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ARTICLE

Fatty acid methyl esters produced by oleaginous yeast *Yarrowia lipolytica* QU21: an alternative for vegetable oils

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ABSTRACT: (Fatty acid methyl esters produced by oleaginous yeast *Yarrowia lipolytica* QU21: an alternative for vegetable oils). Fatty acid methyl esters produced by oleaginous yeast *Yarrowia lipolytica* QU21: an alternative for vegetable oils. An alternative for vegetable oil is microbial oil. There is, nowadays, an increasing interest in microbial lipidic compounds, called *single cell oils* (SCO), due to their several potential biotechnological applications, such as biodiesel production, food ingredients and antimicrobial activity, among others. Oleaginous yeasts are able to accumulate lipids up to 20% of their cellular dry weight, and some species can accumulate up to 70% of lipids. We have screened 86 yeast strains isolated from artisanal cheese using Nile red stain for the detection of intracellular lipid droplets by fluorescence microscopy, and 27% of them were promising for the production of microbial oil. *Yarrowia lipolytica* QU21 was selected for comparison of five different methods of cell wall disruption for lipid extraction at a laboratory scale (dry biomass maceration, lysis with vortex and glass beads, ultrasonic bath and glass beads, maceration using liquid nitrogen, and liquid nitrogen followed by sonication). The method which showed the highest oil yield value (26.5%) was liquid nitrogen with sonication. Oil yield was highly influenced by the method used for cell wall lysis. Fatty acid methyl esters (FAME) composition of strain QU21 after GC analysis was myristic acid (C14:0), myristoleic acid (C14:1), palmitic acid (C16:0), palmitoleic acid (C16:1), heptadecanoic acid (C17:1), stearic acid (C18:0) and oleic acid (C18:1).
Key words: cell disruption, lipid extraction, microbial oil, biodiesel.

RESUMO: (Metil ésteres de ácidos graxos produzidos pela levedura oleaginosa *Yarrowia lipolytica* QU21: uma alternativa para óleos vegetais). Uma alternativa para óleos vegetais é o óleo microbiano. Atualmente, existe um aumento no interesse em compostos lipídicos de origem microbiana, chamados *single cell oils* (SCO), devido ao seu grande potencial de aplicação biotecnológica, como a produção de biodiesel, suplementos alimentares, atividade microbiana, entre outros. Leveduras oleaginosas são capazes de acumular mais de 20% do seu peso seco em lipídios e algumas espécies conseguem acumular mais de 70% de lipídios. Foram analisadas 86 linhagens de leveduras isoladas de queijo artesanal, utilizando o corante vermelho de Nilo para a detecção de gotas lipídicas intracelulares em microscópio de fluorescência. Dessas, 27% se mostraram promissoras para a produção de óleo microbiano. *Yarrowia lipolytica* QU21 foi selecionada para comparar cinco métodos de rompimento da parede celular para a extração de lipídios em escala laboratorial (maceração da biomassa seca, lise com pérolas de vidro em agitador vórtex, lise com pérolas de vidro em banho ultrasônico, maceração utilizando nitrogênio líquido e maceração utilizando nitrogênio líquido seguido por sonicador). O método que apresentou maior rendimento lipídico (26,5%) foi maceração utilizando nitrogênio líquido seguido por sonicador. O rendimento lipídico é amplamente influenciado pelo método utilizado na etapa de lise da parede celular. A composição de metil ésteres de ácidos graxos (FAME) da linhagem QU21 utilizando cromatografia gasosa (GC) foi ácido mirístico (C14:0), ácido miristoleico (C14:1), ácido palmítico (C16:0), ácido palmitoleico (C16:1), ácido heptadecanóico (C17:1), ácido esteárico (C18:0) e ácido oleico (C18:1).
Palavras-chave: rompimento celular, extração de lipídios, óleo microbiano, biodiesel.

Abbreviations: DB, dry biomass maceration; VGB, vortex and glass beads; USGB, ultrasonic bath and glass beads; LN, liquid Nitrogen; LNS, liquid nitrogen with sonication.

