A Comparison of the Oleaginous Yeast, *Candida curvata*, Grown on Different Carbon Sources in Continuous and Batch Culture

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ABSTRACT

The oleaginous yeast, *Candida curvata* D, was grown in both batch and continuous culture on 5 different carbon sources to compare the efficiency of fat production from the various substrates. Maximum lipid accumulation occurred in batch culture with xylose as the carbon source on nitrogen-limited medium reaching a level of 49% (w/w) of the biomass, but this was reduced to 37% at the optimum dilution rate (D = 0.05/hr) in a chemostat. Both the highest biomass and lipid yields were attained in continuous culture with lactose as the sole carbon source at a dilution rate of D = 0.04/hr, giving an efficiency of substrate conversion of 60 g of biomass and 18.6 g lipid per 100 g lactose utilized. The relative proportions of the major fatty acids (16:0, 18:0, 18:1, 18:2) in the lipid were found to vary considerably in batch culture and in continuous culture under carbon-limited conditions. However, on nitrogen-limited media in the chemostat, the fatty acid composition remained relatively constant over the whole range of dilution rates employed. Lipid from xylose-grown cells contained the greatest percentage of stearic acid (18:0) 15% and the lowest linoleic acid (18:2) 4%, whereas lipid from ethanol-grown cells contained elevated levels of oleic acid (18:1) 51% and decreased palmitic acid (16:0) 25%.

Lipids 18:000-000, 1983.

INTRODUCTION

Candida curvata was first described by Hammond and colleagues (1) as an oleaginous yeast capable of efficient conversion of whey permeate, i.e., lactose, to oil. As with most previous work on lipid accumulation (see refs. 2,3), the evaluation of the potential of *C. curvata* was established using batch culture (1,4). However, continuous culture would probably be a more efficient and cost-effective means of cultivating a yeast on a large scale (5) besides being able to give close control over the physiological state of the organism. Under the steady-state conditions of continuous culture, a constant composition of the cells is produced: this includes not only the total amount of lipid within the cells but the fatty acyl moieties of the lipid (6). As a steady state can be maintained indefinitely, the product from the fermenter also remains unchanged. Thus, besides being the method of yeast cultivation in many commercial processes, continuous culture is the ideal laboratory method for making unequivocal comparisons of the same organism grown under different conditions as all conditions, including the growth rate, can be accurately controlled.

The application of continuous culture to lipid accumulation has been examined in this laboratory with respect to *Candida* 107 (6,7), *Rhodotorula gracilis* (8) and *Lipomyces starkeyi* (9). In view of the likely commercial importance of *C. curvata*, we have, with the kind permission of Professor E. G. Hammond, examined the growth of this yeast in continuous culture. This paper reports a comparison of the yeast grown on 5 different carbon sources under both batch and continuous culture; batch culture being used to illustrate the inherent difficulties in attempting to obtain constant cell compositions under different growth conditions. The carbon sources were selected on the basis of what may be reasonable choices in any large-scale process: sucrose, lactose, glucose, xylose and ethanol. They, thus, represented 2 disaccharides, a hexose, a pentose and a C_2 compound.

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METHODS

The yeast, *C. curvata* D, used throughout this study, was kindly supplied by Professor E. G. Hammond, Iowa State University, Ames, Iowa.

Media

The nitrogen-limited media used for lipid accumulation contained (g/l): NH_4Cl, 0.5; KH_2PO_4, 7.0; Na_2HPO_4, 2.0; $MgSO_4.7H_2O$, 1.5; yeast extract, 1.5; $CaCl_2.6H_2O$, 0.1; $FeCl_3.6H_2O$, 0.008; $ZnSO_4.7H_2O$, 0.0001. The carbon sources were added to a concentration of 30 g/l. The carbon-limited media contained the same as above except that NH_4Cl was at 3.0 g/l and carbon source 10 g/l. The medium was adjusted to pH 5.5 with HCl be-

fore sterilization. The medium was sterilized by membrane filtration (pore size 0.25 μm), collected in sterile 20-liter aspirators and kept for 3 days at room temperature to ensure sterility before use.

