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Study of triacylglycerol assembly by Apiotrichum curvatum ATCC 20509 as a model system

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Study of triacylglycerol assembly by Apiotrichum curvatum ATCC 20509 as a model system

by

Inmok Lee

A Dissertation Submitted to the

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GENERAL INTRODUCTION

Although the commercial production of gamma-linolenic-rich oil by fermentation with a mold was reported in England and Japan, production of single-cell oil is marginal economically.

Since its isolation in 1977, Apiotrichum curvatum has attracted attention from several groups, and has been used in biochemical, physiological and microbiological studies as well as studies of feasibility of industrial production of the yeast oil.

Triglyceride assembly is an important step because glyceride structure is important in determining oil quality. Nevertheless, only a few studies have been done on triglyceride biosynthesis by oleaginous yeasts.

Previous studies indicated that A. curvatum incorporated the acyl group of substrate oils into its triglycerides; therefore, growing the yeast on various lipids can provide an excellent means of exploring triglyceride assembly. The yeast also is known to have the acyl composition and glyceride structure typical of vegetable oil; therefore the insights gained from such studies may also be applicable to oil seed plants for which such studies are more difficult.

One possible way to provide economic viability to single-cell oil production might be to transfer the ability to make unusual fatty acids of economic importance to oleaginous yeast. The ability of A. curvatum to tolerate and metabolize such fatty acids can be tested by growing the yeast on the substrate containing those fatty acids. Those studies may also be applicable to oilseed plants as well.

In this study, A. curvatum was grown on various lipids such as pure or mixed fatty acids or triglycerides; the growth and lipid accumulation by the yeast, and the fatty acid composition and distribution of fatty acyl groups in its triglycerides were analyzed to investigate triglyceride assembly by the yeast.

Explanation of dissertation format

This dissertation follows the alternate format, and consists of two major parts. The first part, Triacylglycerol assembly from lipid substrates by Apiotrichum curvatum ATCC 20509, has been submitted as a chapter of the book tentatively titled "Single Cell Oil." The second part includes further investigation of triacylglycerol assembly with the same yeast and has been prepared for a professional journal. The format of the two manuscripts followed those for the book and journal, respectively. The two papers are preceded by a General Introduction and a Literature Review and followed by a General Summary. Literature cited in the entire dissertation are listed in alphabetical order of authors' name and follow the General Summary.

LITERATURE REVIEW

Definition of oleaginous microorganism

Microorganisms that can accumulate a considerable portion of their biomass as lipid are called oleaginous. In defining oleaginous fungi, Weete (1980) used a 20% minimum for the proportion of lipid accumulated under appropriate growth conditions. It is not easy to define "under appropriate conditions," because environmental and nutritional conditions influence lipid accumulation in microorganisms (Rattray, 1985; Weete, 1980).

Ratledge proposed 25% lipid accumulation as the arbitrary line for applying the term "oleaginous." The 25% level was thought to be the dividing line in determining commercial exploitation of the organism. Also, this level limits the number of organisms to be considered, because many organisms have 20 to 25% lipid in their cells (Ratledge, 1982).

Studies on biochemical differences in oleaginous and nonoleaginous yeasts have suggested biochemical definitions of the term "oleaginous." A number of studies suggest that the enzyme adenosine triphosphate (ATP):citrate lyase which cleaves citrate into acetyl coenzyme A (CoA) and oxaloacetate occurs in the cytoplasm of all oleaginous yeasts and is the key enzyme in oleaginicity. The acetyl CoA is used for fatty acid synthesis, and oxaloacetate is converted to pyruvate via malate, producing nicotinamide adenine dinucleotide phosphate (NADPH). The pyruvate then returns to the mitochondria where it is coverted to

citrate that can again migrate to the cytoplasm and repeat the cycle. NADPH is also needed for fatty acid synthesis (Botham and Ratledge, 1979; Boulton and Ratledge, 1981). ATP:citrate lyase has been found in various oleaginous yeasts but not in nonoleaginous yeasts except one nonoleaginous strain of Rhodotorula glutinis (Botham and Ratledge, 1979). Therefore, the presence of the enzyme appears to be an important biochemical criterion of oleaginicity, even if the specific activity of the enzyme does not correlate with the amount of lipid accumulated by the yeast (Evans et al., 1981; Evans et al., 1983a,b; Ratledge, 1986; Ratledge and Gilbert, 1985).

Studies about mechanism of oleaginicity

A great deal of work has been done to understand how an oleaginous organism can accumulate lipid. As a result, the biochemical pathway and mechanism for fatty acid synthesis in oleaginous yeasts are quite well elucidated; however, many questions about regulation of the process remain unsolved.

In early studies, some hypotheses for oleaginicity were proposed, and these theories were tested, mostly by comparison of the biochemical features of oleaginous and nonoleaginous yeasts. Apiotrichum curvatum was used as an excellent model oleaginous yeast. The hypotheses included differences in the control of sugar uptake, regulation of acetyl CoA carboxylase, lipid turnover rate, and the regulatory role of adenine nucleotides. These hypotheses proved inadequate, but it was discovered that acetyl CoA carboxylase from oleaginous yeasts but not

from nonoleaginous yeasts was activated by citrate (Whitworth and Ratledge, 1975; Botham and Ratledge, 1978; Botham and Ratledge, 1979). Other important findings that led to the presently accepted mechanism for oleaginicity were:

1. the presence of an active ATP:citrate lyase in oleaginous yeasts;
2. a low concentration of adenosine monophosphate (AMP) in oleaginous yeasts under nitrogen-limited conditions;
3. the strong dependence of isocitrate dehydrogenase from oleaginous yeasts on AMP (Botham and Ratledge, 1979; Boulton and Ratledge, 1981a; Boulton and Ratledge, 1981b; Evans and Ratledge, 1983b).

After the current mechanism of oleaginicity was proposed, studies followed to prove the hypothesis in detail and to elucidate the control of the process. In 1984, Evans and Ratledge discovered that Rhodosporidium toruloides CBS14 increased its lipid content from 18% to 52% when the nitrogen source was switched from ammonium chloride to glutamate or urea. The yeast provided a good model for the regulation of lipid synthesis by one factor, the nitrogen source.

The metabolic steps that have been studied for possible regulatory roles include: (i) the build-up of ATP and depletion of AMP (Botham and Ratledge,1979); (ii) inactivation of mitochondrial nicotinamide adenine dinucleotide (NAD+): isocitrate dehydrogenase (Evans and Ratledge, 1985b); (iii) transport of citrate across the mitochondrial membrane (Evans et al., 1981; Evans et al., 1983a,b); (vi) enzymatic reaction of ATP:citrate lyase (Boulton and Ratledge,1981a; Evans and Ratledge, 1983b); (v) activation of AMP deaminase and malic enzyme (Evans and

Ratledge, 1985a). Although much has been revealed about the factors that affect each possible regulatory step, it remains unclear how the whole process of lipid biosynthesis is coordinated, and why yeasts can accumulate different amount of lipid under the same growth conditions.

Figure 1 shows the linkage between intermediary metabolism and fatty acid biosynthesis in oleaginous fungi. When the nitrogen supply in the medium is exhausted, the activity of AMP deaminase increases greatly, and the enzyme, which catalyses the reaction AMP -> inosine monophosphate (IMP) + NH_4, creates a low intracellular AMP concentration. At the same time the ATP level in the cell increases as the synthesis of proteins and nucleic acid are impeded by lack of nitrogen (Evans and Ratledge, 1985a). However, it is not clear how AMP deaminase becomes activated by depletion of nitrogen (Ratledge, 1987). NAD^+:isocitrate dehydrogenase in oleaginous yeasts is strongly dependent on the AMP concentration for its activity, and it is inhibited strongly by ATP. Thus, when the mitochondrial enzyme is blocked, citrate accumulates in the mitochondria (Evans et al., 1983a; Botham and Ratledge, 1985).

The accumulated citrate is transported out of the mitochondrion in exchange with malate by citrate-malate translocase (Evans et al., 1983b). Production of malate through the citric acid cycle is blocked by deactivation of NAD^+:isocitrate dehydrogenase. However, malate can be created from pyruvate via oxaloacetate. The reduction of oxaloacetate is effected by mitochondrial malate dehydrogenase (Ratledge, 1987). The malate is believed to move out of the mitochondrion in exchange for pyruvate by a pyruvate-malate translocase system. The pyruvate supply

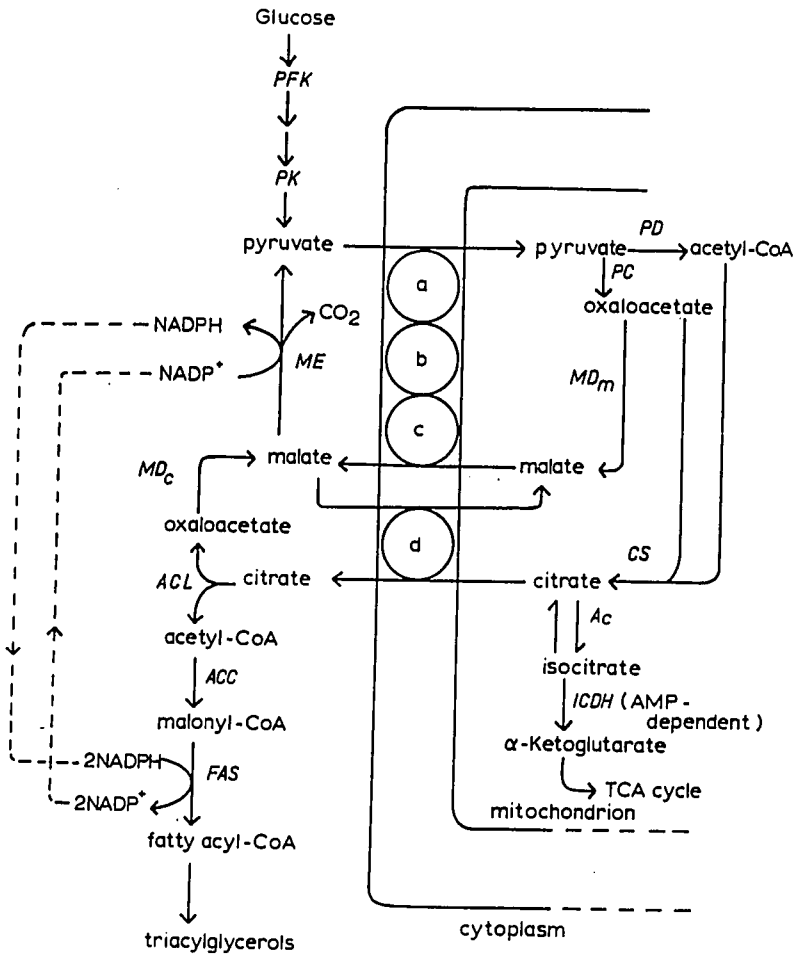

Figure 1. Intermediary metabolism as linked to fatty acid biosynthesis in oleaginous microorganisms. Mitochondrial transport process: a, b, c, interlinked pyruvate-malate translocase systems; d, citrate-malate translocase. Enzymes: AAC, acetyl CoA carboxylase; AC, aconitase; ACL, ATP: citrate lyase; CS, citrate synthase; FAS, fatty acid synthetase complex; ID, isocitrate dehydrogenase; MD_c, malate dehydrogenase (cytosolic); MD_m, malate dehydrogenase (mitochondrial); ME, malic enzyme; PC, pyruvate carboxylase; PD, pyruvate dehydrogenase; PFK, phosphofructo-kinase; PK, pyruvate kinase (Ratledge, 1987)

from sugar continues unabated because the two enzymes regulating glycolysis, phosphofructokinase and pyruvate kinase, are fully active or at least active enough during lipid synthesis (Evans and Ratledge, 1984; Evans and Ratledge, 1985c).

The citrate transferred into the cytoplasm is cleaved into acetyl CoA and oxaloacetate, providing acetyl CoA for fatty acid biosynthesis (Botham and Ratledge, 1979; Boulton and Ratledge, 1981a; Boulton and Ratledge, 1981b; Evans and Ratledge, 1983b). The oxaloacetate in the cytoplasm is converted to malate by cytosolic malate dehydrogenase. A part of the cytosolic malate is transported back to the mitochondrion in exchange for citrate, and extra malate is converted to pyruvate by malic enzyme, which can simultaneously produce the NADPH needed for fatty acid synthesis (Evans and Ratledge, 1985a).

