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Optimizing pressurized liquid extraction of microbial lipids using the response surface method

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ABSTRACT

Response surface methodology (RSM) was used for the determination of optimum extraction parameters to reach maximum lipid extraction yield with yeast. Total lipids were extracted from oleaginous yeast (*Rhodotorula glutinis*) using pressurized liquid extraction (PLE). The effects of extraction parameters on lipid extraction yield were studied by employing a second-order central composite design. The optimal condition was obtained as three cycles of 15 min at 100 °C with a ratio of 144 g of hydromatrix per 100 g of dry cell weight. Different analysis methods were used to compare the optimized PLE method with two conventional methods (Soxhlet and modification of Bligh and Dyer methods) under efficiency, selectivity and reproducibility criteria thanks to gravimetric analysis, GC with flame ionization detector, High Performance Liquid Chromatography linked to Evaporative Light Scattering Detector (HPLC-ELSD) and thin-layer chromatographic analysis. For each sample, the lipid extraction yield with optimized PLE was higher than those obtained with referenced methods (Soxhlet and Bligh and Dyer methods with, respectively, a recovery of 78% and 85% compared to PLE method). Moreover, the use of PLE led to major advantages such as an analysis time reduction by a factor of 10 and solvent quantity reduction by 70%, compared with traditional extraction methods.

1. Introduction

Algae [1,2] and oleaginous microorganisms [3–6] are new routes of major interest for the production of specific lipids from renewable resources, intended, for example, for use as biodiesel. The development of reliable methods for lipid extraction from cells is necessary for the accurate quantification and determination of the composition of all classes of accumulated lipid. The "ideal" extraction method should be quantitative, non-destructive, reproducible, rapid and sparing on solvent. The complexity of the yeast lipidome is linked to the various functions, localizations and chemical structures (acyl chain length, degree of desaturation, phosphorylation and hydroxylation) of compounds. Yeast lipid classes include triacylglycerols, easily soluble in non-polar organic solvents such as n-hexane, and complex lipids, such as phospholipids, glycolipids, partial glycerides and unsaponifable lipids (tocopherols, sterols and carotenoids). These complex lipids are tightly held to the cell components by hydrophobic bonds, Van der Waals forces and hydrogen or ionic bonding. This extraction method requires the use of polar solvents to overcome interactions. Many well-known extraction methods have been applied to food lipids during the last century such as Soxhlet, Bligh and Dyer [7], and Folch [8] processes; in last decades, supercritical fluid extraction and, more recently, pressurized liquid extraction (PLE) have been successfully developed to enhance lipid extraction. Using PLE reduced the extraction time and quantity of organic solvents required under high-temperature and high-pressure conditions. Many applications of PLE methods have been reported such as the extraction of compounds from fruit [9–11], from animal [12–18], from soil [19,20], from algae [21,22] but few from yeast [23–25]. According to the large potential of lipid production with oleaginous microorganisms, the application of PLE methods to lipid extraction from yeast is a major challenge but with promising perspectives.

The purpose of the present investigation was to optimize important operating parameters (duration, extraction temperature and the quantity of diatomaceous dispersant required for efficient extraction) of the PLE procedure. This was done using response surface methodology (RSM) to obtain high lipid extraction yields from oleaginous yeasts with a lipid content ranging from 20% to 70% ($[g_{lip}\, g_X^{-1}]$ lipid mass per dry cell weight). These conditions were suitable to compare performance between PLE method with two conventional extraction methods such as Bligh and Dyer and Soxhlet methods.

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2. Materials and methods

2.1. Reagents

2.1.1. Microorganism

Yeast strain *Rhodotorula glutinis* was supplied by the *Coleccion Española de Cultivo Tipo* (CECT), University of Valencia, Spain.

2.2. Medium and growth conditions

Three pre-cultures were carried out: 8 mL (Lysogony broth-rich medium), 80 mL and 800 mL (mineral medium) in Erlenmeyer flasks containing at 30 °C on a rotary shaker (100 rpm). Mineral medium composition was in g L^{-1}: KH_2PO_4, 4.54; $(NH_4)_2HPO_4$ 0.83; $(NH_4)_2SO_4$, 2.47; $MgSO_4$. 7 H_2O, 1.7; $ZnSO_4$. 7 H_2O, 0.016; $FeSO_4$. 7 H_2O $MnSO_4$. H_2O, 0.0029; $CoCl_2$. 6 H_2O, 0.025; $CuSO_4$. 5 H_2O, 0.0031; Na_2MoSO_4. 2 H_2O, 0.0012; $CaCl_2$. 2 H_2O, 0.018; NaCl, 0.040; and 10 mL vitamin solution. Vitamin solution was prepared with the following composition: d-biotin: 0.05 g L^{-1}, thiamine hydrochloride: 1 g L^{-1}, pantothenic acid: 1 g L^{-1}, pyridoxol hydrochloride: 1 g L^{-1}, nicotinic acid: 1 g L^{-1}, p-aminobenzoic acid: 0.2 g L^{-1}, myo-inositol: 25 g L^{-1}. The pH of this medium was adjusted to 5.5 with phosphoric acid. The glucose concentration was 10 g L^{-1}.

