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Methods for Yeast Lipid Extraction

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The efficiencies of different methods of lipid extraction from a fat yeast, *Lipomyces starkeyi* and a non-fat yeast, *Saccharomyces cerevisiae* were compared. The methods include extraction of cells, (i) directly and (ii) after disruption of cells with HCl, lytic enzymes and mechanical grinding in presence of solvents. Regarding lipid yield, extraction methods involving cell disruption by HCl, lytic enzyme glusulase and mechanical grinding were found to be about equally efficient but more effective than other methods involving direct solvent extraction of intact cells. The residues left after solvent extraction of disrupted cells (except in the case of HCl treatment) were found to contain some lipid (ca. 10%) which can be extracted by HCl treatment followed by solvent extraction. The composition of lipid extracted from these two yeasts and from a *Rhodotorula glutinis* Y_3 strain, another fat yeast, by mechanical grinding and HCl treatment were evaluated. The results revealed that HCl treatment of cells followed by solvent extraction is the most simple and efficient method for fat yeasts like *L. starkeyi* and *R. glutinis* Y_3 where triglyceride is the major lipid component and sterol ester is a minor one. Phospholipids of *L. starkeyi* and *R. glutinis* Y_3 were less degraded by HCl treatment than by mechanical grinding. On the other hand, in non-fat yeast, *S. cerevisiae* where sterol ester and phospholipids are the major lipid components, HCl treatment degrades phospholipid to a larger extent than mechanical grinding. It appears that the lipid components are compartmentalised in cells of the fat yeasts in a different way than in the non-fat yeast.

THE presence of rigid cell wall around yeast cells makes efficient extraction of intracellular components such as lipid difficult[1,2]. Two different approaches towards the solution of this problem have been made : (i) the lipid is extracted from intact wet[3,4] or freeze-dried[3-5,7] yeast cell by solvents and (ii) cell wall is first disrupted followed by solvent extraction. In the latter method cell wall disruption has been accomplished by acid hydrolysis[8] or alkali hydrolysis[10-12], lytic enzyme treatment[5,10] and mechanical cleavage by grinding with glass beads[6,9]. From the above studies it appears that direct extraction by neutral solvents leads to incomplete recovery of lipid from yeast cells. In connection with our studies on the possibilities of using yeast lipid for commercial purposes a convenient method for complete extraction of undergraded lipid from yeast cells was necessary.

In this communication, we wish to report the efficiencies of the different methods of extraction of lipid from two different strains of yeast, *Lipomyces starkeyi*, a fat yeast and *Saccharomyces cerevisiae*, a non-fat yeast. We have determined the composition of the lipids recovered from these two yeasts and also a *Rhodotorula glutinis* Y_3 strain, another fat yeast, by two different methods of cell wall disruption prior to solvent extraction. These methods are : (i) acid hydrolysis, where chances of lipid degradation are high and (ii) mechanical grinding where these chances are low. Our results show that acid treatment prior to solvent extraction is a very efficient method of lipid extraction from fat yeasts (*L. starkeyi* and *R. glutinis* Y_3), but may lead to considerable degradation of phospholipids in non-fat yeast strains like *S. cerevisiae*.

Materials and Methods

1. Microorganism and Culture Conditions :

The yeast strains *Lipomyces starkeyi* 3440 and *Saccharomyces cerevisiae* 3190 were obtained from the National Collection of Industrial Microorganism, National Chemical Laboratory, Poona, India. The *Rhodotorula glutinis* Y_3 strain was isolated from local soil. The genus and species characterisation of this strain was carried out essentially according to the guidelines suggested by Van der Walt[13]. The strain (a) formed red pigments, (b) could not ferment sugars to alcohol, (c) was non-sporulating and multiplied by budding, (d) formed no mycellium (pseudo or true), (e) was capable of growing in vitamin-free medium, (f) was unable to grow at 37°, (g) did not form spores, (h) vigorously assimilated nitrogen-containing compounds, (i) did not produce starch even during growth in strongly acidic medium and (j) could not assimilate inositol. From these properties the strain under study was characterised as a species of the genus *Rhodotorula*. Identification of the species was based mainly on the abilities of different *Rhodotorula* species to assimilate some specific

compounds[14]. This strain could assimilate nitrate, maltose, sucrose, melizitose, raffinose but not lactose or melibiose, indicating the strain to be of the species *Rhodotorula glutinis*. Other properties of the strain like size, shape, growth in malt extract, growth on malt agar and on 50% glucose yeast-extract agar medium also appeared to be in conformity with the above classification. The strain was named as *Rhodotorula glutinis* Y_3 in order to separate its identity with other known *R. glutinis* strains.