INTRODUCTION

Oleaginous microorganisms, such as yeasts, fungi, and microalgae, can accumulate high amounts of neutral storage lipids under appropriate cultivation conditions. There is, nowadays, an increasing interest in microbial lipidic compounds, called single cell oils (SCO), due to their several potential biotechnological applications, such as biodiesel production, food ingredients, antimicrobial activity, and so on (Papanikolaou *et al.* 2004, Li *et al.* 2008, Beopoulos *et al.* 2009, Amaretti *et al.* 2010). Yeasts are a promising source of microbial oil, since they can accumulate up to 70% of their dry weight in lipids (Angerbauer *et al.* 2008), possess a GRAS (generally regarded as safe) status, and are easy to cultivate.

The development of reliable methods for screening oleaginous yeasts is necessary for time optimization and cost reduction in microbial oil production, as well as methods for accurate quantification and determination of the composition of the accumulated lipids. Greenspan *et al.* (1985) examined a variety of dye agents for the observation of lipid droplets, and found that Nile

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red is an excellent stain for the detection of intracellular lipid droplets by fluorescence microscopy and flow citometry. Many well-known extraction methods have been applied to food lipids, such as Soxhlet, Bligh & Dyer (1959) and Folch *et al.* (1957) processes, but extraction of lipids accumulated inside yeast cells needs, as a preliminary step, the disruption of the cell wall. It is known that yeast cell wall is hard to break, and the solvents used for lipid extraction are not sufficient to disrupt the cell wall by their own.

The purpose of the present investigation was to optimize a reliable protocol for screening of oleaginous yeasts and lipid extraction in laboratory scale, and evaluate Brazilian artisanal cheese as a source of oleaginous yeasts. Yeast screening was done using Nile red dye to observe the lipid droplets inside the cell. One oleaginous strain was chosen for the comparison of different methods for cell wall disruption. Lipid extraction was then performed according to Folch *et al.* (1957), and the lipid yield was analyzed in order to evaluate the efficacy of the different methods. Finally, the lipid profile was analyzed by gas chromatograph.

MATERIAL AND METHODS

Yeast screening with Nile red

Microorganism and culture conditions

Eighty six yeast strains, previously isolated from Brazilian artisanal cheeses (Landell *et al.* 2006), maintained in GYP agar (0.5% yeast extract, 1% peptone, 2% glucose, 2% agar) at 4 °C, were inoculated in conical flasks containing 25mL of a culture medium prepared with 10% glucose, 0.1% ammonium sulfate, 0.1% monopotassium phosphate, and 0.05% magnesium chloride hexahydrate (C:N ratio 100:1), and incubated at 150 rpm and room temperature (22-25 °C) during 96 hours.

Yeast molecular identification

Strain QU21 was grown aerobically in GYP broth (2% glucose, 1% peptone, 0.5% yeast extract) at 28 ºC. Total genomic DNA was extracted and purified from 5mL cultures as described by Osorio-Cadavid *et al.* (2009). Sequencing of the D1/D2 domain of the large subunit (26S) ribosomal DNA was performed according to Kurtzman & Robnett (1998) using the primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3'). The amplification conditions were: initial denaturation at 94 ºC for 5 min, 30 cycles of denaturation at 94 ºC for 1 min, annealing at 55 ºC for 30 s, extension at 72 ºC for 1 min, and final extension at 72ºC for 10 min. The PCR product was purified by the polyethylene glycol precipitation method (Lis 1980), and sequenced at the Biotechnology Center of Rio Grande do Sul Federal University (Cbiot/UFRGS), Brazil. The sequence was assembled and compared with sequences reported in GenBank using the basic local alignment search tool (BLAST) algorithm.

Nile red solution

Nile red solution was prepared by dissolving 0.1 mg of Nile red in 1 mL of acetone (Greenspan *et al.* 1985, Kimura *et al.* 2004). Due to the heterocyclic nature of Nile red, it was handled as a carcinogen, though there is no evidence proving it. As Nile red is sensitive to light (Fowler & Greespan 1985), it was always handled with reduced light conditions, and stored at 4 °C.

