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Proceeding Paper

Is There Any Possibility to Use Ultrasounds, High-Pressure Homogenization or Pulsed Electric Field in Single Cell Oil Release from Oleaginous Yeast Cells? †

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Abstract: Microbial oil (SCO) is called lipids accumulated in the cells of oleaginous microorganisms, including yeast, in amount exceeding 20% of dry mass, which are a valuable source of fatty acids in the human diet. In order to facilitate the extraction of storage lipids from cells, methods of physical and chemical pretreatment of biomass are used to break the barrier of the cell wall and membrane of these microorganisms to the action of organic solvents, which are used during traditional extraction. The aim of the study was to evaluate the effectiveness of unconventional methods of extracting microbial oil from *Yarrowia lipolytica* yeast cells. Pulsed electric field (PEF), cell disintegration by ultrasonic waves and high-pressure homogenization (HPH) were used. The use of unconventional methods turned out to be ineffective in the extraction of intracellular lipids of the yeast compared to methods involving organic solvents such as chloroform, methanol and hexane. Nevertheless, the use of a pulsed electric field with a field strength of 200 J/g or high-pressure homogenization (1100 bar) proved to be effective as pre-treatment techniques of *Y. lipolytica* yeast cells (cell permeabilization) for the high yield extraction of intracellular lipids using the extraction method with organic solvents.

Keywords: HPH; PEF; ultrasounds; *Yarrowia lipolytica*; microbial oil

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1. Introduction

Y. lipolytica yeast belongs to the oleaginous yeasts, which means that they have the ability to produce and store intracellular lipids (microbial oil) in the amount of exceeding 20% dry cell mass [1]. Microbial oil is considered a valuable alternative to vegetable and fish oils. Its extraction is an important issue, because this process is usually not very efficient due to the presence of the cell wall of microorganisms [2].

There are many methods for pretreatment of biological material, including methods of mechanical pretreatment e.g., shear forces (high-speed and high-pressure homogenization, microfluidization) and direct energy transfer to cells (laser, ultrasound and microwave) [3]. Physical methods of disintegrating cell walls include decompression, osmotic shock, microwave, pulsed electric field and freeze-drying [4]. Cell permeabilization or

disruption of cells can also be achieved with the use of various types of chemical compounds [4,5]. Most extracting methods for total microbial lipids involve the extraction with an organic solvent using the Soxhlet, Bligh and Dyer and Folch methods [6]. In the study, an attempt was made to evaluate the impact of the unconventional methods of extracting lipids from *Y. lipolytica* yeast cells. The methods used included: pulsed electric field (PEF), ultrasounds (US) and high-pressure homogenization (HPH).

2. Materials and Methods

2.1. Yeast Strain and Culture Conditions

Y. lipolytica strain KKP 379 from the Collection of Industrial Microorganisms at the Prof. Wacław Dąbrowski Institute of Agricultural and Food Biotechnology in Warsaw (Poland) was used in the study. 24h inoculum culture was provided in YPG medium (yeast extract 1%, peptone 2%, glucose 2%) on a rotary shaker at 28 °C for 24 h. The appropriate cultures were carried out in a BioFlo 3000 laboratory bioreactor at 28 °C for 66 h, without pH adjustment, under conditions of minimum 30% oxygenation of the medium with respect to its initial concentration and agitator speed in the range of 200–600 RPM in mineral medium with waste post-frying oil according to Fabiszewska et al. [7]. The biomass obtained after culture in the bioreactor was centrifuged (10 min, 8000 RPM), washed with 0.9% NaCl solution and frozen for further experiments.

