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Lipid profile and antimicrobial activity of microbial oils from 16 oleaginous yeasts isolated from artisanal cheese

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ABSTRACT: (Lipid profile and antimicrobial activity of microbial oils from 16 oleaginous yeasts isolated from artisanal cheese). Microbial oil is becoming an alternative to the increasing cost of vegetable oils, and it can be used for many applications, as biodiesel production and food supplementation. In particular, oleaginous yeasts, being unicellular, devoid of endotoxins, and suitable for large-scale fermentation, are particularly attractive for biotechnological approaches. This work aimed to identify, by molecular analyses, sixteen yeast strains as well as analyze the lipid profile and potential antimicrobial activity of the oil produced by them. All strains were identified as *Yarrowia lipolytica*, a promising single-cell-oil producer. No antimicrobial activity was found for the oil analyzed, although the lipid profile showed interesting results. The major fatty acids identified were oleic (18:1n9) and linoleic (18:2n6c) and the minor fatty acids were palmitic (C16:0), palmitoleic (C16:1), heptadecanoic (C17:1), stearic (C18:0) and α -linolenic (C18:3n3). This last omega-3 fatty acid was identified on two strains (QU22 and QU137), enabling the oil produced by them to be used for dietary applications. Moreover, the oil of the other oleaginous yeasts analyzed in this study appears to be suitable for biodiesel production, since their lipid profiles are similar to the vegetable oils, widely used for that end.

Key words: single-cell-oil, fatty acid, *Yarrowia lipolytica*.

RESUMO: (Perfil lipídico e atividade antimicrobiana de óleos microbianos de 16 leveduras oleaginosas isoladas de queijo artesanal). O uso de óleo microbiano está se tornando uma alternativa ao aumento do custo de óleos vegetais, e podendo ser utilizado para várias aplicações, como a produção de biodiesel e a suplementação de alimentos. Em particular, as leveduras oleaginosas, sendo unicelulares, desprovidas de endotoxinas e adequadas para a fermentação em grande escala, são particularmente atrativas para abordagens biotecnológicas. Este trabalho teve como objetivo identificar, por meio de técnicas moleculares, dezesseis cepas de leveduras e analisar o perfil lipídico e a potencial atividade antimicrobiana do óleo produzido por elas. Todas as cepas foram identificadas como *Yarrowia lipolytica*, uma promissora produtora de óleo microbiano. Nenhuma atividade antimicrobiana foi encontrada para o óleo analisado, porém, o perfil lipídico dos óleos apresentaram resultados interessantes. Os principais ácidos graxos identificados foram oléico (18:1n9) e linoléico (18:2n6c) e os ácidos graxos em menor quantidade foram o palmítico (C16:0), palmitoléico (C16:1), heptadecanoico (C17:1), esteárico (C18:0) e α -linolênico (C18:3n3). Este último ácido graxo ω -3 foi identificado em duas cepas (QU22 e QU137), o que pode permitir que o óleo produzido por estas cepas seja utilizado em aplicações alimentares. Além disso, o óleo das demais leveduras oleaginosas analisadas neste estudo parece ser adequado para a produção de biodiesel, uma vez que o seu perfil lipídico é semelhante ao dos óleos vegetais, largamente utilizados para este fim.

Palavras-chave: óleo microbiano, ácidos graxos, *Yarrowia lipolytica*.

INTRODUCTION

Due to the increasing cost of vegetable oils, many researchers have been developed to evaluate other oil sources. Possible alternatives are the oleaginous microorganisms, such as bacteria, microalgae, yeasts and other fungi. The oil obtained from these microorganisms can be used for many applications, as biodiesel production and dietary supplementation (Ratledge 2004, Li *et al.* 2008, Huang *et al.* 2013, Poli *et al.* 2014). A microorganism is considered oleaginous when it accumulates more than 20 % of the total cellular dry weight of microbial oil. Under a nitrogen limitation condition this value can increase up to 70 % of their biomass or even more (Christophe *et al.* 2012, Koutinas *et al.* 2014).

