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Extraction of lipids from *Yarrowia Lipolytica*[†]

Juan Milanesio,^{a,b,c*} Pablo Hegel,^d Yaocihuatl Medina-González,^{a,b}
Séverine Camy^{a,b} and Jean-Stéphane Condoret^{a,b}

Abstract

BACKGROUND: Microorganisms have often been considered for the production of oils and fats as an alternative to agricultural and animal resources. Extraction experiments were performed using a strain of the yeast *Yarrowia lipolytica* (*Y. lipolytica*), a high-lipid-content yeast. Three different methods were tested: Soxhlet extraction, accelerated solvent extraction (ASE) and supercritical carbon dioxide (SCCO₂) extraction using ethanol as a co-solvent. Also, high pressure solubility measurements in the systems 'CO₂ + yeast oil' and 'CO₂ + ethanol + yeast oil' were carried out.

RESULTS: The solubility experiments determined that, at the conditions of the supercritical extractor (40 °C and 20 MPa), a maximum concentration of 10 mg of yeast oil per g of solvent can be expected in pure CO₂. 10% w/w of ethanol in the solvent mixture increased this value to almost 15 mg of yeast oil per g of solvent. Different pretreatments were necessary to obtain satisfactory yields in the extraction experiments. The Soxhlet and the ASE method were not able to complete the lipid extraction. The 'SCCO₂ + ethanol' extraction curves revealed the influence of the different pretreatments on the extraction mechanism.

CONCLUSION: Evaluating the effectiveness of a given pretreatment, ASE reduced the amount of material and solvent used compared with Soxhlet. In all three cases, the best total extraction performance was obtained for the ethanol-macerated yeast (EtM). Addition of ethanol to the solvent mixture enhanced the oil solubility. Oil can be extracted from *Y. lipolytica* in two different steps: a non-selective ethanol extraction followed by TAG-selective SCCO₂ purification.

Keywords: lipid extraction; lipid solubility; *Yarrowia lipolytica*; supercritical extraction; yeast pretreatments

INTRODUCTION

Microorganisms have often been considered for the production of oils and fats as an alternative to agricultural and animal resources.¹ Single-cell oil (SCO) from yeasts has gained commercial importance mainly due to its similarity in properties to vegetable oils and fats.² Oleaginous yeasts have been reported to grow and accumulate very significant amounts of lipids (60 to 70% w/w of dry mass).² However, even under propitious conditions, only a small fraction of yeast biomass is in the form of lipids. For example, *Saccharomyces cerevisiae* (*S. cerevisiae*) has between 5 and 10% of lipids w/w of dry mass. The main lipidic components of this yeast are triacylglycerols (TAGs) and steryl esters (SEs), but phospholipids (PLs) and free fatty acids are also present.³ Microorganisms may also contain other lipids, like sphingolipids or glycolipids.² These are usually found within special organelles called lipid particles or lipid bodies (LB), which are surrounded by a PL monolayer with embedded proteins.

The yeast *Yarrowia lipolytica* (*Y. lipolytica*) is a high-lipid-content yeast. Several technologies, including various fermentation configurations, have already been used for SCO production by strains of *Y. lipolytica* grown on various agro-industrial by-products or wastes.⁵ The potential applications of these processes include the production of reserve lipids with particular structures (e.g. oils enriched in essential polyunsaturated fatty acids) and the production of non-specific oils for use as renewable starting materials for the synthesis of bio-fuels.⁶

The extraction of lipids from yeasts with various conventional organic solvents, such as chloroform and methanol, hexane and/or petroleum ether, and their application, even at industrial scale,

has already been reported in the literature.² According to the Principles of Green Chemistry, the organic solvents used at pilot and industrial scale have to be replaced in the near future by non-flammable, less toxic and more benign solvents in order to obtain sustainable processes.⁷ Carbon dioxide (CO₂) is an inert, inexpensive, easily available, odorless, tasteless, environment-friendly, ideally suitable for thermally labile natural products and generally regarded as safe (GRAS) solvent.⁸ In addition, its solvent power and selectivity can be adapted according to the operating conditions.

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Extraction of lipids from yeast using SCCO₂ is rarely considered in the literature. To palliate this scarcity of knowledge, an analysis of the data published on oil extraction from seeds could be an interesting starting point. The use of SCCO₂ and co-solvents for the selective extraction of triglycerides has been studied, for example, in the case of canola, soybean lecithin and sunflower oil.^{9–13} To estimate the maximum oil concentration in the solvent that can be expected in the output effluent of a supercritical extractor, oil solubility measurements (phase boundaries) are very helpful. Cocero and Calvo⁹ have shown that, in the case of sunflower oil, the solubility is increased with increasing pressure at a given temperature and is reduced with increasing temperature at constant pressure. Also, the authors showed that the addition of ethanol to the solvent mixture increased the oil solubility at a given temperature and pressure.

The aim of this work is to compare the performance of three different techniques for lipid extraction from a specific yeast material and to estimate, from solubility measurements, the maximum oil concentration that can be expected at the output of the supercritical extractor. The raw material used in this work was a strain of *Y. lipolytica* with high lipid content. It was provided by a major European industrial yeast producer. Different pretreatments were applied in order to increase the yield.

EXPERIMENTAL

Materials

Yeast materials

The yeast *Y. lipolytica* was provided by Lesaffre Group®, after harvesting and preconditioning. The yeast was received in three different forms (raw materials):

1. Compressed yeast (block).
2. Yeast cream (water suspension).
3. Yeast dried by atomization (powder).

The water content of the compressed yeast blocks was 60% w/w. The yeast cream was always centrifuged before the extraction experiments (20 min at 10 000 rpm). The water content of the centrifuged yeasts was 70% w/w.

