Lipid Formation in *Cryptococcus terricolus*

III. Extraction and Purification of Lipids

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Extraction procedures have been developed which release the total lipid content from the yeast *Cryptococcus terricolus* without using hydrolytic or high-temperature treatments. The best results were obtained by extracting freeze-dried yeast material at room temperature. Chloroform-methanol (1:1, v/v) was used as solvent, and the material plus solvent were stirred continuously during three successive three-hour extraction periods. The effectivity of the extraction was found to be dependent on the composition of the solvent mixture and on the length and number of the extraction periods.

An effective purification of the crude lipids was obtained by a thorough mixing of the extract, dissolved in chloroform-methanol (2:1, v/v), with distilled water, followed by separation by centrifugation. The purified lipids were easily redissolved in the usual lipid solvents.

The wash solutions were found to contain only a very small amount of materials of lipid character, varying between 0 and 0.3 % of the lipid content of the organism.

Various methods may be used to separate the lipids from proteins and other components of biological materials. A number of complete procedures for the extraction of lipids from tissues have been described, and a successful extraction of animal preparations is generally obtained with only minor modifications of one of these procedures. However, if a microbial material is to be extracted, it seems difficult to decide which method will give the best result. Various methods have been employed but, in most cases, they include an extreme treatment of the cells, requiring either additions of acids or bases, or extractions at elevated temperatures, or a combination of both treatments. Complete lipid extractions of yeasts have usually been performed by means of such methods. When the interest is concentrated on the determination of the amounts of lipids in phospholipid-poor cell materials, such treatments may be satisfac-

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tory. However, if the extract is to be employed for further analyses to establish the composition of the lipid material, it is essential that the lipids be obtained by the mildest possible procedure.

The main purpose of the present work was to establish an extraction procedure for releasing the total lipid content from *Cryptococcus terricolus* cells without using any undesired pretreatment, and to devise an effective purification procedure for the crude lipid extract. Further analyses of the purified lipid mixture obtained may be performed in the future to give an idea of the composition of the native lipids of this yeast.

EXPERIMENTAL

The yeast was grown in an unbaffled glass fermentation vessel of 10 l capacity. Nutrient solution (7.75 l) of the following composition was poured into the vessel: glucose 40.0 g, KH_2PO_4 1.0 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.0 g, Fe-citrate $\cdot 3\text{H}_2\text{O}$ 0.1 g, and 1.0 ml of metal solution (containing 0.2 mg of zinc as $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 mg of manganese as $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, and 0.01 mg of copper as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) per litre of final solution. The vessel was closed with a stainless steel cover, and autoclaved. 5.4 g of urea in 0.25 l of water was autoclaved separately, and introduced aseptically, together with 1 l of a four-day-old inoculation material, into the fermentation vessel. The vessel was placed in a water bath at 25°C. Compressed air, on being passed through a sterile air filter and a flowmeter at a rate of 1.0 l of air/l of medium/min, was bubbled in continuously at the bottom of the fermentation vessel. In addition, the solution was stirred mechanically throughout the experiment. Foaming was not pronounced, hence no antifoaming agent was added. Intermediate samples of the cell suspension could be taken out for controls and analyses using a sterile pipette. After 3 days, all the sugar had been consumed. The cells were then centrifuged and washed twice with distilled water. One cultivation experiment yielded about 110 g of dry matter. This amount was sufficient for carrying out the investigation. The results from different experiments could thus be directly compared. One part of the cell paste was stored at -20°C. The remainder was immediately freeze-dried to 98–99 % of dry matter and stored in a vacuum desiccator until used.

The following solvents were used for the extractions: diethyl ether anhydrous p.a., petroleum ether (Skellysolve F, residue after evaporation at most 0.0016 %) with a boiling point of 35–60°C, absolute ethanol (99.5 %), chloroform and methanol of analytical grade. The other chemicals used were also of analytical grade.