The oleaginous yeasts, which in most cases are categorized as GRAS (Generally Regarded As Safe) microorganisms, and have fast growth rates, are considered as potential and interesting candidates for the single cell oil production (Papanikolaou & Aggelis 2011b). Although there are more than 600 described yeast species, few are considered oleaginous. Typical oleaginous yeasts genera include *Candida*, *Cryptococcus*, *Lipomyces*, *Rhodotorula*, *Trichosporon* and *Yarrowia* (Beopoulos *et al.* 2011, Ageitos *et al.* 2011).

Lipids produced by yeasts are mainly composed by saturated and mono unsaturated triglycerides. The oil obtained from the yeasts may have a significant variation regarding the lipid profile. Besides the differences between the genera and species, some modifications on the lipid profile between strains are identified. Cultivation

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conditions, such as temperature, pH and oxygen can also affect the lipid profile. In general, if glucose is used as carbon source, the predominant fatty acid is oleic acid, followed by palmitic and linoleic acid (Papanikolaou & Aggelis 2011a, Liang & Jiang 2013).

Fatty acids have been shown to possess antibacterial and antifungal activities against many microorganisms (Liu *et al.* 2008, Altieri *et al.* 2009, Skrivanova *et al.* 2005). Their spectrum of action is influenced by the degree of saturation, length of the carbon chain and orientation of the double bonds. Among the monounsaturated fatty acids, myristoleic (C14:1) and palmitoleic acid (C16:1) are often the most potent, while among the saturated fatty acids, capric acid (C10:0) and lauric acid (C12:0) are the most active. Moreover, the polyunsaturated fatty acids, particularly those with 18 and 20 carbons, are potently antimicrobial (Desbois & Smith 2010, Smith *et al.* 2010). The antimicrobial mode of action of the fatty acids is still poorly understood because there are several ways they can attack the cells. The cell membrane is probably the primary target, and the fatty acids act disrupting the electron transport chain and oxidative phosphorylation. They can also act by inhibition of enzyme activity, peroxidation and auto-oxidation of degradation products, impairment of nutrient uptake, generation of toxic metabolites, or direct membrane disruption causing cell lysis (Desbois & Smith 2010).

The aim of this work was to identify, by molecular analyses, sixteen yeast strains, previously isolated from artisanal cheese and selected as oleaginous yeasts, to analyze the lipid profile of the oil produced by these strains, as well as to evaluate the potential antimicrobial activity of this oil.

MATERIALS AND METHODS

Microorganism and culture conditions

Sixteen yeast strains previously isolated from artisanal cheese in Southern Brazil (Landell *et al.* 2006) were pre-grown on GYP agar (2 % glucose, 1 % peptone, 0.5 % yeast extract) for 24 hours. The pre-culture was performed on an experimental culture medium containing: 10 % glucose (Dinâmica, Brazil), 0.1 % (NH₄)₂SO₄ (Cromoline, Brazil), 0.1 % KH₂PO₄ (Vetec, Brazil) and 0.05 % MgCl₂·6H₂O (Nuclear, Brazil). Cells were grown to an optical density at 600 nm of 1.0 in a rotary shaker (IKA, KS 4000) at 150 rpm and 25 °C. Seed culture (1 %) was inoculated into 250 mL flasks containing 75 mL of the experimental culture medium, and cultivation for lipid accumulation was carried out in a rotary shaker (150 rpm) at 25 °C for 72 hours.

Molecular identification

Total genomic DNA was extracted as described by Osorio-Cadavid *et al.* (2009), with some modifications. Strains were grown in GYP agar (2 % glucose, 1 % peptone, 0.5 % yeast extract) at 28 °C. The biomasses were resuspended in 400 µL of lysis buffer (NaCl 0.5 M, EDTA

10 mM, SDS 2 %, Tris-HCl 50 mM; pH 8) and incubated for 60 min at 65 °C. The other steps were performed as described by Osorio-Cadavid *et al.* (2009).

