Oil Production by Fermentation of Lactose and the Effect of Temperature on the Fatty Acid Composition[1]

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ABSTRACT

Strains of the yeasts *Candida curvata* and *Trichosporon cutaneum* were isolated that were able to ferment the lactose of cheese whey and ultrafiltered whey permeate to produce oil. Rapid fermentation rates and lactose utilization were achieved by the *C. curvata* strains. The fermentation was more efficient on permeate than whey, because the organisms used whey protein very poorly. Methods of extracting the oil were compared, and the best results were obtained by sequential extraction with methanol and benzene or by ethanol and hexane. The fatty acid composition of the yeasts varied with the growth temperature, fermentation time, and medium. The oil was rich in 18:1, 16:0, and 18:0. The process is a technically feasible means of using whey permeate.

INTRODUCTION

The conversion of carbohydrates to fats and oils by fermentation has not been considered seriously as a commercial process for many years, but the problems of disposal of food processing wastes and by-products may make this process attractive again. Cheese whey and especially whey permeate produced by ultrafiltration are typical examples of such food processing by-products. They are dilute and typically contain 6 to 7% solids. Of these solids the chief constituent is lactose. The low intrinsic value of the solids and high energy costs make drying unprofitable. The high biological oxygen demand and water pollution regulations make dumping impossible and costly activated sludge treatments necessary.

The possibility of fermenting whey, processing wastes and other materials to food and feeds has been reviewed (1). For oil-producing fermentations from whey to be practical, several technological and economic conditions must be met: (a) Organisms capable of efficient conversion of lactose to oil must be discovered because nearly all the microorganisms reported to produce oil fail to ferment lactose (2). (b) A new, economically feasible method of oil extraction from yeast is necessary because yeasts are traditionally difficult to extract (3). (c) The medium after fermentation must be very low in biological oxygen demand so that it can be discarded with little further processing to meet water quality standards. Most previous investigators of oil-producing fermentations were unconcerned with this requirement. (d) Fermentation must be economically attractive compared with other methods of disposal.

This paper reports the fermentation of whey and permeate to oil by two yeasts species, the fatty acid composition produced under various fermentation conditions, and the feasibility of the process.

METHODS

Four yeasts able to ferment lactose and produce oil were isolated from cheese plant floors and floor drains. They were identified according to Lodder (4). The cultures were maintained on malt extract agar slants and activated by repeated transfer before use.

A New Brunswick 10-liter fermenter equipped with temperature, aeration, stirring, and pH controls was used. Oxygen consumption was measured with an oxygen electrode and carbon dioxide production by titration of a portion of the exhaust gas. Direct microscopic counts and respiration rates were used to optimize the nutritional and physical conditions for growth and oil production by each organism (5).

Media were dried, whole whey (Formula 521, Associated Milk Producers, San Antonio, TX) reconstituted to 6.5% solids and condensed permeate (Mississippi Valley Milk Producers, Luana, IA). Media were sterilized before fermentation. The permeate was produced from Swiss whey with a Dorr-Oliver ultrafilter (Stamford, CT). The membrane had a 24,000 dalton cutoff. The permeate was condensed and frozen until used.

Periodic samples of fermentation liquor were centrifuged at 9,000 x G for 30 min at 5 C, and the chemical oxygen demand (COD) of the supernatant was measured (6). The precipitated cell paste was used for oil percentage and composition analyses.

For oil composition at 15 C, the yeast was streaked on whey agar (6.5% whey + 1.5% agar) and incubated for 3 weeks. Cells were recovered for analysis by repeated washing of the agar surface and centrifugation of the washings.

Several methods of lipid extraction were compared on wet yeast. To test the extraction methods, yeasts were grown in the fermenter for at least 72 hr, because older cultures were most resistant to extraction. Samples weighed 0.5 g unless they contained less than 50 mg oil, in which case a larger sample was used. The extraction methods were:

1. Chloroform-methanol (10 ml 2:1, v/v) according to Folch et al. (7). The insoluble debris was removed, 2 ml water added to the extract, and the chloroform layer collected and evaporated under nitrogen.
2. Sonication of the sample at maximum intensity for 30 min (Blackstone Instruments, Sheffield, PA) in chloroform-methanol (2:1, v/v). One-minute sonication periods were interspersed with 30 sec cooling in ice water to avoid overheating the sample. After sonication, the extraction was as in method 1.
3. Yeast with an equal weight of ground glass and a few milliliters of chloroform-methanol were treated in a Mickle Disintegrator (Brinkman Instruments, Great Neck, NY) for 30 min. Heating was minimized by using the apparatus in a 4-C room. Insoluble debris was extracted twice more for 30 min with chlorform-methanol so that the final volume of extract was 10 ml. Further treatment of the extract was as in method 1.
4. Cells were digested in 3 ml 2 *N* HCl at 70 C for 5 hr. Digest was extracted twice with 30 ml chloroform-methanol (2:1) for 30 min. The extract was neutralized with 2 *N* NaOH and then treated as in method 1.

