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Lipid Accumulation in an Oleaginous Yeast (*Candida* 107) Growing on Glucose in Single-Stage Continuous Culture

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Lipid accumulation in *Candida* 107, grown at dilution rates from 0.03 to the maximum of 0.21/h, with carbon, nitrogen, phosphate, and magnesium limitations in a chemostat, was maximal at about 40% (wt/wt) with nitrogen-limited medium at a dilution rate of 0.06/h, giving an efficiency of substrate conversion of 22 g of lipid per 100 g of glucose consumed. At higher dilution rates the lipid content decreased. With carbon-limited growth, the highest lipid content (14%, wt/wt) was at the maximum dilution rate. High lipid contents also occurred with phosphate + nitrogen as double limitations of growth, with the lipid content of the yeast (about 35%, wt/wt) continuing to be near maximum at dilution rates also near maximum (0.17/h), thus giving the highest specific rate of lipid formation of any growth conditions (0.059 g of lipid/g of yeast per h). However, the efficiency of substrate utilization was only 5.2 g of lipid formed per 100 g of glucose consumed. The composition of the fatty acyl residues within the lipid remained constant over many weeks if the steady-state conditions remained unchanged. With carbon-limited growth, the degree of unsaturation of the fatty acids markedly decreased as the dilution rate was increased, but with nitrogen limitation the reverse trend was seen. In all cases, linoleic and oleic acids were the principal fatty acyl residues affected, and their relative proportions always varied in opposite directions. When magnesium was a limiting nutrient, there was a considerable increase in the proportion of myristic acid produced within the lipid. Neutral lipids (predominantly triglycerides) varied from 66 to 92% of the total lipid from carbon- and nitrogen-limited growth; phospholipids (varying from 2 to 25%) were highest in nitrogen-limited growth. The fatty acyl residues within each lipid fraction showed the same variations with changing growth rates.

Lipid-producing (oleaginous) organisms have been known for many years, and their potential as alternative sources of animal or plant oils has been periodically assessed (20, 28). Most of the work on these organisms, which usually are selected strains of yeasts or molds, has been carried out with either stationary or shake cultures, though some studies of the factors influencing lipid formation have used stirred fermenters (1, 10, 15). To our knowledge no attempt has been made to examine lipid formation in any oleaginous organism growing in continuous culture, although a good deal of work has been done on the lipids of organisms that are not high lipid producers (2, 4, 7, 13). An objection to attempting work with continuously growing cultures may have been the nature of the lipid accumulation process, which requires the exhaustion of a nutrient, usually nitrogen, to allow excess carbon to be channeled into lipids. This is no reason, however, to doubt that lipid will not accumulate in an organism growing continuously since there have been several reports, especially with bacteria, of reserve lipids and polymers accumulating when the organism has grown at low dilution rates in chemostats limited in the supply of nutrients other than carbon (8, 11, 18, 27).

In this paper we report what we believe is the first time that an oleaginous microorganism has been successfully grown in a single-stage continuous culture producing as high a lipid content as has previously been attained in batch culture. *Candida* 107 has attained a lipid content of about 40% (wt/wt) growing on glucose in a batch culture (19), and it is of interest because of its ability to utilize a range of substrates including *n*-alkanes, lactose, and ethanol while still maintaining a high lipid content (19; unpublished data).

MATERIALS AND METHODS

Medium. *Canadida* 107 (see reference 19) was

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grown on (in grams per liter): KH_2PO_4, 7.0; Na_2HPO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 1.5; yeast extract, 1.5; $CaCl_2 \cdot 6H_2O$, 0.2; $FeCl_3 \cdot 6H_2O$, 0.1; biotin, 0.001; and $ZnSO_4 \cdot 7H_2O$, 0.001; the pH was adjusted to 5.5 with HCl. Glucose and NH_4Cl were supplied for carbon-limited growth at 12 and 3 g/liter, respectively, and for nitrogen-limited growth at 30 and 1.5 g/liter, respectively. Medium was sterilized by membrane filtration (pore size, 0.25 μm), collected in sterile 20-liter aspirators, and kept for at least 3 days at room temperature before use.

Continuous-culture apparatus and operation. A 2-liter bench-top fermenter (MF102, New Brunswick Scientific Co. Inc., New Brunswick, N. J.), fitted with pH and O_2 electrodes, was used. The pH was automatically controlled at 5.5 by adding 2 M NaOH. The temperature control was modified to permit regulation within ± 0.1°C by inserting a thermistor directly into the circulating water line. The volume within the fermenter was kept constant by using an overflow weir (5-mm diameter) connected to a continuously operating flow inducer, which ran 30% faster than the inflow medium inducer pump. All flow pumps were from Watson-Marlow (Falmouth, England), type MHRE. Flow rates were measured independently each day, and fluctuations were less than 2% in any 24-h period. Antifoam (polyglycol P2000, Dow Chemical International S. A., London, England) was maintained at 0.06% (vol/vol) by addition at preset intervals, using an appropriate metering pump and timing clock. Incoming air was passed through two miniature fiberglass filters connected in series (Microflow Ltd., Fleet, Hampshire, England). Evaporation from the fermenter was reduced to less than 1% (vol/vol) per day with a water-cooled condenser (100 by 20 mm) inserted between the outlet port and the air outlet filter.

