyric acid	0	–	300 – 1,200	–	–	–	0	3 – 17	–
Ethanol	154	30 – 450	–	–	–	–	362	173 – 181	–
Methanol	0	340 – 4,550	–	–	–	–	122	–	–
Phenols	609	90 – 3,920	–	–	–	–	5,940	2,231 – 2,551	–
Sulfate	1,225	669 – 3,298	1,400 – 4,500	–	2,300 – 2,900	–	4,986	–	–
TKN*	–	119 – 540	40 – 100	–	–	234.1	–	–	–
Ammonia	–	7.1 – 118	–	1,100	762 – 1,603	10,920	–	–	–
Magnesium	–	467 – 669	–	–	354 – 543	321,250	–	–	–
Calcium	–	292 – 641	–	–	741 – 1,304	828	–	–	–
Potassium	–	3,652 – 1,542	–	3,500	3,147 – 2,827	3,276	–	–	–
Phosphorus	–	44 – 228	9 – 185	–	64 – 111	5,518	–	–	–
Total volatile solids	14,035	4,720 – 26,289	2,900 – 36,700	10,300	–	–	8,560	–	11,396
pH	4.50	4.01 – 6.47	4.7	4.4	4.4 – 4.6	4.6	4.9	4.74	4.18

**CDO: Total chemical oxygen demand; TKN: Total Kjeldahl nitrogen.*

Table 2.Use of *Aspergillus* genus to produce lipids.

Fungus Species	pH	Temperature (ºC)	Time (days)	Growing medium	Lipidcontent (% or g/L)	Source
Aspergillus awamori	5.5	30	2	Commercial	31 %	[23]
Aspergillus oryzae	4.5	30	5	Potato processing wastewater	3.5 g/L	[24]
Aspergillus sp.	5.0	60	2	Corncob waste liquor	23.3 %	[25]
Aspergillus sp.	5.8	28	6	Glucose	3.14 g/L	[26]
Aspergillus carneus	6.0	30	7	Commercial	36.2 %	[27]
Aspergillus flavus	-	28	6	Glucose	Qualitative	[28]
Aspergillus favus	5	30	4	Glucose, urea and KH_2PO^4	40.51 %	[29]
Aspergillus awamori	-	28	5	Czapek-Dox agar	11.3%	[30]

2. MATERIAL AND METHODS

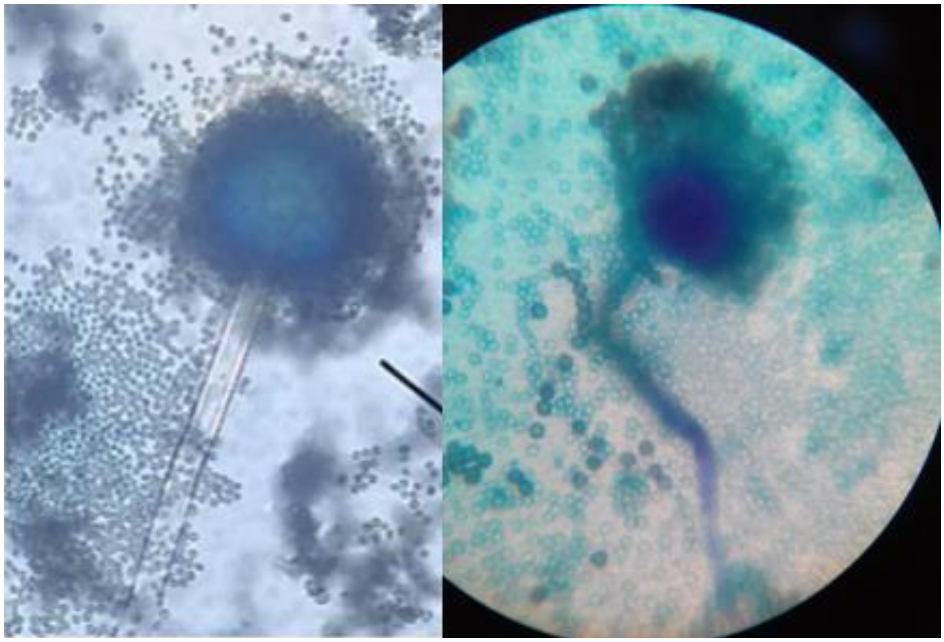
2.1 Species isolations

The culture medium used was commercial PDA (Potato Dextrose Agar) prepared according to the manufacturer's instructions (“Nutrient broth”) in proportions that met the needs of the work. This medium was distributed in Petri dishes to sow the species. After sowing, the plates were kept in an incubator medium at 35°C for seven days, waiting for growth.

