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Use of Wastewaters from Ethanol Distilleries and Glycerol Mixtures for Microbial Oils Production

Evelyn Faife Perez*, Aidin Martínez, Yanay Martínez, Nayra Ochoa, Amaury Álvarez, Juana M. Chanfon, Mayrelis Mesa

Biotechnology Division, Cuban Institute for Research Sugarcane by-Products (ICIDCA), Havana, Cuba

Email address:

evelyn.faife@icidca.azcuba.cu (E. F. Perez), aidin.martínez@icidca.azcuba.cu (A. Martínez), yanay.martinez@icidca.azcuba.cu (Y. Martínez), nayra.ochoa@icidca.azcuba.cu (N. Ochoa), amaury.alvarez@icidca.azcuba.cu (A. Álvarez), juana.chanfon@icidca.azcuba.cu (J. M. Chanfon), mayrelis.mesa@icidca.azcuba.cu (M. Mesa)

*Corresponding author

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Abstract: Biodiesel has become more attractive in recent years because of its environmental benefits. One way to reduce the high cost of biodiesel is by lowering the cost of raw materials specially oils. Recently, much attention has been paid to the development of microbial oils through cultures of oleaginous microorganisms in inexpensive substrates as the wastewaters of some food industries and the crude glycerol from biodiesel production itself. Thus, cultivation of oleaginous yeasts in glycerol-based media is attracting great interest and natural biodiversity is increasingly explored to identify novel oleaginous species recycling this carbon source for growth and lipid production. Identification of oleaginous yeasts and the evaluation in glycerol and vinasse mixtures was employed to produce biomass enriched in microbial lipids and to remove nutrients from vinasse simultaneously. The fatty acid composition of the lipids was similar to that from plant oils and other microbial lipids therefore they can be used as raw material for feed additives and biodiesel production.

Keywords: Vinasse, Glycerol, Lipids, Biodiesel, Oleaginous

1. Introduction

The conventional method for biodiesel production is the transesterification of plant oils with methanol. However, the cost of biodiesel is currently more expensive than that of conventional diesel due to the high cost share (70 - 85%) of the raw material. Increasing interest is being generated to explore ways to reduce the cost of the raw materials.

Recently, the interest in some microorganisms called oleaginous or “oil bearing” has been renewed, as a source for oils and fats. Several algae, yeasts and bacteria are capable of accumulating up to 70% of their dry weight as oil in the form of triacylglycerol. The possibilities of use this oil as a feedstock for biodiesel production is considerable [1].

Vinasses are the major effluent from the bioethanol industry and represent a major environmental problem. This black liquid is produced at a rate of 10 to 15 liters per liter of distilled ethanol and it is a mixture of water, organic and inorganic compounds. The chemical oxygen demand of Cuban vinasse is around 50 000 – 60 000 mg/L.

Currently, most of the vinasse that results from ethanol production is being used as fertiliser due to its organic matter content and nutrients as potassium and nitrogen. A problem occur when the nearest fields to the distillery receive a slop dose that overcomes the assimilation capacity of the soil, reducing the alkalinity destroying the crops, causing manganese deficiency and inhibiting seed germination.

The industrial application of biodiesel production has been stimulated by the production of high amounts of concentrated glycerol residual water, as the main by-product of the trans-esterification process applied in biodiesel. Its disposal constitutes an economic and ecological burden. This aspect can be ameliorated, however, by glycerol recycling to lipid

production.

Therefore, the utilisation of raw glycerol and vinasse as feedstock for microbial oil production seems to be an interesting alternative and "environmentally-friendly" strategy, aiming to control soil deterioration, to improve the economics of biodiesel industry, to produce SCP rich in oils as a supplement in animal feeding as well as to upgrade glycerol waste streams.