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TABLE I

Fermentation of Whey and Whey Permeate under Optimized Conditions[a] with *C. curvata* Strains R and D and *T. cutaneum* 24 and 40

	C. curvata				*T. cutaneum*			
	Whey		Permeate		Whey		Permeate	
	R	D	R	D	24	40	24	40
Fermentation time, hr	60	72	72	72	60	96	72	72
Max. cell no x 10^8/ml	6.5	5.2	8.7	5.2	6.5	3.4	6.5	3.7
Lactose used/liter	48.8	47.5	63.9	56.8	46.5	49.8	45.3	45.9
COD start .10^3 mg/liter	59	65	73	63	60	62	69	59
COD % reduction	71	85	90	95	85	85	52	88
Cell yield/liter	---	---	25.7	26.8	---	---	19.6	23.9
Oil produced/liter	7.3	9.3	13.0	15.6	6.3	7.8	4.0	10.8
Protein produced/liter	---	---	2.25	2.47	---	---	2.92	3.22
Fat coef. g oil/g lactose x 100	15	20	20	27	14	16	9	24

[a]For *C. curvata* R for growth phase, pH 5.8, 30 C, 0.25 liters air/min/liter medium; for fattening phase, pH 5.2, 0.7 liters/min/liter; 155 mg/liter NH_4OH, 2.5 mg $MnSO_4$, 5.3 mg K_2HPO_4. For *C. curvata* D for growth phase, pH 5.4, 28 C, 0.25 liters air/min/liter medium; for fattening phase, pH 5.8, 0.7 liters/min/liter; 46 mg/liter NH_4OH. For *T. cataneum* 24 for growth phase, pH 5.4, 28 C, 0.25 liters air/min/liter medium; for fattening phase, pH 5.8, 0.7 liters/min/liter; 155 mg/liter NH_4OH, 2 mg $MnSO_4$, 3 mg $MgSO_4$, and 10 mg $FeSO_4$. For *T. cutaneum* 40 for growth phase, pH 5.7, 33 C, 0.25 liters air/min/liter medium; for fattening phase, pH 5.6, 0.7 liters/min/liter; 155 mg/liter NH_4OH, 55 mg $MgSO_4$.

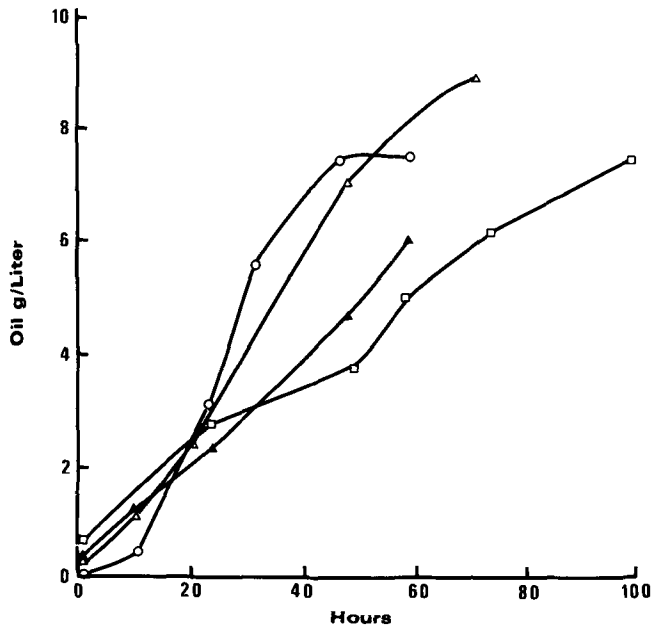

FIG. 1. Production of oil by yeasts when grown in whole whey. ○ *C. curvata* R, △ *C. curvata* D, ▲ *T. cutaneum* 24, □ *T. cutaneum* 40.