The following methods, tested for the purification of the crude lipid extracts, were originally described by Folch *et al.*^{1,2}

Purification by diffusion. A beaker, slightly larger than the volume of extracts to be washed, is submerged into a larger beaker, nine-tenths full of water, and with a capacity of more than ten times the volume of the extract to be washed. The lipid is dissolved in chloroform-methanol (2:1, v/v) and delivered slowly into the small beaker by means of a volumetric pipette. The system thus obtained is allowed to stand overnight.

Purification by centrifugation. The crude extract, dissolved in chloroform-methanol (2:1, v/v), is mixed thoroughly with one-fifth of its volume of either water or a 0.003 N $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ solution, and separated by centrifugation for 15 min at 2 400 r.p.m.

The upper phases obtained with these procedures were removed as completely as possible with a pipette. In the diffusion method, methanol was added to dissolve the cloudy, insoluble matter (fluff) formed at the interface. In the centrifugation procedures, no interfacial fluff was formed. In these cases, the tube wall and the remaining upper phase were washed twice with 1.5-ml portions of a solvent mixture identical with that of the final upper phase.

The lipid samples and the wash solutions were collected and dried on a rotating vacuum drier.

The total carbohydrate content was determined according to the anthrone method described by Trevelyan *et al.*³ The lipid solutions were poured into the tubes and dried before the adding of the reagent. The sugars in the wash solutions could be easily deter-

mined using a Beckman spectrophotometer at 620 $m\mu$. When lipids were present, yellow and brown colours interfered, making the readings very uncertain. The results were referred to a glucose standard.

The phosphorus content was determined with the Fiske-Subbawrow reagent according to the method modified by Bartlett ⁴. Readings were made on a Beckman spectrophotometer at 830 $m\mu$. The lipid solvent was removed on a water bath and any possible residual solvent by repeated blowing with CO_2 .

The nitrogen content was determined by the standard Kjeldahl procedure.

Amino nitrogen was determined according to the ninhydrin method modified for use with phospholipids by Wheeldon and Collins ⁵. Readings were made on a Beckman spectrophotometer at 570 $m\mu$. Serine was used as standard. In preparations rich in fats and free fatty acids, the total amount of material taken for analysis must not exceed 1 mg. If it does, undissolved particles will disturb the spectrophotometric readings.

The Liebermann-Burchard qualitative test for sterols was performed by dissolving a part of the sample in 2 ml of chloroform and adding 10 drops of acetic anhydride plus 2 drops of concentrated sulphuric acid ⁶. Visual readings were made after 2, 10, and 120 min.

RESULTS AND DISCUSSION

When extracting lipids from yeast materials it is usually found that the cells have to be pretreated to give complete extractions.

Table 1 shows the results obtained when a cell paste was extracted directly without any pretreatment. Starkey ⁷ found only 3.1 % of lipids in *Lipomyces starkeyi* by this extraction procedure. Judging from earlier literature, the poor results obtained by direct extraction using diethyl ether are probably due partly to the impermeability of the yeast cell wall to this solvent, and partly to the formation by the lipids of complexes with the cell proteins not released during this treatment.

It has been known for a long time that a treatment of the cell material with hydrochloric acid increases the amount of extractable lipids. A previous work ⁸, showed that autoclaving of the *Cryptococcus terricolus* material for 15 min with 1.2 N hydrochloric acid, followed by extraction according to the methods described by Steinberg and Ordal ⁹, resulted in a complete removal of the lipids (maximum value *ca.* 70 % of the dry matter). The extraction results for *C. terricolus* cells were found to be just as good if the autoclaving was replaced by boiling under reflux for 2 h. This procedure was first introduced by Smedley-MacLean ¹⁰. Milder hydrochloric acid treatments were used successfully by Starkey ⁷ (boiling with 2 % hydrochloric acid under reflux for 4 h) and by Deinema and Landheer ¹¹ (boiling with 0.5 % hydrochloric acid under reflux for 3 h). The last two procedures were quite as effective for pre-

Table 1. Extraction of lipids with diethyl ether from an untreated paste of *Cryptococcus terricolus* cells.

