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Endophytic Fungi as a Source of Biofuel Precursors

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Endophytic fungi, isolated from a number of different species of tropical plants, were investigated for lipid biodiesel precursor production. The extracts produced from liquid cultures of these fungi were subjected to acid-catalyzed transesterification reactions with methanol producing methyl esters and then analyzed through chromatographic (GC–FID) and spectrometric techniques (MS, NMR ^{1}H). The European Standard Method, EN 14103, was used for the quantification of methyl esters extracted from the fungi of the species and genera studied. Xylariaceous fungi exhibited the highest concentrations of methyl esters (91%), and hence may be a promising source for biofuel.

Keywords: Biodiesel, fatty acid methyl esters, endophytic fungi

Throughout modern times, the global energy matrix has been based on nonrenewable resources, especially fossil fuels. During the last few decades, concerns have been raised about the potential impact of this reliance on the environment and global economy, which have led to a rethink regarding the shape of the global energy scene. Consequently, it is widely recognized that there is a need for sustainable energy, and many have been spurred to action by successive increases in oil prices and predictions of the imminent exhaustion of nonrenewable resources [11, 13, 37].

Amongst a number of renewable sources that have been investigated, biodiesel has been shown to be one of the most promising alternatives. The concept and definition of biodiesel is still being discussed. However, the definition adopted by the Brazilian Biodiesel Programme is that it constitutes a fuel obtained from mixtures, in different proportions, of fossil diesel and alkyl esters of vegetable oils or animal fats. In practice, biodiesel is a renewable fuel made from a mixture of alkyl esters obtained by transesterification reactions between vegetable oil or animal fat and alcohol in the presence of a catalyst [21, 22, 29, 31]. Despite the diversity of raw materials used to produce biodiesel, by 2008 it was being sourced mainly from oil and methanol using the reaction presented in Scheme 1 [21, 22].

The increasing number of articles and patents in relation to the subject of biodiesel testifies to the growing interest in this field. Biodiesel has been shown to be a very attractive alternative to petroleum derivatives, and researchers, especially in the USA, Japan, and European countries, have been dedicating considerable effort in order to acquire adequate technological expertise for effective biodiesel production. There are several advantages in the use of biodiesel; it is renewable, produces few pollutants, and is beneficial for the economy and rural development. As the result of a resolution by ANP N°42, commercial diesel in Brazil has been a blend of 5% biodiesel in petrodiesel since January 2010 [31].

Amongst the numerous matrices used to produce biodiesels, approximately 90% are exclusively from plants [4]. In addition, production has been increasing greatly, both in developed and developing countries, leading in some cases to serious negative impacts on ecosystems [18]. One way to more positively meet the demands of industry is to look for potential biodiesel precursors in organisms other than plants. Microorganisms have some clear advantages over plants and animals. Yeasts, fungi, bacteria, and microalgae can accumulate high levels of lipids, do not require land for growth, and do not compete in food production. Furthermore, they can be produced in large-scale fermentation processes, which make them a potentially more viable source for biofuel production. As can be observed in Table 1 [2, 6, 7, 14, 16, 23, 27, 36], many eukaryotic microorganisms (yeast, fungi, microalgae, and bacteria) can accumulate

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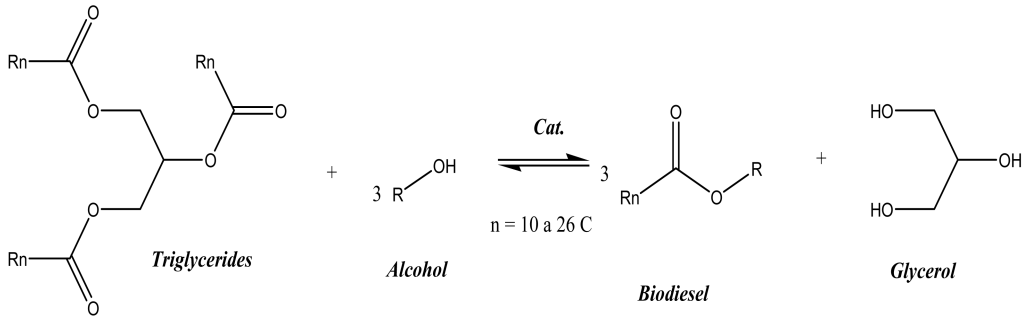

Fig. 1. Reaction of transesterification of biodiesel.

lipid, mainly as a storage material [38]. However, the extent of lipid accumulation differs widely between species, from as little as 3–5% (w/w, dry weight) to in excess of 80% (w/w, dry weight). Table 1 lists several microorganisms that exhibit high levels of lipids.