According to the provider, the *Y. lipolytica* had a TAG fraction of 17.7% w/w of the dry mass. The original raw materials were subjected to different pretreatments as described below:

1. The compressed yeast blocks were dried for 15 h at 60 °C and then milled and sieved (mesh = 1 mm).
2. The yeast cream was centrifuged (20 min at 10 000 rpm), dried for 15 h at 60 °C and then milled and sieved (mesh = 1 mm).
3. The yeast cream was centrifuged (20 min at 10 000 rpm). Then the centrifuged yeast was disrupted by rapid decompression after exposure to SCCO₂ (6 h at 20 MPa and 40 °C) following the procedure of Lin *et al.*¹⁴ After that, the yeast was dried at 60 °C for 15 h, milled and sieved (mesh = 1 mm).
4. The yeast cream was centrifuged (20 min at 10 000 rpm). Then the centrifuged yeast was macerated in ethanol for 20 h (one part of yeast and nine parts of ethanol by weight), dried at 60 °C for 15 h and milled (mesh = 1 mm).
5. The yeast cream was centrifuged (20 min at 10 000 rpm). Then the centrifuged yeast was macerated in methanol for 2 h (one part of yeast and nine parts of methanol by weight), dried at 60 °C for 20 h and milled (mesh = 1 mm).

The milling process was done using a laboratory knife grinder (IKA, Model A11 Basic). The size classification used a sieve

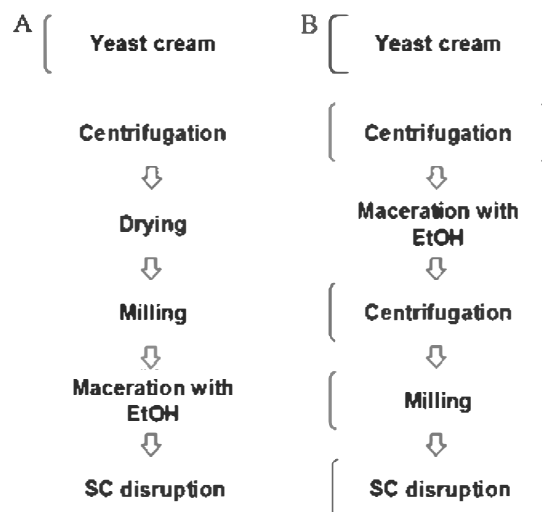


Figure 1. Flow diagrams for the combined yeast pretreatments. Case A: EtMSCD yeast (view Table 1). Case B: WEtMSCD yeast (view Table 1). EtOH: ethanol and SC: supercritical.

with a maximum particle diameter of 1 mm. Also, some of the pretreatments described above were combined to improve the extraction efficiency:

1. The compressed yeast blocks were dried for 15 h at 60 °C and then milled and sieved (mesh = 1 mm). This was followed by an ethanol maceration step (20 h at ambient temperature) and a filtration process. Then, the yeasts were disrupted by rapid decompression after exposure to SCCO₂ (2 h at 20 MPa and 40 °C) following the procedure of Lin *et al.*¹⁴
2. Ethanol maceration for 20 h at ambient temperature followed by centrifugation (only for the yeast cream). The yeasts were then milled and disrupted by rapid decompression after exposure to SCCO₂ (2 h at 20 MPa and 40 °C).

The flow diagrams of Fig. 1 show the different steps for the combined pretreatments described above. The materials used for the extractions and their corresponding acronyms are listed in Table 1.

Solvents

The CO₂ used for the phase equilibrium measurements and for the supercritical extractions was provided by L'Air Liquide with 99.9% purity. The ethanol used for the solubility measurements was absolute ethanol provided by Sigma Aldrich with a minimum purity of 99% by volume. The ethanol used for the supercritical extractions was supplied by Sigma Aldrich with a purity of 96% by volume. The methanol and chloroform used for the Soxhlet extractions and the ASE were of analytical grade. The solvents were supplied by Sigma Aldrich with a minimum purity of 99.9%. The acetone (analytical grade) used to purify the TAG fraction was provided by Scharlau with a minimum purity of 99.5% obtained by GC.

Experimental procedures

Solubility measurements

To estimate the maximum oil concentration in the solvent that can be expected in the output effluent of a supercritical extractor, oil solubility measurements (phase boundaries) were performed. The experimental determination of the phase equilibrium for the

Table 1. Acronyms for the different pretreated materials. The grey filled cells represent the combination of a raw material (in rows) with a given pretreatment (in columns)

Pretreatment Raw material	Centrifugation	Dried (60 C)	Milled (mesh = 1 mm)	SC disruption	MeOH maceration	EtOH maceration	Acronym
Compressed yeast block							DM
Atomized							ATOM
Yeast cream (water suspension)							DM
							SCD
							MeM
							EtM
							EtMSCD
							WEtMSCD

Raw materials are listed in rows and the different pretreatments in columns. When a given raw material was subjected to a given pretreatment, the corresponding box is shaded grey. The last column shows the acronyms used in this work to identify the extraction materials.

'yeast oil + CO₂' binary system and the 'yeast oil + CO₂ + ethanol' ternary system was carried out in a high-pressure variable-volume equilibrium cell (Top Industries S.A., France). The variable-volume windowed equilibrium cell could operate at pressures up to 50 MPa. The variable volume of the cell (13 mL maximum volume) made it possible to independently control pressure and temperature (in a constant volume cell, when temperature is increased, the pressure increase is correlated with it). This set-up was based on the so-called 'synthetic method'.¹⁵ In this simple experimental method, the phase transitions, resulting from pressure variations, are observed by direct visualization. To determine the solubility of oil in the solvent, a gravimetrically determined amount of yeast oil (TAGs fraction of yeast lipids) was placed inside the cell. Then, a given amount of CO₂ was loaded into the cell using a syringe pump (Teledyne Isco, Model 260D). For the ternary system, after the oil had been loaded and before loading the CO₂, a gravimetrically determined amount of absolute ethanol was placed inside the cell. At the end of the loading process, there was a mixture of known composition inside the cell. Next, the system was set under conditions of temperature and pressure such that a homogenous fluid could be observed through the cell window. Then, the pressure was slowly reduced, at constant temperature, until the fluid system became opalescent. This characteristic opalescence corresponded to the formation of a new dense phase. The variable volume equilibrium cell is similar to devices described earlier in the literature.^{16,17} The main elements of the experimental set-up are schematically represented in Fig. 2 and are fully described elsewhere.¹⁸ The temperature was measured with an accuracy of ± 0.2 K and the pressure measurement was accurate to ± 0.02 MPa. Each experimental data point was repeated at least three times and each curve was measured twice. The uncertainties in the oil solubility values were estimated through a very conservative propagation of error analysis, for all systems studied in this work. Overpressures were prevented by a rupture disk.

