Lipid Turnover in Oleaginous Yeasts

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When eight strains of the oleaginous yeasts *Candida curvata*, *Lipomyces starkeyi*, *Rhodospiridium toruloides* and *Trichosporon cutaneum* were starved of carbon after having accumulated lipid up to 34% of their biomass, the lipid was readily converted to new biomass in all cases except the two strains of *L. starkeyi*. When *C. curvata* D was grown in a two-stage chemostat with the second stage as a carbon-starvation vessel (but containing NH_4^+) biosynthesis of new biomass reached 1.9 ± 0.2 g per g lipid utilized. Experiments in a single-stage chemostat undergoing transition from lipid accumulation (nitrogen-limited medium) to carbon-starvation conditions showed that the lipid in *C. curvata* was rapidly mobilized. When the lipid was pre-labelled with ^{14}C , a transitory pool of rapidly metabolizable non-lipid material appeared within 1.5 h of the initiation of starvation. Rates of lipid loss indicated that initiation of lipid degradation occurred immediately carbon was lost from the external medium.

INTRODUCTION

The accumulation of lipid in micro-organisms has been well described from both biotechnical and biochemical viewpoints (Ratledge & Boulton, 1985; Ratledge, 1986). The major lipid which is stored, usually in the form of discrete droplets, is triacylglycerol; it can account for up to 80% of the biomass in certain oleaginous organisms. The accumulation of lipid depends upon there being a surfeit of carbon and a deficit of some other nutrient, usually nitrogen, in the growth medium. It has been presumed that lipid would act as a storage material to be used by the cells, akin to other energy reserves (Dawes, 1985), if they subsequently enter a period of starvation. However, unequivocal evidence for utilization of lipid reserves in starved yeasts has not been presented (see Dawes & Senior, 1973) though Deinema (1961) found several oleaginous yeasts that gradually lost their lipid when starved, and whose viability sharply decreased once the lipid had been consumed.

As this laboratory has an interest in the accumulated lipids of eukaryotic micro-organisms, we have sought to resolve the question of lipid utilization as an endogenous energy and carbon reserve in a number of oleaginous yeasts. Three complementary approaches have been used in case it was argued that lipolysis could be triggered by some unsuspected insult to the cells. If similar conclusions could be drawn from the three separate approaches, we would then feel confident of our interpretations of the data.

METHODS

Yeasts. Yeasts were obtained from culture collections as designated (CBS, Centraalbureau voor Schimmelcultures, Delft, Netherlands; NCYC, National Collection of Yeast Cultures, Norwich, UK; ATCC, American Type Culture Collection, Rockville, Md., USA). *Candida curvata* D (= *Apiotrichum curvatum* ATCC 20599), *C. curvata* R and *Trichosporon cutaneum* 40 were kindly supplied by Professor E. G. Hammond, Iowa State University, Ames, Iowa, USA.

Media. The basal media for batch and continuous culture experiments contained the following constituents (g l^{-1}): KH_2PO_4 , 7.0; Na_2HPO_4 , 2.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.5; yeast extract (Oxoid), 1.5; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.008; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.0001; $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 0.0001; $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$,

0.0001, with, for balanced media, NH_4Cl at 3 g l^{-1} and glucose at 30 g l^{-1} ; for nitrogen-limited media NH_4Cl at 0.5 g l^{-1} and carbon source at 30 g l^{-1} ; for carbon-starvation media NH_4Cl at 3 g l^{-1} and no carbon source. The pH in all media was adjusted to pH 5.5 prior to the addition of carbon source and micronutrients.

Batch culture medium was sterilized *in situ* by autoclaving at 121°C for 15 min. Medium for continuous culture experiments was sterilized by membrane filtration (pore size $0.2 \mu\text{m}$), collected in sterile 20 l aspirators and kept for 3 d at room temperature to ensure sterility before use.

Growth of yeasts. Batch culture experiments. Oleaginous yeasts were cultivated in 1 l vortex-aerated bottles containing 700 ml of medium at 30°C . After 5 d, two duplicate samples (20 ml) were taken to determine biomass concentration and lipid content and then immediately approximately 200 ml of the culture was quickly but aseptically transferred to a similar vessel containing 400 ml of carbon-starvation medium already at 30°C . A further 20 ml sample was taken in duplicate immediately after transfer to calculate the new biomass concentration and thus the volume of culture transferred. Initial glucose and NH_4^+ concentrations were also determined by analysis of the supernatants. Yeasts were grown in the second vessel for a further 36 h prior to determining biomass, lipid content, residual glucose and NH_4^+ concentration.