The assembled vessel was sterilized by autoclaving at 121°C for 1 h, and 1.2 liters of sterile medium was added. A 2% inoculum of *Candida* 107 was added and grown as a batch culture for 24 h before commencing continuous culture. Cultures were grown at 30°C with an aeration rate of 1 volume of air/volume of medium per min and stirred at 1,000 rpm, using two flat-bladed turbine impellers (50 mm in diameter), each fitted with six blades (9.5 by 13 mm), which were held at 90° to the direction of movement and held about 100 mm apart.

Samples of up to 100 ml were removed for analysis through an air-lock device into a screw-top bottle. Steady-state conditions were maintained for at least five complete changes of medium in the vessel. Duplicate samples, taken on 2 consecutive days, were analyzed for yeast dry weights, percentage of lipid, and residual glucose and nitrogen. If these determinations were not in agreement, further samples were taken at daily intervals until they were.

Nomenclature. In a single-stage continuous culture, the dilution rate (D) of the fermenter equals the specific growth rate (μ) of the organism. The output of biomass, or product, from the fermenter is Dx or Dp, where x and p are biomass and product concentration, respectively, in the fermenter (given as grams per liter) at a given dilution rate. The specific rate of product formation is Dp/x or, more correctly, $\mu(\Delta p/\Delta x)$, where Δp is the increase in p product concentration corresponding to an increase Δx in biomass concentration. This gives the rate of product formed independently of the prevailing biomass concentration within the fermenter.

Biomass and lipid yields are used to express amount of yeast or product formed per 100 g of substrate consumed and therefore correspond, in the older nomenclature (28), to the economic and fat coefficients, respectively.

Determination of yeast dry weights. Two 5-ml samples were centrifuged at 5,000 $\times$ g for 10 min at 4°C, washed twice with 10 ml of distilled water, and dried at 80°C over $CaCl_2$ under reduced pressure until a constant weight was obtained (usually 24 h).

Determination of glucose. Glucose was estimated in the supernatant solution (from above) with a glucose oxidase reagent kit (Boehringer Mannheim, GmbH, Mannheim, Germany) after removal of the yeast.

Determination of NH_4^+. NH_4^+ in a culture medium supernatant solution was estimated by the method of Chaney and Marbach (6).

Extract of lipid. Existing methods for removing lipid from yeast (22) are slow, taking about 2 days before the lipid content is finally known. Our methods for lyophilized and acid-hydrolyzed preparations (21, 24) were therefore modified to deal with freshly harvested yeast, and the following procedure was quick and allowed complete lipid extraction. A 50-ml sample was centrifuged at 5,000 $\times$ g for 15 min at 2°C; the yeast was washed twice with 100 ml of distilled water, and then suspended in 10 to 15 ml of distilled water (at 2°C) and passed through a French pressure cell, which was precooled to 2°C, at 35 MPa. The extruded mass was stirred with 300 ml of chloroform/methanol (2:1, vol/vol) at room temperature for 2 to 3 h before filtering through Whatman no. 1 filter paper. The filtrate was washed with 100 ml of 1% NaCl followed by two washes with 100 ml of distilled water. The remaining chloroform layer was then dried with anhydrous $MgSO_4$ before being evaporated under reduced pressure. The lipid extract was dissolved in diethyl ether, and any insoluble material was filtered off. The ether was evaporated under nitrogen, and the lipids were weighed and, if necessary before further analysis, kept at -4°C in the dark.

Fractionation of lipid and analysis of fatty acids (see reference 23). The lipid (about 200 mg) was eluted from a silicic acid column by 100 ml each of chloroform, acetone, and methanol in sequence. The solvent from each fraction was evaporated under reduced pressure, and the weight of the residual lipid was determined. The fractions, in order of elution, were neutral lipids, glycolipids, and finally phospholipids and any other polar lipid.

Fatty acids of each lipid fraction or of the total lipid were analyzed as their methyl esters by gas chromatography (24).

