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Prediction of Quality Properties for Biodiesel Production by Oleaginous Yeast Cultivated in Pure and Raw Glycerol

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Biofuels such as biodiesel are a renewable and environmentally safe alternative to fossil fuels. Besides their production is growing rapidly, leading as a consequence, to large amounts of glycerol, the main co-product generated during the process. There is an increased interest in exploring alternatives for the production of lipids to produce biofuel and also to use glycerol surplus. Oleaginous microorganisms are able to accumulate 20 % or more of their biomass in lipids, mainly in the form of triacylglycerol (TAG), which can be used to produce biodiesel by transesterification process. The properties of various individual fatty esters that comprise biodiesel determine the overall fuel properties. Structural features that influence the physical and fuel properties are chain length, degree of unsaturation and branching of the chain. In this work we studied the production of lipids by *Candida* sp. LEB-M3 cultivated in pure and raw glycerol. Various methods for the prediction as a function of fatty acids were presented and standards used to verify the quality and applicability of this microbial oil as a raw material for biodiesel production. The fatty acid profile showed predominance of oleic acid (C18:1), 57.35 % for cultivation in pure glycerol, and linoleic acid (C18:2), 46.0 % in raw glycerol. Predicted values were (pure - raw): cetane number (56 - 53), heat of combustion (37 - 39 KJ/g), oxidative stability (8.5 - 8 h), kinematic viscosity (3.82 - 3.79 mm^2/s), density (807 - 872 Kg/m^3) and iodine index (74 - 115.5 $g_{I2}/100g$). The results indicate that lipid produced by *Candida* sp. LEB-M3 using raw glycerol is a potential and appropriate source of raw material for biodiesel production according to main current standards.

1. Introduction

Biodiesel has received considerable attention in recent years because it is a biodegradable, renewable and non-toxic fuel, contributing to the environment by emitting less polluting gases in the atmosphere than regular diesel. The traditional production of biodiesel using vegetable oils has economic impacts due to their high costs and the fact that need arable land competing with food sector, thus it is a raw material of low viability (Marchetti et al., 2008). For this reason more researchers are focusing on clean and renewable alternatives, such as microbial lipids, replacing traditional vegetable oils for biodiesel production (Huang et al., 2013).

Microorganisms, which include yeasts, which can accumulate lipids to more than 20 % of their dry weight are considered oleaginous microorganisms (Ratledge, 2005). Currently, microbial lipids have attract much attention because of their bi-function as a supplier of functional oils and feedstock for biodiesel production. However, high fermentation costs prevent their further application, and the possibility of their industrialization (Huang et al., 2013). On the other hand, yeasts can grow well on a variety of substrates, even inexpensive materials such as nutritional residues from agriculture and industry. Various low-cost substrates, like raw glycerol have received attention (Rahuet et al., 2013).

Moreover, biodiesel produced from vegetable oils generates about 10% glycerol as the main by-product, whose generation in excess may represent an environmental problem, since currently the market does not absorb the entire production (Silva et al., 2009). According to ANP (Agência Nacional do Petróleo, Gás Natural e Biocombustíveis) in 2012 Brazilian market produced 2.7 Mm^3 of biodiesel, which resulted over than 273 Mm^3 of raw glycerol. When it comes to use of the residual glycerol from biodiesel in culture media, without prior purification, advantages over the traditional use of pure glycerol as a substrate are

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observed, mainly with respect to lower cost and higher lipid production. Besides crude glycerol contains not only glycerol but also impurities such as potassium and sodium salts, methanol, and non-glycerol organic matter that can be beneficial to the yeast metabolism (Duarte et al., 2013).
The suitability of microorganism-based biodiesel for application as a fuel must follow certain requirements, as with traditional biodiesel, and must comply with the specifications of some parameters stipulated by current standards. These parameters may be influenced by the individual characteristics of FAMEs (fatty acid methyl esters), according to the structure of the constituent fatty acids (Gopinath et al., 2009).Therefore, it is important to know how the various fatty acid profiles of different oil sources can influence the overall properties of the fuel. Ester chain length, degree of unsaturation and branching are the main characteristics which contribute to determining the quality of biodiesel (Knothe, 2005). While many of these specifications are related to fuel quality issues, such as completeness of the transesterification reaction or storage conditions, several parameters directly depend upon the fatty acid composition of the biodiesel fuel. Among these specifications are cetane number, kinematic viscosity, oxidative stability, and cold-flow properties in form of the cloud point or cold-filter plugging point. Other important feature to consider that are influenced by fatty ester composition but are not contained in biodiesel standards are exhaust emissions and lubricity (Knothe, 2008).