Continuous Culture Operations

A one-liter (working volume) chemostat was used (LHE 500 series II, LH Engineering, Stoke Poges, Bucks, England) for all experiments. pH was maintained at 5.5 by the automatic addition of NaOH; temperature was controlled at 30 C. Addition of antifoam was maintained at preset intervals using an appropriate metering pump and timing clock. Incoming air was passed through 2 fiberglass filters connected in series; the aeration rate was maintained at 1 vol air/vol medium/min. The volume within the fermenter was kept constant by using an overflow weir (5 mm diameter). The assembled vessel was sterilized by autoclaving at 121 C for 45 min. The chemostat was erected and filled with 1000 ml of sterile medium to which was added a 2% (v/v) inoculum of *C. curvata* D. Agitation was by means of flat bladed impellers and operated at 500 rpm. The fermenter was used for batch culture by switching off the medium pump and run without pH control for 96 hr.

Samples of 50-100 ml were removed for analysis through an air-lock device into a sterile screw-top bottle. Steady-state conditions were maintained for at least 6 complete changes of medium in the vessel. Duplicate samples, taken every other day, were analyzed for yeast dry weight, percent lipid (w/w) and residual NH_4^+ and carbon source in the culture filtrates until steady-state samples were in agreement. NH_4^+ in culture filtrates was determined by the method of Chaney and Marbach (10). Glucose was estimated using the glucose oxidase-peroxidase method (Boehringer-Mannheim). Sucrose and xylose were estimated using methods based on Herbert et al. (11). Lactose and ethanol were determined using standard reagent kits from Boehringer-Mannheim GmbH.

Dry Weight Determination

Samples (10 ml) were centrifuged at 5000 × g for 5 min in preweighed, dried tubes and washed twice with 10 ml of distilled water. The pellets were dried at 80 C over P_2O_5 in a vacuum oven until constant weight (ca. 48 hr).

Lipid Estimation

Total lipid was determined by a method based on that of Folch et al. (12): lipid was extracted from freeze-dried cells with chloroform/methanol (2:1), filtered and washed with 0.9% naCl and distilled water. The extract was dried over anhydrous $MgSO_4$ and evaporated to dryness using a rotary evaporator. The lipid was redissolved in diethyl ether and transferred to a preweighed vial. The ether was removed in a stream of nitrogen and dried in a vacuum dessicator for 1 hr. This method is not the same as that advocated by Moon and Hammond (4) for maximum lipid extraction from this yeast but it is a procedure which we have found to be convenient and reproducible.

Fatty Acid Analysis of the Lipid

Fatty acid methyl esters were prepared using sodium methoxide (13). The total lipid sample was dissolved in hexane to give a final concentration of 75 mg/ml. One hundred μl sample was removed and added to 2 ml methanol, followed by 1 ml sodium methoxide and heated 60 C for 15 min. The contents were cooled and acidified with 10% H_2SO_4 in methanol using bromothymol blue as indicator. Two ml hexane were added and mixed and the upper layer removed for analysis using gas chromatography.

Gas Liquid Chromatography (GLC)

Samples were analyzed in a Pye Unicam series 104 gas chromatograph (Cambridge, England). Samples were separated on a glass column (1.5 m × 4 mm) pretreated with dichloromethyl silane and packed with 5% diethyleneglycol succinate on Chromosorb 6 W H-P (100-200 mesh). The carrier gas was nitrogen at a flow rate of 50 ml/min and the column was held at 185 C. Peaks were identified by comparison of their retention times with those of authentic fatty acid methyl standards.

RESULTS

The growth of *C. curvata* in batch culture and continuous culture on glucose, sucrose, lactose, xylose and ethanol is shown in Figure 1 (a-o). The patterns observed were similar to those observed previously with oleaginous yeasts in both batch (1,4,14) and continuous culture (6-9).

In batch culture (Fig. 1, a-e), with a high C:N ratio to ensure high lipid accumulation, the nitrogen was consumed after ca. 30 hr growth in each case (not shown). The amount of cellular lipid then increased from ca. 10% of the biomass to between 30 and 35% after 90 hr as the carbon continued to be assimilated and metabolized to lipid. The efficiencies of the conversions of carbon source into both biomass and lipid are summarized in Table 1. Lactose was