Preparation of new strains:

- *Aspergillus niger*: An onion was exposed to air for seven days, the time necessary for the fungus to develop, then the fungus was isolated. Pieces of this food were transferred for cultivation on PDA plates at 28ºC for seven days. The colonies with fungal characteristics identified under the microscope were picked on another plate with PDA to obtain the isolates.
- *Aspergillus flavus*: An orange was collected in the decomposition phase, which contained the desired fungus, and it was used to perform the isolation. Pieces of this food were transferred for cultivation on plates with PDA medium and kept at 28ºC for seven days. Colonies characteristic of the fungus identified under the microscope were picked on another PDA plate to obtain the isolates.

The species were identified through their macroscopic (colony color and texture) and microscopic characteristics, according to [31] in Figure 1.



(a) **(b)**

Fig. 1. Microscopic visualization of Aspergillus niger from [2] (a) and authorial (b), respectively.

2.2Commercial Cultivation

Commercial cultivation was carried out using “Nutrient Broth” as recommended by the manufacturer in proportions that met the needs of the work. The broth was used pure and in a mixture of broth and vinasse v:v (1:1).

2.3Home Cultivation

The cultivation preparation was carried out according to [2]: 800 g of potatoes with skins, which were well washed and cut into small pieces, were used. 4000 ml of tap water was added to this potato. Then, it was boiled for approximately 40 minutes and filtered using gauze. The acquired extract was diluted to a volume of 4000 ml, and 80g of dextrose was added with a pH of around 7.0 without needing a solution to correct it, followed by sterilization in an autoclave at 121 °C for 15 minutes. The homemade broth was used pure and mixed with vinasse in different dilutions: 10 mL, 20 mL, 30 mL, 40 mL, and 50 mL vinasse in 100 ml. The steps for preparing homemade broth are shown in Figure 2.

(a) **(b)** **(c)** **(d)**

Fig. 2. Step-by-step preparation of homemade PDA broth: (a) weighing the cut potato; (b) Cooking the potatoes; (c) Addition of dextrose; (d) Ready broth.

2.4Processing the use of vinasse as a growing medium

The processing of vinasse as a cultivation medium was carried out in both cultivation media (commercial and homemade). Initially, the dilution was carried out in 250ml flasks containing 100 ml of the medium with regressive dilutions ranging from 100 ml to 10 ml of sterile crude vinasse. A standard was adopted from these dilutions, depending on the adaptation of the fungus to the medium, and the 1:1 (v:v) dilution of medium (traditional/homemade) and vinasse was then standardized. The vinasse was also tested purely to observe its resistance to fungal growth. The inoculation period was 4 to 7 days at 28 ± 2°C.

2.5Obtaining the dry mass

As the fungi grew in the broth (commercial and homemade), forming a supernatant material, it was necessary to perform vacuum filtration (Figure 3) followed by drying in the oven at a temperature of 110°C for 3 hours. After drying, the sample was crushed in a mortar to obtain particulate material.

Fig. 3. Vacuum filtration of the fungus in a homemade medium.

2.6Pre-Treatment for extraction

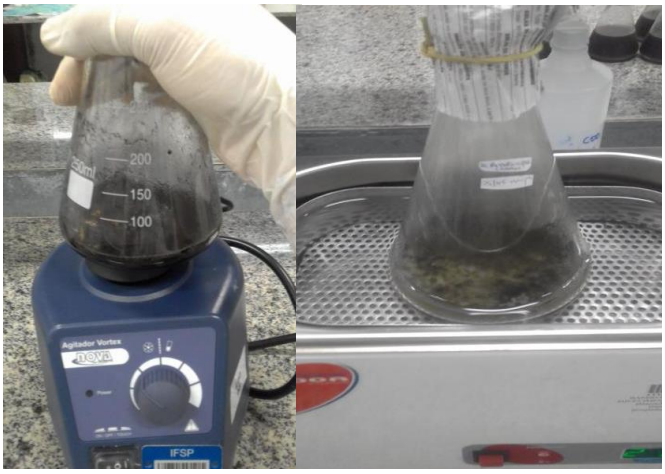
The Bead Mill (BM) technique was performed for samples grown in traditional media. The particulate material obtained after filtration and drying was transferred to a test tube. For every 1.0 g of fungal mass, 3.0 ml of distilled water was added, along with some glass beads [32]. This mixture was stirred for 10 min in a Vortex shaker, as shown in Figure 4. Then, the samples were dried in an oven at 110ºC for 3 hours.

Fig. 4. Realization of the traditional Bead Mill technique.

For samples grown in a homemade medium, the BM technique and ultrasound technique (UL) were adapted from the protocol [32]. The two pretreatmenttechniques were carried out to compare the efficiency of the technique in the yield of extraction of lipids from fungi. For the Ultrasound technique (UL), it was necessary to remove excess broth to reduce the

volume by half (50 ml) through filtration, and then the concentrate was added to the ultrasonic bath for 25 minutes.