RESULTS

Consistency of fatty acid composition. At a constant specific growth rate of 0.15/h, the fatty acids of *Candida* 107 remained almost un-

changed for many weeks (Table 1). Subsequent cultivation of the yeast under the same steady-state conditions, carried out over 1 year later, brought about the same fatty acid profile. Thus, in the following work, any particular steady-state could, if needed, have been held and used to give a continuous supply of yeast with an unchanging fatty acid composition over an indefinite period of time.

Effect of carbon limitation on fatty acid composition. Limitation of growth by the supply of carbon occurred when glucose was at 12 g/liter and NH_4Cl was at 3.0 g/liter. The yeast biomass was constant between specific growth rates of 0.06 and 0.21/h (Table 2). Above μ = 0.21/h, washout commenced. The lipid content of the yeast decreased with decreasing growth rate, probably due to diversion of a greater percentage of the glucose to energy production and maintenance of the organism at the slower growth rates.

Under these conditions, the minimum lipid content of the yeast was 9% (wt/wt) and the maximum was 14% (wt/wt). The maximum rate of lipid output from the fermenter was 0.18 g/liter per h at a yeast concentration of 6 g/liter, i.e., giving a specific rate of lipid formation of 0.03 g of lipid/g of yeast per h.

The lipid from the yeast grown at each dilution rate was fractionated into: (i) neutral lipids, of which the major component (>98%) is triglyceride (24); (ii) glycolipids; and (iii) phospholipids. None of these fractions was further purified, and the nature of the glycolipid was not examined. At all dilution rates the major component was the neutral lipid fraction (Table 2). The increased content of lipid in the yeast grown at the higher dilution rates was principally due to the increased synthesis of neutral lipids. The amounts of glycolipid and phospholipid varied considerably, but both were at higher relative proportions in the total lipid at low dilution rates than at high rates. This is probably a reflection of the cell size changing with the growth rate (see "Morphology of *Candida* 107"); small oval cells, such as exist at low growth rates, will need relatively more structural and functional lipids than longer cells. The difference in roles of the three lipid fractions (phospholipids, glycolipids, and neutral lipids) is further evidenced by the considerable variations in the proportions of fatty acyl groups associated with them (Table 2).

The proportions of fatty acids in all three lipid fractions varied considerably with the growth rate of the yeast (Table 2). Short-chain fatty acids (C_{10} and C_{12}) only occurred in the glycolipid and reached a maximum proportion at μ = 0.17/h. Of all the acids, linoleic acid ($C_{18:2}$) showed the greatest variation in concentration; in rapidly growing cultures it was present only as a small percentage of phospholipids, whereas in the slowest growing cultures over 57% of the total lipids was linoleic acid. Although the concentration of oleic acid ($C_{18:1}$) showed the opposite trend, the net effect on the degree of unsaturation (calculated as the number of double bonds per mole of lipid, i.e., Δ/mole) was that all three lipid fractions were more than twice as unsaturated at the slowest growth rates as at the fastest growth rates.

Effect of nitrogen limitation on fatty acid composition. To achieve concentrations of yeast in nitrogen-limited cultures equivalent to those in carbon-limited cultures, the concentration of NH_4^+ was made equal to the maximum consumption of nitrogen by the carbon-limited yeast, i.e., NH_4Cl at 1.5 g/liter.

In nitrogen-limited cultures, both the biomass and quantity of glucose consumed increased with decreasing dilution rate, reaching a maximum at D = 0.06/h (Table 3). (The spe-

TABLE 1. *Consistency of fatty acid composition in Candida 107 growing for several weeks in a single-stage, glucose-limited chemostat at a dilution rate of 0.15/h*

Elapsed time (weeks)	Dry wt (g/liter)	Lipid content (% dry wt of yeast)	Relative fatty acid composition (%, wt/wt)									
			14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	22:0	24:0
0	5.5	11.1	T[a]	19.4	1.5	5.9	42.7	23.6	T	T	2.2	1.3
1	6.5	9.5	1.6	21.6	1.8	8.0	35.5	24.6	2.0	T	3.2	T
2	5.3	11.3	T	19.8	1.3	7.3	40.1	24.3	T	1.9	2.0	1.6
6	5.9	10.6	T	20.6	1.3	7.4	41.6	22.0	T	2.0	T	1.2
11	6.6	9.8	T	18.0	1.4	7.5	43.1	23.5	T	1.8	2.9	T
13	7.0	10.1	1.1	20.5	1.6	8.1	40.1	22.6	T	2.1	3.1	2.0
15	5.7	9.4	T	21.9	T	8.0	37.8	25.1	T	T	1.9	1.7
21	6.1	10.2	T	21.7	T	7.9	37.5	24.7	T	2.3	1.4	1.2
Avg	6.1	10.25	T	20.4	1.5	7.5	39.8	23.7	T	2.0	2.3	1.5

[a] T, Trace.

