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Fractionation process for the protective isolation of ergosterol and trehalose from microbial biomass

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Fractionation process for the protective isolation of ergosterol and trehalose from microbial biomass

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Abstract

A new process is described for the two phase extraction of ergosterol and trehalose from microbial biomass. Baker's yeast was used as a model organism to develop the method, which was then applied for extracting 13 oleaginous microbes. Major findings of the study were that the ergosterol content was not dependent on intracellular oil content and that 1-butanol and alkaline pH were needed to protect ergosterol. Saponification for 3–4 hours at 85–100 °C followed by extraction of the reaction mixture with toluene gave the maximal ergosterol yield. Trehalose was stable at this temperature and remained in water solution, but the maximal yield was obtained after a shorter reaction time at lower alkalinity. Although trehalose alone is stable at alkaline pH, extraction yields of trehalose from yeast decreased with increasing alkalinity. This finding led us to propose a two-step process in which trehalose is separated in the first step and ergosterol in the second. The possibility to apply this method to fractionate oleaginous microbes in process scale is discussed from technical viewpoints.

Keywords: ergosterol, trehalose, yeast, microbial oil, extraction, fractionation

1 Introduction

Microbial biomass contains variable amounts of valuable compounds such as lipids, proteins, carbohydrates and sterols. Ergosterol is an example of sterols that are uneconomical to produce via chemical synthesis, but can be produced in microbes.[1,2] It is a membrane sterol in certain groups of fungi, such as *Aspergillus* and *Penicillium*, and in yeasts particularly in the genus *Saccharomyces*. [3,4] It can be found in intracellular oil droplets where it is stored in esterified form and in the plasma membrane, where it influences fluidity, permeability of the membranes and the activity of membrane bound proteins.[5,6] Due to its aforementioned role in cellular biochemistry, ergosterol has been a subject of interest from the viewpoint of increasing ethanol tolerance of *S. cerevisiae*. [7–9]

The structure of ergosterol allows its use in microbial conversions to produce similar intermediates as are formed from phytosterols and cholesterol.[10] Although ergosterol is a possible precursor for steroids,[11] fractionation of abundant residual yeast biomass from industrial side-streams to ergosterol and other products has not been studied to a great extent. Steroidal derivatives can be used in cancer prevention and prostate problem protection,[12] and as a consequence the sterol markets are expected to increase remarkably within the next five years.

Ergosterol exists in yeasts both as steryl esters and as a free sterol.[3] Isolation of ergosterol from various microbial biomasses has been reported from an analytical perspective. These methods are based on the alkaline hydrolysis of cells in ethanol containing reaction mixtures at elevated temperatures, most often 80–90 °C for several hours. The scission of ergosterol esters is followed by extraction of ergosterol from the water suspension with organic

solvents.[13] The amount of sterols in yeasts is usually below 1% of cell dry weight (CDW).[2,14] Therefore, isolation of other valuable fractions is needed.

Trehalose is a disaccharide composed of two glucose units linked with an α -1,1 bond. It is widely distributed in nature and can be found in many organisms where it serves as an energy source and protects against dryness, freezing or osmotic pressure.[15,16] In the food industry trehalose is used as a low-calorie sweetener. Its sweetness is about 45% of that of sucrose.[17] Trehalose is produced industrially from starch using an enzymatic process,[15] but by fractionation of microbial biomass, co-production of trehalose with other products may be a viable alternative.[18] Yeasts are also suitable raw materials for the isolation of food-grade protein and carbohydrate fractions.[19] Moreover, microbial oil production based on oleaginous yeasts and process integration with butanol fermentation have been evaluated as possible routes to transportation fuels.[20] In addition to these relatively low-value fractions more value-added products are needed for better economical profitability. Achieving this goal requires process development for the isolation of intracellular biochemicals.

This report summarizes the development of a fractionation procedure liberating ergosterol and trehalose from yeasts. Ergosterol and trehalose yields from *S. cerevisiae* are compared to those of oleaginous yeasts. In addition, we determined the stabilities of ergosterol and trehalose, and propose an improved extraction process that takes into account the product recovery and recycling of solvents.

2 Materials and methods

2.1 Chemicals

Reagent grade chemicals were used in this work. The solvents used were methanol (Rathburn), ethanol (Primalco), 1-propanol (LAB-Scan), isopropanol (Lab-Scan), 1-butanol (Merck), iso-butanol (Fluka), 1-pentanol (Merck), isopentanol (Merck), 1-hexanol (Fluka), n-heptane (Riedel-de Haën), toluene (Sigma-Aldrich), dichloromethane (Rathburn), petroleum ether 40-60 (Riedel-de Haën), each with a purity of $\geq 98\%$. Moist commercial baker's yeast (Suomen hiiva Oy, Finland) mass was used in testing yeast fractionation and developing the fractionation procedure. Ergosterol (purity $> 98\%$) was purchased from Fluka, D(+)-trehalose (α -D-glucopyranosyl- α -D-glucopyranoside) dihydrate isolated from *Saccharomyces cerevisiae* was from Sigma, and D(-)-mannitol was from Merck.

2.2 Determination of solubility and stability properties of ergosterol

Solubility of ergosterol was determined in 11 organic solvents that were saturated with ergosterol at 40 °C. The solutions were let to cool to room temperature (20 °C) and allowed to stand for additional 20 h before analysis of ergosterol. Solubility of ergosterol at elevated temperatures was tested using 1-butanol, iso-butanol, 1-pentanol and iso-pentanol. These solvents were saturated with ergosterol at 50 °C or 70 °C and the solutions were allowed to stabilize for 20 h at 40 °C or 60 °C before analysis.

To find the optimum temperature and duration of extraction, ergosterol was dissolved in toluene. Two ml of this solution was mixed with 2 ml of 18 % (w/v) NaOH or 2 mL of water and the mixture was incubated at 85 and 100 °C for 0.5–4 hours. After the heating period

ergosterol was analyzed from the organic phase. To achieve better phase separation this procedure was modified by solubilizing ergosterol in 1-butanol/toluene 1:1 (v/v) mixture.

