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Unconventional Extraction Methods of Oleaginous Yeast Cell Pretreatment and Disruption

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Featured Application: Results of this study, especially those obtained for the pulsed electric field, can be applied as an unconventional method for yeast biomass pretreatment in the conventional procedure of microbial lipid extraction with organic solvents.

Abstract: Extraction is one of the most commonly used methods for obtaining and purifying chemical compounds for commercial usage. The aim of this study was to evaluate the effect of unconventional permeabilization and cell disruption methods on the yield of lipid extraction from cells of the oleaginous yeast *Yarrowia lipolytica*. Batch cultures in a medium with molasses and waste post-frying oil were carried out. The biomass was subjected to pulsed electric field (PEF), high-pressure processing (HPP), ultrasounds (US), and several conventional processing techniques with chemical and mechanical agents (glass beads, acetone, Triton and Tween surfactants). The effectiveness of the applied methods, either on cell permeabilization or cell disruption, was investigated by analyzing the oil and total protein extraction yield and oil leaching efficiency, as well as by using microscope images. The PEF and US treatments proved to be effective permeabilization methods as a step of sample pretreatment for extraction. These unconventional physical methods could efficiently increase intracellular lipid extraction yield in solvent applications.

Keywords: microbial oil; cell permeabilization; cell disintegration; pulsed electric field; ultrasounds; *Yarrowia lipolytica*

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1. Introduction

Microbial lipids (SCO: single-cell oil) are produced by oleaginous bacteria, yeast, molds, and algae, so named because of their ability to accumulate lipids above 20% of cell dry weight. The type of lipids extracted from microorganisms depends mainly on the culture conditions and the microorganism species [1]. Plant sources can provide lipids of up to 50% of dry weight, while the bacterial species *Rhodococcus opacus* can provide up to 87% of cellular dry weight, and the wild yeast strain *Yarrowia lipolytica* up to 48% [2,3].

Studies on the use of SCOs for biodiesel production are in progress [4–6]. The addition of SCOs has the potential to enrich and increase the nutritional value of food products as well as replace vegetable lipids (e.g., cocoa butter or palm oil) and certain free fatty acids (FFAs). Microbial lipids have been used as stabilizers, thickeners, water binders, and emulsifiers [7]. SCOs are a rich source of essential FFAs, e.g., docosahexaenoic acid and

arachidonic acid in infant formulas [8]. Microbial lipids are considered to play the role of soothing agents, emollients, and laxatives. SCOs are also ingredients in products used in enteral and parenteral nutrition therapy [9].

Y. lipolytica is a dimorphic oleogenic species, growing as mycelium and yeast-like cells. *Y. lipolytica* has a high ability to utilize hydrophobic substrates, which makes it an interesting species for industrial applications. Relatively little is known about the structure of its cell wall, especially the cell wall proteins. The structure and composition of the yeast cell wall have been studied mainly in *Saccharomyces cerevisiae*. The cell wall is composed mainly of glucans, chitin, and glycoproteins. This structure shows high resistance to shear forces, indicating that its components are tightly linked [10,11].

Extraction of lipids from plant and animal matrixes as well as microbial biomass can be carried out using a number of methods (Figure 1). Soxhlet developed a hexane-based extraction method that is widely used despite the major drawback of using organic solvents or the potential emission of toxic compounds during the procedure [12,13]. The Bligh and Dyer method and Folch method are two of the best-known lipid extraction methods involving the use of a chloroform and methanol mixture [14,15]. These methods are used to extract lipids from many food products such as avocados, eggs, mayonnaise, and fish [14,16].

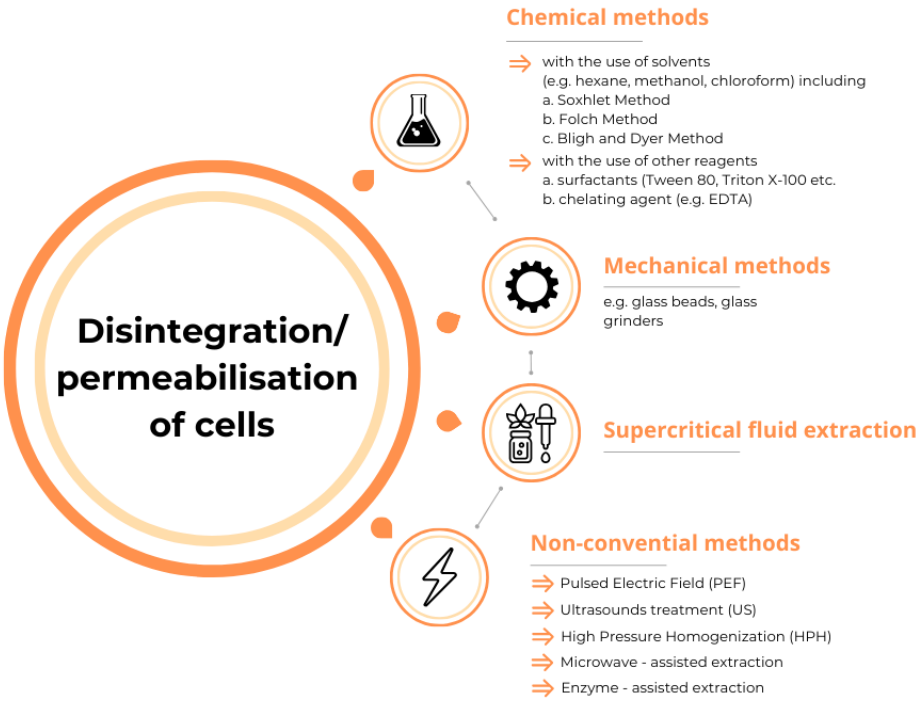


Figure 1. Methods for disintegrating cell walls to extract lipid compounds.

Still, a growing interest in unconventional extraction methods can be observed. Pulsed Electric Field (PEF) is a technology where the delivery of a specific portion of energy creates pores in the cell membrane, which contributes to its permeability and increases the extraction efficiency of valuable components such as dyes, flavors, proteins, lipids, and antioxidants present in the organelles of living cells [17,18]. Microwave-assisted extraction is used to heat solvents in contact with the sample, with better simultaneous recovery of analytes compared to conventional extraction techniques [19,20]. The use of ultrasound (US) also contributes to accelerating the extraction process. The increase in efficiency is mainly due to the mechanical effects of acoustic cavitation, which increases both solvent penetration into and release of intracellular products by disrupting cell walls [21,22]. A method that is highly energy efficient and also results in minimal product degradation is HPP (high-pressure processing). The mechanisms of cell destruction are variously

attributed to fluid shear stress [23]. This method may be practically applied to large-scale processing of microbial biomass, only if the energy requirement is low enough [24].