Commercial potential of single cell oil production

It was in the early 1870s that an ergot fungus was observed to contain 30% of its biomass as fat. During the following half century, a number of microorganisms, mostly fungi, that accumulated significant amounts of lipid were isolated and charaterized (Woodbine, 1959).

The two world wars provided a great impetus for research in Germany on the production of oil from microbial sources, but this did not result in commercially successful production (Ratledge, 1984). In 1959, Woodbine (1959) reviewed the potential for commercial production of microbial oil. However, it has been only in the last ten years that interest in microbial oil has resurfaced, and extensive research has

been performed. The renewed interest in oleaginous fermentations appears to be indebted to successful commercialization of single cell protein production as well as realization of the need for oils from alternative sources by the painful experiences of the petroleum crisis in 1970s (Ratledge, 1982). In 1982, Ratledge compared the research of microbial lipid to "insurance" against possible soaring prices for conventional fats and oils. He also concluded that "events far outside the realms of the fermentation laboratory" would decide the feasibility of commercial-scale microbial lipid production (Ratledge, 1982).

Bacteria generally do not accumulate much lipid, and their lipid usually is mainly phospholipid and glycolipid. Some bacteria accumulate waxes as well as neutral lipids, and bacterial lipid from some genera is suspected of being toxic or allergenic. Various unusual fatty acids such as hydroxy, branched-chain, and cyclopropanoid fatty acids have been reported from bacterial oil, however, the polyunsaturated fatty acids typically found in vegetable oils usually were not identified (Waywan et al., 1984).

Microalgae have attracted attention because of the exceptionally great photosynthetic productivity of their cell mass; however, their lipid content is generally only 15-20%, except for some species that grow very slowly. Also, algae contain very high proportions of nonneutral oil, and refining their oils would need complex and expensive downstream processes (Shifrin, 1984).

Many fungi are able to accumlate more than 60% of their biomass as lipid, of which 80-90% is triglyceride. Fungal lipids are similar to

vegetable oils, being composed of palmitic, stearic, oleic, linoleic and linolenic acids (Glatz et al., 1984). Generally fungi, especially yeasts, are regarded as the most likely candidates for edible oil production because the technology for large-scale production of single cell protein (SCP) can be adapted to oil production, and yeasts generally are accepted in human food (Murali et al., 1987). Among the yeasts studied, A. curvatum has become the most extensively investigated for commercial potential because of its ability to utilize cheese whey, a byproduct of cheese making (Moon and Hammond, 1978).

The more serious attempts to achieve commercial production of microbial oils have been done in European countries and New Zealand, where indigenous oil sources are limited but supplies of relatively low-priced carbon sources are available (Moreton, 1987; Stobart and Stymne, 1987; Floetenmeyer et al., 1985; Davies, 1987).

A basic question in pursuing the commercial production of microbial oil is whether such oil can compete with typical agriculturally-produced oils and, if this is not the case, are there circumstances where microbial oil could compete in the oil market. Two papers suggest that the microbial oil would have to be worth over $5,000/ton to be produced commercially in European countries (Moreton, 1987; Sinden, 1987). This price was about 10 times greater than the current price of soybean oil. This calculation indicated that microbial oil would have to find markets comparable to expensive specialty oils such as cocoa butter.

Production costs, of course, can vary with costs of raw materials, equipment and labor. According to Floetenmeyer et al. (1985), the

economically feasible cost in the United States, when whey permeate is utilized as a substrate in a continuous culture of A. curvatum (Floetenmeyer et al., 1985) would be $680/ton. However, this cost does not include the downstream processes of extraction and refining.

The Department of Scientific and Industrial Research of New Zealand has been actively pursuing the scale-up of single cell oil processes because of New Zealand's limited domestic vegetable oil supply and geographical situation. Davies has provided extensive information about the industrial-scale production of yeast oil based on studies in a 500-L pilot-scale fermentor with A. curvatum on a whey substrate (Davies, 1987). Among the various methods examined for downstream processing, cell concentration with a Sharples decanter and extraction of the spray-dried yeast with hexane (20% w/w) in a bead mill were the best options. To be economically attractive, the price of product yeast oil should be more than $1,000/ton, according to his assessment. Table 1 shows the current prices of some vegetable oils. Further process development and optimization of downstream processes were reported; however, commercial production of the process has not been realized yet (Davies, 1991).

The first commercial production of microbial oil was achieved in 1987. A British company announced the production of an oil rich in gamma-linolenic acid by fermentation with Mucor javanicus. A glucose-based defined medium was used to carry out the fermentation in 220-m^3 fermentors. The oil was extracted using organic solvents and used as a substitute for evening primrose oil, which contains 8-10% gamma-linolenic acid and is sold at $56,000/ton in England (Sinden, 1987).

Table 1. Wholesale prices of various agricultural oils in the United States (June, 1991)[a]

Item	Price ($/ton)
Castor oil	805
Cocoa butter	4500
Coconut oil, crude	478
Corn oil	653
Cottonseed oil, crude	448
Linseed oil, raw	794
Palm oil	428
Peanut oil, crude	932
Rapeseed oil	419
Safflower oil	1080
Soybean oil, crude	433
Sunflower oil	523
Tung oil	1356

[a]From "Oil Crops: Situation and Outlook Yearbook" (1991, USDA).

Another commercial production of gamma-linolenic-rich oil by fermentation with Mortierella species was reported in Japan (Ratledge, 1987). However, the market for specialty oils such as gamma-linolenic-

rich oil is small and its future is unclear.

Studies on A. curvatum

Since isolation of A. curvatum in 1977, this oleaginous yeast has been investigated by several groups. In this section, the studies of several of these groups will be reviewed chronologically.

At Iowa State University

A. curvatum, formerly known as Candida curvata, was originally isolated from the Iowa State University dairy plant (Moon, 1977). A search was made for microorganisms that could grow on whey and produce oil. A. curvatum, one of the strains that was selected, utilized lactose efficiently and grew well on both whey and whey permeate as a substrate with only ammonium hydroxide added. Optimum physical conditions for batch fermentation in a 14-L fermentor were: pH between 5.4 and 5.8; temperature, 28°C; aeration rate, more than 0.7 L/min/L medium. Under optimized conditions A. curvatum could produce 15.6 g/L of oil and 26.8 g/L of biomass (57% oil content) from Swiss cheese whey permeate in 72 hrs, and the chemical oxygen demand (COD) of the whey permeate was reduced by 95% (Moon et al., 1978). The fatty acid composition of the yeast oil varied with growth temperature, fermentation time and medium composition (Moon and Hammond, 1978).

Later, extensive examination of oil from A. curvatum grown on whey permeate (Choi, 1980) showed that the lipid consisted of 80 to 90% triglyceride. The fatty acid composition of the triglyceride was 30.4%

palmitic, 0.8% palmitoleic, 11.4% stearic, 51.0% oleic, 6.2% linoleic and 0.4% linolenic acid. Glyceride structure analysis showed that saturated acyl groups were almost completely excluded from the sn-2 position of the glycerol. Major components of the polar lipids and unsaponifiable lipids were identified as phosphatidylcholine and squalene, respectively. Among the various extraction methods tried, a sequential extraction with ethanol, hexane and benzene was the most effective. By recrystallization of the yeast oil, a fraction that contained 33% of the original weight was obtained and the fraction was reported to have a melting range and glyceride structure similar to those of cocoa butter (Hammond et al., 1981).

Teasdale (1981) tried to improve the economics of A. curvatum fermentations. He confirmed that the ratio of carbon to nitrogen was a very important parameter for production of biomass and oil and for COD reduction in the fermentation process. Attempts to reduce the fermentation time by using high inoculum levels and condensed permeate were not successful. However, fermentations with both unheated and pasteurized (63°C, 30min) permeate were successful when the inoculum size was 10^6 cells/ml or greater, but unpasteurized medium gave lower biomass and oil yields. Permeate from Swiss, Cheddar, blue, cottage wheys and milk showed potential as substrates for the fermentation (Hammond et al., 1981).

Attempts were also made to alter the characteristics of the yeast by mutagenesis to improve the economics of the fermentation process (Baehman, 1983). Mutants of A. curvatum that could grow faster, at

lower pH, and at lower or higher temperature than usual were targets of the mutagenesis. Mutants were generated by exposure of cells to ultraviolet light; however, the mutants that were isolated were not superior in their performance, and mutants tended to revert to the wild type when they were cultured in broth medium (Glatz et al., 1984).

Potential uses of A. curvatum in treatment of other waste products from food and agricultural processing also were studied. Floetenmeyer (1983) tried various simple or complex carbohydrates as carbon sources for the fermentation. The yeast grew well on mono- and disaccharides such as glucose, galactose, xylose, mannose, fructose, ribose, maltose, cellobiose, sucrose and lactose but not on arabinose. Cellulose-rich waste material such as corn cobs, wood cellulose and oat hulls showed limited growth and no lipid accumulation. Good yield of biomass but poor lipid accumulation were observed when blanch water from bean processing and soluble starch were used as carbon sources. Ripe bananas were good substrates for both cell growth and lipid accumulation (Floetenmeyer, 1983; Glatz et al., 1984).

Generally continuous culture systems are considered more commercially attractive. Floetenmeyer et al. (1985) also proved that continuous culture fermentation was much more effective in lipid accumulation than was a batch system when heat-sterilized whey permeate was used as growth medium. In a system with a 350-ml working volume, the optimum dilution rate was 0.05 hr^{-1}, at which rate 0.306 g/L/hr of lipid was produced. This rate can be compared with 0.095 g/L/hr as the best for a batch system. The yield of biomass was 0.665 g/L/hr

(Floetenmeyer, 1985).

In 1983, A. curvatum was first reported to have moderate exolipase activity and to be able to grow on corn oil as sole carbon source in shake flasks. This result was observed during searches for oleaginous yeasts that were able to modify fats and oils by fermentation. These results showed that the yeast could grow on fatty acids as substrates (Bati, 1983). Kinetic aspects of the fermentation with A. curvatum were also investigated (Brown, 1984). Kinetic studies of cell growth and lipid accumulation provide useful information in optimizing process conditions and in assessing the potential of the process. A model was devised for both batch and continuous fermentation in a 7-L or 14-L fermenter, and the model described some phenomena of the fermentation satisfactorily. From the model it also could be concluded that residence time of a two-stage continuous fermentation system would not be significantly shorter than that of a single-stage system (Brown, 1984; Glatz et al., 1984).

In 1985, mutagenesis of A. curvatum was again attempted (Li, 1985). Efforts were focused on isolation of mutants with higher temperature tolerance that were expected to shorten the fermentation time and to produce oil with a more desirable composition. Mutagenesis was done by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), raising the selection temperature 0.2°C at a time. A mutant was obtained that could grow at 37.5°C in shake flasks with nitrogen-limited medium (150mg/L) and was stable. However, the lipid yield of the mutant at the elevated temperature was not better than that of the wild type at 30°C. Also the

fatty acid profiles of the lipids from the two fermentations were not significantly different. Growth of A. curvatum on corn oil as a carbon source was reported in one of the tests for biomodification of oils with the yeast. This resulted in a lipid yield of 7.29 g/L; dry cell weight, 11.17 g/L; % lipid, 65.3. Fatty acid composition of the yeast oil was similar to that of substrate corn oil; however, the oil from the harvested cell was not completely free of substrate oil.

As a previous study had indicated, ripe bananas were a good substrate for lipid production by A. curvatum (Floetenmeyer, 1983), More extensive studies were performed to optimize conditions for the fermentation of ripe banana juice (Vega, 1987). In this study with a 500-ml working volume fermentor, optimum conditions for growth and oil accumulation were: temperature, 30°C; efficient aeration (but not with pure oxygen); banana juice concentration, 20%; heat treatment of the juice (less than sterilization); asparagine, 1.4 g/L; mineral salts (mg/L: $MgSO_4.7H_2O$, 1000; $Fecl_3.6H_2O$, 20; $CaCl_2.2H_2O$, 200; $ZnSO_4.7H_2O$, 1; $MnSO_4.H_2O$, 2;NaCl, 60; and $CuSO_4.7H_2O$, 0.1). A fed-batch fermentation in which sterile banana juice was added as the culture density increased, yielded cell dry weight three times greater than in batch fermentation. However the cellular lipid content was low (30%) and utilization of COD was poor (32% utilized in 82 hr) (Vega et al., 1988).