Endophytic fungi have been studied by our group for more than a decade. In the last few years, these microorganisms have been attracting researchers' attention all over the world because of their important and unique characteristics. Our understanding of the chemistry and biology governing the relationship between endophytic fungi and their hosts is just beginning, and some theories already indicate that the host plant (macrophyte) protects and feeds the endophyte, which "in return" produces bioactive metabolites to enhance the growth and competitiveness of the host and to protect it from herbivores and plant pathogens. These microorganisms that colonize the internal tissue of a particular host plant during its life cycle [28], are in this sense, great producers of secondary metabolites that show a great diversity of biological activities and applications in several industrial areas [1, 8, 9, 12, 30, 32]. Therefore, the aim of this paper has been to evaluate, characterize, and quantify the capacity and content of lipid, in the form of methyl esters, from endophytic fungi. This is a novel matrix for this kind of application, with an end goal of establishing a newly designed protocol for use in the biofuel industry.

Table 1. Lipid content of microorganisms.

Microoganisms		Lipid content (%)	Reference
Microalgae	*Chlorella vulgaris*	40–60	[16]
	Chlorella emersonii	63	[16]
	Nannochoris sp.	38–68	[23]
	Schizochytrium sp.	50–77	[16, 23]
Fungi	*Aspergillus oryzae*	57	[3, 23]
	Cunninghamella echinulata	40–47	[6, 7]
	Mortierella isabellina	68–86	[6, 23, 27]
	Mucor circinelloides	20	[35]
Yeast	*Lypomices starkegi*	64	[23]
	Rhodotula glutinis	72	[23]
Bacteria	*Gordonia* sp.	93	[14]
	Rhodococus opacus	96	[14]

Materials and Methods

Preparation of Cultivation Medium

The fungi *Penicillium brasillianum*, *Penicillium griseoroseum*, *Xylaria* sp. (NICl3), *Xylaria* sp. (NICL5), *Penicillium* sp. (PAOE), and various species of *Trichoderma* were cultured in flasks with the liquid medium Czapeck for a period of 14 to 21 days. Each flask was filled with 150 ml of 15 g dextrose, 1.5 g $NaNO_3$, 0.5 g KH_2PO_4, 0.25 g $MgSO_4$, 0.005 g $FeSO_4 \cdot 7H_2O$, 0.25 g $MgSO_4$, and 0.25 g KCl dissolved in 1.0 l of distilled water and 20 g of yeast extract.

Transesterification Reaction Catalyzed by Acid and Extraction of Lipid Compounds

The mycelium was subjected to transesterification reaction in acid catalysis. For this, we used mycelial mass and added 150 ml of MeOH and 10 ml of HCl. The reaction mixture was left to reflux for 24 h. After that period, the reaction mixture was filtered and concentrated in a rotatory evaporator. The lipid compounds were then extracted, by liquid–liquid extraction using heptanes, and concentrated in a rotatory evaporator.

Sample Preparation for GC-FID Analysis

Biodiesel samples of endophytes were weighed (250 mg) in a 10 ml vial and dissolved in 5 ml of an internal standard solution of methyl heptadecanoate in heptane. Methyl heptadecanoate 99.5% (Fluka) was used as the internal standard and heptane HPLC (TEDIA) as the solvent.

Chromatographyc Analysis and Nuclear Magnetic Resonance

Analyses were performed on a gas chromatograph SHIMADZU GC 2010 FID equipped with a flame ionization detector and column STABILWAX-DA (30 m × 0.32 mm × 0.25 mm). A volume of 1.0 µl of sample solution was automatically injected in Splitless mode. The temperature of the injector was set at 250°C and the detector at 250°C. The oven was operated at 200°C.

The analyses by mass spectrometry were performed with a gas chromatograph CARLO ERBA GC 8000 series equipped with a J&W Scientific DB1 column (30 m × 0.25 mm × 0.25 mm). The oven

was operated initially at 90°C isotherm for 5 min and subsequently heated to 170°C, with increases of 5°C/min, then increasing the temperature again to 300°C at increases of 7°C/min, and finally raising the temperature to 320°C at 2°C/min. The injector was operated at 250°C and the interface at 280°C/min. The mass spectra were obtained on a MICROMASS Platform II equipped with electron ionization. The ionization source was operated at 180°C and the mass scan was performed in a range *m/z* 50–750.

Analyses by NMR spectroscopy were performed on a BRUKER ARX 200 spectrometer operating at 4.7 Tesla. The sample was then dissolved in 99.9% $CDCl_3$ (MERCK).

RESULTS AND DISCUSSION

In most cases, oil from microorganisms comes in the form of triglycerides, which are also the main component of vegetable oils and animal fats. Therefore, microbial lipids can potentially be used as a raw material for biodiesel production using the common method for the production of methyl esters by the biodiesel industry, namely a transesterication reaction with methanol in the presence of a basic catalyst such as sodium and potassium hydroxide, or sodium methoxide. However, the use of basic catalysts in the transesterification of vegetable oils and animal fats carries with it several drawbacks, such as a low yield of biodiesel production and complications in the steps for purification and separation due to soap formation in the reactions of neutralization [34]. However, this can be solved with the use of acid catalysts such as hydrochloric acid and sulfuric acid. An acid catalysis in the presence of methanol produces a large amount of methyl esters, increasing the yield of biodiesel [10].