The aim of the present study was to evaluate the effectiveness of using unconventional methods to extract microbial oil from *Y. lipolytica* yeast cells cultured in media with waste carbon sources (namely post-frying rapeseed oil or molasses). Selected mechanical and chemical methods were studied for comparison purposes. The effects of yeast cell treatment on the increase in cell wall permeability (a permeabilization effect) and on cell structure disruption (a disintegration effect) were investigated. Both phenomena were studied with microscopic and analytical techniques.

2. Materials and Methods

2.1. Microorganism and Culture Conditions

The yeast strain *Y. lipolytica* KKP 379, which was purchased from the Collection of Industrial Microorganisms of the Prof. Wacław Dąbrowski Institute of Agricultural and Food Biotechnology—State Research Institute (Warsaw, Poland), was used for experiments. Inoculum culture was carried out for 24 h, in YPG medium (2% peptone, 2% glucose, 1% yeast extract), on a rotary shaker, at 28 °C. Yeast cultivations in a BioFlo 3000 bioreactor (New Brunswick Scientific, Nürtingen, Germany) were started by adding 1.5 mL of inoculum to the appropriate medium. Cultures were provided at 28 °C, for 66 h, without pH regulation, under conditions of a minimum 30% oxygenation of the substrate with respect to its initial concentration and stirring speed in the range of 300–600 rpm. Biomass yield was determined using the weight method. Batch cultures were provided in media with waste oil—post-frying rapeseed oil (WOM) or molasses (MM)—according to Wierzchowska et al. [25]. Yeast biomass from cultures was mixed in order to use an averaged probe for experiments.

2.2. Experimental Design

2.2.1. Mechanical and Chemical Methods for Lipid Extraction

The biomass was subjected to different pretreatment methods (Figure 2). For the mechanical method, glass beads and 50 mL of water were added, while for the chemical technique, a surfactant was used, and instead of water, 50 mL of 5% solution of Triton X-100 was added. The use of surfactant was aimed at reducing the surface tension of the liquid and cell permeabilization. The contents of flasks were vortexed for one hour on an IKA shaker (Warszawa, Poland) at room temperature. In the next step, flask contents were separated by centrifugation, after which the biomass was dried and divided into two identical parts. One of them was used to perform extraction using the Folch method, and the other was used for oil extraction (leaching) using hexane. For SEM images, additional yeast biomass was subjected to 5% Tween 80 solution and Y-PER Yeast Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA).

2.2.2. Pulsed Electric Field

Effects of PEF on yeast cells were investigated using the Elea PEF system (Elea Vertriebs und Vermarktungsgesellschaft mbHVer, Quakenbrück, Germany), which provides 2 Hz pulses with a frequency with exponential decay (monopolar signal, width 40 ms). The yeast biomass was placed in a cell of the PEF system with water of 220 µS/cm conductivity and a temperature of 21 ± 1 °C. The electrodes were made of stainless steel, and the slot between them was 28 cm. The samples were subjected to specific energies of 1 and 3 kJ/kg at an equal electric field strength of 1.07 kV/cm. The applied average energy for individual PEF application was 100–120 J/g of solution (4% solution of *Y. lipolytica* yeast biomass in distilled water).

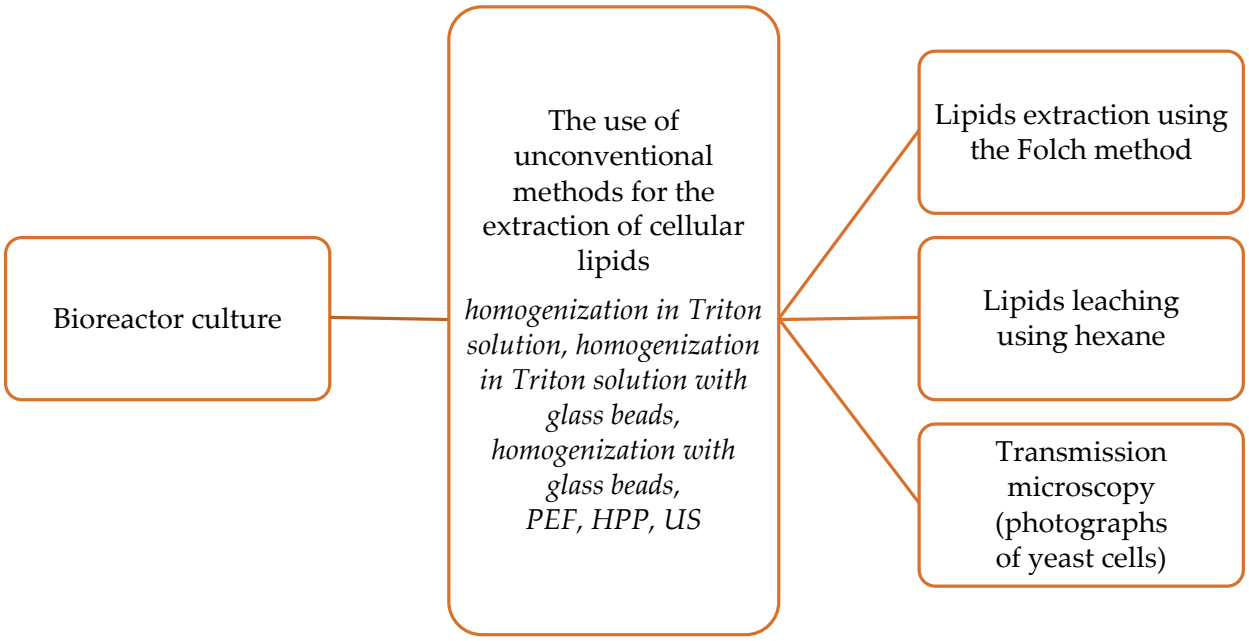


Figure 2. Experimental diagram for yeast biomass pretreatment and oil extraction from previously treated yeast cells.

2.2.3. Ultrasound Treatment

Yeast cells suspended in distilled water were treated with ultrasonic waves using UP400S ultrasonic homogenizer (Hielscher Ultrasonics, Teltow, Germany). The obtained suspension was subjected to US for 45 min at a vibration amplitude of 210 µm, a duty cycle of 80%, with a frequency of 21 kHz, and a generator power of 400 W.

2.2.4. High-Pressure Processing

Samples were pressurized at 400 MPa for 5 min at 5 °C using a using a U5000/120 apparatus (Unipress, Institute of High Pressures of the Polish Academy of Sciences, Poland). The high-pressure treated cells (maximum pressure of 600 MPa, temperature scale from 4 to 60 °C) were filled with water as a pressure transfer medium.

