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Short Communication

Exploration of Brazilian biodiversity and selection of a new oleaginous yeast strain cultivated in raw glycerol

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HIGHLIGHTS

- Yeasts were selected using a qualitative technique by staining with Sudan Black B.
- We evaluated the lipid production by *Candida* sp. using pure and raw glycerol.
- The profile of fatty acids produced using raw glycerol was similar to vegetable oils.
- The yeast *Candida* sp. was able to accumulate about 56% of lipids.

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ABSTRACT

The objective of this study was to use glycerol generated from the synthesis of biodiesel to study the oleaginous potential of wild yeasts. An initial selection was performed via a rapid and qualitative technique by staining with Sudan Black B. Initially 129 yeasts were present, from which 5 were selected and cultivated in liquid medium containing pure or raw glycerol. The yeast LEB-M3, isolated from the Pantanal, presented lipid content of 20.46% and 56.58% for cultivation in pure and raw glycerol, respectively. This strain was genotypically identified as *Candida* sp. The fatty acid profile showed predominance of oleic acid (C18:1), 57.35% for cultivation in pure glycerol, and in raw glycerol linoleic acid (C18:2) was predominant (46.0%). It was possible to select a yeast with high lipid concentrations 9.14 g/L and fatty acid profile similar to vegetable oils commonly used in the synthesis of biodiesel.

1. Introduction

Climate changes are provoked mainly by the burning of fossil fuels, directing studies to seek alternative sources with lower environmental impact (Costa and Morais, 2011). Biodiesel is a biofuel which has physical characteristics similar to those of petroleum diesel, and can be used in diesel engines without any adaptation to reduce the emission of pollutant gases in the atmosphere (Castro et al., 2004). Acquiring new sources of lipid material is of great importance due to the increasing incentives for biofuel production, since vegetable oil, expensive and a source of low viability (Marchetti et al., 2008). Microbial lipids are among the most promising feedstock sources for biodiesel production because they present composition similar to vegetable oils (Dai et al., 2007; Ratledge, 2005), as well as advantages of not depending on arable land and independent of climatic factors (Li et al., 2007; Saenge et al., 2011).

Brazil presents the greatest biodiversity on the planet and a large microbiota is still unknown. Yeasts represent a part of the microflora of natural ecosystems, which may be promising sources for the production of various compounds including lipids. Recently, bioprospecting techniques were used for isolating microbial strains capable of converting glycerol to ethanol (Choi et al., 2011) and lactic acid (Hong et al., 2009). Alternatives for utilization of increasing glycerol production are necessary, its application as a substrate in cultivation of microorganisms is one alternative and therefore it is important to obtain microbial strains that efficiently metabolism glycerol. With rapid selection techniques it is possible that bioprospecting of microbial diversity may result in the discovery of microorganisms with desirable technological characteristics (Almeida et al., 2009).

Evans et al. (1985) used a staining technique with Sudan Black B, seeking a rapid method for qualitative estimation of oil content accumulated in the microbial cells and select oleaginous yeasts. Other studies have also used Sudan Black B to select oleaginous microorganisms (Pan et al., 2009; Li et al., 2011) on different carbon sources. The objective of this study was to select wild yeasts

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isolated from different Brazilian ecosystems, capable of metabolizing glycerol generated in the synthesis of biodiesel as an alternative and low cost carbon source for the production of lipids, so that they may be used as part of a sustainable biodiesel production chain.

2. Methods

2.1. Yeasts

A pool of 129 wild yeasts was studied which were isolated by (Maugeri and Hernalsteens, 2007) from soil, stems, fruits and flowers collected from different Brazilian regions: Atlantic Forest, Cerrado, Pantanal and the Amazon Forest. The microorganisms were maintained on GYMP agar slants at 5 °C as stock cultures. Before cultivation, colonies were reactivated and incubated at 30 °C for 48 h.