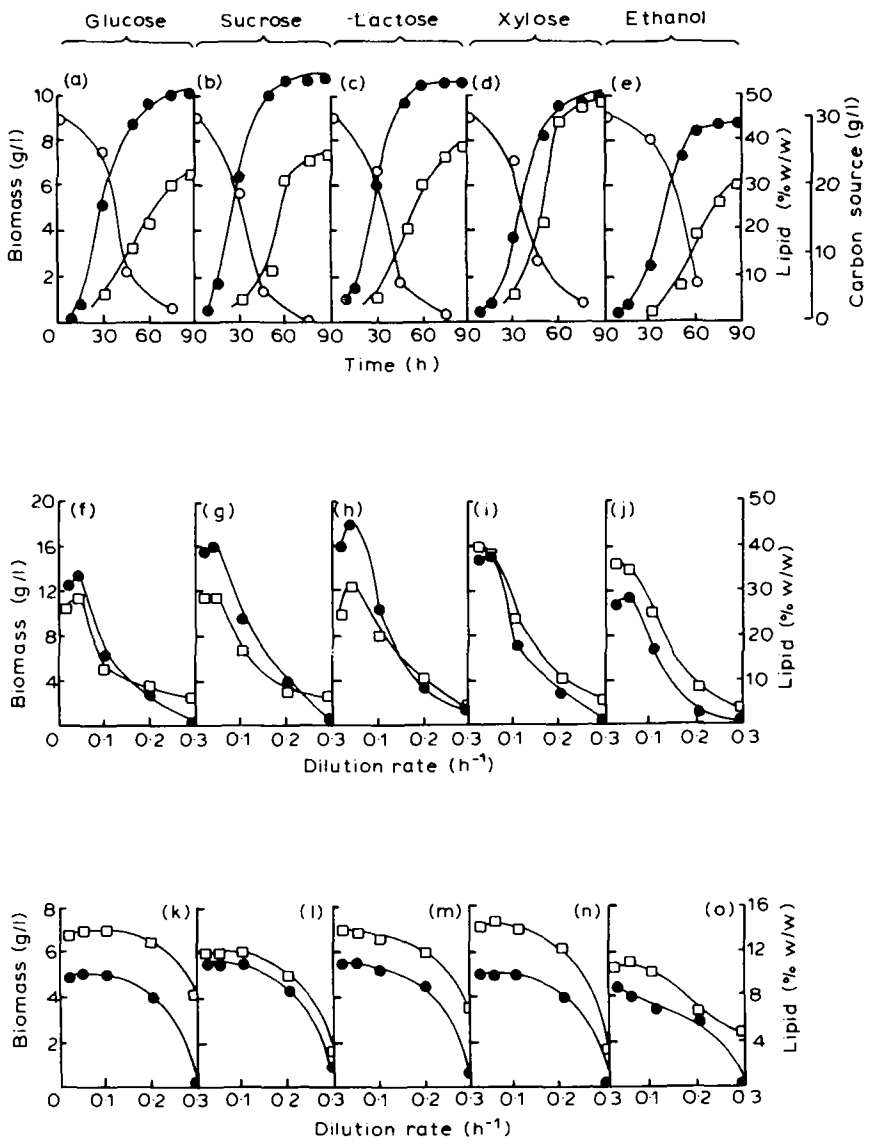

FIG. 1. Lipid and biomass production by *C. curvata* growing on glucose, sucrose, lactose, xylose and ethanol: • biomass (g/l); □ lipid % of biomass; ○ carbon source (g/l). Figs. a-e, growth in batch culture; f-j, growth in continuous culture under nitrogen-limited conditions; k-o, grown in continuous culture under carbon-limited conditions.

the most effective carbon source for biomass production; xylose was the most efficient for lipid production. Ethanol was the least efficiently utilized carbon source for both biomass and lipid production.

In continuous cultures, under carbon-limited conditions (Fig. 1, k-o), lipid content of the cells did not exceed 15% of the biomass. Beyond the critical dilution rate (i.e., at the point beyond which wash-out of the cells begins to occur), the lipid content of the cells declined abruptly with all 5 substrates. Under these growth conditions, there was insufficient carbon available to allow lipid accumulation to occur. However, under nitrogen-limited conditions (Fig. 1, f-i), sufficient excess carbon was available to allow lipid accumulation and in all 5 cases maximum lipid accumulation occurred at a dilution rate of 0.04-0.05/hr. Wash-out of the cells was complete at a dilution rate (=

TABLE 1

Lipid Production by *C. curvata* Grown in Batch Culture for 90 hr[a]