Sequencing of the D1/D2 domain of the large sub-unit (26S) ribosomal DNA was performed according to O'Donnell (1993) using the primers NL-1 and NL-4. The PCR mix contains Taq polymerase (1U) (Invitrogen), 1X Buffer, MgCl₂ (3 mM), primers (0.64 pmol/µL), dNTPs (10 µM) and DNA (1 ng/µL). The amplification conditions were: initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 15 s, 55 °C for 45 s, extension at 72 °C for 90 s, and final extension at 72 °C for 6 min. The sequences were obtained by the automated sequencer ABI-PRISM 3100 Genetic Analyzer (Life Technologies Corp., USA), and the protocols established by the company Ludwig Biotech Brasil (Alvorada, RS, Brazil) were used. The sequences were assembled and compared with sequences reported in GenBank using the basic local alignment search tool (BLAST) algorithm.

Determination of dry biomass

Cells were harvested by centrifugation (5000 rpm, 10 minutes), and washed twice with distilled water. Pellets were stored overnight at -30 °C, and freeze-dried at -47 °C for 24 h. Biomass was expressed as gram of dry biomass per liter of medium (g/L).

Lipids extraction and gas chromatography analysis

Lipids were extracted from biomass using a modified Bligh & Dyer (1959) method. Aliquots of 4 mL of methanol and 4 mL of hexane were added to the tubes containing the dry biomass. The tubes were vortexed and transferred to Ultrasonic bath for 30 minutes. After that, 2 mL of water were added and the hexane was removed. Two additional hexane washes were carried out, one using 4 mL hexane and the other 2 mL of the solvent.

The extracted lipids were converted to their methyl-esters in a two-step reaction with methanolic potassium hydroxide (0.5 M) and methanolic sulphuric acid (1.0 M), both reactions at 80 °C for 60 minutes. Fatty acid methyl esters (FAME) were analyzed in a gas chromatograph (GC 17A, Shimadzu) equipped with FID (flame ionization detection) and the capillary column Supelco SP2340 (60 m x 0.25 mm x 0.2 µm). Temperatures from the detector and injector were 260 °C and 240 °C, respectively. The following temperature program was used for the separation of FAME: 120 °C for 5 minutes, with a gradual increase of 4 °C per minute until the final temperature of 240 °C, where it was held for 5 minutes. The carrier gas was H₂ with constant flow (17 mL/min). Volume of injection was 1 µL with split rate of 1:100. The qualitative and quantitative composition of fatty acids were determined comparing the retention time and the area of the peak samples with fatty acid methyl esters standards (47885-U FAME mix, Supelco, EUA).

Antibacterial activity

To analyze the antibacterial activity the following

organisms were used in the growth inhibition experiments, *Escherichia coli* ATCC 23229, *Salmonella* Typhimurium ATCC 14028, *Listeria monocytogenes* ATCC 1531, *Staphylococcus aureus* ATCC 14458, *Enterococcus faecalis* ATCC 29212 and *Lactobacillus plantarum* ATCC 10012. They were all kept frozen (-20°C) as stock cultures in skimmed milk 5% sucrose (W/V). To prepare working cultures, stock cultures were standardized through two successive 24 h growth cycles in BHI broth, aerobically without agitation. Cells from the standardized cultures were then inoculated in fresh medium and incubated (35 °C without agitation) for 12-18 h to obtain working cultures containing approximately 10⁷ colony forming units (CFU)/mL. *L. plantarum* was inoculated and grown on Lactobacilli MRS Broth (Himedia, Brazil), in an atmosphere enriched with hydrogen and carbon dioxide (Gaspak Anaerobic System; Becton Dickinson, Cockeysville, MD, USA).

Sixteen different yeast oils were resuspended in 100 µl of dimethyl sulfoxide (DMSO) and the amount of oil in each one varied from 227 µg/mL (strain QU137) to 931 µg/mL (strain QU123).

The experiment was performed in 96 well microplates, in a final volume of 100 µL of Mueller Hinton Broth or lactobacilli MRS Broth and 10⁵ UFC/mL of each bacteria were tested individually with 50 µg/mL of each yeast oil. The *L. plantarum* microplate was incubated in an atmosphere enriched with hydrogen and carbon dioxide (Gaspak Anaerobic System; Becton Dickinson, Cockeysville, MD, USA) at 35 °C for 48 h. The other microplates were incubated in aerobic conditions at 35 °C for 48 h. After incubation, 1 % 2,3,5-Triphenyl-tetrazolium chloride solution was added to the wells, in order to verify the presence of viable cells. The absence of viable cells on the wells was considered as an inhibitory effect. The positive controls for growth consisted of Mueller Hinton or Lactobacilli MRS Broth without yeast oil, inoculated with the diluted working cultures. Uninoculated broth containing only yeast oil was used as negative controls.