2.3. Determination of Oil Content

2.3.1. Evaluation of Fat Content in Yeast Cells Using the Folch Method

The dry biomass was crushed, weighed, and transferred to a Falcon-type tube. Guiding the extraction of lipids, for every 1 g of dry biomass, 10 mL chloroform:methanol mixtures (in a 2:1 ratio) were added. The samples were subjected to centrifugation (10 min, 8000 rpm) and the supernatant was filtered through filter paper. The procedure was provided 4 times. The solvent was evaporated.

2.3.2. Leaching of Intracellular Oil from Yeast Cells

The dried biomass was crushed, weighed, and transferred to conical flasks. For every 1 g of biomass, 10 mL of hexane was added. The whole mixture was stirred in a reciprocating shaker (150 rpm) for 60 min at room temperature. Then, the mixture was filtered through filter paper and the solvent was evaporated.

2.4. Protein Determination Using the Lowry Method

In order to measure the amount of protein in the solutions after treatment of yeast cells, 1 mL of the supernatant was added to a test tube. For the control sample, 1 mL of deionized water was measured instead of the solution. Then, 5 mL of copper reagent was added, consisting of 2% Na_2CO_3 in 0.1 M NaOH, 1% $CuSO_4$, and 2% potassium sodium tartrate in a volume ratio of 100:1:1. After 10 min., 0.5 mL of Folin–Ciocalteu reagent was

added, and after 30 min, the intensity of the solutions' coloration was measured at 750 nm using a spectrophotometer. A standard curve using bovine serum albumin was used to determine the protein concentration of the solutions.

2.5. Microscopy Techniques

An Olympus Fluoview FV3000 confocal laser microscope (Olympus Corporation, Tokyo, Japan) was used to image the morphology of the yeast *Y. lipolytica*. Nile Red solution (Nile Red, Sigma-Aldrich, St. Louis, MO, USA), which was used to stain lipid bodies, was prepared in ethanol at a concentration of 1 mg/mL. Observations were made at an excitation wavelength of 488 nm using a 63× objective (oil immersion). In this study, scanning electron microscopy techniques were also used according to the methods described by Kapturowska et al. [26]. Cells were fixed in a solution of glutaraldehyde and then in osmium tetroxide. After fixation, the suspension was filtered with a carbon filter and dehydrated in an increasing concentration gradient of ethanol and acetone. The grids on which yeast biomass was applied were dried at the critical point and sputtered with a layer of gold as a conductive layer. The images were taken at the Analytical Center of Warsaw University of Life Sciences using Quanta FEI Electron Optics Scanning Electron Microscope.

2.6. Statistical Analysis

Statistical analysis was performed in the STATISTICA 13.3 program (Tibco Software, Palo Alto, CA, USA). Results were compiled using one-way analysis of variance. Homogenous groups, statistically not significantly different in terms of mean, were identified using Tukey's test at a significance level of $p \leq 0.05$. The normality of the distribution was assessed using the Shapiro–Wilk test.

3. Results

3.1. Effects of Conventional and Unconventional Extracting Methods for Y. lipolytica Yeast Cells

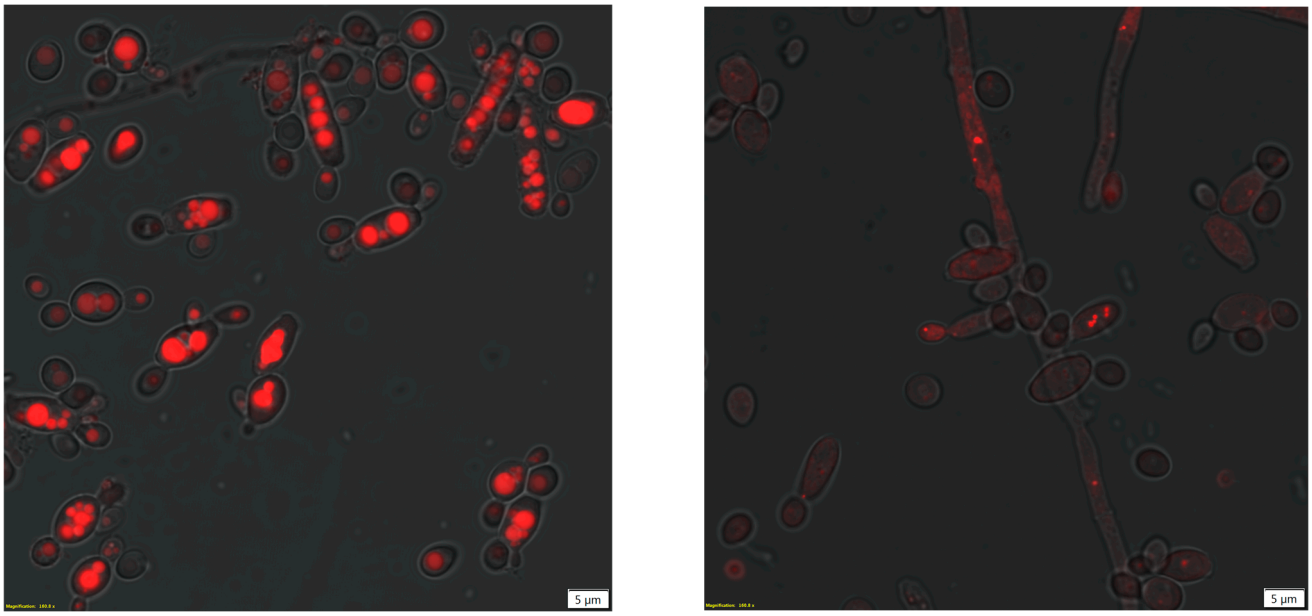
3.1.1. Comparison of Lipid Extraction and Leaching Efficiencies for Yeast Storage Lipids

The preliminary studies were performed and described in the paper of Wierzchowska et al. [27] and allowed for choosing non-conventional methods for further investigations. The effectiveness of the PEF, US, and HPP methods for extraction of microbial oil from *Y. lipolytica* yeast cells was studied, and it has been proven that the use of PEF with a field strength of 200 J/g or HPP (1100 bar) can be an effective pretreatment technique for *Y. lipolytica* yeast cells for the high yield extraction of intracellular lipids, providing the extraction method with organic solvents.