To reduce energy consumption and loss of fungal biomass in the transfer processes, the samples grown in the homemade medium did not go through the drying process; pretreatments were carried out directly in the cultivation flasks, as shown in Figures 5 (a) and (b). After these steps, the samples were dried in an oven at 110 ºC for a period of 3 hours.



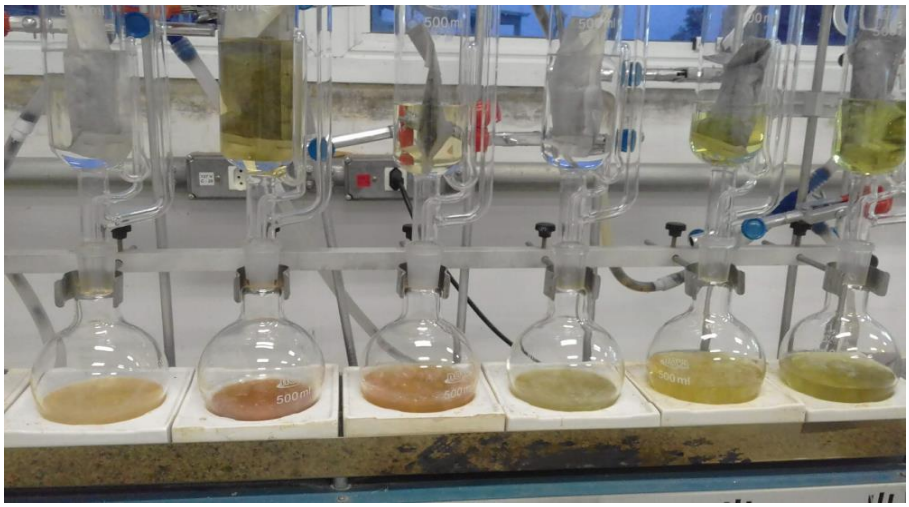
(a) **(b)**

Fig. 5. (a) Pre-treatment by adapted Bead Mill. (b) Adapted Ultrasound pre-treatment.

2.7Extraction of lipids by Soxhlet

The Soxhlet extraction method, adapted from [32], consisted of the use of 4 solvents with different polarities: ethyl acetate, hexane, chloroform, and dichloromethane/methanol (9:1) in the proportion of 100 ml for each 0.1 g of particulate material samples, maintaining reflux for 3 hours, the extraction system is represented in Figure 6. Based on the results, a new extraction was carried out with a 1:1 mixture of the solvents that obtained better yields, demonstrated in Figure 7. This extraction with a mixture of solvents has the purpose of verifying the presence of synergism between the solvents, verified only in samples from traditional cultivation.

Fig. 6. Initial extraction in Soxhlet with different solvents.



(a) (b)(c)(d) (e) (f)

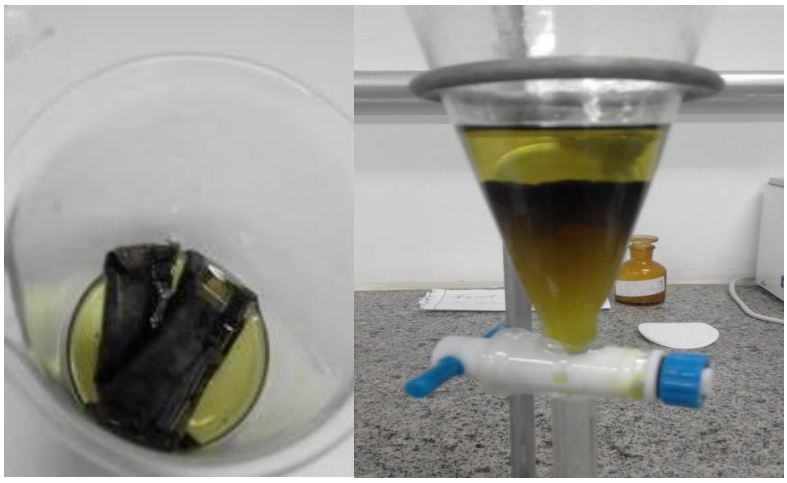
Fig. 7. Extraction by Soxhlet with the best solvents, with (a) *A. flavus* grown in broth extracted with dichloromethane/methanol/chloroform; (b) *A. flavus* grown in broth + vinasse (1:1) extracted with dichloromethane/methanol/chloroform; (c) *A. flavus* grown in pure vinasse extracted with dichloromethane/methanol/ethyl acetate; (d) *A. niger* grown in acetate/chloroform-extracted broth; (e) *A. niger* grown in broth + vinasse (1:1) extracted with acetate/hexane; (f) *A. niger* grown in pure vinasse extracted with acetate/chloroform.

