Studies on the 'Osmophilic' Yeast *Saccharomyces rouxii* and an Obligate Osmophilic Mutant

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(Received 19 April 1974; revised 11 October 1974)

SUMMARY

An obligate osmophilic mutant (strain B1/4) of *Saccharomyces rouxii* has been isolated that fails to grow at osmotic pressures corresponding to 20 % (w/v) sucrose or less. In 30 % sucrose the yeast is filamentous and grows slowly. In 40 % sucrose it is mainly filamentous and has over twice the normal diameter. In 60 % sucrose it grows in the yeast form with a growth rate twice that of the culture in 40 % sucrose. This mutant is lysed by a sudden drop in the osmotic pressure of the environment. Cell envelopes of the parent strain contained glucose and mannose in the ratio 1.2:1 and contained 3.8 % (w/v) hexosamine, whereas the envelopes of the mutant contained 0.8 % hexosamine. Cell envelopes of the mutant grown in 40 % sucrose contained glucose and mannose in the ratio 1.9:1, whereas for envelopes of the yeast grown in 60 % sucrose the ratio was 1.2:1. Neutral lipids from whole cells and those from the envelopes of the mutant strain generally contained more unsaturated fatty acids than the corresponding fractions from the parent strain.

INTRODUCTION

Obligate osmophilic mutants of the yeast *Saccharomyces rouxii* have been isolated as reported in an accompanying paper (Koh, 1975). These mutants require an environment of high sugar or salt concentration for growth. The changes in the growth characteristics, morphology and chemistry of the mutant B1/4 and its parent strain, as functions of the concentration of sugar present during growth, were studied. Cell envelopes were isolated and their chemical composition determined.

METHODS

Materials. All reagents used were of Analar grade, and organic solvents used for isolation of lipids were redistilled and stored in dark bottles in the dark. All percentages are weight per volume unless otherwise stated.

Organisms, growth media and conditions of growth. A strain of *Saccharomyces rouxii* Boutroux (sa 24), isolated from Mauritius sugar by Dr M. P. Scarr (Scarr & Rose, 1966), and the mutant strain B1/4 derived therefrom (Koh, 1975) were used in this work. Stock cultures were maintained on agar slopes (Davies, 1964) containing 0.2 % yeast extract (Difco) supplemented with 10 % (w/v) glucose and 40 % (w/v) sucrose. The cultures were stored at 4 °C and transferred to fresh slopes every six months. For use in experiments, organisms were grown at 30 °C in a defined medium (Davies, 1964) normally supplemented with 2 % glucose as sole carbon source (D medium) with or without sucrose at the concentrations stated in the text.

A small loopful of yeast (approx. 10^8 cells) from a slope was inoculated into 100 ml of

defined medium in a 250 ml conical flask. The culture was shaken at 120 oscillations/min and allowed to grow to early stationary phase. For *S. rouxii* this took 48 h and for mutant B1/4 approximately 100 h. These organisms were then subcultured into 1 l batches of media contained in 2 l conical flasks, to give cell densities of approximately 7×10^{-3} mg dry wt/ml, and the cultures incubated in an orbital shaker at 200 oscillations/min until the cultures were in mid-exponential phase of growth (0.3 to 1.0 mg/ml). This required 15 h for the parent yeast and 40 to 60 h for strain B1/4.

Cell concentrations were measured by diluting samples with isotonic KCl and measuring turbidity with a Spekker absorptiometer (Hilger). Growth characteristics were followed by measuring turbidity, which was related to dry weight by calibration curves determined as described below.

Harvesting and preparation of cell suspensions. Cells grown in high sucrose concentrations were harvested by diluting the cultures with an equal volume of cold isotonic KCl to reduce the viscosity and centrifuging at 4 °C for 10 min at 22000 g. The pellets were washed twice by suspending in isotonic KCl and centrifuging for 5 min at 12000 g. The cells thus obtained were resuspended in an appropriate buffer at the required concentration.

Preparation of lyophilized cells for chemical studies. The KCl-washed yeast was further washed with 100 ml cold distilled water, resuspended in water, frozen and lyophilized. The water wash removed not more than 2 % of the total cell mass as estimated by assay of protein, carbohydrate, nucleic acids and inorganic phosphate. Salt-washed preparations of the mutant yeast (100 to 200 mg/ml) were filtered, under suction, through glass-fibre filters mounted on sintered-glass filters. Surplus KCl was washed away quickly with 2 to 5 ml cold distilled water. The cells were then lyophilized. This procedure was adopted to minimize leakage of intracellular material by sudden changes of osmotic pressure, and it was found that no more than 2 % of the cell material was present in the filtrate in the form of protein, carbohydrate and nucleic acids.