2.2. Screening of the yeasts strains with potential of high oil content

Screening was performed by the Sudan Black B staining technique (Evans et al., 1985). For this purpose, 20 colonies of each microorganism were inoculated on plates with culture medium proposed as Evans and Ratledge (1983) using pure glycerol as the carbon source and 2% (w/v) agar, for 96 h, at 30 °C until the colonies reached 2–3 mm in diameter. The technique was performed in triplicate.

2.3. Preparation of inoculum

Two tubes of the reactivated microbial culture were scraped with 10 mL solution of 0.1% peptone in water for removal of microorganism cells and transferred into Erlenmeyer flasks containing 180 mL of the culture medium composed of (g/L): 30 glycerol, 7 KH_2PO_4, 2.5 Na_2HPO_4, 1.5 $MgSO_4.7H_2O$, 0.15 $CaCl_2$, 0.15 $FeCl_3.6H_2O$, 0.02 $ZnSO_4.7H_2O$, 0.06 $MnSO_4.H_2O$, 0.5 $(NH_4)_2SO_4$ and 0.5 yeast extract, pH 6.0 (Papanikolaou and Aggelis, 2002). The inoculum was cultivated at 28 °C in shaken flasks at 185 rpm. The cell concentration was monitored by counting in a Neubauer chamber until reaching approximately 1×10^8 cells/mL (Zhang et al., 2005).

2.4. Shake flasks

The experiments were carried out using Erlenmeyer flasks containing 200 mL of culture medium, the same as that used for inoculum, in which the carbon source was pure or raw glycerol (42.4% (w/v) obtained by transesterification of soybean oil with methanol, without any previous treatment), which was supplied by SP-BIO (Sumaré-SP, Brazil). The amount of raw glycerol added to the medium was determined by considering the desired concentration of the carbon source. Flasks were incubated at 28 °C and 185 rpm (10% v/v of inoculum). All tests were performed in triplicate in order to treat the data by ANOVA and Tukey test for determining the significant differences between yeasts and between types of glycerol, at 95% of confidence ($p \leqslant 0.05$) using the software Statistica 7.0 by Statsoft.

2.5. Identification of the oleaginous yeast

Genotypic characterization was performed by sequencing the D1/D2 domains of the gene encoding subunit 26S of ribosomal DNA. The universal primers NL1F (5′GCATATCAATAAGCGGAG GAAAAG3′) and NL4R (5′GGTCCGTGTTTCAAGACGG3′) were used for D1/D2 amplification, according to the methodology described by Kurtzman and Robnett (1998). The DNA was extracted according to (Ausubel et al., 1998) then it was amplified and sequenced. The nucleotide sequences obtained were analyzed and compared with the sequences deposited in the NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/), using the tool Basic Local Alignment Search Tool (BLAST).

2.6. Analysis

Cells were harvested by centrifugation at 785*g* for 10 min and washed, then suspended in water for spectrometric analysis at 600 nm (Zhang et al., 2005). Cellular lipids were extracted according to Bligh and Dyer (1959). Glycerol concentrations were determined by HPLC with a RI detector, a HPX-87H column, H_2SO_4 0.005 N, pH 2.6 was used as mobile phase. Lipids were converted to their methyl-esters (Metcalfe and Schmitz, 1966) and analyzed in a gas chromatograph equipped with a FID detector and Carbowax column, N_2 was used as a carrier gas. Fatty acids were identified by comparison of with Sigma–Aldrich standards and quantified by area normalization.

3. Results and discussion

3.1. Selection of oleaginous yeasts

Among the yeasts studied, 42 presented a blue color which indicated, according to the methodology utilized, the potential for lipid accumulation, where 5 were selected and characterized with respect to the number of colonies that showed bluish tint and intensity of the color formed. Although this technique does not allow a precise response to the cellular lipid content of the yeasts, it does provide partial information regarding the ability of the microorganisms to accumulate lipids. According to the results (Table 1), it is possible to observe that among the pre-selected microorganisms are yeasts isolated from different Brazilian ecosystems: Cerrado, Amazon Forest and Pantanal.