2.3 Development of the extraction procedure

The procedure investigated for ergosterol and trehalose isolation from yeasts was based on the simultaneous extraction and alkaline hydrolysis of membrane lipids and ergosterol esters in the presence of 1-butanol. Toluene was used in the second step to extract ergosterol from the water phase in which trehalose remains. To study the effect of extraction temperature, moist baker's yeast (0.3–0.5 g dry material) was mixed with 1 ml of aqueous 18 % (w/v) NaOH (w/v) and 1 ml of 1-butanol. The reaction mixtures were incubated at 85 and 100 °C for 0.5–4 hours. The tubes were mixed with a vortex mixer for 10 s occasionally during the incubation and after the addition of 1 ml of toluene. The extraction mixtures were centrifuged using Eppendorf 5084 centrifuge at 5000 rpm for 5 minutes. The samples were analyzed for ergosterol by HPLC after diluting 0.6 ml of the clear toluene/1-butanol phase with 0.6 ml of methanol. The above described procedure was also carried out either without 1-butanol or NaOH. In addition to 1-butanol, iso-butanol, pentanol and iso-pentanol were also tested as co-solvents at 85 °C.

The effect of alkalinity on ergosterol yield was studied by adding sodium hydroxide in concentrations of 0.1, 1.0, 10 and 15 % (w/v) to the extraction solvent system. In this case, 2 ml of aqueous sodium hydroxide and 2 ml of 1-butanol were added to 1 g of yeast (0.3 g as dry weight). This mixture was heated to 85 °C for 0.5–4 hours, and the subsequent extraction was carried out with 2 ml toluene as described above. Additionally, extraction of yeast was carried out according to the previously published method (Tan et al., 2003). Wet baker's yeast (0.05 g dry weight) was first incubated at 85–90 °C for 3 h in 32 mL of 25% (w/v) potassium hydroxide

in 60% (v/v) aqueous ethanol solution. The cooled suspension was extracted with 20 mL of petroleum ether, and ergosterol was analyzed from the sample withdrawn from the organic phase.

2.4 Extraction of ergosterol and trehalose from microbial biomass

Cells of 13 oleaginous yeasts were extracted with the method developed. The microbial biomass materials were derived from fed-batch fermentor cultivations conducted essentially as previously described.[20] The cells were harvested by centrifugation, washed with deionized water, and lyophilized for the determination of the lipid contents. The wet cell samples were stored at -22 °C until extraction. An aliquot of about 1 g of cell dry weight was weighed in a glass tube and 2 ml of 18 % NaOH (w/v) solution was added, followed by the addition of 2 ml of 1-butanol. These mixtures were vortex mixed for 15 seconds and the tubes were incubated at 90–100 °C for 0.5–4 hours. After the incubation, 2 ml of toluene was added over the water phase. All tubes were centrifuged with an Eppendorf 5804 centrifuge at 5000 rpm for 5 minutes. Ergosterol samples for HPLC analysis were taken from the organic phase. Trehalose was determined from the water phase. Experiments were performed as duplicates.

2.5 Analytical methods

Ergosterol was analyzed using an HP 1100 HPLC equipment with 100 % methanol as the eluent at a flow rate of 0.4 ml/min. The analytical column was Waters NovaPak 250 x 4.6 mm packed with C18 material having 4 µm particle size. The column oven temperature was 50 °C. Ergosterol was identified and quantified with a UV-detector at the wavelength of 282 nm against external standard calibration.

Trehalose was analyzed using a Shimadzu HPLC with the refractive index detector RID-10A. The analytical column used was SP0810 Shodex 8.0 mm ID x 300 mm with a Bio-Rad deashing guard column. The eluent was deionized water delivered at a flow rate of 0.7 ml/min and the column oven (CTO 20A) temperature was 60 °C. Trehalose was identified and quantified using authentic reagents for external standard calibration using the Class-VP program (Shimadzu).

Total fatty acid (FA) analysis of lyophilized yeast cells was carried out as previously described.[21] In this procedure, the fatty acids were saponified with 3.7 M NaOH in 49% (v/v) methanol at 100 °C for 30 min and then methylated with 48% (v/v) methanol in 3.3 M HCl at 80 °C for 10 min. Extraction of the aqueous suspension with hexane/methyl-tert-butyl ether (1:1, v/v) isolated methyl esters in the organic phase, which was washed with 0.3 M aqueous alkali. Nonadecanoic acid methyl ester (Sigma) was added to the samples as an internal standard. Analysis of the fatty acid methyl esters was performed with a Hewlett-Packard model 6890 gas chromatograph using an HP-FFAP column (25 m, 0.2 mm i.d., 0.33 µm film thickness) with a flame ionization detector (FID). The column temperature was programmed from 70 °C to 200 °C at a rate of 25 °C min⁻¹ and the total run time was 30 min. The individual fatty acids were identified from their GC peak retention times relative to fatty acid methyl ester standards (Sigma).

3 Results and discussion

This investigation was carried out to develop a scalable extraction process for the isolation of biochemicals from microbial biomass. The main motivation was to determine isolation yields

of ergosterol and trehalose, which are common intracellular components. The alkaline extraction procedure selected for these experiments leads to three phases that can be further treated to separate the products. Ergosterol can be recovered by extraction with an organic solvent, while trehalose and other water soluble components remain in the water phase and the residual yeast biomass remains as the solid phase.

3.1 Solubility and stability of ergosterol

A series of experiments was carried out to test the solubility and stability of ergosterol in various solvents and temperatures to find suitable extraction conditions. Stability of pure ergosterol without microbial biomass at elevated temperatures was investigated in solvent mixtures comprising different fractions of water, toluene, aqueous NaOH, and 1-butanol. In the toluene/deionized water system 43% of ergosterol was lost during 4 h incubation at 100 °C (Table 1). Degradation occurred to lower extent in the presence of alkali or 1-butanol; one fourth of the initial amount of ergosterol was lost after 4 hours incubation at 100 °C. However, when using a solvent system containing both aqueous alkali and 1-butanol it was possible to achieve unaltered concentration (within experimental error) of ergosterol after 4 h incubation. It is also noted that the stabilizing effect of aqueous alkali and 1-butanol on ergosterol could be verified at 85 °C and using a lower initial ergosterol concentration of 1 g/L (95.7% recovery with alkaline water and butanol compared to 68.6% without alkali after 3 h incubation). **The stabilizing effect of alkaline pH is in agreement with earlier literature.[22] Pure ergosterol has been found stable in methanol for 6 months at room temperature,[23] whereas photocatalytic reactions occur in the course of several days under ambient temperature and daylight[24] and dibutylhydroxytoluene has been used to prevent heat-induced oxidative degradation of ergosterol in 1-propanol.[25]**

Table 1. Percentage recovery of pure ergosterol when treated in various solvent mixtures at 100 °C for 0–4 h. The initial concentration of ergosterol was 8 g/L.

