An Acad Bras Cienc (2023) 95(4): e20191256 DOI 10.1590/0001-3765202320191256
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

MICROBIOLOGY

Evaluation of cell disruption methods in the oleaginous yeasts *Yarrowia lipolytica* QU21 and *Meyerozyma guilliermondii* BI281A for microbial oil extraction

CARINA A. TIMOTHEO, MARIANA F. FABRICIO, MARCO ANTÔNIO Z. AYUB & PATRICIA VALENTE

Abstract: The interest for oleaginous yeasts has grown significantly in the last three decades, mainly due to their potential use as a renewable source of microbial oil or single cell oils (SCOs). However, the methodologies for cell disruption to obtain the microbial oil are considered critical and determinant for a large-scale production. Therefore, this work aimed to evaluate different methods for cell wall disruption for the lipid extraction of *Yarrowia lipolytica* QU21 and *Meyerozyma guilliermondii* BI281A. The two strains were separately cultivated in 5 L batch fermenters for 120 hours, at 26 ºC and 400 rpm. Three different lipid extraction processes using Turrax homogenizer, Ultrasonicator and Braun homogenizer combined with bead milling were applied in wet, oven-dried, and freeze-dried biomass of both strains. The treatment with the highest percentage of disrupted cells and highest oil yield was the ultrasonication of oven-dried biomass (37-40% lipid content for both strains). The fact that our results point to one best extraction strategy for two different yeast strains, belonging to different species, is a great news towards the development of a unified technique that could be applied at industrial plants.

Key words: cell disruption, lipid extraction, microbial oil, oleaginous yeast.

INTRODUCTION

In recent years, concerns about the increase in oil prices and environmental pollution caused by petroleum-based fuels have led efforts for searching alternatives, such as the use of lipids from microbial sources, to produce biodiesel (Maza et al. 2020, Shi & Zhao 2017, Spagnuolo et al. 2019). However, the reality of using single cell oils (SCOs) for production of bioproducts is far from being achieved (Martínez et al. 2015, Ratledge 2004). Many studies have exploited microbial oil due to the similarity in composition to vegetable oils and, consequently, the possibility of using it for various purposes, such as the use in biodiesel and other biochemicals (Rosa et al. 2015). However, carbon sources, recovery of biomass and extraction of microbial lipids are determinants for the reduction of the operational costs of SCOs-based biorefineries (Probst et al. 2015, Yousuf et al. 2017).

There are many oleaginous microorganisms that are potential sources for SCOs production (Athenaki et al. 2017). Among the most promising, oleaginous yeasts are considered excellent candidates as they are capable of accumulating elevated lipid levels over 20% in their dry biomass weight (Gao et al. 2013, Papanikolaou & Aggelis 2011, Tapia et al. 2012).

Traditionally, yeasts are microorganisms known to be used in fermentation processes and many of them are capable of producing several bioproducts for industrial use simultaneously or after modification of cultivation conditions, being considered true cell factories (Beopoulos et al. 2011, Rosa et al. 2015). Different strains have already been tested as platforms for obtaining oleochemicals and other bioproducts (Zhou et al. 2016). In addition, oleaginous yeasts could be implemented as cell factories to improve the existing biofuels production plants (Kavšček et al. 2015).

Microbial lipids are generally similar to lipids from plant sources like soybean and olive oils, which are mainly composed by neutral lipids such as triacylglycerols (TAG) and steryl esters (SE). SCOs have several advantages over vegetable oils, for example, rapid growth in a short period of time, climate and local independence, and do not require large areas for their cultivation and production (Dey & Maiti 2013, Chemat et al. 2017), being therefore a more efficient and sustainable source of oil.

Although obtaining lipids produced by microorganisms is considered a sustainable alternative, the high cost and the efficiency of the methodologies for cell disruption and oil extraction are considered determinants for industrial large-scale production (Mendoza-López et al. 2016). Generally, the extraction of the microbial oil may be accomplished by various approaches such as mechanical methods, chemical methods or a combination of them. In addition, the oil can be extracted from fresh or dried biomass (Poli et al. 2014). Therefore, this work aimed to evaluate different methodologies of cell lysis for lipid extraction in two yeast strains belonging to different species, in order to select the most efficient extraction method with the best oil yield.