Soxhlet extractions and characterization of Soxhlet extracted lipids

Soxhlet extraction is a widely used and suitable method for determining the lipid content of a wide variety of solid matrices. However, it is a time-consuming technique which requires large volumes of organic solvents. For instance, using a solvent mixture of chloroform and methanol (2:1 by volume), good results were obtained for a number of food products.¹⁹

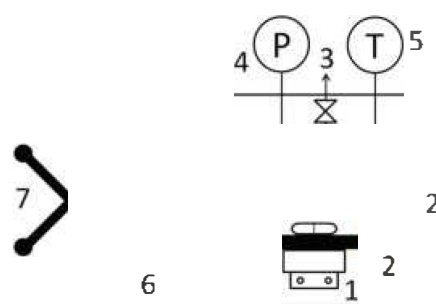


Figure 2. Schematic experimental setup of the high pressure phase equilibrium cell: 1. magnetic stirrer, 2. sapphire windows, 3. pressure valve, 4. pressure transducer, 5. thermometer, 6. thermostatic bath, 7. screw pump.

Soxhlet extractions were carried out for 8 h with a chloroform:methanol solvent mixture (2:1 by volume). After the extraction, the solvents were recovered in a rota-evaporator, which was operated with a vacuum pump.

The crude lipidic extract obtained from the Soxhlet extractor contained carbohydrates, proteins, and other unwanted materials, which were separated by a method based on the work of Hubbard *et al.*¹⁹ The first step of the method consisted in re-dissolving the crude lipidic extract in 50 mL of the original solvent mixture used for the Soxhlet extraction (chloroform:methanol, 2:1 by volume) and then adding 12 mL of a solution of sodium chloride (NaCl) in distilled water (1% w/w). A separating funnel was used to decant the two-phase mixture for 2 h. The chloroform-rich heavy phase contained a mixture of TAGs and PLs. The chloroform was separated from the lipids using a rota-evaporator. In the lipid mixture, the PL fraction was separated from the TAGs and quantified by adding cold acetone, which dissolves neutral lipids only. The acetone mixture was decanted into a separate beaker. This method is employed to enrich the PL content in commercial soybean lecithin²⁰ and for oil recovery and lecithin production using water degumming sludge of crude soybean oils.²¹ The lipid fraction that was insoluble in acetone was assumed to be the PL fraction. Lipids that were soluble in acetone were assumed to be TAGs and free fatty acids. The extraction and the purification processes were repeated at least twice. The experimental error caused by the presence of ethanol remaining in the total extract or acetone in the TAG fraction was considered negligible. Each extraction yield was noted as the mean value \pm standard deviation.

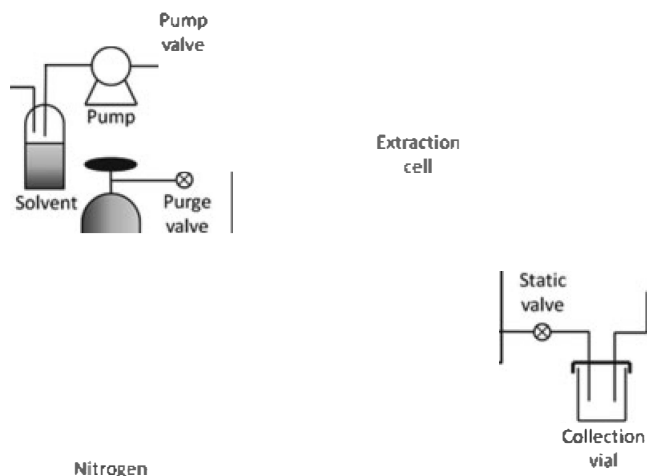


Figure 3. Schematic for experimental setup of the accelerated solvent extractor.

Accelerated Solvent Extractions

A recently developed method to determine lipid content is accelerated solvent extraction (ASE[®]), a technique registered by Dionex, Germany.²² This technique uses liquid solvents at high temperatures and pressures. The solvents are those normally used for standard liquid extraction techniques such as Soxhlet. The ASE technique reduces the volume of solvent required and shortens the sample extraction time compared with Soxhlet. ASE yields are comparable with those obtained with the Soxhlet method.²³ Nevertheless, there is evidence that ASE efficiencies depend on specific lipid-matrix interactions.²³

Yeast lipid extraction was performed by the ASE technique²² with a chloroform:methanol solvent mixture (1:1 by volume, using the same solvents as for the Soxhlet extractions). High pressure and temperature operation accelerated the extraction kinetics. ASE can be used to replace Soxhlet extraction for the determination of oil content in conventional matrices, such as oil seeds. An automated extraction system for accelerated solvent extraction (Dionex Model ASE 200, Germany) was used to perform the extractions. The ASE system (Fig. 3) consists of a stainless steel sample cell with electronically controlled heaters and pumps to maintain extraction parameters (pressure, temperature, volume of solvent, extraction time) at the programmed set points. The upper temperature limit at the extraction system is 200 °C. To begin the extraction, a 2 g yeast sample was placed in an 11 mL stainless steel cell and then the cell was filled with the extraction solvent mixture. A static extraction cycle began at high temperature (80 °C) and pressure (10 MPa). The different yeast samples were subjected to five static extraction cycles of 8 min each. The extract was filtered with a cellulose filter and a stainless steel frit at the outlet, and finally collected in a 40 mL glass vial. The extraction conditions for the yeast samples are shown in Table 2. Each run was repeated three times. The method was found to be very reproducible. Each extraction yield was noted as the mean value \pm standard deviation.

Supercritical extractions

Supercritical CO₂ has been successfully used to extract lipids from oil seeds. This has been one of the most important and most-studied applications of this technology since the pioneering works on this topic were published.^{24,25} Supercritical studies

Table 2. Extraction conditions for the ASE technique

Cell volume [mL]	11
Mass of yeast loaded [g]	≈ 2
Pressure [MPa]	10
Temperature [°C]	80
Static extraction time [min]	8
Number of cycles	5
Chloroform–methanol volume ratio	1:1
Amount of solvent renewed on each cycle [%]	100

have been reported for the extraction of high added-value products from yeast, such as astaxanthin and squalene.^{1,26,27} More studies can be found for SCCO₂ lipid extraction from algae material²⁸ but few references^{1,29} can be found in the literature about the detailed extraction of lipids from yeasts with CO₂ and co-solvents, especially taking into account the yeast pretreatment, which has a marked influence on the extraction efficiency.

