Quantification of fatty acids from oleaginous yeasts with potential for biodiesel production

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Abstract: Oleaginous microorganisms have been studied to be used as potential raw material for the production of biofuels. The main objective of this work is the development of a safe methodology for the quantification of fatty acids from oleaginous yeasts by Chromatography Coupled to Mass Spectrometry, in order to test their capacity for lipid accumulation and lead to the characterization of fatty acids accumulated by them, since the lipid composition influences the final quality and fuel performance to verify which of the oleaginous microorganisms tested is the most appropriate as raw material to biodiesel production.

Keywords: Oleaginous microorganisms, biodiesel, quantification, fatty acids.

1. INTRODUCTION

Fossil fuels make up a network of great influence on the geopolitical relations, since petroleum are the basic element of industrial energetic matrix and can lead to trade, financial and diplomatic disputes such as wars and violent conflicts between countries [1]. Besides, the depletion of raw materials of fossil origin over time implies increasing costs of production [2], while the environment has been receiving more attention, due to the implications and consequences resulted from fossil fuels use [3], such as global warming, attributed to the excessive use of fossil fuels and CO_2 production by its burning [4]. As a consequence, many studies are being conducted to the use of alternative energy sources, such as biofuels [5, 6]. These fuels are produced predominantly from biomass [7], which is a renewable energy source, obtained from agricultural products like sugar cane, oil plants and other organic materials, and may be used as pure fuels or mixed with conventional fuels[8].

Biodiesel is a fuel produced mainly from vegetable oils, but it can also be produced from animal fat, through esterification of free fatty acids or transesterification of triglycerides. This fuel can

be defined as a mix of alkyl esters of long chain fatty acids produced through chemical reactions [9]. Because it comes from renewable raw material, it emits less polluting gases than diesel, such as carbon dioxide or sulfur into the atmosphere during its burning [3].

Analytical parameters are adopted aiming at the control over fuel performance and can be influenced by molecular structures of alkyl esters constituents of biodiesel. These include: specific mass, kinematic viscosity, iodine number, fraction of distillates, cetane number, cloud point – CP, (cold-filter plugging point – CFPP, pour point – PP [10]. Thus, the raw material that will be used for the production of biodiesel must produce oil capable of passing through all these parameters in order to generate a good quality biodiesel

Oleaginous microorganisms, such as bacteria, yeasts, fungi and microalgae are being studied to convert lipid bodies into biodiesel [11]. A microorganism must be able to accumulate at least 20% of its biomass in lipids to be considered an oleaginous microorganism [3, 12].

Yeasts can use low cost culture media for fermentation using industrial and agriculture residues[13], such as lignocellulosic biomass and other low cost materials, like glycerol [14]. Oleaginous yeasts can accumulate up to 70% of its biomass in lipid bodies during metabolic stress, such as an increase in the C/N ratio, which can be achieved either by nitrogen limitation or carbon excess [15, 16].

With the need to show quality in chemical measurements, through its comparability, traceability and reliability, the validation of an analytical method is being increasingly recognized and required. Unreliable analytical data can lead to disastrous decisions and irreparable financial losses. To ensure that a new analytical method generates reliable and interpretable information about the sample, it must be validated [17]. This way GC-MS will be utilized to lipid quantification, and parameters such as selectivity, linearity, deteccion limit, quantification limit, accuracy/bias must be evaluated to method validation[18] assuring confiability and traceability of the results.

In the present study, we evaluated different species of yeasts and its lipids production in restrict growth condition to discover which oleaginous yeast tested can accumulate more lipid corpuscles, besides to verify the fatty acids types accumulated by them.

2. MATERIALS AND METHODS

2.1. *Strains and growth conditions*

Four yeast species were used in this study: *Rhodotorula glutinis*, *Candida albicans*, *Yarrowia lipolytica* and *Rhodothorula slooffiae.* With exception of *Rhodotorula slooffiae* that was collected from the soil of Nacional Institute of Metrology, Quality and Technology – Xerém, RJ- Brazil, the other three strains were obtained from the collection of Nacional Institute of Health Quality Control/Oswaldo Cruz Foundation – Manguinhos, RJ – Brazil. The cryopreserved strains were cultivated on Petri dishes containing Sabouraud Dextrose Agar medium (Himedia) for 5 days at 30°C, being then transferred to 200 mL of Sabouraud Dextrose Broth (Himedia) and maintained for 5 days at 30 ° C in a skaker-type incubator at 200 rpm. After that, an aliquot of each culture was centrifuged at 2500 xg for 5 minutes. The inoculum grown on Sabouraud Dextrose Broth medium was transferred to a nutrient restricted medium