Carbon source (30 g/1)	Biomass (g/1)	Lipid (% w/w)	Substrate utilized (g/1)	Biomass yield (g/100 g substrate utilized)	Lipid yield (g/100 g substrate utilized)
Glucose	10.2	33.2	28.3	36.0	11.9
Sucrose	11.2	37.4	28.0	40.0	14.8
Lactose	12.5	39.2	29.6	42.2	16.5
Xylose	9.9	48.6	27.3	36.3	17.4
Ethanol	8.5	30.1	25.5	33.3	10.0

[a]Culture conditions as described in Materials and Methods. Biomass (10 ml) and lipid (100 ml) samples were removed and treated in duplicate. Carbon sources were assayed as in Methods.

TABLE 2

Efficiency of Lipid Production by *C. curvata* Grown in Continuous Culture on Various Carbon Sources under Nitrogen-Limited Conditions

	Carbon source (30 g/1)				
	Glucose	Sucrose	Lactose	Xylose	Ethanol
Dilution rate (per hr)	0.04	0.04	0.04	0.05	0.05
Residence time (hr)	25	25	25	20	20
Substrate utilized (g/1)	29.8	29.6	29.8	29	30
Biomass (g/1)	13.5	16	18	15	11.5
Rate of biomass synthesis (g/1/hr)	0.54	0.63	0.72	0.75	0.58
Biomass yield (g biomass/100 g substrate)	45	53	60	51	38
Lipid (% of biomass w/w)	29	28	31	37	35
Total lipid produced (g/1)	3.94	4.54	5.6	5.5	4.0
Rate of lipid synthesis (g/1/hr)	0.16	0.18	0.22	0.27	0.2
Lipid yield (g lipid/100 g substrate)	13.1	15.1	18.6	18.3	13.3
Specific rate lipid production (g lipid/100 biomass/hr)	0.012	0.011	0.0124	0.018	0.017

Analyses carried out as given in Table 1.

maximum specific growth rate) of 0.30/hr. Table 2 summarizes the data for the efficiencies of conversions of carbon source to biomass and lipid in the 5 cases. The most efficiently used carbon source for biomass was, as with batch culture, lactose. But in contrast with batch culture results (Table 1), lactose was also the most effective substrate for lipid production. Xylose, however, was just slightly less efficient and, within the limits of experimental error, should probably be regarded as equally good as lactose.

One of the advantages of continuous culture over batch cultivation methods can be seen from a comparison of Tables 1 and 2. With all 5 substrates, the efficiencies of conversion of carbon to both biomass and to lipid were higher with the former method of growth. The steady states engendered by the chemostat allow the cell to achieve optimum dynamics of its biochemistry and this is reflected by the cell achieving maximum growth (i.e., cell yield) per mole of substrate utilized. It can also be seen from Tables 1 and 2 that the productivity (g product per liter fermenter volume per unit time) of the continuous procedures were up to 5 times higher than the batch culture methods: with lactose, for example, the rate of biomass production was 0.72 g/l/hr in continuous culture but was $12.5 \div 90 = 0.14$ g/l/hr in batch culture. These figures become even more in favor of continuous culture if one adds to the fermentation time of the batch culture (i.e., 90 hr) a period for "turn around" of the fermenter (say 24 hr for cleaning, resterilizing and medium addition). The rate of lipid production (g/l/hr) also can be shown to be 4 to 5 times faster in continuous culture than in batch.

The non-steady state of a batch culture is shown when successive samples of the cells are taken during growth as often considerable variations in cell composition can be seen (see also ref. 4). It was, therefore, no surprise to see vari-