2.3. Culture

Fed-batch experiments were performed in a 20 L fermentor using the Braun Biostat E fermenting system (Braun, Melsungen, Germany) [26]. The temperature was regulated at 30 °C, and the pH at 5.5 with the addition of ammonia solution or KOH solution (10 mol L^{-1}). An overpressure of 0.3 bar was maintained in the reactor. The fermentor was supplied with 3 sterile feeds using a peristaltic pump (Masterflex and Gilson). The glucose feed concentration was 740 g L^{-1}. The second feed was concentrated salts with the following composition in g L^{-1}; $MgSO_4$. 7 H_2O, 5.278; $MnSO_4$. H_2O, 0.009; $CaCl_2$. 2 H_2O, 0.051; Na_2MoO_4. 2 H_2O, 0.0360; $FeSO_4$. 7 H_2O, 0.215; H_3PO_4, 28.620; $ZnSO_4$. 7 H_2O, 0.078; H_2SO_4, 16.370; $CuSO_4$. 5 H_2O, 0.009; H_3BO_3, 0.0025; $CoCl_2$. 6 H_2O, 0.158; KCl, 7.450 g L^{-1}. The third feed was the nitrogen source: a 5 mol L^{-1} ammonia solution. Vitamin solution was added correlated to biomass growth (10 mL of vitamin mixture were added when 10 g_X L^{-1} of biomass was formed). The fermentor was connected to a computer. Homemade software enabled on-line acquisition, monitoring and regulation of operating parameters (stirring rate, pH, temperature, partial pressure of dissolved oxygen (DO), base and antifoam additions, etc.). The mass of glucose added to the fermentor was estimated on-line by weighing (CPA16001S, Sartorius (Goettingen, Germany). Outlet gas was analysed by mass spectrometry after the gas condenser. The mass spectrometer (Prima 600s; VG Gas, Manchester, United Kingdom) was used for its accuracy to measure CO_2, O_2, N_2, and Ar compositions. O_2 consumption rate and CO_2 production rate were calculated from mass balances, taking into account the evolution of the gas volume in the reactor, inlet airflow (measured by a mass flowmeter, Brooks, USA), temperature, humidity and pressure. The glucose concentration in the fermentor was evaluated by homemade software based on mass balance on carbon taking into account on-line acquisition data (glucose precision scale, gas analysis and inlet/outlet gas flow). This software was coded in TurboPascal version 4.

2.4. Extraction methods for total lipids

For biomass samples from yeast culture, cells were washed twice with saline (NaCl 9 g L^{-1}) and then lyophilized (Serail, RP35). Two reference methods were used to evaluate the lipid extraction yield of the PLE method: the modification of the Bligh and Dyer procedure and the Soxhlet method. While non-polar solvents like hexane or chloroform are excellent solvents for non-polar organics like triacylglycerols, their ability to extract polar organics such as phospholipids is often poor. Binary solvent mixtures such as chloroform–methanol were therefore used for all methods.

2.4.1. Modified Bligh and Dyer method

The modified Bligh and Dyer method used a procedure improved by Cot et al. [27]: 500 mg of cell dry mass were suspended in 15 mL of solvent mix in a tube. After the first extraction by methanol/chloroform (2:1, v/v), the remaining cell lipids were further extracted successively with two methanol/chloroform mixtures (1:1, v/v) and (1:2, v/v). Each extraction step consisted of incubation for about 24 h at room temperature on a roller mixer. Finally, the three organic phases were brought together.

2.4.2. Soxhlet method

Linked to extraction cell size, 3 g of sample were used for this extraction method mixed with 2 g of hydromatrix placed in a 22 mm × 80 mm extraction thimble and extracted with 150 mL mixture for 15 h in a Soxhlet apparatus with a frequency of 5 cycles h^{-1}. The same solvent mixtures used with modified Bligh and Dyer method were tested with three successive 15 h Soxhlet extractions.