The objectives of this study is evaluate the potential of selected oleaginous yeasts to growth and accumulate lipids in vinasse and glycerol mixtures to propose this media as a feedstock for microbial oil production and as a solution to environmental contamination

2. Materials and Methods

2.1. Screening of Oleaginous Yeasts

The samples were collected from sugarcane fields and molasses from a sugar mill in the central region of Cuba. Twenty (20) g of samples were added to 100 mL of distilled water (DW). Mixtures were homogenised during 30 minutes at 150 rpm and 30°C. Serial dilutions in distilled water were made until 10^{-3} and then 0.1 mL diluted culture was isolated on YPG agar medium using spread plate technique for 48 – 72 h at 30°C. Interesting colonies were examined in a microscope, isolated and purified by Harrigan method [2].

Total yeast strains were stained with Sudan Black B technique [3, 4] and observed under a phase contrast microscope on oil immersion for the presence of blue or greyish coloured fat globules within the cell lipids. The yeast strains showing fat globules within the cells were preselected for further assays.

2.2. Identification of Oleaginous Yeasts

A primary identification was performed using two chromogenic media: Sabouraud and a chromogenic medium designed for some *Candida* species by BioCen (Cuba), similar to CHROMagar Candida®, [5]. Yeasts were cultured at 30°C during 24 h in this medium and identification was determined by visual inspection of blue and pink colours.

Identification of preselected yeast strains was made by API 20C System. Molten (50°C) API basal medium ampoules were inoculated with yeast colonies, and the suspension was standardised to a density below 1+ (lines can be clearly distinguished) on a Wickerham card.

Each cupule was inoculated, and the trays were incubated for 72 h at 30°C. Cupules showing turbidity significantly heavier than that of the negative control cupule (0 cupule) were considered positive. Identification was made by generating a microcode and using the API 20C and software ApiWeb StandAlone®.

2.3. Yeast Inoculation, Flasks and Bioreactor Culture

The oleaginous yeast colonies were initially streaked onto YEPD slant and grown for 2 days. After that, cells were transferred to 250-mL Erlenmeyer flasks containing 50-mL of inoculation medium (YPG) at 30°C on a rotary shaker at 150 rpm for 48 hours.

10 mLs of inoculum was transferred to 90 ml of a primary medium compound by pure glycerol medium (20 g/l glycerol, purity 99.5%, (BDH Prolabo), 10 g/l yeast extract, 20 g/l peptone) to propagate cells for different assays. All experiments in flasks and bioreactors were made using a 10% (v/v) of cells grown in exponential phase in this medium (10^8 cell/mL and 95% of viability)

The effect of glycerol concentration at 20, 40, 60, 80, 100 and 120 g/L as carbon source on cell growth of selected yeast strains in spread-plates and flasks was investigated. Plates were inoculated with 20 mL of 10^{-4}, 10^{-5} and 10^{-6} diluted pre-cultures inoculums of 24 h and incubated during 24 - 72 h at 30°C. The study in 500 mLs erlenmyer flasks was made by triplicate at at 30°C and 150 rpm during 120 hours.

Taking advantage of the experience of ICIDCA (Cuba) in the industrial production of single cell protein (SCP) using molasses/vinasse mixtures as culture medium [6-9], experiments in 4 L bioreactors (Marubishi MD 5, Tokyo, Japan) were done using a nitrogen-limited medium (30% of nitrogen requirement) containing $(NH_4)_2SO_4$ 0.92; $(NH_4)_2HPO_4$ 1.22 in g/L, prepared with a vinasse/glycerol mixture adjusted to 75 000 mg/L of total (COD with a 70:30 ratio, where 70% was the vinasse contribution and 30% of glycerol).

Fermentation parameters were fixed at 600 rpm, air supply of 1.2 vvm (air volume per medium volume per minute) and pH 4.0. The experiments were performed during 24 h with two replicates and samples analysed per duplicate for dry biomass, chemical oxygen demand and lipid content.

2.4. Determination of Yeast Dry Biomass

Aliquots of 5-mL cultures were harvested by centrifugation at 5000×*g* for 5 min. Collected biomass was washed twice with 5-mL of distilled water. The biomass was determined gravimetrically in an infrared balance (Sartorius GmbH, Germany) at 105°C.