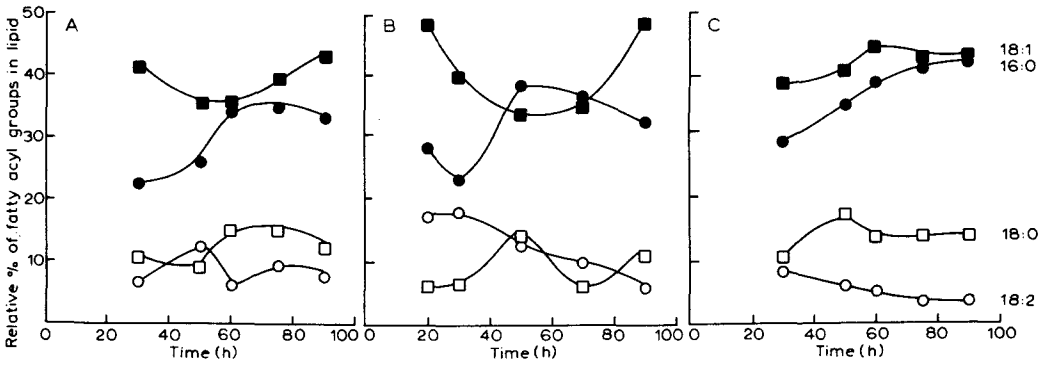

FIG. 2. Fatty acyl composition (relative % w/w) of lipid of *C. curvata* grown in batch culture with: A, glucose; B, lactose; C, xylose. • = 16:0; □ = 18:0, ■ = 18:1; ○ = 18:2. Traces of 14:0 and 16:1 were seen in most cases.

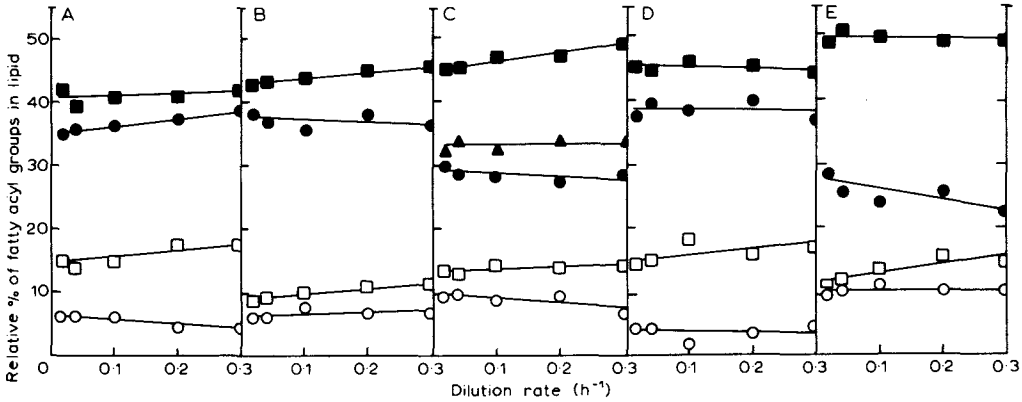

FIG. 3. Fatty acyl composition (relative % w/w) of lipid of *C. curvata* grown in continuous culture under nitrogen-limiting conditions with: A, glucose; B, sucrose; C, lactose; D, xylose; E, ethanol. • = 16:0, □ = 18:0; ■ = 18:1; ○ = 18:2. Traces of 14:0 and 16:1 were seen in most cases.

ations in the fatty acyl composition of the cell lipid of *C. curvata* when grown in batch culture on glucose, lactose and xylose (Fig. 2). (Data for the fatty acyl groups from sucrose- and ethanol-grown cultures was not obtained except after 96 hr–see Table 3.) With each substrate, oleic acid (18:1) was the major fatty acid, although palmitic acid (16:0) was almost as abundant. Linoleic acid (18:2) and stearic acid (18:0) were the only other 2 major fatty acids, although traces of myristic acid (14:0) and palmitoleic acid (16:1) were seen in most analyses. Stearic acid was almost never higher than 15% of the total fatty acids. A comparison of the final fatty acyl composition taken at the end of growth (96 hr) for the yeast grown on all 5 substrates is given in Table 3. This showed that xylose produced the oil with the lowest degree of unsaturation (Δ/mole–ref. 15) while ethanol produced the one with the highest degree of unsaturation. The oils, though, in all 5 cases were not widely different.

In continuous culture, the composition of the fatty acyl groups remains constant under any one set of growth conditions (6). Thus, samples of *C. curvata* taken from the chemostat running with each of the 5 substrates at the same dilution rate can be accurately compared. Thus, at the dilution rate where maximum lipid accumulation occurred (i.e., 0.04/hr under nitrogen-limited conditions, see Fig. 1, f-i), the fatty acyl groups from the 5 cultures showed differences which could be asserted to be slight but significant (see Table 3). As with the batch cultures, xylose produced the lipid with the lowest degree of unsaturation and ethanol the one with the highest. The oils produced from glucose and sucrose were similar to each other as were those from lactose and ethanol.