Time (h)	Composition of the solvent mixture			
	A	B	C	D
0	100.0	100.0	100.0	100.0
0.5	97.2	96.9	99.0	96.0
1.0	95.9	96.4	94.3	98.7
2.0	90.1	93.9	85.2	98.6
3.0	61.5	82.9	85.7	97.8
4.0	57.3	74.4	75.2	98.4

A: water : toluene (1:1 v/v); **B:** 18% (w/v) NaOH (aq) : toluene (1:1 v/v); **C:** water : toluene : butanol (2:1:1 v/v/v); **D:** 18% (w/v) NaOH (aq) : toluene : butanol (2:1:1 v/v/v).

Solubility of ergosterol was determined in 11 organic solvents to guide the selection of the extraction solvent. The solubility tests were first conducted at 20 °C. At this temperature the straight chains alcohols were better solvents than the corresponding iso-alcohols. The solubility of ergosterol increased with the carbon chain length of 1-alcohols until pentanol. Hexanol was less powerful solvent than butanol or pentanol. The best solvent for ergosterol was pentanol dissolving 35 g/l at 20 °C while the second best solvent butanol dissolved 27 g/l. Two other good solvents were iso-pentanol and iso-butanol, dissolving 24 g/l and 20 g/l, respectively. It was recently shown that 1-alcohols are good solvents for extracting lipids from microbial cells.[26] Alcohols containing less than four carbons are miscible in water but alcohols with ≥ 4 carbons are slightly soluble in water and are good ergosterol solvents. Overall, the alcohol functionality and octanol/water partition coefficient ($\text{Log } K_{ow}$) of 1–1.5 correlated with

enhanced dissolution of ergosterol as its solubility was much lower in dichloromethane, toluene, and n-heptane.

The partition coefficient influences the ease of solvent recycling and thus it was a positive finding that the water-miscible alcohols were not the best solvents for ergosterol. On the basis of the results presented in Table 2 four best solvents were tested further at elevated temperatures, 40 °C and 60 °C to assess the possibility of isolating ergosterol by cooling crystallization.

Table 2. The measured solubility (*S*) of ergosterol at 20 °C in various anhydrous solvents with differing properties.

Solvent	<i>S</i> (g/L)	Solvent properties			References
		bp. (°C) ^a	Log K _{ow} ^b	Water solubility at 25 °C (g/L)	
Methanol	0.8	65.0	-0.520	miscible	[27]
Ethanol	1.5 ^c	78.5	-0.300	miscible	[28]
1-Propanol	12.0	97.4	0.300	miscible	[29]
<i>iso</i> -Propanol	8.3	82.4	0.050	miscible	[29]
1-Butanol	27.0	117.3	0.930	72.5	[27,30]
<i>iso</i> -Butanol	19.9	108.0	0.760	81.0	[29,31]
1-Pentanol	32.3	137.3	1.480	21.7	[32,33]
<i>iso</i> -Pentanol	24.0	128.5 ^d	1.21 ^e	27.0	[31]
1-Hexanol	21.0	158.0	1.990	6.18	[32,34]
Dichloromethane	7.9	40.0	1.07 ^e	17.0	[35]
Toluene	10.9	110.6	2.680	0.53	[35,36]
n-Heptane	1.5	98.4	4.660	0.003	[37,38]

^a: Boiling points were retrieved from the Handbook of Chemistry and Physics, CRC Press, 1975. ^b:

Logarithm of the partition constant in 1-octanol/water. ^c: Source: Merck Index. ^d: determined at 750

mm Hg pressure. ^e: Obtained using the method of Broto et al. (1984).

Figure 1 shows the increasing solubility of ergosterol in response to elevated temperature. At 60 °C 1-butanol dissolved 87.6 g/l and 1-pentanol 107.5 g/l of ergosterol. The solubility data

in different temperatures are important in crystallization and purification of extracted ergosterol. The results indicate that when these solvents are used, 70% of ergosterol can be precipitated from saturated solutions of 1-butanol or 1-pentanol by cooling crystallization from 60 °C to 20 °C. The results at elevated temperatures confirmed that straight chain alcohols are better in dissolving ergosterol than iso-alcohols with the same carbon number. This could be explained by steric factors such as the larger space occupied by the hydrocarbon chain of the iso-alcohol when the OH groups of ergosterol and the solvent interact via weak interactions.

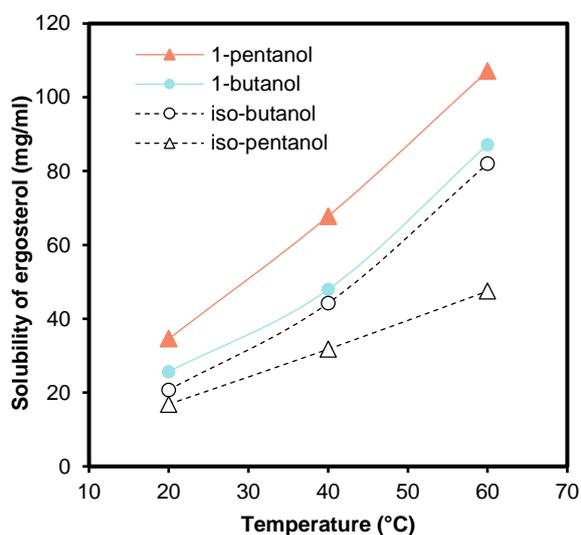


Figure 1. Solubility of ergosterol in non-aqueous alcohol solvents as a function of the temperature.

