hydrocarbon carotenoids.

In this paper we have separated and identified 11 hydrocarbon carotenoids, including seven not previously isolated, in palm oil processed fractions. The increasing interest in the medical applications of this class of compounds makes their isolation and characterization crucial. Palm oil, one of the world's most widely consumed vegetable oils and a proven non-toxic food (16), could become an important commercial source of carotenoids. Future work will focus on high pressure liquid chromatographic analysis of these compounds and their potential anti-carcinogenic and/or antioxidative effects.

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Effect of Culture Conditions on Fatty Acid Composition in Lipids Produced by the Yeast *Cryptococcus albidus* var. *albidus*

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The influence of culture conditions on the fatty acid composition in lipids produced by *Cryptococcus albidus* var. *albidus* CBS 4517 was studied.

The major fatty acids in *C. albidus* var. *albidus* were oleic (18:1), linoleic (18:2) and palmitic (16:0) acid. The relative amounts of fatty acids produced varied considerably during growth and lipid accumulation phases in nitrogen-limited as well as excess-nitrogen cultures. The degree of unsaturation correlated to the lipid content in the biomass and decreased with increasing amounts of cellular lipid. After glucose exhaustion, no further changes in the fatty acid composition nor in the lipid content of the cells were observed. A number of carbon and nitrogen sources could be utilized for lipid synthesis, but they influenced the fatty acid composition only to a minor extent.

Lipid production from microorganisms provides ample opportunity for research activities, and the subject recently has been reviewed by Ratledge (1). A great deal of the work has been performed on lipid-accumulating yeast strains because a number of them are capable of accumulating large amounts of intracellular lipids (50-70%, w/w) (2,3). The lipid fraction usually has a great similarity to plant oils (1,4), and with the aim of producing substitutes for some of the more expensive types, it is important to gain increased understanding and control over fatty acid biosynthesis in microorganisms.

The yeast *Cryptococcus terricolus* Pedersen (now re-named *C. albidus* var. *albidus* (5) has been reported to have a high lipid-accumulating capacity, which is not dependent on a high C/N ratio in the medium (2,6). This is a unique characteristic because lipid accumulation in oleaginous yeasts is regarded as a two-stage process where lipid starts to be produced from excess carbon after exhaustion of nitrogen (or other nutrient except carbon) from the medium. Despite its interesting properties as a lipid producer, very little work has been reported on this strain of *C. albidus* in recent years. Boulton and Ratledge (6) compare their results concerning the constitutive lipid-producing capacity with those of Pedersen (2,7), whereas Krylova et al. (8,9) deal with lipid synthesis in *C. albidus* var. *aerius* IBFM y-229 using ethanol as the carbon source. Results from

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our laboratory (10) confirm that *C. albidus* var. *albidus* can produce substantial amounts of lipids in the presence of excess nitrogen, but the lipid productivity was found to be markedly increased under nitrogen-limited conditions, suggesting an intermediate lipid accumulation pattern between the traditional oleaginous yeasts and the originally described *C. terricolus*.

No investigation has, so far, been reported concerning the effect of culture conditions on the fatty acid composition in *C. albidus* var. *albidus*. Such knowledge is of interest, as control of certain growth parameters may provide a means to influence the final quality and value of the product.

In this work we report on the effects of carbon source, nitrogen source and temperature on fatty acid composition in *Cryptococcus albidus* var. *albidus*. Alterations in the various fatty acids during cultivation under nitrogen-limited and excess-nitrogen conditions are described, as are a correlation between the degree of unsaturation in the lipid fraction and the lipid content of the cells.

EXPERIMENTAL PROCEDURES

Microorganism. Cryptococcus albidus var. *albidus* CBS 4517 was used. *C. albidus* var. *albidus* was kept on YM-agar slants at 8 C and was transferred every second month.