2.2. Lipids Extraction

The dry biomass was grinded, weighed and transferred to a falcon-type tube. For every 1 g of dry biomass, 10 cm^3 of a chloroform:methanol mixture in 2:1 (*v*/*v*) ratio was added, conducting a two-fold extraction of lipids according to the Folch method. The samples were centrifuged (10 min, 8000 RPM). Into a weighed round-bottom flask, the liquid obtained after centrifugation was filtered on a filter paper strainer. The procedure was repeated four times. The solvent was evaporated. For the determination of lipid content in solutions after treating cells with ultrasound, PEF or HPH, the procedure was identical, with the addition of 10 cm^3 of the solvent mixture for every 20 cm^2 of solution. To evaluate the effect of permeabilization on Folch extraction of lipids, yeast biomass was shaken with hexane for 60 min at room temperature, the solvent was evaporated, and the biomass was subjected to Folch extraction.

2.3. Non-Conventional Methods for Yeast Cell Treatment

The unfrozen yeast cells were suspended in saline. The resulting solutions were sonicated using a Hielscher UP400S ultrasonic homogenizer (Germany) for 10 min under the conditions shown in Table 1.

Table 1. Parameters of sonication.

No.	Yeast Content in Solution [% m/v]	Cycle [1] [%]	Amplitude [μm]
1	10	100	105
2	30	100	105
3	10	100	210
4	30	100	210
5	10	50	105
6	30	50	105
7	10	50	210
8	30	50	210

[1] the percentage of the experiment duration that has been used for the sonication.

Yeast cells were also treated with PEF according to the conditions shown in Table 2. For this purpose, the conductivity of the solutions was measured, and then the cells were transferred to the chamber of the Elea GmbH (Germany) pulsed electric field application system.

Table 2. Conditions for the application of a pulsed electric field.

Form of Yeast Cell Biomass	Yeast Content in Saline Solution [% m/v]	Electrical Conductivity of the Solution [mS/cm]	Average Energy at Individual PEF Application [J/g]	Electric Voltage [kV]	No. of Pulses
Lyophilized	4	2.300	420.00	10	600
Raw	4	0.821	200.00	10	52,000

A 25% (m/v) suspension of yeast cells was passed through a Niro Soavi NS 1001 L2 PANDA high-pressure homogenizer (GEA, Italy) under the variable pre-treatment conditions shown in Table 3.

Table 3. Conditions for yeast treatment with high pressure homogenization.

No.	Pressure [bar]	Times Yeast Treated by HPH
1	300	1
2	300	2
3	150	1
4	700	1
5	1100	10