2.4.3. PLE method

The PLE system, ASE 300, was provided by Dionex. The sample was placed in a stainless steel cell (11 mL) linked to electronic controllers which maintain extraction parameters (pressure, temperature, volume of extraction solvent and extraction time) at the programmed set points. The extraction solvent was pumped through the extraction cell, fitted with a cellulose filter and a stainless steel frit at the outlet. Because of the texture of the freeze-dried yeast powder, the pressure applied during PLE compressed the powder preventing effective extraction. A dispersant was added to avoid this.

Several cycles of extraction with different solvent mixtures were used. This method was the modified Bligh and Dyer method adapted for yeast lipid extraction with three extraction cycles using three different chloroform/methanol solvent mixtures: 1:2; 1:1 and 2:1 (v/v). For each solvent mixture, 2 static cycles were applied.

For each experiment, 0.4–0.7 g of lyophilized biomass was subjected to PLE extraction. Up to 1 g of Hydromatrix® (Varian) was used as a dispersant [28] in the extraction cell.

2.4.4. Washing

All extraction methods extract non-lipid components, such as sugars, amino acids, proteins and salts. In order to remove these molecules, the organic phase was mixed with a KCl solution (0.08 g L^{-1}, pH 1) for 15 min on a roller mixer; then centrifuged (5000 × *g*, 10 min) to recover lipids as dry material after evaporation of the solvent with Genevac EZ-2 plus® (35 °C, 200–5 mbar).

2.5. Analyses of total lipids

2.5.1. Gravimetry

Total lipid content was quantified by weight after total drying (variation minus 0.2 mg between two successive evaporations).

2.5.2. GC with an extraction step

In order to quantify different fatty acids present in lipid extracts, free or linked fatty acids were methylated into fatty acid methyl esters (FAME) using trimethyl sulfonium hydroxide (TMSH, 0.2 M in methanol, Macherey-Nagel, Germany) [29]. GC analysis was carried out on a Hewlett-Packard 5890 gas chromatograph equipped with

Table 1
Evolution of mobile phase composition (v/v) during HPLC-ELSD vs. time. The eluents were (A) acetonitrile, (B) water + trifluoroacetic acid (0.1%, v/v) and (C) hexane + isopropanol (4/5, v/v).

Time (min)	A (%)	B (%)	C (%)
0	70	30	0
15	100	0	0
30	50	0	50
40	50	0	50

a 50 m × 250 μm × 0.25 μm WCOT fused silica column with the polar bonded phase CP-Select CB for FAME (Varian) and a flame ionization detector with Chromeleon® (Dionex) acquisition software, under the following conditions: carrier gas N_2 flow 1 mL min^{-1} column pressure 2.05 bar oven temperature 50–75 °C at 9 °C min^{-1}, 75–140 °C at 13 °C min^{-1}, 140–180 °C at 1.5 °C min^{-1} and finally 180–240 °C at 4.5 °C min^{-1}, injector temperature 140 °C, detector temperature 250 °C with 40 mL min^{-1} H_2 flow and 450 mL min^{-1} air flow. Identification and quantification of methyl esters were based on the comparison of retention times and peak areas of serial dilutions of commercial standards. Internal standards were C9:0 for short carbon chain length fatty acids and C19:0 for medium carbon chain length fatty acids.

2.5.3. GC without an extraction step

Fatty acids from crude yeast oil were directly converted into their methyl esters using the method of Browse [30] and analysed by gas chromatography. This method is called digestion method.

2.5.4. HPLC ELSD

Mono, di and triacylglycerols were quantified by HPLC separation with an evaporative light scattering detector (ELSD). HPLC analyses were carried out on an Ultimate 3000 (Dionex) liquid chromatograph equipped with a 250 mm × 4 mm × 5 μm Prontosyl C30 column (ICS) and a 380-LC ELSD (Varian). The analysis conditions were: column flow 1 mL min^{-1}, oven temperature 40 °C, nitrogen flow 1 mL min^{-1}, nebulisation temperature 40 °C, evaporation temperature 60 °C.

A miscible gradient of 3 solvents mixtures ((A) acetonitrile, (B) water + trifluoroacetic acid (0.1%, v/v) and (C) hexane + isopropanol (4/5, v/v) was used to elute lipids with variable polarity. Gradient evolution is reported in Table 1.