Cultivation medium. The nitrogen-limited medium contained, per liter: NH_4Cl, 1.0 g; yeast extract, 1.0 g; tryptone, 1.0 g; KH_2PO_4, 3.0 g; $MgSO_4 \cdot 7H_2O$, 1.0 g; $FeCl_3 \cdot 6H_2O$, 15 mg; $ZnSO_4 \cdot 7H_2O$, 7.5 mg, and $CuSO_4 \cdot 5H_2O$, 0.5 mg. The excess-nitrogen medium was the same as above, but the NH_4Cl concentration was 5 g/l. The glucose concentration was 30 g/l in fermentor cultures and 20 g/l in shaker flask experiments. No pH adjustments were performed in shaker flask cultures; in order to shorten the cultivation time at decreased pH, the initial glucose concentration was lowered in these experiments. In the nitrogen-limited fed-batch culture, additional amounts of glucose in concentrated solutions were added at 40 and 65 hr of cultivation to give a concentration of 30 g/l.

When growing the inoculum, NH_4Cl concentration was 2.5 g/l and glucose concentration was 15 g/l. The medium was otherwise the same as above. Neither ammonium ions nor glucose were exhausted at the time for inoculation. About 70 ml inoculum (cell suspension) from the logarithmic growth phase was used per liter fermentor volume, resulting in an initial optical density of $\sim$0.6.

Investigations concerning effects of different carbon or nitrogen sources were performed in nitrogen-limited shaker flask cultures. All flasks contained 0.67 mole carbon/l (corresponding to 20 g/l glucose), and the nitrogen source to be tested was added to give a concentration of 0.019 mole nitrogen/l (corresponding to 1 g/l NH_4Cl).

The pH was adjusted to 5.5 with NaOH. The carbon source and the magnesium solution were autoclaved separately. Urea and amino acids were sterilized by membrane filtration (0.2 μm).

Cultivation equipment and conditions. Fermentor cultures were performed in a fermentor (Chemoferm AB, Hägersten, Sweden) with a working volume of 3.0 l. The cultivation temperature was 20 C, and pH was kept constant at 5.5 by titration with 1.0 M NaOH. Air was sparged through the broth with a flow rate of 0.5 VVM (volume/volume, min). The dissolved oxygen tension was measured continuously with a galvanic oxygen electrode (11). No oxygen limitation occurred during the cultivations. The stirrer speed was 350 rpm. Foaming was controlled by addition of Adekanol LG-109 (Asahi Electro Chemical Co., Japan).

Shaker flask experiments were performed in one-l baffled flasks, containing 150 ml of cell suspension, on a water bath rotary shaker.

In the investigation of temperature effects the inoculum was grown at 22.5 C and then used for cultures at 20, 25 and 30 C. In all other experiments, the incubation temperature was 20 C for inoculum as well as for subsequent cultures.

Growth measurements. Cell growth was followed by measuring optical density (OD) at 620 nm in a Turner photometer (G.K. Turner Associates, Palo Alto, California). The dry weight of biomass was determined by centrifuging 15 ml of cell suspension, washing the pellet with 0.9% NaCl and drying at 105 C for 17 hr.

Lipid determination. 100 ml (OD$\leq$10) or 50 ml (OD$>$10) of cell suspension was removed from the culture. Each sample was centrifuged at 20,000 xg for 10 min at 10 C and washed once in 0.9% NaCl. The pellet was kept in a sealed centrifuge tube and stored at -20 C.

Before extraction of lipids was performed, the cells were disintegrated. Two ml of water was added to the thawed pellet and the cell suspension was sonicated for 6 min on ice in an MSE Soniprep 150 Ultrasonic Disintegrator (MSE, Crawly, England). Thereafter, 5 g of glass beads ($\varnothing$ = 0.5-0.75 mm) were added and the cells were disrupted in a Lab-Line Multiwrist Shaker (Lab-Line Instr., Inc., Melrose Park, Illinois) at maximum intensity for 1.5 hr.

Lipid extraction was performed with n-butanol as the solvent. 10 ml of butanol was added to the cell suspension, and the tube was shaken, as described above, for 20 min. The butanol phase was separated from cells and glass beads by centrifugation. The extraction procedure was repeated twice, and the butanol extracts were pooled. The extracts were washed according to the method of Folch et al. (12) by shaking with 5 ml of water for 5 min. After centrifugation, the butanol phase was transferred to a weighed vessel and evaporated under vacuum. The lipid was dried over anhydrous P_2O_5 in an evacuated desiccator, and the amount of lipid was determined gravimetrically.