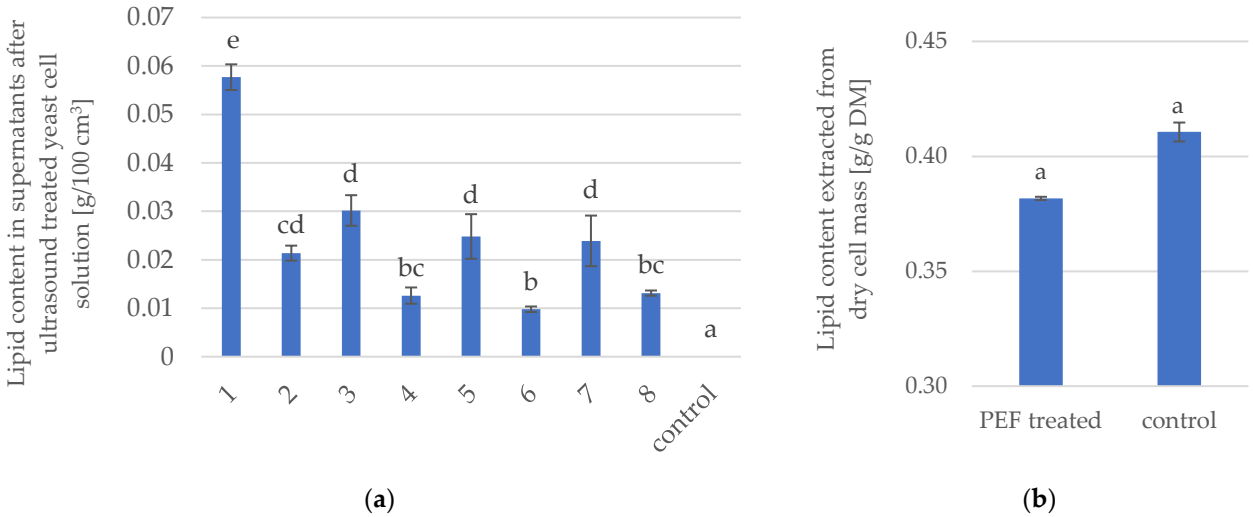
2.4. Statistical Analysis

Statistical analyses were performed of repeated measurements with the one-way ANOVA followed by Tukey's multiple comparison test using STATISTICA 13.3 (Statsoft, Poland). P-values lower than 0.05 were considered to be statistically significant.

3. Results

Figure 1a shows the average content of microbial oil extracted from the solution after ultrasonic disintegration of *Y. lipolytica* yeast KKP 379. For the control sample, not treated with ultrasounds, microbial oil was not detected in yeast solution. It should be noted that sample 1. had the highest percentage of extracted oil, followed by samples 3, 5 and 7. As in the case of extraction from dried biomass, these results indicated that the sonication process proceeded more efficiently when the concentration of cells suspended was lower (10%). It is worth noting that all sonicated samples were characterized by the presence of microbial oil in the solutions. This demonstrated the permeabilization of yeast cell structures took place, intracellular lipids were released into the external environment.

Preliminary experiments using a pulsed electric field (data not shown) concluded that the higher the applied average field energy the higher the extraction efficiency of microbial oil from yeast biomass and probably the better the permeabilization effect of cell structures. It would have been beneficious to use the highest electric field voltage of 17 kV, but at this value during the initial experiment, the electric pulse application system was discharged due to too high solution conductivity. In the next experiment, an electrical voltage of 10 kV and an average energy of about 100 J/g of solution was applied (Figure 1b). Non-significant differences were observed in the content of microbial oil extracted from PEF-treated and untreated freeze-dried biomass.



(**a**) (**b**)

Figure 1. (**a**) Lipid content in supernatants after ultrasound treated yeast cells [g oil/100 cm³]. (**b**) Microbial oil content extracted from dry biomass of PEF-treated *Y. lipolytica* yeast. Homogeneous groups designated on the basis of Tukey's test were identified by letters and different posts. No of treatment refers to individual process parameters according to Table 1 (a) or Table 2 (b). Control are untreated yeast cells.

Figure 2. shows the average content of oil extracted from *Y. lipolytica* yeast biomass after high-pressure homogenization. It should be noted that the average contents of extracted oil from samples 1, 2 and 4 did not show a significant difference from the control sample, while the average content of lipids obtained from sample 3 is even lower compared to the control sample. It was also evaluated the effects of extracting microbial oil into solutions from *Y. lipolytica* yeast cells subjected to high pressure homogenization. A small amount of microbial oil (2%) could be extracted only from the variant with the highest applied pressure (700 bar, data not shown). Thus, it was confirmed that extraction of lipids from HPH-treated biomass at the assumed high pressure homogenization conditions were most likely insufficient to induce perforation of *Y. lipolytica* yeast cell structures and lipid leakage.