2.8Lipid extraction by Bligh-Dyer

The Bligh-Dyer extraction method, adapted from [33], was carried out on samples grown in homemade broth and in fractions to optimize an experiment to determine the ideal amount of solvent for the process.

For *A. niger* grown in homemade broth, using the BM technique for membrane disruption, three consecutive extractions were performed on the same sample. The sample was kept inside a sealed filter paper system and placed in a beaker, designated as the primary system. To this system (Figure 8 (a)), 10 ml of methanol + 10 ml of chloroform were added and manually stirred for 3 minutes. After this period, another 10 ml of chloroform was added and transferred to a separation funnel called the secondary system (Figure 8 (b)). Then, 10 ml of distilled water was added, forming the two-phase system.

For *A. niger* grown in broth and vinasse, the BM technique was used, with only two extractions, which have the same characteristics as the extraction of this species in homemade broth, but with the proportion of 20 ml of chloroform plus 20 ml of methanol in the primary system and 20 ml of chloroform plus 20 ml of water in the secondary system. The other samples, including A. flavus with the UL technique, went through the same extraction process as *A. niger* from broth and vinasse, with the same proportions of chloroform-methanol-water. The chloroform phase with the lipid was isolated, and the lipid content was quantified after rota-evaporation of the chloroform.



(a) **(b)**

Fig. 8. (a) Primary extraction system; (b) Secondary extraction system.

2.9Quantification of lipid content and determination of the fatty profile of lipidfractions in each extraction

The yield of the extraction process was calculated by the relationship between the fungal mass (X) and the lipid oil mass (Y) obtained after the rote evaporation process. The lipid content was determined using the mathematical expression demonstrated in Equation 1.

$$T = \frac{Y}{X} \times 100 \qquad (1)$$

Where:
T: lipid content.
Y: lipid mass.
X: dry fungal mass.

To determine the profile of fatty acids, present in lipid samples extracted from fungi in different cultivation media, the BS EN 14103:20 Standard - Fat and oil derivatives - Fatty Acid Methyl Esters (FAME) - Determination of ester and linolenic acid methyl contents, in gas chromatography – FID, equipment from Thermo. The parameters used were initial temperature 250 ºC, heating ramp: 120 ºC; 180 ºC - 10 min; 230 ºC – 5 min; 300 ºC – 20, final temperature 300 ºC, 5% diphenyl dimethyl polysiloxane column – 30 m, He carrier gas, Split injection mode 100:1, carrier gas linear velocity 45.0 cm/sec.; carrier gas flow 3.0 mL/min, total analysis time 30 min. The methodology was applied, so the samples needed to be esterified. For the esterification reaction, 10 mL of methanol, 0.2 g KOH (85% purity), and 2 mL oil were used. The analysis conditions are 1 µL of the sample, using methanol as a solvent for dilution in a ratio of 1:100 (v:v).

After the analyses, the areas of the chromatogram peaks were integrated, and the relative percentage in the area of each component in the sample was defined (semi-quantitative analysis).

3. RESULTS AND DISCUSSION

Both fungi, *Aspergillus niger* and *Aspergillus flavus*, showed favorable growth in commercial and homemade environments. Dilutions with vinasse with commercial broth also showed favorable growth, as did dilutions with homemade medium. As there was favorable development of the fungi in the different dilutions of homemade broth with vinasse, the 1:1 dilution (100 ml broth+100 ml vinasse) was preserved to standardize and facilitate the quantification of dry mass/culture medium as well as yield lipid.

Although both media showed favorable growth, the homemade medium provided an increase in growth and consequently in the resulting dry mass, which can be seen in Figure 9, which shows the values in grams obtained in each 200 ml of commercial broth (CB) and homemade broth (HB) and its dilutions (1:1) with vinasse.