There were bioreactor batch cultures provided in which two different carbon sources were used. In post-frying rapeseed oil medium, waste oil medium (WOM) cells accumulated storage lipids via an ex novo pathway, and in cultures with molasses (MM), there was expected de novo lipid synthesis. Figure 3 presents a microscopic image of colored yeast cells from both cultures. In the case of the WOM culture, the content of cellular lipids after 40 h was 0.21 g/g d.m., while in the case of the culture of MM, the value reached a lower value of 0.13 g/g d.m. Analysis of the preparation from culture WOM shows a higher amount of lipid bodies, in which lipids are accumulated. The obtained image confirms the obtained trend of intracellular lipid content in both of these culture variants.

The results shown in Figure 4 present the efficiencies of yeast lipid extraction obtained after using the mechanical and chemical methods PEF, US, and HPP. The first scientific hypothesis assumed that those methods can effectively disrupt yeast cells. The control variant was an untreated biomass. The highest efficiency of microbial oil extraction using the Folch method was obtained after the previous treatment of the biomass with a PEF (28%). The use of Triton solution (chemical method with the use of surfactant), Triton in combination with shaking with glass beads (combined chemical and mechanical techniques), as well as high-pressure processing made it possible to achieve an efficiency of 6–11%. The use of glass beads (8%) and US (9%) did not increase the amount of lipids extracted. None of the methods applied in the study allowed for the extraction of lipids

from the interior of the disrupted cells. It was concluded that these methods cannot be considered as extraction methods but they are worth investigation as permeabilization techniques in pretreatment steps.



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

Figure 3. Microscopic images of stained *Y. lipolytica* yeast cells with visible red lipid bodies, magnification x = 160.8: (**a**) cultured in post-frying rapeseed oil medium (WOM—waste oil medium); (**b**) cultured in medium with molasses (MM—molasses medium). Images were taken with confocal laser microscope.

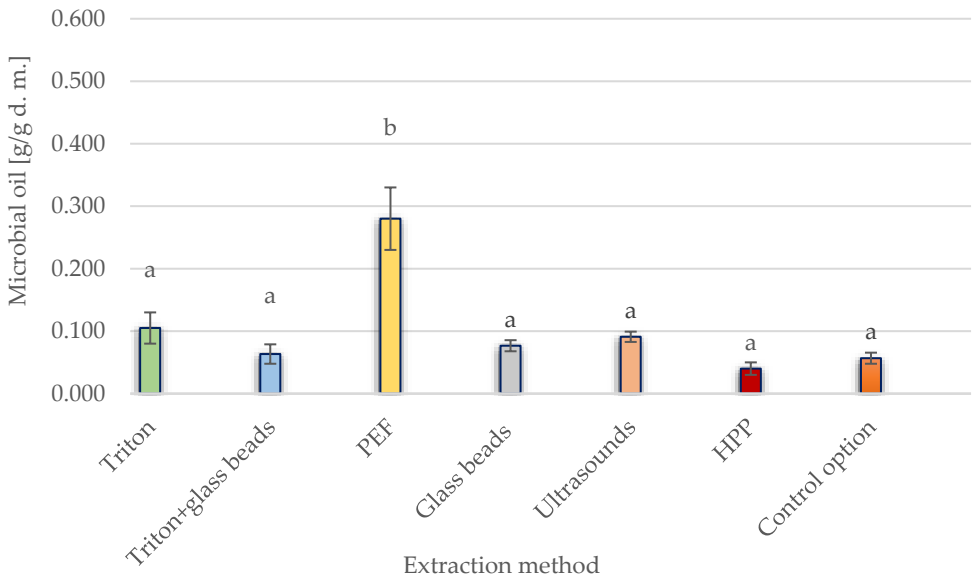


Figure 4. Lipid extraction yield from yeast cells pretreated with different methods. Means with the same capital letter (a, b) did not differ significantly ($\alpha = 0.05$).

This statement was studied on the basis of comparing the leaching efficiency of microbial lipids measured for yeast cells treated with the same techniques (Figure 5). As in the case of Folch extraction, six experimental options and a control variant were performed. The highest efficiency of lipid leaching using hexane was obtained by application of PEF. The yield obtained was 28%. The use of the other treatments made it possible to amount

leaching efficiencies for storage lipids at a higher or the same efficiency as the variant control, which was ca. 6%.

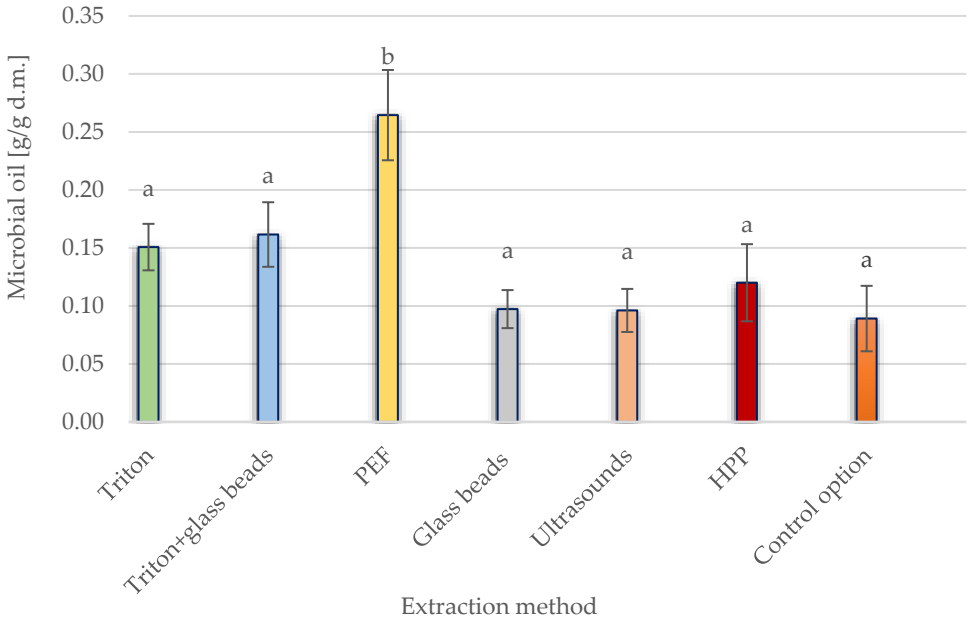


Figure 5. Comparison of leaching yield of intracellular lipids from yeast cells pretreated with different methods. Means with the same capital letter (a, b) did not differ significantly ($\alpha = 0.05$).

Finally, the efficiency of lipid extraction and leaching yield using the Folch method were compared (Figure 6). The PEF method had the highest result (92.86%). Cells treated with US were also characterized by a high ratio value (94.63%). On the other hand, the methods using glass beads, as well as surfactants, had a slightly higher ratio value when entrained with the control variant (63.59%). The use of a combination of glass beads with Triton, as well as high-pressure homogenization, did not improve the efficiency of lipid extraction from yeast cells.