More detailed physiological studies of A. curvatum were performed in a chemically defined medium by Park (Park, 1989; Park et al., 1990). The yeasts that were grown at various C/N ratios varied in growth, lipid accumulation and cell composition. Park reported that the yeast could

utilize not only endogenous lipid but also endogenous carbohydrate as carbon and energy sources when it was starved for carbon, and that intracellular carbohydrate was an intermediate for both lipid accumulation and lipid turnover. Strong induction of catalase activity was reported when the yeast was manipulated to utilize endo- and exogenous lipid, which implied the importance of peroxisomal beta-oxidation and the glyoxylate cycle in lipid degradation. Proliferation of peroxisomes also was demonstrated by electron microscopy. Purified peroxisomes but not mitochondria from A. curvatum showed high activity of several key enzymes of beta-oxidation and the glyoxylate cycle. Park suggested an essential role of peroxisomes in lipid metabolism in the oleaginous yeast A. curvatum.

At the University of Hull, England

In 1981, Ratledge and his associates began studies on A. curvatum. This group had already been investigating microbial production of lipid and biochemical explanations of lipid synthesis in other oleaginous microorganisms (Whitworth and Ratledge, 1975; Botham and Ratledge, 1978; Botham and Ratledge, 1979; Ratledge, 1982). A. curvatum provided an excellent model for correlating lipid accumulation with the activities of various enzymes envolved in lipid biosynthesis. Along with A. curvatum, Rhodosporidium toruloides, Hansenula saturnus, Trichosporon cutaneum, Candida 107, Rhodotorula graminis, Rhodotorula glutinis, Lipomyces starkeyi, Lipomyces lipofer, also were studied, and compared with nonoleaginous yeasts such as Candida utilis, Saccharomyces

cerevisiae and Saccharomyces uvarum. Their research in this area was discussed earlier in this literature review. The discussion here will be restricted to papers which dealt with A. curvatum.

In 1983, Evans and Ratledge anticipated the likely commercialization of the yeast and performed fermentation studies with A. curvatum. Various carbon sources such as glucose, sucrose, lactose, xylose and ethanol were tested for efficiency of fat production in batch and continuous systems. Ethanol was reported as the least efficient substrate. On the other hand, xylose and lactose were the best substrates for lipid accumulation in batch and continous modes, respectively. The optimal dilution rate was also determined for each substrate. The fatty acid profile of the yeast varied with substrate and whether the fermentation was batch or continuous. Some of the fermentation results are summarized in Table 2.

The potential utilization of food waste products by this organism prompted more detailed biochemical studies (Evans and Ratledge, 1983b). Examination of the various enzymes for lipid biosynthesis in A. curvatum gave results that generally corresponded with those of other oleaginous yeasts. Citrate was found in the culture medium when nitrogen became limiting.

The physiological function of the accumulated lipid in oleaginous yeasts was not clear, and the Ratledge group also addressed the question of possible utilization of endogenous lipid. In a 1979 paper, Botham and Ratledge reported that lipid turnover did not occur or occurred at an immeasurably slow rate in a steady-state culture of the oleaginous

Table 2. Comparison of growth and lipid accumulation of A. curvatum produced under various cultural conditions

Culture Mode	Carbon Source	C/N	Dilution Rate (hr^{-1})	DCW (g/L)	%lipid	Y[a] (g/L/hr)
Batch[b]	Whey permeate	-	-	26.8[c]	57	0.217
Continuous[d]	Whey permeate	-	0.05	13.1	45	0.306
Batch[e]	Lactose	-	-	12.5	39.2	0.054
Continuous[e]	Lactose	-	0.04	18.0[f]	31	0.220
Continuous[g]	Whey permeate	43	0.04	24.8	37.7	0.374
Batch[h]	Banana juice	-	-	11.6[i]	71.2	0.088
Batch[j]	Whey permeate	40	-	21.6[k]	36	0.199
Continuous[j]	Whey permeate	20	0.07	21.0	20	0.294
Continuous[j]	Whey permeate	40	0.053	20.0	36	0.382
Fed-batch[l]	Whey permeate	40	-	85.0[m]	35	0.372
Partial[n] recycling	Whey permeate	40	0.033	91.4	33	0.995

[a]Lipid yield.
[b]Moon et al., 1978.
[c]Culture for 72 hr.
[d]Floetenmeyer et al., 1985.
[e]Evans and Ratledge, 1983.
[f]Culture for 90 hr.
[g]Davies, 1988.
[h]Vega et al., 1988.
[i]Culture for 96 hr.
[j]Ykema et al., 1988.
[k]Culture for 39 hr.
[l]Ykema et al., 1988; A recycle unit was attached to a batch culture after all lactose was consumed (total feed flow rate = 0.15 L/hr).
[m]Culture for 70 hr.
[n]Ykema et al., 1988; A recycle unit was attached to a batch culture after all lactose was consumed (total feed flow rate = 0.15 L/hr); when a dry cell weight of about 90 g/L was reached an extra pump withdrawing culture (bleed flow rate = 0.033 L/hr) was added.

yeast Candida 107 on a nitrogen-limited medium at a dilution rate of 0.05 h. In a 1988 paper, Holdsworth and Ratledge reported that most oleaginous yeasts were able to convert their accumulated lipid to new biomass if they then were starved for carbon. A. curvatum produced 1.9 ± 0.2 g of new biomass per g lipid utilized. In contrast, Lipomyces starkeyi could not use accumulated lipid to produce new biomass. The onset of the lipid turnover seemed to occur immediately after carbon was removed, and in the early stages of turnover, rapidly metabolizable nonlipid materials were detected.

Further studies followed to understand the control of lipid turnover. These studies involved examining the activities of six key enzymes of lipid metabolism (Holdsworth et al., 1988). Two important enzymes for lipid biosynthesis, ATP:citrate lyase and malic enzyme, showed diminished activity during lipid utilization. Two peroxisomal enzymes, carnitine acetyl transferase and isocitrate lyase showed considerable increase in activity during lipid utilization; however, $NADP^+$-dependent isocitrate dehydrogenase increased slightly. The activity of catalase varied among yeast species. Proliferation of peroxisomes was demonstrated in the cells that utilized endogenous lipid. Also, they observed greater changes of enzyme activities in yeasts utilizing exogenous lipid than in those utilizing endogenous lipid.

Holdsworth and Ratledge (1991) also were involved in the biochemical study of triacylglycerol biosynthesis in the oleaginous yeast A. curvatum. Review of this work will be found in a later section

(page 32).

Vrije Universiteit, The Netherlands

This Dutch group became involved in studying lipid production by A. curvatum in 1986. They noticed that the carbon to nitrogen ratio (C/N ratio) of the growth medium greatly affected the yield coefficient and fermentor productivity, parameters that are vital to the economics of microbial oil production. They constructed a mathematical model to describe the influence of C/N ratio on lipid production with oleaginous yeasts, and the model was tested by continuous fermentation of A. curvatum in a fermentor of 1-L working volume that contained a semi-defined medium with glucose as the carbon source. They concluded that their model applied well to the experimental results (Ykema et al., 1986).

This group also studied the optimization of lipid production by A. curvatum on whey permeate as a substrate. Four different culture modes were tested: batch, fed-batch, continuous and partial culture recycling. Various C/N ratios were adjusted by adding glucose or ammonium chloride, and each fermentation mode was optimized. The optimum C/N ratio was reported to be 30-35 for all the culture systems. When cell-recycling systems were applied in the fed-batch and partial recycling modes, both attained high cell densities. A high lipid production rate (0.995 g/L/h) was observed in the partial recycling mode. They concluded that culture modes capable of attaining high cell densities were desirable (Ykema et al., 1988). Their results, together with ones from the other

research groups, are summarized in Table 2 on page 20. However, low specific growth rate (μ) often is encountered in situations with high cell densities; therefore, the group studied growth and lipid accumulation at low μ values, ranging from 0.15 hr^{-1} to 0.004 hr^{-1}. Maintenance coefficients of A. curvatum (1.3-21.8 μmole glucose/g biomass/hr) were lower than those for other fungi such as Sacharomyces cerevisiae. They observed no lipid production at growth rates below 0.02 h^{-1}, and stressed this value as an important parameter to consider in processes with high cell density such as fed batch systems (Ykema et al.,1989).

The group also studied ways to improve the quality of the lipid produced by A. curvatum. In an attempt to make a cocoa butter substitute from A. curvatum, they isolated unsaturated fatty acid (Ufa) mutants that could not convert stearic acid to oleic acid. They suggest this sort of mutant because the lipid of wild-type A. curvatum has higher oleic acid and lower stearic acid contents than cocoa butter. The mutants were obtained by treatment with MNNG, and selected as oleic acid auxotrophs. Some of the mutants were reported to be stable over at least 50 generations. The mutants required oleic acid in their medium, and the concentration of oleic acid affected the growth, lipid production and fatty acid composition of the yeast oil. Comparison of growth and lipid accumulation with wild type was not done. However, the oil from the mutants was reported to have a percentage of saturated fatty acids similar to that of cocoa butter. Table 3 summarizes fatty acid profiles of cocoa butter and yeast lipids produced under various

Table 3. Fatty acid compositions of various yeast lipids resembling cocoa butter

Yeast Strains (Treatments)	Fatty Acids						%SFA[a]
	16:0	16:1	18:0	18:1	18:2	18:3	
Cocoa butter	25.8	0.3	34.5	35.3	2.9	-	60.3
A. curvatum, wild-type[b]	30.4	0.8	11.4	51.0	6.2	0.4	41.8
A. curvatum, wild-type[b] (fractionation)	35.3	-	17.0	43.6	4.2	0.5	52.3
cA. curvatum, Ufa33[c] (oleic acid, 1 g/L)	22.0	1.9	36.7	24.8	8.4	1.8	58.7[d]
A. curvatum, F33[e] (hybrid by spheroplast fusion)	25.9	-	20.9	36.6	7.1	-	53.3[f]
A. curvatum, R25.75[g]	28.8	-	29.5	28.4	7.7	0.9	62.7[h]
Candida 107[i] (Sterculia oil, 0.8 ml/L)	29.8	<1.0	28.0	11.1	22.7	<1.0	57.8
T. cuteneum[j] (Sterculia oil, 1.2 ml/L)	35.3	<1.0	22.9	24.1	13.5	1.7	58.2

[a]Percentage saturated fatty acids (% w/w).
[b]Hammond et al., 1981.
[c]Mutant with defective desaturase; Ykema et al., 1990.
[d]Contains 1.8% C24:0.
[e]Mutant with partially blocked desaturase; Verwoert et al., 1989.
[f]Contains 3.3% C24:0.
[g]Revertant of an Ufa mutant; Ykema et al., 1990.
[h]Contains 2.1% C24:0.
[i]Moreton, 1985; Sterculia oil contained 49.8% sterculic and 4.8% malvalic acids which have a cyclopropene group which inhibit desaturation of stearic acid.
[j]Trichosporon cutaneum; Moreton, 1985.

conditions (Ykema et al., 1989b).

The Ufa mutants needed at least 0.6 g/L of relatively expensive oleic acid in the growth medium for appropriate growth and lipid accumulation. Therefore, they attempted to isolate strains with partially blocked desaturase activity, that could grow without oleic acid supplement in the medium. Spheroplast fusion between methionine auxotrophic mutants and Ufa mutants were reported to be successful. The stability of the hybrids was not mentioned, but the hybrids could grow on whey permeate and most of them showed comparable growth and lipid accumulation to the wild type (Verwoert et al., 1989). Fatty acid profiles of the lipids from some hybrids are shown in Table 3. Other attempts to get strains with partially blocked desaturase activity were based on selecting revertants of Ufa mutants able to grow without oleic acid supplementation. Fatty acid compositions of lipids from the revertants before and after growth for 50 generations were used as a criterion of stability of the revertants. Lipids from the revertants ranged from 27 to 86% in their percentage of saturated fatty acid (%SFA). Two of the revertants yielded lipids that had a %SFA and melting point similar to cocoa butter except for the existence of a minor fraction with a higher melting temperature (Table 3). The revertants grew well on whey permeate, but their growth rate was slower than the wild type (Ykema et al., 1990).