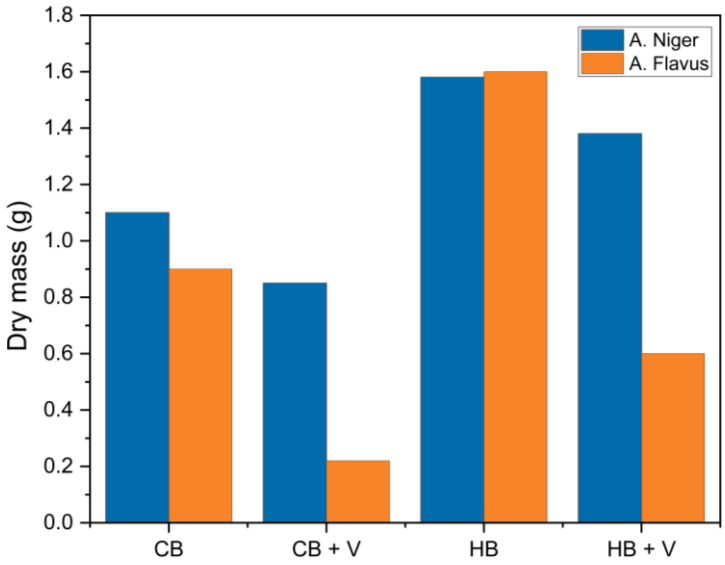


Fig 9 Dry mass (g) of crops in commercial and homemade media and in mixture with vinasse. CB: commercial broth; HB: homemade broth; V: Vinasse.

A blank extraction was carried out in both extraction techniques, with the solvents used in the processes, and the values obtained in these extractions demonstrate the margins of error that may occur. The results obtained from this extraction are represented in Table 3. With the values of the contamination/impurities margin of the solvent used, it was possible to correct the values resulting from the extractions.

Table 3. Blank extraction results.

Solvents	Impurity
Ethyl acetate	0.02%
Hexane	0.2%
Chloroform	0%
Dichloromethane/methanol (9:1)	0.02%

3.1 Results of fungal cultivation in commercial mixture

The percentage lipid content of the fungal samples calculated using equation 1, in the different culture media and the four solvents used are presented in Table 4.

Table 4. Lipid percentage in different solvents.

Fungus/ cultivation medium	Hexane (%)	Chloroform (%)	Ethyl acetate (%)	Dichloromethane /methanol (9:1) (%)
A. niger/ Commercial broth	3.40	17.70	18.78	12.08
A. niger/ Commercial broth + Vinasse	0.90	1.05	2.26	1.28
A. niger/ Vinasse	6.20	4.90	8.28	5.35
A. flavus/ Commercial broth	18.80	32	24.98	32.98
A. flavus/ Commercial broth + Vinasse	7.30	28.71	9.52	17.16
A. flavus/ Vinasse	0.75	5.14	9.45	9.75

The best result found in the extraction was with*Aspergillus flavus*, using dichloromethane/methanol in a culture medium containing commercial broth, with a lipid content of 32.98%. The mixture of these solvents provides a double polarity (dichloromethane-nonpolar and methanol-polar). This data aligns with [32], which worked with microalgae using a non-polar solvent (petroleum ether), which did not result in good yield. However, when subjected to extraction with the Bligh Dyer technique, the yields were more promising, using polar and nonpolar solvents. A high lipid content was obtained for this same fungus cultivated in the broth, around 30%, using pure chloroform. This solvent has a nonpolar character and is an aprotic solvent. This solvent has small dipole moments and does not have hydrogen, capable of forming hydrogen gasses. The solvent effect shows the presence of polar and non-polar compounds extracted from fungi.

With the results of the samples obtained from the fungi in their different cultivation media and different solvents, a new extraction was carried out, this time using a mixture of the best solvents for each fungus. The solvents, the cultivation medium, the fungus, and the results obtained are described in Table 5.

Through the results, it can be seen that the yield is linked to the cultivation medium, as well as the solvent used for extraction. According to [34], using a single solvent is not recommended for extracting lipids of animal origin. However, in this work, the mixture of solvents did not result in synergism, except for the mixture of dichloromethane/methanol (9:1) for the fungi *A. flavus* cultivated in broth and broth and vinasse with lipid content of 32.98 and 17 .16% respectively, from Table 4 and for *A. niger* grown in broth and vinasse extracted with ethyl acetate/hexane (1:1) with lipid content of 15.12%, from Table 5.

Table 5. Lipid percentage in solvents mixture.

Fungus/ cultivation medium	Solvents mixtures (1:1)	Lipid percentage (%)
A. niger/ Commercial broth	Acetate/ Chloroform	4.98
A. niger/ Commercial broth + Vinasse	Acetate/ Hexane	15.12
A. niger/ Vinasse	Acetate/ Chloroform	0.25
A. flavus/ Commercial broth	Dichloromethane/ Methanol/ Chloroform	4.98
A. flavus/ Commercial broth + Vinasse	Dichloromethane/ Methanol/ Chloroform	14.71
A. flavus/ Vinasse	Dichloromethane/ Methanol/ Ethyl Acetate	10.42

The lowest values were those when pure hexane was used as solvent regardless of the species and cultivation medium. According to [35], this solvent is usually used to extract oil from oilseeds due to its non-polarity, facilitating its interaction with the oil and making the extraction process faster. However, this solvent did not provide satisfactory results for extracting lipids from microorganisms. This data is according to[36], where the lowest results found for extractions were those carried out with hexane, which proved that the amount of non-polar lipid components such as triacylglycerols in the sample is low.