Two-stage continuous culture experiments.

C. curvata D was grown in a two-stage continuous culture with the first vessel being a 1.5 l chemostat with an operating volume of 1.25 l. The second vessel was a similar unit with a working volume of 3.75 l. The vessels were connected in such a way that the entire output of vessel 1 was pumped directly into vessel 2. The hold-up time of the yeast in the connecting tube was about 50 s. Fresh, carbon-free medium was also pumped into vessel 2. The yeast was grown at 30°C and the cultures in both vessels were maintained at pH 5.5 by automatic addition of KOH. Foaming was controlled by the timed addition of antifoam (polyglycol P2000; Bevaloid Ltd, Beverley, UK).

Steady-state conditions in vessel 1 were unchanged during the entire course of the experiment. After a change in the steady-state conditions in vessel 2, at least five changes of medium occurred before samples were taken on two successive days. If the biomass values did not agree, further samples were taken until they did. The dilution rate in vessel 2 was varied by altering the flow rate of carbon-free medium into stage 2. The concentration of glucose in stage 2 remained at about 0.025 g l^{-1} and that leaving stage 1 was at a steady-state concentration of 2 g l^{-1} .

The increase in biomass (or decrease in lipid) per litre of medium in vessel 2 was calculated as

$$x' - xf/f'$$

where x and x' are the biomass (or lipid) concentrations (g l^{-1}) entering and leaving vessel 2 at flow rates f and f' (l h^{-1}) respectively. The specific growth rate of the yeast in the second stage was calculated as

$$\mu' = \frac{f'x' - fx}{v_2x'}$$

where v_2 is the volume of medium in vessel 2.

Single-stage chemostat transition studies. *C. curvata* D was grown at 30°C on nitrogen-limited media in a chemostat, working volume 1.25 l, at a dilution rate of 0.05 h^{-1} with the pH and foaming controlled as given above for the operation of the two-stage chemostat. When at steady state, the incoming media was switched from nitrogen-limited to carbon-starvation (see above) to give the transition point, this being recorded as zero time. In a further series of experiments, *C. curvata* D was grown in a single stage as before. Sodium[1- ^{14}C]acetate [1 ml; 125 Ci (4.63 TBq); specific activity $60 \mu\text{Ci } \mu\text{mol}^{-1}$ ($2.22 \text{ MBq } \mu\text{mol}^{-1}$)] was added to the culture via a syringe being inserted into the medium itself. After 45 min equilibration, the incoming media was switched from nitrogen-limited to carbon-starvation. (The expelled air from the chemostat was passed through two Drechsel bottles containing 20%, w/v, KOH in order to absorb any labelled CO_2 before being led to the atmosphere.) Samples were periodically taken in duplicate from the chemostat for appropriate analyses.

Analyses. Dry weight. Samples (10 ml) were centrifuged at 5000 g for 5 min in pre-weighed, dried tubes and washed twice with 10 ml distilled water. The pellets were dried at 80°C over P_2O_5 in a vacuum oven until constant weight (about 48 h).

Glucose. The concentration of glucose in medium samples was determined using the glucose-oxidase-peroxidase method (Boehringer).

Ammonia. The concentration of NH_4^+ in the medium samples was determined by the method of Chaney & Marbach (1962).

Lipid. Total lipid was determined by a method based on that of Folch *et al.* (1957). Lipid was extracted from freeze-dried cells with chloroform/methanol (2:1, v/v), filtered and washed with 0.9% NaCl and then distilled water. The extract was dried over anhydrous MgSO_4 , and the solvent evaporated using a rotary vacuum evaporator. The lipid was redissolved in diethyl ether and transferred to a pre-weighed vial. The ether was removed in a stream of nitrogen and the residue dried in a vacuum desiccator for 1 h.

Glycogen. Glycogen content of cells was determined by the method of Gunja-Smith *et al.* (1977).

Determination of radioactivity. In lipid. Lipid was extracted as before and finally taken up in 1 ml toluene. A sample (100 μ l) was transferred to 10 ml scintillation fluid (4 g PPO in 1 l toluene) and counted.