Relationship between dry weight and turbidity. Since the mutant yeast cells were lysed by a sudden decrease in osmotic pressure, a procedure for estimating their dry weight was devised such that a minimum of intracellular material was lost. The yeast was diluted with an equal volume of isotonic KCl and washed twice by centrifugation at 30 °C with isotonic potassium phosphate buffer at pH 7. Thus the concentration of the solution was 0.4 M for yeast grown in the presence of 40 % sucrose and 0.6 M for yeast grown in 60 % sucrose. No crystallization of potassium phosphate occurred at 30 °C. Pelleted yeast was resuspended in 3 to 5 ml of the same buffer. Duplicate 1 ml samples were transferred to tared crucibles, dried at 105 °C for 48 h, cooled *in vacuo*, and weighed. The mass due to buffer constituents was determined by measuring the phosphate contents of the yeast suspensions after dilution with isotonic KCl and removal of cells by centrifugation, and the mass due to the yeast obtained by subtracting this from the total weight. To determine the relationship between cell mass and turbidity, yeast suspensions of known dry wt/ml were diluted either with KCl isotonic to the growth medium or with appropriate mixtures of KCl and sucrose.

Osmotic fragility. The yeast was grown in D medium plus sucrose and harvested as described above. Duplicate samples (10 to 50 mg dry weight) were centrifuged at 900 g for 10 min. One sample was resuspended in KCl solution isotonic to the growth medium and the other sample was suspended in 3 ml distilled water. The samples were left at 20 °C for 10 min and the yeast harvested by centrifugation. The supernatant solutions were recentrifuged to remove any unpelleted yeast. This procedure was repeated twice. The yeast pellets from a given sample were combined, resuspended in 3 ml distilled water, heated at 100 °C for 10 min and undissolved material removed by centrifugation. The cold and hot extracts

were diluted appropriately and their E_{260} measured. The relative amount of u.v.-absorbing material released in the cold was taken as a measure of osmotic fragility. Protein was assayed (Lowry *et al.* 1951) without prior trichloroacetic acid precipitation.

Isolation of yeast envelopes. The yeast was suspended (50 to 150 mg dry wt/ml) in KCl solution isotonic to the growth medium and containing 10 mM-tris-HCl buffer at pH 7.2. The suspension (5 to 10 ml) was mixed with an equal volume of No. 12 Ballotini beads in a 25 ml masticator (MSE 77313) and blended at 9000 rev./min in an ice/salt mixture for 15 min by means of an MSE microhomogenizer. By this procedure, over 98 % cell breakage was usually obtained. Otherwise, the suspensions were centrifuged and the pellets subjected to a further 5 min homogenization with Ballotini beads, after resuspension in buffered isotonic KCl.

Yeast envelopes were separated by the method of Nurminen, Oura & Suomalainen (1970), involving centrifugation at 600 g. The product was either lyophilized or used directly for lipid extraction and chemical analysis. The supernatant solutions, after removal of the envelopes, were centrifuged at 10000 g for 10 min. The pellets were suspended in small volumes of 10 mM-tris-HCl pH 7.2 for the assay of succinic dehydrogenase (SDH).

Purity of the yeast envelope preparations was based on the following criteria: (i) appearance under phase-contrast microscope and on negative staining; (ii) absence of SDH activity – an indication of freedom from mitochondrial contamination; (iii) constancy in chemical composition – an indication of homogeneity.

Succinic dehydrogenase assay. SDH was assayed by the method of Duncan & Mackler (1966), involving phenazine methosulphate-dependent reduction of dichlorophenyl indophenol (DCPIP).

Chemical composition

For estimation of protein and carbohydrate, cells and cell envelopes (approx. 5 mg) were digested with N-NaOH at 70 °C for 10 min to solubilize proteins and disperse polysaccharides. For nucleic acid estimation samples were extracted three times with 0.5 N-perchloric acid at 70 °C for 30 min and the extracts pooled.

Total carbohydrate was estimated by procedure 3 of Devor (1950). Standard curves were prepared by using glucose and mannose (0 to 100 µg). E_{570} for glucose was 1.32 times the mannose equivalent. For determination of the composition of whole yeasts, the relative amounts of these sugars were assumed to be 1:1, and for yeast envelopes the mannose:glucose ratios were determined by gas-liquid chromatography and the appropriate mixture of mannose and glucose used as the standard for carbohydrate estimation.

Protein was estimated by the method of Lowry *et al.* (1951) on NaOH extracts.

RNA was estimated from the E_{260} of 1 N-NaOH or 0.5 N-perchloric acid extracts. Absorption due to protein was corrected for by assuming that 1 µg protein/ml increases E_{260} by 0.001 unit. A standard curve for yeast RNA hydrolysed in 0.5 N-perchloric acid showed that E_{260} was 1.0 at 38.7 µg RNA/ml.

DNA in perchloric acid extracts was estimated by the Giles & Meyer (1965) modification of the Burton (1956) method. Standard curves were made by using calf-thymus DNA.

Total hexosamine. Samples of lyophilized yeast envelope (2 to 4 mg) were digested and samples were withdrawn and assayed by the Morgan-Elson reaction as described by Ghuysen, Tipper & Strominger (1966). A calibration curve was obtained by subjecting 0 to 500 nmol of glucosamine to the same hydrolysis treatment in the presence of 2 mg glucose. In the absence of glucose, glucosamine was destroyed by the hydrolysis treatment and the development of chromogen was much reduced. The presence of glucose prevented this, but

the absorbance of the blank was increased to about 0.15 units, which was low compared with the experimental values obtained. Inclusion of glucose in the standards corrects for interference due to the carbohydrate content of the samples. Some yeast envelopes were assayed in the presence of added glucose and, after correction for interference of carbohydrates, gave similar results to those obtained in the absence of added glucose.