3.2 Effect of extraction solvents on the recovery of ergosterol and trehalose from baker's yeast

The objective of these experiments was to determine the extraction conditions for intracellular ergosterol and trehalose from yeast cells. For this, baker's yeast was extracted with aqueous sodium hydroxide, one of the various C4 or C5 alcohols, and toluene at 85 °C and the yields of ergosterol in the organic phase and trehalose in the water phase were determined. The highest extraction yields of ergosterol were obtained with 1-butanol and then with iso-butanol (Fig. 2).

Majority of ergosterol was released during the first two hours and the yield started to level-off towards four hours of extraction. Lower yields were obtained with 1-pentanol and iso-pentanol, which on the other hand showed a slow and almost linear release of ergosterol from 30 min to 4 h extraction (Fig. 2).

The finding that 1-pentanol extracted less ergosterol from yeast cells than 1-butanol did might be a result of the broader biphasic solubility of 1-butanol.[26] The solvent needs to possess sufficient aqueous solubility to dissolve in the cytosol and solubilize ergosterol released from saponified membranes. In contrast, the release of trehalose from the cytosol would be facilitated by the dispersing of saponified yeast intracellular molecules such as the fatty acid linoleic acid which is known to associate with trehalose.[15] This hypothesis accords well with the most prominent release of trehalose with iso-butanol that has the highest water-solubility among the alcohols (Table 2). Compared to the yields with other solvents, with iso-butanol 50% higher trehalose yield were obtained, and two hours was sufficient for extraction (Fig. 2). A schematic model showing for the extraction of ergosterol from yeast cells is shown in Fig. 3. The extraction is initiated by alkaline hydrolysis of the yeast cell wall and cell membrane. The extraction of the permeabilized cells follows in three steps (i) permeation of alcohol into the cytosol; (ii) solubilization of ergosterol released from cell membranes; (iii) leakage of alcohol solution of ergosterol from cells. Because the alkaline hydrolysis appears to be the key initial reaction, it was considered important to study how much sodium hydroxide is required in the extraction process.

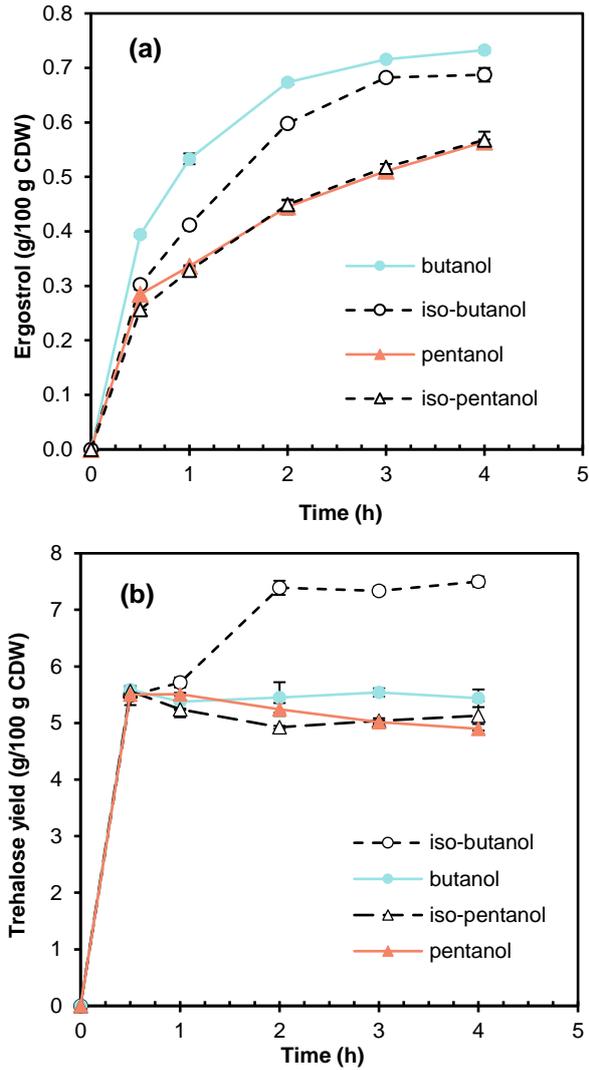


Figure 2. Yield of ergosterol (a) and trehalose (b) from baker's yeast extracted for 0.5–4 h at 85 °C with aqueous sodium hydroxide (15%) containing 1-butanol, iso-butanol, 1-pentanol, or iso-pentanol at the volume ratio of 1:1.

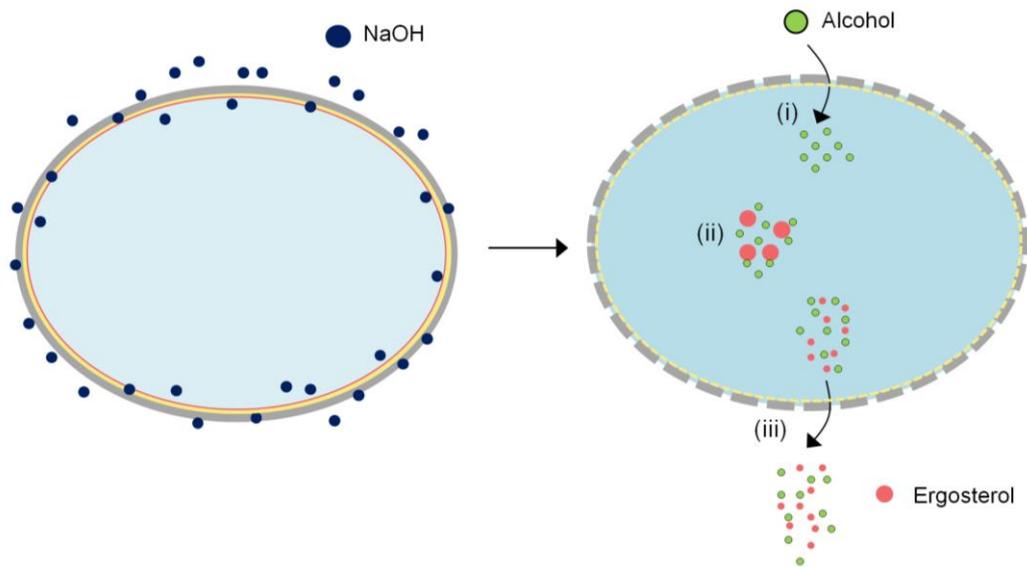


Figure 3. A model for alkaline solvent extraction of yeast cells. Saponification of lipid layers renders the cell membrane more permeable for extraction with alcohols in five stages: (i) permeation of alcohol through the permeated cell wall and cell membrane; (ii) solubilization of ergosterol by the alcohol; (iii) leakage of alcohol solution of ergosterol from cells.