According to [4], a microorganism must have 20-25% lipids in its biomass to be a favorable candidate for commercial interests. Therefore, the species of *Aspergillus flavus* cultivated in commercialbroth, using ethyl acetate (24.28%), chloroform (32%), and dichloromethane/methanol (32.98%) as solvents, can be considered favorable, and the same, cultivated in a mixture of commercial broth + vinasse (1:1), using chloroform (28.71%) in the extraction.

Aspergillus niger showed better results when cultivated in commercial broth and extracted with ethyl acetate (18.78%) and chloroform (17.7%). However, in cultures using vinasse, the lipid content was considerably low. The best result was from the extraction of ethyl acetate and hexane (15.12%) from Table 5. This solvent mixture has low polarity.

The chromatograms obtained from the samples showed distinct fatty acid profiles, confirming the culture medium's influence on the fungus's lipid metabolism. The retention time of the compounds was compared with the retention time of fatty acid esters present in a sample of methyl soy biodiesel and with the BS EM 14103:2011 Standard. Figure 10 presents this profile and the samples' percentage of fatty acid esters.

The results obtained for the sample of *A. flavus* cultivated in media containing commercial broth + vinasse and pure vinasse indicate the chromatographic profile of predominance of saturated fatty acids (C14:0, C16:0 and C≥20:0). The samples of *A. niger* and *A. flavus* grown in commercial broth, showed a predominance of esters with a carbon chain above 20 saturated carbon atoms (C>20:0) with a concentration of 23.60% and 13.74% respectively. These results confirm the efficiency of extraction with non-polar and slightly polar solvents (chloroform and dichloromethane/methanol). Lipids from *A. flavus* fungi can be considered

raw material for biodiesel production, and, according to their lipid profile, they present good oxidative stability due to the saturation of carbon compounds [36].

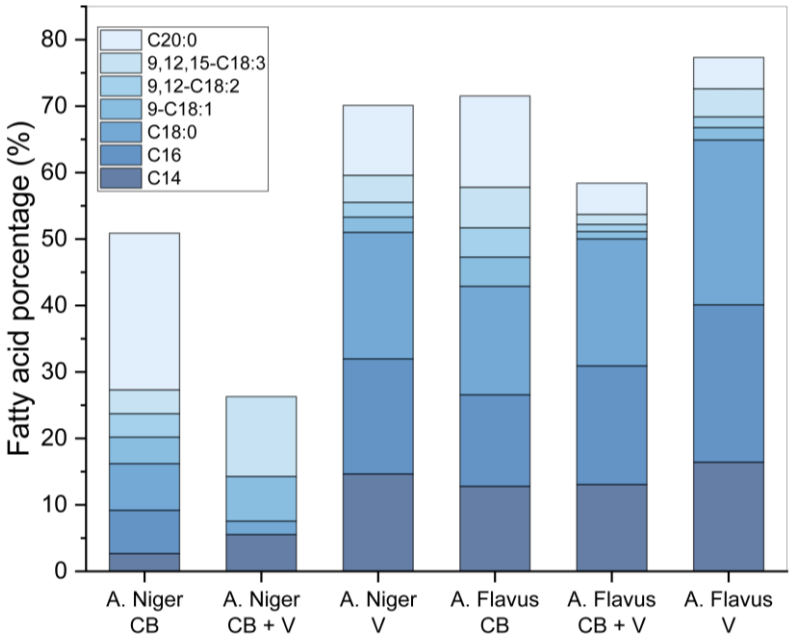


Fig. 10. Profile of fatty acid. CB: commercial broth; V: Vinasse.

Although the yield in the extraction of lipids from *A. niger* in broth was not satisfactory, the fatty acid profile showed a percentage of esters with more than 20 carbon atoms that were entirely satisfactory at 23.60%, which can be seen in Figure 10 a presence of C14, C16, and C18:0, with the predominance of formation of saturated esters. It is also possible to observe, although, in percentages lower than 5%, the presence of unsaturated fatty acid esters such as oleate (9-C18:1), linoleate (9.12-C18:2), and methyl linolenate (9,12,15-C18:3). The sample of A. niger grown in broth + vinasse was the only one that showed a predominance of unsaturated fatty acid esters, with 12.03% of Linolenate (9,12,15-C18:3), the predominance of unsaturated fatty acids for Biodiesel production requires careful quality control, as they are more susceptible to oxidation and thermal decomposition reactions. Samples of *A. niger* grown in pure vinasse present a profile of saturated fatty acids with a predominance of C14, C16, C18, and C>20:0 and can be used to produce biodiesel with more excellent oxidative stability.