2.5. Chemical Oxygen Demand

It was determined according to Conde method [10]. Briefly, 1-mL of centrifuged fermented medium was poured into a reflux conical flask of 25-mL and 0,04 g of $HgSO_4$ (II). Finally, 1-mL of 0.04 M of potassium dichromate and several glass pearls were added, the latter to avoid bumping, and slowly, 3-mL of sulphuric acid (d=1.84) with 9,9 g/L of $AgSO_4$. The reaction mixture is refluxed for 12-15 min, and, after cooling, 100-mL of distilled water was added. The excess of dichromate is determined with diammonium ferrous sulphate with ferroin as indicator. A blank with 1-mL of distilled water was run in parallel.

2.6. Determination of Lipid Content

Lipids were extracted, dried and weighed and the method of Bligh and Dyer [11] with slight modifications was used. A 50-mL sample was centrifuged at 5000x*g* for 5 min, after

which the yeast was washed twice with 50-mL of distilled water and dried overnight, then 1 gram of dry biomass was added into 10-mL of 4 M HCl, and incubated at 60°C for 1 to 2 h. The acid-hydrolysed mass was stirred with 10-mL of chloroform/methanol mixture (1:3) at room temperature for 2 to 3 h, followed by centrifugation at 5000x*g* for 5 min to separate the aqueous and organic phases. Finally, the lower phase containing the lipids was recovered with a Pasteur pipette, and evaporated under vacuum for 10 min. The dry lipids were weighed.

2.7. Fatty Acid Derivatisation for Gas Chromatography

A sample of 150 mg of oil was poured into a dry test tube and 5 mL of 10% methanol solution of sodium methoxide were added and hermetically sealed. Then, the tube is then placed in a thermostatic water bath at 85^{o}C for 2 h with occasional vigorous stirring. After that, the tube is cooled at room temperature and 5 mL of n-hexane and 5 mL of distilled water were added and the sample stirred for 1 min. The mixture is separated into two phases and 4 mL-aliquot of the upper phase is extracted and placed in a dry tube. The lower phase is discarded. The extract is mixed with 5 mL of n-hexane and 5 mL of N methanol solution of sodium hydroxide, and then the tube is sealed and placed in a rotary shaker for 1 min. The solution splits into two phases and from the upper one a 2 mL-aliquot was extracted and the solvent evaporated under vacuum in a centrifuge. Finally, the fatty acid methyl esters were dissolved in acetone for gas chromatography.

For fatty acid characterisation, a GC-17A Shimadzu chromatograph provided with AOC-20S auto-injector coupled to a MS-QP5050A mass spectrophotometer were employed. The instrument has an electronic ionisation source and a quadrupole analyser. A DB-1 non polar 30 m x 0.25 mm capillary column was used.

3. Results and Discussion

3.1. Screening and Identification of Yeasts

Seventy three yeast strains were isolated from soils and molasses and cultivated in YPG medium at pH (4-5.5) with chloramphenicol 1%. Seven yeast strains were found positive from results of Sudan Black B technique. L/3-74-21 and L/3-74-22 yeasts developed a blue colour when were culture in BioCen medium and L/24-25-1, L/24-26-1, L/24-26-2 and L/24-26-3 shown a pink coulor in Sabouroud medium, wich are typical colour of *Candida sp.* and *Rhodotorula sp.* in these media, respectively (Figure 1).

According to the results of identification by API 20 AUX System most of the strains belong to *Candida* and *Rodotorula sp*. (Table 1). This amount is probably related to the adaptability of these genus to many ecosystems [12]. The capacity of these genus to produce lipids has been previously reported by several researchers [13-19].

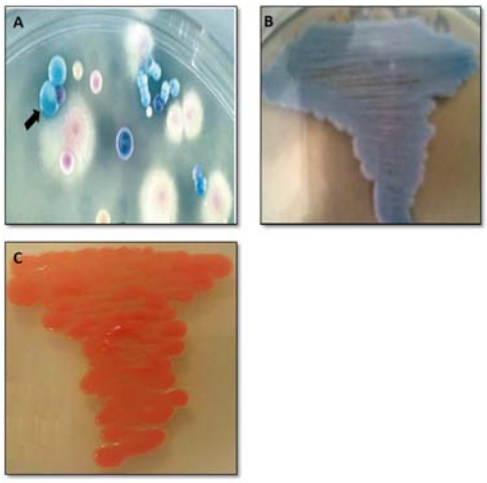


Figure 1. *Growth in plates of the strains in Sabouraud and BioCen media (A: isolated strain yeasts from molasse, B: purified L/3-74-22 strain in BioCen medium and C: purified L/24-26-1 strain in Sabouraud medium).*