MATERIALS AND METHODS

Microorganisms

The strains used in this study were retrieved from the Laboratory of Microbiology of the Universidade Federal do Rio Grande do Sul (Porto Alegre, Brazil). The strains, *Yarrowia lipolytica* QU21 (Poli et al. 2013, 2014) and *Meyerozyma guilliermondii* BI281A (Ramírez-Castrillón et al. 2017) were selected as previous studies have identified them as oleaginous yeasts.

Culture conditions

Activation of yeast cells was carried out in 250 mL Erlenmeyer flasks containing 50 mL of GYP medium (0.5% yeast extract, 2% glucose, 1% peptone), grown for 24 h at 26°C and 150 rpm. Cell growth was measured using a spectrophotometer using a wavelength of 660 nm (O.D. = 1). A $1x10^6$ cell/mL standardized inoculum was transferred to the pre-inoculum containing medium C (0.5% yeast extract, 0.7% KH_2PO_4, 0.25% Na_2HPO_4, 0.15% $MgSO_4.7H_2O$, 0.015% $CaCl_2$, 0.002% $ZnSO_4.7H_2O$, 0.05% $(NH_4)_2SO_4$, 5% glucose) at pH 6, and the same medium was used in the batch fermentation. The yeast cells were grown in a BIOSLAT B 5 L batch system (B. Braun Biotech International, Germany) equipped with temperature control, pH, agitation and aeration. The cultivation was carried out for 120 h and the operating conditions were 26° C, agitation of 400 rpm, aeration rate of 10 $L.min^{-1}$. The pH was not controlled during the process and to avoid foaming 0.1 $g.L^{-1}$ defoamer (Antifoam 204, Sigma-Aldrich, St. Louis, USA) was added.

Determination of yeast biomass

After fermentation in the bioreactor, the cells of each yeast strain were transferred to 50 mL falcon tubes and centrifuged at 5000 rpm for 10 min. The supernatants were discarded, and the pellet washed twice with distilled water. The cells then received three different pre-treatments prior to

oil extraction. Some cells were kept fresh, where only the supernatant was removed, and the pellet stored at -80°C. Other samples were oven dried at 40°C for 24 h or freeze-dried (Liotop L1001) at -30°C for 24h. The biomass was weighed (grams) using an analytical balance (Shimadzu AY220). All experiments were performed in technical triplicates.

Cell disruption methods

Turrax homogenizer

After 120h of fermentation, samples of both yeasts were transferred into falcon tubes (50 mL) and centrifuged at 5000 rpm for 10 minutes. The supernatant was discarded and the biomass was resuspended in the solvent mixture, chloroform:methanol (2:1, v/v). Cell lysis was performed with the Turrax homogenizer tool (*BASIC ULTRA-TURRAX T18; IKA*), with homogenization cycles for 6 minutes at 4000 rpm and cooling with ice every 2 min to avoid the heating of the sample.

Ultrasonic sonicator

The same procedure as described above was applied for biomass collection. Then, the samples were subjected to ultrasonic assisted extraction performed using the Ultrasonic Sonicator (Qsonica, Sonicator Ultrasonic Processor Q700) for 6 minutes with pulses of 60 seconds, followed by a pause of 60 seconds at 30 kHz. The flasks with the samples were kept on ice throughout the extraction process to avoid heating and consequently sample modification/degradation.

Braun MSK cell homogenizer and bead milling

The supernatant was discarded, and the biomass was resuspended in the solvent mixture (chloroform/methanol 2:1, v/v) and a volume of 0.3 g glass bead milling (diameter 200 mm) was added. The samples of both yeast strains were subjected to cell disruption using the Braun cell homogenizer tool (MSK 953030, B. Braun Biotech International) for 6 minutes with pause every 2 minutes so that the sample vial was refrigerated on ice to avoid heating. After extraction, bead milling was carefully separated from the sample prior to separation by centrifugation.

