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Biomodification of Fats and Oils: Trials with *Candida lipolytica*[1]

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ABSTRACT

Various oil-accumulating yeasts were tested for their ability to produce lipase and live on fats and oils as carbon sources. Of these, *Candida lipolytica* seemed most promising, and the possibility was explored of modifying fats and oils by fermenting them with *C. lipolytica* and extracting the modified oil deposited in the yeast cells. Oxygen was required for the growth of yeast on fats and oils, but unless the oxygen level was controlled at a low value after cell populations peaked, most of the substrate oil was converted to citrates rather than accumulating as oil. Oil accumulation by *C. lipolytica* from a corn oil substrate was slightly depressed by excess nitrogen in the medium. The yeasts were able to use about 18 g/l of oil in 72 hr. At substrate oil levels greater than 18 g/l, the dry yeasts were 60% oil, and about 45-57% of the substrate oil was recovered as yeast oil. The fatty acid composition of the yeast oil was quite similar to that of the substrate oil under optimum conditions of deposition. Sterols, but not tocopherols, were transferred from the substrate to the yeast oil. *Candida lipolytica* oil was high in free fatty acids. The greatest potential for biomodification by fermentation with *C. lipolytica* seems to be in altering glyceride structure.

INTRODUCTION

Fat and oil technologists have several techniques available for modifying compositions of raw materials, namely, blending fats and oils from diverse sources, hydrogenation, fractional crystallization, interesterification and plant breeding (1,2). These techniques make available a wide range of lipid products and have made it possible to substitute fats and oils for each other in many instances. But some advantageous techniques are missing. For example, we have as yet no good way to remove the linolenic acid selectively from an oil. We cannot reverse the hydrogenation reaction and convert tallow into an oil rich in linoleic acid. We have only limited ability to modify glyceride structure.

We look with envy on biological systems with their precise control of chain length, unsaturation and glyceride composition, and we ponder the possibility of using enzymes to modify lipids, but these systems seem discouragingly complex and fragile. There is, of course, a kind of biomodification that we have been using for years. We convert oils in feed grains into animal fats. This is not very efficient, but it is known that microorganisms do bioconversions quite efficiently. Usually, when fermentations are considered in regard to fats and oils, they have been used to convert carbohydrates to oils, but microorganisms are known that will use fats and oils as carbon sources and also will accumulate oil (2,3,4).

These experiments were conducted to explore the possibility of biomodifications of fats and oils by the yeast, *Candida lipolytica.* Fermentation conditions were optimized, and the yeast oil that accumulated was examined.

Methods

Yeast cultures were obtained from the collection of the Iowa State University Food Technology Department; from the USDA Northern Regional Research Center, Peoria, IL, and from Dr. C. Ratledge, University of Hull, England. The cultures were maintained on agar slants containing 0.3% yeast extract, 0.3% malt extract, 0.5% peptone (Difco Laboratories, Detroit, MI) and 1% glucose.

Selection for organisms able to use fat as a carbon source and accumulate fat was made on plates containing 5% corn oil, 0.67% yeast nitrogen base (Difco) and 2% agar. Colonies were stained with Oil red O (Pfaltz and Bauer, Inc., Flushing, NY) by saturating an isopropanol solution with the dye, diluting this with an equal volume of water and using it as a wet-mount medium. To test for lipase production, tributyrin agar plates were prepared (5), and the cell pastes to be tested were added to wells cut in the plate and incubated for 48 hr at 37 C. The agar was inspected for clear zones around the wells.

Fermentations were done in a 750-ml fermenter (Multigen, New Brunswick Scientific Co., New Brunswick, New Jersey) with a 450-ml working volume. Air flow and agitation rates could be controlled. The pH was controlled by a TTT2 automatic titrator (Radiometer, Copenhagen, Denmark) with the addition of 3 N NaOH. Dissolved oxygen was measured with an electrode prepared according to Johnson et al. (6) and recorded with a DO-50 dissolved oxygen recorder (New Brunswick). Fermentations were at 30 C,

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with an aeration rate of 0.5 L/min and agitation rate of 435 rpm for the first 22 hr to maintain dissolved oxygen at 80% saturation and at 212 rpm from 22 to 72 hr to maintain dissolved oxygen near 0% saturation. The inoculum (50 ml) was grown in a 250-ml shake flask on the medium of Gill et al. (7) with 2% corn oil as a carbon source instead of sugar. The fermentation medium was the same, with the amount and kind of fat or oil and nitrogen adjusted as indicated below.