2. Materials and Methods

2.1 Microorganism

The culture was isolated from flowers found in the Pantanal by Maugeri and Hernalsteens (2007) and screened as a oleaginous yeast strain identified of Candida sp. LEB-M3 (Laboratory of Bioprocess Engineering, UNICAMP, Brazil) in previous work (Duarte et al., 2013). The yeast was maintained on GYMP (yeast, malt, glucose, peptone) agar slant at 5 °C, as stock cultures. Before each culture, colonies were reactivated on GYMP slants composed of (g/L): 20.0 glucose, 5.0 yeast extract, 10.0 malt extract, 2.0 KH_2PO_4 and 20.0 agar, pH 5.5 and incubated at 30 °C for 48 h.

2.2 Preparation of inoculum

Two tubes of the microbial culture on slant GYMP were scraped with 10 mL of 0.1 % peptone water for removal of the microorganism cells and transferred into Erlenmeyer flasks containing 180 mL of culture medium composed of (g/L): 30.0 glycerol, 7.0 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 (New Brunswick Scientific model Innova 4430, Edison, NJ, USA) at 185 rpm. The cell concentration was monitored by counting in a Neubauer chamber until reaching approximately $1x10^8$ cells/mL.

2.3 Shaken flasks

The experiments were carried out using Erlenmeyer flasks containing 200 mL of the culture medium as proposed by (Papanikolaou and Aggelis, 2002), the same as that used for inoculum, in which the carbon source was pure or raw glycerol (from the synthesis of biodiesel), composed of 42.4 % (w/v) glycerol obtained by transesterification of soybean oil with methanol, without any previous treatment, which was kindly supplied by SP-BIO (Sumaré-SP, Brazil). The amount of raw glycerol added to the culture medium was determined by considering the desired concentration of the carbon source in the substrate. Flasks were incubated at 28 °C and 185 rpm with 10 % v/v of inoculum.

2.4 Prediction of biodiesel quality parameters

From the percent composition of FAMEs from the lipid material extracted in different culture (both kind of glycerol), some parameters governing the quality of biodiesel were estimated. Prediction was performed based on individual characteristics of each methyl ester that are fundamental to predict the main properties of biodiesel (Knothe, 2005). The quality parameters evaluated were cetane number (CN), density (ρ), heat of combustion (ΔHC), oxidative stability index (OSI), kinematic viscosity (υ) and iodine index (II). Properties of the ester mixture (biodiesel) can be predicted by the linear mixing rule where the properties of each component are multiplied by concentration of the components in the mixture (Pratas et al., 2011). This prediction method showed a good correlation between the measured and predicted values for CN, ΔHC (Pereyra-Irujo et al., 2009), ρ (Pratas et al., 2011), and OSI (Knothe, 2005). For the prediction of viscosity (υ), a logarithmic equation was obtained and the iodine index (II) was directly related to the number of double bonds present in the oil (Pereyra-Irujo et al., 2009). The prediction models of the quality parameters analyzed are shown in Table 1 where the fractions of each methyl ester present in biodiesel are represented by %ME. The properties CN, ρ, ΔH, OSI, υ, II, MM when applied to each methyl ester present in biodiesel correspond to the acronym “me”. DB is the number of double bonds present in the oil

and 253.82 is the atomic weight of two iodine atoms which are theoretically added to the double bond. [a](Ramos et al., 2009); [b](Pereyra-Irujo et al., 2009).

Table 1: Prediction models of the biodiesel quality parameters in function of the FAME composition

Parameter	Model
Cetane number[a]	$NC = \sum \frac{CNme \cdot \%ME}{100}$
Density[b]	$\rho = \sum \frac{\rho me \cdot \%ME}{100}$
Heat of combustion[b]	$\Delta H_C = \sum \frac{\Delta HC_{me} \%ME}{100}$
Oxidative stability index[a]	$OSI = \sum \frac{OSIme \cdot \%ME}{100}$
Kinematic viscosity[b]	$Ln\ (\upsilon) = \sum \frac{\ln(\upsilon me) \%ME}{100}$
Iodine index[b]	$II = \sum \frac{253,82 \cdot \%DB \cdot \%ME}{MMme}$