3.2Results of growing fungi in homemade mixture

Dilutions were made in the medium containing vinasse to determine the best proportion. However, as there was growth in the different dilutions, the 1:1 ratio was preserved (100 ml broth + 100 ml vinasse) to standardize and facilitate the quantification of dry mass/medium of cultivation and lipid yield. Both species showed satisfactory growth in the homemade medium and the same containing vinasse.

The homemade broth made observing a significant increase in the medium's dough possible. The values obtained were compared with the results of fungi grown in the traditional culture medium and are represented in Figure 11.

The lipid yields of the two species of fungus, in different cultivation media (homemade and vinasse), using the Bead Mill (BM) technique are described in Table 6.

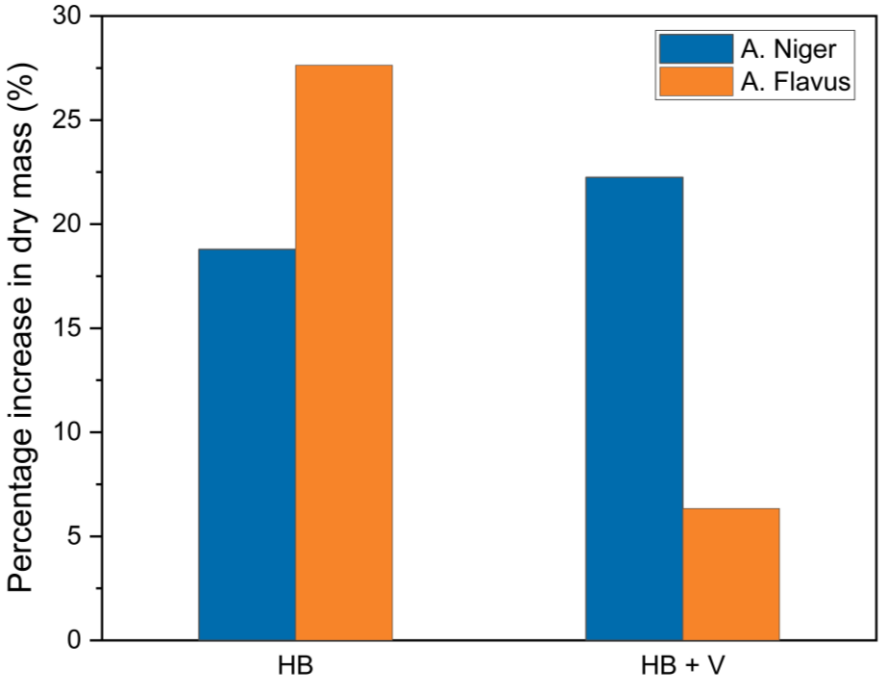


Fig. 11. Percentage increase in dry mass comparing homemade broth (HB) and homemade broth + vinasse (V).

Table 6. Lipid percentage of fungi using BM Technique.

Species	Cultivation medium	Number of extractions	1ª Extraction (%)	2ª Extraction (%)	3ª Extraction (%)
A. niger	HB	3	36.98	33.96	4.83
A. niger	HB + V	2	15.08	10.13	---
A. flavus	HB	2	18.35	11.19	---
A. flavus	HB + V	2	20.80	27.36	---

HB: Homemade broth; V: Vinasse.

When comparing the values in Table 6 with those of the traditional cultivation medium in Table 4, an increase of approximately 58% for *A. niger* in homemade broth and 24.16% in the broth-vinasse mixture can be observed. For *A. flavus* in broth, there was a decrease of 2.46%; in the broth-vinasse mixture, there was an increase of 19.45%. According to[34], methods based on the binary mixture of chloroform and methanol can extract both neutral lipids (fatty acids, triacylglycerols) and polar lipids (glycerophospholipids, glycolipids) efficiently, thus characterizing an increase in cold method extraction.

Extraction of the fungus using the ultrasound technique (UL) only for *A. flavus* cultivated in the broth-vinasse mixture due to greater mass availability. The values obtained were 61.48% for the first extraction and 56.85% for the second. Those values show an increase of 18.63% and 29.49% compared to extractions of the same fungus when using the BM technique. They reflect the efficiency of the UL technique compared to BM for this type of sample. The lipid percentages of the samples represented in Figure 12 (a) for *A. niger* e Figure 12 (b) for *A. flavus.*