Supercritical extractions were carried out in the pilot plant (Separex Chimie Fine Model SF 300, France) represented in Fig. 4. Supercritical 'CO₂ + ethanol' extraction conditions were selected according to previous works^{9,30} in order to obtain selectivity towards TAGs. The pilot plant has two cyclone separators downstream of the extractor. The maximum CO₂ flow rate was $1.6 \times 10^{-3} \text{ kg s}^{-1}$. All the experiments were performed using a 54 mL tray to hold the yeast sample. The yeast was placed inside the tray, which had an upper and a lower stainless steel frit to allow the solvent to flow through the yeast sample. Subcooled liquid CO₂ was pumped by a volumetric membrane pump (Dosapro Milton Roy, Model MB), then heated to the desired temperature and continuously introduced into the extractor. The maximum operating pressure was 30 MPa and the maximum temperature around 60 °C. The experimental procedure has been described in previous publications.^{11,31} Briefly, the heating system was set at the desired temperature and the extraction cylinder was filled with a given amount of yeast and placed inside the extractor vessel. Once the high pressure extractor vessel was closed, CO₂ was pumped into the extractor until the operating pressure was reached. A co-solvent was used to increase the solvent power of CO₂ and to facilitate the mechanical recovery of the extract in the separators. A given flow-rate of ethanol was pumped into the vessel to obtain the desired solvent mixture. Eight minutes were necessary to stabilize the process conditions. The extractor and the separator pressures were maintained using three back pressure regulators (BPRs).

Lipids + ethanol samples were taken from the separators every 5 or 10 min during the total extraction time (120–180 min). They were collected from the bottom of the separators in 100 mL glass flasks. Then the ethanol was separated from the lipids in a rota-evaporator at 60 °C operated with a vacuum pump. For each sample, the amount of extracted lipids was quantified gravimetrically after the separation. Each extraction curve was repeated twice. The mean extraction yield was noted.

The mass of yeast loaded was usually around 20 g. The operating conditions were: 20 MPa in the extractor, 6 MPa in the first separator and 2 MPa in the second separator, a temperature of 40 °C for the extractor and the separators, a flow-rate of $30 \pm 2 \text{ g min}^{-1}$ of CO₂ and a flow rate of $3 \pm 0.2 \text{ g min}^{-1}$ of ethanol. The experiments were carried out for 120–180 min.

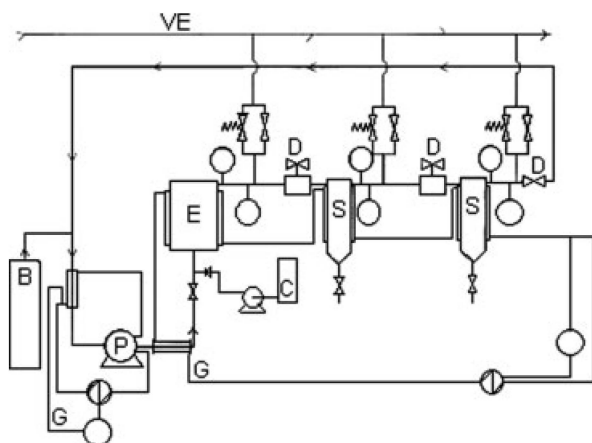


Figure 4. Simplified flow sheet of the pilot plant for the supercritical extraction experiments: B, CO₂ cylinder; E, extraction vessel; S, separators; P, pump; G, cooling and heating group; D, back pressure regulator valves; C, co-solvent; VE, venting system.

RESULTS

Solubility results

In order to determine the maximum amount of oil that can be dissolved by the pure CO₂ or 'CO₂ + ethanol' mixture, the yeast oil solubility was measured as a function of pressure, at three different temperatures. Here the term 'yeast oil' corresponds to the extract obtained from the Soxhlet apparatus, then purified by acetone (i.e. it corresponds to the TAG and free fatty acid fraction).

Figure 5 shows the solubility data obtained in the present work as a function of pressure for the system 'yeast oil + CO₂' at 40, 50 and 60 °C. The dashed lines in Fig. 5 correspond to smoothed data and have been added to facilitate the visualization of the trends. The results in Fig. 5 show that the solubility increased with the increase of pressure at a given temperature. On the other hand, the solubility decreased with the increase of temperature at constant pressure. This phenomenon is called 'retrograde solubility' and has often been reported in the literature.³² Also, Fig. 5 shows that 10.35 ± 0.09 mg of yeast oil per g of pure CO₂ can be dissolved at 40 °C and 20.52 MPa. Raw experimental data for this system are reported in Table 3.

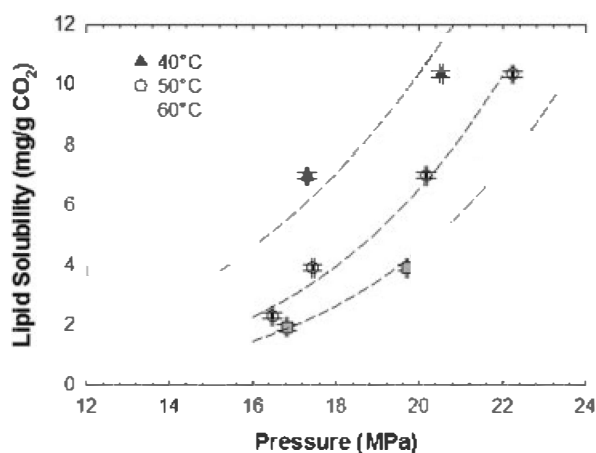


Figure 5. Experimental solubility values as a function of pressure for the system 'yeast oil + CO₂' at three different temperatures. Filled triangles: 40 °C; empty circles: 50 °C; grey squares: 60 °C. Triangles, circles and squares: raw experimental data (this work). Dashed lines: smoothed data.