Material	Extraction method	Extracted lipids. Per cent of dry matter
Wet cell paste	Separatory funnel	7.1
Dried cell paste	Soxhlet	5.1

extraction treatments of *C. terricolus* cells as the stronger methods used previously. Extraction of the hydrolyzed material with petroleum ether in separatory funnels, or extraction of dried hydrolyzed material in Soxhlet extractors with petroleum ether or diethyl ether gave almost identical results.

Direct saponification with ethanolic KOH, followed by acidification of the soap solution and extraction, has also been employed for yeasts¹². This method, however, was not tried for *C. terricolus* cells.

Large amounts of non-lipids were always extracted whenever the treatment involved a hydrolysis of the cells. The crude extract obtained was exceedingly difficult to purify. After a most particular purification, the lipid extract still had a dark and, as will be shown, unnaturally brown colour. For theoretical reasons, it was also considered desirable to avoid hydrolysis, since destructions of and alterations in the lipid structure most probably increase during such treatments. It is a well known fact that a hydrolytic pretreatment destroys the neutral lipids, especially the phospholipids. Lipids obtained after such treatments are, therefore, poorly suited for further qualitative separations.

Different combinations of solvents were tried for the direct extraction of untreated, dried cell paste in a Soxhlet apparatus. Chloroform¹³, ethanol-diethyl ether (3:1, v/v)¹³, chloroform-methanol (2:1, v/v)¹³, ethanol-benzene (4:1, v/v)¹⁴, and methanol-petroleum ether (1:10, v/v)¹⁵ were used. 20–30% of the lipids were extracted at the most. Anyhow, compared with the diethyl ether extraction, this was at any rate an improvement since greater amounts of the lipids could be extracted directly without pretreatment.

Refluxing the wet cell paste directly with ethanol-diethyl ether as solvent, released still more of the lipids. A combination of the extracts from a 3 hours' reflux treatment with ethanol-diethyl ether (3:1, v/v), followed by two re-extractions with diethyl ether under reflux, each for 2 h, resulted in 45.9 % of extracted matter, corresponding to approximately 3/4 of the lipid content. To get the remainder extracted, a treatment with hydrochloric acid was necessary. It is, in fact, in most cases, advisable to subject the solvent-extracted residue to hydrolysis, followed by new extractions, to check that the extracted material represents the total lipids.

A direct extraction of disintegrated cells was tried in order to obtain a total removal of the cell lipids without hydrolytic pretreatment. The cells in

Table 2. Extraction of an untreated and a disintegrated wet cell paste of *Cryptococcus terricolus*. Refluxed with ethanol-diethyl ether (3:1, v/v), and thereafter subjected to two diethyl ether extractions. The extracted residue hydrolyzed and extracted with petroleum ether.

Material	Lipids. Per cent of dry matter		
	Direct extraction	Rest extracted	Total
Untreated	45.9	13.6	59.5
Disintegrated	50.5	9.2	59.7

a wet cell paste were successfully disintegrated, using a procedure described by Edebo¹⁶. Microscopic examinations of the material after disintegration indicated a practically total breakage of the individual cell walls. On extraction, the interior of the cells probably came in a more direct contact with the solvents. Table 2 shows that a greater part of the lipids are extracted from a disintegrated material, about 9 % being left for hydrolytic treatment. Even with the solvent mixture used here, the cell wall constitutes a barrier to the solvents, in spite of the fact that diethyl ether, to which the dehydrating agent ethanol has been added, penetrates the wall more effectively than does diethyl ether alone.

Like hydrolysis of the cell material, it is also desirable to avoid the use of elevated temperatures. Hanahan¹⁷ preferred to use extraction at room temperature or at most one at 30–35°C. He found phospholipids from yeasts to be severely affected by extractions with ethanol-diethyl ether (3:1, v/v) at 55–60°C. By the use of this last procedure, they were obtained as very dark, viscous oils, while the extract at 25°C had a light yellow colour.

An attempt was made to extract the disintegrated material directly at room temperature by shaking it in separatory funnels. Large amounts of emulsions were formed, rendering a separation into two phases practically impossible. Lipid extraction, without heating, but by means of continuous stirring with different solvent mixtures, was tried. The first extract, after having been filtered, was found to contain large amounts of contaminations. Upon purification in separating funnels, emulsions were formed which resulted in a difficult separation also in this case.