Table 1. *Results of the yeast strains identification by API 20C System according to different substrates consumption.*

Carbon Source	L/3-74-21	L/3-74-22	L/25-7-20	L/24-25-1	L/24-26-1	L/24-26-2	L/24-26-3
Glucose	+	+	+	+	+	+	+
Glycerol	+	+	-	+	+	+	+
L-Arabinose	-	-	-	+	+	+	+
Xylose	-	+	-	+	+	+	+
D-galactose	-	+	+	-	+	+	+
D-Lactose	-	-	-	+	-	-	-
D-Sucrose	-	+	+	+	+	+	+
Identification	*A*	*A*	*B*	*C*	*D*	*D*	*D*

3.2. Influence of Glycerol Concentration

It has been demonstrated that glycerol is a good substrate for different yeast species [20-23]. Others researchers found that only a few species of *Candida, Cryptococcus*, *Pichia, Kluyveromyces and Saccharomyce.,* are capable to consume more than 90% glycerol and were also able to accumulate lipids [24]. Recently some experiments demonstrated the assimilation capacity of crude glycerol from biodiesel industrial waste of some yeasts species as *Rhodotorula glutinis*, *Candida aloleophila*, *Candida curvata*, *Candida pulcherrima*, *Yarrovia lipolytica, Cryptococcus curvatus* and *Pichia membranifaciens* [22, 25-27].

Visual inspection of the plate cultures using glycerol as unique carbon source shown that there was no growth inhibition until 120 g/L of glycerol concentration. One *C.*

tropicalis strain (L/3-74-22) and one *R. mucilaginosa* (L/24-26-1) strain were selected to continue the experiments. The biomass concentration, substrate yields and specific growth rate of *C. tropicalis* and *R. mucilaginosa* cultured in flasks after 24 h are shown in Table 2. The results shown that *R. mucilaginous* strain reached the highest value of biomass concentration, yield and specific growth rate in evaluated glycerol concentrations.

This results matches with a screening reported by Taccari [28] using anothers *C. tropicalis* and *R mucilaginosa* strains where *R mucilaginousa* cell densities were almost double respect *C. tropicalis* on pure glycerol at 20 /L. Specific growth rate value of *C. tropicalis* L/3-74-22 strain at 20 g/L of glycerol was lower than others values of *Candida sp.* (0,14 – 0,17 h^{-1}). In case of *R. mucilaginosa* L/24-26-1, the specific growth rate reported was very similar to *Rhodotorula mucilaginosa* DiSVA 6094 (0,14 h^{-1}) and *Rhodotorula mucilaginosa* DiSVA C7.1 (0,16 h^{-1}).

Several researchers have demonstrate that cell growth and lipid content of different oleaginous yeast cells is lower in glycerol respect to other carbon source to the same concentration and showed their positive effect when it is used simultaneously with another energy source as xylose [29, 30].

Table 2. *Comparative fermentation parameters of C. tropicalis L/3-74-22 and R. mucilaginosa L/24-26-1 strains a in the presence of different glycerol concentration in 500 ml flasks and 30°C.*

Glycerol conc.	Biomass conc. (g/l)		Yx/s		μ (h^{-1})	
(g/l)	L/3-74-22	L/24-26-1	L/3-74-22	L/24-26-1	L/3-74-22	L/24-26-1
control	10,6 ± 1,8	12,2 ± 0,4	0,530 ± 0,03	0,496 ± 0,02	0,11 ± 0,03	0,25 ± 0,05
20	4,9 ± 1,1	7,34 ± 0,3	0,245 ± 0,12	0,279 ± 0,01	0,07 ± 0,02	0,15 ± 0,01
40	7,5 ± 1,3	9,15 ± 0,2	0,156 ± 0,05	0,189 ± 0,03	0,09 ± 0,01	0,16 ± 0,01
60	9,1 ± 2,2	10,3 ± 1,6	0,122 ± 0,03	0,144 ± 0,05	0,10 ± 0,01	0,18 ± 0,02
80	10,2 ± 2,1	12,1 ± 1,9	0,107 ± 0,04	0,133 ± 0,02	0,10 ±0,01	0,22 ± 0,01
100	11,6 ± 0,2	15,7 ± 1,4	0,101 ± 0,01	0,143 ± 0,04	0,11 ±0,01	0,20 ± 0,03
120	12,7 ± 0,8	17,7 ± 2,1	0,100 ± 0,02	0,137 ± 0,01	0,12 ±0,02	0,26 ± 0,02