Procedure

The samples were prepared as described below. Aliquots of 1 mL of each inoculum were centrifuged at 2000 rpm during five minutes. The supernatant was removed and the cells were resuspended with 1 mL of PBS buffer 10 mM (pH 7.4). The centrifugation and resuspension steps were repeated twice, and a smear was prepared spreading aliquots of 10 µL of the final cell suspension in a glass slide, which was air dried to fix the cells. 10 µL of Nile red solution were added to the smear and kept at room temperature for five minutes. The excess of Nile red was removed with PBS buffer 10 mM (pH 7.4), the glass slide was covered with a coverslip, and analyzed in a fluorescence microscope using lenses with wavelengths ranging between 450-500 nm. Lipids were observed as yellow gold droplets, whose size was visually estimated in relation to cell area.

Lipid extraction

Five different methods to disrupt yeast cell wall were tested. Lipid extraction was done according to Folch *et al.* (1957) using a mixture of chloroform and methanol in a proportion of 2:1. The procedure and steps are described below.

Microorganism and culture conditions

The yeast strain QU21 was chosen to analyze the total lipid content. It was maintained in GYP agar, inoculated in conical flasks containing 150mL of the culture medium described above, and incubated at 150rpm and room temperature (22 – 25 °C) for 72 hours.

Chemical Solutions

Pure solvent to upper and lower phase: This solution was prepared by mixing chloroform, methanol and distilled water in 8:4:3 proportions, respectively. A biphasic system is obtained and the two phases shall be collected and stored at amber glass bottles separately. The upper phase shall be denominated *pure solvent to upper phase* and the lower phase shall be denominated *pure solvent to lower phase*. These solutions shall be stored at room temperature.

Methods for yeast cell wall disruption

Dry biomass (DB): After 72 hours, the inoculum was filtered in a Kitassato flask with a membrane of 0.45 µm

pore diameter. The membrane containing the biomass was incubated at 40 ºC during two hours for drying. Then, dry biomass was transferred into a mortar and macerated with 20 mL chloroform and methanol solution with a pistil during 30 minutes.

Vortex and glass beads (VGB): After 72 hours, the inoculum was transferred into falcon tubes (15mL) and centrifuged at 5000 x g in a refrigerated centrifuge for 20 minutes. The supernatant was discarded, and 1 mL of chloroform and methanol solution, and a volume of 0.2 g of glass beads (diameter 200 μm) were added to the biomass in the falcon tubes. The mixture was vigorously vortexed during 15 minutes. Then, biomass and solvent were transferred into a mortar (at this step, it was necessary to wash the falcon tubes several times with the chloroform and methanol solution to remove total biomass without transferring the glass beads, which pelleted at the bottom of the tube), and macerated with 20mL chloroform and methanol solution with a pistil during 30 minutes.

Ultrasonic bath and glass beads (USGB): After 72 hours, the inoculum was transferred into falcon tubes (15 mL), and centrifuged at 5000 x g in a refrigerated centrifuge for 20 minutes. The supernatant was discarded, and 1 mL of chloroform and methanol solution and a volume of 0.3 g of glass beads (diameter 200 μm) were added to the biomass in the falcon tubes. The tubes were vortexed and transferred to Ultrasonic bath (Model USC700) with ice for two hours. Then, biomass and solvents were transferred into a mortar (at this step it was necessary to wash the falcon tubes several times with the chloroform and methanol solution to remove total biomass without transferring the glass beads, which pelleted at the bottom of the tube). The maceration step was not performed due to the amount of solvent needed to remove the glass beads, but the biomass remained in contact with the chloroform and methanol solution during the same 30 minutes used in the other treatments.

Liquid Nitrogen (LN): After 72 hours, the inoculum was filtered in a Millipore filtration system with a membrane of 0.45 μm pore diameter. The biomass retained in the membrane was transferred into a mortar. Liquid nitrogen was added to biomass, which was macerated with a pistil to break the cells. Then, 20 mL of chloroform and methanol solution was added, and the biomass with the solvents was macerated during 30 minutes.

Liquid Nitrogen with Sonication (LNS): After 72 hours, the inoculum was filtered in a Millipore filtration system with a membrane of 0.45 μm pore diameter. The biomass retained in the membrane was transferred into a mortar. Liquid nitrogen was added to biomass, which was macerated with a pistil to break the cells. After this step, the biomass was transferred into a falcon tube, distilled water was added to the biomass and cells were sonicated ten times for 30 seconds (Vibra Cell Sonicator, Model VC601, Sonics and Materials Inc.). Falcon tubes remained on ice during sonication. Sonicated cells remained in contact with 20 mL chloroform and methanol solution during 30 minutes.