Fatty acid composition. The fatty acid composition was determined by gas chromatography of the fatty acid methyl esters. The lipids to be transesterified were dissolved in hexane, and methyl esters were prepared according to the procedure of Glass et al. (13).

The instrument used was a Varian 3700 Gas Chromatograph. Samples were separated on a stainless steel column (1.8 m $\times$ 2 mm) containing 10% DEGS liquid phase coated on Chromosorb W HP (80-100 mesh). Nitrogen with a flow rate of 50 ml/min was used as the carrier gas. The oven temperature was programmed from 130 to 180 C at the rate of 2 C/min. Peaks were identified by means of fatty acid methyl ester

standard FO6 (Larodan Fine Chemicals, Analytical Standards AB, Kungsbacka, Sweden).

The total amount of fatty acids in the lipid fraction constitutes 100%. Besides those fatty acids that were identified by using the FO6 standard (14:0, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3), a small part, less than 4% of the fraction, consisted of other fatty acids, viz. 15:0, 17:0, 17:1, 20:0, 20:1, 20:2, 20:3, 22:0, 22:1, 24:0, and 24:1. Those were identified in a few samples by capillary column chromatography by AB Karlshamns Oljefabriker. Unsaturated fatty acids from this part of the lipid fraction were not included in the calculation of the degree of unsaturation.

The degree of unsaturation (Δ/mole) in the lipid fraction was calculated according to Kates and Baxter (14). The formula used is:

$$\Delta/\text{mole} = [1.0\,(\%\ \text{monoene}) + 2.0\,(\%\ \text{diene}) + 3.0\,(\%\text{triene})]/100$$

Analyses of glucose and NH_4Cl. All assays were performed on samples where cells had been removed from the cultivation broth by centrifugation and membrane filtration. Glucose was measured by using a Boehringer test kit (Boehringer, Mannheim, Federal Republic of Germany), and NH_4Cl was assayed as ammonium ions according to Chaney and Marbach (15).

RESULTS AND DISCUSSION

Effect of carbon source, nitrogen source and growth temperature on fatty acid composition. The influence of different carbon sources on growth and fatty acid composition was investigated in nitrogen-limited (1 g/l NH_4Cl) shaker-flask cultures. The cells were harvested in the stationary phase, and dry weight, lipid content and fatty acid composition were determined (Table 1). *C. albidus* var. *albidus* was able to utilize all the carbon sources tested except ethanol, but very poor growth took place on glycerol. It is clear from Table 1 that only minor changes were observed in the fatty acid composition, and consequently in the degree of unsaturation (Δ/mole).

The stability in fatty acid composition regardless of the carbon source utilized is in agreement with results reported for *Candida* 107 (16,17) and *Rhodotorula gracilis* Lundin R-gl 172 (18). The most pronounced alterations in fatty acid composition as a function of carbon source have been reported for n-alkanes (1,19). The ability among yeasts to utilize n-alkanes is, however, restricted to a limited number of strains.

The effect of different nitrogen sources on the fatty acid composition is shown in Table 2. The carbon source was glucose, 20 g/l. The growth curves (not shown) were almost identical regardless of the nitrogen source used. A drop in pH, most marked in NH_4Cl, $(NH_4)_2SO_4$ and L-ornithine cultures, was observed. *C. albidus* var. *albidus* has, however, previously been found to have a broad pH-optimum for growth (20, and Hansson and Dostálek, unpublished results) and because growth rates were not affected, the changes in pH were assumed not to affect markedly the results presented below.

The relative amount of stearic acid (18:0) was about 5.6% when the cells were grown on inorganic and ~10.5% on organic nitrogen sources. A slight decrease in palmitic acid (16:0) was observed when L-arginine or L-ornithine was used, and an increase in linoleic acid (18:2) was noted when cells were grown on any of the amino acids. The degree of unsaturation was in the same order of magnitude for all the nitrogen sources tested.