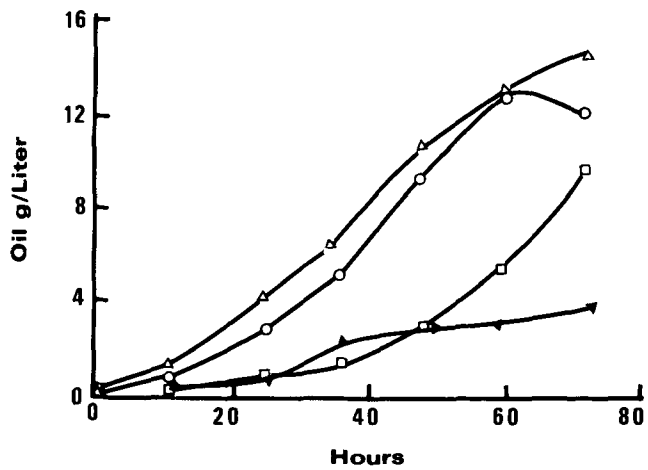

FIG. 2. Production of oil by yeasts when grown in ultrafiltered whey. ○ *C. curvata* R, △ *C. curvata* D, ▲ *T. cutaneum* 24, □ *T. cutaneum* 40.

5. Extraction with 5 ml methanol, 5 ml benzene-methanol (1:1, v/v), and 5 ml benzene according to Sobus and Holmlund (8). Each extraction was for 20 min. The extracts were pooled and evaporated under nitrogen.
6. Same as method 5 except hexane and ethanol were used.
7. Cells were digested with 5 ml alcoholic KOH (12% v/v, 12% w/v, respectively, in water) at 70 C for 2 hr in a sealed tube. The pH was adjusted to 3 with concentrated HCl, and the fatty acids were extracted twice with hexane (5 ml, then 2 ml). The hexane was evaporated under nitrogen.

Method 7 was used to extract cells for fatty acid analyses. The fatty acids were converted to methyl esters according to Luddy et al. (9) About 5 mg fatty acids and 0.3 ml 14% BF_3 methanol were heated to 65 C under nitrogen in a sealed vial. The vial contents were shaken with 0.5 ml Na_2CO_3 solution, 3 ml water, and 1 ml hexane. The hexane layer was used for gas chromatography on a Beckman GC-5 instrument equipped with a flame ionization detector and a 180 x 0.3 cm stainless steel column packed with 15% EGSSX on 100/120 mesh Chromosorb P (Applied Science Laboratories, State College, PA). Helium was the carrier gas at 50 ml/min, and the column temperature was 185 C. Peak areas were integrated with an Autolab 6300 instrument (Spectra-Physics, Mountain View, CA). Instrument accuracy was verified with a standard methyl ester mixture.

Thin layer chromatography was on Silica Gel G plates 0.25 mm thick development with 15% ether in hexane. Spots were viewed under ultraviolet light after spraying the plate with 0.2% 2',7'-dichlorofluorescein in ethanol.

RESULTS AND DISCUSSION

Four yeasts that efficiently fermented lactose to oil were identified as two strains each of *Candida curvata* and *Trichosporon cutaneum*. Fermentations were under optimum nutritional and physical conditions (5). Table I shows the maximum rates of fermentation, efficiency of oil production, and final cell composition. On both media, the *C. curvata* strains were more successful than the *T. cutaneum* strains. The *C. curvata* strains gave more complete COD reduction and had greater numbers of cells and greater oil yields. Compared to whole whey, whey permeate gave greater fermentation yields and efficiencies, probably

TABLE II

Extraction of Fat[a] from *Candida curvata R* after 72 hr Fermentation of Whey Permeate

Method of extraction	% Fat dry weight
Methanol:benzene	51.1
Methanol:hexane	36.1
Ethanol:benzene	31.2
Ethanol:hexane	50.2
Chloroform:methanol	10.2
Chloroform:methanol (sonicated cells)	10.2
Chloroform:methanol (Mickle disentegrator)	41.8
Acid hydrolysis	43.7
Alcoholic KOH	50.2

[a]Results are averages of at least two trials using duplicate samples.