Neutral sugar. Cell envelopes (approx. 5 mg) were hydrolysed by the method of Saeman *et al.* (1954). The neutralized deionized product was dried *in vacuo* at 45 °C and dissolved in 1 ml of pyridine dried over molecular sieve type 4A. Isomeric forms of sugars were equilibrated by heating at 100 °C for 30 min. They were then trimethylsilylated by the method of Sweeley *et al.* (1963). The dried products were dissolved in 2 ml petroleum spirit (b.p. 60 to 80 °C). Samples (1 to 5 µl) were analysed by gas-liquid chromatography on a column of 10 % polyethylene glycol adipate (Pye) on 100 to 120 mesh celite (BDH), by means of a Pye 104 argon chromatograph with dual flame ionization detectors. The argon and hydrogen flow rate was about 40 ml/min and that of air was about 600 ml/min. The temperature was programmed for the range 90 to 190 °C at 4 °C/min. Trimethyl silylated inositol was used as a marker for identification of peaks. The relative amount of each sugar was estimated from the peak areas.

Lipids. (i) Extraction, purification and total lipid estimation. Lyophilized yeast or yeast envelopes (50 to 100 mg) were resuspended in distilled water (1 to 20 ml), dispersed by brief sonication, and lipid extracted by the method of Suomalainen & Nurminen (1970). Extracts were then purified by the method of Wells & Dittmer (1963). The eluates were dried *in vacuo* and weighed.

(ii) Fractionation. This was done by the column chromatographic method described by Rouser, Kritchevsky & Yamamoto (1967) for separation of lipids into neutral, glyco- and phospholipids. Silicic acid (Mallinckrodt, 100 mesh) was mixed with celite (coarse grade from BDH) in a ratio of 1:1 (w/w) to facilitate elution by increasing the flow rate of solvents. Each eluate was dried under vacuum at 40 °C and the content of each class of lipid was estimated as described for total lipids. Each fraction was redissolved in CHCl_3 and stored under N_2 at -20 °C until used for further analyses.

(iii) Thin-layer chromatography. All thin-layer plates were coated with Kieselgel G (Merck) at a thickness of 0.25 mm. Neutral lipids were chromatographed by using a solvent system of petroleum ether (b.p. 40° to 60° C)-diethyl ether-formic acid (85:15:1, by vol.), phospholipids in CHCl_3 -MeOH- H_2O (65:25:4, by vol.) and glycolipids in CHCl_3 -MeOH-acetic acid (100:25:8, by vol.). Amino groups (on phosphatidyl ethanolamine) were detected by spraying the plates with 1 % ninhydrin in 96 % ethanol, phospholipids by the molybdate stain of Dittmer & Lester (1964), and glycolipids by a diphenylamine spray containing 20 ml alcoholic 10 % diphenylamine, 100 ml conc. HCl and 80 ml acetic acid. Lipids and other organic materials were revealed by spraying with chromic acid (0.6 %, w/w, $\text{Na}_2\text{Cr}_2\text{O}_7$ in 55 %, w/w, H_2SO_4) and charring at 160 °C for 15 min. Lipid spots for fatty acid analysis were revealed by spraying with Rhodamine 6G (0.5 % in 96 % ethanol) and exposure to u.v. light. Samples (1 to 5 mg) of lipids were chromatographed. Ergosterol, triacylglycerols and free fatty acids were estimated by the colorimetric methods described by Moore & Baumann (1952), Van Handel & Zilversmit (1957) and Heinen & De Vries (1966), respectively.

(iv) Fatty acid analysis. Lipids from t.l.c. plates were transmethylated by the method of Nichols & Moorhouse (1969). The resulting fatty acid methyl esters were dissolved in small volumes of petroleum spirit (b.p. 40 to 60 °C) and analysed by gas-liquid chromatography as described above for neutral-sugar analysis but with the column at 190 °C throughout. The relative amount of each fatty-acid methyl ester was determined from the peak areas.

Fig. 1. Mutant B1/4 grown in (a) 2 % glucose plus 40 % sucrose, and (b) 2 % glucose plus 60 % sucrose.

RESULTS

Cell morphology

The same length (5 to 15 μm) and ovoid shape of the parent strain of *Saccharomyces rouxii* was observed at high and low concentrations of sucrose. Mutant B1/4 was unable to grow in defined liquid medium at or below 20 % sucrose. In 30 % sucrose it grew slowly and most of the yeasts were filamentous. When grown in 40 % sucrose a large proportion of the cells were filamentous with large bulbous regions 15 to 20 μm in diameter (Fig. 1a). When grown in the presence of 60 % sucrose the cells were morphologically similar to those of the parent strain (Fig. 1b). Sudden reduction in osmotic pressure caused the yeasts to extrude membranous and granular material. Some, but not all, of them became phase-dark on extrusion of intracellular material.