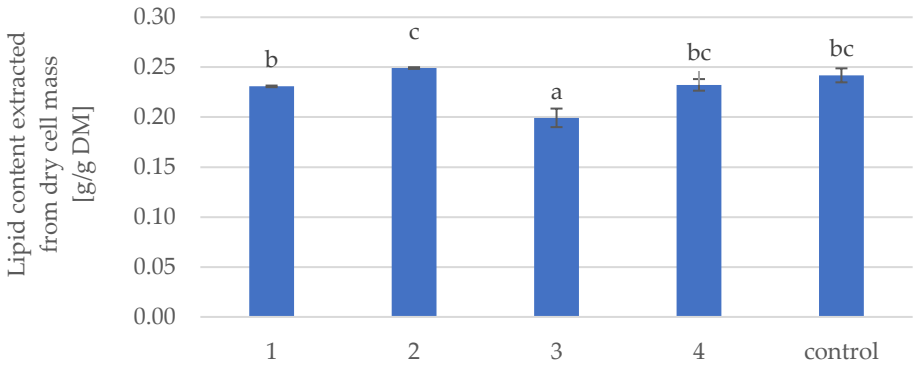


Figure 2. Average microbial oil content extracted by the Folch method from dried *Y. lipolytica* yeast biomass after high-pressure homogenization treatment according to parameters in Table 3. Homogeneous groups designated on the basis of Tukey's test were identified by letters and different posts. Control are untreated yeast cells.

Given the unsatisfactory results of intracellular lipid extraction by the methods described above, their suitability as a form of pretreatment of yeast cells was evaluated. The degree of permeablization of the membrane and cell wall structure assessed by the amount of lipids eluted from cells by extracting with hexane (Table 4). The application of

a pulsed electric field with a field strength of 10 kV (200 J/g) (Table 2, parameters for raw biomass) and high-pressure homogenization (Table 3, 10—times application of 1100 bar pressure) proved to be effective methods for the preparation of *Y. lipolytica* yeast cells (cell permeabilization) for the proper extraction of intracellular lipids by the Folch method (with a mixture of chloroform and methanol solvents).

Table 4. Impact of HPH and PEF on yeast permeabilization. Homogeneous groups designated on the basis of Tukey's test were identified by letters and different posts.

Pretreatment	Content of Extracted Intracellular Lipids [g/100 g DM]		Lipid Washout Efficiency [%]
	Total Lipids (Folch Method)	Lipids Washout (hexane Extraction)	
-	30.1 ± 0.3 (a)	20.2 ± 0.1 (a)	67.1
PEF	36.5 ± 0.1 (b)	26.4 ± 0.1 (b)	72.3
HPH	45.7 ± 0.4 (c)	27.0 ± 0.1 (b)	59.1

4. Discussion

Sonication is a good laboratory method for permeabilizing cell walls and releasing proteins from the cell [8]. This indicates that, the use of ultrasounds causes a high degree of loosening of the yeast cell wall and membrane. In the experiment, a higher degree of intracellular lipids extraction from yeast cells was achieved relative to the control, confirming that the use of ultrasounds has a positive effect on destroying cell structures and improving the efficiency of microbial oil extraction, although the result was far from satisfactory.

Based on the literature, using high-pressure homogenization of 2000 bar, with 15 passes of the sample through the homogenizer, the efficiency of lipid extraction from *Saitozyma podzolica* DSM 27,192 yeast cells after Folch extraction was 37.8% [9]. There are other studies that also used high-pressure homogenization against a 15% solution of *Y. lipolytica* JMY5578 yeast cells. The biomass solution was passed through a homogenizer 20 times at a pressure of 1500 bar. The conditions used resulted in an oil extraction of 83.9% from the dried biomass compared to a control of 19.8% [10]. This may indicate that the high-pressure homogenization conditions used in the present study were too mild to perforate yeast cell structures and consequently increase the efficiency of intracellular oil extraction by the Folch method. Only the use of high-pressure homogenization (10 × 1100 bar) could be considered as a method for preparing yeast biomass for proper intracellular lipid extraction.

A slightly lower, but also significant difference in microbial oil extraction relative to the control was observed in the sample after application of pulsed electric field action. As reported in the literature, other experiments using pulsed electric field also increased the extraction efficiency of microbial oil from dry biomass, but not as significantly as other techniques. In one study, the average content of intracellular fats extracted from dry biomass after PEF application increased from 19.8% to 29.4% [10]. The use of this method probably allowed permeabilization and/or disintegration of yeast cell walls and membranes, resulting in a slight increase in extraction efficiency relative to the control.

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