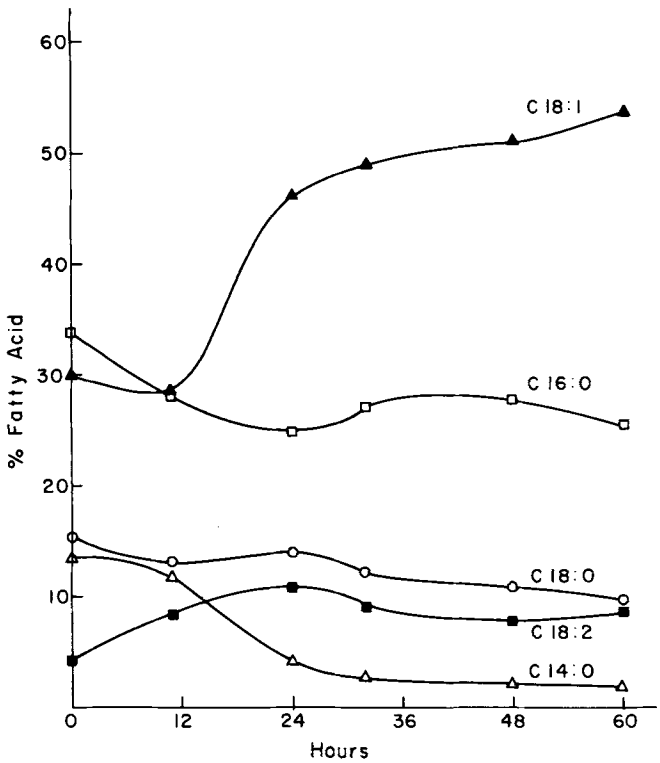

FIG. 3. Changes in fatty acid composition during the fermentation of whey by *C. curvata* R.

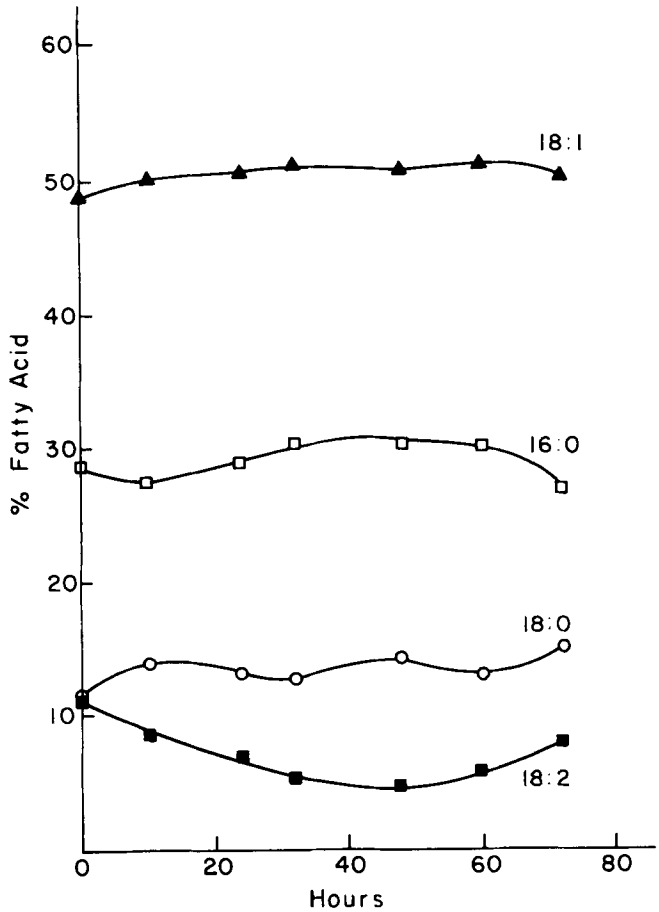

FIG. 4. Changes in fatty acid composition during the fermentation of whey permeate by *C. curvata* R.

because of greater concentrations of lactose in the permeate. Also, poor utilization of whey proteins resulted in greater terminal CODs.

All yeast strains fermented lactose in two phases consisting of a growth phase with little fat accumulation followed by a fattening phase. This two-stage fermentation results from depletion of growth nutrients and conversion of residual carbohydrates to oil (2). Figures 1 and 2 show the production of oil by these yeasts when grown in whey and whey permeate. Oil production and fermentation efficiencies of *C. curvata* were comparable to the best results reported for any yeast grown on glucose media (10). The oil coefficient was high and approached the theoretical maximum of 37.

Lipid extraction from yeast is difficult, seemingly because of characteristics of the cell walls (3). Several methods have been compared for *Saccharomyces cereviseae* (8), and sequential extraction with methanol, methanol-benzene (1:1, v/v), and benzene was most efficient. We found this method more effective than chloroform-methanol even when chloroform-methanol was supplemented with sonication, abrasion, or acid digestion (Table II). Methanol-benzene gave slightly higher values than alcoholic KOH digestion because methanol-benzene should also extract the glycerol and polar substituents of complex lipids. Methanol and benzene are expensive and toxic solvents to consider for extraction of edible oils. Ethanol-hexane was almost as good, but surprisingly, methanol-hexane and ethanol-benzene mixtures proved less effective.