The nitrogen source used might affect the fatty acid composition by differences in the uptake rates for organic and inorganic nitrogen or in the nitrogen catabolism. The intracellular NH_4^+ concentration recently was reported to affect lipid production rate and the regulation of lipid biosynthesis in *Rhodosporidium toruloides* Banno CBS 14 (21,22). *Rh. toruloides* contained, in contrast to *C. albidus* var. *albidus*, higher amounts of lipid when grown on organic nitrogen sources than on inorganic. No data concerning fatty acid

TABLE 1

Fatty Acid Composition in *Cryptococcus albidus* var. *albidus* Grown on Different Carbon Sources in Nitrogen-Limited Cultures (1 g/l NH_4Cl)[a]

Carbon source	Biomass (g/l)	Lipid in biomass (%, w/w)	Relative fatty acid composition[b] (%, w/w)						Degree of unsaturation (Δ/mole)
			16:0	16:1	18:0	18:1	18:2	18:3	
xylose	7.1	33.0	21.9	1.7	4.3	54.5	13.8	1.1	0.87
glucose	8.3	40.1	18.6	1.2	5.4	60.9	11.2	1.1	0.88
maltose	8.2	37.7	22.2	1.2	6.0	53.9	13.2	1.2	0.85
lactose	6.5	26.3	25.8	1.2	5.1	47.4	16.6	1.4	0.86
sol. starch[c]	4.7	33.1	22.0	0.9	5.1	51.9	16.1	1.5	0.90
mannitol	7.8	43.5	22.3	0.9	8.2	55.4	10.4	1.3	0.81
glycerol[c]	1.5	43.8	20.5	1.1	5.9	52.4	14.8	1.3	0.87
ethanol[d]	no growth	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

[a]Initial carbon source concentration was 0.67 mole carbon/l unless otherwise indicated.
[b]Small amounts of other fatty acids also were detected, see "Experimental Procedures."
[c]Initial carbon source concentration, 15 g/l.
[d]Initial carbon source concentration, 10 g/l.
N.D., not determined.

TABLE 2

Fatty Acid Composition in *Cryptococcus albidus* var. *albidus* Grown on Different Nitrogen Sources[a]

Nitrogen source	Biomass (g/l)	Lipid in biomass (%, w/w)	Relative fatty acid composition[b] (%, w/w)						Degree of unsaturation (Δ/mole)
			16:0	16:1	18:0	18:1	18:2	18:3	
NH_4Cl	8.4	42.0	18.3	1.3	5.5	60.7	11.0	1.5	0.89
$(NH_4)_2SO_4$	8.6	40.2	17.4	1.3	5.7	59.7	11.3	2.7	0.92
urea	8.4	35.2	18.5	0.8	11.2	54.1	11.2	1.8	0.83
L-glutaminate	10.0	30.3	21.5	1.2	10.1	48.0	14.3	2.1	0.84
L-arginine	9.2	30.8	15.4	0.7	11.4	52.4	14.9	2.0	0.89
L-ornithine	9.0	34.4	15.4	0.8	9.8	53.0	15.8	2.1	0.92
L-proline	9.7	30.8	19.1	1.1	10.1	51.1	14.2	2.5	0.88

[a]The initial concentration of the nitrogen source to be tested was 0.019 mole nitrogen/l. Glucose (20 g/l) was the carbon source.
[b]Small amounts of other fatty acids also were detected, see "Experimental Procedures."

TABLE 3

Influence of Growth Temperature on Fatty Acid Composition in Nitrogen-limited Cultures of *Cryptococcus albidus* var. *albidus*

Cultivation temperature (°C)	Biomas (g/l)	Lipid in biomass (%, w/w)	Relative fatty acid composition[a] (%, w/w)						Degree of unsaturation (Δ/mole)
			16:0	16:1	18:0	18:1	18:2	18:3	
20	8.8	44.4	18.0	1.3	5.2	60.3	11.7	1.3	0.89
25	7.8	44.0	17.1	1.2	5.3	60.0	10.9	2.1	0.89
30	no growth	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

[a]Small amounts of other fatty acids also were detected, see "Experimental Procedures."
N.D., not determined.

composition were given.

C. albidus var. *albidus* was grown at 20, 25 and 30 C in a medium containing glucose and NH_4Cl as carbon and nitrogen sources. As can be seen in Table 3, no significant differences in fatty acid composition or in the lipid content of biomass were observed at 20 as compared to 25 C. No growth occurred at 30 C.