When the specific growth rate (= dilution

TABLE 3

Fatty Acid Composition of Lipid from *C. curvata* Grown on Different Carbon Sources

Carbon source	Batch culture[a]					Continuous culture[b]				
	16:0	18:0	18:1	18:2	Δ/mol[c]	16:0	18:0	18:1	18:2	Δ/mol[c]
Glucose	33.0	12.0	42.9	7.3	0.59	35.8	13.7	39.7	6.4	0.55
Sucrose	32.4	11.3	42.0	6.7	0.57	36.6	9.6	43.5	5.9	0.57
Lactose	32.5	11.0	49.0	6.0	0.63	28.0	13.2	46.9	10.8	0.69
Xylose	41.2	14.0	43.0	3.5	0.51	29.5	15.0	44.5	3.9	0.52
Ethanol	26.5	12.5	49.0	8.9	0.69	25.7	12.0	51.2	10.0	0.72

[a]Samples after 96 hr growth.
[b]Samples from dilution rate = 0.04/hr.
[c]Δ/Mol = degree of unsaturation (ref. 15).

rate) was changed in each chemostat, there was almost no change in lipid composition when cultures were run with nitrogen-limiting medium. Figure 3 shows the remarkable constancy of the fatty acyl groups in going from a very slow specific growth rate, D = 0.02/hr, to the very fastest one, D = 0.3/hr. However, when the fatty acids of the lactose culture grown under carbon-limited conditions were examined, their composition changed quite markedly in going from the slowest specific growth rate to the highest one (Fig. 4); the relative proportion of palmitic acid decreased by nearly 50% while that of oleic acid increased by over 50%; linoleic acid decreased to a very low amount at the high dilution rates while stearic acid reached a maximal level. In spite of these large changes, the degree of unsaturation of the total fatty acids remained unchanged throughout the whole range of dilution rates.

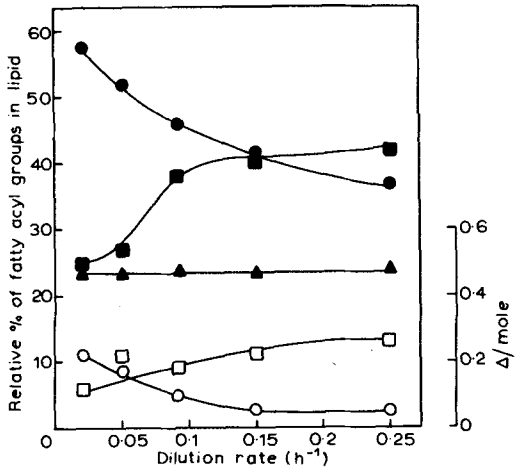

FIG. 4. Fatty acyl composition of *C. curvata* grown on lactose under carbon-limited conditions. ○ = 16:0; □ = 18:0; ■ = 18:1; ○ = 18:2; ▲ = Δ/mole–see ref. 15.

DISCUSSION

C. curvata is an oleaginous yeast of some potential. Its efficient conversion of lactose to oil has already been amply recorded by Hammond and associates (1,4,16) and in this paper we have shown that it is almost as equally efficient in converting other substrates–glucose, sucrose, xylose and ethanol–into lipid and biomass. The efficient utilization of xylose is noteworthy in view of the current interest in this material as a substrate for microbial fermentation process (17). Xylose is readily available in large quantities from the chemical hydrolysis of hemicellulose which, along with cellulose, is the most abundant renewable resource in nature. Ethanol was the poorest of the 5 substrates tested in producing lipid and this can probably be attributed to it inducing expression of the enzyme isocitrate lyase. Such enzyme activity would decrease the amount of isocitrate acid, and thence citric acid, which was accumulated. This usually occurs by isocitrate dehydrogenase being the sole enzyme for isocitrate dissimilation and it being dependent upon AMP for activity (18). As AMP is at a low concentration in oleaginous yeasts, a mechanism is provided which leads to the accumulation of citrate which is then the immediate precursor, by the action of ATP:citrate lyase, of acetyl-CoA. Ethanol, by inducing an alternative route for isocitrate metabolism, is therefore not a good substrate for achieving high lipid accumulation in spite of it appearing to be, at first inspection, an excellent substrate to promote lipid accumulation as it would be expected to be readily metabolized (via acetaldehyde and acetate) to acetyl-CoA. Clearly, though, this is not sufficient metabolic reason to engender high lipid levels. However, not all yeasts show this loss of

efficiency; *R. gracilis* has been reported to have only slightly less better yields of biomass and lipid in changing from glucose to ethanol (19).