Thin layer chromatography of a methanol-benzene extract of cells taken at the end of fermentation indicated that the lipid was primarily triglyceride and that very little free fatty acids, complex lipids, or sterols were present. The triglyceride accumulation in the cells could be observed as a large single refractile droplet by phase microscopy.

The fatty acid composition of the cells varied with growth temperature, fermentation time, and medium (Table III and Fig. 3-10). All four organisms were rich in octadecenoic acid (presumably oleic acid) and contained considerable amounts of palmitic and stearic acids. These

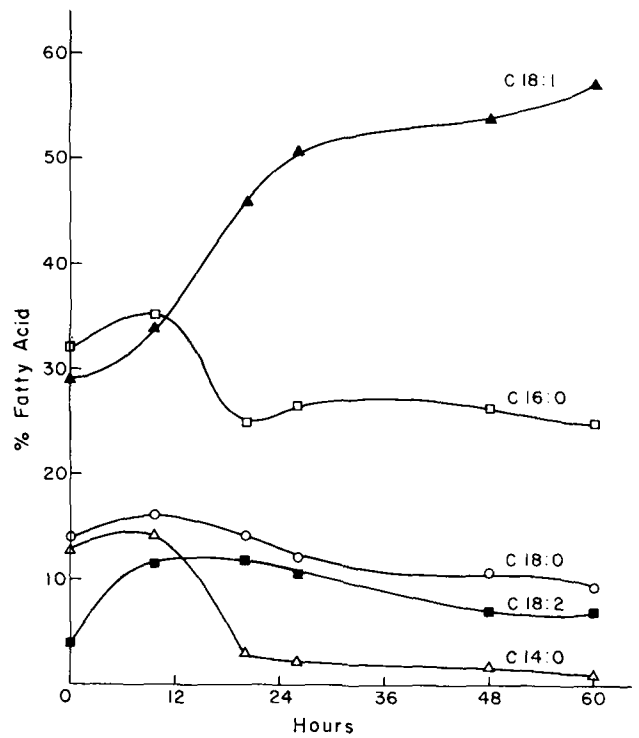

FIG. 5. Changes in fatty acid composition during the fermentation of whey by *C. curvata*D.

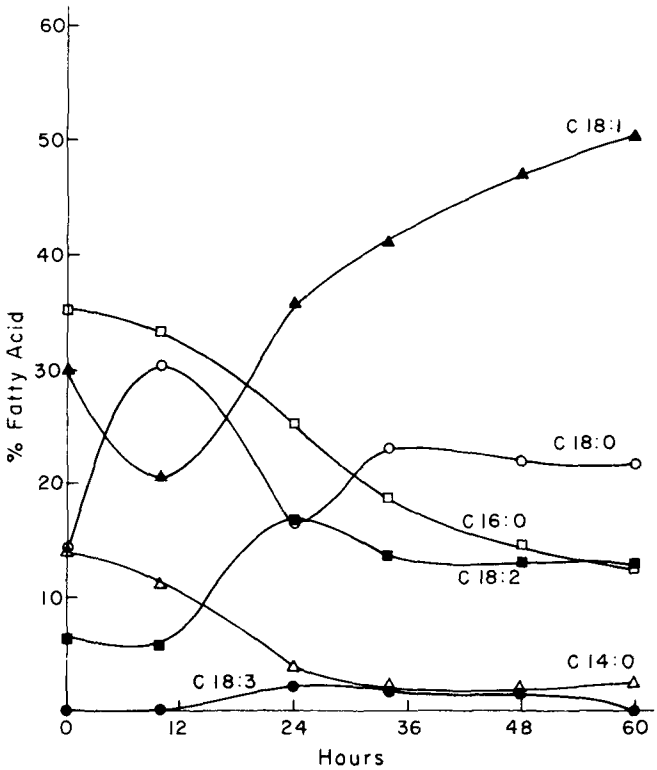

FIG. 7 Changes in fatty acid composition during the fermentation of whey by *T. cutaneum* 24.