TABLE 2. *Growth, lipid yields, and lipid and fatty acid*

Dilution rate (per h)	NH_4Cl remaining[a] (g/liter)	Biomass (g/liter)	Biomass yield (g of yeast/g of glucose used)	Lipid content (% biomass)	Lipid yield (g of lipid/100 g of glucose used)	Lipid output rate (g of lipid/liter per h)	Specific rate of lipid formation (g of lipid/g of yeast per h)	Lipid fractions[b] (% lipid)		
								P	G	N
0.03	1.52	5.7	47.5	9.2	4.3	0.016	0.003	25	9	66
0.06	1.50	6.0	50.0	9.4	4.7	0.034	0.005	11	21	68
0.10	1.49	6.1	50.8	9.0	4.6	0.055	0.009	22	10	68
0.14	1.49	6.2	51.6	9.7	5.0	0.084	0.014	17	14	69
0.17	1.65	6.2	51.6	12.2	6.3	0.14	0.023	5	12	83
0.19	1.74	6.0	50.0	13.3	6.7	0.15	0.025	5	5	90
0.21	1.80	6.0	50.0	14.2	7.1	0.18	0.030	2	6	92

[a] At 3.0 g/liter in incoming medium.
[b] Abbreviations: P, phospholipid; G, glycolipid; N, neutral lipid (mainly triglyceride).
[c] Calculated by the formula of Kates and Baxter (14).
[d] T, Trace.

cific rate of glucose utilization [grams of glucose per gram of yeast per hour], however, increased with increasing growth rate as might be expected; these values are not calculated in Table 3.) The increase in biomass at low dilution rates was mainly due to the lipid produced: at $D = 0.06$/h the lipid content of the yeast was 37% of the dry weight, with a lipid yield (grams of lipid produced per 100 g of glucose consumed) of 22. The biomass yield at this growth rate was 60, with all the glucose within the chemostat having been consumed. However, if the concentration of glucose in the incoming medium was raised from 30 to 60 g/liter, 36 g of glucose per liter was now consumed at the same dilution rate but gave only 15 g of yeast per liter, although this contained 42% of its weight as lipid. The metabolic status of the yeast is thus influenced by the concentration of available glucose in the medium, and further work on this aspect is currently in progress.

The maximum lipid output rate was 0.40 g of lipid per liter per h at $D = 0.06$/h, with a yeast concentration of 18 g/liter. The maximum specific rate of lipid formation, however, was at the maximum dilution rate ($= 0.21$/h), though this gave a poor conversion of substrate to lipid.

The content of neutral lipid in the total lipid varied with the dilution rate: when lipid accumulation was highest, the amount of neutral lipid was only about 70% of the total lipid, but this increased to over 80% at higher dilution rates. The amount of glycolipid was appreciable at all dilution rates, whereas the phospholipids were often at very low concentrations, about 0.4% of the biomass in some cases (Table 3).

Although the proportion of linoleic acid in the neutral lipid increased with increasing growth rate, its effect on the overall degree of unsaturation was modulated by an opposite variation in oleic acid concentration. Highly unsaturated phospholipids were maximal at the times of greatest lipid accumulation, which may indicate a need for such acids in the synthesis of the membranes of vacuoles used for storing the lipid.

Effect of phosphate limitation on fatty acid composition. For phosphate-limited growth, KH_2PO_4 at 0.05 g/liter, with no Na_2HPO_4 added, was the sole source of phosphate. Glucose and NH_4Cl were at 30 and 1.5 g/liter, respectively. Cultures were not limited by the supply of K^+, since adding K_2SO_4 at 4 g/liter to maintain the K^+ concentration at its previous level did not change the yeast concentration within the chemostat. At the extremes of growth rate ($0.07\text{/h} < \mu > 0.15\text{/h}$) there was an unexplicable increase in demand by the yeast for nitrogen. Even at the highest dilution rate (0.17/h), when the biomass had dropped to 2.2 g/liter, no NH_4^+ could be detected in the medium. How this amount of NH_4^+ was being utilized was not investigated. At both high and low dilution rates cultures consequently grew with dual limitations of both phosphate and nitrogen. Since accumulation of lipid was not as high as with nitrogen-limited growth (Table 4), further investigations using modified media to achieve only phosphate limitation at these dilution rates were not pursued. However, the amount of lipid that did accumulate under these conditions of dual limitation was higher than experienced when either nitrogen or phosphate was the sole limitation (cf. Tables 3 and 4). Over 44% of the yeast biomass was lipid at $\mu = 0.075$/h; furthermore, at $\mu = 0.15$ and 0.17/h the lipid contents were still very high (about 35%). Thus high concentrations of lipid were produced at growth rates approaching maximum, but at these high dilution rates the efficiency of carbon utilization dropped dramatically: biomass yields were less than 40% of those achieved at other dilution rates and the lipid yields were correspondingly very low.