All the strains were grown up to stationary phase at 25° with constant aeration in a medium of the following composition : 2% glucose, 0.5% peptone, 0.3% yeast extract and 0.3% malt extract ; *p*H was adjusted between 6.4 and 6.6. The cells were collected by centrifugation, washed twice with 0.1% Na_2HPO_4 solution and stored in deep-freeze before lipid extraction.

2. Extraction methods :

The extracted lipid was dried and weighed in all the cases.

(i) Extraction of wet cells : 2.5 g wet cells were suspended in 5 ml water and the lipid extracted according to the method of Bligh and Dyer as modified by Kates[6] using methanol-chloroform (2 : 1 v/v) and methanol-chloroform-water (2 : 1 : 0.8 v/v) mixtures.

(ii) Extraction of vacuum dried cells : 2.5 g wet cells were spread on a petri-dish and dried under vacuum at 25° to a thin sheet. The sheet was ground, and lipid extracted according to method (i) but using only the methanol-chloroform-water mixture (2 : 1 : 0.8 v/v) twice.

(iii) Hot methanol treatment : 2 g cells were suspended in 8 ml water, 20 ml methanol was added and refluxed on a steam-bath for 15 mins. 10 ml chloroform was added (i.e., methanol : chloroform : water = 2 : 1 : 0.8) ; rest of the procedure was as in method (i).

(iv) HCl treatment : To cells (1 g dry wet) suspended in 5 ml of water an equal volume of 2*N* HCl was added and the mixture was heated on a steam-bath for 15 mins. The treated cells were freed from acid by centrifugation followed by washing twice with water. Lipid extraction was according to method (i). The optimum conditions regarding digestion time and strength of HCl were determined in prior experiments.

(v) Mechanical grinding with glass beads : Yeast pellet (5 g wt cell) together with twice its volume of micro glass beads (0.1 mm) were blended (Omnimixer, Sorvall) in cold, twice with 15 ml methanol-chloroform mixture (2 : 1) and twice with 15 ml methanol-chloroform mixture (1 : 2). Duration of each blending operation was 3 min. The mixture was centrifuged after each blending and the supernatants were pooled, mixed with chloroform and water, each 1/5 of the total pooled volume and the phases were allowed to separate. The lower chloroform layer was taken, and the lipid recovered by the evaporation of the solvent.

(vi) Protease digestion : Cell suspension containing 2.5 g wet cells in 10 ml water was vortexed with 2.5 ml of toluene for 2 min, centrifuged and washed with 10 ml of 0.001*M* tris-HCl buffer (*p*H 7.5) containing 0.0115*M* $CaCl_2$. To the residue was added 0.2 ml of 0.001*N* HCl and 1 ml of protease (Sigma, Type VI., 4 unit/mg solid, 1 unit will hydrolyse casein to produce colour equivalent to 1 μ mole of tyrosine / min at *p*H 7.5 at 37°) solution in 0.001*M* Tris-HCl buffer (*p*H 7.5) containing 1 mg protease per ml, mixed well and incubated at 37° for 1 h. The lipid was extracted from the suspension according to method (i).

(vii) Glusulase treatment : Spheroplasts were prepared according to Hutchinson and Hartwell[15] with glusulase (Endo Laboratories, New York, U. S. A.). Cells were taken at a concentration of 10^8 cells per ml. After incubation for 2 h almost 99% of the cells became spheroplasts as was observed under a phase contrast microscope. Lipid was extracted out of this suspension according to method (i).

(viii) HCl treatment of the residues left after lipid extraction : In each of the above methods, except in method (i), the lipid was extracted, dried and estimated as in method (iv). This fraction is termed as 'residual lipid' in the text and the lipid extracted by methods (i) to (vii) is referred as 'readily extractable lipid'.