3.3. Evaluation in Vinasse/Glycerol Mixture with Nitrogen Limitation

The evaluation of *C. tropicalis* and *R. mucilaginosa* strains in this mixture showed that both yeasts were able to grow and accumulated lipids under these culture conditions, nevertheless their cell growth parameters were very different. The results of cell growth parameters and lipid content shown in Table 3 demonstrated that there was a marked difference between the behaviour of each strain. *C. tropicalis* achieved higher values of biomass concentration, specific growth rate and COD removal while the lipid content were higher in *R. mucilaginosa*.

Oleaginous microorganisms start to produce lipids when a growth required nutrient, mostly nitrogen, is exhausted in the medium [31, 32]. The excess carbon is then channeled into lipid production. Accordingly, it has been shown that the lipid content is constantly low when nitrogen is present in the culture medium. Thus, the carbon to nitrogen ratio (C/N ratio) of the culture medium is a very important parameter for the potential lipid production. Some researchers have demonstrated that oils content increase from 18 to 46% when C/N ratio increase from 25 to 70 [33].

Many factors including medium components, such us carbon and nitrogen sources as well as culture conditions (temperature, dissolved oxygen and pH) have also a significant influence on biomass and oil accumulation [34-36].

The pattern of a *R. glutinis* strain in different wastewaters from fruit juice, potato and lettuce processing was described by Schneider et al. [37]. None of the wastewaters allowed for significant lipid production by *R. glutinis* due to the most important limiting factors for lipid production in the screened wastewaters seem to be the low content of available carbon sources in fruit juice and lettuce processing waters and the high content of nitrogen in potato processing waters, respectively.

Even at 30 hours, the lipid content is not significant in both strains because most of the available carbon was exhausted before nitrogen became the growth limiting factor, these results are enough to select *R. mucilaginousa* as a better candidate to reached a high lipid production in less time under this culture condition. To enhance lipid concentration and lipid productivity is necessary to increase C/N ration in the mixture.

Table 3. *Kinetic parameters of L/3-74-22 (C. tropicalis) and L/24-26-1(R. mucilaginousa) strain in vinasse/glycerol mixture with COD ratio 70:30 in 4L bioreactors experiments at 30 h.*

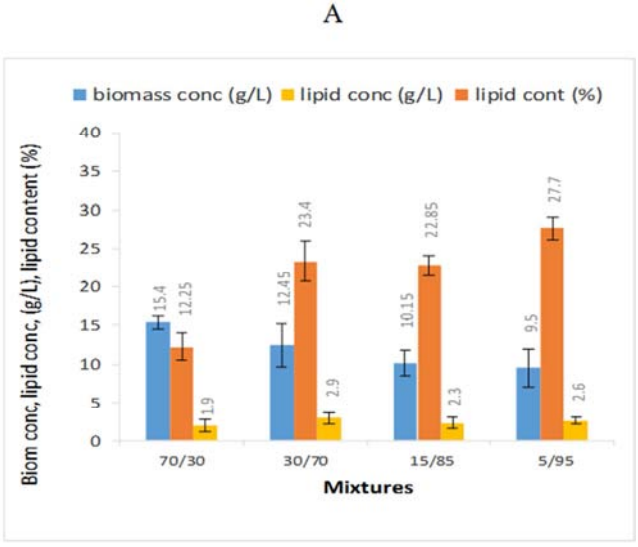
Strain	Biomass conc (g/L)	μ (h^{-1})	Yield $_{X/DQO}$	COD removal (%)	Lipid Content (%)
L/3-74-22	20,9 ± 5,4	0,25 ± 0,06	0,39 ± 0,09	59,2 ± 10,7	13,6 ± 1,40
L/24-26-1	12,1 ± 2,5	0,15 ± 0,05	0,32 ± 0,08	47,5 ± 17,7	20,3 ± 0,15