2.5.5. Thin layer chromatography

In order to check that the extraction method was not selective regarding the lipid species, the different lipid classes were analysed by Thin Layer Chromatography (TLC) on 60F254 silica gel plates (10 cm × 20 cm, 250 μm, Merck, Darmstadt, Germany). The mobile phase was a hexane/methyl tert-butyl ether (MTBE)/acetic acid mixture (70 mL/30 mL/0.2 mL). Migration was stopped at half of the plate, and followed by a hexane migration. Hexane was used to separate free fatty acids, esterified fatty acids, diglycerides, triglycerides, ergosterol, lanosterol, squalene and sterol esters. Phosphatidylinositol, phosphatidylserine, phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, cardiolipin and phosphatidic acid were separated by a single migration with a chloroform/acetone/methanol/glacial acetic acid/water mixture (50 mL/15 mL/10 mL/10 mL/5 mL). Calibration was performed using a standard solution containing 0.5–4 mg of standards of each of the above compounds. Lipids separated on TLC plates were developed by spraying a 10 g L^{-1} $CuSO_4 \cdot 5\,H_2O$ solution made up in 8 g L^{-1} H_3PO_4 and heating at 180 °C until the appearance of brown spots. The different lipid standards were all prepared at the same concentration. Quantification was performed by image analysis. The image sample spots were compared to the image of different reference compound spots under criteria of number of pixels area. Standard curves were realized with five different concentrations.

Table 2
Ranges of the three independent variables used in RSM. Dispersant quantity % [$g_H\, g_X^{-1}$] (mass of dispersant [g] per gram of dry cell weight).

Factors	Definition	Levels				
		$-\alpha$	−1	0	1	α
X_1	Time [min]	5	10	20	30	35
X_2	Temperature [°C]	54	67	87	107	120
X_3	Dispersant quantity % [$g_H\, g_X^{-1}$]	0	26	72	118	144

Vitamins and lipid standards were all purchased from Sigma–Aldrich Chimie (Lyon, France) with a purity of at least 99.9%. All others chemicals (mineral medium, solvent and mobile phases) were all purchased from VWR (Fontenay sous bois, France) with a purity of at least 99%.

3. Results and discussion

3.1. Experimental design and optimization of PLE method

The extraction process is significantly influenced by various physical and chemical parameters. The first step in process optimization is screening of the most important parameters with an estimation of their optimal levels. According to preliminary studies (not shown), pressure, preheat time, flush ratio and purge time were found to have a minor effect on extraction yield. In the range of pressure tested (75–150 bar), no influence of pressure on extraction efficiency was detected (data not shown). Therefore, an operation pressure of 100 bar was maintained for all the trials undertaken to keep the solvent in the liquid state at high temperatures. Moreover, a short sample preheating phase of 5 min was imposed in order to limit spoiling of analyte. The flush ratio was fixed at 80% of cell volume with a purge time of 300 s in order to collect whole lipid extract. By quantifying lipid amount after two, three, four static cycles, results show that the benefit of a third or a fourth static cycle was about +0.4% of recovery compared to two static cycles. Therefore static cycles number was set at two. Moreover, the previously obtained results showed that, temperature, time and the relative quantity of dispersant were the most critical factors affecting extraction efficiency.

The conventional approach to optimize processes is to investigate one factor at a time, while keeping the others constant. However, this approach is time consuming and does not take into account interactions among factors. Response surface methodology (RSM), which combines statistical and mathematical techniques, is useful for developing, improving and optimizing processes [31–34]. So, initial screening factorial design and response surface analysis were used to determine the optimum values of the factors studied.

RSM was employed to optimize the three most significant factors (time (A), temperature (B), relative quantity of dispersant (C)) to enhance lipid extraction yield. The three independent variables were studied at five different levels ($-\alpha$, −1, 0, 1, α) (Table 2). A total of twenty combinations including five replicates at the centre point with each value coded as 0 (to get a good estimate of experimental error (pure error)) were chosen in random order according to central composite design configuration (CCD configuration) for the three factors (Table 3). The levels of the factors were chosen according to previous experiments. The statistical software package 'Design Expert 7.0' was used to analyse the experimental data [35]. All variables were taken at a central coded value of zero.

The average of three results of extraction coming from gravimetric determination (lipid content, [$g_{lip}\, g_X^{-1}$]) was taken as the

Table 3
Experimental plan for optimization of lipid extraction using RSM (X_1: time [min]; X_2: temperature [°C]; X_3: dispersant quantity % [$g_H\,g_X^{-1}$] (mass of dispersant [g] per gram of dry cell weight).