Hartman *et al.*¹⁵, in extracting freeze-dried *Rhodotorula graminis* material in a Soxhlet apparatus with methanol and light petroleum, obtained 88 % of the total lipids. Judging from these results, freeze-drying seems to have a favourable effect on the extraction procedure. Material obtained from *Cryptococcus terricolus* was freeze-dried and extracted with different solvents and solvent mixtures under continuous stirring at room temperature. The results

Table 3. Extraction of freeze-dried *Cryptococcus terricolus* material under continuous stirring at room temperature with different solvents. Three successive extraction periods of three hours. Extracted residue re-extracted with chloroform-methanol (1:1, v/v) under reflux.

Solvent	Lipids. Per cent of dry matter	Residual lipids. Per cent of dry matter	Total lipids. Per cent of dry matter
Petroleum ether *	58.2	2.5	60.7
Petroleum ether	36.0		
Ethanol-petroleum ether (3:1, v/v)	50.2	7.4	57.6
Diethyl ether	32.5		
Ethanol-diethyl ether (3:1, v/v)	41.6	12.3	53.9
Chloroform	37.5		
Chloroform-methanol (2:1, v/v)	54.5	5.0	59.5

* Material autoclaved before extraction in separatory funnels.

are given in Table 3, and compared with the amount of lipids extracted from an autoclaved material.

Extraction with diethyl ether, petroleum ether, or chloroform alone, released from 54 to 61 % of the total extractable lipids. Consequently, it may be assumed that freeze-drying makes the cell walls more permeable to the various solvents. If dehydrating agents, such as ethanol or methanol, are added, the extraction becomes, of course, more complete. Apart from additional effects upon the cell walls, this might indicate that the lipids are partly present as lipoproteins. Water molecules play an important role in the binding between lipids and proteins¹⁷. The addition of the dehydrating agent ruptures the lipid-protein linkage, thus making the extraction possible. The best results were obtained with chloroform-methanol, which permitted the extraction of *ca.* 90 % of the lipids, while ethanol-diethyl ether released only 69 % of the lipids. "Maximum extractable lipids" in Tables 4 and 5 give the amounts obtained after re-extraction with chloroform-methanol (1:1, v/v) of the autoclaved, petroleum ether-extracted residue.

It is a well-known fact that the optimum ratio for the solvents may vary considerably from one material to another. Consequently, different chloroform-methanol mixtures were tested.

Table 4. Extraction of freeze-dried *Cryptococcus terricolus* material by continuous stirring at room temperature with different chloroform-methanol mixtures. Three successive extraction periods of three hours.

Chloroform-methanol (v/v)	Lipids. Per cent of dry matter	Percentage of maximum extractable lipids
3:1	44.6	73.5
2:1	54.5	89.8
1:1	60.6	99.8
1:2	52.3	86.2

Table 4 shows that the extraction is practically complete if chloroform-methanol (1:1, v/v) is used as a solvent. The total extraction period comprises nine hours. It would be convenient if this time could be shortened. As shown in Table 5, however, the long extraction period has to be retained in order to obtain satisfactory results.

Table 5. Extraction of freeze-dried *Cryptococcus terricolus* material by continuous stirring at room temperature with chloroform-methanol (1:1, v/v). Length and number of extraction periods varied.

Number of periods	Length of periods in hours	Lipids. Per cent of dry matter	Percentage of maximum extractable lipids
3	1	55.3	91.1
3	2	57.2	94.2
3	3	60.6	99.8
2	3	58.0	95.6
1	3	54.2	89.3

The material obtained after these extraction procedures was of a light yellow colour. This is due to the absence of elevated temperatures during the extractions as well as to the presence of relatively small amounts of contaminants. Sugars in particular, give a brown colour upon heating. However, to avoid misleading results in the subsequent chromatographic separations, the non-lipid contaminants had to be further removed.