Table 3. Experimental phase equilibrium data for the systems 'CO₂ + yeast oil' and 'CO₂ + ethanol + yeast oil' (this work)

CO ₂ + yeast oil	
T = 40 °C	
Pressure [MPa]	Solubility [mg oil g ⁻¹ solvent]
13.76	2.3 ± 0.1
14.9	3.9 ± 0.1
17.31	6.98 ± 0.1
20.52	10.35 ± 0.09
T = 50 °C	
16.48	2.3 ± 0.1
17.44	3.9 ± 0.1
20.16	6.98 ± 0.1
22.23	10.35 ± 0.09
T = 60 °C	
16.83	1.93 ± 0.1
19.71	3.9 ± 0.1
21.68	6.98 ± 0.1
23.57	10.35 ± 0.09
CO ₂ (90% w/w) + ethanol (10% w/w) + yeast oil	
T = 40 °C	
10.18	1.99 ± 0.1
13.26	4.44 ± 0.1
13.73	6.70 ± 0.1
16.09	12.55 ± 0.1
T = 50 °C	
12.32	1.99 ± 0.1
14.40	4.44 ± 0.1
15.05	6.70 ± 0.1
17.31	12.55 ± 0.1
T = 60 °C	
14.41	1.99 ± 0.1
16.43	4.44 ± 0.1
16.82	6.70 ± 0.1
18.12	12.55 ± 0.1

Figure 6 displays the experimental solubility data for the 'yeast oil + CO₂ + ethanol' system using a solvent mixture with 10% w/w of ethanol. The dashed lines in Fig. 6 correspond to smoothed data and have been added to facilitate visualization. The trends are similar to those presented for the 'yeast oil + CO₂' system. The solubility increased with increasing pressure at a given temperature and decreased with increasing temperature at constant pressure. The addition of ethanol to the solvent mixture increased the amount of oil that could be dissolved. According to Fig. 6, 12.55 ± 0.1 mg of yeast oil per g of solvent can be dissolved at 16.09 MPa and 40 °C. This value implies that, at 20 MPa and 40 °C, more than 12.55 ± 0.1 mg of yeast oil per g of solvent can be dissolved (around 15 mg of yeast oil per g of solvent, following the tendencies of Fig. 6). Cocero and Calvo⁹ measured solubility data for the 'sunflower oil + CO₂ + ethanol' using a solvent mixture with 10% w/w of ethanol and the results obtained in the present work are consistent with theirs regarding the effect of pressure and temperature on the total amount of oil

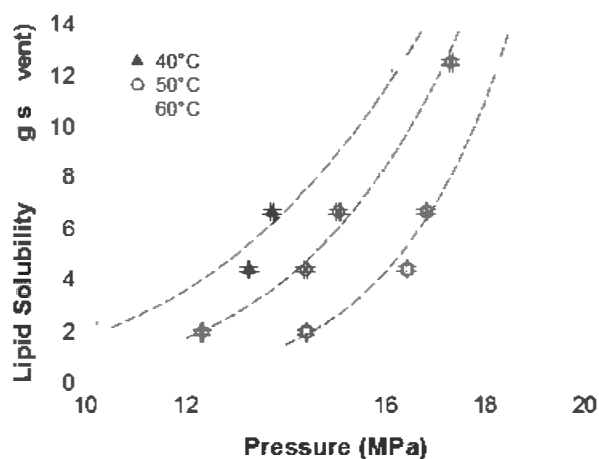


Figure 6. Experimental solubility values as a function of pressure for the system 'yeast oil + CO₂ + ethanol' with a solvent mixture of 90% w/w of CO₂ and 10% w/w of ethanol at three different temperatures. Filled triangles: 40 °C; empty circles: 50 °C; grey squares: 60 °C. Triangles, circles and squares: raw experimental data (this work). Dashed lines: smoothed data.

dissolved. Raw experimental data for this system are reported in Table 3.

In their study of *S. cerevisiae* SCCO₂ extraction, using the same experimental set-up, Hegel *et al.*³⁰ reported that, with this flow configuration, the maximum lipid concentration in the output fluid phase was about 4.5 mg of yeast oil per g of CO₂. The maximum oil concentration in the CO₂-rich fluid phase was significantly lower than the solubility observed in the present phase equilibrium results. This result shows that saturation of the solvent mixture was not reached in this case, probably because of hydrodynamic or mass transfer limitations in the extraction cylinder since channeling, entrance and exit flow perturbations and/or free convection effects could be occur there.

Extraction results

Soxhlet extractions

Table 4 shows the results in terms of total extract, PL and TAG fractions after the Soxhlet extractions for the different yeast samples. These results were obtained after the separation procedure described above. The maximum amount of TAGs extracted was obtained for the ethanol-macerated (EtM) yeast. Also, the total extract shows a maximum for this pretreatment, reaching 29.7 ± 2.5% w/w of dry mass. The unexpectedly high mass of total extract found in this case could be associated with a high water-content in the matrix, due to an incomplete drying process. For the methanol-macerated (MeM) yeast, the dried and milled (DM) yeast and the supercritical disrupted (SCD) yeast, similar results were obtained for the TAG fraction (between 7.4 ± 0.6 and 8.4 ± 0.7% w/w of dry mass). The worst extraction performance was obtained with the atomized (ATOM) yeast. Figures 7 and 8 display the total extract yields and the TAG fraction yields respectively.

The TAG fraction given by the provider was never obtained, as can be seen in Table 4. The procedure used by the provider involved much longer extraction times (24 h) and successive changes of the solvent mixture. The results obtained in this work by the Soxhlet procedure show that a strong lipid-matrix interaction is present. The different pretreated materials gave different extraction results. As was shown by Hegel *et al.*³⁰ for *S. cerevisiae*, the key step for yeast oil extraction is the pretreatment

Table 4. Soxhlet lipid extraction results using 2 chloroform:1 methanol (by volume) mixture as solvent

	Total extract yield [% w/w of dry mass]	PL fraction yield [% w/w of dry mass]	TAG fraction yield [% w/w of dry mass]
DM	11.9 ± 1.2	1.2 ± 0.1	7.4 ± 0.6
ATOM	9.9 ± 1.0	1.1 ± 0.1	4.9 ± 0.5
MeM	14.7 ± 1.4	0.7 ± 0.1	8.4 ± 0.7
EtM	29.7 ± 2.5	3.1 ± 0.3	13.4 ± 1.2
SCD	15.5 ± 1-5	2.0 ± 0.2	7.8 ± 0.7