Procedure

After the step of yeast cell wall disruption, the technique of lipid extraction was performed according to Folch *et al.* (1957). The mixture of biomass and chloroform/methanol solution was filtered through a filter paper MN-615 (Macherey-Nagel), and the dry weight of the biomass was determined. The filtered (crude) extract was collected in a graduate cylinder, its volume was determined and it was added 0.2 times its volume of distilled water, vigorously mixed in a flat bottom flask and transferred back into the graduate cylinder. The graduate cylinder with the sample was kept at rest until complete separation of the biphasic system. According to Folch *et al.* (1957), this step can be performed centrifuging the sample, but we were not able to successfully separate the phases by centrifugation (data not shown). The upper phase was removed with a pipette without disturbing the interface. The graduate cylinder walls were carefully washed several times with pure solvent to upper phase avoiding interface disturbance, and the excess of solvent was removed with a pipette. The upper phase was discarded, and 10 mL of methanol was added to the lower phase, which contains the extracted lipids, mixed in order to homogenize the phases, and transferred into a rotary evaporator. The graduate cylinder walls were washed several times with pure solvent to lower phase, and the solvents were eliminated with a flash evaporator at these conditions: 40 °C and 120 rpm. After evaporation of solvents, lipids were removed from the evaporating flask with a pipette and transferred into a falcon tube (50 mL). The residual sample was removed from the rotary evaporator with the help of chloroform and methanol solution until complete removal. The falcon tube was incubated at 37-40 ºC for complete evaporation of the solvent, and weighted (final weight).

In the LN and LNS procedures for cell wall disruption, the crude extract was collected in a 50 mL falcon tube. The procedure for lipid extraction was as described above, except that the tubes were incubated at 37-40 ºC, without using rotary evaporator.

Quantification of total lipids was estimated according to the difference between the final weight and the weight of the falcon tubes and filter paper used, allowing the estimation of the proportion of accumulated lipids in relation to dry biomass (oil yield).

Statistical analysis

All values shown for the yeast cell wall disruption methods are the average of two separate experiments. The mean values are reported and compared by analysis of variance (One-way ANOVA) ($P \leq < 0.05$). Difference among the observed averages of the treatments were tested using the Tukey test ($P < \leq 0.05$).

Lipid profile

In order to obtain the lipid profile (qualitative analysis), the lipids extracted from strain QU21 were methylated according to Hartman & Lago (1973) to obtain fatty acid methyl esters (FAME). FAME were analyzed in a gas chromatograph equipped with FID (flame ionization detection) and the capillary column DB-Wax (60 m x 0.25 mm x 0.2 µm). Temperatures from the detector and injector were 250 ºC. The following temperature program was used for the separation of FAME: 100 ºC for 1 minute, with a gradual increase of 2 ºC per minute until the final temperature of 240 ºC, where it was held for 10 minutes. The carrier gas was H_2 with constant flow (20 mL/min). Volume of injection was 1 microliter with split rate of 1:100. The identification of FAME was evaluated using Supelco 37 Component Fame Mix (10 mg/mL – SIGMA) as standard.

RESULTS

Yeast screening with Nile red

All yeasts analyzed (n = 86) presented lipid droplets in different proportions, whose size was visually estimated in relation to cell area, based on the yellow gold fluorescence emitted by neutral lipids after treatment with Nile red dye (Fowler & Greespan 1985). Of these, 32 and 31 yeast strains accumulated lipids up to 30% and between 30 to 50% of the cell area, respectively. However, 23 yeasts strains filled more than 50% of cell area with lipid droplets, and were selected as potentially oleaginous yeasts. Yeast strains representative of each of the three lipid accumulation groups after screening with Nile red dye can be visualized in Figure 1.

Molecular identification of strain QU21

Based on the results obtained in the screening step using Nile red dye, strain QU21 was chosen for lipid extraction, as it belonged to the group in which lipid droplets filled more than 50% of cell area. The 26S rDNA D1/D2 region of this strain showed a 99% sequence identity with *Yarrowia lipolytica*, therefore strain QU21 was identified as belonging to this species.