Trial	X_1	X_2	X_3	Lipid contents (%)	
				Observed	Predicted
1	5	87	72	0.280	0.301
2	20	87	72	0.424	0.420
3	20	120	72	0.436	0.413
4	20	54	72	0.360	0.359
5	10	107	26	0.241	0.243
6	20	87	72	0.424	0.420
7	35	87	72	0.433	0.392
8	20	87	72	0.424	0.420
9	30	107	118	0.491	0.506
10	20	87	72	0.424	0.420
11	10	67	118	0.423	0.402
12	30	67	118	0.487	0.485
13	20	87	72	0.424	0.420
14	10	107	118	0.483	0.463
15	20	87	72	0.424	0.420
16	30	67	26	0.243	0.265
17	20	87	144	0.530	0.526
18	20	87	0	0.175	0.156
19	30	107	26	0.246	0.268
20	10	67	26	0.211	0.200

response (Y). A multiple regression analysis of data was carried out to calculate the coefficients of the second order polynomial equation proposed to correlate the response to the three parameters:

$$Y = k_0 + \sum_{i=1}^{3} k_i * X_i + \sum_{i=1}^{3} k_{ii} * X_i X_i + \sum_{i<j}^{3} k_{ij} * X_i X_j \quad (1)$$

where Y is the predicted response, k_0 the intercept, k_i linear coefficients, k_{ii}, squared coefficients, k_{ij}, interaction coefficients and X_i, X_iX_i, and X_iX_j are combinations of the independent variables. The response surface curves were obtained using 'Design Expert' software to determine the optimum levels of variables for maximal lipid extraction yields.

3.2. Optimization of screened components

The statistical mean predicted and observed responses are presented in Table 2.

The response lipid amount of 20 sets of variable combinations was obtained (Table 3). Data of test of significance for regression square showed a standard deviation 0.022 g g^{-1} equivalent to a 5.82% coefficient of variation with a mean of 0.38 g g^{-1} for the response.

The ANOVA data were analysed to evaluate the significance of the different models equations (linear, quadratic, cubic, etc.) associated with models parameters established by regression calculations to fit all of the polynomial models to the selected response. The goodness of fit of models was checked by determination of coefficient R^2. Quadratic model with the highest adjusted R squared was selected. The value for the coefficient of determination (R^2) around the mean value was 97.7%. This value indicates adequacy of the applied model. 'Adj R^2' was adjusted for the number of terms in the model. The 'Pred R^2' of 0.8258 is in reasonable agreement with the 'adj R^2' of 0.9566. 'Adeq precision' is a signal-to-noise ratio: it compares the range of predicted values at fixed levels to average prediction error. The adequate precision of 24.294 indicates an adequate signal. The model can be used to navigate the design space [36].

Estimated values of regression coefficients were also obtained, and the regression model, a function of time, temperature and relative dispersant quantity, was predicted as:

Table 4
Test of significance for regression coefficient (X_1: time [min]; X_2: temperature [°C]; X_3: dispersant quantity % [$g_H\,g_X^{-1}$] (mass of dispersant [g] per gram of dry cell weight).

Source	Sum of squares	Mean square	F-value	P-value
Model	0.21	0.023	47.5	< 0.0001
X_1	9.83E−03	9.83E−03	20.17	0.0012
X_2	3.71E−03	3.71E−03	7.61	0.0202
X_3	0.17	0.17	356.21	< 0.0001
X_1X_2	8.76E−04	8.76E−04	1.8	0.2097
X_1X_3	1.62E−04	1.62E−04	0.33	0.5769
X_2X_3	1.31E−04	1.31E−04	0.27	0.6151
X_1X_1	0.01	0.01	20.57	0.0011
X_2X_2	2.01E−03	2.01E−03	4.12	0.0697
X_3X_3	0.011	0.011	23.04	0.0007

$$Y = 0.42 + 0.027 * X_1 + 0.016 * X_2 + 0.11 * X_3 - 0.026 * X_1^2 - 0.012 * X_2^2 - 0.028 * X_3^2 - 0.010 * X_1 * X_2 + 4.5 \times 10^{-3} * X_2 * X_3 + 4.5 \times 10^{-3} * X_1 * X_3 \quad (2)$$

The predicted values of lipid yields were calculated by using the predicted regression model and compared with experimental values (Table 3).