2.5 Analysis

Cells were harvested by centrifugation at 785 g for 10 min and washed twice with distilled water. Cellular lipids were extracted by a mixture of chloroform and methanol according to a known procedure (Bligh and Dyer, 1959). 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 (30.0 m x 0.25 mm x 0.25 µm). N_2 was used as a carrier gas, at a flow rate of 1.6 mL/min, and split ratio of 1:100. The oven temperature program initiated at 140 °C where it was held for 20 min, increased to 220 °C at a rate of 2.5°C/min and then maintained at 220°C for 10 min. The injector and the detector temperatures were adjusted to 230 °C and 250 °C, respectively. Fatty acids were identified by direct comparison of retention times with Sigma-Aldrich standards and quantified by area normalization.

3. Results and Discussion

The determination of some biodiesel quality properties by the experimental procedure can be an expensive and lengthy process, therefore studies based on the effect that the fatty acid profile exerts on these properties are needed to reduce costs and analysis time, and have been developed by several research groups. Biodiesel quality is positively influenced by saturated and long fatty acids, because they are associated with increased calorific value, cetane number and oxidation stability. On the other hand, shorter and more unsaturated chains increase the viscosity and flow characteristics at low temperatures, which are desirable characteristics of fuels. To obtain a biodiesel with appropriate quality characteristics, an appropriate ratio between saturated and unsaturated fatty acids should be maintained (Knothe, 2005).
Fatty acid composition (Table 2) of the lipid fraction of the yeast *Candida* sp. LEB-M3, cultivated in medium containing pure glycerol, consisted of 17.1 % saturated fatty acids (SFA). The content of monounsaturated fatty acids (MUFA) was 73.65 %, highlighting oleic acid (C18:1) which accounted for 57.35 % and the content of polyunsaturated fatty acids (PUFA) represented 9.38 %.

Table 2: Fatty acid profile obtained on different cultivations

Fatty acids	Pure glycerol (%)	Raw glycerol (%)
Palmitic acid - C16:0	14.8	13.5
Stearic acid - C18:0	2.21	3.04
Palmitoleic acid - C16:1	8.88	0.51
Oleic acid - C18:1	57.35	33.6
Linoleic acid C18:2	8.90	46.0
Linolenic acid - C18:3	0.48	2.69
others	7.38	0.66

For cultivation in raw glycerol the proportion of saturated fatty acids was 16.54 %, 34.72 % monounsaturated fatty acids and 48.69 % polyunsaturated fatty acids, highlighting linoleic acid (C18:2) which made up 46.0 %. The lipids produced by cultivation in glycerol generated from the biodiesel synthesis 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. In previous work this yeast *Candida* sp. LEB-M3 presented potential to produce high content of lipids higher than 50 % (m/m) when cultivated in raw glycerol (Duarte et al., 2013).
Table 3 presents datas from literature for methyl esters of different methyl fatty acids obtained for *Candida* sp. LEB-M3. This parameters were using to predict the quality of biodiesel produced by this yeast
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Table 3: Parameters from literature for methyl esters of different fatty acids

FAME	Formula	MM (g/mol)	ρ (Kg/m^3)	υ (mm^2/s)	ΔH_C (KJ/g)	OSI (h)	CN	Lub (μm)
Methyl palmitate	$C_{17}H_{34}O_2$	270.46	852[a]	4.32[b]	39.47[b]	>40[c]	74.5[b]	357[c]
Methyl palmitoleate	$C_{17}H_{32}O_2$	268.44	875[a]	3.67[c]	39.32[b]	2.1[c]	51[b]	246[c]
Methyl stearate	$C_{19}H_{38}O_2$	298.51	850[a]	4.74[b]	40.10[b]	>40[c]	101[b]	322[c]
Methyl oleate	$C_{19}H_{36}O_2$	296.49	874[a]	4.51[b]	39.93[b]	2.5[c]	59.3[b]	290[c]
Methyl linoleate	$C_{19}H_{34}O_2$	294.48	889[a]	3.27[b]	39.72[b]	1.0[c]	42.2[b]	236[c]
Methyl linolenate	$C_{19}H_{32}O_2$	292.46	895[a]	3.14[b]	39.37[b]	0.2[c]	22.7[b]	183[c]

[a] (Lapuerta et al., 2010); [b](Knothe, 2005); [c](Moser, 2009); [d]data obtained as the mean between C16:0, C16:1 and C18:0 and C18:1.