Bioprospecting, exploration and investigation of resources from the fauna and flora for acquisition of new products with commercial or scientific value (Staley et al., 2010) have received attention in recent years, and have therefore stimulated the study of microorganisms isolated from natural environments with ability to produce compounds of interest. In this sense, (Dai et al., 2007) isolated yeasts from flowers collected in China and selected, using a staining technique similar to that in this study, yeasts with potential for lipids production. The use of techniques that reduce the number of microorganisms under study and also indicate those with greatest potential for a determined target product is of great importance in the selection of microorganisms.

3.2. Selection of oleaginous yeasts in agitated flasks

When observing the growth kinetics of the selected yeast in media containing pure and raw glycerol Fig. 1 (a) and (b), it appears that the two substrates enabled cell growth, and in the

Table 1
Quantitative and qualitative[a] analysis of five selected yeasts using the staining technique with Sudan Black B.

Microorganisms	Staining (average)	Color intensity[a]
LEB-M3 (Pantanal)	17	+++/++/+++
LEB-AQ5 (Amazon Forest)	16	++/+++/+++
LEB-AJ10 (Amazon Forest)	20	++/+++/+++
LEB-AAN1 (Cerrado)	14	++/++/+++
LEB-AAI4 (Cerrado)	20	+++/++/+

[a] Different coloration intensities of the yeasts were indicated qualitatively by: + weak, ++ average and +++ intense, for each of the triplicate repetitions.

cultivation medium containing raw glycerol cell multiplication was highest for three yeasts. The greater microbial growth in raw glycerol may be related mainly to the impurities present in this co-product that act as nutritional elements that can be assimilated by microorganisms during cultivation, such as phosphorus, calcium, nitrogen and sodium (Thompson and He, 2006). (Chi et al., 2007; Saenge et al., 2011) also reported the positive influence that these inorganic salts have on biomass production. Yeast LEB-M3 showed the highest biomass production (11.86 ± 0.08 and 16.12 ± 0.91 g/L) when grown in media containing pure and raw glycerol, respectively, differing statistically from the other yeasts studied (Table 2). In other studies using raw glycerol, production of 9.3 g/L of biomass was reported for *Candida curvata* NRRL-Y 1511 and 11 g/L for *Candida pulcherrima* LFMB 1 (Chatzifragkou et al., 2011).

For the majority of yeasts that grow aerobically, the biomass yield (Yx/s) is typically between 0.4 and 0.8 g/g. Yields lower than 1 indicate that the carbon source is used not only for biomass production but also for production of energy, the main product, by-products and for maintenance (Taccari et al., 2012). The Yx/s of the yeast LEB-M3 (0.61 ± 0.03 g/g) in raw glycerol was smaller compared to the yeasts AAN1 and AQ5, indicating in this case that glycerol was used in greater quantity for the accumulation of lipids. The concentration of 30 g/L of raw glycerol inhibited growth of the yeasts AJ10 and AAI4, which also showed lower glycerol consumption.

When comparing the two substrates it was observed that the use of pure glycerol in the culture medium resulted in greater glycerol consumption only for LEB-M3, for the other crops there was no significant difference between the two substrates. Glycerol is initially used for cell growth which is faster at the beginning of cultivation, since at the end biomass production decreases due to lack of nutrients in the medium (Ratledge, 2005). These changes in cell metabolism occur after a period of time and promote greater efficiency in production of lipids due to increased synthesis of enzymes that convert carbon (Dyal and Narine, 2005). The lower consumption of raw glycerol, in some cases, may be related to impurities of the glycerol that are consumed together.