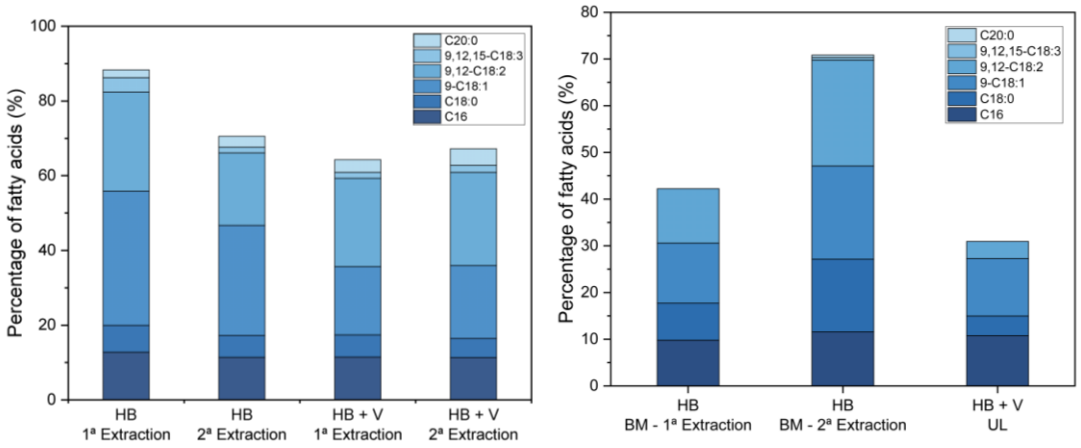


Fig. 12. Percentage of fatty acids after extractions, comparing homemade broth (HB) and homemade broth + vinasse (V), for (a) A. niger by BM technique and for (b) A. flavus by BM and UL techniques.

The results obtained for the sample of *A. flavus* cultivated in homemade culture medium using the Bead Mil (BM) technique showed the chromatographic profile of predominance of chain unsaturated fatty acids (C18:1 and C18:2). In contrast, the sample grown in homemade cultivation medium + vinasse using the ultrasound technique (UL), it showed a higher concentration of carbon chain lipids (C16:0 and C18:1). The 2nd extraction of *A. flavus* was the most efficient with a percentage of carbonic chain fatty acids (C16:0, C18:0, C18:1, C18:2) confirming the effectiveness of these lipids to produce biodiesel. These results also confirm the efficiency of the extraction process with non-polar solvent (chloroform).

For *A. niger* samples, only the Bead Mill technique was used for both homemade culture medium and a mixture of homemade medium + vinasse. All extractions showed a predominance of unsaturated carbon chain esters (C18:1 and C18:2), as shown in Figure 12.

The amounts of lipids extracted depend on the species and substrates. The gas chromatography results demonstrate that *A. flavus* has potential for producing biodiesel. A. niger, with a predominance of unsaturated fatty acids, tends to present physicochemical parameters that ensure the quality of biodiesel in terms of its viscosity but require care regarding its oxidative stability [37].

4. CONCLUSION

Through the results, it was possible to conclude that the Aspergillus species adapted to the different cultivation media to which they were subjected. However, the homemade cultivation medium provided an increase in the growth of fungi. There was no relevant interference in the results in the medium containing vinasse. Thus, it proves to be a good alternative as a supplement to the cultivation medium as it helps reduce costs and is a way of directly contributing to preserving the environment using industrial waste.

The pre-treatment prior to lipid extraction is also directly associated with lipid yield, and the ultrasound technique proved to be more satisfactory. The most satisfactory lipid yields were those subjected to extraction using the technique Bligh-Dyer, reaching an increase of up to 58% compared to extraction by Soxhlet.

The chromatographic profile varies not only for each species but also for each cultivation medium. *A. niger* presented a profile with a predominance of unsaturated fatty acids, which require care in the quality control of biodiesel due to its susceptibility to oxidation reactions. On the other hand*, A. flavus* presents a profile with a predominance of saturated fatty acids and can be used as raw material to produce biodiesel as they have good oxidative stability due to the saturation of carbonic compounds.

The use of filamentous fungi of the genus Aspergillus can be a good alternative to produce biodiesel, considering their speed of generation, where they can produce in a limited area the same amount of lipids as a vegetable that requires an area of very large soil. A. flavus was the most favorable fungus for the proposed purpose, considering its lipid content and its chromatographic profile compatible with a sample of methyl soy biodiesel.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTIONS

All authors participated in the conception of the main idea of the work, contributing to constructing the necessary analyses of the process. All experimental data from the production was made available by Renata Pinto Ferreira Zaguini. Ana Bárbara Moulin Cansian managed the literature searches. Ana Bárbara Moulin Cansian, Jane Karla de Faria Borges, and Caroline Peters Pigatto De Nardi process data analyses where the graphs and tables were produced, and the experimental improvement proposals were reviewed. Finally, all authors participated in the text construction and review process, approving the final manuscript.

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