Citrate production was verified by Bergmeyer's method (8). The amount of citrate produced was estimated from the pH, the amount of NaOH added and the dissociation constants of citric acid. At the usual pH 5.5, citrate in grams = 0.098 x meq. NaOH.

Yeast cells were harvested by centrifugation at 7000 rpm for 30 min at 15 C in a RC2-B centrifuge (Ivan Sorvall, Newtown, Connecticut). To remove and measure unassimilated oil, hexane was layered onto the upper part of the centrifuge tube, recovered and evaporated. Lipid was extracted and measured by extraction with ethanol, hexane and benzene according to Hammond et al. (9). The portion insoluble in solvents was dried 24 hr at 50 C in a vacuum oven and weighed.

Lipids were fractionated and analyzed according to Hammond et al. (9) except for glyceride structure analysis, which was by the method of Pan and Hammond (10).

TABLE I

The Ability to Grow on Corn Oil as a Carbon Source and Lipase Production of Several Oleaginous Yeasts

Strains		Growth	Lipase
Candida curvata	D[a]	+	++
	R[a]	++	++
	3529[b]	–	–
	4325[b]	–	–
	3528[b]	∓	∓
	6791[b]	–	–
Candida	107[c]	–	∓
Pigmented unknown yeast[a]		++	++
Lipomyces tetrasporous	11563[b]	–	∓
	11562[b]	–	∓
Lipomyces starkii	11389[b]	–	+
	11560[b]	–	∓
Rhodotorula glutinis	1596[b]	–	–
	2502[b]	–	–
Candida lipolytica	1094[b]	+++	+++
	1095[b]	+++	+++
Rhodotorula rubra	2505[b]	+	+
	1591[b]	+	+

[a]Source, Iowa State University Food Technology Department.
[b]Source, USDA Northern Regional Research Center.
[c]Source, Dr. Colin Ratledge, Univ. of Hull, England.
– no growth or lipase; ∓ questionable; + trace; ++ moderate growth and lipase; +++ good growth and lipase.

RESULTS AND DISCUSSION

Table I shows that many oil-accumulating yeasts are able to grow on corn oil as a sole carbon source. This ability is highly correlated with lipase production. On the basis of these tests, *C. lipolytica* 1094 was selected for further tests.

Examination by electron microscopy of *C. lipolytica* 1094 growing on corn oil showed that oil droplets accumulated on the outer cell wall of the yeast cells from whence they were transferred to invaginations on the outside of the cytoplasmic membrane. The oil droplets eventually were transferred to a large oil storage depot in the center of the cell.

Table II shows that the dissolved oxygen level was critical for the efficiency of oil accumulation by *C. lipolytica.* This organism is known to produce citric and isocitric acids, and its exploitation for this purpose has been suggested (11). The presence of citric acid was confirmed when this organism was grown on corn oil as a sole carbon source. The presence of oxygen during the growth phase was necessary for *C. lipolytica* to use the reduced carbon source, but after about 22 hr, if oxygen was supplied in abundance, citrate and isocitrate were major products. The time of 22 hr seemed to coincide with maximum cell growth, but it was difficult to count cells accurately in the presence of corn oil. After 22 hr, the rate of base utilization accelerated rapidly. The deposition of lipid was enhanced by reducing the dissolved oxygen level to nearly zero during this latter stage of fermentation. Cell nonlipid yields also were increased by decreasing aeration during the latter stage of the fermentation.

The dissolved oxygen is much more sensitive to the agitation rate than to aeration rate, so this parameter was controlled by agitation. Reducing aeration to 0.01 L/min while maintaining the agitation gave too great a dissolved-oxygen value. The agitation rate also is important in maintaining the dispersion of the oily substrate. Possibly, efficiency could be increased by maintaining agitation and aerating with a mixture of air and nitrogen.