Other groups

As mentioned previously, the Department of Scientific and Industrial Research in New Zealand has also been working on A. curvatum. They studied the scale-up of downstream processes as well as the fermentation. Detailed data for commercial-scale production of the yeast oil were provided from their pilot-scale studies (Davies, 1988; Davies, 1991).

A Polish group at Agricultural and Technical University of Olsztyn also has been involved in the study of oil production with the yeast A. curvatum. Beet molasses was tested as the substrate for the culture in shake flasks, and optimal physical conditions were determined: pH, 5.4; temperature, 30°C. Under these condition, beet molasses had a lower lipid yield coefficient than synthetic medium in which the sucrose level was adjusted to be the same as molasses. When a mixture of beet molasses-whey (5:95 w/w) was used in the medium, the yield coefficient was high (0.225 g lipid/g sugar), and the cell mass and lipid content were 29.5 g/L and 40.2%, respectively (Bednarski et al., 1986). Deproteinized whey, obtained by thermal and acid coagulation of the whey, was also tested as a substrate, and it gave lower yield and lipid content (0.088 g/L/hr and 29.3%, respectively) than those reported by Moon et al (1978); however, fatty acid compositions were similar to those previously reported (Leman et al., 1987). Further study showed that the proportion of lipid fractions and fatty acid composition of the lipid varied with culture temperature, pH and medium substrates (Leman et al., 1990).

Studies on triacylglycerol assembly in oleaginous yeasts

In the early 1950s sn-glycerol-3-phosphate and phosphatidic acids were found to be important intermediates for the synthesis of glycerolipid in animal liver, and thioesters of long-chain fatty acids were shown to be involved in the process (Kennedy, 1953; Kornberg and Pricer, 1953a,b). Since then, numerous studies have been performed to understand the pathway and regulation of triacylglycerol biosynthesis, mostly in plant and animal cells. The glycerol-3-phosphate pathway to triglycerides (Kennedy pathway) was originally established with animal tissues (Kennedy, 1961) and then with plant tissues (Barron and Stumpf, 1962). The nonoleaginous yeasts Saccharomyces cerevisiae and Candida tropicalis were the only yeasts studied for triacylglycerol assembly, a process of conversion of fatty acid acyl CoA esters into triacylglycerol (Holdsworth and Ratledge, 1991). The pathways in yeasts appeared similar to those in animal and plant cells, and are shown in Figure 2.

In S. cerevisiae, the enzymes responsible for each reaction have been identified, purified and characterized (Hosaka and Yamashita, 1984a,b). From the study correlating enzyme activities to accumulation of triacylglycerol, Hosaka and Yamashita (1984b) reported that phosphatidate phosphatase, which catalyzed the formation of diacylglycerol, controlled the rate of triacylglycerol assembly in the yeast S. cerevisiae. They also reported that the biosynthesis of individual fatty acid did not regulate the rate of triacylglycerol assembly.

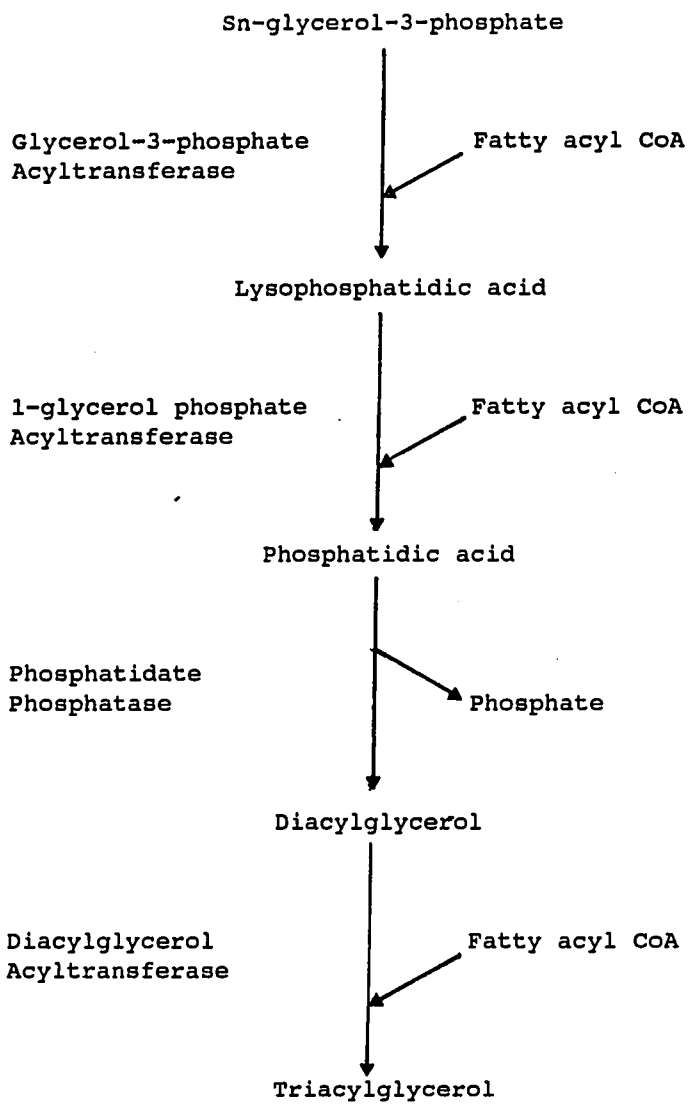

Figure 2. Major pathways of triacyl glycerol biosynthesis in plants (Harwood and Russell, 1984)

Especially in oleaginous yeast, triacylglycerol assembly is an important process because glyceride structure, a major factor for oil quality, is determined in this step. However, triacylglycerol assembly in oleaginous yeast has not been studied much. Studies on the modification of lipids by microorganisms indicated that the microorganisms not only utilized the substrate lipid in catabolic processes but also incorporated the substrate's carbon skeleton directly into triacylglycerol reserves (Bati, 1983; Li, 1983; Koritala et al., 1987). Bati et al. (1984) showed that the fatty acid composition of the yeast oil from C. lipolytica was similar to that of the substrate corn oil, but there were significant changes in the distribution of the fatty acids. Noguchi et al. (1982) used various esters of different fatty acids as carbon sources for the culture of several species of Rhodotorula and Candida to study the regulation of yeast lipid formation. They demonstrated that the fatty acid composition of the yeast oil varied greatly with the esters presented in the substrate oil. Also, they observed a difference between yeast lipids obtained from ethyl stearate and butyl stearate as substrates (Table 4). They did not study the fatty acid distribution on the glycerol in the yeast oil.

Because alkane is converted to fatty acids before being utilized in the cells of some microorganisms, it has also been used as a useful substrate to study the metabolic and physiological features of some oleaginous yeasts such as C. lipolytica (Fukui and Tanaka, 1981; Tanaka et al., 1982; Fukui, 1988). Recently, Efremenko et al. (1990) reported their study on the regulation of lipid in the yeast Candida rugosa with

Table 4. Effect of substrate esters on growth, lipid and fatty acid composition of lipid from Candida I-31[a] (Noguchi et al., 1982)

Carbon source	DCW[b] (g/L)	LC[c] (%)	Fatty acid composition								
			10:0	12:0	14:0	14:1	16:0	16:1	18:0	18:1	18:2
Ethyl caprate	7.7	20.8	0.6		0.3		21.1	5.0	13.8	52.8	5.6
Ethyl laurate	14.2	33.1	0.2	20.4	2.2	5.2	15.7	5.5	11.0	41.4	3.2
Ethyl myristate	16.4	32.9			35.8		15.7	10.3	4.1	25.6	2.6
Methyl palmitate	17.8	41.0					62.1	16.6	3.5	14.5	2.0
Ethyl palmitate	18.2	46.2					60.1	16.6	3.0	16.1	2.2
Isopropyl palmitate	15.3	52.0					60.7	15.9	3.8	16.1	2.2
Ethyl stearate	16.7	44.3					15.6	0.7	43.5	33.5	5.1
N-butyl stearate	8.8	42.0					41.7	8.1	19.3	23.8	2.8
Ethyl oleate	16.1	53.6					5.8	7.0	3.3	75.7	6.1

[a]The culture was incubated for 5 d at 35°C (stearate esters), 30°C (other esters) in 500-ml flasks containing 80 ml of the culture medium in reciprocating shaker (145 oscills/min, 7cm stroke).

[b]Dry cell weight.

[c]Lipid content.

alkanes as carbon sources in the presence of cerulenin (2 mg/L) which inhibited de novo synthesis of fatty acid. Thus, the yeast lipid was necessarily determined by the substrates used. The yeast could survive on octadecane, eicosane and docosane as their only carbon source when their fatty acid synthesis was blocked by cerulenin (Table 5); however, the yeast could not grow on hexadecane even when glucose was added as a carbon source. They concluded that the yeast was not able to synthesize functional membranes from palmitic acid, the fatty acid corresponding to hexadecane. Because the fatty acid composition in yeast lipid and membrane lipid produced from various chain length alkanes was relatively constant, they suggested that the yeast had a mechanism to maintain the consistency of its membrane lipids, and that the mechanism was based on the substrate specificity of the enzymes controlling fatty acid metabolism. They believed that peroxisomes and mitochondria were involved in membrane lipid production. They demonstrated that entry of eicosanoic acid into mitochondria was considerably greater than that of stearic acid. However, the substrate specificities of the enzymes involved were not investigated. Obviously, the experiments of Efremenko et al. (1990) do not seem to be an appropriate system to study triacylglycerol assembly, because the yeast was harvested before the stage of rapid accumulation of yeast lipid, and the lipid was extracted with a chloroform-methanol mixture (1:2 w/w) which favored the extraction of polar lipids. However, there are interrelationships between the metabolic pathways of neutral lipids and phospholipids (Rattray et al., 1975; Harwood and Russell, 1984).

Table 5. Fatty acid composition of lipids from C. rugosa on various alkanes as carbon sources in or without the presence of cerulenin[a] (Efremenko et al., 1990)

Carbon source	Fatty acid composition							
	13:0	16:0	16:1	16:2	18:0	18:1	18:2	18:3
C_{18}		17.8	3.8		10.4	49.0	19.0	tr[b]
C_{18} + cerulenin		18.4	3.6		8.9	45.0	24.1	tr
C_{20}	2.9	20.3	8.7	tr	7.1	39.2	20.9	tr
C_{20} + cerulenin	tr	19.5	7.5	4.6	4.0	25.3	31.8	5.2
C_{22}	tr	23.1	10.1	6.1	1.9	43.9	14.3	tr
C_{22} + cerulenin	tr	20.2	8.6	5.8	3.0	36.7	19.1	tr

[a]The culture was grown to the middle of the exponential phase in Erlenneyer flasks (alkane, 10 g/L).

[b]Trace.

A paper on triacylglycerol synthesis in the oleaginous yeast A. curvatum was published recently by Holdsworth and Ratledge (1991). From a study with cell-free extracts and spheroplasts of the yeast grown on nitrogen-limited medium, they reported much lower activity of the fatty acyl CoA synthetase with stearate than with palmitate, oleate and linoleate. Also stearate and stearyl CoA were poorly incorporated into lipids. Another oleaginous yeast, Lipomyces starkeyi, did not show such a difference in the activity of its fatty acyl CoA synthetase. As is the case in S. cerevisiae, the rate-limiting step of lipid biosynthesis

in A. curvatum was reported to be phosphatidic acid phosphatase, according to this study.

Identification of the site of lipid synthesis in A. curvatum also was investigated. Christiansen (1978) had isolated lipid particles from a homogenate of S. cerevisiae, and reported that the lipid particles were associated with triacylglycerol-synthesizing enzymes. From the study with subcellular fractionation of spheroplasts of A. curvatum, Holdsworth and Ratledge (1991) reported that the activity of fatty acyl CoA synthetase appeared mainly in the lipid bodies, and the activity for phosphatidic acid formation was in the mitochondria. For the restoration of triacylglycerol synthesizing activity of the cell, all the fractions had to be recombined.