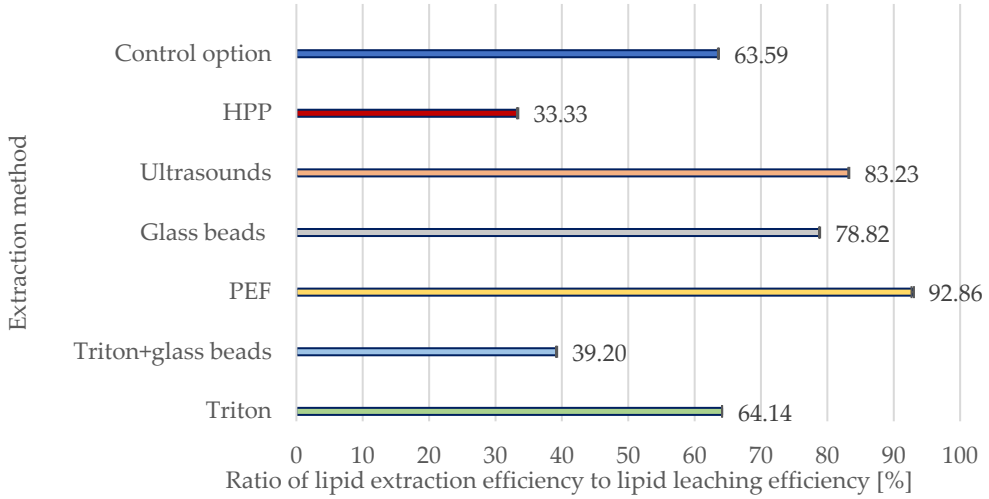


Figure 6. Comparison of the performance of lipid extraction using the Folch method with leaching of these components using hexane.

3.1.2. Protein Release from Yeast Cells after Conventional and Unconventional Treatment

Proteins and lipids are biomolecules of several nm to hundred µm. Still, lipid bodies can achieve a larger size than proteins, which do not usually form any aggregates. The aim of the experiment was to compare the permeability of the cell wall and cell membrane

of permeabilized yeast cells treated with different methods. That is why, apart from lipid extraction, protein release was measured during experiments. Figure 7 presents the protein concentration in the supernatant remaining after different permeabilization processes performed on yeast cells. Comparing protein leakage from treated cells helped to prove the efficiency of the conventional and unconventional treatments performed.

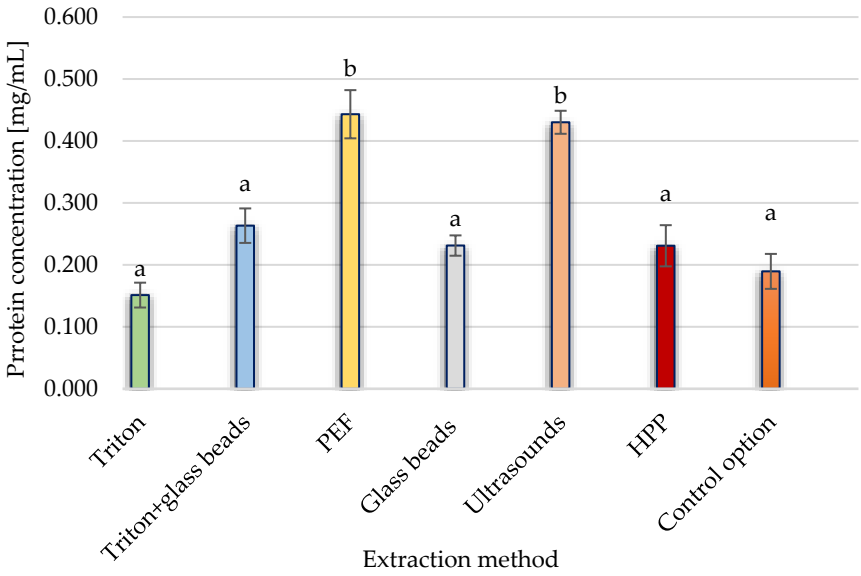
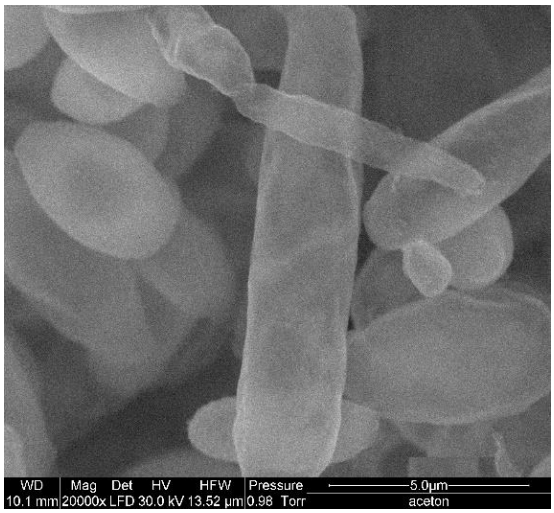


Figure 7. Protein concentration in yeast culture residual fluid. Means with the same capital letter (a, b) did not differ significantly ($\alpha = 0.05$).

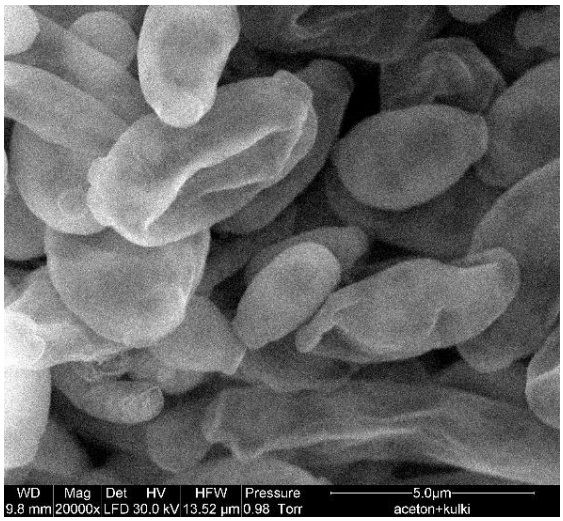
The highest protein content was measured for the supernatant in which PEF-treated biomass was suspended (0.443 mg/mL). Similarly, a high concentration of this component was obtained when US were applied (0.430 mg/mL). Using pretreatment in a Triton solution with glass beads, it was possible to obtain a concentration of protein at a level higher than that in the control variant (0.263 mg/mL and 0.250 mg/mL, respectively). The lowest protein concentration was obtained by treatment of the yeast biomass with Triton (0.151 mg/mL). Possibly, Triton is a surfactant that does not affect the cell wall morphology, so there is no increase in protein permeability [28]. Therefore, it can be concluded that the highest protein content was obtained after PEF and US application. In contrast, the use of Triton X-100 solution and glass-bead shaking or glass bead treatment and high-pressure processing yielded a comparable protein content, which is higher than for the control variant.