supplemented with glycerol containing: 0.4 g/L KH_2PO_4; 0.3 g/L yeast extract; 2.0 g/L NH_4Cl; 40 g/L glycerol 85%; pH 6.5. The cultures were maintained for 20 days at 30 °C and 200 rpm.

2.2. ***Lipid extraction with organic solvents***

For lipid extraction, cultures were lyophilized and 6 mg of each species were used. First, cells were broken with zirconia beads (0.5 mm) using a Mini-Beadbeater – a mechanic method to cell disruption. After that, the lipid extraction was performed using Bligh & Dyer method [19], a liquid-liquid separation using methanol, chloroform and water (2:1:0.8). The samples were kept at 4 ^oC for 24 h, and then, cells debris were separated by centrifugation at 2.000 xg for 20 min. Chloroform and water were added in the same proportion (1:1) to separate the organic fraction, which was dried under nitrogen atmosphere.

2.3. ***Lipid Esterification***

Lipid esterification was performed by adding 5 mL of methanol containing 2% of sulfuric acid as a catalyst. This mixture was maintained at 60°C for 2 h, followed by the addition 2 mL of petroleum ether and 2 mL of water to separate the organic fraction, which was dried under nitrogen atmosphere, as described in 2.2.

2.4 ***Fatty Acid Methyl Esters (FAMEs) analysis***

For FAMEs analysis, esterified lipid samples were diluted (1:10) and GC-MS analysis were conducted using the conditions: capillary column: DB-5 30 m x 0.25 mm, 0.25 µm (Agilent); injector temperature: 250°C; detector temperature: 300°C; carrier gas: helium; injection mode: splitless. The programming of the oven temperatures is shown in table 1.

Table 1: GC-MS oven programing

Rate °C	Value °C	Hold
-	120	1
40	180	1
20	190	1
0,4	195	5
20	300	3

2.4.1 Validation of GC-MS method to lipid quantification

2.4.2 ***Linearity***

Linearity was tested using 5 concentration levels of fatty acids standards - palmitic, linoleic, oleic and stearic- Sigma Aldrich. Linearity got through external standardization and formulated with the mathematical expression y=ax+b. An analytical curve was obtained using the concentrations:10 µg/mL, 7µg/mL, 5 µg/mL, 3 µg/mL and 1 µg/mL. Each concentration level was injected three times on GC-MS. Linearity was evaluated graphically and by correlation coefficient (R).

2.4.3. ***Selectivity***

The selectivity was tested using MS (mass spectrometry), which is a technique able to differentiate the analyte of interest of the others. The analytes of interest are yeasts fatty acids, such as palmitic, linoleic, oleic and stearic acids.

2.4.3.1 ***Matrix effect***

For the matrix effect analysis, samples from the 7^{th} and 20^{th} culture days were used from all the four species. The lipid extraction and esterification were performed as described in 2.2 and 2.3. Each sample was submitted to standard addition following the concentrations cited in 2.4.2. Statistical analysis was performed using t test with excel.

The quantification was performed together with matrix effect.

2.5 *Results*

2.5.1 *Linearity*

Linearity was evaluated by the correlation coefficient (R). This parameter was larger than 0.95 for all fatty acids (table 2). An analytical curve was obtained by a gravimetric method to determinate the real concentrations (Table 3) used to build the curve.