composition of Candida 107 grown under carbon-limited conditions

Relative proportion of fatty acids (%, wt/wt)																																		Δ/mol[c]		
10:0			12:0			14:0			16:0			16:1			15.0 + 17.0			18:0			18:1			18:2			20:0			22:0					
P	G	N	P	G	N	P	G	N	P	G	N	P	G	N	P	G	N	P	G	N	P	G	N	P	G	N	P	G	N	P	G	N	P	G	N
						0.8	1.2	2.6	16.3	22.1	20.8	1.0	1.1	1.3	1.1	1.8	2.7	2.7	4.6	4.2	11.7	14.7	16.3	64.8	54.5	48.1				1.5		3.2	1.42	1.25	1.14
			0.6	1.7		1.0	1.4	1.3	27.2	22.4	27.8	2.1	2.2	1.8				4.1	5.5	7.4	31.2	37.4	33.0	33.3	28.1	27.4	1.2	2.3	0.5				1.00	0.96	0.90
	T[d]			T		0.5	1.6	1.0	22.6	25.9	27.6	1.6	1.3	1.4	0.7			2.8	6.5	6.5	12.0	21.0	28.2	59.0	42.5	33.0			0.8				1.31	1.07	1.11
	5.4		T	6.6		T	1.4	1.2	26.3	25.0	31.0	3.6	2.2	2.1	0.8			2.7	4.2	7.3	24.9	32.6	41.8	40.7	21.8	16.6	0.7		1.1				1.10	0.78	0.77
	14.1			14.1		5.4	3.5	3.0	38.0	21.5	31.2	5.4	5.7	6.5			1.3	9.0	3.5	4.6	36.4	36.3	49.0	5.9	1.2	1.2		0.9			1.5		0.54	0.44	0.58
	2.4			2.4		4.1	5.0	2.1	41.3	52.4	35.8	4.8	4.8	2.8				5.2	8.0	8.2	36.4	22.0	42.9	7.3	3.0	5.4		1.5	0.8				0.56	0.33	0.57
	6.2			6.2		4.7	3.3	2.7	30.8	35.8	26.7	3.0	3.8	4.7				8.2	8.8	7.2	40.4	36.9	53.8	12.9				3.3	0.5		1.7	0.69	0.41	0.59

The formation of linoleic acid was favored by high dilution rates, and this was unchanged when phosphate as the sole nutrient limitation gave way to phosphate and nitrogen as dual limitations of growth. In this respect the production of linoleic acid was similar to that observed for nitrogen-limited growth (cf. Table 3).

The lipid from the yeast grown at $D = 0.1$/h with phosphate as the limiting nutrient, when fractionated according to Barron and Hanahan (3), was not depleted in phospholipid content when compared with the lipid from nitrogen-limited growth (Table 3). Instead, a slight decrease in the content of sterol esters (about 5% of the lipid from nitrogen-limited *Candida* 107) to about 2 to 2.5% was found.

Effect of magnesium limitation on fatty acid composition. For magnesium-limited growth, $MgSO_4 \cdot 7H_2O$ was added at 0.05 g/liter. Concentrations of glucose and NH_4Cl were unchanged from phosphate-limited conditions. Adding Na_2SO_4 at 0.8 g/liter to restore sulfate ions to their former concentration did not increase the biomass within the fermenter. Magnesium-limited growth was impossible to achieve at every dilution rate without constantly reformulating the medium. Presumably, as with phosphate-limited medium, this is because the metabolic status of the yeast alters with changing growth rates and thus the demand for other nutrients is not constant. Accordingly, both nitrogen and magnesium were limiting at specific growth rates below 0.07 and above 0.1/h (Table 5). As the amount of lipid within the yeast was not as high as with nitrogen limitation, reformulation of the medium to give only magnesium limitation was not carried out. As before, we are at a loss to explain why so much NH_4^+ was being used by cultures at a high dilution rate when there was less biomass to support.

Unlike phosphate-plus-nitrogen-limited growth, magnesium-plus-nitrogen limitation did not sustain as high a lipid content of the yeast at the higher dilution rates. At $D = 0.125$/h, however, the lipid content of the yeast was over 50% greater than with only magnesium limitation at $D = 0.1$/h. The fatty acids produced under these conditions (Table 5) were unusual for their higher-than-normal content of myristic acid ($C_{14:0}$). At $D = 0.15$/h the amount of this acid constituted over 13% of the total acids. Traces of lauric acid ($C_{12:0}$) were also seen in most samples. The occurrence of these shorter-chain acids appeared in both magnesium-limited and magnesium-plus-nitrogen-limited conditions and was thus probably a consequence of the low magnesium concentration.