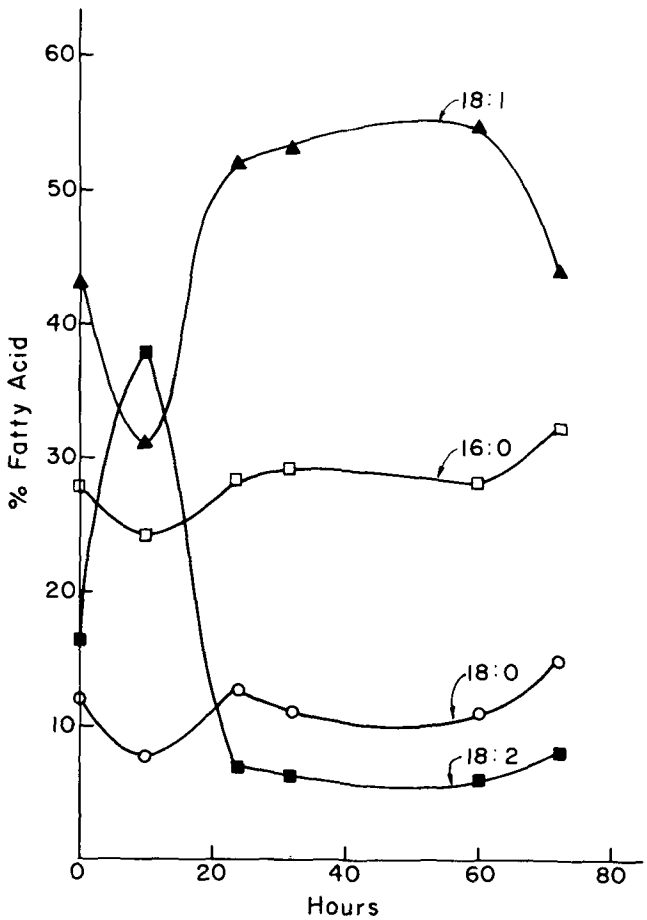

FIG. 6. Changes in fatty acid composition during the fermentation of whey permeate by *C. curvata* D.

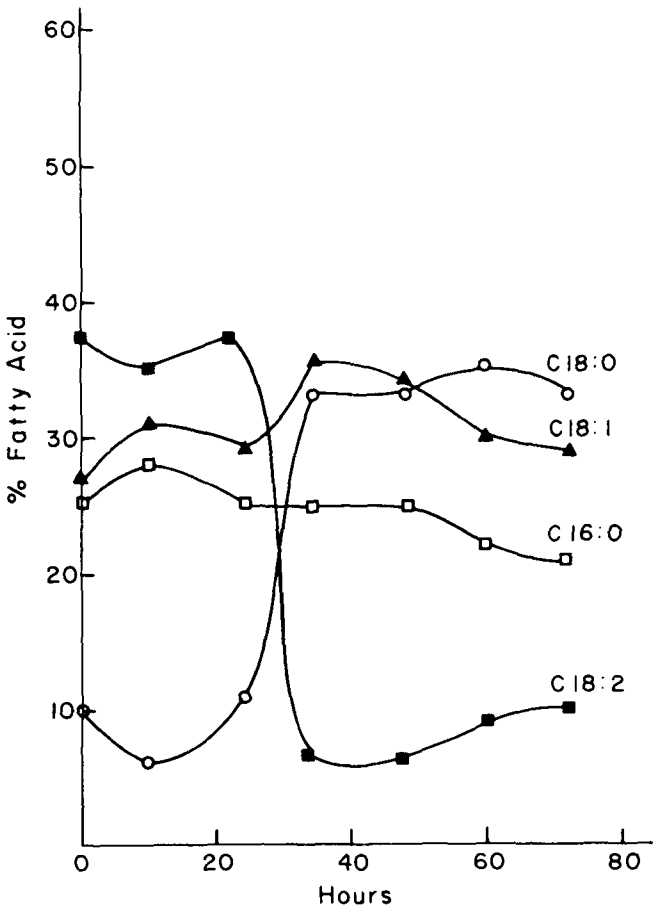

FIG. 8. Changes in fatty acid composition during the fermentation of whey permeate by *T. cutaneum* 24.