PART I.

TRIACYLGLYCEROL ASSEMBLY FROM LIPID SUBSTRATES BY *APIOTRICHUM CURVATUM* ATCC 20509

ABSTRACT

The oleaginous yeast Apiotrichum curvatum was grown on various lipids as carbon sources. When commercial animal and vegetable oils were used as substrates, the yeast triglyceride had a fatty acid profile similar to that of the substrate oil, but the glyceride structures were altered. Saturated free fatty acids with less than 14 carbons could not be used as carbon sources by the yeast. An emulsion of palmitic acid was well utilized by the yeast, but it was extensively desaturated before being deposited in the yeast triglyceride. Stearic and arachidic acid emulsions gave very limited and no growth, respectively. Oleic acid supported very good yeast growth; eicosenoic acid supported limited growth; erucic acid gave very poor growth. The yeast grew on petroselinic acid and deposited it extensively in its triglyceride. When supplemented with 1000 ppm butylated hydroxyanisole, linoleic and linolenic acids showed excellent growth and lipid accumulation. The yeast deposited ricinoleic acid, eleostearic and vernolic acids in its triglyceride when the yeast was grown on triglycerides containing these fatty acids, but the yeast triglyceride contained less of these conjugated and oxygenated acids than were found in the substrate oils. When crambe oil was used as a carbon source for the yeast, fatty acids with 20 or more carbons were concentrated in the residual substrate oil while those with 16 and 18 carbons were almost completely utilized. When cholesterol was incorporated in the growth medium, the yeast incorporated very little of it into its depot fats.

INTRODUCTION

Production of single-cell oil is marginal economically (1, 2), and most studies of such processes have focused on using inexpensive substrates such as wastes or by-products from the food industry (3, 4, 5, 6) or production of high-priced lipid products (7, 8, 9, 10, 11, 12).

The prices of fats and oils hinge on their chemical, nutritional and physical properties, and these are determined by their fatty acid composition and glyceride distribution. The fatty acid composition of microbial oils can be varied to some extent by manipulating growth temperature, pH, culture time, and medium composition (4, 13, 14). More extensive modification of the fatty acid composition has been achieved with an inhibitor of acyl desaturase (15), by addition of fatty acids to the growth medium (8, 16) and by selection of mutants with altered ability to synthesize fatty acids (7, 16, 17).

When grown on sugar substrates, Apiotrichum curvatum accumulates triglycerides with an acyl composition typical of vegetable oils (1). The glyceride structure also is like a typical vegetable oil with saturated acyl groups confined to the sn-1 and sn-3 positions. Because A. curvatum will use fatty acids and triglycerides as substrates, this yeast provides a simple and convenient means of exploring triglycerides assembly. The insights gained from such studies may be applicable to oilseed plants for which such studies are more difficult.

There is considerable interest in transferring the ability to make unusual fatty acids of economic importance into domesticated oilseed

crops (18, 19, 20). Oils that contain fatty acids that have industrial uses, such as eleostearic, ricinoleic or vernolic acids, command a price significantly higher than typical edible oils. The transfer of the ability to make such fatty acids to an oleaginous yeast also might provide an economically viable fermentation process. The ability of A. curvatum to assimilate fatty acids from its medium provides a convenient method of testing the ability of an oleaginous yeast to tolerate and metabolize such exotic fatty acids, and the insights that are gained may also be applicable to oilseed plants as well.

We have observed the ability of A. curvatum to alter and incorporate into its triglycerides various lipid substrates, and the stereospecific distribution of the acyl groups in the resulting triglycerides has been determined. These studies have included a number of fatty acids not normally produced by A. curvatum.

MATERIALS AND METHODS

A. curvatum was maintained on refrigerated slant cultures of yeast extract/dextrose/peptone/agar (1/2/2/1.5 %w/w), transferred monthly (21). The basal medium (Table 1) was adjusted to pH 5.5 and supplemented with 18 g/L of substrate lipid.

Pure fatty acids and castor oil were purchased from Sigma Chemical Co. (St. Louis, MO). Soybean oil, corn oil, lard (fortified with butylated hydroxyanisole, propyl gallate and citric acid) and tung oil were purchased from local stores. Crude pressed crambe oil was provided by the Center for Crops Utilization Research at Iowa State University. Vernonia anthelmintica seed oil was extracted after deactivation of lipase of the seeds according to the method by Ayorinde et al. (22). In the experiments with combinations of lactose and corn oil as carbon sources, the amount of oil in the medium was reduced 1.8 g for every 4 g of lactose added.

A seed culture was prepared by inoculating about 2% of the yeast washed from a slant culture into 100 ml of heat-sterilized basal medium with corn oil as a carbon source. The culture was grown in 250-ml flasks in a Labline orbital shaker (Melrose Park, IL) at 32°C and 180 rpm. The seed culture was in logarithmic growth after about 2 days, and its optical density (OD) at 440 nm was normally 9 to 10. About 1 ml of seed culture was used as an inoculum for 100 ml of medium containing the substrate lipid to be tested. The test cultures were grown for 7 days under the same conditions used for the seed culture.

Leftover substrate was separated from the culture in a separatory

Table 1. The composition of the basal medium

	g/L		mg/L
KH_2PO_4	2.5	NaCl	60
$MgSO_4.7H_2O$	1.0	$FeCl_3.6H_2O$	20
Asparagine	0.8	$MnSO_4.H_2O$	2
$CaCl_2.2H_2O$	0.2	$ZnSO_4.7H_2O$	1
		Thiamine-HCl	1
		$CuSO_4.5H_2O$	0.1

funnel if the substrate was liquid at room temperature. Oil extraction from the yeast was according to Hammond et al. (23), which involved extraction in sequence with ethanol, hexane and benzene. Fatty acids having melting points above 32°C were emulsified into the medium with 5 g/L gum acacia (8). After heat-sterilization of the mixture of gum acacia, fatty acid and basal medium in a blender jar, emulsion was made in situ by blending at high speed for 1 min and transferred aseptically into the culture flasks. When emulsified substrates were used, agitation was decreased to 120 rpm to minimize destabilization of the emulsion. The yeast cell mass could not be separated completely from the emulsified fatty acid by centrifugation, so after removal of as much

of the cell mass as possible by centrifugation, the supernatant was evaporated in a rotary vacuum evaporator, and the residue was pooled with the cell mass recovered by centrifugation. Extraction of lipid in this residue was accomplished as before.

Dry weight of the cell residue (DCW) after lipid extraction was determined by drying the residue at 110°C for 2 hr and weighing it. The amount of triglyceride in yeast oil was determined by thin-layer chromatography (TLC). Aliquots of the ethanol extract and pooled hexane and benzene extracts were applied to TLC plates 1.0 mm thick. The plates were developed in hexane/ether/acetic acid (85/15/1, v/v/v), and bands were visualized by spraying with 0.2% dichlorofluorescein in ethanol and viewing under ultraviolet light. Triglycerides were eluted from the plates with three 20-ml portions of diethyl ether, and the residue was weighed after evaporation of the ether under nitrogen. Stereospecific analysis was done according to Fatemi and Hammond (24). For fatty acid analyses, glycerides were transesterified by the method of Frey and Hammond (25), and the methyl esters were separated on a Varian Model 3700 Gas Chromatograph equipped with a 1.8 M x 3.3 mm column of 10% SP-2330 on Chromosorb WAW and a flame ionization detector (FID). Verification of oleic, petroselinic and cis-vaccenic acid was done isothermally at 190°C on a Hewlett Packard Model 5890 Gas Chromatograph equipped with FID and a fused silica column (15 M x 0.254 mm) with 0.25 μ of DB-23 as a liquid phase (J & W Scientific, Folsom, CA).

For cholesterol analysis, 0.5 to 1 g of lipid was refluxed with 1.5

ml of 60% w/w aqueous potassium hydroxide and 25 ml of ethanol for 30 min. The reaction mixture was extracted with four 50-ml portions of diethyl ether, and the ether extracts were combined and washed three times with 25-ml portions of water. The ether layer was washed twice with a sequence of 20 ml of 0.5 N aqueous potassium hydroxide and 25 ml of water. Additional washing with water was continued until the washings did not change the color of 1% phenolphthalein indicator to pink. The washed ether layer was evaporated under nitrogen in a rotary vacuum evaporator, and the residue was transferred to a vial with several portions of chloroform. The solvent was again evaporated to dryness under nitrogen; then, 1 ml of internal standard solution (400 ppm of 5-α-cholestane in ethyl acetate) was added to the vial. The ethyl acetate solution was injected into a Varian Model 3700 Gas Chromatograph equipped with a 30 m x 0.32 mm capillary column SPB-1 (Supelco, Bellefonte, PA), a direct capillary injector and a flame ionization detector. The concentration of cholesterol was calculated from the peak areas. Yeast was grown on a medium containing 18 g/L of oleic acid spiked with 2234 ppm of cholesterol.

All reported results are the average of two replicate measurements except those of stereospecific anylyses, which represent a single measurement. However, the errors for stereospecific analyses were less than 7% in determining the whole, sn-1 and sn-2 compositions.

RESULTS AND DISCUSSION

Modification of oils by A. curvatum

Table 2 shows that triglycerides isolated from A. curvatum grown on corn oil had a fatty acid composition almost identical with that of corn oil. However, significant changes in the glyceride structure were observed. These results are similar to those obtained by Bati et al. (26) with Candida lipolytica. The yeast shows much less tendency to place linoleyl groups on the sn-2 position compared with corn oil. The yeast favored placing oleyl groups on sn-2 to a greater extent than is found in corn oil. Palmityl groups were placed on sn-1 more readily than on sn-3 by the yeast.

Similar trends were observed with lard as a substrate, but the yeast oil contained fewer saturated and more unsaturated acyl groups than the substrate fat. The elevated concentration of palmityl groups on the sn-2 position of lard had been redistributed to the sn-1 and -3 positions by the yeast, but sn-1 was favored over sn-3. Oleyl groups are concentrated on the sn-2 position of the yeast oil, and polyunsaturated acyl groups are favored on sn-3. Seemingly, the yeast hydrolyzes and absorbs the medium oil and redistributes the fatty acids according to the specificity of its enzymes for triglyceride biosynthesis. Inasmuch as the yeast triglyceride serves as an energy reserve (21), it is advantageous for the yeast to deposit the medium fat or oil with minimal change in its fatty acid composition. The fatty acid composition of the triglycerides of yeast that were grown on

Table 2. Stereospecific analysis of yeast oil triglyceride compared with its substrate oil

	16:0	18:0	18:1	18:2	18:3
On corn oil					
Substrate	11.4	2.1	26.7	58.8	1.1
sn-1	21.3	2.9	23.6	50.8	1.5
sn-2	1.3	0.3	26.7	70.6	1.0
sn-3	11.6	3.1	29.8	55.0	0.8
Yeast oil	8.8	2.8	27.3	60.1	1.0
sn-1	17.1	2.6	18.6	61.0	0.7
sn-2	0.4	0.2	41.3	57.8	0.4
sn-3	8.9	5.6	22.0	61.5	1.9

	14:0	16:0	16:1	17:0	17:1	18:0	18:1	18:2	18:3	20:0
On lard										
Substrate	1.3	24.6	2.8	0.5	0.4	14.8	42.8	11.2	0.6	1.0
sn-1	0.8	12.8	2.6	0.7	0.3	24.0	46.0	10.5	0.9	1.5
sn-2	3.6	66.6	5.4	-	0.5	3.3	14.9	5.6	-	-
sn-3	*	*	0.4	0.8	0.4	17.1	67.5	17.5	0.9	1.5
Yeast oil		18.5	2.3	-	-	7.1	53.6	18.0	0.5	-
sn-1		32.9	3.4	-	-	8.3	42.1	13.4	-	-
sn-2		3.2	0.7	-	-	0.5	81.2	14.1	0.3	-
sn-3		19.4	2.8	-	-	12.5	37.5	26.5	1.2	

*Negative value.

various amounts of both lactose and corn oil as carbon sources are shown in Figure 1. The more lactose there was in the medium, the more closely the fatty acid profile resembled that of yeast oil grown on lactose alone as the carbon source. The linearity of the response to corn oil and lactose suggests that A. curvatum can use these carbon sources equally well.