Fermentor cultures under nitrogen-limited or excess-nitrogen conditions. In order to investigate how the fatty acid composition in lipids varied during growth and lipid production phases, fermentor experiments were performed in nitrogen-limited and in excess-nitrogen media. Data from these cultures are shown in Figure 1. The highest values of biomass and lipid content were observed in the nitrogen-limited culture, but considerable amounts also were produced under conditions with excess nitrogen. The dominant fatty acids in the lipid fraction of *C. albidus* var. *albidus* are, as in most other oleaginous yeasts (1), palmitic, oleic and linoleic acid. Changes in the fatty acid composition with cultivation time are shown in Figure 2. An increase in linoleic (18:2) and linolenic acid (18:3) and a corresponding decrease in oleic (18:1), palmitic (16:0) and stearic (18:0) acid occurred during the first phase of both cultivations (to about the 25th hour). As can be seen in Figure 1, this time interval corresponds to a period of fast cell growth where the lipid content in the cells actually shows a slight but reproducible decrease. During the lipid accumulation phase of the cultivations (from around 25 hr until exhaustion of glucose) the relative amount of the different fatty acids changes conversely. Once glucose had been used up, no further significant change was observed in the relative amount of either of the fatty acids or, as can be seen in Figure 1, in the lipid content of the cells.

Variations in fatty acid composition have been reported previously for other oleaginous yeasts, for example *Trichosporon cutaneum* (23) and *Candida curvata* (23,24). Because different organisms and media were used, direct comparisons are not meaningful.

Correlation between lipid content and degree of unsaturation. The correlation between lipid content in the biomass and the degree of unsaturation (Δ/mole) in the lipid fraction is shown in Figure 3. The data originate from the fermentor cultures described above and from a nitrogen-limited fed-batch culture (see Experimental Procedures). The fatty acids are of a more unsaturated composition in cells containing low amounts of lipid, and the degree of unsaturation decreases with increasing lipid content. In Figure 4, the relative amounts of the three dominant fatty acids, palmitic, oleic and linoleic acid, are plotted against the lipid content in the cells. The changes observed in the respective fatty acids with increasing cellular lipid content are similar under nitrogen-limited and excess-nitrogen conditions. A slight increase in palmitic acid with increasing lipid content was observed, whereas the increase in oleic acid was more pronounced. However, the dramatic decrease

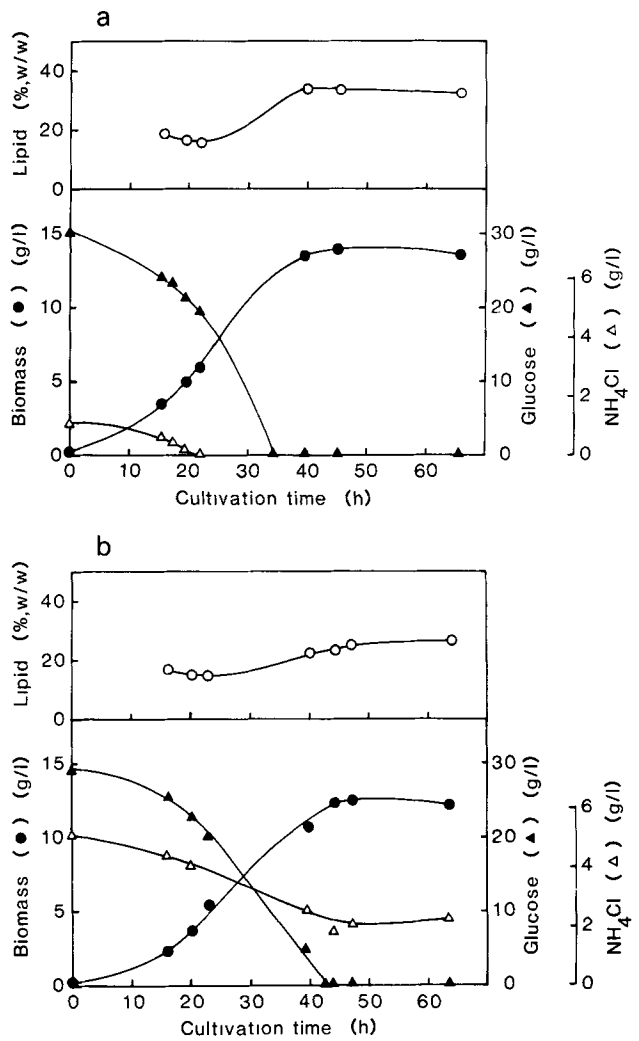

FIG. 1. Cell growth (●), consumption of nutrients (glucose - ▲, NH_4Cl - △) and lipid content in biomass (○) in nitrogen-limited (Fig. 1a) and excess-nitrogen (Fig. 1b) cultures of *Cryptococcus albidus* var. *albidus*.