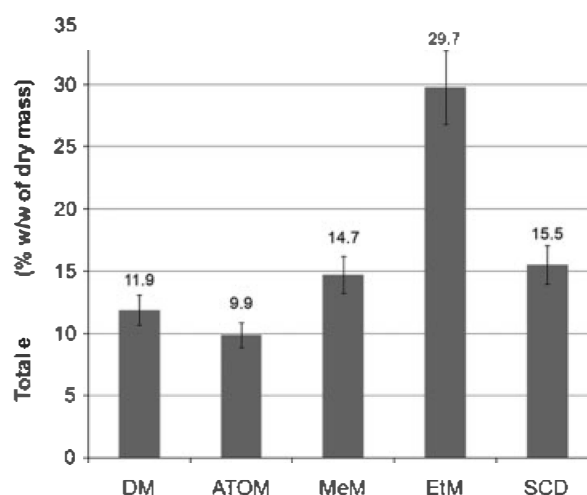


Figure 7. Experimental total extract yields for the Soxhlet method (in % w/w of dry mass). The bars correspond to the different pretreated yeast materials (Table 1). Dried and milled yeast (DM); atomized yeast (ATOM); methanol macerated yeast (MeM); ethanol macerated yeast (EtM); supercritical disrupted yeast (SCD).

of the matrix. The Soxhlet technique was unable to complete the lipid extraction in almost all the cases studied.

Accelerated solvent extractions

In the present work, the aim of the ASE experiments was to assess the influence of the different pretreatments and not to perform complete lipid extraction. For each extraction, 40 min and less than 40 mL of solvent mixture were used. The experimental results obtained are shown in Table 5. Figure 9 displays a bar graph with the total extract yield. Due to the small amounts of extract obtained in each ASE run, it was not possible to differentiate the lipid composition (TAGs, PLs) in the total extract (following Hubbard *et al.*'s purification procedure¹⁹). Here also, the pretreatments have a remarkable effect. The highest yield was obtained for the EtM yeast. The total extraction yield reached 27.7 ± 0.3% w/w of dry mass. This result is consistent with the one obtained for the Soxhlet extraction. The unexpectedly high mass of total extract could be associated with an incomplete drying process. The MeM yeast and the ATOM yeast showed similar results with a total extraction yield of 16.2 ± 0.3% w/w of dry mass and 15.6 ± 0.3% w/w of dry mass, respectively. The lowest total extraction yields were obtained for the SCD yeast and the DM yeast with values of 11.5 ± 0.3% w/w of dry mass and 11.6 ± 0.3% w/w of dry mass respectively. According to these results, ASE extraction performance is comparable with that of Soxhlet extraction but saving time and materials.

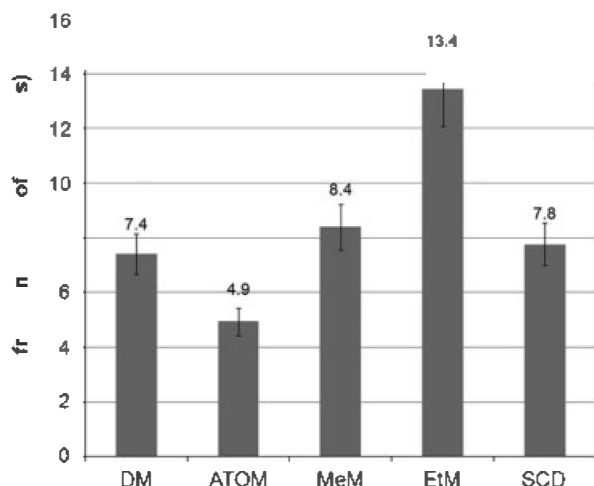


Figure 8. Experimental TAG fraction yields for the Soxhlet method (in % w/w of dry mass). The bars correspond to the different pretreated yeast materials (Table 1). Dried and milled yeast (DM); atomized yeast (ATOM); methanol macerated yeast (MeM); ethanol macerated yeast (EtM); supercritical disrupted yeast (SCD).

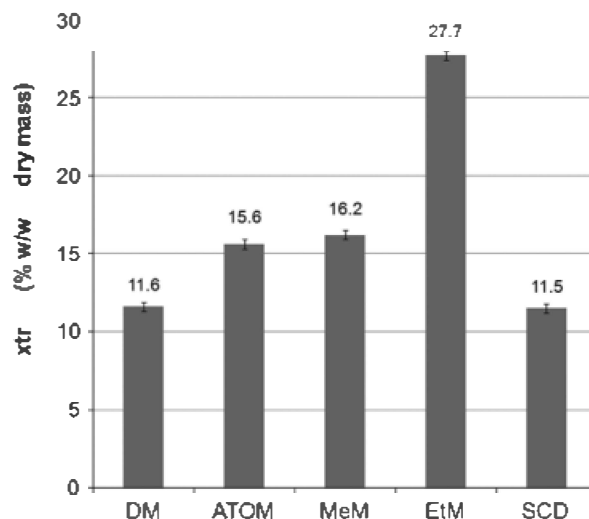


Figure 9. Experimental total extract yields for the ASE method (in % w/w of dry mass). The bars correspond to the different pretreated yeast materials (Table 1). Dried and milled yeast (DM); atomized yeast (ATOM); methanol macerated yeast (MeM); ethanol macerated yeast (EtM); supercritical disrupted yeast (SCD).

Table 5. ASE results. The extraction conditions are listed in Table 1

	Total extract yield [% w/w of dry mass]
DM	11.6 ± 0.3
ATOM	15.6 ± 0.3
MeM	16.2 ± 0.3
EtM	27.7 ± 0.3
SCD	11.5 ± 0.3

Supercritical extractions

Figure 10 shows the results of the extraction for the different pretreated yeasts. The residence time of the solvent was kept constant in all the experiments. The comparative results of the 'SCCO₂ + ethanol' extractions are shown in Fig. 10. The dashed lines in Fig. 10 correspond to smoothed data and have been added to facilitate visualization of the trends.

In the first part of the extraction curves the solute concentration in the fluid phase is generally high, as can be seen from the high slopes of the curves. The different pretreatments can affect the interactions between the lipids and other biomolecules. In the first extraction period, the accessible free solute can easily be removed by the solvent. The amount of free solute depends on the efficiency of the pretreatment. High lipid concentration in the output fluid phase can be found in this extraction step. In the second period of the extraction curves, the solute concentration in the output fluid phase decreases significantly. This is a diffusion-controlled extraction stage characterized by a strong solute–matrix interaction.