Growth of Yeast on Various Fatty Acids

Growth of the yeast on saturated short-chain fatty acids (C_4 to C_{14})

The yeast grew just as well on the free fatty acids isolated from vegetable oils as on the oils themselves, and yeast oils from these two substrates gave identical fatty acid profiles and glyceride structures. When the yeast was grown on free fatty acids and extracted sequentially with ethanol, hexane and benzene (23), the ethanol extract generally was rich in free fatty acids, with only traces of triglyceride. The hexane and benzene extracts contained primarily triglyceride with only traces of fatty acid, and generally, the benzene extract contained less free fatty acid than the hexane extract. A. curvatum grown on sugar contains almost no free fatty acid (13), so it was assumed that the free fatty acid recovered from the yeast was substrate and that the triglyceride was yeast oil. It was not possible to make this simplifying assumption when the yeast was grown on triglyceride, so yield data were not available when triglyceride substrates were used. However, it seems a safe assumption that the final benzene extract from such yeast represents yeast triglyceride that is essentially free of substrate

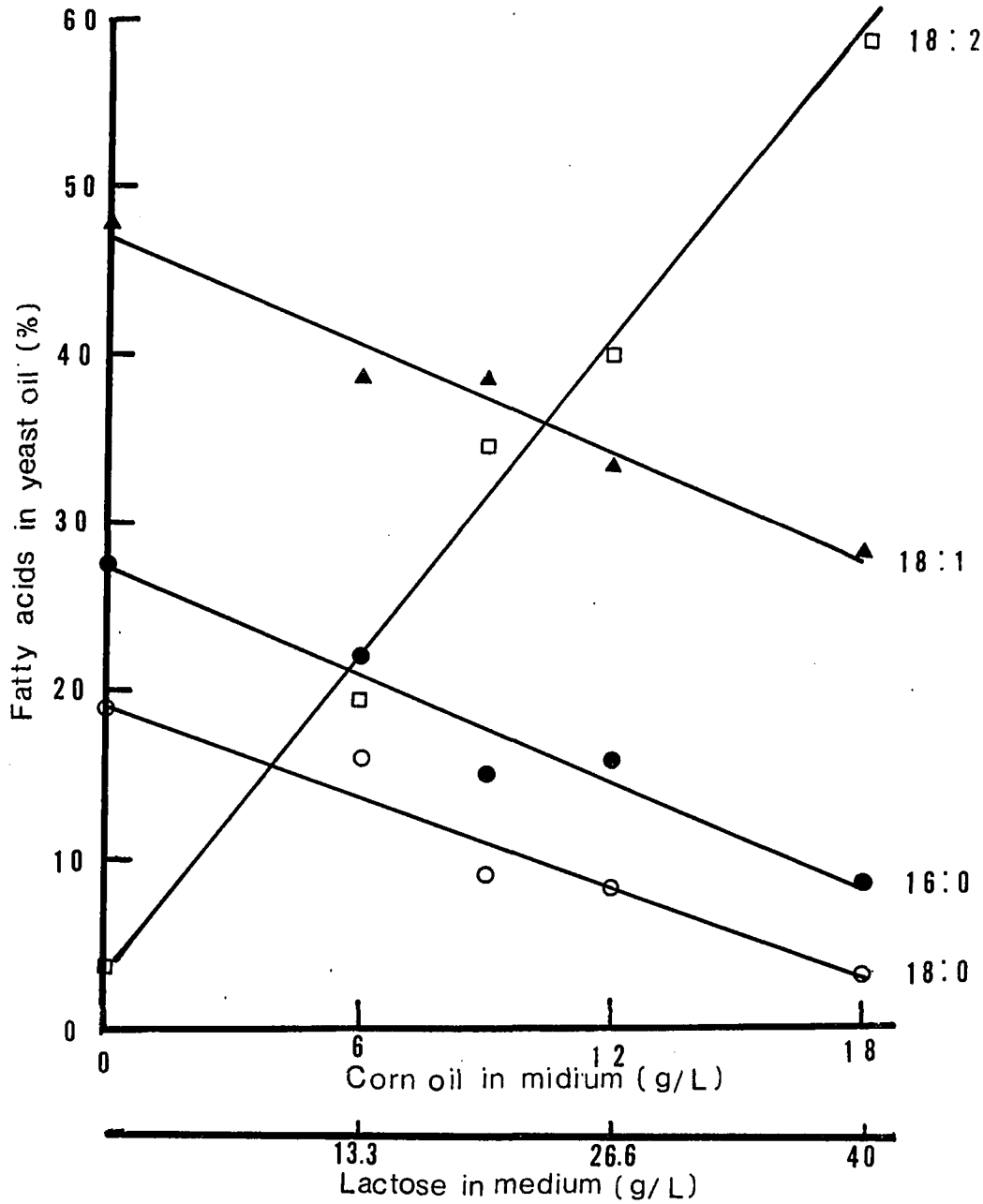

Figure 1. Fatty acid compositions of yeast triglycerides from A. curvatum grown on various amounts of both lactose and corn oil as carbon sources. The medium with 0 g/L of oil contained 40 g/L of lactose, and the lactose was decreased 13.3 g/L for each 6 g/L of corn oil added

contamination.

Short-chain fatty acids, which have melting points lower than the incubation temperature (32°C), could be added to the medium directly. None supported the growth of the yeast, but when glycerol tributyrate and tricaprate were used as substrates, the yeast grew. The yeast oil recovered from the benzene extract of yeast grown on tributyrin had the same composition as yeast grown on sugar (Table 3). Seemingly, the yeast could not incorporate butyryl groups into its triglycerides but could use the butyric acid and/or glycerol in tributyrin as a carbon substrate. On the other hand, capric acid was detected in the yeast oil grown on tricaprin. Seemingly, on a triglyceride substrate, the yeast was able to incorporate capric acid into its triglycerides as well as elongate it and use caprate (or glycerol) as a carbon source.

The fatty acids longer than capric were solids at the growth temperature of the yeast (32°C), so they were emulsified with gum acacia to test their ability to support yeast growth. There was no sign of growth or fat production by the yeast on gum acacia alone. Lauric acid, like the shorter-chain fatty acids, did not support growth of the yeast, but growth was observed with myristic acid as a carbon source. On myristic acid, the cell mass and amount of yeast oil recovered were quite small, but the yeast triglyceride was more than 90% myristic acid. Because the melting point of alpha-form trimyristin is 33°C (27), the yeast oil must be very near its melting point at the culture temperature.

Table 3. Fatty acid composition of triglycerides from A. curvatum grown on lactose, glycerol, tributyrin and tricaprin as carbon sources

	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2
Lactose			0.8	28.6	0.8	19.6	45.4	4.7
Glycerol			0.9	30.7	-	20.9	43.0	4.5
Tributyrin			0.8	31.1	1.3	15.5	47.5	3.9
Tricaprin	15.3	3.8	3.3	21.1	2.2	8.7	38.5	7.0

Growth of the yeast on long-chain saturated fatty acids

Table 4 shows the fatty acid composition and stereospecific analysis of the triglyceride from yeast grown on palmitic acid for 3.5 and 7 days. The yeast grew well on palmitic acid and produced 7.6 g/L of fat, which is comparable to that produced on oleic acid. As the culture aged, the yeast converted more of the palmitic acid to oleic acid. Considerable proportions of the palmitic acid are converted to palmitoleic, oleic and linoleic acids, presumably by desaturation and elongation. The octadecenoic acid was verified as oleic rather than cis-vaccenic, which would be produced by elongation of palmitoleic acid, by its retention time in the gas chromatograph. This suggests that desaturation followed elongation to 18 carbons.

Table 4. Stereospecific analysis of triglyceride from A. curvatum grown on palmitic acid as a carbon source for 3.5 and 7 days

	16:0	16:1	18:0	18:1	18:2
3.5d (TG[a])	65.2	16.1	0.4	15.7	2.6
sn-1	83.2	9.4	0.5	5.7	1.2
sn-2	15.9	34.8	-	42.6	6.7
sn-3	96.5	4.1	0.7	*	*
7d (TG)	61.3	13.9	0.8	21.5	2.6
sn-1	82.4	11.9	-	5.7	-
sn-2	11.5	24.2	-	56.7	7.6
sn-3	89.9	5.7	2.4	2.0	0.1

[a]Triglyceride.

*Negative value.

Normally the yeast almost completely excludes saturated acyl groups from the sn-2 position of glycerol, but stereospecific analysis of the yeast oil showed that 11 to 16% of the acyl groups at sn-2 were palmitoyl (Table 4). Seemingly, the unusual carbon source forced the yeast to place more saturated acyl groups on the sn-2 position than it normally does.

The emulsion of stearic acid gave very limited growth and lipid accumulation (1.35 g/L of fat). Table 5 shows the fatty acid composition and sn-2 composition of triglycerides from yeast grown on stearic acid. The stearic acid seems to be desaturated to oleic and linoleic acid, and more oleic acid is produced on stearic acid as a substrate than with palmitic acid. There is relatively little change in the length of the fatty acid chain. Much less saturated acid was found on the sn-2 position than was found with palmitic as substrate. The yeast could not grow on the emulsion of arachidic acid.

Growth of the yeast on monounsaturated fatty acids

Fatty acids with one double bond were more convenient carbon sources than long-chain saturated acids. The monoenes caused no oxidation problems and required no emulsification. Substrate fatty acid left over after incubation could be removed easily and fairly completely from the cell mass so that accurate data about cell growth and lipid accumulation could be obtained. Table 6 compares the growth of A. curvatum and accumulation of yeast oil with various monounsaturated fatty acids as substrates. The yeast grew well on hexadecenoate and octadecenoate, regardless of the position of double bonds. The cis-11-eicosenoate, gondoic acid, gave poorer growth and less oil accumulation than the hexadecenoate and octadecenoates, and the amount of cell mass produced on the docosaenoate, erucic acid, was too small to be recovered and weighed.

Table 5. Fatty acid composition of A. curvatum triglycerides and the sn-2 position from yeast grown on stearic acid as a carbon source

	16:0	18:0	18:1	18:2
TG	0.9	48.0	46.1	5.1
Sn-2	0.2	3.0	87.9	8.9

The stereospecific analysis of the triglycerides from yeast grown on the various monounsaturated fatty acids as carbon sources is shown in Table 7. For palmitoleic (cis-9-hexadecenoic) and oleic acids, the triglyceride contained about 90% of the substrate fatty acid; however, for petroselinic (cis-6-octadecenoic) acid, 84.2% of the acyl groups was petroselinate. Oleoyl groups were favored more than palmitoyl or petroselinoyl groups on the sn-2 position. Yeast grown on petroselinic acid had significantly more palmitoyl groups than those grown on oleic or palmitoleic acids, and produced a considerable proportion of oleoyl groups in its oil. The yeast grown on oleic acid contained more linoleoyl groups in its oil than those grown on palmitoleic or petroselenic.

When gondoic acid was the substrate, only 67.5% was recovered unchanged in the yeast triglycerides, and considerable proportions of oleoyl, linoleoyl, and palmitoyl groups were produced. Although the

Table 6. Cell growth and lipid accumulation with different monounsaturated fatty acids as carbon sources

	Lipid-extracted DCW[a] (g/L)	Triglyceride produced	Total DCW (g/L)	% Oil
Palmitoleic acid	7.49	8.92	16.41	54.4
Oleic acid	7.66	8.02	15.68	51.6
Petroselinic acid	7.07	8.52	15.58	54.4
Gondoic acid	5.63	3.71	9.34	39.8
Erucic acid	Minute quantity (could not determined)			

[a]Dry cell weight.

amounts of oleoyl and gondoyl groups on the sn-2 position were equal, oleoyl groups must be favored in the sn-2 position because the total oleoyl content is about one-third of that of gondoyl group.

The small amount of yeast oil produced with erucic acid as substrate contained only about 3% erucic acid. Undecylenic acid (10-undecenoic acid) did not support yeast growth.