In whole cells. A known volume of culture from the chemostat was centrifuged at 10000 g for 5 min, washed once with water and resuspended in a minimum volume of water. The pellet was dried at 121 °C over P₂O₅ in a vacuum oven until most of the water had evaporated. Soluene-100 (Packard) (1 ml) tissue solubilizer was added, and the vial was incubated at room temperature for 2 h; 10 ml of scintillation fluid (4 g PPO, 60 g naphthalene, 20 ml ethanediol, 100 ml methanol and 880 ml dioxan) was then added. The vials were stored in a dark cupboard overnight to reduce chemiluminescence and then counted.

In aqueous samples. Aqueous radioactive samples were counted by adding 1 ml to 10 ml of dioxan-based scintillation fluid (as above) held in a plastic vial. A standard to give counting efficiency and a blank to give background information were also counted for all determinations.

RESULTS

Lipid utilization in starved oleaginous yeasts growing in batch culture

The effect of transferring cells already having accumulated lipid in a nitrogen-limited growth medium to a carbon-starvation medium led to significant utilization of lipid in all but two cases (Table 1). With all yeasts except the two strains of *Lipomyces starkeyi*, the depletion of endogenous lipid was accompanied by an increase in cell biomass. Both strains of *L. starkeyi* failed to show any biomass increase during the 36 h of starvation and one strain (CBS 1809) only utilized its accumulated lipid very poorly with less than 20% of the available lipid being consumed. The other strain examined (CBS 2512) consumed less than 40% of its stored lipid.

Thus, three of the four species of yeast examined, *C. curvata*, *Rhodospiridium toruloides* and *Trichosporon cutaneum*, utilized the stored lipid with an accompanying increase in biomass. As the results with these three yeasts were not dissimilar, further studies were done with *C. curvata* D.

Lipid utilization in two-stage continuous culture

C. curvata D, when grown in a two-stage chemostat with the first stage being nitrogen-limited and the second stage being a carbon-starvation environment, utilized its lipid reserves in proportion to the time that the cells were held in vessel 2, i.e. the residence time (Table 2). In all cases, the final lipid content of the starved cells was 6% or less of the biomass which represented better than 80% utilization of the total lipid.

The calculations for the conversion of lipid into new biomass (Table 2) take into account that the original biomass entering vessel 2 consumes its own lipid and any increase in biomass which occurs is therefore effectively from the lipid-free biomass value. An additional factor has also been taken into account in these calculations which allows for some of the biomass increase to occur from the residual glucose being transferred from vessel 1 into vessel 2. (It has been assumed that the 2 g glucose l⁻¹ entering vessel 2 would have yielded 1 g new cells l⁻¹.)

The conversion of stored lipid into new biomass was, apart from the cells being held in vessel 2 with the longest residence time, remarkably constant at 2.6 ± 0.2 g biomass per g lipid consumed. As this value exceeds the approximate theoretical value of about 1:1.7 (w/w) for the conversion of triacylglycerol to biomass, some of this biomass must have arisen from the yeast extract (at 1.5 g l⁻¹) in the incoming carbon-starvation medium. This, we assume, may have accounted for approximately 0.7 g cells, thus leaving 1.9 ± 0.2 g cells which could only have been derived from the stored lipid being used in conjunction with the external NH₄⁺ supply.

C. curvata D was subsequently found to accumulate, in addition to the lipid, about 10% (w/w) of its biomass as glycogen. Upon starvation for 24 h in batch culture (see above) this decreased to about 3% (w/w) and was thus evidently a second storage material for the cells. However, this glycogen could not have accounted for any of the biomass increase recorded in Table 2 as even optimal utilization of glycogen could only have yielded an equal weight of biomass (if, for example, it was converted to cell envelope polysaccharide) and would, if converted to other cell intermediates, lead to a net depletion of the biomass. The contribution of glycogen (positive or negative) has thus been ignored in our calculations.

Table 1. *Effect of carbon starvation on the biomass and lipid content of oleaginous yeasts in batch culture*

Yeasts were grown for 5 d on nitrogen-limited media (700 ml) in 1 l vortex-aerated vessels at 30 °C (stage 1). A volume of culture was then aseptically transferred to a similar vessel containing carbon-free medium (400 ml) and growth allowed to continue for a further 36 h (stage 2). Experiments were done three times; the results are means of the three.