When comparing the lipid content, it was found that all yeasts studied differed significantly, where higher values were obtained when grown on glycerol from biodiesel (Table 2). However the strain LEB-M3 presented increased lipids production compared to others, both in the medium containing pure glycerol (20.46 ± 0.64%) and in raw glycerol (56.58 ± 5.62%). The lipids concentration obtained also showed to be greater for cultivation in raw glycerol, reaching a concentration of 9.14 ± 1.28 g/L for the yeast LEB-M3. Yeast LEB-AJ10 was highly inhibited by raw glycerol, causing stress which led to the accumulation of lipid, but low concentration and lipid yield. DAI et al. (2007) assessed yeasts isolated from flowers, selected the most promising for lipid production which produced 5.55 g/L identified as *Rhodotorula glutinis*.

Lipid yield on glycerol obtained in other studies showed similar results as compared with the present study (0.04–0.17 g/g) (Papanikolaou et al., 2008; Fakas et al., 2009). However only the yeast LEB-M3 showed higher lipid yield (0.33 g/g) when compared to literature data. This may be due to impurities in raw glycerol, with were converted into lipids and so increasing the yields. Different tolerances to raw glycerol have been observed during growth of microorganisms in high concentrations of this substrate (Papanikolaou et al., 2000). It should be noted that different glycerol purities are obtained after biodiesel production, varying according to the process and type of treatment carried out with this co-product. In the present study no treatment was performed and glycerol presented high levels of impurities that proved beneficial for the growth of biomass and accumulation of lipids.

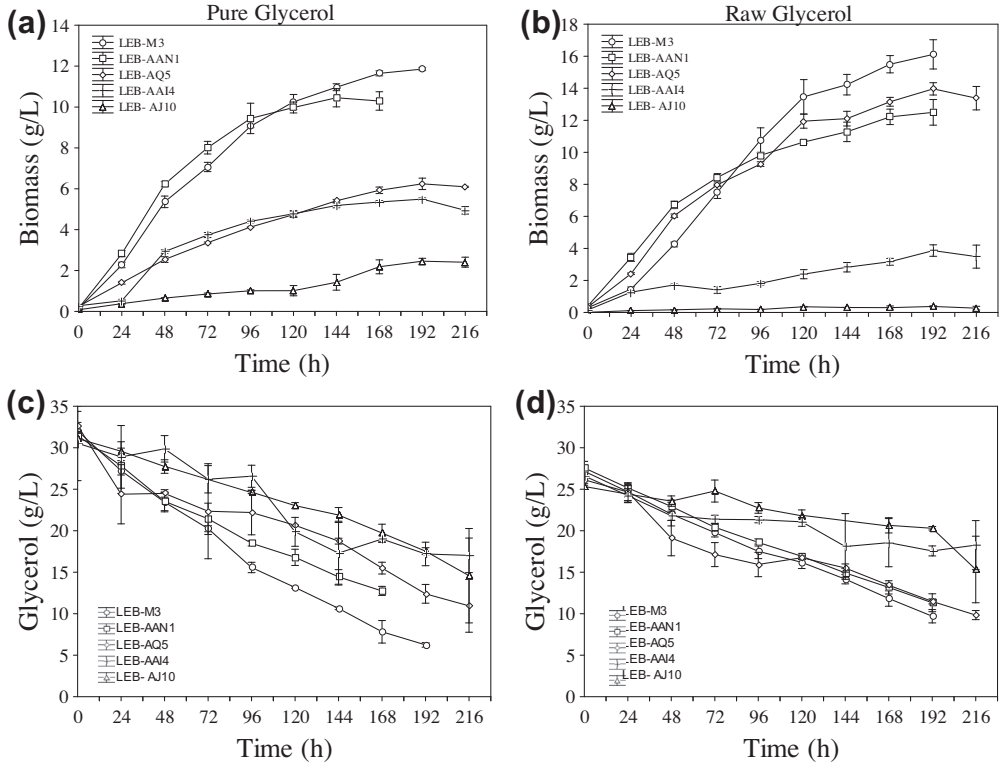

Fig. 1. Biomass concentration during cultivation of yeasts in (a) pure and (b) raw glycerol, and consumption of (c) pure and (d) raw glycerol: (o) LEB-M3, (□) LEB-AAN1, (◇) LEB-AQ5, (+) LEB-AAI4 and (Δ) LEB- AJ10.