3.3 Effect of alkalinity on ergosterol and trehalose yields from baker's yeast

The concentration of sodium hydroxide had a clear impact on the yield of ergosterol from yeast cells (Fig 4). Ergosterol yields were on the same level when alkalinity was varied between 10–15%, but notably lower yields were obtained in the case of 1% and 0.1 % alkalinity. Possible explanation is that under low alkalinity all lipids and steryl esters are not fully hydrolyzed. The lipid content of baker's yeast is approximately 4%, but in the case of oleaginous microbes intracellular storage lipids can consume notable amounts of alkali in the saponification reaction. Higher concentration of sodium hydroxide could thus be needed in the extraction of oleaginous microbes.

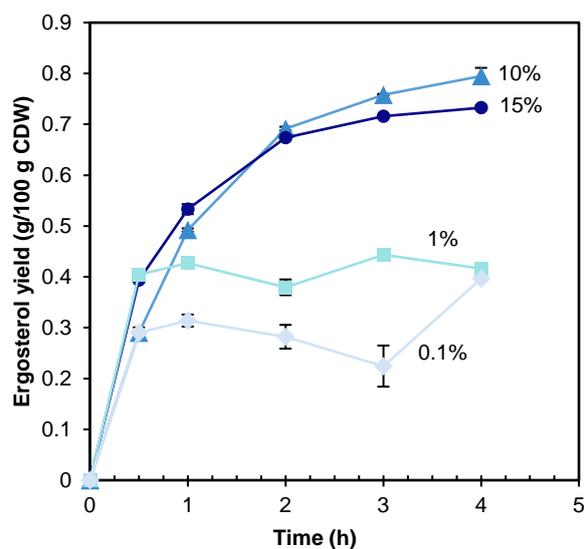


Figure 4. The effect of sodium hydroxide concentration on extraction yield of ergosterol from baker's yeast. About 300 mg baker's yeast (cell dry weight, CDW) was extracted at 85 °C with a biphasic solvent mixture comprising 2 mL of aqueous NaOH and 2 mL of 1-butanol. The released ergosterol was determined from the organic phase that formed when 2 mL of toluene was added to the system.

Compared to the yield of ergosterol increasing alkalinity had an opposing effect on the detected amounts of trehalose released from baker's yeast. The highest trehalose yields were obtained at 0.1% NaOH concentration at room temperature (Fig. 5). Increasing the extraction temperature to 85 °C and the alkalinity to 10% yielded only half of the maximum amount of trehalose obtained at the mildest alkalinity. The mechanism on how trehalose concentration in the extract is decreased at high alkalinity is not understood at the moment, but it may involve reactions or associations with proteins or other intracellular substances, since pure trehalose was found to be stable under the extraction conditions. Altogether, these results indicate that trehalose leaks out from yeast cells even at low alkalinity (0.1%). This behavior is different from that of ergosterol, suggesting that it would be beneficial to separate trehalose before extracting the remaining solids to obtain ergosterol. By extracting the biomass early on at low alkalinity and temperature, the loss of trehalose and leaching of other intracellular compounds that may hamper crystallization of trehalose from the water phase would be avoided.

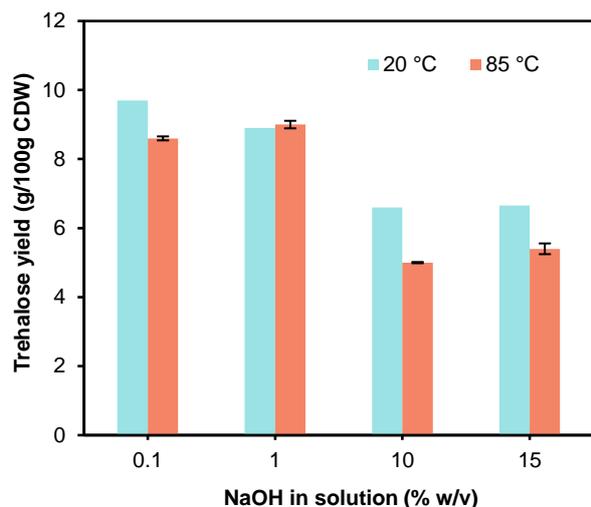


Figure 5. The effect of the strength of alkaline solution on trehalose content in water phase when yeast cells were extracted at 20 or 85 °C for four hours. The extraction conditions were otherwise similar to those given in the caption to Figure 4.

To get understanding over the efficiency of the extraction method developed here, it was compared to the literature method.[13] The results shown in Fig. S1 in the supplementary material indicate that when starting from baker's yeast the final ergosterol content in the organic phase increased within three hours to 0.8% from CDW, but remained in the range 0.4–0.6% with the reference method using ethanol/petroleum ether as solvents. **The higher yield obtained with the former procedure results from the stabilizing effect of 1-butanol on ergosterol, as it was found to be a better solvent for ergosterol (Table 2), and being more lipophilic than ethanol it probably facilitated extraction by disrupting the yeast cells more efficiently.**

3.4 Ergosterol and trehalose contents of oleaginous microbes

Biomass samples from 13 fed-batch cultivations of oleaginous yeasts were extracted for ergosterol and trehalose. The lipid contents of the microbes ranged from 4.3% in *S. cerevisiae* to 40% in the oleaginous yeast *C. curvatus* CBS 5324 (Table 3). The detected ergosterol content was in all cases below 1% of CDW. The highest ergosterol content (0.8%) in *R. paludigenum*

CBS 6566 was similar to that in baker's yeast. Trehalose contents higher than 10% of CDW were found in four yeast species. *Rhodotorula glutinis* VTT-C-01432 contained the highest trehalose content of 15 % of CDW. The ergosterol concentrations showed weak but statistically significant correlations (t-test, $p < 0.005$) with trehalose and lipid contents among these microbes. Ergosterol concentration has been shown to correlate with biomass concentration in fed-batch cultivation of *S. cerevisiae*, [39] whereas trehalose is produced as a means to counter osmotic or thermal stress. [40] Marked differences in intracellular concentrations of ergosterol, trehalose and lipids among the microorganisms listed in Table 3 suggest that the sales price of these chemicals would have to be taken into account, when the biomass cultivation process is optimized.