Since glucosamine is a component of yeast cell envelopes (Bacon *et al.* 1966; Cabib & Bowers, 1971; Sentandreu & Northcote, 1968) it is possible that the observed weakness of mutant B1/4 envelope and the morphological changes are due to an inability to synthesize glucosamine. Supplementing the defined growth medium with 0.1 % glucosamine did not allow growth of mutant B1/4 in 20 % sucrose. Yeasts grown in medium containing 0.1 % glucosamine, 40 % sucrose and 2 % glucose were unable to adapt to growth in medium containing 0.1 % glucosamine, 20 % sucrose and 2 % glucose.

Fig. 2. $\diamond$, $\triangle$, $\circ$, $\square$, Growth curves of mutant B1/4 in D medium plus various concentrations of sugars: $\diamond$, 2 % glucose plus 40 % sucrose; $\triangle$, 2 % glucose plus 60 % sucrose; $\circ$, 10 % glucose plus 40 % sucrose; $\square$, 10 % glucose plus 60 % sucrose. $\bullet$, Growth curve of parent *S. rouxii* in D medium plus 2 % glucose.

Growth characteristics of the parent strain of S. rouxii and mutant B1/4

The growth curves of both strains in liquid medium containing various solute concentrations are shown in Fig. 2. The parent *S. rouxii* grew to approximately the same turbidity in stationary phase irrespective of the sugar concentration used. Mutant B1/4 also grew to a turbidity that was roughly independent of the sugar concentration and smaller than the final turbidity of the parent strain.

The growth rate of the parent strain of *S. rouxii* was relatively unaffected by changes in the concentrations of glucose, sucrose or KCl, whereas the growth rate of mutant B1/4 was lower in 2 % glucose plus 40 % sucrose (filamentous form) than in 2 % glucose plus 60 % sucrose (yeast form).

Fragility of the yeasts

A sudden reduction in osmotic pressure (osmotic shock) resulted in considerable release of protein and u.v.-absorbing material from mutant B1/4 but a much smaller release from the parent *S. rouxii* (Table 1). KCl, isotonic to the growth medium, caused release of some protein and u.v.-absorbing material. Soluble protein released from the mutant constituted 6 to 13 % of the total cell protein as measured by the Lowry-Folin method (Lowry *et al.* 1951).

Table 1. Release of u.v.-absorbing material from parent *S. rouxii* and mutant B1/4 on resuspension in isotonic KCl and in water

Percentages are expressed as % of total material absorbing at 260 nm released on boiling the yeast in water for 10 min.

	Parent <i>S. rouxii</i> grown on:		Mutant B1/4 grown on:	
	2 % Glucose	2 % Glucose + 40 % sucrose	2 % Glucose + 40 % sucrose	2 % Glucose + 60 % sucrose
U.v. absorbing material (%) in:				
3 washes in KCl	17	14	28	13
3 washes in H ₂ O	21	25	72	65

Table 2. Percentage composition of parent *S. rouxii* and mutant B1/4 grown in D medium plus various concentrations of sucrose

Values are expressed as % cell dry wt; each batch was analysed in triplicate.

	Parent <i>S. rouxii</i>			Mutant B1/4	
	0 %	40 %	60 %	40 %	60 %
Sucrose concn in D medium ...	0 %	40 %	60 %	40 %	60 %
No. of batches analysed ...	4	3	2	2	2
Protein	40 ± 3	47 ± 4	54 ± 12	46 ± 3	52 ± 3
Carbohydrate	44 ± 3	32 ± 1	25 ± 1	29 ± 8	25 ± 4
Lipids	4.0 ± 0.7	4.4 ± 0.7*	8 ± 2	14 ± 2	11 ± 2
RNA	5 ± 1	6.7 ± 0.2	6 ± 1	7 ± 2	5.5 ± 0.1
DNA	0.3 ± 0.1	0.26 ± 0.09	0.23 ± 0.02	0.32 ± 0.07	0.18 ± 0.06
Polyphosphate	0.9	1.9	—	—	—
Total phosphorus	1.85 ± 0.03	1.85 ± 0.02	—	—	—
Sum of fractions (%)	94 ± 9	92 ± 6	93 ± 16	96 ± 15	94 ± 9

* Four batches analysed.

SDH activity of the various fractions recovered during isolation of yeast envelope

Yeast envelope fractions contained less than 2 % of total SDH and hence were presumably free from contamination by mitochondria. Most of the SDH activity was located in the 10000 g supernatant fractions. The total SDH activity of mutant B1/4 (0.24 to 0.42 μ mol DCPIP reduced/min/mg cells) was significantly lower than that of the parent strain of *S. rouxii* (1.6 to 2.1 μ mol DCPIP reduced/min/mg cells).

Chemical compositions of whole yeasts and yeast envelopes

Whole yeasts of the parent strain varied in total carbohydrate content, depending on the sucrose concentration of the growth medium (Table 2). The carbohydrate content of yeast grown in high sucrose concentrations was lower than that of yeast grown in the absence of sucrose. This change was accompanied by a corresponding increase in protein and lipid content. Whole cells of mutant B1/4 showed no significant variation in content of these components when grown in media of different sucrose concentrations, and the values were similar to those for the parent strain grown in 60 % sucrose. Neither strain showed significant changes in RNA or DNA content, either between strains or as a result of changes in sucrose concentration in the growth medium.