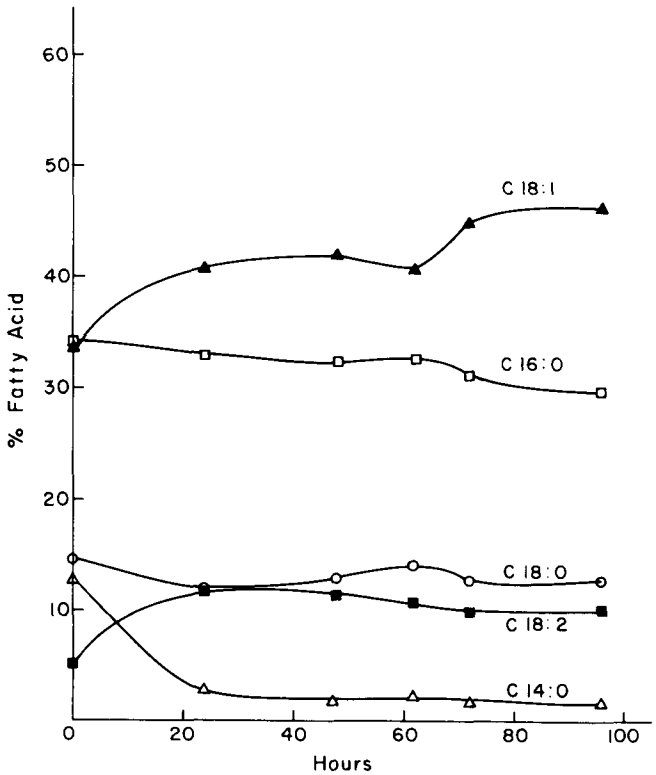

FIG. 9. Changes in fatty composition during the fermentation of whey by *T. cutaneum* 40.

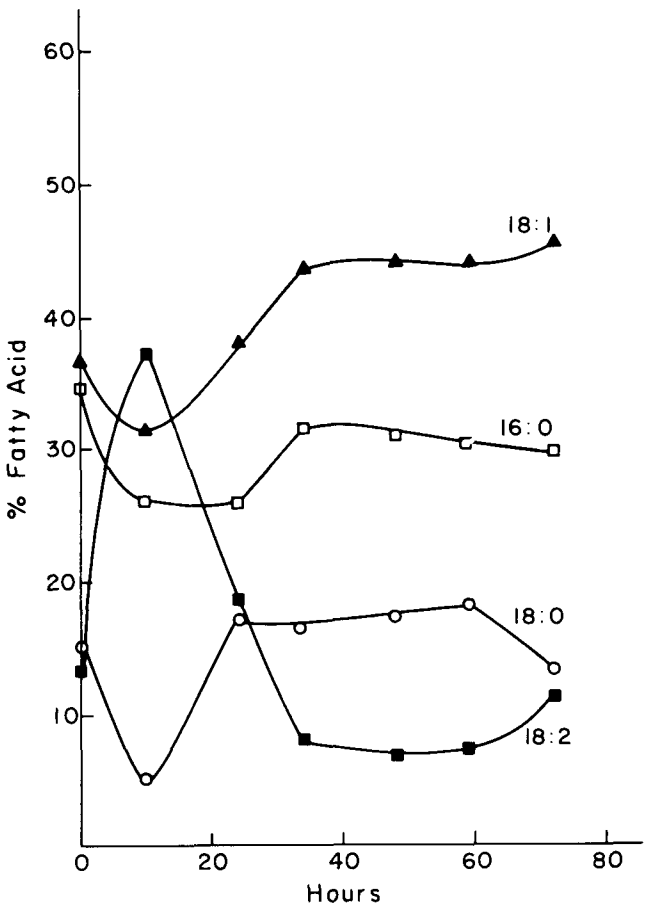

FIG. 10. Changes in fatty acid composition during the fermentation of whey permeate by *T. cutaneum* 40.

results are similar to those reported for other yeast (11), although other species of *Candida* can produce more linoleic and less oleic acid (12). The low mean unsaturation, 0.5-0.7, was unusual for yeast; a value of 1.3 is typical for fungi grown at 30 C (13). These low mean unsaturation values are more typical of thermophilic organisms grown at 45 C (14).

When the growth temperature of an organism is lowered, unsaturation of fatty acids usually increases to maintain lipid fluidity (15). Fatty acids produced at 15 C were slightly more unsaturated than at the optimum temperature, except for *T. cutaneum* 40, but the mean unsaturation was still quite low compared with the typical value of 1.5 for other yeast at 15 C (12). It is not clear if the *C. curvata* and *T. cutaneum* strains maintain their oil in a liquid state, and if so, how they do it.

The fatty acid composition varied with the age of the culture. During the initial growth phase, little oil accumulated, but when maximum cell numbers were reached, excess sugar was converted to oil (5). In the growth phase, the degree of unsaturation and/or the content of shorter acids was greatest. During this stage, the cells probably were richer in phospholipids than triglycerides. When oil production began after about 10 hr, the proportion of $C_{18:1}$ dramatically increased as particularly noticed for *T. cutaneum* 24 grown on permeate (Fig. 8).