	Stage 1			Stage 2					
	Dry wt (g l ⁻¹)	Lipid (%, w/w)	Vol. of medium transferred to stage 2 (ml)	Initial glucose concn (g l ⁻¹)	Final glucose concn (g l ⁻¹)	Dry wt (g l ⁻¹)	Lipid (%, w/w)	Increase in total biomass after 36 h (g)	Decrease in total lipid after 36 h (g)
<i>Candida curvata</i> D	9.2	34	240	0.6	0.025	4.48	8	0.67	1.29
<i>Candida curvata</i> R	8.2	28	215	0.045	0.03	3.28	11	0.25	0.91
<i>Lipomyces starkeyi</i> CBS 2512	10.8	31	230	0.054	0.022	3.95	18.8	0.01	0.31
<i>Lipomyces starkeyi</i> CBS 1809	10.3	34	222	0.072	0.027	3.7	28.0	0.01	0.14
<i>Rhodospiridium toruloides</i> CBS 14	9.1	24	209	0.043	0.033	3.86	2.8	0.45	0.39
<i>Rhodospiridium toruloides</i> ML 2590	7.5	7	223	0.47	0.039	3.14	0.4	0.28	0.11
<i>Rhodospiridium toruloides</i> ATCC 26217	9.4	22	201	0.25	0	3.7	1.7	0.33	0.38
<i>Trichosporon cutaneum</i> 40	11.9	23	233	0.27	0.035	4.91	5.9	0.33	0.46

Table 2. Conversion of lipid to new biomass with *C. curvata* D growing in two-stage continuous culture with varying times of residence in vessel 2

Subscripts to columns A, B and C (e.g. A₁) indicate data from vessel 1 or vessel 2.

				A	B	C	D	E	F	G	H	
								Biomass net of lipid consumed + biomass derivable from residual glucose (D + 1)	Biomass corrected for dilution in vessel 2 (E × <i>f/f'</i>)	Effective biomass increase net of lipid consumed (A ₂ - F)	Decrease in lipid (g l ⁻¹) C ₂ - C ₁ (<i>f/f'</i>)	Increase in biomass per decrease in lipid (g g ⁻¹) (G/H)
Vessel	Dilution rate (h ⁻¹)	Specific growth rate (<i>f/f'</i>)*		Biomass (g l ⁻¹)	Lipid in biomass (%, w/w)	Lipid (g l ⁻¹)	A ₁ - $\frac{A_1(B_1 - B_2)}{100}$					
1†	0.05	0.05		13.1	29.5	3.86						
2	0.036	0.005	0.48	7.34	4.1	0.30	9.77	10.77	5.17	2.17	1.56	1.4
2	0.043	0.01	0.39	7.06	4.25	0.30	9.79	10.79	4.21	2.85	1.04	2.7
2	0.053	0.013	0.32	5.95	3.0	0.18	9.63	10.63	3.40	2.55	1.06	2.4
2	0.063	0.016	0.27	5.04	4.3	0.22	9.80	10.80	2.92	2.12	0.84	2.5
2	0.072	0.021	0.24	4.44	5.9	0.26	10.01	11.01	2.64	1.80	0.68	2.6
2	0.077	0.024	0.21	4.01	6.1	0.25	10.03	11.03	2.32	1.69	0.61	2.8

* Dilution factor, where *f* and *f'* are the flow rates of culture entering and leaving vessel 2, respectively.

† Mean values pertaining in vessel 1, acting as a feed vessel over course of run: biomass, ± 0.7 g l⁻¹; percentage lipid, ± 1.5; residual glucose, 2 g l⁻¹.

Fig. 1. Distribution of ^{14}C , derived from [^{14}C]acetate, between lipid and lipid-free biomass during transition of *C. curvata* D from nitrogen-limited medium to carbon-starvation medium in a single-stage chemostat. $\square$, d.p.m. per mg lipid; $\blacksquare$, d.p.m. per mg lipid-free biomass. (This experiment was done in duplicate; the results are means of the two.)

To confirm that direct utilization of lipid into biomass was occurring further experiments were done using a transition, single-stage chemostat culture involving the use of ^{14}C -labelled lipid.