The results in Fig. 10 show that the best performance at the end of the extraction run was obtained for the EtM yeast. In this case, the solute concentration in the fluid phase reached a value of 4.3 mg of lipids per g of solvent in the first part of the extraction curve. The total extraction yield at the end of the extraction was 10.8% w/w of dry mass. For the ATOM yeast, the total extract yield reached almost 8% w/w of dry mass but the shape of the extraction curve is markedly different. The initial solute concentration is very low (0.25 mg of lipids per g of solvent) and increases during the

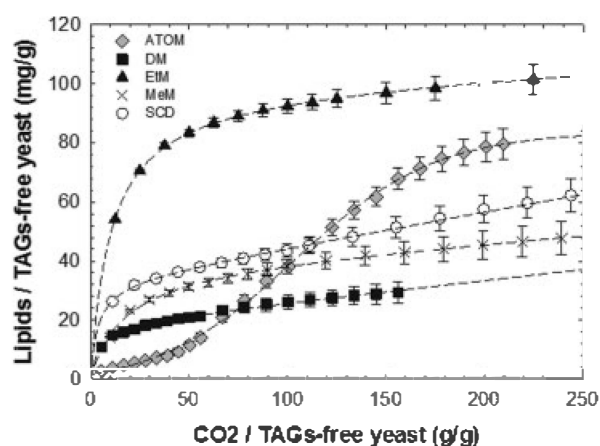


Figure 10. SCCO₂ + cosolvent experimental extraction curves for different pretreated *Y. lipolytica* yeast samples. Extraction conditions: 30 g CO₂ min⁻¹, 3 g EtOH/min, 40 °C and 20 MPa in the extractor. Dashed lines: smoothed data. Filled squares: dried and milled yeast (DM); X: methanol macerated yeast (MeM); empty circles: supercritical disrupted yeast (SCD); grey rhombuses: atomized yeast (ATOM); black triangles: ethanol macerated yeast (EtM). The retention time was the same for all the experiments.

extraction. The highest concentration observed along the curve is around 0.5 mg of lipids per g of solvent. In the final part of the extraction curve, the solute concentration in the fluid phase decreases again. For the SCD yeast, the initial slope is 2.4 mg of lipids per g of solvent. In the second period of the extraction curve, a relatively high oil concentration, compared with the other curves (0.25 mg of lipids per g of solvent), is observed for this extraction stage. This relatively high slope is evidence of incomplete solute extraction caused by a strong matrix–lipid interaction. The yield at the end of the extraction reaches a value of 6.4% w/w of dry mass. The MeM yeast and the DM yeast show low initial concentrations, less than 2 mg of lipids per g of solvent, and also low total extract yield at the end of the extraction, less than 5% w/w of dry mass.

To improve the extraction yields, two combinations of different pretreatments were used (described in Fig. 1). A maceration step

Table 6. Extraction results for the combined pretreatments listed in Table 1

	EtMSCD total extract yield [% w/w of dry mass]	WEtMSCD total extract yield [% w/w of dry mass]
Pre-extraction (maceration) with ethanol	4.1 ± 0.2	22.2 ± 1.0
SC extraction	3.3 ± 0.2	3.2 ± 0.2
Total	7.4 ± 0.3	25.4 ± 1.0

was combined with a drying and milling process and a supercritical disruption in different sequences. In case A of Fig. 1, the centrifuged cream yeast was subjected to a drying process (15 h at 60 °C) followed by a milling step (mesh = 1 mm). The dried material was macerated with ethanol for 20 hours at ambient temperature. Finally, the material was filtered and placed in the extractor. Then a supercritical disruption (2 h at 40 °C and 20 MPa and suddenly decompressed) was performed before the extraction. To quantify the amount of lipids extracted by the ethanol in the maceration step, the solute was separated from the solvent in a rota-evaporator, operated with a vacuum pump. In Table 1, the acronym used for this pretreated material is EtMSCD. In case B of Fig. 1, the centrifuged cream yeast was not dried before maceration with ethanol. The wet yeast was left in contact with ethanol for 20 h at ambient temperature and then centrifuged again. The amount of lipids extracted by the ethanol in the maceration step was quantified, separating the solute from the solvent in the ethanol-rich liquid phase using the same rota-evaporator described above. The yeast gel obtained after maceration was milled and placed in the extractor. A supercritical disruption (2 h at 40 °C and 20 MPa and suddenly decompressed) was performed before the extraction. The advantage of this combination of pretreatments is that it can be done *in situ* in an industrial extractor, because no drying step is involved. In Table 1, the acronym used for this pretreated material is WEtMSCD.

The extraction results for these combined methods are shown in Table 6. The corresponding extraction curves are presented in Fig. 11. The EtMSCD pretreated material exhibited low yield (7.4 ± 0.3% w/w of dry mass). It is very likely that part of the lipids was extracted by ethanol during the maceration step because, in this case, ethanol was separated from the solid by filtration, entraining the dissolved lipids. Regarding this result, the ethanol maceration step not only modified the matrix, allowing the extraction of bonded lipids, but it constituted a pre-extraction step. More than 50% of the total extract was obtained from the maceration step (determined by evaporation of the filtrate). The last experimental points of the extraction curve for this material show a solute concentration in the fluid phase (i.e. the slope of the extraction curve) of 0.12 mg of lipids per g of solvent. This value of concentration demonstrates that solute extraction was not complete at the end of the experiment and that there is a strong interaction between the solute and the matrix.

For the WEtMSCD pretreated material, similar behavior was observed but with an increase in the yield. The total extraction yield reached 25.4 ± 1.0% w/w of dry mass. In Fig. 11, the extraction curve for this material shows asymptotic behavior. The slope of the curve at the end of the experiment is almost zero, showing that solute extraction was complete. The fraction of the total extraction