Lipid extraction

Lipid extraction was done according to Bligh & Dyer (1959) and Folch et al. (1957), with modifications as described in Ramírez-Castrillón et al. (2017). The biomass was suspended in chloroform:methanol (2:1, v/v) and cell disruption occurred using three methods (Turrax homogenizer, Ultrasonic and Braun homogenizer combined with bead milling), explained below. After each cell disruption method, the mixture was shaken for 30 minutes at 150 rpm and an additional 1:1 dilution in chloroform and anhydrous sodium sulphate 1.5% was done. Then, the biomass was separated from the solvents by centrifugation at 5000 rpm for 5 minutes, the upper aqueous layer containing methanol, water and non-lipid compounds was discarded and the lower layer was recovered using Pasteur pipette, filtered on Whatman filter paper containing 1.0 g of anhydrous sodium sulphate and collected in pre-weighed falcon flasks. The bottom phase was collected, and the evaporation of the solvents was carried out in a rotary evaporator (Laborota 4000eco) at 60°C for 24 h. The weight of the extracted lipids was measured using analytical balance. Lipid concentration was calculated as a function of volume of culture medium ($g.L^{-1}$), lipid yield as a function of biomass weight ($g.g^{-1}$), and lipid content was determined as percentage (%) of lipid weight in relation to biomass weight. Each extraction was done in triplicate.

Statistical analysis

All data was analysed through analysis of variance (ANOVA) and the means of treatments were compared by Tukey's test at the 5% level of significance using PAST software package (Hammer et al. 2001).

RESULTS

The biomass concentration was 0.79 $g.L^{-1}$ for *Yarrowia lipolytica* QU21 and 0.9 $g.L^{-1}$ for *Meyerozyma guilliermondii* BI281A in wet biomass. The concentration values of dry and lyophilized biomass were 0.4 to 0.5 $g.L^{-1}$ for both strains (Table I). The highest lipid yield ($g.g^{-1}$) for both strains was obtained with the ultrasonic sonicator extraction from the oven-dried biomass (0.368 $g.g^{-1}$ for BI281A and 0.369 $g.g^{-1}$ for QU21, Table II), while the lowest yield was from the extraction using the Braun homogenizer combined with bead milling using the wet biomass (Table II).

Ultrasonication was the procedure that obtained the best lipid yields, followed by the method using the Turrax homogenizer and the Braun homogenizer with bead milling, which were less efficient for lipid extraction. The ultrasonication treatment in oven-dried biomass proved to the most efficient extraction method (37-40%) for both strains (Table II). Although wet biomass produced low contents compared to oven-dried and freeze-dried biomass, application of the ultrasonication treatment to wet biomass resulted in lipid contents higher than 20%.

Table I. Biomass weight of each yeast strain in the three different biomass treatments.

Strain	Wet biomass ($g.L^{-1}$)	Oven-dried biomass ($g.L^{-1}$)	Freeze-dried biomass ($g.L^{-1}$)
BI281A	0.939	0.536	0.465
QU21	0.795	0.520	0.438

DISCUSSION

Oil-producing microorganisms have been the subject of several studies in recent decades. Microbial oil is interesting mainly for the possibility of substitution of animal and vegetable oils, and can be used for biodiesel production (Ratledge 2002, Rosa et al. 2015). The oil from oleaginous yeasts is particularly attractive for industrial applications because of their high capacity for synthesis and accumulation of intracellular lipids (Beopoulos et al. 2011, Garay et al. 2016).

In the present work, different biomass treatments (wet, oven-dried, freeze-dried) prior to cell lysis were used with the purpose of increasing lipid yields and minimizing costs related to energy demand. Some studies have shown that dry biomass extraction is more efficient compared to wet biomass for lipid recovery as the presence of water influences the efficiency of solvent-based extraction processes, which can reduce the mass transfer and increase formation of emulsion (Dong et al. 2016). However, the biomass drying step prior to extraction is economically costly for large-scale applications (Dong et al. 2016, Yellapu et al. 2016).