Lipid utilization in a single-state transition chemostat

Use of [^{14}C]acetate. A culture of *C. curvata* D growing in a single-stage chemostat under nitrogen-limited conditions was labelled with [$1\text{-}^{14}\text{C}$]acetate (see Methods). Previous work (Botham & Ratledge, 1979) had shown that, following injection of [^{14}C]acetate into a chemostat culture of an oleaginous yeast growing under the same conditions, the label was readily incorporated into lipid and reached a steady-state distribution within 15 min with no subsequent turnover. Accordingly, with the culture of *C. curvata*, the medium was changed 45 min after the presentation of [$1\text{-}^{14}\text{C}$]acetate to carbon-starvation medium. At this point, about 96% of the total radioactivity had become incorporated into the lipid, the remaining 4% was in the remainder of the cells and negligible ^{14}C remained in the medium.

Upon the onset of starvation, ^{14}C was lost from the lipid and after 1.5 h (the first time of sampling) 40% of the label in the lipid had been lost but was recovered in the cell biomass (Fig. 1). However from 1.5 h to 3.0 h, the ^{14}C within the lipid declined less rapidly but a substantial part of the ^{14}C which had been incorporated into the biomass was now lost, presumably as $^{14}\text{CO}_2$. This loss is greater than the simple subtraction of the two consecutive values given in Fig. 1 as obviously ^{14}C in lipid would have continued to have been transferred into the remainder of the biomass during the intervening 1.5 h. Evidently, the ^{14}C in the biomass was only partially incorporated into new cell material with the majority being in a readily metabolizable pool.

After 3 h of starvation, the rates of ^{14}C loss from the lipid and biomass were approximately that due to wash-out of the cells from the chemostat. However, towards the end of the starvation period (24–30 h), ^{14}C began to accumulate in the biomass indicating that a gradual movement was occurring in the relocation of the ^{14}C from its readily metabolizable pool into more permanent macromolecules.

Turnover in unlabelled cells. Using the same protocol as above, but omitting the presentation of [^{14}C]acetate prior to initiating starvation, the changes in biomass and lipid content of *C. curvata* were followed over 36 h (Fig. 2a). The lipid content of the yeast fell rapidly over the first 4 h, decreasing from 30 to 21% (w/w) of the biomass; this was followed by a fairly steady drop from

Fig. 2. Changes in the biomass and lipid content of *C. curvata* D growing in a chemostat at $D = 0.05 \text{ h}^{-1}$ following transition from nitrogen limitation to carbon starvation. (a): NH_4^+ in medium (●); biomass (○); lipid in cells (■); glucose in medium (□). (b) Exponential rates of loss of lipid (■) and biomass (○). (The experiment was done in duplicate with concurring results; the data are for one experiment.)

21 to 10% (w/w) over the next 12 h. The lipid content then remained constant at about 10% (w/w). The biomass of the yeast fell slowly over the first 1.5 h compared with the decrease in lipid level. This was followed by a gradual decrease from 12.6 to 2.7 g l⁻¹ over the next 32 h.

The glucose concentration in the medium fell immediately after the onset of starvation to reach a negligible value (<0.1 g l⁻¹) after 1.5 h. The NH_4^+ concentration (which was initially zero) rose to reach a steady value of 1.5 g l⁻¹ after 24 h.

As the wash-out of cells from the chemostat must be taken into consideration in calculating the rate of lipid turnover, the data of Fig. 2(a) were recalculated as a log_e plot of the fraction of lipid (or biomass) remaining in the chemostat; i.e. $\log_e(l_t/l_0)$ [or $\log_e(x_t/x_0)$] where l_t (and x_t) are the concentrations of lipid (or biomass) in the chemostat at time t , and l_0 (and x_0) are the initial values. This recalculation (Fig. 2b) showed that the rate of lipid loss was biphasic: from zero time up to 17 h the rate of loss corresponded to 0.12 h⁻¹ and afterwards was 0.041 h⁻¹ and approximately equal to the dilution rate of 0.05 h⁻¹. The rate of loss of biomass was constant throughout the period of the experiment (-0.048 h⁻¹).

DISCUSSION

Lipid may accumulate to over 70% of the biomass in several oleaginous yeasts and it has always been presumed that this is a storage material which confers some advantage to the cell should it subsequently enter a period of starvation. Although some yeasts do readily degrade their stored lipid, and use it to provide carbon for cell growth, not all do so rapidly; both strains of *Lipomyces starkeyi* showed only a poor ability to use their accumulated lipid.