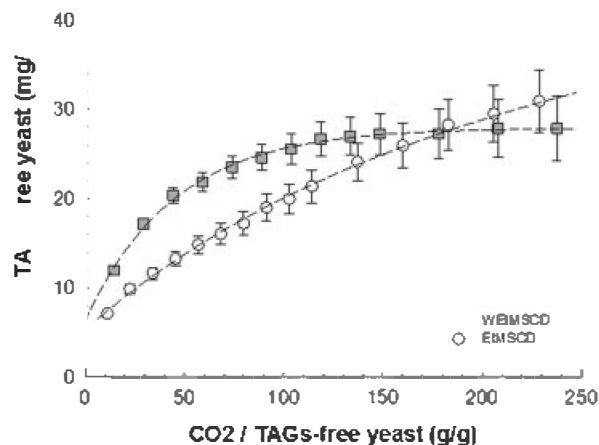


Figure 11. SCCO₂ + cosolvent experimental extraction curves for the different combined pretreatments in *Y. lipolytica* yeast samples. Extraction conditions: 30 g CO₂ min⁻¹, 3 g ethanol min⁻¹, 40 °C and 20 MPa in the extractor. Dashed lines: smoothed data. Empty circles: EtMSCD yeast (see Table 1). Grey squares: WEtMSCD yeast (see Table 1). The retention time was the same for all the experiments.

yield corresponding to the ethanol maceration is higher than 85%. As was shown for the EtMSCD yeast, the maceration constituted a pre-extraction step. Nevertheless, the TAG fraction in the ethanol soluble extract was 30%. The ethanol-maceration pre-extraction step is not selective towards TAGs.

These results indicate that oil can be extracted from yeast in two different stages, non-selective ethanol extraction followed by TAGs-selective SCCO₂ purification.

DISCUSSION

The results shown above with the different extraction techniques indicate that the key step for complete lipid extraction is pretreatment of the matrix to weaken the interaction between lipids and biopolymers present in the membranes.

Drying is a thermal pretreatment usually applied in the edible oil industry in order to coagulate proteins, to decrease oil affinity for solid surfaces and to agglomerate the oil into larger droplets.³³ The extraction curve for the DM pretreated material (Fig. 10) showed that soluble lipids still have a strong interaction with the matrix at the start of the extraction process. The output fluid phase concentration in the first extraction period was less than 2 mg of lipids per g of solvent. This value is lower than the initial output fluid phase concentration obtained in previous work by Hegel *et al.*³⁰ for the extraction of TAGs from *S. cerevisiae* with the same operating conditions in the same extractor, using oil-impregnated glass beads. Also, this value is much lower than the thermodynamic solubility, which was experimentally determined in our high pressure cell. This low yield could be attributed to the coagulation and agglomeration of proteins, which encapsulate the lipids to be extracted. These phenomena are caused by exposure to high temperature. Also, the incomplete drying of the material could lower the extraction efficiency due to hydrophilic hindrance of the lipids to be extracted. However, the ethanol presence could help to dehydrate the material.

The hypothesis for the low yield attributed to the coagulation of proteins in the drying process at 60 °C can be confirmed by looking at the results for the ATOM yeast. In this case, the drying of the material was produced by rapid pulverization in heated air. According to the cumulative extraction curve, we can see that,

at the end of the process, a larger amount of solute could be extracted from the ATOM yeast than the DM yeast. Nevertheless, the extraction curve of this material shows an unexpected shape, with a low solute concentration obtained in the first period of the extraction. This peculiar behavior is still not explained.

The SCD yeast was prepared following the procedure described by Lin *et al.*¹⁴ According to these authors, the pressurized CO₂ penetrates into the cells and breaks the cell walls during the releasing process. However, SCF disruption of the yeasts barely improved the results, when compared with the other pretreatments. The TAG fraction of extracted lipids was also found to be similar to our previous results.

Maceration in polar solvents, like alcohols, makes it possible to disrupt the interaction between lipids and other biopolymer molecules containing hydrogen bonds (COOH, NH₃, and polar functional groups of proteins). The extraction yields from this material revealed the noteworthy impact of the pretreatment. The fraction of non-bonded lipids in the membrane was significantly increased after this pretreatment. The initial output fluid phase concentration was 4.3 mg of lipids per g of solvent. This increase in the concentration of the output extract indicates the presence of larger amounts of free-oil, compared with other pretreatments.

The combined pretreatment EtMSCD demonstrates that the drying process at 60 °C can coagulate the yeast proteins, leading to a decrease in the total extract yield. Without the drying process, as can be seen for the WEtMSCD pretreated yeast, the ethanol-maceration pre-extraction step yields a large amount of extract. This combined pretreatment also demonstrates that the yeast lipids are soluble in ethanol and that the SCCO₂ extraction step can be useful for a TAG purification process.

CONCLUSION

Extraction of lipids from a non-conventional matrix, the yeast *Y. lipolytica*, has been carried out using three different extraction techniques: (i) Soxhlet extraction, (ii) ASE, and (iii) SCCO₂ + ethanol high pressure extraction. In order to determine the maximum possible solute content at the output of the supercritical extractor, yeast oil solubility in pure CO₂ and in 'CO₂ + ethanol' was measured in a high pressure cell. In both cases, the oil solubility increased with increasing pressure at a given temperature. Also, a retrograde solubility phenomenon was observed in the range of pressures tested. Roughly, at the conditions of the supercritical extractor (40 °C and 20 MPa), a maximum concentration of 10 mg of yeast oil per g of solvent can be expected in pure CO₂. The addition of ethanol increases this value to almost 15 mg of yeast oil per g of solvent.

The Soxhlet extraction using a chloroform:methanol (2:1 by volume) solvent mixture showed different yields depending on the type of pretreatment. The maximum TAG fraction and total extract were obtained for the EtM yeast. At the conditions tested in this work (8 h), this technique was unable to complete the lipid extraction in all the cases studied. The ASE results were similar to those obtained for the Soxhlet extraction. The highest yield was obtained for the EtM yeast with a total extract yield of almost 27.7 ± 0.3% w/w of dry mass. ASE reduced the time and the material needed to evaluate the effectiveness of the different pretreatments compared with Soxhlet. 'SCCO₂ + ethanol' supercritical extraction curves revealed the extraction mechanism. In the first part of the extraction curves, generally, the solute is not bound with other biomolecules and its concentration in the fluid phase has the

highest value of the entire extraction curve. The second part of the extraction curves corresponds to a diffusion-controlled extraction stage, where the solute concentration in the fluid phase decreases significantly. The best total extraction performance was obtained for the EtM yeast. In all cases studied a strong solute-matrix interaction was observed and the performance of the extraction was markedly dependent on the pretreatment of the material. Combined pretreatments revealed that the alcohol maceration constituted a pre-extraction step not selective toward TAGs. Oil can be extracted from *Y. lipolytica* in two different steps: a non-selective ethanol extraction followed by TAG-selective SCCO₂ purification.

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