Oil composition changed significantly when the yeast

TABLE III

The Fatty Acid Composition of Two Strains Each of *C. curvata* and *T. cutaneum* in Different Media and at Different Temperatures

Organism	Medium	% Fatty acid					Double bonds /molecule[a]
		14:0	16:0	18:0	18:1	18:2	
C. curvata R	Whey[b]	2	26	10	54	9	0.72
	Whey permeate[b]	Tr[c]	31	12	51	6	0.63
	Whey 15 C[d]	Tr	19	6	62	10	0.82
C. curvata D	Whey	1	25	10	57	7	0.71
	Whey permeate	Tr	32	15	44	8	0.60
	Whey 15 C	Tr	22	6	67	5	0.77
T. cutaneum 24	Whey	3	13	22	50	13	0.63
	Whey permeate	Tr	21	33	29	10	0.49
	Whey 15 C	Tr	20	11	59	10	0.79
T. cutaneum 40	Whey	2	30	13	46	10	0.66
	Whey permeate	Tr	30	13	46	11	0.68
	Whey 15 C	Tr	25	22	51	2	0.55

[a][(% Monoenes) + 2 (% dienes) + 3 (% trienes)/100].
[b]Whey and whey permeate at optimum temperature for each organism: Strain R, 30; D, 28; 24, 28; 40, 33 C.
[c]Tr = detected in amounts less than 1% of total fatty acids.
[d]Whey agar medium incubated at 15 C.

was grown in permeate rather than whey (Table II). This may be related to the increased yield of oil on permeate. Except for *T. cutaneum* 24, the oil produced on permeate was more saturated than that produced on whey.

The fatty acid composition of *C. curvata* R did not change as much during fermentation as that of the other yeasts when grown in permeate (Fig. 3 and 4). In contrast, the oil increased in oleic acid during fermentation of whey. Possibly *C. curvata* R was well into the fattening phase when it was inoculated into the permeate and continued to make fat while growing. The percentage of oil in this organism never really dropped to less than about 15% (dry weight), suggesting continual lipid accumulation (5). The other organisms were about 10% oil during the growth phase.

The cost of doing a fermentation of permeate with *C. curvata* D on a scale of 2.3 x 10^5 liters/day, the size needed for a typical cheese plant, would be $3,250/day according to a study by R. Fournier and C.E. Glatz, of the Department of Chemical Engineering, Iowa State University. One would produce 3.4 metric tons/day of oil, 0.55 of proteins and 2.15 of other cellular material, which we presumed to be chiefly cell wall carbohydrate. If one assumes the protein will compete with soy protein in feed at 44 cents/kg and the cell carbohydrate has no value, the oil would have to be worth 88 cents/kg to break even with no return on capital. Oil prices have seldom approached these levels in the last few years.

But the study suggests some promising ways of cutting fermentation costs. One way would be to reduce the fermentation time, possibly by adjusting the inoculum level.

Almost 50% of the fermentation costs are for power for agitation and aeration. Theoretically little aeration and agitations should be needed in the fattening stage of fermentation, but this was not observed (5). Possibly, high aeration was needed during fattening to remove CO_2 which would otherwise inhibit the organisms. More data on this requirement might significantly reduce power costs.

If the permeate were condensed, more carbohydrate would be put in the fermenter, and if fermentation times were not increased correspondingly, a greater throughput could be achieved. The costs of condensing to double strength was estimated at $525/day (16).

It may be possible by selecting other organisms or by mutation of the present ones to achieve oils of different compositions and economic values. To a limited extent composition can be controlled by fermenter temperature. Selection for organisms that make less cell wall should also be desirable.

It is unlikely that people with iron fermenters will ever convert sugar to oil as cheaply as can green plants, but when dilute wastes rich in carbohydrates must be disposed of, fermentation may be less costly than other means and become profitable. Currently whey is dried, and permeate is usually condensed and forced back on the milk producers. When true costs are calculated, these procedures do not break even most of the time.

These experiments show that it is possible to convert lactose to oil efficiently and reduce the COD of the medium to an acceptable value. A reasonably efficient means of extracting the oil was found. Further research should improve the efficiency of fermentation. The real barriers to this technology may be institutional ones. The costs of establishing the safety of yeast oil as a food grade oil may be significant. The small scale of producing oil from whey and its wide dispersion geographically will make yeast-oil extraction unattractive to oil processors. For example, if the $1.3 \cdot 10^7$ metric tons of sweet cheese whey produced annually in the United States (17) were all converted to oil by the process reported here, the $2 \cdot 10^5$ metric tons of oil produced would be only 5.4% of the estimated edible oil production of the United States in 1977 (18).

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