The envelopes from the parent and the mutant organisms contained similar amounts of total carbohydrate, RNA and DNA. Envelopes from mutant B1/4 grown in 60 % sucrose had a higher lipid content than envelopes from 40 % sucrose (Table 3). Both strains had more protein in the envelope at the higher sucrose concentrations.

Table 3. *Percentage composition of cell envelopes isolated from parent S. rouxii and from mutant B1/4 grown in D medium plus various concentrations of sucrose*

Values are expressed as % dry wt of cell envelope; each batch was analysed in triplicate.

Sucrose concn in D medium ... No. of batches analysed ...	Parent <i>S. rouxii</i>		Mutant B1/4	
	0 % 2	40 % 5	40 % 5	60 % 3
Percentage composition of envelopes:				
Protein	8 ± 1	13 ± 3§	10 ± 2	18 ± 2
Glucose:mannose ratio	1:1	1:1	2:1	1:1
Total carbohydrate	84 ± 2	78 ± 5	82 ± 6	77 ± 8
Lipids	3 ± 1	3.7 ± 0.8	4 ± 1	9 ± 2
RNA	0.9	1.4 ± 0.8	0.9 ± 0.5	1.3 ± 0.4
DNA	< 0.006	< 0.02	< 0.02	< 0.06
Hexosamine	3.9 ± 0.4	3.7 ± 0.3†	0.8 ± 0.1*	0.7 ± 0.1*
Total percentage	100 ± 5	99 ± 10	100 ± 10	106 ± 13
Percentage sugar in total neutral sugars				
Glucose	55.5 ± 0.5	54 ± 2†	66 ± 3‡	55 ± 1†
Mannose	44.5 ± 0.5	46 ± 2†	34 ± 3‡	45 ± 1†
Glucose:mannose ratio	1.25:1	1.17:1	1.94:1	1.22:1

* Two batches analysed.

† Three batches analysed.

‡ Four batches analysed.

§ Six batches analysed.

The envelopes from the parent yeast contained approximately 4 % hexosamine whereas mutant B1/4 contained only 0.8 % (Table 3), irrespective of the sucrose concentration.

In both strains, glucose and mannose were the only neutral sugars in the envelopes detected by gas-liquid chromatography. Envelopes of the parent strain had a glucose:mannose ratio of 1.2:1 (Table 3) when grown either in the absence or presence of 40 % sucrose. When mutant B1/4 was grown in 40 % sucrose (filamentous form), this ratio was 1.9:1.0, whereas in 60 % sucrose (the yeast form), it was 1.2:1.0, as in the parent strain.

Lipids in whole yeasts and yeast envelopes

Neutral lipid and phospholipid were the major components of these fractions. Neutral lipid constituted 30 to 70 % and phospholipid 20 to 60 % of the total lipid in whole yeasts of both strains, the proportions being highly variable in different batches. In the envelopes, neutral lipid constituted 55 to 85 % and phospholipid 15 to 45 % of the total lipid. Glycolipid occurred in small amounts (5 %) and could not be estimated gravimetrically because the redistilled acetone used to elute the material contained non-volatile residues.

The neutral lipid fraction

Microdensitometry traces of chromic-acid charred thin-layer chromatography plates showed the major fractions to be triacylglycerols, sterol esters and free fatty acids in both whole yeast and yeast envelope lipids. This method of determining relative quantities is subject to considerable error; the intensity of charring varies with degree of cyclization and unsaturation. By colorimetric assay, the neutral lipid from whole yeasts of both strains (11 to 18 % of the total) was found to contain approximately 10 times more ergosterol than that from envelopes (0.6 to 2.6 % of the total). Envelope neutral lipid contained more free fatty acids (6 to 13 %) than that from whole yeasts (2 to 5 %) in both strains. Triacylglycerols

Table 4. *Percentage fatty acid content of whole-cell neutral lipids*

Sucrose concn in D medium	Parent <i>S. rouxii</i>				Mutant B1/4	
	0 %*	0 %†	40 %*	60 %*	40 %*	60 %*
Fatty acids						
14:0	Tr	Tr	Tr	2.1	0.5	Tr
15:1	1.1	0.4	0.2	0.3	Tr	—
16:0	6.0	8.1	10.6	15.8	9.4	8.2
16:1	17.4	9.9	10.6	18.3	18.2	21.4
17:0	—	—	1.3	—	3.1	—
18:0	1.9	1.7	2.4	6.5	3.8	1.7
18:1	41.7	47.6	27.6	47.6	31.8	45.6
18:2	31.0	32.7	47.3	9.4	33.3	23.2
16:0/16:1	0.35	0.82	1.00	0.86	0.52	0.38
18:0/18:1	0.05	0.04	0.09	0.14	0.12	0.04
18:2/18:1	0.75	0.69	1.75	0.20	1.05	0.51
Unsaturated fatty acids (% of total)	91.2	90.6	85.7	75.6	83.3	90.2

Tr, trace amounts present (< 0.2 %).

* Mid-exponential phase of growth.

† Late exponential phase of growth.