Table 3. Lipid content and results of two phase extraction for ergosterol and trehalose contents in oleaginous yeasts.

Microbe species	Lipid content (% of CDW) ^a	Ergosterol content (% of CDW)	Trehalose content (% of CDW)
<i>R. paludigenum</i> CBS 6566	15	0.83	1.03
<i>R. kratochvilovae</i> CBS 332	37	0.15	7.68
<i>Rhodotorula glutinis</i> VTT-C-01423	25	0.20	15.4
<i>R. toruloides</i> CBS 12050	29	0.30	4.30
<i>R. toruloides</i> VTT-C-00393	31	0.10	12.3
<i>R. lusitaniae</i> CBS 7604	25	0.20	2.02
<i>R. kratochvilovae</i> CBS 7293	28	0.32	1.80
<i>R. toruloides</i> CBS 350	13	0.54	3.38
<i>R. diobovatum</i> CBS 11125	10	0.21	0.97
<i>R. toruloides</i> CBS 12015	35	0.31	3.49
<i>Cryptococcus curvatus</i> CBS 5324	22	0.20	12.2
<i>C. curvatus</i> CBS 5324	40	0.16	11.4
<i>Lipomyces starkeyi</i>	28	0.00	bdl ^b
<i>Baker's yeast (S. cerevisiae)</i>	4.3	0.81	5.4

^a: The weight percentage of fatty acids released from yeast cell dry weight (CDW) and analysed as fatty acid methyl esters.[21] ^b: bdl, below detection limit.

3.5 Process proposal for the isolation of ergosterol and trehalose from microorganisms

In this investigation we were primarily interested in separating ergosterol and trehalose simultaneously from microbial cells. The process conditions need to be protective to avoid

degradation of trehalose and unwanted reactions of ergosterol such as formation of Diels-Alder adducts or vitamin D₂.^[41,42] The proposed two phase extraction process is schematically shown in Figure 6. The two phase system was most effective when 1-butanol/toluene/NaOH (0.1%) combination was used at 20 °C for one hour for separation of trehalose (Fig. 5). Solid residual biomass could be further extracted at 85 °C in the same solvent combination but with higher NaOH concentration of 10–15 %. This two-stage extraction process gives three fractions and the needed products can be further purified from each fraction separately. *In the case of trehalose, several purification methods have already been described, for example using zeolites for enrichment of trehalose^[43] or combining microfiltration and crystallization to recover pure trehalose dihydrate.^[44]*

When using baker's yeast, the mass yield of each fraction was analyzed gravimetrically. It was found that the organic phase contained 8% of the initial CDW. *Fractional crystallization of ergosterol^[45] can be achieved by lowering the temperature of the organic fraction to 20 °C or below.* The evaporated organic solvent can be filtered and recycled. An advantage of this method is the good phase separation in addition to the fact that 1-butanol dissolves in toluene nearly completely when the volume ratio of water/toluene is close to 1:1. Water-soluble materials like sugars and glycerol remain in alkaline water where 55 % of the initial baker's yeast CDW can be found. The solid residues make together 37 % of the initial CDW.

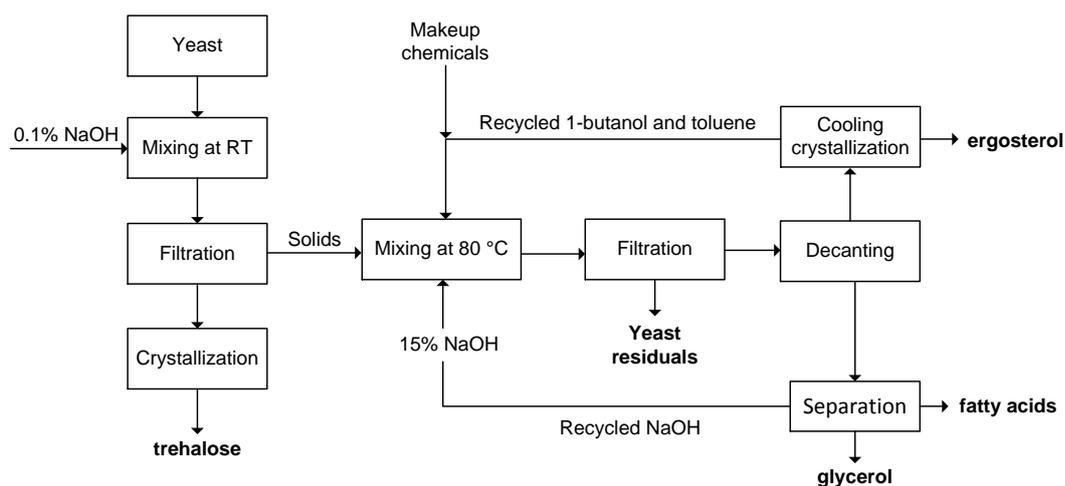


Figure 6. Schematic presentation of the proposed two phase extraction process.

4 Conclusions

This report suggests that the optimal conditions are different for the isolation of ergosterol and trehalose from microbial biomass. It is important to separate trehalose first at low alkalinity to obtain high yield. After this initial and short extraction, ergosterol can be protectively extracted using 10–15% sodium hydroxide water solution in the presence of 1-butanol. The maximal ergosterol yield is reached after 3 hours at 80–85 °C. In this method 1-butanol and toluene are used to give good phase separation and only low amount of 1-butanol remains in the water phase. These solvents have high boiling points and are inconvenient to distill, but ergosterol can be easily crystallized from the organic phase by lowering the temperature. This makes it possible to recycle the solvents into the extraction step and this holds promise for the proposed extraction process. From the process economy stand point, further work is needed to establish optimal yields of ergosterol, trehalose, lipids, and insoluble biomass fractions.