Antifungal activity

The antifungal activity of yeast oil extracts was evaluated against filamentous fungi and yeasts of medical importance. The technique used for the tests was broth microdilution following the CLSI M38-A2 protocol (CLSI, 2008). To analyze the susceptibility of filamentous fungi *Aspergillus fumigatus* ATCC 13073, *A. niger* ATCC 9028, *Fonsecaea monophora* 69704, *F. pedrosoi* ATCC 46428, *Sporothrix schenckii* ATCC 201679, *S. schenckii* ATCC 201681, *Trichophyton mentagrophytes* 60809 and *T. rubrum* Control Lab®1 were used. For the yeasts, the methodology described in the CLSI M27-A3 protocol (CLSI, 2008) was followed, and the strains *Cryptococcus neoformans* ATCC 32045, *Candida albicans* ATCC 10231, and *C. krusei* ATCC 6258 (quality control) were used. The concentration range of the yeast oil extracts was from 0.5 to 512 µg/mL. The minimum

inhibitory concentration (MIC) was defined as the lowest concentration able to inhibit fungal growth visually. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

Molecular identification

All the strains had 99% sequence identity with the type strain of *Yarrowia lipolytica*, thus being identified as belonging to this species. *Y. lipolytica* is a well-known oleaginous yeast, and is considered a GRAS and “safe to use” microorganism, confirming the potential application of the strains as oil producers (Groenewald *et al.* 2014). Table 1 shows the accession number of the oleaginous yeasts at the Collection of Microorganisms, DNA and Cells of Universidade Federal de Minas Gerais (CM-UFMG), Brazil.

Biomass content

The total biomass produced by the strains ranged from 1.02 g/L (QU137) to 2.39 g/L (QU38) (Table 1). This result was not as high as expected, since *Y. lipolytica* is widely studied as a microbial oil producer, and usually produces high biomass yields on glucose-based media at laboratory scales, as shown by Papanikolaou *et al.* (2009), Tsigie *et al.* (2012) and Tai & Stephanopoulos (2013), who obtained 5.9 g/L, 9.3 g/L and 10.3 g/L of total biomass, respectively. A high biomass and lipid content were not the aim of this study, but our results can be improved by the manipulation of culture conditions, such as the nitrogen source used, shaking speed, pH and temperature.

Lipid profile

The fatty acid profile of the oils obtained from the yeast strains cultivated on a glucose-based medium can be seen in Table 2. It was found that lipid samples contained palmitic (C16:0), palmitoleic (C16:1), heptadecanoic (C17:1), stearic (C18:0), oleic (18:1n9),

Table 1. Culture collection identification (ID) and biomass produced by the 16 yeasts analyzed.

Strain	Culture collection ID*	Biomass (g/L)
QU11	UFMG-CM-Y324	1.49
QU16	UFMG-CM-Y326	1.09
QU21	UFMG-CM-Y327	1.91
QU22	UFMG-CM-Y328	2.13
QU29	UFMG-CM-Y329	1.65
QU31	UFMG-CM-Y330	1.54
QU36	UFMG-CM-Y332	1.90
QU38	UFMG-CM-Y333	2.39
QU46	UFMG-CM-Y334	1.70
QU50	UFMG-CM-Y335	2.00
QU69	UFMG-CM-Y336	1.25
QU77	UFMG-CM-Y337	1.47
QU111	UFMG-CM-Y338	1.11
QU123	UFMG-CM-Y339	2.27
QU125	UFMG-CM-Y340	1.48
QU137	UFMG-CM-Y341	1.02

* Collection of Microorganisms, DNA and Cells of Universidade Federal de Minas Gerais (UFMG-CM).

Table 2. Fatty acid profile of the oil extracted from the 16 yeast strains cultivated on a glucose-based medium for 72 hours¹.