The biomass yield of 42 g cells/100 g substrate for the yeast grown on lactose in this present work was slightly less than the value of 46% calculable from the data reported by Moon and Hammond (4). However, our lipid yields were substantially less than theirs: 16.5% as compared to their value of 27%. This could be due to the differences in lipid extraction techniques but is probably attributable to their use of whey or whey permeate which contains many nutrients besides lactose and which must be supposed to be beneficial to lipid production.

Although there was a slight decrease in the percentage of lipid in the biomass in going from batch culture to continuous culture, the efficiency of utilization of the substrate increased considerably with all 5 substrates tested and thus resulted in much higher biomass and lipid yields.

The merits of continuous culture were also reflected in the production of a product of unvarying composition. This is an obvious advantage of continuous culture if considering any commercial development. The relative content of the major fatty acids in the lipid produced from the hexoses was ca. palmitic (36%), stearic (13%), oleic (44%) and linoleic (6%), which is similar to that reported for *Candida* 107 (6) but markedly different to that of *R. glutinis* (8). The ability of this yeast to produce a good quality oil from a wide selection of substrates indicates the potential economic viability of a process utilizing such a yeast. The profitability of this process obviously depends on the utilization of a substrate of little intrinsic value to produce an oil of a much higher value. The use of continuous culture techniques to minimize operating costs should be of considerable relevance to the overall economics of the process.

ACKNOWLEDGMENT

This work was supported by a Co-operative Award in Science and Engineering from the Science and Engineering Research Council (U.K.).

REFERENCES

1. Moon, N.J., Hammond, E.G., and Glatz, B.A. (1978) J. Dairy. Sci. 61, 1537-1543.
2. Woodbine, M. (1959) Prog. Indust. Microbiol. 1, 179-245.
3. Ratledge, C. (1982) Prog. Indust. Microbiol. 16, 119-206.
4. Moon, N.J., and Hammond, E.G. (1978) J. Am. Oil Chem. Soc. 55, 683-688.
5. Tempest, D.W., and Wouters, J.T.M. (1981) Enzyme Microbial Technol. 3, 283-290.
6. Gill, C.O., Hall, M.J., and Ratledge, C. (1977) Appl. Environ. Microbiol. 33, 231-239.
7. Hall, M.J., and Ratledge, C. (1977) Appl. Environ. Microbiol. 33, 577-584.
8. Ratledge, C., and Hall, M.J. (1979) Biotechnol. Lett. 1, 115-120.
9. Boulton, C.A., and Ratledge, C. (1981) J. Gen. Microbiol. 127, 169-176.
10. Chaney, A.L., and Marbach, E.G. (1962) Clin. Chem. 8, 130-132.
11. Herbert, D., Phipps, P.J., and Strange, R.E. (1972) in Methods in Microbiology (Norris, J.R., and Ribbons, D.W., eds.) Vol. 5B, pp. 210-344, Academic Press, London and New York.
12. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
13. Marientti, G.V. (1962) J. Lipid Res. 3, 1-20.
14. Kessell, R.H.J. (1968) J. Appl. Bacteriol. 31, 220-231.
15. Kates, M., and Baxter, R.M. (1963) Can. J. Biochem. Physiol. 40, 1213-1227.
16. Hammond, E. G., Glatz, B.A., Choi, Y., and Teasdale, M.T. (1981) in New Sources of Fats and Oils (Pryde, E.H., Princen, L.H., and Mukherjee, K.D., eds.) Am. Oil Chem. Soc., Monograph no. 9, pp. 171-187.
17. Lee, Y.Y., Lin, C.M., Johnson, T., and Chambers, R.P. (1979) Biotechnol. Bioeng. Symp. 8, 75-83.
18. Botham, P.A., and Ratledge, C. (1979) J. Gen. Microbiol. 114, 361-375.
19. Krumphanzl, V., Gregr, J.V., Pelechova, J., and Uher, J. (1973) Biotech. Bioeng. Symp. 4, 245-256.

[Received March 28, 1983]