3.2. Impact of Conventional and Unconventional Methods of Cell Permeabilization and/or Disruption on its Morphology

Changes in the cell morphology of the *Y. lipolytica* KKP 379 strain are presented in Figure 8a–i. The greatest degree of cell membrane disintegration was observed in Y-PER lytic preparation-treated (Figure 8h) and US-treated biomass (Figure 8i). US-treated cell microphotographs confirmed the results of protein leakage to the yeast culture residual fluid (Figure 7). A significant degree of cell wall disruption and morphological changes were also noted when using combination methods with acetone and glass beads (Figure 8b), Tween-80 and glass beads (Figure 8d), and Triton X-100 and glass beads (Figure 8f). Nevertheless, the effect was noticeable to a lesser extent than when using the solvent acetone (Figure 8a) or the surfactants Tween 80 (Figure 8c) and Triton X-100 (Figure 8e) individually.



(**a**)



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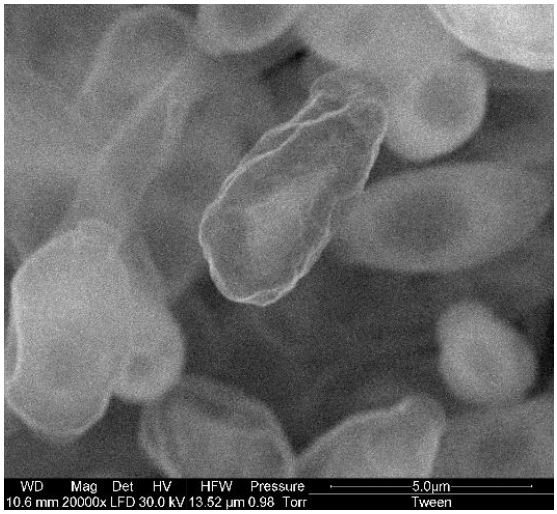
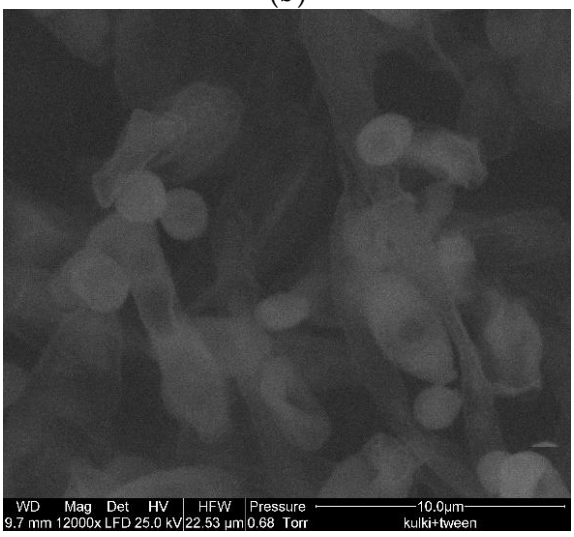


(**c**)



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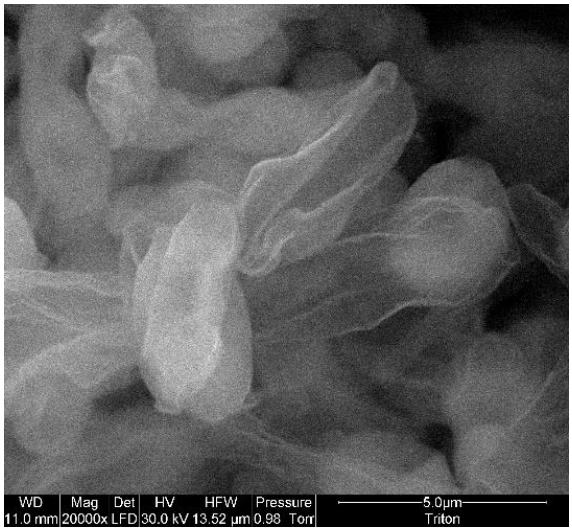

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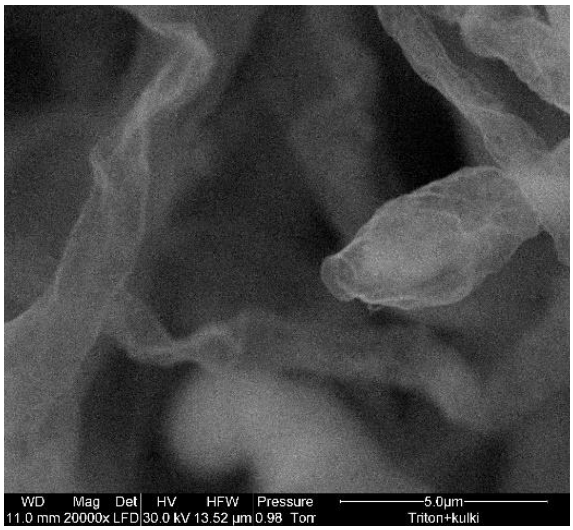

(**f**)

Figure 8. *Cont*.