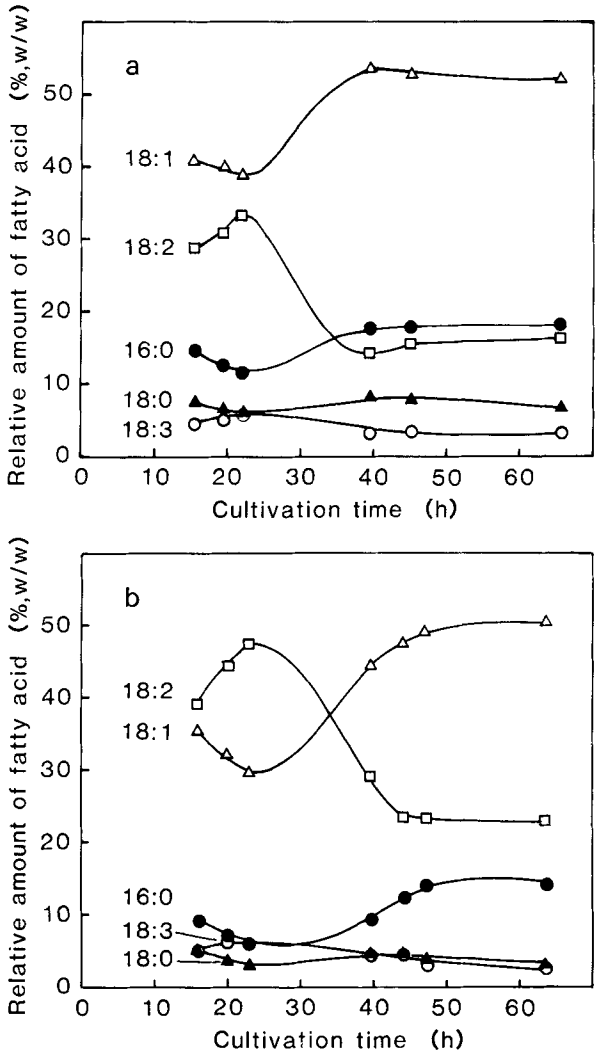

FIG. 2. Changes in the fatty acid composition in lipids produced by *Cryptococcus albidus* var. *albidus* during nitrogen-limited (Fig. 2a) and excess-nitrogen (Fig. 2b) cultures. Small amounts of other fatty acids also were detected; see "Experimental Procedures."

in linoleic acid is the major influence on the Δ/mole value. These results are in agreement with the conclusion reached by Enebo and Iwamoto (25), i.e., the composition of microbial lipids varies with the extent of lipid accumulation.

In batch cultures, the concentration of nutrients in the medium decreases simultaneously with the increase in lipid concentration in the biomass, and the growth rate declines. Fast growing cells, originating from acceleration and logarithmic growth phases, contained low amounts of lipid, and the degree of unsaturation of the fatty acids was high. In declining and stationary phases, however, when the cells grew very slowly or not at all, the lipid content was high and the degree of unsaturation low. Thus, growth conditions may also influence lipid composition, and the growth rate may be used—in chemostat cultures for example—to control fatty acid composition.