Growth on polyunsaturated fatty acids

Although the yeast grew well on mixtures containing substantial proportions of polyunsaturated fatty acids when these substrates were

Table 7. Stereospecific analyses of A. curvatum triglycerides grown on different monounsaturated fatty acids as carbon sources

On palmitoleic acid

	16:0	16:1	18:0	18:1	18:2
Whole	0.5	89.5	2.8	7.1	0.2
Sn-1	1.7	91.9	3.1	3.1	0.2
Sn-2	0.1	83.4	0.5	15.4	0.5
Sn-3	*	93.2	4.8	2.8	*

On oleic acid

	16:0	18:0	18:1	18:2
Whole	0.8	0.7	92.2	6.3
Sn-1	2.4	0.6	91.1	5.9
Sn-2	-	-	94.0	6.0
Sn-3	-	1.5	91.5	7.0

On petroselinic acid

	16:0	18:1(Δ6)	18:1(Δ9)	18:2
Whole	2.8	84.2	10.4	2.6
Sn-1	5.9	82.9	6.9	4.3
Sn-2	3.2	65.3	24.0	7.6
Sn-3	*	104.4	0.3	*

*Negative value.

Table 7. (continued)

	16:0	16:1	18:0	18:1	18:2	20:1	20:2
On gondoic acid							
Whole	4.7	0.4	0.8	22.0	3.7	67.5	0.9
Sn-1	12.5	1.2	1.6	21.5	5.0	57.1	1.0
Sn-2	0.2	-	0.1	45.6	7.2	45.6	1.3
Sn-3	1.4	-	0.7	*	*	99.8	0.4

isolated from soybean or corn oil, the yeast did not grow well on purified linoleic acid or mixtures of purified fatty acids containing more than 20% linoleic acid or more than 15% linolenic acid. Oxidation seemed to be the major problem, but the addition to the medium of up to 1500 ppm of alpha-tocopherol or of the unsaponifiables collected from corn oil were not effective in producing yeast growth on purified substrates rich in polyunsaturated fatty acids. If media made from purified polyunsaturated fatty acids were supplemented with 1000 ppm butylated hydroxyanisole (BHA), the yeast grew. Evidently, antioxidant factors other than tocopherols in the natural fatty acid mixtures were necessary to support growth in media rich in polyunsaturated fatty acids. Table 8 shows the growth of A. curvatum on various mixtures of oleic, linoleic and linolenic acid. With 1000 ppm BHA, the yeast gave

Table 8. Cell growth and lipid accumulation with various mixture of oleic, linoleic and linolenic acid with 1,000 ppm of BHA as carbon sources

	Lipid-extracted DCW (g/L)	Triglyceride produced (g/L)	Total DCW (g/L)	%Oil
Oleic acid	7.10	6.57	13.67	48.1
O:L[a] = 2:1	6.93	7.75	14.68	52.8
O:L = 1:2	8.06	9.88	17.94	55.0
Linoleic acid	8.13	10.57	18.70	56.3
Linolenic acid	6.19	8.83	15.02	58.8

[a]O:L = oleic:linoleic.

slightly less growth and lipid accumulation than without BHA, but the antioxidant did not change fatty acid composition of the yeast oil. More linoleic acid in the medium gave greater dry cell mass, primarily due to the increased amount of yeast oil. Linolenic acid gave greater dry cell mass than oleic but not as much as linoleic acid. Linolenic acid-grown yeast had the greatest oil content among all the fatty acids tested. Table 9 gives a stereospecific analysis of the triglycerides isolated from the yeast grown on linoleic and linolenic acid. The substrate fatty acids constituted most of the acyl groups in the yeast oil. Oleoyl and linoleoyl groups were favored at sn-2. On linolenic acid substrates, the yeast

Table 9. Stereospecific analysis of yeast triglyceride grown on linoleic and linolenic acid as carbon sources

On linoleic acid					
	16:0	18:0	18:1	18:2	
TG	0.9	1.5	1.3	96.3	
Sn-1	2.3	1.5	0.7	95.5	
Sn-2	-	-	2.2	97.8	
Sn-3	0.4	3.0	1.0	95.6	
On linolenic acid					
	16:0	18:0	18:1	18:2	18:3
TG	1.9	1.6	7.6	0.5	88.3
Sn-1	3.3	1.5	5.3	-	89.9
Sn-2	0.8	-	18.7	1.4	79.1
Sn-3	1.6	3.3	*	0.1	95.9

*Negative value.

produced considerable amounts of oleate.

Utilization of conjugated and oxygenated fatty acids

Attempts to grow yeast on the fatty acids isolated from tung oil were unsuccessful, probably because the fatty acids polymerized rapidly.

The yeast grew on tung oil, but at a slower rate than on corn oil. Because the substrate was a triglyceride, the lipid accumulation in the yeast was difficult to quantify exactly, but microscopic examination showed good fat accumulation inside the yeast cells. The dry cell weight after lipid extraction was 8.2 g/L, which is almost as good as that on corn oil fatty acids. Table 10 shows the fatty acid profile of the tung oil substrate, the triglyceride recovered from the benzene extract of the yeast and the composition of the sn-2 position of the yeast triglyceride. About 50% of the yeast oil was eleostearic acid compared with 80.6% in the substrate oil; however the two isomers, alpha- (cis-9-,trans-11,trans-13-) and beta-eleostearic (trans-9,trans-11,trans-13-octadecatrienoic), were not resolved. The eleostearate esterified in the sn-2 position was much less than its percentage in the yeast oil.

V. anthelmintica seed, which contains vernolic acid (12,13-epoxy-cis-9-octadecenoic acid) was extracted and used as a carbon source in the yeast medium. The seed oil allowed moderately good growth and yielded 5.2 g/L of dry cell weight after oil extraction. The seed oil was composed of 73.8% vernolic acid. Triglycerides from the benzene extracts of the yeast contained 44% vernolic acid.

Castor oil and its chief component, ricinoleic acid (12-hydroxy-cis-9-octadecenoic acid), were used as carbon sources in yeast media. The yeast could grow on both substrates, but the growth with ricinoleic acid was much slower than that with castor oil. Ricinoleic acid constituted 85.5% of the castor oil, but the triglyceride extracted

Table 10. Fatty acid composition of tung oil substrate, the oil from yeast grown on tung oil and its sn-2 position

	16:0	16:1	18:0	18:1	18:2	18:3	18:3[a]
Tung oil	2.3	-	2.5	6.8	7.2	0.8	80.4
Yeast oil	6.9	0.8	1.2	23.6	14.7	0.7	52.1
Sn-2	1.4	1.2	0.1	55.0	32.8	0.4	9.0

[a]Eleostearic acid.

(benzene extract) from the yeast grown on castor oil contained only 29.2%.

Eleostearic, ricinoleic and vernolic acids have frequently been suggested as targets for gene transfer experiments because oils containing them have industrial uses and are relatively expensive (18, 28). These results show that the yeast is able to accumulate significant amounts of these acids in its triglyceride, but their concentrations in the yeast triglycerides were always less than in their substrates, and growth on these carbon sources was less than for substrates such as corn oil. These observations suggest a reluctance on the part of the yeast to include these acyl groups in its oil depot. Of the exotic fatty acids that we have tested (i.e., those that are not accumulated when the yeast grows on sugar), petroselinic seems the most acceptable to the yeast as a component of its triglycerides.

Some application of A. curvatum grown on lipid substrates

Separation of erucic acid from crambe oil The poor utilization of erucic acid by A. curvatum suggested that it might be left unutilized and concentrated in the medium when erucic acid-containing oils were used as carbon sources by the yeast. Table 11 shows the results when crude, pressed crambe oil was used as a carbon source for yeast grown for 7 and 14 days. The yeast obviously discriminated against C_{20}, C_{22} and C_{24} fatty acids so that there were smaller proportions of these in the yeast oil, and more of them accumulated in the residual medium oil. However, the incorporation of C_{20} to C_{24} fatty acids in the yeast oil was not completely avoided, and the proportion of these fatty acids in yeast oil increased with incubation time as alternative fatty acids were depleted. Thin-layer chromatography showed that most of the residual lipid in the medium was free fatty acid. Table 12 shows the amounts of fatty acids shorter than C_{20} and those of fatty acids $\geq$ 20 in the various oil phases from this crambe oil experiment.

Cholesterol incorporation by A. curvatum There is considerable interest in the elimination or reduction of cholesterol in animal fats. Choi (29) reported that the major sterols in A. curvatum grown on a sugar-substrate media were sitosterol and stigmasterol. No cholesterol was found. This suggests that, if A. curvatum were grown on animal fats as a carbon source, the fatty acids might be deposited in the yeast and the cholesterol accumulated in the residual medium fat. Bati et al.(26) reported that cholesterol was discriminated against when Candida lipolytica was grown on substrates of lard and tallow, but some

Table 11. Fatty acid composition of oil phases from experiments with crambe oil as a carbon source

	Substrate crambe oil	Leftover oil phase		Yeast oil (benzene extract)	
		7 day	14 day	7 day	14 day
16:0	2.8	1.0	0.2	4.7	4.4
16:1	0.4	-	-	0.8	0.5
18:0	1.2	0.9	0.6	1.6	1.5
18:1	22.0	15.1	6.0	34.1	37.2
18:2	11.9	5.8	1.7	25.8	23.8
18:3	6.9	2.7	1.6	13.7	9.7
20:1	5.2	5.3	4.3	4.5	4.5
22:0	1.2	2.1	3.0	-	0.2
22:1	46.9	64.7	78.8	14.7	18.3
22:1 -	1.6	2.4	3.8	-	-
≥20:1	54.9	74.5	89.9	19.2	23.0

cholesterol was found in the yeast oil. It is not certain, however, that all the external fat was removed in their experiments.

We grew A. curvatum on a synthetic mixture of oleic acid containing

Table 12. The amounts of fatty acids in the oil phases from experiment with crambe oil as a carbon source

Fatty acids	In substrate crambe oil (g/L)	In leftover oil phase (g/L)		In yeast oil (g/L) (benzene extract)	
		7 day	14 day	7 day	14 day
<20:1	8.12	2.93	0.80	1.87	4.13
≥20:1	9.88	8.55	7.09	0.44	1.23

cholesterol. The yeast was washed with ethanol until no oleic acid was found in the washings. This required eight washings with ethanol. The residual triglycerides in the yeast were extracted with hexane and benzene as usual. Of 2234 ppm of cholesterol in the medium, the yeast triglyceride contained only 45 ppm. The concentration of cholesterol in the residual substrate was 4692 ppm. However, a considerable portion of the yeast triglyceride was extracted along with the free fatty acid during the eight washings. After first and second washings with ethanol, the yeast oil contained 844 ppm and 168 ppm of cholesterol, respectively. A. curvatum discriminates against cholesterol in its medium effectively, but it will be necessary to find better ways of separating external and internal fat to make this a practical method of removing cholesterol from animal fats.

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PART II.

TRIACYLGLYCEROL ASSEMBLY

BY *APIOTRICHUM CURVATUM*

FROM BINARY MIXTURES OF FATTY ACIDS

ABSTRACT

The oleaginous yeast Apiotrichum curvatum was grown on various binary mixtures of palmitic, stearic, oleic and linoleic acids as carbon sources. When oleic-linoleic acid mixtures of various ratios were used as substrates, the yeast grew well, and triglycerides with a wide range of acyl group compositions were obtained. Oleic acid was favored over linoleic acid at the sn-2-position of the glycerol. When the percentages of oleate and linoleate at the three glycerol positions were plotted versus the percent of the acyl groups in the whole yeast, linear relations were observed for most of the range, and the sum of the intercepts and slopes of the three lines of each fatty acids was 0 and 3, respectively. A simple mathematical model of triglyceride assembly was proposed to explain these observations. With more palmitic and stearic acids in the medium, the yeast accumulated less oil. Incorporation of stearic acid into the triglyceride also was very limited. When mixtures of palmitic-oleic and palmitic-linoleic acids were used as substrates, the yeast oils of cultures grown on the ratios of 50:50 and 25:75 had similar acyl group profiles. Possibly the yeast had more limited access to solid fatty acid substrates than to liquid fatty acids.