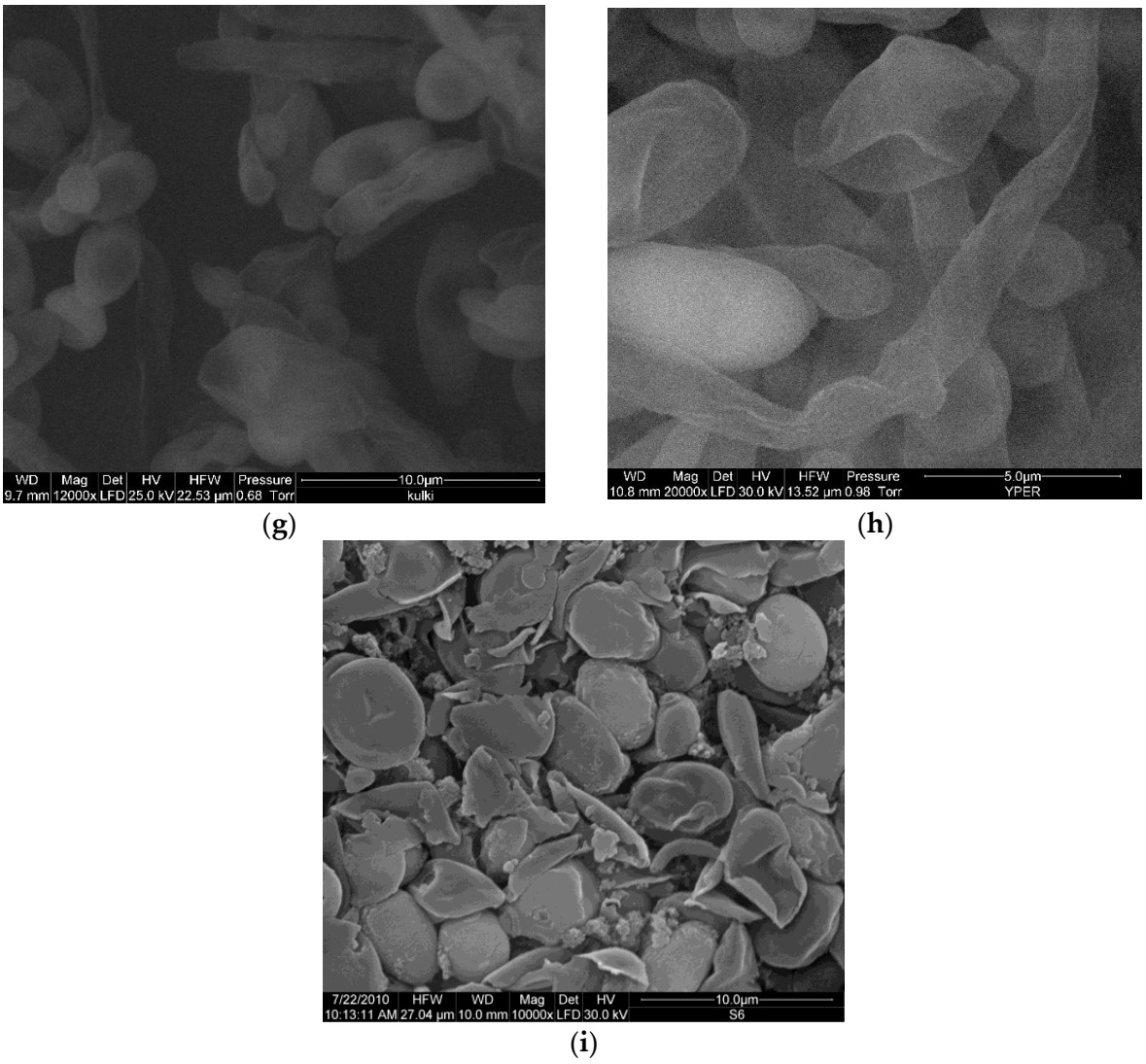

Figure 8. *Yarrowia lipolytica* KKP 379 yeast cells treated with (**a**) acetone; (**b**) acetone and glass beads; (**c**) Tween-80 surfactant; (**d**) Tween-80 surfactant and glass beads; (**e**) Triton X-100; (**f**) Triton X-100 and glass beads; (**g**) glass beads; (**h**) Y-PER lytic preparation; (**i**) ultrasonic treatment. Images were taken with scanning electron microscope.

4. Discussion

The biomass of the yeast *Y. lipolytica*, which has the GRAS status, was used in the described experiments. Importantly, following the Commission Implementing Regulation (EU) 2019/760 of 13 May 2019, the biomass of this yeast species is authorized as a novel food and may be used as an ingredient in human nutrition [29]. It is a well-known fact that *Y. lipolytica* garnered plenty of attention in the scientific community and constitutes a perfect platform for the biosynthesis of numerous, extremely valuable secondary metabolites with their potential application in the food, cosmetic, and pharmaceutical industries [30].

Regarding research on one of them, more precisely microbial oil—which is accumulated intracellularly—it is necessary to select and use an appropriate extraction method. In fact, extraction is one of the most commonly used methods for obtaining and purifying chemical compounds. Unconventional extraction methods not only shorten the process time and reduce energy consumption but also eliminate the use of harmful solvents and improve the quality of the final product [31]. In the current study, the following methods for the improvement of the microbial oil extraction process were applied: PEF, US, and HPP.

Comparing the extraction efficiencies of intracellular lipids from yeast cells treated with different permeabilization methods, it can be concluded that the highest efficiency was achieved by using PEF. When yeast cells are exposed to a voltage higher than their natural potential, both the cell wall and the membrane become permeable, and because of the electric current, the formation of pores on the cell surface occurs, i.e., the so-called electroporation [32]. The application of PEF for the extraction of intracellular metabolites including proteins or polysaccharides from yeast has been studied several times [33]. The use of PEF also allows some lipid molecules to pass through the pores in the membrane, and thus, PEF can be considered a good method for the permeabilization of yeast cells; in the current study, this was acknowledged both in the case of lipid extraction and the measurements of protein concentration in the remaining liquids after oil extraction experiments.

In recent years, scientists have attempted to use PEF treatment for lipid extraction (Table 1). Gorte et al. [34] applied PEF as a pretreatment step in ethanol-hexane-mediated lipid extraction from *Saitozyma podzolica*. The authors emphasized the importance of lower conductivity on the efficiency of PEF treatment, and moreover, the biomass was not subjected to any processing such as freezing or freeze-drying. Washing steps for yeast biomass both reduced the conductivity, as well as increased the efficiency of lipid extraction by 54% [34]. Drévillon et al. [35] also evaluated numerous cell disruption methods for the improvement of oil extraction from *Y. lipolytica*. The authors applied, e.g., high PEF, high voltage electrical discharges, US, and high-pressure homogenization. Compared to 19.8% for control samples, the authors observed an increase in the oil extraction yield to 29.4% when 20 kV/cm of electrical field strength was used. In addition, in the case of microalgae lipids, Zhang et al. [36] observed increased lipid extraction yield by up to 166.67% in *Chlorella* cells when the PEF treatment was applied compared to control samples without any disruption method. The authors also claimed that the too-high voltage caused a decrease in the quality of the extracted oil [36].

There were also provided experiments on PEF-assisted protein extraction. In the study of Liu et al. [37], PEF was used as a method of extracting proteins from waste brewing yeast. By applying 2% yeast biomass solution and an electric voltage of 10 kV, the authors observed 2.788% protein extraction efficiency. The results obtained within the current work allowed the extraction of almost 0.5% of protein from yeast cells using PEF but the physiology and cell wall structure of the yeast itself may be a cause of the differentiated results.

US also cause cell wall disintegration, contributing to the release of proteins and lipids from cells. The mechanisms of ultrasound-assisted extraction are based on fragmentation, erosion, sonoporation, and detexturation [38]. The mass transfer to the liquid phase is improved due to the collapse of cavitation bubbles, and moreover, this process is characterized primarily by a much shorter time and lower energy consumption compared to conventional extraction methods [39].