Lipid formation from carbohydrates by yeast usually requires the absence of an essential nutrient such as nitrogen (2,4,12). Table III shows that high nitrogen levels in the medium did not block lipid accumulation by *C. lipolytica.* At the two lowest nitrogen levels, cell dry weights and lipid accumulation are restricted. The optimum nitrogen level was about 124 mg/l; higher levels depressed cell dry-weight production and restricted lipid accumulation. This result was unexpected. Perhaps the higher levels of ammonium sulfate inhibited culture growth.

Table IV shows the effect of the concentration of substrate oil on the accumulation of oil by the yeast in a 72-hr fermentation. When more than 18 g/l of oil was supplied, not all could be assimilated in 72 hr, but more nonlipid was

TABLE II

Effect of Dissolved Oxygen on Lipid Accumulation and Citrate and Isocitrate Formation by *C. lipolytica*

Dissolved O_2 22-72 h % saturation	Citrates g/l	Yeast nonlipid g/l	Yeast lipid g/l	Yeast lipid %
80	9.4	4.28	2.58	37.6
40	9.0	4.76	3.10	39.4
20	7.0	4.58	3.30	41.9
15	3.3	4.70	4.31	47.8
5	2.3	5.47	6.42	54.0
0	1.2	5.35	6.48	54.8

Fermentation conditions: 30C; pH 5.5; aeration 0.5 L/min; agitation 435 rpm 0-22 hr, 212-260 rpm 22-72 hr; carbon source, corn oil 18 g/l; nitrogen 124 mg/l.

TABLE III

Effect of the Amount of Nitrogen Added to the Medium on Lipid Accumulation by *C. lipolytica*

N mg/l	Citrates g/l	Yeast nonlipid g/l	Yeast lipid g/l	Yeast lipid %
0[a]	–	1.28[b]	3.09[b]	70.7
62	1.12	4.72	5.63	54.4
124	1.10	6.00	6.36	51.5
247	1.18	5.64	6.35	53.0
492	1.18	4.26	6.01	58.5
636	0.91	3.91	5.90	60.1
848	1.12	3.88	5.52	58.7
1166	1.03	3.60	5.40	60.0

[a]The medium contained 1% yeast extract, which supplied some N.
[b]These yields may be low because of difficulty in separating the cells from residual oil. In other trials, the residual oil was less than 0.1 g at 72 hr.
Fermentation conditions: 30C; pH 5.5; aeration 0.5 L/min; agitation rate 435 rpm 0-22h, 212 rpm 22-72 hr; carbon source 18 g/l corn oil; nitrogen source, $(NH_4)_2SO_4$.

TABLE IV

Effect of the Amount of Substrate Corn Oil on Oil Accumulation by *C. lipolytica.*

Substrate oil g/l	Citrates g/l	Unassimilated oil g/l	Yeast lipid g/l	Yeast nonlipid g/l	Yeast lipid %	Substrate oil recovered as yeast oil (%)
18	1.33	0.05	6.36	5.99	51.5	35.3
22.5	1.62	2.85	10.11	8.44	55.0	44.9
25	1.53	2.95	12.80	8.71	59.5	51.2
27	1.25	3.10	15.50	9.66	61.4	57.4
37.75	1.62	7.00	17.97	11.20	61.6	47.6

Fermentation conditions: 30C; pH 5.5; aeration 0.5 L/min; agitation 435 rpm 0-22hr, 212 rpm 22-72 hr; carbon source, corn oil; nitrogen 124 mg/l.

TABLE V

Influence of pH on Lipid-Accumulating Fermentations by *C. lipolytica*

pH	Citrates g/l	Unassimilated oil g/l	Yeast nonlipid g/l	Yeast lipid g/l	Yeast lipid %
4.7	1.25	3.75	8.85	10.5	53.3
5.5	1.20	3.51	8.44	10.1	55.0
5.7	0.52	3.20	8.53	10.3	54.7
6.5	2.28	3.46	8.70	8.3	49.0

Fermentation conditions: 30C; aeration 0.5 L/min; agitation 435 rpm, 0-22 hr, 212 rpm 22-72 hr; carbon source, corn oil 22.5 g/l; nitrogen 124 mg/l.

produced and more oil was accumulated by the yeast as the substrate oil concentration increased. Under the best conditions, 57% of the substrate oil was recovered as yeast oil.

Variation of the pH from 4.7 to 5.7 did not influence the efficiency of the fermentation appreciably, but at pH 6.5, less lipid accumulated (Table V).