3. Analysis of the composition of the lipid components :

To 10 ml of the culture (stationary phase) 2×10^5 cpm of ^{14}C-acetate (4×10^6 cpm/μ mole) were added and incubated with aeration. After 5 h the cells were harvested, washed with water and divided into two parts. Lipids were extracted from one part by method (iv) and from the other by method (v) followed by HCl treatment of the residue. The labelled lipid was redissolved in 1 ml chloroform, 2 μl of this solution was used for the measurement of total activity. Another aliquot was applied as band on 0.5 mm thick 14×20 cm silica gel G (E. Merck) TLC plate. The chromatogram was developed with a mixture of petroleum ether (40-60°), diethylether and acetic acid (80 : 20 : 1.5). The bands were visualised by a brief exposure to iodine vapour and marked. After removal of iodine under low pressure individual bands were scraped off the plate to counting vials, 5 ml 0.4%. Omniflor (New England Nuclear Corp., U. S. A.)

in toluene was added to each vial and the activity counted in a scintillation counter (Beckman, Model LS 100). The bands were identified by comparing their R_f values with authentic compounds like digalactosyldiglyceride, phosphatidylcholine, cholesterol, oleic acid, triolein, diolein and cholestryloleate (Applied Science Laboratories Inc., State College, P. A., U. S. A.) spotted on the same plate. The composition was calculated from the total count and those due to individual component bands.

Results and Discussion

Table 1 represents the amounts of readily extractable and residual lipid in the different methods tried. In both *S. cerevisiae* and *L.starkeyi* extraction of wet cells by methanol-chloroform mixture produced the lowest amount of readily extractable lipid as also observed by other workers[4,5,8,12]. With dried cells, however, the yield of this fraction using the same solvent increased over twofold and became comparable with that of hot methanol treatment. These results conform with the idea that water molecules play an important role in the hydrophobic binding between cellular lipid and proteins and removal of this water by drying or hot methanol treatment facilitates extraction of lipid by solvents. It is also clear from Table 1 that the extraction by hot methanol or a

TABLE 1—EXTRACTION OF LIPID BY DIFFERENT METHODS FROM *Saccharomyces cerevisiae* AND *Lipomyces starkeyi*

Method	*Saccharomyces cerevisiae*		*Lipomyces starkeyi*	
	Readily extractable lipid	Residual lipid	Readily extractable lipid	Residual lipid
	(lipid in g/100g dry cell)			
(i) Extraction of wet cells	1.4	4.0	7.8	18.2
(ii) Extraction of vacuum-dried cells	2.6	2.8	17.5	7.3
(iii) Hot methanol treatment	2.4	2.6	14.6	10.9
(iv) HCl treatment	5.4	–	25.0	–
(v) Mechanical grinding with glass beads	4.5	0.7	25.1	2.7
(vi) Protease digestion	1.7	3.4	12.9	7.2
(vii) Glusulase treatment	4.5	0.8	24.2	2.9

methanol-chloroform mixture does not lead to complete extraction of lipid, and it seems quite probable that in yeast there are barriers other than hydrophobic interaction responsible for this incompleteness of lipid extraction by solvents. The results also show that cell wall disruption methods produced higher amounts of extractable lipid than direct solvent extraction methods[8,9] or protease digestion, which lead to the idea that carbohydrate rich cell wall is the major barrier towards the extraction of lipid from yeast cells.

The results in Table 1 point to the fact that some residual lipid is always left in mechanical grinding procedure or glusulase treatment, which can only be extracted by solvent after HCl treatment. This points to the possibility that these residual lipid fractions are bound to cell in a different fashion from the readily extractable fractions. The composition of the lipid extracted out of *S. cerevisiae*, *L. starkeyi* and *R. glutinis* Y_3 cells by HCl treatment and mechanical grinding methods are presented in Table 2. For the mechanical grinding method, compositions of readily extractable and residual lipids are presented. The results show that from the compositions of the lipids the strains can be divided into two groups. The 1st group consisting of *L. starkeyi* and *R. glutinis* Y_3 is characterised by high triglyceride, and very low sterol ester contents of the lipid. In *L. starkeyi* the levels of triglyceride and sterol ester obtained by HCl treatment are 78.0% and 0.2% respectively whereas in *R. glutinis* Y_3 these are 42.0% and 1.6% respectively. In contrast, in *S. cerevisiae* which falls in the 2nd group, the lipid contains higher amount of sterol esters (30.3%) and lower amount of triglycerides (24.8%) than the 1st group. The lower level of triglycerides (46%) and somewhat higher levels of free fatty acids (31.6%) and polar lipid (18.0%) in *R. glutinis* Y_3 are due to the fact that these cells were harvested earlier in the log phase than the other two strains. The composition of the lipid harvested in later period becomes very similar to that of *L. starkeyi* (Table 2).