Table 5. *Percentage fatty acid content of whole-cell sterol esters from organisms grown in D medium plus various concentrations of sucrose*

Sucrose concn in D medium	Parent <i>S. rouxii</i>				Mutant B1/4	
	0 %*	0 %†	40 %*		40 %*	60 %*
			Expt 1	Expt 2		
Fatty acids						
15:1	12.2	4.3	1.6	6.8	2.2	0.3
16:0	16.5	21.8	30.2	21.9	3.7	0.8
16:1	12.2	13.0	—	15.5	20.2	6.1
17:0	—	—	—	—	0.7	—
18:0	8.0	19.6	27.7	7.8	2.7	—
18:1	35.2	37.0	15.1	36.9	29.7	59.8
18:2	11.0	4.3	5.0	5.9	35.8	33.0
20:1	5.0	—	20.2	3.0	4.9	—
16:0/16:1	1.35	1.67	—	1.41	0.17	0.13
18:0/18:1	0.23	0.53	1.83	0.21	0.09	0.00
18:2/18:1	0.31	0.12	0.33	0.16	1.20	0.55
Unsaturated fatty acids (% of total)	75.6	58.6	41.9	68.5	92.8	99.2

* Mid-exponential phase of growth.

† Late exponential phase of growth.

constituted by far the largest fraction of the neutral lipids (80 to 90 %) in both the whole cell and the cell envelopes of both strains.

Fatty acids in neutral lipid esters. In the parent strain of *S. rouxii* and in the mutant B1/4 the major fatty acid components of all the neutral lipid fractions were the unsaturated C_{16:1}, C_{18:1} and C_{18:2}. Both strains contained relatively little C_{18:0} fatty acid (Table 4). The C_{16:0} to C_{16:1} ratio for whole-cell neutral lipid from mutant B1/4 was lower than that for the parent yeast grown in defined medium containing 2 % glucose and 40 or 60 % sucrose. Both strains had higher ratios in 40 % than in 60 % sucrose. When the parent strain was grown in 2 % glucose alone, the ratio was similar to that of the mutant.

Table 6. Ratios of fatty acids in various neutral lipid fractions of whole cells and envelopes of the parent yeast and its mutant BI/4 grown in *D* medium plus various concentrations of sucrose

The numbers of batches analysed are given in parentheses.

Lipid	Sucrose concn (%)	Parent <i>S. rouxii</i>			Mutant BI/4		
		16:0/16:1	18:0/18:1	18:2/18:1	16:0/16:1	18:0/18:1	18:2/18:1
Cell-envelope sterol esters	40	0.32 ± 0.03 (2)	0.10 ± 0.06 (2)	0.3 ± 0.2 (2)	0.29 ± 0.07 (3)	0.07 ± 0.02 (3)	0.08 ± 0.02 (3)
	60	—	—	—	0.09 ± 0.02 (2)	0.02 ± 0.01 (2)	0
Whole-cell triacylglycerol	0	0.6 ± 0.3 (2)	0.06 ± 0.01 (2)	0.74 ± 0.02 (2)	—	—	—
	40	1.65	0.11	0.83	0.47	0.04	0.97
	60	1.32	0.10	0.31	0.47	0.09	0.50
Cell-envelope triacylglycerol	40	1.3 ± 0.4 (3)	0.2 ± 0.1 (3)	0.4 ± 0.2 (3)	0.9 ± 0.2 (3)	0.08 ± 0.04 (3)	0.24 ± 0.08 (3)
	60	—	—	—	0.81 ± 0.04 (2)	0.06 ± 0.01 (2)	0.28 ± 0.02 (2)
Whole-cell free fatty acids	40	2.18	0.15	0.97	3.26	0.21	0.41
	60	—	—	—	2.98	0.05	0.18
Cell-envelope free fatty acids	40	5 ± 2 (2)	0.22 (2)	0.11 ± 0.04 (2)	1.5 ± 0.8 (3)	0.17 ± 0.04 (3)	0.14 ± 0.02 (3)
	60	—	—	—	1.52 ± 0.01 (2)	0.10 ± 0.01 (2)	0.15 ± 0.04 (3)

Sterol esters from whole cells showed a great difference in $C_{16:0}$ to $C_{16:1}$ ratio between the parent and the mutant BI/4, and a smaller but significant variation with respect to the ratios $C_{18:0}$ to $C_{18:1}$ and $C_{18:2}$ to $C_{18:1}$ (Table 5). Mutant whole-cell sterol esters contained less $C_{16:0}$ and $C_{18:0}$ but more $C_{18:2}$ fatty acids. These variations were not observed in the cell envelope sterol esters which generally contained much less $C_{18:2}$ fatty acid.

The triacylglycerol fraction of whole-cell lipids from the mutant had lower $C_{16:0}$ to $C_{16:1}$ ratios and slightly higher $C_{18:2}$ to $C_{18:1}$ ratios than the corresponding fractions from the parent yeast grown in similar media (Table 6). Omitting sucrose lowered the $C_{16:0}$ to $C_{16:1}$ ratio for the parent strain, mainly because the $C_{16:0}$ content fell. There was no meaningful change in the $C_{18:0}$ to $C_{18:1}$ ratio between the strains. The $C_{18:2}$ to $C_{18:1}$ ratio decreased when both strains were grown in 60 % sucrose as compared with 40 %.