Meullemiestre et al. (2016) investigated different forms of lipid extraction in *Yarrowia lipolytica* to also maximize the oil extraction yield. The authors tested extraction techniques like those we used, including ultrasonication, Turrax homogenizer with bead milling and microwaves, to enhance the efficiency of lipid recovery. Additionally, various pre-treatments were applied, such as freeze/thaw, freeze-drying, bead milling and microwaves to facilitate cell wall disruption and release of the microbial oil. The results were similar to ours, where the extraction in freeze-dried biomass using Turrax homogenizer and bead milling represented the

Table II. Lipid concentration ($g.L^{-1}$), yield ($g.g^{-1}$) and contens (%) obtained in strains BI281A and QU21 from wet, oven-dried and freeze-dried biomass.

	BI281A			QU21		
Treatment	**Concentration ($g.L^{-1}$)**	**Yield ($g.g^{-1}$)**	**Content (%)**	**Concentration ($g.L^{-1}$)**	**Yield ($g.g^{-1}$)**	**Content (%)**
UWB	0.197 ± 0.012^{a}	$0.210\pm0.016^{b,c}$	21	0.186 ± 0.031^{a}	0.235 ± 0.048^{b}	23
UOB	0.197 ± 0.014^{a}	0.368 ± 0.032^{a}	37	0.206 ± 0.003^{a}	0.396 ± 0.006^{a}	40
UFB	0.089 ± 0.000^{b}	$0.192\pm0.001^{b,e}$	19	$0.104\pm0.011^{b,c}$	0.237 ± 0.032^{b}	24
TWB	0.127 ± 0.005^{b}	$0.135\pm0.006^{d,e}$	14	0.167 ± 0.009^{a}	$0.210\pm0.014^{b,c}$	21
TOB	0.139 ± 0.025^{b}	0.260 ± 0.058^{b}	26	0.119 ± 0.002^{b}	0.229±0.005b	23
TFB	$0.095\pm0.011^{b,c}$	$0.204\pm0.029^{b,d}$	20	$0.086\pm0.004^{b,d}$	$0.196\pm0.011^{b,d}$	20
BWB	0.093 ± 0.005^{b}	0.099 ± 0.006^{f}	10	$0.076\pm0.004^{c,d}$	0.095 ± 0.006^{e}	10
BOB	0.072 ± 0.005^{c}	$0.135\pm0.011^{d,e}$	14	$0.076\pm0.007_{c,d}$	$0.145\pm0.017^{d,e}$	15
BFB	0.066 ± 0.003^{c}	$0.143\pm0.009^{c,d,e,f}$	14	$0.067\pm0.005^{c,d}$	$0.154\pm0.013^{c,d,e}$	15

UWB=Ultra-sonication in Wet Biomass, UOB=Ultra-sonication in Oven-dried Biomass, UFB=Ultra-sonication in Freeze-dried Biomass, TWB=Turrax in Wet Biomass, TOB=Turrax Oven-dried Biomass, TFB=Turrax Freeze-dried Biomass, BWB=Braun in Wet Biomass, BOB= Braun Oven-dried Biomass, BFB= Braun Freeze-dried Biomass. All values are mean ± standard deviation of triplicates. Means among the same strain within a column that have no common superscript letter are significantly different ($p<0.05$).

highest lipid content (13.56%), followed by the ultra-sonication (8.10%) and microwave (7.13%). The authors also noted that the difference in microwave extraction might be due to a degradation of lipids with the heat conditions employed during the process.

In previous studies by our research group, Poli et al. (2013) observed similar results with the strain *Y. lipolytica* QU21, the same we used, and five different lipid extraction methods. The highest lipid yield was 26.5%, using liquid nitrogen pre-treated biomass and maceration followed by ultrasonication extraction. In the cell disruption processes with liquid nitrogen and maceration, the values corresponded to 14.3% and 12.8%. Poli et al. (2014) increased oil yield from strain QU21 by supplementation of culture medium with glycerol and use of ultrasonication for microbial oil extraction, obtaining 30.1% of maximum lipid content. The higher oil contents we obtained for QU21 (up to 40%) with the ultrasonication may be explained by the use of a culture medium enriched in micronutrients when compared to the medium used by Poli et al. (2013, 2014).