Strain	Palmitic acid (C16:0)	Palmitoleic acid (C16:1)	Heptadecanoic acid (C17:1)	Stearic acid (C18:0)	Oleic acid (C18:1n9t)	Linoleic acid (C18:2n6c)	α -Linolenic acid (C18:3n3)
QU11	4.42	7.43	3.54	2.21	61.13	21.27	0.00
QU16	10.84	0.00	0.00	0.00	44.25	44.91	0.00
QU21	6.32	8.56	4.09	4.09	57.19	21.55	0.00
QU22	7.96	0.00	0.00	0.00	39.77	40.44	11.83
QU29	4.91	7.84	3.43	0.00	55.96	27.85	0.00
QU31	5.09	11.55	0.95	1.52	66.41	14.03	0.00
QU36	5.64	8.49	2.45	2.45	47.55	35.86	0.00
QU38	4.97	9.81	1.63	1.66	66.49	14.82	0.00
QU46	5.58	5.98	3.18	0.33	50.27	33.63	0.00
QU50	6.75	7.96	0.00	0.00	49.35	35.94	0.00
QU69	8.66	11.68	0.00	1.78	49.78	28.11	0.00
QU77	7.23	14.47	1.73	2.05	51.85	22.67	0.00
QU111	4.89	8.02	3.75	1.89	58.81	22.64	0.00
QU123	6.81	9.94	3.94	1.89	47.22	30.20	0.00
QU125	6.50	10.47	3.09	2.22	58.71	19.01	0.00
QU137	10.20	0.00	0.00	0.00	54.47	28.67	6.67

1. Results are percentage of each fatty acid identified.

linoleic (18:2n6c) and α -linolenic (C18:3n3) acids, but some notable differences were found between the strains. All the strains belong to the same species (*Y. lipolytica*), indicating that distribution of the fatty acids was strain-dependent.

The oleic acid (18:1n9) was the major fatty acid, with values varying from 39.77 % (QU22) to 66.49 % (QU38), except for the strains QU16 and QU22, in which linoleic acid (C18:2n6c) was a major component of the oil. The saturated fatty acids identified were palmitic and stearic acid and the total saturated fatty acids ranged from 4.91 % (QU29) to 10.84 % (QU16) (Table 3). These two saturated fatty acids are in fact the most common among oleaginous yeasts (Beopoulos *et al.* 2009).

Total monounsaturated fatty acids (MUFA) ranged from 39.77 % (QU22) to 78.91 % (QU31), with oleic and palmitoleic acids being present in all the strains (Tables 2 and 3). Beopoulos *et al.* (2009) mention a work with *Y. lipolytica* where the total MUFA is 34 % while Papanikolaou *et al.* (2009) found 78.3% of MUFA. Both studies were performed with glucose as carbon source, emphasizing that fatty acids profile is strain-dependent.

Total polyunsaturated fatty acids (PUFA) ranged from 14.03 % (QU31) to 52.27 % (QU22). When glucose was used as carbon source, Kanti *et al.* (2013) found a total PUFA of 12.5% for *Candida orthopsilosis*, while Lin *et al.* (2011) found a total PUFA of only 2.7% for *Lipomyces starkeyi*. Considering studies that used *Y. lipolytica* growing in a glucose-based medium, Papanikolaou *et al.* (2009) found a maximum total PUFA of 20.2 %, while Katre *et al.* (2012) found 82.4 % cultivating *Y. lipolytica* in wheat straw hydrolysate as carbon source. Yu *et al.* (2011) found 20.9% of total PUFA. PUFA composition on oleaginous yeasts is generally represented by the linoleic acid (LA), an omega-6 (ω -6) fatty acid. Few works also identify the presence of α -linolenic acid (ALA), an omega-3 (ω -3) fatty acid, but in a small amount (0.1 to

2.02 %) (Katre *et al.* 2012, Galafassi *et al.* 2012, Chang *et al.* 2013). In the present work the oil from the strains QU22 and QU137 showed the presence of ALA at the concentration of 11.83 % and 6.67 %, respectively. LA and ALA, are essential fatty acids, which the human body cannot produce, therefore, both are entirely derived from the diet and necessary for human health. In addition, LA is the parent omega-6 fatty acid and ALA is the parent omega-3 fatty acid, meaning that they are the fatty acids that our body needs to produce the derivative fatty acids, such as arachidonic acid (AA) and eicosapentaenoic acid (EPA). A ω -6: ω -3 fatty acid ratio of 5:1 or less is desired, as suggested by nutrition experts (WHO/FAO, 1994). However, studies show that this ratio can reach values up to 25:1 in diets based on fast-foods (Kamei *et al.* 2002). This shows the importance of finding alternative sources of ω -3 fatty acids to produce supplemented foods and reach a good ω -6: ω -3 ratio, since this fatty acid is nowadays produced mainly from fish oils (Rubio-Rodríguez *et al.* 2010).