Morphology of *Candida* 107. Under carbon- and nitrogen-limited growth conditions at dilution rates near maximum ($D = 0.21$/h), the yeast was elongated and formed extensive pseudomycelia. As the dilution rate decreased the yeast shortened, and at $D = 0.14$/h the average length was 25 to 30 μm. Under carbon-limited conditions, the yeast continued to shorten as the dilution rate decreased until at $D = 0.03$/h it was only about 10 μm long. When nitrogen was the limiting nutrient, lipid accumulation became extensive at the lower growth rates (see Table 3) and the yeast did not shorten to much less than 20 μm. Extensive lipid vacuoles were seen and cells often contained five or more discrete vacuoles, thus assuming a "peas-in-a-pod"-like appearance. Similar changes in cell length, though not as extensive and without the formation of lipid vacuoles, have been reported in *Saccharomyces cerevisiae* grown at different dilution rates (4, 17).

DISCUSSION

Lipid accumulation in *Candida* 107 growing in continuous culture follows a course that could be predicted from its pattern in batch culture, which, like that in other oleaginous yeasts, can be viewed as a two-stage process: the first stage is the multiplication of cells; the second, caused by the limitation of growth by exhaustion of a nutrient, is the conversion of

TABLE 3. *Growth, lipid yields, and lipid and fatty acid composition of Candida 107 grown under nitrogen-limited conditions*

Dilution rate (per h)	Glucose remaining[a] (g/liter)	Biomass (g/liter)	Biomass yield (g of yeast/100 g of glucose used)	Lipid content (% biomass)	Lipid yield (g of lipid/100 g of glucose used)	Lipid output (g of lipid/g of yeast per h)	Specific rate of lipid formation (g of lipid/g of yeast per h)	Lipid fractions[b] (% lipid)			Relative proportion of fatty acids (%, wt/wt)																		Δmol[c]		
											10:0		12:0		14:0		16:0		16:1		18:0		18:1		18:2		20:0		22:0			
								P	G	N	P	G	N	P	G	N	P	G	N	P	G	N	P	G	N	P	G	N	P	G	N
0.03	0	16.4	54.5	21.8	11.9	0.11	0.007	2	2	3	75		5.6	6.4	10.7	1.5	2.7	0.9	43.8	40.9	32.3	1.2	0.6	0.6	11.7	17.6	17.5	24.4	15.4	43.2	9.0	2.8	0.4	2.0	3.7	5.0				0.44	0.22	0.45
0.06	0	18.1	60.2	37.1	22.4	0.40	0.022	2	3	0	68		4.9	1.6	10.2	1.0	2.6	0.8	33.6	50.4	36.9	1.2	1.1	0.7	4.8	10.2	13.6	30.9	17.5	35.6	27.0	3.1	8.3			4.2				0.86	0.25	0.53
0.08	0	12.9	43.1	32.3	13.9	0.33	0.026	8	1	4	78		14.4	0.5	21.2	0.4	5.1	0.8	15.5	24.8	29.5	1.4	1.2	1.3	1.6	6.7	8.4	23.9	25.1	47.3	56.4	0.5	9.7		0.9	2.9				1.38	0.27	0.68
0.1	0.75	14.0	40.8	27.9	13.3	0.39	0.028	6	1	0	84		11.8	1.5	22.6	0.3	3.4	1.0	19.0	22.6	32.1	1.5	1.1	1.2	2.3	4.4	13.0	27.5	21.6	40.6	47.4	11.4	10.0		1.1	1.4				1.24	0.46	0.62
0.13	7.5	11.7	52.2	23.2	12.1	0.35	0.030	2	1	5	83		17.9	2.1	11.6	1.6	1.9	1.3	37.0	39.9	45.9	0.7	0.8	0.7	7.6	9.5	13.0	32.6	13.2	29.3	17.5	5.2	6.2	1.0		3.6				0.68	0.24	0.42
0.177	13.5	8.5	52.8	20.5	10.6	0.31	0.036	1	6	1	0	7	4	0.4	6.1	0.6	2.4	0.9	26.7	36.5	30.6	1.2	1.7	1.2	2.0	8.0	10.8	23.8	25.7	41.3	45.1	16.4	12.5		1.7	2.8				1.15	0.60	0.68
0.21	15.5	7.15	49.3	19.5	9.5	0.29	0.041	2	2	1	77	0.7	6.0	1.8	4.4	1.5	1.5	1.0	36.4	40.5	40.1	1.2	1.2	1.8	4.3	9.8	10.6	21.8	22.8	27.6	29.8	11.8	17.4	0.9	1.9	1.6		0.9		0.83	0.48	0.64