For cell-envelope triacylglycerol, the $C_{16:0}$ to $C_{16:1}$ ratio for mutant BI/4 was approximately 65 % that of the parent strain (Table 6). The $C_{18:0}$ to $C_{18:1}$ ratio was also generally lower for mutant BI/4, while the $C_{18:2}$ to $C_{18:1}$ ratio showed no significant variation. For whole yeasts and cell envelopes percentages of $C_{16:0}$, $C_{16:1}$, $C_{18:0}$ and $C_{18:1}$ fatty acids in triacylglycerol were similar in both strains. There was a decrease in the percentages of $C_{18:2}$ fatty acid in the envelope triacylglycerol compared with whole-cell triacylglycerol.

There is a general tendency for the $C_{16:0}$ to $C_{16:1}$ and $C_{18:0}$ to $C_{18:1}$ ratios to be lower in mutant BI/4 neutral lipids than in parent strain neutral lipids, from both whole cells and cell envelopes. Also there is a general tendency for neutral lipids from mutant BI/4 to have a higher content of unsaturated fatty acids than do the lipids from the parent strain.

Free fatty acids in the neutral lipid fraction. Whole-cell free fatty acids of mutant BI/4 showed higher $C_{16:0}$ to $C_{16:1}$ ratios than did the parent strain, whereas the $C_{18:2}$ to $C_{18:1}$ ratios were lower for the mutant (Table 6). The free fatty acids of mutant BI/4 cell envelope had lower $C_{16:0}$ to $C_{16:1}$ and $C_{18:0}$ to $C_{18:1}$ ratios than those of the parent strain, mainly due

to a decrease in $C_{16:0}$ and $C_{18:0}$ content. Both strains had a similar $C_{18:2}$ to $C_{18:1}$ ratio in the envelope free fatty acids. The $C_{18:2}$ fatty acid was relatively constant in both strains. The difference in $C_{18:0}$ to $C_{18:1}$ ratios is very slight but the trend is again towards lower ratios for mutant B1/4.

Fatty acids of the phospholipid fraction

Phospholipid was fractionated into cardiolipin, phosphatidyl ethanolamine, phosphatidyl choline plus phosphatidyl serine, and phosphatidyl inositol by thin-layer chromatography as described. The fatty acids from each fraction were subjected to gas-liquid chromatography which showed the major fatty acids in both parent and mutant B1/4 to be $C_{16:0}$, $C_{18:0}$, and $C_{18:1}$. There were no obvious differences between the two strains in the ratios $C_{16:0}$ to $C_{16:1}$, $C_{18:0}$ to $C_{18:1}$ and $C_{18:2}$ to $C_{18:1}$.

DISCUSSION

The fragility of mutant B1/4 when suspended in media of low osmotic pressure, its filamentous form in 40 % sucrose and the low hexosamine content of the envelope (Table 3) suggest that the mutation(s) may affect the synthesis of envelope components and of bud scars which are centres of chitin accumulation (Bacon *et al.* 1966; Cabib & Bowers, 1971). The growth rate in medium supplemented with 40 % sucrose (filamentous form) was approximately 50 % of that in 60 % sucrose (yeast form), or 10 % glucose plus 60 % sucrose (Fig. 2). This variation in growth rate with solute concentration was not observed with the parent strain; this suggests that at low external osmotic pressures the functions of the cytoplasmic membrane were impaired, which resulted in an impaired uptake of nutrients, a leakage of low molecular weight substances from the cell pool, or impaired wall synthesis. Since the yeast suspended in isotonic KCl did not leak more material than did the parent strain (Table 1), leakage of low molecular weight material from the cell pool is unlikely to have caused the variation in growth rate. It is apparent that one cause (and possibly the most important one) of obligate osmophilism in mutant B1/4 is the inability of the yeast to synthesize a wall capable of protecting the plasma membrane in environments of low osmotic pressure.

The chemical composition of whole yeasts (Table 2) showed that growth of the parent strain of *S. rouxii* in high sucrose concentrations led to a reduction in the total carbohydrate content. This could be due to a decrease in accumulation of storage carbohydrates, caused by an increase in fermentation rate. Sucrose would not be directly involved in this phenomenon, since the cells are impermeable to this sugar and lack surface invertase (Scarr, 1951).

The chemical compositions of the envelopes of both the parent and the B1/4 strains were similar. These results are, with some exceptions, in general agreement with those reported for *S. cerevisiae* by Falcone & Nickerson (1956), Eddy (1958) and Northcote & Horne (1952). The hexosamine content found for *S. rouxii* (approx. 4 %) is 1.5 to 2.0 times that found by Falcone & Nickerson (1956), Eddy (1958) and Northcote & Horne (1952) for *S. cerevisiae*. Estimates of carbohydrate content are in general agreement with those of Falcone & Nickerson (1956) but higher than those of Northcote & Horne (1952).