Table 4 shows the quality parameters predicted in the different glycerol conditions, and Table 5 shows the limits required by the biodiesel standards. The parameters were compared with the limit values stipulated by current standards ASTM-D6751 (EUA), EM 14213, EM 14214 (Europe) and ANP 255/2003 (Brazil).

Table 4: Biodiesel quality parameters predicted by the FAME properties obtained at different cultivation

Glyerol	ρ (Kg/m^3)	υ (mm^2/s)	ΔH_C (KJ/g)	OSI (h)	CN	II (g_{I2}/100g)
Pure	807	3.82	37	8.5	56	74.0
Raw	872	3.79	39	8.0	53	115.5

Table 5: Limits established by the standards ASTM, EM and ANP for biodiesel commercialization

Standards	ρ (Kg/m^3)	υ (mm^2/s)	ΔH_C (KJ/g)	OSI (h)	CN	II (g_{I2}/100g)
ASTM D6751	--	1.9-6.0	--	Min 3	Min 47	--
EM 14213	860-900	3.5-5.0	35	Min 4	--	Max 130
EM 14214	860-900	3.5-5.0	--	Min 6	Min 51	Max 120
ANP 255/2003	--	--	--	--	Min 45	--

The cetane number (CN) is a dimensionless number for diesel just as the octane number is for gasoline and is associated with ignition delay time, i.e., the time between fuel injection the beginning of ignition. The shorter the ignition time, the higher the cetane number. CN value was higher in the assays with pure glycerol (56), but very similar to cultivation in raw glycerol (53). This behavior is due to the high fraction of SFA and longer chain FAMEs obtained from the conditions of cultivation in pure glycerol. It should be noted that both indicated CN values which add quality to the lipid material and the corresponding biodiesel

is within the limits of current standards, according to Table 5, which require a minimum CN of 45 for Brazilian limits and higher values for Europen and American standards. This parameter is one of the most important property for biodiesel quality.

The oxidative stability index is measured in hours and is related to the number and position of unsaturations that when broken cause biodiesel oxidation. This value were similar for both kind of glycerol (8.5 - 8.0 h) and high to the minimum established for standars.The iodine index (II) refers to the tendency of biodiesel to react with oxygen at room temperature. The low iodine index indicates less susceptibility to oxidation by oxygen. The higher II was obtained in raw glycerol,114.5 g_{I2}/100g, and for pure glycerol was 74 g_{I2}/100g and this is less susceptible to the oxidation by oxygen, but both are within the limits. Density values greater than those considered adequate may increase the required fuel/air mixture, which increases emission of pollutants including hydrocarbons, carbon monoxide and particulate matter. Low values may favor the formation of a lean air/fuel ratio, leading to loss of engine power and consequent increased fuel consumption (Pratas et al., 2011).

Experimental data showed that biodiesel density decreased with increasing unsaturation levels of FAMEs (Pratas et al., 2011) according to verified in this work when the cultivation in raw glycerol density was higher (872 Kg/m^3) than pure glycerol (807 Kg/m^3) due to the high fraction of PUFA. Kinematic viscosity and heat of combustion were virtually the same in both conditions, approximately 3.8 mm^2/s and 38 KJ/g, respectively and within of the limits. It is important to highlight that linolenic acid C18:3 is limited to 12 % by EN14214 due to its high rate of oxidation and the consequent reduction in biodiesel quality, where none of the assays exceeded 2.69 % of this compound. These predicted parameters give an indication of the applicability of lipid material obtained on cultivation in a coproduct of biodiesel production.

3. Conclusions

The predicted parameters studied from lipidic material of the yeast *Candida* sp. LEB-M3 were within the stipulated by current standards. The analysis of fatty acid profile indicated that the following predicted values were (pure - raw): cetane number (56 - 53), heat of combustion (37 - 39 KJ/g), oxidative stability (8.5 - 8 h), kinematic viscosity (3.82 - 3.79 mm^2/s), density (807 - 872 Kg/m^3) and iodine index (74 - 115.5 g_{I2}/100g). The data presented in this paper regarding biodiesel quality indicate the feasibility of applying the yeast *Candida* sp. LEB-M3 for biodiesel synthesis. The study of biodiesel synthesis and increase of process scale may permit the application of this new lipid source.

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