3.4. Evaluation in Mixtures Slops/Glycerol with Different COD Ratios

In order to know the influence of the composition of the vinasse/glycerol mixture and considering the availability of vinasse and glycerol, the evaluation of vinasse/glycerol COD ratios of 70/30; 30/70; 15/85 and 5/95 was carried at lab scale (by triplicate) using *C. tropicalis* and *R. mucilaginosa* strains. The initial COD was adjusted to 60 000 mg/L and 1 g/L of ammonium phosphate was supplemented to all media. Duplicated samples were analysed for dry mass, chemical oxygen demand and percentages of lipids. Figure 4 (A and B) shows a resume of the obtained results.

Unlike the behavior of these strains in glycerol, in these mixtures *C. tropicalis* cells reached higher concentrations relative to *R. mucilaginousa*. The maximum biomass concentration (15,4 and 10,7 g/L) and the minimum lipid content (12,2 and 18,5%) of both strains were achieved in presence of 70/30 vinasse/glycerol mixture due to a higher nitrogen concentration in this mixture. Nevertheless, there was not significative variation between lipid concentrations of each strain in the different mixtures. Considering that mixture with 5:95 COD ratio has the higher C/N molar ratio, it is expected that the maximum lipid content of both strains was reached in this mixture (27,7 and 36,8%, respectively) while the maximum lipid concentration was reached in mixture with 30/70 COD ratio (Figure 2).

The higher values of COD removal were attained in mixtures with higher vinasse proportion in both strains (Table 3). These results responded to the fact that there are different compounds in vinasse as glucose, ethanol, fructose and arabinose among others that could be more easily assimilated by these yeasts instead glycerol due to the different metabolic pathways involved in their assimilation [38].



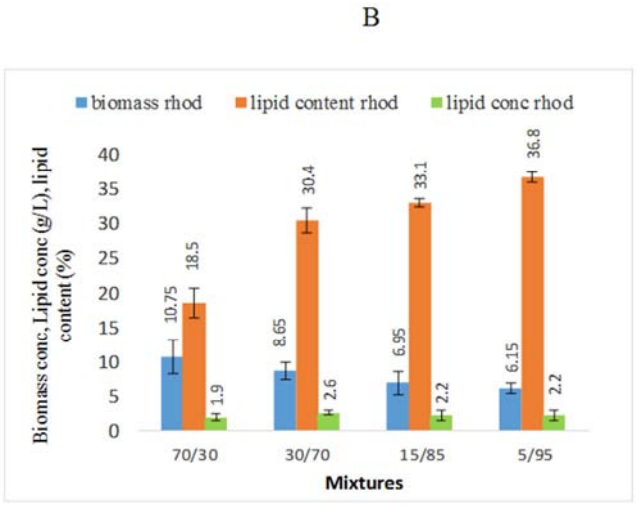


Figure 2. *Biomass concentration, lipid concentration and lipid content comparison between different vinasse/glycerol mixture compositions. A: C. tropicalis, B: R. mucilaginousa.*

According with results showed in Table 3, *C. tropicalis* strain achieved higher COD removal respect to *R. mucilaginousa* during these assays, although the API results showed fewer assimilated substrates by *C. tropicalis* strain. These results is expected considering that *C. tropicalis* reached higher cell concentrations due to probably differences in the anabolic and catabolic process during cultivation of each specie yeasts. The presence of inhibitory compounds from vinasse as acetic acid, furfural, hydroxymethyl furfural,vanillin, and syringealdehyde could have toxic effects on microbial cell growth, metabolism, as well as on lipid accumulation, presenting challenge in biological conversion of biomass.[39-42]. It is demonstrated that furfural and its derivatives imposed major inhibition on cell growth and lipid production of *R. toruloides* Y4.