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5 References

- [1] H. Gao, T. Tan, Fed-batch fermentation for ergosterol production, *Process Biochem.* 39 (2003) 345–350. doi:10.1016/S0032-9592(03)00076-1.
- [2] F. Shang, S. Wen, X. Wang, T. Tan, High-cell-density fermentation for ergosterol production by *Saccharomyces cerevisiae*., *J. Biosci. Bioeng.* 101 (2006) 38–41. doi:10.1263/jbb.101.38.
- [3] K. Athenstaedt, Neutral Lipids in Yeast: Synthesis, Storage and Degradation, in: T.J.P. Smyth, A. Perfumo, S. McClean, R. Marchant, I.M. Banat (Eds.), *Handb. Hydrocarb. Lipid Microbiol.*, 2010: pp. 1–6. doi:10.1007/978-3-540-77587-4.
- [4] X. He, X. Guo, N. Liu, B. Zhang, Ergosterol production from molasses by genetically modified *Saccharomyces cerevisiae*, *Appl. Microbiol. Biotechnol.* 75 (2007) 55–60. doi:10.1007/s00253-006-0807-6.
- [5] X. He, B. Zhang, H. Tan, Overexpression of a sterol C-24(28) reductase increases ergosterol production in *Saccharomyces cerevisiae*, *Biotechnol. Lett.* 25 (2003) 773–778. doi:10.1023/A:1023572403185.
- [6] E.L. Dulaney, E.O. Stapley, K. Simpf, Studies on ergosterol production by yeasts., *Appl. Microbiol.* 2 (1954) 371–379.
- [7] S.J. Dong, C.F. Yi, H. Li, Changes of *Saccharomyces cerevisiae* cell membrane

- components and promotion to ethanol tolerance during the bioethanol fermentation, *Int. J. Biochem. Cell Biol.* 69 (2015) 196–203. doi:10.1016/j.biocel.2015.10.025.
- [8] A. Borrull, G. López-Martínez, E. Miró-Abella, Z. Salvadó, M. Poblet, R. Cordero-Otero, N. Rozès, New insights into the physiological state of *Saccharomyces cerevisiae* during ethanol acclimation for producing sparkling wines, *Food Microbiol.* 54 (2016) 20–29. doi:10.1016/j.fm.2015.11.001.
- [9] K. Zhang, M. Tong, K. Gao, Y. Di, P. Wang, C. Zhang, X. Wu, D. Zheng, Genomic reconstruction to improve bioethanol and ergosterol production of industrial yeast *Saccharomyces cerevisiae*, *J. Ind. Microbiol. Biotechnol.* 42 (2014) 207–218. doi:10.1007/s10295-014-1556-7.
- [10] Y. Al Jasem, M. Khan, A. Taha, T. Thiemann, Preparation of steroidal hormones with an emphasis on transformations of phytosterols and cholesterol -a review, *Mediterr. J. Chem.* 3 (2014) 796–830. doi:10.13171/mjc.3.2.2014.18.04.15.
- [11] H.N. Bhatti, R.A. Khera, Biological transformations of steroidal compounds: A review, *Steroids.* 77 (2012) 1267–1290. doi:10.1016/j.steroids.2012.07.018.
- [12] W. Wei, S.Y. Fan, F.Q. Wang, D.Z. Wei, Accumulation of androstadiene-dione by overexpression of heterologous 3-ketosteroid Δ^1 -dehydrogenase in *Mycobacterium neoaurum* NwIB-01, *World J. Microbiol. Biotechnol.* 30 (2014) 1947–1954. doi:10.1007/s11274-014-1614-3.
- [13] T. Tan, M. Zhang, H. Gao, Ergosterol production by fed-batch fermentation of *Saccharomyces cerevisiae*, *Enzyme Microb. Technol.* 33 (2003) 366–370. doi:10.1016/S0141-0229(03)00132-7.
- [14] B.G. Adams, L.W. Parks, Isolation from yeast of a metabolically active water-soluble form of ergosterol., *J. Lipid Res.* 9 (1968) 8–11.
- [15] T. Higashiyama, Novel functions and applications of trehalose, *Pure Appl. Chem.* 74

- (2002) 1263–1269. doi:10.1351/pac200274071263.
- [16] E.P. Feofilova, A.I. Usov, I.S. Mysyakina, G.A. Kochkina, Trehalose: Chemical structure, biological functions, and practical application, *Microbiology*. 83 (2014) 184–194. doi:10.1134/S0026261714020064.
- [17] L.C. Stewart, N.K. Richtmyer, C.S. Hudson, The Preparation of Trehalose from Yeast, *J. Am. Chem. Soc.* 329 (1950) 8–10.
- [18] M. Lamacka, J. Sajbidor, Ergosterol determination in *Saccharomyces cerevisiae*. Comparison of different methods, *Biotechnol. Tech.* 11 (1997) 723–725. doi:10.1023/A:1018484015806.
- [19] D. Otero, Mi; Vasallo, M; Verdecia, O; Fernandez, V; Betancourt, A Process for the Complete Fractionation of Baker ' s Yeast, *J. Chem. Technol. Biotechnol.* 67 (1996) 67–71.
- [20] M.H. Sipponen, V. Pihlajaniemi, H. Vainio, E. Palonen, S. Hokkanen, M. Vahvaselkä, O. Pastinen, A. Nyssölä, S. Laakso, Integrating the opposites of biofuel production: absorption of short-chain alcohols into oleaginous yeast cells for butanol recovery and wet-extraction of microbial oil, *Green Chem.* 18 (2016) 2775–2781. doi:10.1039/C5GC03008K.
- [21] M. Suutari, K. Liukkonen, S. Laakso, Temperature adaptation in yeasts: the role of fatty acids, *J. Gen. Microbiol.* 136 (1990) 1469–1474. doi:10.1099/00221287-136-8-1469.
- [22] M.O. Gessner, A.L. Schmitt, Use of solid-phase extraction to determine ergosterol concentrations in plant tissue colonized by fungi, *Appl. Environ. Microbiol.* 62 (1996) 415–419. doi:December, 21 2012.
- [23] M.W. Davis, R.T. Lamar, Evaluation of methods to extract ergosterol for quantitation of soil fungal biomass, *Soil Biol. Biochem.* 24 (1992) 189–198. doi:10.1016/0038-0717(92)90218-M.