Table 3. Total saturated fatty acids, monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) of the 16 yeast strains analyzed.

Strain	Saturated (%)	MUFA (%)	PUFA (%)
QU11	6.63	72.10	21.27
QU16	10.84	44.25	44.91
QU21	10.41	69.84	21.55
QU22	7.96	39.77	52.27
QU29	4.91	67.23	27.85
QU31	6.61	78.91	14.03
QU36	8.09	58.49	35.86
QU38	6.63	77.93	14.82
QU46	5.91	59.43	33.63
QU50	6.75	57.31	35.94
QU69	10.44	61.46	28.11
QU77	9.28	68.05	22.67
QU111	6.78	70.58	22.64
QU123	8.70	61.10	30.20
QU125	8.72	72.27	19.01
QU137	10.20	54.47	35.34

Besides this potential nutritional application, the oil from some of the investigated oleaginous yeasts are suitable for biodiesel production, considered as one of the most important renewable energy sources due to its economic and environmental benefits (Papanikolaou & Aggelis 2011b). In fact, for an oil to be considered as a suitable feedstock for biodiesel, the composition of fatty acids is an important criterion, with high contents of long chain saturated and/or monounsaturated fatty acids (Katre *et al.* 2012, Poli *et al.* 2013). This potential can be explained by the similar fatty acid composition of the oil obtained from some of the studied yeasts (Tables 2 and 3) to the vegetable oils usually applied as biodiesel feedstocks.

Antimicrobial activity

The yeast oil extracts are mixtures of multiple components, active and non-active compounds. There are no validated criteria for the MIC end points for *in vitro* testing of extracts. However, Aligiannis *et al.* (2001) has proposed a classification for extracts based on MIC results, where strong inhibitors exhibit MIC up to 500 µg/mL. From these extracts there are greater chances of obtaining active molecules for the development of antimicrobial drugs. Under this criterion the yeast oil extracts tested failed to inhibit the bacteria growth, and viable cells of the tested bacteria were observed for all of them. Therefore the minimal inhibitory concentration assay with various yeast oil concentrations was not performed. Also, no fungi growth inhibition was observed for all the oils analyzed.

The absence of antimicrobial activity of the oils might be due to their fatty acids profile, where the major components were oleic acid (C18:1n9t) and linoleic acid (C18:2n6c). It has been reported that Gram negative bacteria were resistant to the inhibitory effects of medium and long chain fatty acids and their derivatives (Russel 1991, Ouattara *et al.* 1997, Sheu & Freese 1997). It has also been reported that some Gram positives (*Staphylococcus aureus* and *Listeria monocytogenes*) can be resistant to long chain fatty acids like oleic, palmitic, stearic and myristic acids (Ouattara *et al.* 1997, Harrison *et al.* 2013). Similarly, Liu *et al.* (2008) analyzed the antifungal activity of oleic acid at high concentrations and no inhibitory effect on the mycelial growth of the fungi analyzed was detected. The absent antimicrobial activity of the oleic acid can be explained by its low unsaturation level, since C18 fatty acids with more than one double bond have their toxic nature increased as the number of unsaturations increases in their chains (Wang & Jonhson 1992).

CONCLUSIONS

Microbial oils have many technological applications. Despite no antifungal or antibacterial activities were found for the oil extracts analyzed, some notable results regarding the oil lipid profile can be highlighted. The strains QU22 and QU137 seem to be more suitable for nutritional applications, as they produce LA and ALA fatty acids, and were

identified as *Y. lipolytica*, a “safe-to-use” microorganism. The oil from the other strains can be effective for industrial use, such as for biodiesel production, since they showed a desirable fatty acid profile for this purpose.

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