Table 4. *Effect of vinasse/glycerol COD ratio over growth, COD remotion and lipid concentration of L/3-74-22 (C. tropicalis) and L/24-26-1(R. mucilaginouse) strains at 48 h.*

vin/glyc COD ratio	Lipid conc (g/L)		COD removal (%)	
	L/3-74-22	L/24-26-1	L/3-74-22	L/24-26-1
70/30	1,9 ± 0,8	1,9 ± 0,5	77,5 ± 5,4	52,3 ± 3,2
30/70	2,9 ± 0,7	2,6 ± 0,3	76,7 ± 3,8	49,5 ± 1,4
15/85	2,3 ± 0,7	2,2 ± 0,7	60,0 ± 8,2	45,8 ± 2,7
5/95	2,6 ± 0,5	2,2± 0,8	54,6 ± 3,4	40,1 ± 3,6

3.5. Analysis of Fatty Acid Composition

Table 5 summarizes the fatty acid composition of oils obtained from *C. tropicalis* L/3-74-22 and *R. mucilaginouse* L/24-26-1 in vinasse/glycerol mixtures with 70/30 and 5/95 COD ratios in order to compare them with vegetable oils percentage range and to evaluate the influence of the mixture composition on it. The results indicated that fatty acids composition of lipids extracted from both strains comprising the range established of ASTM D6751 standard for vegetable oils used for biodiesel production.

Most of the lipids from both strain correspond to oleic (18:1) and linoleic (18:2) acids followed by palmitic (16:0 and stearic acid (18:0) suggesting that these mixtures vinasse/glycerol could be used as appropriated medium for biodiesel production from these microbial oils. This profile is similar to that of *Rhodosporidium toruloides* Y2 cultured in bioethanol wastewater and of *Jatropha curcas L.*, *Cryptococcus curvatus* and *R. toruloides* Y4 oils [43]

Oleaginous strains can accumulate different type of lipids because according to the medium composition they can substitute their fatty acids in triglyceride and change their lipid composition [44].

Table 5. *Fatty acids composition (%) of lipids extracted from C. tropicalis and R. mucilaginousa strains cultured in 70/30 and 5/95 vinasse/glycerol mixtures compared with vegetable oils used for biodiesel production.*

C16 – C18 fatty acids (%)	*C. tropicalis*		*R. mucilaginousa*		Vegetable oils for biodiesel
	70/30	5/95	70/30	5/95	
Palmitic acid (C16:0)	21,5	8,57	12,77	12,6	3,5 – 42,6
Palmitoleic acid (C16:1)	5,19	nd	0,62	0,56	0,1 – 0,7
Stearic acid (C18:0)	3,6	7,45	7,58	5,18	0,9 – 24,5
Oleic acid(C18:1)	35,12	38,9	36,05	49,37	13,3 – 54,1
Linoleic acid (C18:2)	22,9	43,43	41,04	28,45	10,0 – 77,0
Linolenic acid (C18:3)	5,18	nd	nd	nd	0,2 – 6,3
Total (%)	93,49	98,35	98,4	95,61	

nd: not detectable

4. Conclusions

Evaluation of *C. tropicalis* and *R. mucilaginousa* strains in vinasse/glycerol mixtures demonstrated that it is possible to develop a fermentation strategy where different composition of vinasse/glycerol mixtures could be used for microbial oil production reaching COD removal around a 50% in batch operation. Future research efforts should be done in order to further increase the lipid production focused on the optimization of media and fermentation conditions to increase lipid production and COD removal, evaluation of fed batch and continuous fermentation and developing a strategy to pick up strains with high potential to assimilate inhibitors present in vinasse and raw glycerol.

Abbreviations

TAG: Triacylglycerol

SCP: Single Cell Protein

API: Analytical Profile Index

YPG: yeast extract, 10 g/L; peptone, 20 g/L and glucose 20 g/L

COD: Chemical oxygen demand

GC: Gas chromatography

$Y_{X/DQO}$: Biomass coefficient, grams of biomass/grams of chemical oxygen demand

C/N: Carbon/Nitrogen Molar ratio

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