The synthesis of fatty acids in yeasts results mainly in the formation of palmitic (16:0) acid and, to a minor extent, of stearic (18:0) acid, from which the unsaturated fatty acids are formed (26,27). It seems that fast-growing cells of *C. albidus* var. *albidus* rapidly convert palmitic and stearic acid to oleic (18:1) acid, which in turn is converted to linoleic (18:2) and linolenic (18:3) acids. In slow-growing cells, palmitic and oleic acids are accumulated at the expense of linoleic and linolenic acids. Thus, it seems that the synthesis of polyunsaturated fatty acids is gradually reduced during lipid accumulation. This reduction might be caused by the lack of a co-factor needed for desaturation (molecular oxygen and NADH or NADPH [26]). It might also be advantageous for the cell to accumulate more saturated lipids for storage and future use as an energy deposit, because more steps are involved in the β-oxidation of unsaturated fatty acids compared to saturated fatty

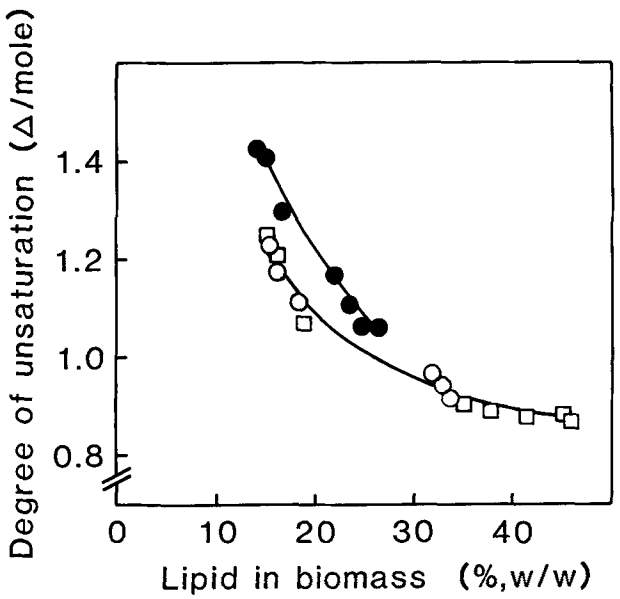

FIG. 3. Correlation between the lipid content in biomass and the degree of unsaturation in the lipid fraction. *Cryptococcus albidus* var. *albidus* was grown in nitrogen-limited batch (○), nitrogen-limited fed-batch (□) and excess-nitrogen batch (●) cultures.

acids. These assumptions concerning the effect of growth rate and the correlation of lipid content in biomass on fatty acid composition have been confirmed in continuous cultures of *C. albidus* var. *albidus* in our laboratory (28).

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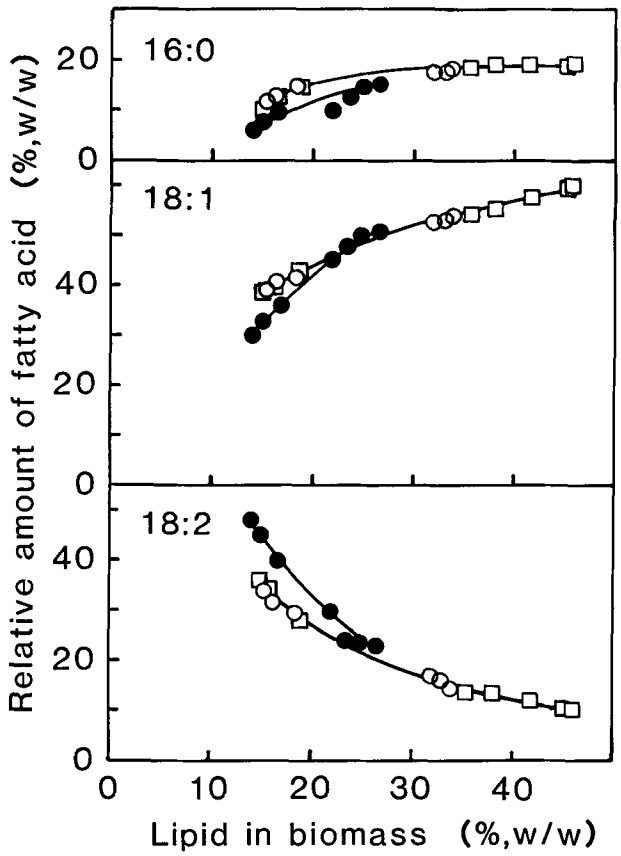

FIG. 4. Relative amounts of palmitic (16:0), oleic (18:1) and linoleic (18:2) acids as a function of the lipid content in cells of *Cryptococcus albidus* var. *albidus*. The following symbols are used: nitrogen-limited batch (○), nitrogen-limited fed-batch (□) and excess-nitrogen batch (●) cultures.

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