Several techniques have been employed in the determination of the ester content present in biodiesels. Gas chromatography with a flame ionization detector (GC–FID), gas chromatography-mass spectrometry (GC–MS), and Nuclear Magnetic Resonance (^{1}H and ^{13}C NMR) are some commonly used examples [17, 22, 24].

The extracts obtained as products of the transesterification reaction, produced by different species of endophytic fungi in acid catalysis, were subjected to ^{1}H NMR analysis, allowing for their characterization. The analysis revealed several signals indicating the hydrogen characteristic of the methyl esters. A singlet seen at δ 3.67 is related to the presence of methoxyl hydrogens. A triplet noted at δ 0.89 indicates the presence of the terminal methyl group. Singlets were also observed at δ 1.30 and 1.26, due to the presence of methylene hydrogens. A triplet noted at δ 2.30 relates to α-carbonyl methylene hydrogens. In addition to these signals, it was possible to identify a multiplet at δ 5.35 in relation to the presence of vinylic hydrogens, which confirms the presence of unsaturated methyl esters in the reaction product. The same NMR profile was observed in all of the extracts of fungi examined.

Mass spectrometry data obtained by electron impact ionization confirmed the methyl ester production. The presence of *m/z* 74 and *m/z* 87 ions, produced by a MacLafferty type rearrangement and a 1-2-hydrogen shift followed by a cleavage of the adjacent bond, respectively, proved methyl ester structures. The ion of *m/z* 143 produced by a 1-6-hydrogen shift followed by a cleavage of the adjacent bond is also a characteristic fragmentation of fatty acid methyl esters under electron ionization conditions, and are the purest form of biodiesel [19].

Through GC–MS analysis, it was possible to identify some of the esters obtained from the endophytic fungi samples and compare them based on the levels found in each of the microorganisms. Hence, methyl esters such as palmitic, stearic, oleic, linoleic, and linolenic acids, which are considered the most important methyl esters in biodiesel from plants like soybeans, were identified [15]. Table 2 lists the characteristics of the methyl esters of the different species of endophytic fungi.

Amongst the endophyte samples, there were some fungi that were identified as promising sources of methyl esters, with similar concentrations of methyl esters seen from

Table 2. Average composition of methyl esters of fatty acids of endophytic fungi.

Methyl esters	*Xylaria* (NICL3)	*Penicillium* (PAOE)	*Penicillium brasilianum*	*Penicillium griseoroseum*	*Xylaria* (NICL5)	*Trichoderma* T19	*Trichoderma* T25	*Trichoderma* T27	Soy biodiesel [20]
Palmitic acid (C 16:0)	21.60%	33.25%	26.40%	29.90%	15.50%	53.05%	31.20%	30.94%	11.29%
Stearic acid (C 18:0)	2.45%	5.20%	6.04%	7.56%	8.76%	–	3.39%	1.59%	3.54%
Oleic acid (C 18:1)	22.40%	13.80%	13.90%	10.10%	26.50%	10.66%	28.55%	21.74%	22.45%
Linoleic acid (C 18:2)	43.79%	43.51%	44.60%	29.45%	49.58%	1.41%	23.33%	13.00%	54.62%
Linolenic acid (C 18:3)	5.28%	2.94 %	0.94%	2.10%	7.78%	0.92%	4.73%	0.34%	8.11%

soybeans. Variations in the content of methyl esters were observed for the different species of endophytic fungi being used as an important taxonomic classifier for microorganisms [2]. The endophytic fungus, coded by *Xylaria* (NICL5), showed concentrations of methyl esters such as palmitic acid (15.50%), stearic acid (5.20%), oleic acid (26.50%), linoleic acid (48.58%), and linolenic acid (7.78%) at high levels, similar to those found in soybean oil (11.29%, 3.54%, 22.45%, 54.62%, and 8.11% for the respective methyl esters). The endophytic fungus identified as *Penicillium* (PAOE) also showed methyl ester concentrations at high levels (33.25%, 5.20%, 13.80%, 43.51%, and 2.94%, respectively). The latter values were also seen to be near to those observed for soy biodiesel. Therefore, a search for different *Xylaria* and *Penicillium* strains, and the optimization of the transesterification reaction and culture conditions in order to maximize lipid production, would be very promising avenues for future research to increase biofuel production. Other endophytes studied exhibited comparatively lower levels of methyl ester concentrations.