The effect of HCl treatment was widely different in the two groups. In *S. cerevisiae*, the polar lipids were hydrolysed during HCl treatment as indicated by the lower percentage (13.8%) of this fraction in comparison to that obtained by mechanical grinding (33.5%) in one hand and the concomitant increase in the percentage of free fatty acids (15.2%) as against 3% obtained in the latter method on the other. Phospholipids were preferentially hydrolysed as indicated from the results of estimation of total phosphorus in the lipid of *S. cerevisiae* obtained by the two methods. In the case of 1st group no hydrolysis of polar lipid occurred due to HCl treatment as indicated from the results in Table 2. In *R. glutinis* Y_3 as a matter of fact some phospholipids were degraded during mechanical grinding in presence of solvents. This degradation was probably due to the release of hydrolytic enzymes during grinding and their activation by the solvents[17]. In HCl treatment these enzymes were most probably inactivated. This discrepancy, in the effects of acid treatment and mechanical grinding in presence of solvents towards the degradation of phospholipid in these two different groups of yeast, indicates a

TABLE 2—COMPOSITION OF READILY EXTRACTABLE AND RESIDUAL LIPID OF YEAST BY HCl TREATMENT AND MECHANICAL GRINDING

Yeast	Lipid extraction method	Lipid yield (g/100 g dry cell)		Composition of lipid (wt%)				
				polar[a] lipid	sterol+ diglyceride	free fatty acids	triglyceride	sterol ester
Saccharomyces cerevisiae	HCl treatment		5.0	13.8[b]	10.1	15.2	24.2	36.7
	Mechanical grinding	Readily extractable	4.5	33.5[c]	8.3	3.1	24.8	30.3
		Residual	0.7	21.5	23.4	15.7	20.3	19.1
Lipomyces starkeyi	HCl treatment		25.0	6.3	6.8	8.7	78.0	0.2
	Mechanical grinding	Readily extractable	25.1	6.3	6.2	13.6	73.6	0.3
		Residual	2.8	3.4	4.4	2.1	89.8	0.3
Rhodotorula glutinis Y_3	HCl treatment		18.8[d]	18.8	14.2	23.4	42.0	1.6
	Mechanical grinding	Readily extractable	18.0	13.8	7.0	31.6	46.4	1.2
		Residual	1.8	18.3	11.4	6.8	61.3	2.2

[a]—Contains both phospholipid and glycolipid,

[b,c]—Phosphate estimation in lipid was done according to Ames[16] by converting organic phosphate to Pi by $Mg(NO_3)_2$ digestion and estimating it colorimetrically using ascorbic acid-molybdate reagent. Pi values were multiplied by appropriate factor to obtain phospolipid percentage in the sample. b—contains 12.9%, c—contains 29.1% phospholipid.

[d]—Composition of lipid by HCl treatment from *R. glutinis* Y_3 cells harvested at late log phase—polar lipid 6.2%, diglyceride and sterol 3.3%, free fatty acids 6.9%, triglycerides 83.3% and sterol esters 0.3%.

probable difference in the compartmentalisation of the phospholipids in the two cell systems. It may be pointed out that the compositions of the residual lipid in the mechanical grinding method in the 1st group of yeasts were composed mainly of triglycerides[18]. According to Itoh and Kaneko[9] grinding in the presence of solvent is the most efficient extraction method for yeast lipid but we have found that some phospholipids are liable to be degraded during a similar procedure by us (method v). In our case, the amount of residual lipid is also higher than that of the above workers[19], which may be due to their using a more effective grinder (Cell Homogeniser, Braun).

In our case, HCl treatment followed by solvent extraction, i.e. method (iv) proves to be a simple and efficient method for lipid extraction from fat yeasts like *L. starkeyi* and *R. glutinis* Y_3 containing large amount of triglyceride and can be effectively used for large scale lipid extraction for commercial purposes from these types of yeast strains. Longer treatment of *L. starkeyi* with conc. HCl as done by Suzuki *et al*[5] caused considerable hydrolysis of polar lipids with little increase in total yield (25.99 g/100 g cell) whereas, our method yielded nearly same quantity of lipid (25.0 g/100 g cell) with very little degradation of polar lipids.

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