Table 1. Oil yield obtained using five different methods to disrupt the *Yarrowia lipolytica* QU21 cell wall.

Methods for yeast cell wall disruption	Dry biomass (g)	Total lipid (g)	Oil yield (%)
LNS*	0.34	0.09	26.5 A**
DB	0.28	0.04	14.3 B
LN	0.17	0.02	12.8 B
USGB	0.41	0.03	7.3 C
VGB	0.27	0.01	3.7 C

Abbreviations: LNS, liquid nitrogen with sonication; DB, Dry biomass maceration; LN, liquid nitrogen; USGB, ultrasonic bath and glass beads.VGB, vortex and glass beads.

**Values followed by the same letter do not differ statistically by the ANOVA Test ($P \leq 0.05$).

Lipid extraction comparing different methods for yeast cell wall disruption and lipid profile of strain QU21

We tested five different methods for disruption of yeast cell wall before lipid extraction using the oleaginous strain QU21, selected after screening with Nile Red. USGB and VGB showed the lowest values of oil yield, with 7.3 and 3.7% of the dry biomass composed by oil, while DB and LN showed similar values with 14.3 and 12.8% of oil yield (Table 1). The method showing the highest oil yield value was LNS with 26.5% of oil yield, with significant difference compared with any other method tested here. The lipid profile of strain QU21 can be seen in Table 2.

DISCUSSION

The treatment with Nile Red proved to be efficient for a preliminary large scale screening of oleaginous yeasts. The whole screening process lasted 96 hours for yeast cultivation, and 20 to 30 minutes per sample for Nile Red smear preparation and fluorescence detection. This process would greatly benefit from a reduction in the

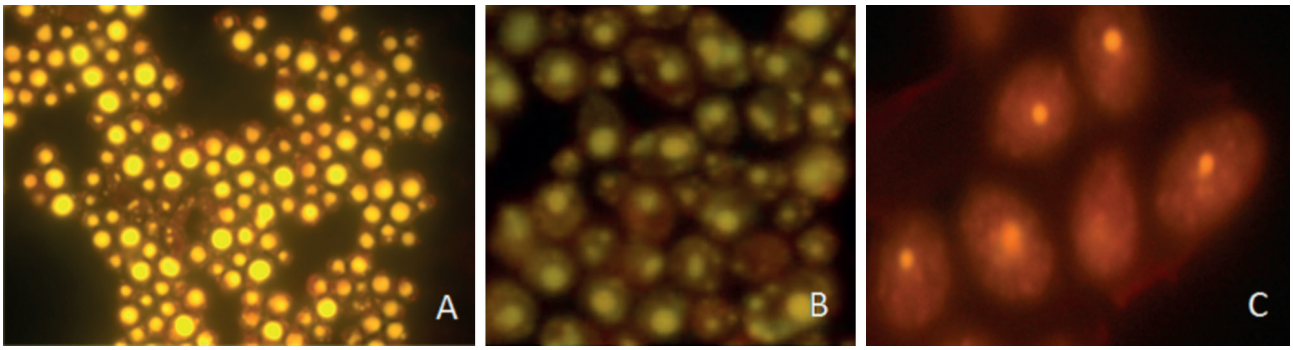

Figure 1. Representative photos of yeast strains classified according to the area covered by lipid droplets (yellow-gold) inside the cell visualized with the oil immersion objective lenses (100x). A. Lipid droplets filling more than 50% of cell area. B. Lipid droplets filling between 30 to 50% of cell area. C. Lipid droplets filling less than 30% of cell area.

Table 2. Long chain fatty acid profile of *Yarrowia lipolytica* QU21 and its comparison with two types of vegetable oils usually applied in biodiesel production.