The other oleaginous yeasts examined, *C. curvata*, *Rhodospiridium toruloides* and *Trichosporon cutaneum* all readily used their lipid reserves upon starvation and could evidently produce some new biomass from the lipid. More extensive work with *C. curvata* using one- and two-stage chemostat cultures then confirmed that lipid could indeed be degraded to yield new biomass. The conversion of lipid to new biomass appeared to be high with our calculations indicating that up to 1.9 g new cells could be produced from 1 g lipid. It should though be appreciated that these starved cells did have NH_4^+ (and O_2) available to them with which to synthesize new proteins, nucleic acids etc. When ^{14}C -labelled lipid was created, the label was readily lost upon the cells being starved; the released ^{14}C appeared to go through a transitory, metabolizable pool – perhaps intermediates of the tricarboxylic acid cycle – before eventual incorporation into the macromolecules of the cell. When the rate of lipid turnover was examined in a continuous culture undergoing transition from nitrogen-limitation (i.e. lipogenic conditions) to carbon-starvation, two pools of lipid could be discerned. Approximately 70% of the total lipid was lost at a steady but rapid rate (approximately 2.5 times faster than the growth rate) over the first 17 h; this we presume corresponds to utilization of the triacylglycerols which constitute the bulk of the stored lipid (though this was not checked by lipid analysis). The remaining 30% of the lipid (= 9% of the cell biomass) which did not turn over would probably be structural lipid (i.e. mainly phospholipid, sterols and sterol esters). (In the two-stage chemostat experiments, the lipid decreased to between 4 and 6% in the starved cells with residence times of between 28 and 13 h. The difference between the two results is attributed to differences in the protocols between the two experiments though it may be inferred that some of the so-called 'structural' lipid may be capable of degradation.)

Our results have shown that lipid degradation begins very rapidly after the onset of carbon exhaustion from the medium. Determinations done 1.5 h after initiation of starvation clearly showed that lipid had already begun to be consumed. One may presume this had commenced much earlier than this, possibly within a few minutes of loss of carbon from the medium. The factors which would trigger the appropriate intracellular lipases into such an immediate response are unknown but one is probably dealing with activation of the enzymes rather than waiting for enzyme induction.

Lipid degradation, besides being of importance in the physiology of the yeast cell, also is important when considering potential industrial processes for lipid production. Lipid breakdown could obviously be triggered during the processing of a yeast and lengthy periods without a metabolizable external carbon source must obviously be avoided as our results would suggest that breakdown of stored lipid is a readily instigated event.

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REFERENCES

- BOTHAM, P. A. & RATLEDGE, C. (1979). A biochemical explanation for lipid accumulation in *Candida* 107 and other oleaginous micro-organisms. *Journal of General Microbiology* **14**, 361–375.
- CHANEY, A. L. & MARBACH, E. P. (1962). Modified reagents for the determination of urea and ammonia. *Clinical Chemistry* **8**, 130–132.
- DAWES, E. A. (1985). Starvation, survival and energy reserves. In *Bacteria in their Natural Environments*, pp. 43–79. Edited by M. Fletcher and G. D. Floodgate (Special Publication no. 16 of the Society for General Microbiology). London: Academic Press.
- DAWES, E. A. & SENIOR, P. J. (1973). The role and regulation of energy reserve polymers in micro-organisms. *Advances in Microbial Physiology* **10**, 135–266.
- DEINEMA, M. H. (1961). *Intra- and extra-cellular lipid production by yeasts*. PhD thesis, Agricultural University, Wageningen, The Netherlands.
- FOLCH, J., LEES, M. & SLOAN-STANLEY, G. H. (1957). A simple method for the isolation and purification of total lipid from animal tissues. *Journal of Biological Chemistry* **226**, 497–509.
- GUNJA-SMITH, Z., PATIL, N. B. & SMITH, E. E. (1977). Two pools of glycogen in *Saccharomyces*. *Journal of Bacteriology* **130**, 818–828.
- RATLEDGE, C. (1986). The potential of micro-organisms for oil production – a review of recent publications. In *Proceedings of the World Conference on Emerging Technologies in the Fats and Oils Industry*, pp. 318–330. Edited by A. R. Baldwin. Champaign, Illinois: American Oil Chemists' Society.
- RATLEDGE, C. & BOULTON, C. A. (1985). Fats and Oils. In *Comprehensive Biotechnology*, vol. 3, pp. 983–1003. Edited by S. Drew, D. I. C. Wang & H. Blanch. Oxford: Pergamon Press.