The most interesting changes in the cell envelope, due to mutation, were:

(i) A decrease in hexosamine content. This is at variance with the findings of Bartnicki-Garcia & Nickerson (1962) and Chattaway, Holmes & Barlow (1968) on the filamentous and yeast forms of *Mucor rouxii* and *Candida albicans* respectively. These authors found that the hexosamine contents of the walls of the filamentous forms of their organisms were

greater than those of the yeast forms. Chattaway *et al.* (1968) showed that this hexosamine was mainly chitin. However, Kobayashi & Guiliacci (1967) found that the wall from the yeast form of *Histoplasma capsulatum* contained more hexosamine than the wall of the filamentous form.

Most of the hexosamine of *S. rouxii* envelopes is probably a component of mannan-protein complexes, similar to those isolated by Kessler & Nickerson (1959) and Korn & Northcote (1960) from *S. cerevisiae*, where the hexosamine serves as a link between the mannan and the protein moieties of the macromolecule (Sentandreu & Northcote, 1968). These complexes may confer mechanical strength on the envelope of the parent strain of *S. rouxii*. In mutant B1/4, the low hexosamine content of the cell envelope and the extrusion of intracellular material in environments of low osmotic pressure suggest that the mutant may be unable to synthesize, or assemble, components of these mannan-protein complexes.

(ii) The variation in the ratio of glucose to mannose when the yeast was grown in various sucrose concentrations. This ratio (1.2:1.0) for the envelopes was the same for *S. rouxii* grown in 2 % glucose or in 2 % glucose plus 40 % sucrose and for the mutant grown with 60 % sucrose. The value is similar to that reported by Northcote & Horne (1952) for *S. cerevisiae*. After growth in 2 % glucose plus 40 % sucrose (filamentous form), the glucose to mannose ratio for the mutant cell envelopes was 1.9:1.0. This suggests that there had been a loss of some mannose component (25 % of total neutral sugars), an increase in the glucose component, or a combination of both factors. Since one of the outer layers of the yeast envelope may be composed of mannans (Northcote, 1963) or mannan-protein complexes, it is possible that this layer is not synthesized when the mutant is grown under these conditions. Alternatively, this layer could be synthesized but fail to be incorporated in the envelope in the absence of sufficient hexosamine, and hence be secreted into the growth medium.

The difference in hexosamine content between the mutant B1/4 envelope and that of the parent strain could not have resulted from differences in growth rate, because the envelope of the mutant B1/4 grown in the presence of 40 or 60 % sucrose had the same hexosamine content although the growth rates (0.078 and 0.127 h⁻¹, respectively) were different. Similarly, the elevated glucose:mannose ratio for mutant B1/4 grown in the presence of 40 % sucrose (1.9:1.0) was not solely due to a reduction in growth rate; the glucose:mannose ratio for the mutant grown in 60 % sucrose was the same (1.2:1.0) as that for the parent strain, whether the latter was grown without sucrose or in 40 % sucrose, although there were significant differences in the growth rates of these two strains.

(iii) Changes in lipid composition. Neutral lipids were a major component of this fraction in both whole cells and cell envelopes. Triacylglycerol was the major component of neutral lipids – in agreement with the findings of Hunter & Rose (1972) and Suomalainen & Nurminen (1970) for *S. cerevisiae*. These triacylglycerols appear to be major components either of the yeast plasma membrane or, at least, of that part of the envelope external to this membrane. The occurrence of free fatty acids in the lipids of envelopes has been suggested by Eddy (1958), and Hunter & Rose (1972) found that about 10 % of whole-cell neutral lipids was present as free fatty acids.

The fatty acid compositions of neutral lipids showed that the major fatty acids were C_{16:0}, C_{16:1}, C_{18:1} and C_{18:2}, and it is possible that changes in the ratios of these fatty acids might cause changes in the properties of the cell membranes. That the properties of the cell membrane depend on the nature of its fatty acid content has been reported by Proudlock, Haslam & Linnane (1969) for yeast mitochondrial oxidative phosphorylation, by Schairer & Overath (1969) for thiomethyl galactoside transport in an *Escherichia coli* mutant auxo-

trophic for oleic acid, and by Garland & Cori (1972) for rat-liver microsomal glucose-6-phosphatase activity. The present study showed that the $C_{16:0}$ to $C_{16:1}$ ratios for neutral lipids, sterol esters and triacylglycerols of whole yeasts, and for triacylglycerols and free fatty acids of envelopes were lower for mutant B1/4 than for the parent, *S. rouxii*, when this was grown in high sucrose concentrations. Generally there was a greater content of unsaturated fatty acids in lipids from mutant yeasts and their envelopes. Increase in content of unsaturated fatty acids in lipids from the mutant could result in an increase in fluidity of the cell membranes. Changes in force-area diagrams indicate that there is an increase in fluidity of artificial membranes caused by an increase in the content of unsaturated fatty acids (Demel, Van Deenen & Pethica, 1967). In mutant B1/4, this increase in membrane fluidity could have resulted in the structural and enzymic changes reflected in the following observations: ease of lysis; reduction in succinic dehydrogenase activity; changes in the glucose/mannose ratio of the envelope; filamentous growth in 40 % sucrose.

The author is grateful to the Wellcome Foundation for a research studentship, Dr R. Davies who supervised the work, and members of this sub-department for much useful discussion and encouragement.

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