LCFAs	QU 21 yeast strain	Soybean oil*	Rapeseed oil*
Myristic acid (C14:0)	✓	✓	
Myristoleic acid (C14:1)	✓	✓	
Palmitic acid (C16:0)	✓	✓	✓
Palmitoleic acid (C16:1)	✓	✓	✓
Hexadecanoic acid (16:2)		✓	
Heptadecanoic acid (C17:1)	✓		
Stearic acid (C18:0)	✓	✓	✓
Oleic acid (C18:1)	✓	✓	✓
Linoleic acid (C18:2)		✓	✓
Linolenic acid (C18:3)		✓	✓
Arachidic acid (20:0)		✓	
Eicosenoic acid (C20:1)			✓
Behenic acid (22:0)		✓	
Erucic acid (22:1)		✓	

✓ presence of fatty acid.

* Data from Downey & Craig 1964, Goodrum & Geller 2005, Saka & Kusdiana 2001, Koutb & Morsy 2011.

cultivation time, especially if a high throughput methodology is established. The strain QU21 was chosen for lipid extraction, as it belonged to the group in which lipid droplets filled more than 50% of cell area, as well as it was identified as belonging to *Y. lipolytica* species. *Y. lipolytica* is largely known as an oleaginous yeast (Beopoulos *et al.* 2009, Papanikolaou *et al.* 2011a, Papanikolaou *et al.* 2011b) proving that the screening step with Nile red was efficient.

Lipid extraction from oleaginous yeasts is an extremely important issue. Unfortunately, it is generally accepted that there is no extraction method capable of resulting in 100% oil yield (Jacob 1992, Ageitos *et al.* 2011). In our preliminary experiments, we observed that there was little correspondence between the fluorescence image after staining with Nile Red and the lipid yield obtained after extraction according to Folch *et al.* (1957). We tentatively explained this lack of correspondence by an inefficient cell wall breakage prior to lipid extraction with solvents. According to Jacob (1992), yeasts have several disadvantages for lipid extraction, including the presence of a thick cell wall that renders the yeast cells resistant to many solvents, as well as the possible presence of lipases in their cell extract. The inefficiency in this step could act as an important barrier during the process of intracellular lipid extraction.

Of the five different methods for disruption of yeast cell wall before lipid extraction using the oleaginous strain QU21, LNS yielded the highest oil content (26.5%). Oleaginous yeasts are able to accumulate more than 20% of their cellular dry weight in lipids (Ageitos *et al.* 2011, Koutb *et al.* 2011). Therefore, strain QU21 can be considered an oleaginous yeast. Examples of oleaginous yeasts besides *Y. lipolytica* include the species *Rhodotorula glutinis*, *Rhodosporidium toruloides Lipomyces starkeyi*, and *Cryptococcus curvatus* (Ageitos *et al.* 2011). Some yeast species, like *Lipomyces starkeyi*, can accumulate oil up to 70% of their cellular dry weight (Angerbauer *et al.* 2008).

Lipids produced by yeasts are classified as neutral (triglycerides) and polar (glycolipids and phospholipids), and are mainly composed by saturated and monounsaturated triglycerides. Microbial oil can be an alternative to vegetable oil in some industrial processes, such as biodiesel production, thus a comparative lipid profile between QU21 oil and two types of vegetable oils usually applied in biodiesel production can be seen in Table 2 (Downey *et al.* 1964, Goodrum *et al.* 2005, Clemente & Cahoon 2009, Saka *et al.* 2001). In the conditions analyzed in this work, strain QU21 does not accumulate two types of polyunsaturated fatty acids (Linoleic and Linolenic acid). According to Papanikolaou & Aggelis (2011a), the oil obtained from yeasts is less unsaturated than oil from other oleaginous fungi. Biodiesel constituted by unsaturated methyl esters, especially poly-unsaturated methyl esters, such as methyl linoleate (C18:2) and methyl linolenate (C18:3) is most susceptible to oxidation (Xin *et al.* 2009). Therefore, the oil obtained with yeast strain QU21 is advantageous for biodiesel production, since it is devoid of polyunsaturated fatty acids.

In summary, cheese proved to be a promising source of oleaginous yeasts, and the Nile red dye was an adequate tool for rapid screening of these yeasts. The oil yield obtained after lipid extraction was highly dependent on the method used for disrupting yeast cell wall. In the conditions we used, the most efficient method for laboratory scale use was Liquid Nitrogen with Sonication. The lipid profile from *Yarrowia lipolytica* QU21 shows that this strain should be thoroughly investigated due to its potential industrial application, such as oil source for biodiesel production.

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