Table 2
Mean ± standard deviation values for biomass concentration, consumption of glycerol, cell yield (Yx/s), lipid content, lipid concentration and lipid yield at the end of cultivation in pure and raw glycerol, with statistical analysis of the data for different yeasts.[a]

Yeast	Biomass (g/L)		Glycerol consumption (%)		Cell yield (g/g)	
	Pure	Raw	Pure	Raw	Pure	Raw
M3	11.86 ± 0.08^{aB}	16.12 ± 0.91^{aA}	80.50 ± 0.97^{aA}	64.31 ± 2.53^{aB}	0.45 ± 0.01^{aB}	0.61 ± 0.03^{bA}
AAN1	10.30 ± 0.45^{bA}	12.50 ± 0.80^{bA}	59.49 ± 1.10^{abA}	58.90 ± 4.26^{aA}	0.54 ± 0.02^{aB}	0.64 ± 0.04^{abA}
AQ5	6.10 ± 0.03^{cB}	13.38 ± 0.73^{bA}	65.97 ± 5.49^{abA}	62.37 ± 1.77^{aA}	0.27 ± 0.01^{abB}	0.80 ± 0.06^{aA}
AAI4	4.95 ± 0.18^{dA}	3.49 ± 0.72^{cB}	42.75 ± 14.97^{bA}	31.23 ± 12.04^{bA}	0.41 ± 0.22^{abA}	0.27 ± 0.13^{cB}
AJ10	2.41 ± 0.24^{eA}	0.27 ± 0.11^{dB}	49.01 ± 14.47^{bA}	39.43 ± 16.02^{abA}	0.16 ± 0.03^{bA}	$0.03 \pm <0.01^{dB}$
	Lipid content (%)		Lipid concentration (g/L)		Lipid yield (%)	
	Pure	Raw	Pure	Raw	Pure	Raw
M3	20.46 ± 0.64^{aB}	56.58 ± 5.62^{aA}	2.43 ± 0.09^{aB}	9.14 ± 1.28^{aA}	7.57 ± 0.19^{aB}	33.67 ± 4.67^{aA}
AAN1	10.71 ± 0.84^{bB}	37.73 ± 7.22^{bcA}	1.10 ± 0.05^{bB}	4.70 ± 0.82^{bA}	3.50 ± 0.18^{bB}	17.12 ± 3.53^{bA}
AQ5	7.04 ± 0.73^{cB}	28.84 ± 4.07^{bcA}	0.43 ± 0.05^{cB}	3.84 ± 0.38^{bA}	1.32 ± 0.19^{cB}	14.7 ± 1.39^{bcA}
AAI4	11.69 ± 0.25^{bB}	40.96 ± 3.68^{bA}	0.29 ± 0.05^{cB}	0.81 ± 0.07^{cA}	0.97 ± 0.21^{cB}	3.06 ± 0.24^{cA}
AJ10	5.95 ± 1.22^{cB}	23.97 ± 4.75^{cA}	0.28 ± 0.02^{cA}	0.10 ± 0.04^{cB}	0.90 ± 0.08^{cA}	0.43 ± 0.14^{cB}

Culture conditions: initial glycerol concentration 30 g/L, initial pH 6.0, agitation 185 rpm, incubation temperature 28 °C.
Cell yield (g biomass/g glycerol), Lipid yield (g lipid/100 g glycerol).
[a] Equal lower case letters indicate no significant difference between lines, and equal upper case letters indicate no significant difference between columns, for each response, both at 95% of confidence ($p < 0.05$).

3.3. Molecular identification of oleaginous yeasts

The nucleotide sequence obtained by sequencing of the D1/D2 domain of rDNA was compared with the data made available by NCBI using the BLASTn tool. The fragment of 550 bp obtained showed high similarity with strains of the genus *Candida*, being 100% identity similarity with the strain *Candida* sp. ST-358 (DQ404510.1, query coverage 97%, e-value 0.0) and 99% with the strain *Candida tenuis* strain ZIM 232 (FR690080.1, query coverage 100%, e-value 0.0). Due to this phylogenetic similarity, the microorganism LEB-M3 was classified as *Candida* sp. LEB-M3.