Table 4 sums up the test of significance for regression coefficient. The Model F-value of 47.5 implies the model is significant. There is only a 0.1% chance that a "Model F-value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. The "Lack of Fit F-value" of 26.08 implies the lack of fit is significant. There is only a 0.14% chance that a "Lack of Fit F-value" this large could occur due to noise. Analysis of variance indicated that the variables selected explained significantly the distribution of total lipid content. In this case, the three variables are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The interactive effects, time–temperature, temperature–dispersant and time–dispersant are not significant model terms.

The response model is mapped against two experimental factors while the third is held constant at its central level. The relationship between variables is illustrated by these three-dimensional plots. Fig. 1 shows the three dimensional plots of the effect of the independent variables time and temperature on the lipid content measured with PLE extraction with 144% of relative quantity of dispersant. Fig. 2 shows the three dimensional plots of the effect of the independent variables temperature and relative dispersant quantity on the lipid content measured with PLE extraction with

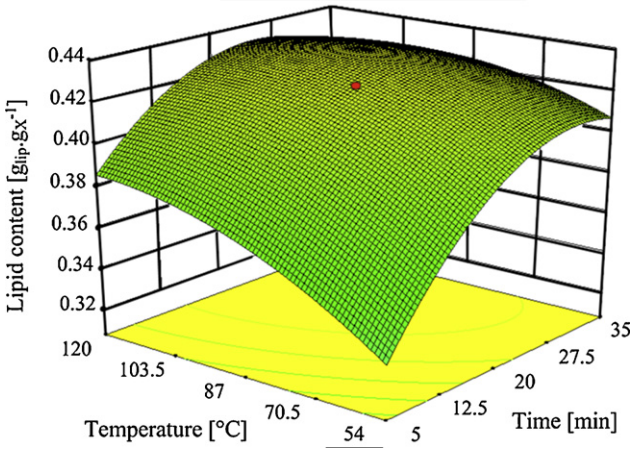

Fig. 1. Three dimensional plots of the effect of the independent variables time and temperature on the lipid content for a relative dispersant quantity of 144%.

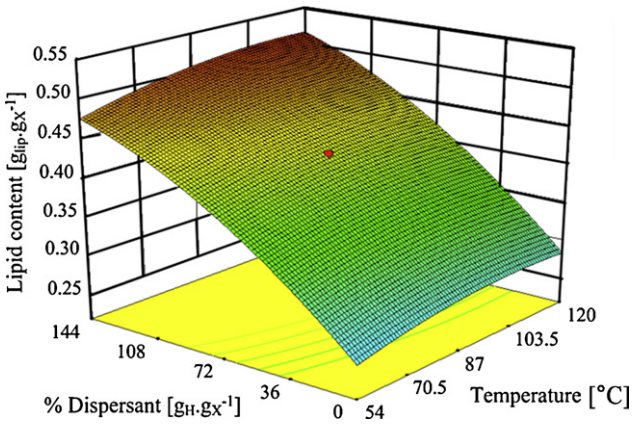

Fig. 2. Three dimensional plots of the effect of the independent variables temperature and relative dispersant quantity on the lipid content for a time of 25 min.

20 min of time. Fig. 3 shows the three dimensional plots of the effect of the independent variables time and relative dispersant quantity on the lipid content measured with PLE extraction at 100 °C.

Fig. 1 shows that with a relative quantity of dispersant of 144%, the lipid extraction yield was maximal when the temperature was between 90 °C and 110 °C during from 20 min to 27.5 min. By using equation 2, with a relative quantity of dispersant of 144%, the maximum lipid extraction yield was obtained at 102 °C and with an extraction time of 24.6 min. By decreasing the extraction duration from 25 min to 15 min, the average decrease of lipid extraction yield is lower than 5% of the maximum extraction yield. As this value is similar to experimental uncertainty, extraction duration can be reduced to 15 min.

These observations could be correlated to an increase of solubility, diffusion rates and mass transfer of lipid to solvent mixture vs. temperature, whereas viscosity and surface tension of the solvents are lower than at room temperature. Furthermore at higher operating temperatures, the activation energy of desorption is more readily overcome, and the kinetics of desorption and dissolution are also improved. Pressure helps to force liquid into pores and to keep the solvent in the liquid state at higher operating temperatures [37].