Table 2: Mathematical parameters

	Mathematical function	**R^2**	**R**
Palmitic	y= (4.04E+6) – 2.3E+6	0.9977	0.9989
Linoleic	y= (2.26E+6) – 2.4E+6	0.9824	0.9912
Oleic	y= (4.1E+6) – 3.06E6	0.9969	0.9984
Stearic	y= (3.97E+6) - 2.94E+6	0.9967	0.9983

Table 3: Determination of fatty acid standard concentration

Teoric Concentration (µg/mL)	**Actual Concentration (µg/g)**			
	Palmitic	Linoleic	Oleic	Stearic
1	0.694	0.703	0.700	0.693
3	2.139	2.167	2.158	2.137
5	3.626	3.673	3.658	3.623
7	5.116	5.183	5.162	5.113
10	7.229	7.324	7.294	7.224

2.5.2 *Selectivity*

The mass spectrum obtained from each fatty acid chromatograms were compared to the *NIST* library using the included in the GC analysis software. The comparison between mass spectrum resulting of standards and fatty acids found in yeasts resulted in data that showed us that MS is a technique able to differentiate each compound through mass spectrum resulting.

2.5.2.1 *Matrix Effect*

Matrix effect was tested for all species at the 7th and 20th day of cultivation. Statistical analysis (table 4) showed matrix effect in almost all species tested, with the exception of *Rhodotorula glutinis* in the 7th day of culture.

Table 4: t test results

Matrix Effect - Statistical analysis					
Species	t calculated				t tabulated
	Palmitic	Linoleic	Oleic	Stearic	2.086
RG 7	0.772	1.530	0.239	1.551	
RG 20	2.676	3.773	2.370	2.781	
RS 7	3.198	2.618	2.967	2.667	
RS 20	4.3	3.62	4.106	3.978	
CA 7	2.620	2.871	1.355	2.465	
CA 20	4.72	4.160	3.559	4.418	
YL 7	3.037	3.148	0.760	2.682	
YL 20	4.202	3.787	3.104	3.916	

Legend: RG- *R. glutinis*; RS- *R.slooffiae*; CA- *C. albicans*; YL- *Y. lipolytica*; 7- 7th cultive day; 20- 20th cultive day.

2.5.3 *Fatty acids quantification*

Quantification of fatty acids by GC-MS demonstrated that *Rhodotorula glutinis* in the 7th day of cultivation was able to accumulate more fatty acids than all the other species, being considerate the best yeast to accumulate lipid (table 5).

Table 5: Determination of fatty acids accumulated by yeasts

	Fatty acids concentration (µg/mL)			
	Palmitic	Linoleic	Oleic	Stearic
RG 7	93.48	98.97	220.79	20.23
RG 20	61.80	37.61	117.58	44.74
RS 7	15.08	3.13	96.40	10.77
RS 20	18.33	4.21	98.85	22.36
CA 7	43.20	18.63	164.38	44.45
CA 20	39.84	15.11	153.10	40.11
YL 7	48.64	10.22	246.13	45.81
YL 20	41.43	8.16	207.86	44.51

2.6 *Conclusion*

Oleaginous yeasts were tested in this study to verify which specie of yeast can be considerate the best potential raw material for biodiesel production. With the quantification by GC-MS we can suggest that *R. glutins* is the best specie, but in the 7th day of cultivation. On the other hand, *Y. lipolytica* can accumulate a little bit more oleic and stearic acid than *R.glutinis*. The results of partial method validation showed that linearity was good for all fatty acids, because all analytical curve showed a R > 0,95. MS is a selective method, which can be used to identify all analytes of interest. Because fatty acids are intracellular products, we tested the influence of matrix on the analytical curve, by doing a standard addition. Our results showed that only *R.glutinis* was not influenced by interferers contained in the matrix. Limits of detection and quantification have not yet been determined as well as accuracy and bias, but these are the next steps. We are going to evaluate detection and quantification limits through signal/noise relation with successive injections on GC-MS with low standards concentrations. Accuracy and bias are going to evaluate through recovery trial, so that we will verify how much of the sample is lost during chemical processes and whether the method used is efficient for this type of analysis.

The study have to aim demonstrate which yeast that was tested can accumulate more lipids in metabolic stress. The qualitative and quantitative analysis were performed using GC-MS, an analytical technique capable separate, identify and quantify molecules generation reliable data through validation of analytical method. Validate the qualitative and quantitative method of lipid compounds became important to assist the biodiesel industry. Thus, the project will provide the analysis of the fatty acid profiles present in the samples, in order to evaluate the adequacy of the lipid composition in relation to the current national and international standards for the maintenance of biodiesel quality. Therefore we will be able to know which microorganism tested is the best to be utilized as raw material to biodiesel production in terms of the production and molecular structure of fatty acids.

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