3.4. Fatty acid profile

Fatty acid composition of the lipid fraction of the yeast *Candida* sp. LEB-M3 cultivated in pure glycerol, consisted of 17.1% saturated fatty acids. The content of monounsaturated was 73.65%, highlighting C18:1, which accounted for 57.35% and the content of polyunsaturated represented 9.38%. For cultivation in raw glycerol the proportion of saturated fatty acids was 16.54%, 34.72% monounsaturated and 48.69% polyunsaturated, highlighting C18:2, which made up 46%. The lipids produced by cultivation in raw glycerol was positive for production of polyunsaturated fatty acids, showing resemblance to the composition of vegetable oils normally used for production of biodiesel (Easterling et al., 2009), such as soybean oil (Fig. 2). Papanikolaou and Aggelis (2002) used raw glycerol as substrate for the cultivation of *Yarrowia lipolytica* and report the accumulation of 43% lipids, where the fatty acid profile consisted predominantly of C18:1(47%) and C18:2 (21%).

The fatty acid profile of soybean oil consists of a higher quantity of linoleic acid present in its composition, very similar to that obtained by the yeast *Candida* sp. LEB-M3 cultivated in raw glycerol. According to CONAB (National Supply Company), the 2011/2012 soybean harvest in Brazil reached a production of 71 million tons for an area of 24.63 million planted hectares, with total yield of about 3000 kg/ha. Knowing soybean oil accounts for roughly 20% of the total weight, 1 hectare produces about 600 kg of oil per year. Assuming a microbial lipid production of 9 g/L, the value obtained for cultivation of *Candida* sp. LEB-M3 in raw glycerol,

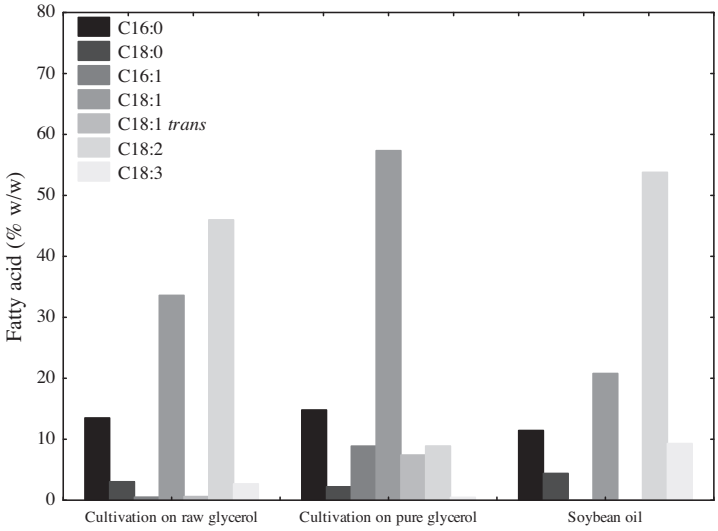

Fig. 2. Results of the mainly fatty acid (FA) composition (% w/w of total lipid) from lipids produced during growth of *Candida* sp. LEB-M3 on pure and raw glycerol utilized as substrate compared to fatty acid profile from soybean oil (Akoh et al., 2007).

roughly 66 m^3 of culture medium would be required for the production of oil equivalent to 1 hectare, while emphasizing that the soybean crop is annual and microorganism based oil production occurs in approximately 10 days.

4. Conclusion

The results of this study indicate that the yeast isolated from the Pantanal has great potential for the accumulation of lipids, reaching concentrations in excess of 50% while utilizing glycerol derived from biodiesel production, indicating that it is possible to use this co-product as a substrate for production of lipids with high yield. This study contributes to a better utilization of raw glycerol and confirms the potential for using microbial lipids in the biodiesel production process.

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