Under fermentation conditions in which efficient oil accumulation occurred, the yeast oil had a fatty acid composition almost identical to that of the original corn oil except that the yeast oil had slightly less palmitic acid and a small amount of palmitoleic acid. This is in contrast to the shake-flask experiments reported for *C. lipolytica* by Glatz et al. (2) in which significant decreases in saturation occurred. Probably the shake-flask experiments differed from those in fermenters because of less complete assimilation of the oil and poorer stirring and aeration in the shake-flasks.

Thin-layer chromatography (TLC) revealed that the yeast lipid was rich in triglyceride but also contained considerably more free fatty acid than did the original corn oil. A stereospecific analysis of the yeast oil triglycerides showed that there were significant changes in the distribution of fatty acids (Table VI).

TABLE VI

Stereospecific Analysis of Yeast Oil Triglycerides Compared With That of the Corn Oil Substrate

	16:0	16:1	18:0	18:1	18:2	18.3
Corn oil	11.1	–	1.8	24.5	61.3	1.4
Sn-1	16.9	–	1.3	23.8	58.0	–
Sn-2	1.5	–	–	23.3	73.8	1.4
Sn-3	14.9	–	3.7	26.1	52.6	2.6
Yeast oil	7.5	5.6	0.7	21.5	58.8	5.9[a]
Sn-1	7.8	6.6	1.2	23.0	61.0	0.4
Sn-2	2.7	–	–	32.2	65.1	–
Sn-3	11.9	10.3	0.8	9.4	50.6	17.4[a]

[a]This is a combination of 18:3 and an unknown fatty acid that was poorly resolved. It is estimated that about half is 18:3.

No tocopherol was detected in the yeast oil. The sterol fraction from yeast oil contained a number of unknown compounds, but when the yeasts were grown on corn oil as a carbon source, peaks typical of corn oil sterols were present in the yeast lipid. When lard or tallow was substituted as a carbon source, the peaks typical of corn oil sterols disappeared, and a cholesterol peak could be observed. Seemingly, the yeasts deposit sterols from the medium oil to some extent.

These experiments illuminate the possibilities of biomodification of fats and oils by fermentation. The possibility of modifying fatty acid composition seems limited. Our results agree with those of Ozawa et al. (3) and Noguchi et al. (4) that yeasts accumulating oil from a fat or oil substrate deposit the fatty acids they are fed. This is reasonable because, if to the yeast the substrate represents only an energy source that can be stored, there is little motive for modifying its fatty acid composition. However, the shake-flask results reported previously with *C. lipolytica* (2) suggest that, under some conditions, considerable specific hydrogenation is possible. Possibly yeasts more fastidious about the oil they store may be found as well.

C. lipolytica seems a poor choice for an oil-accumulating organism because the oil it stored contained considerable free fatty acid. It should be possible to find organisms that do not store much free fatty acid. *Candida curvata* D, for example, was moderately good at growing on corn oil as a carbon source, but it does not store much free fatty acid (9), at least when growing on sugars.

It does not seem that biomodification of animal fats by fermentation will remove cholesterol. Considerable amounts of the substrate are diverted into the production of nonlipid biomass. This material probably can be used as a feed supplement after the oil is extracted, but, considering the nitrogen used, it probably is only ~20% protein. The time and expense of the fermentation probably can be reduced considerably by using a continuous fermentation, but even so, the process will be expensive.

Because the yeast oil retains the glyceride structure typical of oleaginous yeasts (9), the most interesting possibility is to convert a saturated low-cost product such as tallow into an edible cocoa butter substitute. Here, the tendency for the organism to absorb and deposit unaltered the fatty acids it is fed may be an advantage because, in oil deposition from sugar by yeasts, one is dependent on the particular mix of fatty acids that the yeast makes, which usually is too rich in palmitic acid to make a good cocoa butter substitute (9). But to do this successfully, one needs a yeast able to flourish at higher temperatures. On tallow and lard substrates, *C. lipolytica* was not able to digest the saturated triglycerides well at 30 C, and the fermentation was quite inefficient.

Finally, it seems possible that *C. lipolytica* might produce citric and isocitric acids from oils efficiently, but the oils probably would be more expensive than the hydrocarbon substrates that can be used.

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