[a] Glucose at 30 g/liter in incoming medium.
[b, c] See Table 2.

the excess carbon to lipid. From a physiological viewpoint, however, lipid accumulation is not caused by nitrogen depletion suddenly inducing a much higher rate of lipid synthesis (15) but by the synthesis of other cell constituents such as protein and nucleic acids, which require nitrogen, ceasing. It is the rate of lipid synthesis relative to the rate of synthesis of other cell components, therefore, that determines whether lipid accumulates in microorganisms. To achieve this situation in continuous culture, the growth rate of the organism must be kept considerably less than maximum, and under such conditions lipid accumulation in *Candida* 107 reached the same concentration that it achieved in batch culture (20). Lipid accumulation is therefore no different from the observed accumulation of various storage polymers in bacteria growing in continuous culture (see Introduction) and should be attainable with most other oleaginous organisms if grown in continuous culture. The conclusion reached by Krumphanzl et al. (16), after following lipid accumulation in *Rhodotorula gracilis*, that a two-stage system would be necessary to make this process continuous is evidently incorrect. Such a two-stage chemostat system does indeed work but offers no practical advantage over a single-stage process (M. J. Hall and C. Ratledge, unpublished data).

A continuous-culture system has many advantages over a batch production system, not least of which is the production of a product that has an unvarying composition. In batch culture, the fatty acid composition of a microbial oil can vary between quite wide limits (10, 15), but in continuous culture we have shown that under steady-state conditions the lipid composition, as reflected by its fatty acid composition, will remain almost unchanged over many weeks of operation. This clearly is an essential prerequisite for any commercial development of such a process. Such variations as are encountered (see Table 1) can probably be attributed to minor changes in different batches of medium and to experimental error and variation. Thus, a desirable fatty acid composition, once attained, could be maintained indefinitely, and a product of known and predictable composition could be produced for as long as the steady-state conditions could be maintained.

Minor changes in the growth rate (= dilution rate) would probably be the greatest influence on the constancy of fatty acid composition, but these changes are unlikely to be excessive (see Tables 2–5). However, the variations in the fatty acid composition of *Candida* 107 with varying growth rates did show distinct differ-

TABLE 4. *Growth, lipid yields, and lipid and fatty acid composition of Candida 107 grown under phosphate-limited conditions*

Dilution rate (per h)	Glucose remaining (g/liter)	Biomass (g/liter)	Lipid (% biomass)	Biomass yield (g of yeast/100 g of glucose used)	Lipid yield (g of yeast/100 g of glucose used)	Lipid output (g/liter per h)	Specific rate of lipid formation (g of lipid/g of yeast per h)	Relative percentage of fatty acids in total lipids (%, wt/wt)								Δ/mol
								14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	
0.075[a]	0.2	8.79	44.5	29.5	13.1	0.29	0.033	0.9	26.7	4.9	5.0	41.2	21.2	T[b]	T	0.85
0.075	10.0	7.43	28.0	37.2	10.4	0.17	0.023	0.9	28.2	3.1	4.6	44.7	18.5	T	T	0.85
0.09	14.0	7.78	31.0	48.6	15.1	0.22	0.028	0.7	26.0	3.6	4.2	44.7	20.8	T	T	0.89
0.1	11.5	6.98	26.1	37.7	9.8	0.18	0.026	T	30.0	3.0	4.8	38.4	22.1	0.8	0.9	0.85
0.12	7.0	6.01	18.7	26.1	4.9	0.13	0.022	T	26.9	3.0	3.6	38.8	27.6	T	T	0.97
0.15	10.0	4.16	36.2	20.8	7.5	0.23	0.055	T	29.9	3.0	4.2	39.2	25.3	T	T	0.93
0.17[a]	15.5	2.2	34.8	15.2	5.2	0.13	0.059	1.8	32.0	2.0	3.9	33.2	27.1			0.89

[a] Dual growth limitation of nitrogen and phosphate.
[b] T, Trace.

TABLE 5. *Growth, lipid yields, and lipid and fatty acid composition of Candida 107 grown under magnesium-limited conditions*