Fig. 2 shows that in the range of 5–40 min of extraction time, the extraction efficiency was increased by increasing the relative quantity of dispersant. This behavior may be connected to the decrease of density of lyophilized biomass improving solvent penetration and circulation of solvent in the lyophilisate matrix. In the range 0–144%

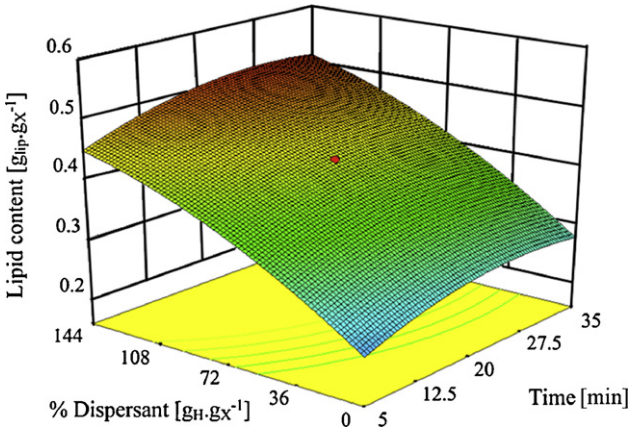

Fig. 3. Three dimensional plots of the effect of the independent variables time and relative dispersant quantity on the lipid content for a temperature of 100 °C.

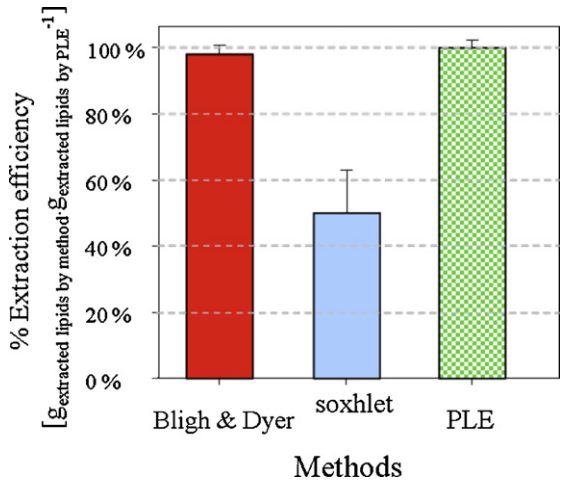

Fig. 4. Total lipid contents quantified by gravimetric measurements for different extraction methods normalized with respect to values obtained with PLE method.

of dispersant, the extraction efficiency was improved by increasing extraction time until 15 min, for longer extractions, the benefit of time increase was not significant compared to uncertainty of lipid quantification method.

The response surface methodology allowed us to simulate the effect of extraction time, operating temperature and relative dispersant quantity on lipid extraction yield with PLE. The following operating conditions: 15 min cycle time, at 100 °C with a relative dispersant quantity of 144% were selected in order to combine optimized extraction lipid yield with a short duration procedure.

3.3. Comparison of the PLE method with reference methods

Lipid extraction efficiency was compared between PLE and traditional methods using samples of *R. glutinis* cells with different lipid contents (8%, 13%, 35%, 46% and 65%); an average of two trials for each level of lipid was used for comparison.

Firstly, as shown in Fig. 4, with the same solvent mixtures, the difference of extraction efficiency between the modified Bligh and Dyer method and the PLE method was not significant whereas that obtained using the Soxhlet apparatus only reached 50% of the PLE value. This difference could be explained by the non-attendance of pressure during extraction compared to PLE method and the lack of mixing compared to modified Bligh and Dyer method.

The composition of lipid extracted using the modified Bligh and Dyer, the Soxhlet and the PLE methods was analysed by HPLC-ELS and the two TLC techniques. The results are shown in Fig. 5. For all lipid categories or subcategories, Bligh and Dyer and PLE methods were more efficient than Soxhlet method in term of yield. These methods extracted all lipid categories with the same efficiency. About 60% of polar lipids and 40% of non-polar lipids were extracted with Soxhlet method compared to PLE extraction method yield. A selectivity was observed with Soxhlet extraction method: it could be suggested that during Soxhlet extraction, due to the difference of chloroform and methanol volatility, a solvent composition variation occurred in the extraction thimble and affected extraction efficiency. Lack of extraction efficiency could arise from difference of solvent gradient composition. Only diacylglycerols, cardiolipid and phosphatidylcholine were better extracted with the modified Bligh and Dyer method but the difference was very low and included in the uncertainty of measurement method (1.5%).