Some authors [26] stated that the use of US causes a high degree of loosening of the yeast cell wall and membrane, which is also very useful during the extraction of microbial oil. In the current study, the US did not have an impact on the improvement of lipid extraction yield but proved its use in protein release from yeast cells. On the other hand, application of US for *Trichosporon* sp. Biomass proved to be the most efficient method for lipid extraction with the obtained lipid yield of 43% (Table 1). The achieved results were higher than those obtained with Soxhlet (30%) and Folch (36%) methods [40]. Selvakumar and Sivashanmugam [41] compared lipid recovery with the use of different biomass concentrations of oleaginous yeast *Naganishia liquefaciens* NITTS2, the chloroform: methanol ratio, and ultrasound power density. The recovery of 99.3 ± 0.1% was achieved after 30 min at 30 °C when 60 g/L of biomass, a solvent ratio of 1:1, and 0.6 W/mL of US power were used.

Table 1. Efficiency of microbial lipid extraction by application of unconventional permeabilization and cell disruption techniques.

Method Applied for Extraction	Microorganism	Experimental Conditions	Lipid Extraction Efficiency [%]	Reference
PEF treatment	*Saitozyma podzolica* yeast	suspension with a conductivity at 20 °C of either 10.14 ± 0.63 or 1.27 ± 0.09 mS/cm; the output impedance of generator (50 Ω); electric field intensity was varied between 1.4 and 4 MV/m	54.0	[34]
	Yarrowia lipolytica yeast	20 kV/cm of electrical field strength	29.4	[35]
	Chlorella algae	max. 35 kV, the pulse width of the square wave pulse from 2 to 99 µS at a pulse repetition rate of 1~1 kHz.	increase by 166.7%	[36]
US treatment	*Trichosporon* sp.	Ultrasonic bath working at 520 kHz with an intensity of 40 W	43.0	[40]
	Naganishia liquefaciens yeast	US-assisted extraction of 0.6 W/mL of US power combined with solvents	97.1	[41]
HPP	*Yarrowia lipolytica* yeast	298 K, 1500 × 10^5 Pa with 20 passes	83.8	[35]
	Saitozyma podzolica yeast *Apiotrichum porosum* yeast	2000 bar, 5 min, with 15 passes	95.0 * 53.0 *	[42]

* Disruption rate.

Sonication is a valuable method for permeabilizing the yeast cell wall and releasing intracellular components like proteins. The biomass of *Saccharomyces cerevisiae* 2200 was subjected to the US treatment and the content of protein released from the cells reached 320 mg/g of the dry yeasts [26].

The third unconventional method used in the current study was HPP. According to the obtained results, it can be seen that the use of HPP as a permeabilization method did not improve the lipid extraction yield, hence it can be stated that such pretreatment is not suitable for *Y. lipolytica* cells. The use of high-pressure processing is rather scarce for yeast biomass treatment and more often high-pressure homogenization was applied for metabolite extraction. HPH was used in the previously mentioned preliminary studies [27], as well as in the papers of Gorte et al. [42] and Drévillon et al. [36]. As a result of using homogenization at a high pressure of 2000 bar for 5 min with 15 passes of the sample through the homogenizer, 95% disruption efficiency was observed for *S. podzolica* and only 53% for *Apiotrichum porosum*, which in the case of the selected extraction methods, i.e., Folch, Bligh–Dyer, or ethanol-hexane mixture, translated into 37.8–46.9% and 14.1–15.0% of whole lipid/CDW, respectively [42].

In Drévillon et al.'s [36] research, the use of HPH was the most efficient method for *Y. lipolytica* cell disruption, which resulted in 83.8% oil extraction yield with only 19.8% for the control method (Table 1). In the case of their study, a cell suspension of 15% DM was passed through the homogenizer 20 times at a pressure of 1500 bar. The obtained results were 2- to 4-fold higher in comparison with the application of US or PEF.

Selecting the appropriate permeabilization method is a crucial step before extraction, as described in the above discussion. On the other hand, selecting the extraction method itself is another key factor when obtaining valuable substances from the inside of yeast cells. Milanesio et al. [43] compared three different extraction methods, i.e., Soxhlet lipid extraction using chloroform: methanol (2:1, *v*/*v*), accelerated solvent extraction (conducted in high temperature and pressure), and supercritical carbon dioxide extraction with ethanol as co-solvent. Moreover, the authors decided to compare permeabilization methods for the biomass of *Y. lipolytica*. The following pretreatments were applied: drying and milling; yeast atomization; methanol maceration; ethanol maceration; and supercritical disruption of yeast cells. In the case of Soxhlet and accelerated extraction, the application of ethanol

to biomass maceration proved to be the best pretreatment method, which resulted in the highest total extract yields (TAGs and polar lipids). For the former method, 29.7% lipid of total dry mass was obtained, and in the latter, it was 27.7%. The extract yield of 22.2% was obtained when the authors used the combined method, that is, wet yeast were macerated in ethanol for 20 h, and then subjected to supercritical disruption for 2 h at 40 °C, and 20 MPa was conducted [43].

In addition, switchable hydrophilicity solvents were applied as an alternative to conventional organic solvent extraction by Yook et al. [44]. The authors used *N*-ethylbutylamine (EB), *N*-dipropylamine (DP), and *N,N*-dimethylcyclohexylamine (DMCHA) as the lipid extraction solvents. Interestingly, EB and DMCHA allowed achieving up to 13% better results in comparison with Folch extraction. Importantly, the extraction time of 3 h was enough to obtain equal or higher lipid yields compared to the conventional method.

5. Conclusions

The aim of this research was to evaluate the impact of several methods of permeabilization and disintegration of cell wall structures and cell membranes on the efficiency of microbial oil extraction from oleaginous yeast cells of the *Y. lipolytica* species. The research conducted in this study indicated that none of the unconventional methods used, such as US, PEF, or HPP, led to effective disintegration and release of lipid bodies from cells. Extraction of microbial lipids was possible only after using solvent extraction. However, it can be clearly stated that as a result of using appropriate techniques for processing biological material (such as PEF or US), a better efficiency of microbial oil extraction from yeast cells can be achieved. Still, optimization experiments should be further conducted in order to enhance the permeabilization effect. Based on the results published by other authors, there is an unreached potential for unconventional methods. In the future, it would be advisable to conduct research to answer the question of whether the use of PEF in the flow would improve the permeabilization effect. Moreover, some of the methods tested in this work, such as the use of sonication to improve the extraction of microbial oil from yeast cells, require further experiments. It seems that the use of ultrasonics may bring better results in terms of extraction of cell contents after applying more stringent process conditions.

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