- [24] P. Anderson, C.M. Davidson, D. Littlejohn, A.M. Ure, C.A. Shand, M. V. Cheshire, Extraction of ergosterol from peaty soils and determination by high performance liquid chromatography, *Talanta*. 41 (1994) 711–720. doi:10.1016/0039-9140(93)E0007-Z.
- [25] S.S. Geras'kina, M. V. Mukhina, Stabilization of Pirovitamins of the D Group, *Khimiko-Farmatsevticheskii Zhurnal*. 14 (1981) 90–92.
- [26] M.H. Sipponen, V. Pihlajaniemi, H. Vainio, E. Palonen, S. Hokkanen, M. Vahvaselkä, O. Pastinen, A. Nyssölä, S. Laakso, Integrating the opposites of biofuel production: absorption of short-chain alcohols into oleaginous yeast cells for butanol recovery and wet-extraction of microbial oil, *Green Chem.* (2016) 2775–2781. doi:10.1039/C5GC03008K.
- [27] E.M. Cornford, Correlation between lipid partition coefficients and surface permeation in *Schistosoma japonicum*., *J. Membr. Biol.* 64 (1982) 217–224. doi:10.1007/BF01870888.
- [28] J.W. Roddy, Distribution of Ethanol-Water Mixtures to Organic Liquids, *Ind. Eng. Chem. Process Des. Dev.* 20 (1981) 104–108.
- [29] E.O. Dillingham, R.W. Mast, G.E. Bass, J. Autian, Toxicity of Methyl- and Halogen-Substituted Alcohols in Tissue Culture Relative to Structure–Activity Models and Acute Toxicity in Mice, *J. Pharm. Sci.* 62 (1973) 22–30. doi:10.1002/jps.2600620103.
- [30] A.E. Hill, W.M. Malisoff, The mutual solubility of liquids. III. The mutual solubility of phenol and water. IV. The mutual solubility of normal butyl alcohol and water, *J. Am. Chem. Soc.* 48 (1926) 918–927. doi:10.1021/ja01415a011.
- [31] A.F.M. Barton, Solubility data series, Pergamon Press, Oxford, 1980. doi:10.1016/0021-9614(80)90193-7.
- [32] Y. Yonezawa, Y. Urushigawa, Chemico-biological interactions in biological purification systems V. Relation between biodegradation rate constants of aliphatic

- alcohols by activated sludge and their partition coefficients in a 1-octanol-water system, *Chemosphere*. 8 (1979) 139–142. doi:10.1016/0045-6535(79)90062-6.
- [33] R. Ginnings, PM; Baum, Aqueous Solubilities of the Isomeric Pentanols, *J. Am. Chem. Soc.* 59 (1937) 1111–1113.
- [34] D.J. Donahue, F.E. Bartell, U. Michigan, A. Arbor, The boundary tension at water-organic liquid interfaces, *J. Phys. Chem.* 56 (1952) 480–484. doi:10.1021/j150496a016.
- [35] A. Nahum, C. Horváth, Evaluation of octanol-water partition coefficients by using high-performance liquid chromatography, *J. Chromatogr. A.* 192 (1980) 315–322. doi:10.1016/S0021-9673(80)80006-9.
- [36] H. Sanemasa, I; Araki, M; Deguchi, T; Nagai, Solubility measurements of benzene and the alkylbenzenes in water by making use of solute vapor, *Bull. Chem. Soc. Jpn.* 55 (1982) 1054–1062.
- [37] Y.B. Tewarl, M.M. Miller, S.P. Wasik, D.E. Martire, Aqueous Solubility and Octanol/Water Partition Coefficient of Organic Compounds at 25.0 °C, *J. Chem. Eng. Data.* 27 (1982) 451–454. doi:10.1021/je00030a025.
- [38] C. McAuliffe, Solubility in Water of Paraffin, Cycloparaffin, Olefin, Acetylene, Cycloolefin, and Aromatic Hydrocarbons, *J. Phys. Chem.* 70 (1966) 1267–1275. doi:10.1021/j100876a049.
- [39] F. Shang, Z. Wang, T. Tan, High-cell-density cultivation for co-production of ergosterol and reduced glutathione by *Saccharomyces cerevisiae*, *Appl. Microbiol. Biotechnol.* 77 (2008) 1233–1240. doi:10.1007/s00253-007-1272-6.
- [40] C.G. Hounsa, E.V. Brandt, J. Thevelein, S. Hohmann, B.A. Prior, Role of trehalose in survival of *Saccharomyces cerevisiae* under osmotic stress, *Microbiology.* 144 (1998) 671–680. doi:10.1099/00221287-144-3-671.
- [41] J.G. Martin, R.K. Hill, Stereochemistry of the Diels-Alder Reaction, *Chem. Rev.* 61

- (1961) 537–562. doi:10.1021/cr60214a001.
- [42] M.F. Holick, Vitamin D: A millenium perspective, *J. Cell. Biochem.* 88 (2003) 296–307. doi:10.1002/jcb.10338.
- [43] M. Boy, M. Pompejus, D. Klein, M. Volkert, J.R. Brown, METHODS FOR THE ENRICHMENT OF TREHALOSE USING ALUMINOSILICATES, US 2008/0108113 A1, 2008.
- [44] N. Kinouchi, METHOD OF ISOLATION AND PURIFICATION OF TREHALOSE, US005441644A, 1995.
- [45] L.M. Pruess, W.H. Peterson, E.B. Fred, Isolation and identification of ergosterol and mannitol from *Aspergillus fischeri*, *Jounal Biol. Chem.* 97 (1932) 483–489.