In terms of fatty acid selectivity of the extraction method, the digestion method combined with GC measurement was used to normalize all the results. Relative extraction yield of PLE method

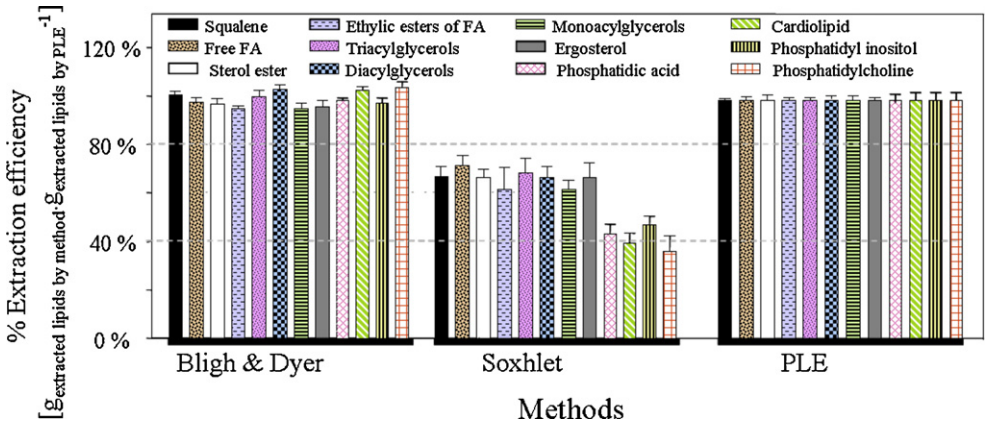

Fig. 5. Lipid classes contents quantified by HPLC-ELS and TLC methods for different extraction methods normalized by values obtained with PLE method.

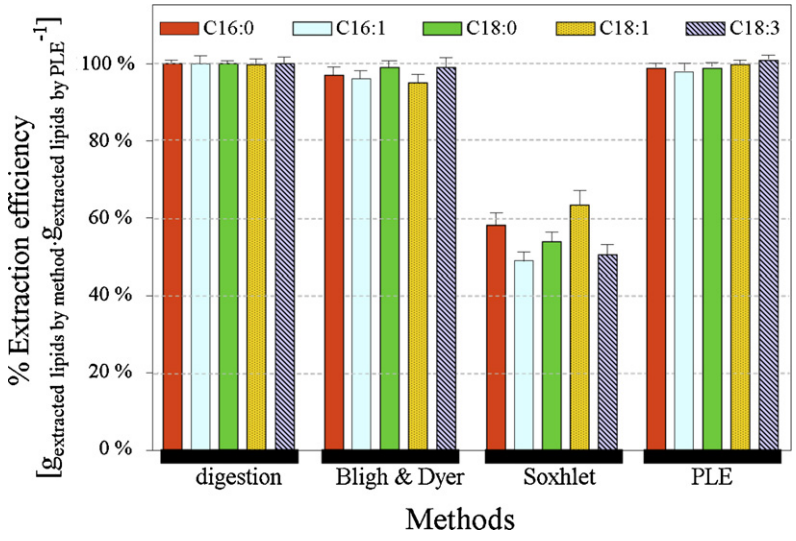

Fig. 6. Fatty acid contents for different extraction methods normalized with respect to yield obtained with digestion method.

ranged from 98% to 101% and from 95% to 99% for Bligh and Dyer method compared to digestion method extraction yield. Soxhlet method demonstrated the highest heterogeneity of extraction efficiency with a maximum of efficiency of 56% for palmitic acid and 43% for palmitoleic acid compared to digestion method extraction yield. The optimized PLE method gave the best global efficiency without selectivity for extracted lipids (Fig. 6).

4. Conclusions

Pressurized liquid extraction was applied to recover the total lipids of *R. glutinis* including triacylglycerols, phospholipids, sterols, etc. In this work, the effects on extraction efficiency of extraction cycle duration, extraction temperature and relative quantity of dispersant were studied and a polynomial model was established by means of response surface methodology. It showed that a cycle time of 15 min, a temperature of 100 °C and 144% dispersant were the best combination of operating parameters (criteria of reproducibility and extraction yield) to extract lipids from oleaginous yeast cells. The presence of the Hydromatrix® dispersant also avoided the compression of the cell lyophilisate and improved solvent access to the lipids within the cells. The composition of yeast lipid extracted with PLE was similar to that obtained with the modified Bligh and Dyer method, demonstrating the non-selectivity of PLE concerning lipid classes with PLE. As a conclusion, PLE was 5-fold faster and required 20-fold less solvent compared to Bligh and Dyer or Soxhlet methods. That is why PLE method constitutes an ideal alternative extraction method of yeast lipid. Moreover, the PLE system can be entirely automated.

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