Lipid preparations have a remarkable ability to solubilize many non-lipid components. In general, sugars, free amino acids, urea, and also inorganic substances, may be found as contaminations. Partly, the contaminating material is soluble in the lipid solvent used, but mostly the contamination is due to the complexing ability of the various types of phospholipids. For this reason, selective precipitations are not applicable as purification procedures. Liquid-liquid partition methods are effective and widely favoured although they may lead to losses of the most polar lipids. Purification of the crude extract has to be carried out at room temperature.

Re-extraction of the crude extracts in separatory funnels was tried in order to prepare a purified lipid material. The water and solvent phases could hardly be separated after shaking, owing to the formation of large amounts of apparently permanent emulsions. Another method tested consisted in shaking a lipid extract redissolved in chloroform-methanol (2:1, v/v) with a 0.9 % NaCl solution. The formation of emulsion was in this case more moderate and temporary, thus making separation possible, but after drying, large amounts of NaCl crystals were found to be present in the lipids. It was very difficult to free the extract from the NaCl, since, upon repeated shakings with water, the stable emulsions were, to a certain extent, regenerated. Moreover, in this case the addition of the salt increased the problem of contamination.

Reproducible methods for the removal of many of the impurities in a crude lipid extract, are the washing techniques of Folch *et al.*^{1,2}, mentioned briefly

Table 6. Effect of different washing procedures on the crude lipid extract of *Cryptococcus terricolus* material.

Solutes	Crude extract. Amount	Washed by diffusion in beaker		Washed by centrifugation with H ₂ O		Washed by centrifugation with 0.003 N MgCl ₂	
		Amount left	Per cent removed	Amount left	Per cent removed	Amount left	Per cent removed
Total solutes (mg)	221.0	211.5	4.3	214.0	3.2	214.0	3.2
Total nitrogen (mg)	0.42	0.35	16.7	0.32	23.8	0.32	23.8
Amino nitrogen (mg)	0.25	0.17	32.0	0.16	36.0	0.16	36.0
Phosphorus (μg)	126.4	108.5	14.2	83.6	33.9	92.6	28.7
Carbohydrates (as glucose) (μg)	2500	1525	39.0	250	90.0	250	90.0

The qualitative sterol test was negative in the washing solutions removed.

above. These methods were originally applied to brain tissues and some other animal tissues. Folch and his co-workers found that these washing procedures removed the non-lipid contaminants from the extracts, with a concomitant loss of lipids ranging from 0 to at most 0.6 %.

The crude *C. terricolus* lipid extract was purified directly after extraction, without any intermediate drying. The results are given in Table 6.

Washing by means of the two centrifugation procedures seems to have the same effect, 3.2 % of the crude extract materials being removed. The greater loss in total solutes by the beaker diffusion method is probably due to difficulties encountered in removing the upper phase without simultaneously taking away traces of the interfacial fluff. This is the most probable explanation, since the removal of the other compounds investigated was less in this procedure. Carbohydrates were ineffectively removed by the diffusion method, while by the centrifugation methods, the carbohydrate content could be reduced to only 0.1 % of the lipid content. The contents of the other compounds investigated were also very low. The phosphorus content was found to be only about 0.05 %, which indicates a low phospholipid content. The low phospholipid content was verified by preliminary chromatographic separations of the major lipid groups. With the small nitrogen quantities in this material, the determination of the total nitrogen content is, of course, accompanied by relatively large titration errors. From a comparison of the phosphorus and nitrogen contents, it seems likely that the nitrogen contents obtained were too high, especially as the lecithin content according to preliminary chromatographic separations was found to be very low. The amino nitrogen constituted approximately half of the nitrogen found in the purified materials. The losses in total nitrogen during the washing procedures can be accounted for by the removal of amino group-containing compounds.

The washed extract was easily dissolved in the usual lipid solvents without leaving any measurable, undissolved residue. On re-extraction, the wash solutions from the two centrifugation procedures contained at the most 0.3 % of extractable materials of lipid character. In many experiments, no losses at all were detected. The losses of lipids during the washing procedures have consequently been kept at a satisfactory low level.

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