Ramírez-Castrillón et al. (2017) studied the production of lipids by *Meyerozyma guilliermondii* BI281A, the other strain we used, in a culture medium with glycerol as carbon source. Lipid extraction was done using oven dried biomass and the Turrax homogenizer. Lipid contents obtained were among 34.97% and 52.38% of the total biomass weight, while we obtained 26% in the same extraction conditions. The differences in culture medium between Ramirez-Castrillón et al. (2017) and the present work, added to the use of antifoam and air by us can alter the conditions and may explain the higher yield values obtained by Ramirez-Castrillón et al. (2017).

Cultivation conditions such as carbon sources, nitrogen availability and mineral concentrations influence the accumulation of lipids in oleaginous yeasts (Beopoulos et al. 2011, Castanha et al. 2014, Ratledge & Wynn 2002, Signori et al. 2016). Nitrogen deprivation

is an important factor as when the nitrogen is exhausted, the biomass production decreases and cells start to accumulate lipids intracellularly (Ochsenreitheri et al. 2016, Polburee et al. 2015, Qin et al. 2017). Therefore, the lipids obtained from different oleaginous yeast species may be influenced by different culture media and conditions, and should be optimized for each strain.

Based on the current literature, there is no extraction method that is 100% effective in yielding oils derived from microorganisms (Ageitos et al. 2011, Jacob 1992, Ochsenreitheri et al. 2016). Extraction of lipids from oleaginous yeasts is often limited by cell wall resistance, lipid accessibility and mass transfer. The use of alternative pre-treatments prior to lipid extraction (freezing/defrosting, cold drying, bead milling, microwave, etc) may turn the lipid structure more accessible to the solvents, minimizing the greatest limitation step in the process, which is the diffusion of solvent into the raw material (Meullemiestre et al. 2016). In addition, the amount of biomass may interfere with the extraction of lipids, requiring more cycles for cell disruption (Ageitos et al. 2011). Therefore, cultivation and lipid extraction strategies are very important to obtain better yields of microbial oil for future use in biotechnological processes. The fact that our results point to one best extraction strategy (ultrasonication of oven-dried biomass) for two different yeast strains, belonging to different species, with different cell morphologies, and possibly different cell wall compositions, is a great news towards the development of a unified technique that could be applied at industrial plants.

Acknowledgments

The authors would like to thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes) for the scholarship granted to C.A.T., M.A.Z.A. and P.V. also thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the researcher grants.

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How to cite

TIMOTHEO CA, FABRICIO MF, AYUB MAZ & VALENTE P. 2023. Evaluation of cell disruption methods in the oleaginous yeasts *Yarrowia lipolytica* QU21 and *Meyerozyma guilliermondii* BI281A for microbial oil extraction. An Acad Bras Cienc 95: e20191256. DOI 10.1590/0001-3765202320191256.

Manuscript received on October 11, 2019;
accepted for publication on March 9, 2020

CARINA A. TIMOTHEO[1]
https://orcid.org/0000-0003-2147-8006

MARIANA F. FABRICIO[2]
https://orcid.org/0000-0001-8148-9570

MARCO ANTÔNIO Z. AYUB[2]
https://orcid.org/0000-0003-4410-6336

PATRICIA VALENTE[1]
https://orcid.org/0000-0001-5374-0991

[1]Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Departamento de Microbiologia, Imunologia e Parasitologia, Laboratório de Micologia, Rua Sarmento Leite, 500, 90050-170 Porto Alegre, RS, Brazil

[2]Universidade Federal do Rio Grande do Sul, Instituto de Ciência e Tecnologia, Laboratório de Biotecnologia e Engenharia Bioquímica, Av. Bento Gonçalves, 9500, 91501-970 Porto Alegre, RS, Brazil

Correspondence to: **Patricia Valente**
E-mail: patricia.valente@ufrgs.br

Author contributions

P.V and M.A.Z.A. supervised the project. C.A.T and M.F.F. carried out the experiments. C.A.T wrote the manuscript with support from M.F.F., P.V. and M.A.Z.A.