The National Petroleum Agency, Natural Gas and Biofuels of Brazil (ANP), has been enacting resolutions, regulations, and laws concerning the characterization and marketing of biodiesels. ANP Resolution 7 establishes the methods to determine the characteristics of biodiesels produced in Brazil [4]. Among other properties, the resolution requires that the quantification of fatty esters content does not have a fixed minimum amount. The chromatographic method with internal standardization and calibration by one point, described in European Standard Method EN 14103, has been adopted as a quantitative technique [5]. In 2008, a new resolution was passed that established a minimum purity of 96.5% methyl esters in a biodiesel as a requirement, and enforced the addition of 3% biodiesel to diesel, showing the increasing importance for its development as a new energy source [33].

Among the techniques used for the quantification of methyl esters, the European Standard Method EN 14103 utilizes gas chromatography with a flame ionization detector (GC–FID), using an internal standard and quantification by a point. Generally, this method uses methyl heptadecanoate as the internal standard and quantifies methyl esters with C_{14} – C_{24} saturated or unsaturated carbon chains [33].

Quantification of methyl esters was performed according to the European Standard Method EN 14103. Through this methodology, the chromatogram was integrated in a time interval from 1.0 to 20.0 min, corresponding to the retention times of methyl myristate (C 14:0) and nervonate methyl (C 24:1), respectively. This range covers the peaks of most parts of the esters derived from grease sources and is considered the optimal interval for biodiesel compounds [33].

The concentrations of methyl biodiesel were estimated by the following equation, where A_t is the total area integrated, A_{pi} is the area of the peak regarding the internal standard, and C_{pi} is the concentration of the internal standard.

$$C_{biodiesel} = \frac{(A_t | A_{pi})}{A_{pi}} \times C_{pi}$$

Table 3. Concentrations of biodiesel from endophytic fungi.

Endophytic fungi	Concentration of Biodiesel (%)
Xylaria (NICL3)	66.7
Penicillium PAOE	83.1
Penicillium brasilianum	50.8
Penicillium griseoroseum	40.5
Xylaria (NICL5)	91.0
Trichoderma T19	67.8
Trichoderma T25	11.6
Trichoderma T27	40.1
Trichoderma harvezionum	40.4
Soy biodiesel [20]	90.7

The quantitative data obtained from the endophytic fungi samples is presented in Table 3 and was compared with soybean biodiesel, one of the best known biodiesels made from vegetable sources [20]. The data of Table 3 show that the vast majority of endophytes did not exhibit methyl ester concentrations of satisfactory levels to be considered as suitable biofuels. However, different biodiesel production optimizations involving culture condition variations (pH, temperature, inductive mediums) and transterification reaction parameters should be evaluated in further investigations. A Xylariaceous fungus, coded as *Xylaria* (NICL5), nevertheless showed a very high concentration (91%) near to what is expected from an ideal biodiesel (96.5%). The data show that the methodology was effective for the extraction of methyl esters of endophytic fungi and this should motivate other similar studies of various diverse Xylariaceous fungi for use as a biodiesel. In addition, *Penicillium* PAOE presented a satisfactory concentration of methyl esters (83.1%), motivating the search for other species of fungi for the production of biodiesel.

Although the process of fatty acid biosynthesis is well known, the genetic factors that control the extent to which lipid accumulates in a particular organism are far from clear. More recently, the importance of malic enzymes (MEs) in the accumulation of lipid content by fungi has been reported [38]. It is described that some microorganisms could significantly reduce their total lipids, diminishing malic enzyme activity either by mutating the gene or inhibiting the enzyme [39]. ME seems to play a key role in provision of reduced nicotinamide adenine dinucleotide phosphate (NADPH) for both fatty acid biosynthesis and fatty acid desaturation [38]. Inhibitors of ME, such as sesamol, lead to decreased lipid accumulation [39]. Therefore, the optimization of microbial culture conditions and the further study of the malic enzyme and possible inductors

could improve the production of lipids and thus increase the yield for the production of methyl esters for the production of biofuels.

It should be noted that manipulation by genetic engineering techniques has been used in fungi to increase the microbial lipids for biofuel production. For instance, the oleaginous fungus *Mucor circinelloides*, which was the first microorganism used for commercial production of microbial lipids, had its genome sequenced, and a large collection of genetic engineering techniques for its manipulation have been applied for the optimization of biofuel production [34], such as gene expression using autoreplicative plasmids and inactivation of genes by disruptions [25] or gene silencing (RNAi) [26].

In conclusion, The methodology and analytical techniques used were satisfactory in the production and identification of the fatty acid methyl esters obtained by transesterification. The use of European Standard Method EN 14103 to quantify the methyl esters of the endophytic fungi showed some concentrations similar to those obtained in plants. The quantification of the methyl esters indicates that some endophytic fungi possess a lipid matrix at high concentrations and are promising sources of biofuels.

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