Dilution rate (per h)	Glucose remaining (g/liter)	Biomass (g/liter)	Lipid (% biomass)	Biomass yield (g of yeast/100 g of glucose used)	Lipid yield (g of lipid/100 g of glucose used)	Lipid output (g of lipid/liter per h)	Specific rate of lipid formation (g of lipid/g of yeast per h)	Relative proportion of fatty acid in total lipid (%, wt/wt)							Δ/mol
								14:0	16:0	16:1	18:0	18:1	18:2	18:3	
0.05[a]	4.4	8.75	26.4	34.5	9.10	0.116	0.013	2.0	35.4	1.9	7.7	34.8	16.8	1.4	0.75
0.075	10.9	6.92	23.3	36.2	8.4	0.121	0.018	1.7	29.6	1.4	7.8	40.2	17.8	1.4	0.81
0.10	14.4	6.50	20.0	41.4	8.3	0.130	0.020	4.5	27.7	1.0	6.7	34.2	22.8	2.9	0.90
0.125[a]	15.5	4.72	32.3	32.6	10.4	0.191	0.040	4.3	27.8	0.5	5.9	33.2	27.0	1.3	0.92
0.15[a]	11.8	7.22	22.9	39.6	9.1	0.249	0.034	13.2	25.9	1.5	5.0	38.5	24.6	1.3	0.83
0.175[a]	12.5	6.45	13.4	36.9	4.9	0.152	0.024	4.7	30.5	1.3	3.2	33.3	24.5	2.5	0.91
0.20[a]	16.5	4.08	16.9	30.2	5.1	0.138	0.034	ND[b]	ND	ND	ND	ND	ND	ND	ND

[a] Dual nutrient limitation of magnesium and nitrogen.
[b] ND, Not determined.

ences, depending on which nutrient was made limiting. With carbon limitation, fatty acids in all three lipid fractions became more unsaturated with decreasing growth rate, whereas with limitations of all other nutrients the reverse tended to occur. Variations in the growth rate of carbon-limited cells appeared to produce the greatest variations in both lipid and fatty acid composition. For example, neutral lipids varied from 66 to 92% of the total lipid, and the degree of unsaturation of the fatty acids varied from 0.57 to 1.14 double bonds/mol. This is probably due to these carbon-limited cells containing much less lipid than any of the other types of cell examined here, and consequently we may deduce that structural lipids (phospholipids and perhaps glycolipids) can be much more readily influenced by the growth conditions, maybe by having a higher turnover rate, than storage lipid. The acid principally affected in all cases was linoleic acid ($C_{18:2}$). Since there has been very little work done on the effect of growth rate on fatty acid composition with different nutrient limitations (12, 22), these effects may be peculiar to *Candida* 107 and, until more work has been done, cannot be taken as a general trend. The production of relatively high concentrations of myristic acid under magnesium-limited conditions is unusual and may indicate some dependency upon this cation for the fatty acid synthetase complex to complete the final stage of chain elongation.

Lipid accumulation in *Candida* 107 was highest when the limiting nutrient was nitrogen rather than either phosphate or magnesium. However, the dual limitation of nitrogen with phosphate induced even higher concentrations of lipid, and, more importantly, the lipid concentration was still high in the yeasts growing close to its maximum growth rate. Under these circumstances, utilization of carbon and nitrogen was less efficient and both biomass and lipid yields were poor. The factors causing lipid to accumulate may therefore be different with different limiting nutrients, but to substantiate this a firmer understanding of the biochemical basis of lipid accumulation in oleaginous organisms is needed (25, 26).

The rate of lipid output from a fermenter is an important factor to be considered when eval-

uating the potential of a microbial lipid production process. To quote such rates as a simple output rate (i.e., grams of lipid per liter per hour) is misleading since this does not take into account the biomass concentration, which may be any concentration up to an amount dictated by the limits of the fermenter. Thus specific rates of lipid production must be calculated. The maximum specific rate of lipid production with *Candida* 107 with growth limitations of carbon, nitrogen, phosphate (plus nitrogen), and magnesium (plus nitrogen) were, respectively, 0.030, 0.041, 0.059, and 0.040 g of lipid/g of yeast per h, although these rates do not coincide with the highest lipid content of the yeast that can be achieved with a particular nutrient limitation and are achieved without efficient utilization of the substrate. Although rates of lipid formation of *Candida* 107 growing in batch culture are unknown, these rates are higher than those calculated by Kessell (15) for batch-grown *R. gracilis*, which were only about 0.015 g of lipid/g of yeast per h.

The efficiency with which lipid is synthesized, which is expressed here as "lipid yield" and elsewhere as "fat coefficient" (28), is extremely important if the cost of substrate is of prime consideration in the evaluation of a microbial lipid process. With carbohydrate as substrate, lipid yields in batch cultures are rarely over 20% with any microorganism (20, 28), although Duncan (9) has recorded an unprecedented 25% conversion with *Penicillium lilacum*. Since *Candida* 107 has reached lipid yields in excess of 20% on many occasions when growing in continuous culture, this type of cultivation clearly brings about an efficient utilization of substrate and, coupled with the reproducibility of the fatty acid composition, makes continuous culture an obvious choice for any future development. However, some compromise between efficient substrate utilization and lipid productivity rates will probably be needed to optimize the dynamics of this system.

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