INTRODUCTION

Triglyceride structure is considered to be associated with the stability (1) and nutritional value (2) as well as the physical properties (3) of fats and oils. Stereospecific analysis of animal (3) and plant (4) triglycerides revealed that the distribution of their acyl groups is not random. Since Christie and Moore (5) first suggested linear relations in the plots of percentages of fatty acids on the glycerol positions vs. the percentage of the fatty acids in the whole triglyceride, such linear relations have been reported in soybean (6, 7), oat (7) and peanut (8, 9) oils. However, no such studies have been done with yeast oils.

When the oleaginous yeast Apiotrichum curvatum was grown on binary mixtures of oleic and linoleic acids as carbon sources, the yeast yielded triglycerides with a wide range of acyl group composition. These triglycerides provided a good model to study the characteristics of fatty acyl distribution by the yeast.

In a previous paper, we reported the effects of different pure free fatty acids and neutral oils on growth and lipid accumulation by the yeast Apiotrichum curvatum ATCC 20509, and on the distribution of fatty acyl groups in its triglycerides. The effect of using binary mixtures of the four major fatty acids of yeast oil as substrate is reported in the present paper.

MATERIALS AND METHODS

A. curvatum was maintained as refrigerated slant cultures on yeast extract/dextrose/peptone/agar (1/2/2/1.5 %w/w), transferred monthly. The constituents of the basal medium were (g/L): KH_2PO_4, 2.5; $MgSO_4 \cdot 7H_2O$, 1.0; asparagine, 0.8; $CaCl_2.2H_2O$, 0.2; NaCl, 0.06; $FeCl_3.6H_2O$, 0.02; $MnSO_4.H_2O$, 0.002; $ZnSO_4 \cdot 7H_2O$, 0.001; thiamine-HCl, 0.001; $CuSO_4 \cdot 5H_2O$, 0.0001 (10). The basal medium was adjusted to pH 5.5 and supplemented with 18 g/L of substrate lipid. Pure fatty acids were purchased from Sigma Chemical Co. (St. Louis, MO).

A seed culture was prepared by inoculating about 2% of the yeast washed from a slant culture into 100 ml of heat-sterilized basal medium with the fatty acids isolated from corn oil as a carbon source. The culture was grown in 250-ml flasks in a Labline orbital shaker (Melrose Park, IL) at 32°C and 180 rpm. The seed culture was in logarithmic growth after about 2 days, and its optical density at 440 nm was normally 9 to 10. One milliliter of seed culture was used as an inoculum for 100 ml of medium containing the substrate lipid to be tested. The test cultures were grown for 7 days under the same conditions used for the seed culture.

Residual substrate was separated from the culture with a separatory funnel in the experiments with oleic-linoleic acid mixtures. Oil extraction from the yeast was according to Hammond et al. (11), which involved extraction in sequence with ethanol, hexane and benzene. Fatty acid mixtures having palmitic and stearic acid were emulsified into the

medium with 5 g/L gum acacia. After heat-sterilization of the mixture of gum acacia, fatty acid and basal medium in a blender jar, emulsion was made in situ by blending at high speed for 1 min, and the emulsified medium was transferred aseptically into the culture flasks. When emulsified substrates were used, agitation was decreased to 140 rpm to minimize destabilization of the emulsion. The yeast cell mass could not be separated completely from the emulsified fatty acid by centrifugation, so after removal of as much of the cell mass as possible by centrifugation, the supernatant was evaporated in a rotary evaporator, and the residue was pooled with the cell mass recovered by centrifugation. Extraction of lipid in this residue was accomplished as before.

The amount of triglyceride in yeast oil was determined by thin-layer chromatography (TLC). Aliquots of the ethanol extract and pooled hexane and benzene extracts were applied to TLC plates 1.0 mm thick. The plates were developed in hexane/ether/acetic acid (50/50/1, v/v/v), and bands were visualized by spraying with 0.2% dichlorofluorescein in ethanol and viewed under ultraviolet light. Triglycerides were eluted and the residue was weighed after evaporation of the ether under nitrogen. For further lipid analysis, triglycerides were separated from the pooled hexane and benzene extracts by the same TLC method described above.

Stereospecific analysis was done according to Fatemi and Hammond (6). For fatty acid analysis, glycerides were transesterified by the method of Frey and Hammond (12), and the methyl esters were separated on

a Varian Model 3700 Gas Chromatograph equipped with a 1.8 M x 3.3 mm column of 10% SP-2330 on Chromosorb WAW and a flame ionization detector.

All reported results are the average of two replicate measurements except those of stereospecific analyses, which represent a single measurement. However, the errors for stereospecific analyses were less than 7% in determining the whole, sn-1 and sn-2 compositions.

RESULTS AND DISCUSSION

Structure of triglycerides from A. curvatum grown on oleic-linoleic acid mixtures

Table 1 shows the fatty acid composition and stereospecific distribution of fatty acids in triglyceride from A. curvatum grown on pure oleic and linoleic acids and on mixtures of oleic and linoleic acids as carbon sources. Saturated acyl groups in the yeast triglyceride was <3% in all instances. Figure 1 shows the percentage of oleate and linoleate in the yeast triglycerides plotted versus the percentage of the fatty acids in the substrate used. The percentage of linoleate in triglyceride from the yeast grown on pure oleic acid as a substrate was plotted vs the zero percent in the substrate for the line of linoleic, and vice versa for the oleic acid line in the Figure 1. Different intercepts were observed from the lines of the two fatty acids possibly because of the desaturation of oleate to linoleate by the yeast or possibly because linoleate is accumulated slightly better than oleate. Almost the same slopes (0.892 and 0.900 for oleic and linoleic acids, respectively) were noted from the plots. Correlation coefficients of the plots were 0.9988 and 0.9984 for oleic and linoleic acids, respectively.

The percentage of each fatty acid on the three glycerol positions was plotted vs the percentage of the acyl groups in the whole yeast triglyceride in Figures 2 and 3. The percentages of oleate and linoleate on the sn-1-, sn-2- and sn-3-positions were linearly related

Table 1. Stereospecific analysis of triglycerides from A. curvatum grown on different ratios of oleic:linoleic acid

		Fatty acid composition			
		16:0	18:0	18:1	18:2
O:L[a] = 100:0	TG[b]	0.8	0.7	92.2	6.3
	Sn-1	2.4	0.6	91.1	5.9
	Sn-2	-	-	93.5	6.0
	Sn-3	-	1.5	91.6	7.0
O:L = 94:6	TG	3.4	2.6	83.5	10.5
	Sn-1		N.A[c]		
	Sn-2	-	-	92.9	7.1
	Sn-3		N.A		
O:L = 85:15	TG	1.3	1.0	76.9	20.8
	Sn-1	3.4	1.4	66.0	29.3
	Sn-2	0.5	0.2	86.6	12.6
	Sn-3	-	1.4	78.1	20.5
O:L = 76:24	TG	1.4	1.1	65.7	31.9
	Sn-1	2.9	1.4	56.9	38.9
	Sn-2	-	-	80.8	19.2
	Sn-3	1.3	1.9	59.4	37.6
O:L = 67:33	TG	1.1	1.3	57.1	40.5
	Sn-1	2.0	1.2	50.3	46.6
	Sn-2	-	-	73.0	27.0
	Sn-3	1.3	2.7	48.0	47.9
O:L = 45:55	TG	0.8	0.8	39.6	58.8
	Sn-1	1.5	0.9	30.9	66.7
	Sn-2	0.2	0.1	56.1	43.5
	Sn-3	0.7	1.4	31.8	66.2

[a]Oleic : linoleic.

[b]Triglyceride.

[c]Not available.

Table 1. (continued)

O:L = 33:67	TG	0.8	1.0	29.0	69.2
	Sn-1	1.6	0.9	22.8	74.8
	Sn-2	-	-	43.8	56.2
	Sn-3	0.8	2.1	20.4	76.6
O:L = 15:85	TG	1.3	1.3	13.8	83.6
	Sn-1	3.6	2.1	8.7	85.7
	Sn-2	0.5	0.3	27.6	71.6
	Sn-3	-0.2	1.5	5.1	93.5
O:L = 10:90	TG	1.2	1.6	9.0	88.2
	Sn-1	3.6	2.4	7.5	86.5
	Sn-2	-	-	14.7	85.3
	Sn-3	-	2.4	4.8	92.8
O:L = 0:100	TG	0.9	1.5	1.3	96.3
	Sn-1	2.3	1.5	0.7	95.5
	Sn-2	-	-	2.2	97.8
	Sn-3	0.4	3.0	1.0	95.6

to the total percentage of the fatty acids in triglyceride for a certain range of each fatty acid: oleic, approximately 15-70%; linoleic, approximately 25-80%. The three lines in Figures 2 and 3 represent plots from five different triglycerides in the range. The slopes, intercepts and correlation coefficients are listed in Table 2.

In the triglycerides from the yeast grown on oleic-linoleic mixtures, oleate was favored at the sn-2-position compared with linoleate. The same trend was reported in oat oil. On the other hand, linoleate was favored over oleate in soybean and corn oil (7). The sign

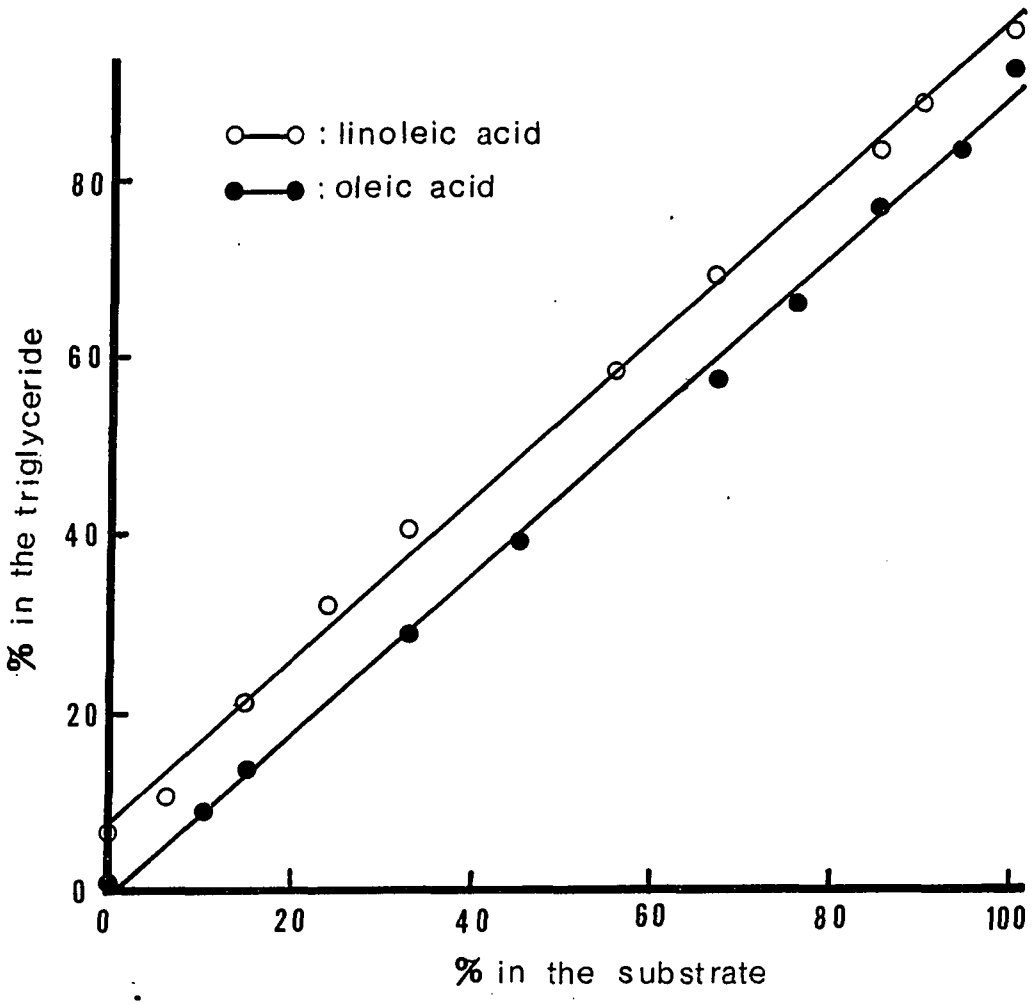

Figure 1. The percentage of fatty acids in the yeast triglycerides vs the percentage of those in the substrates

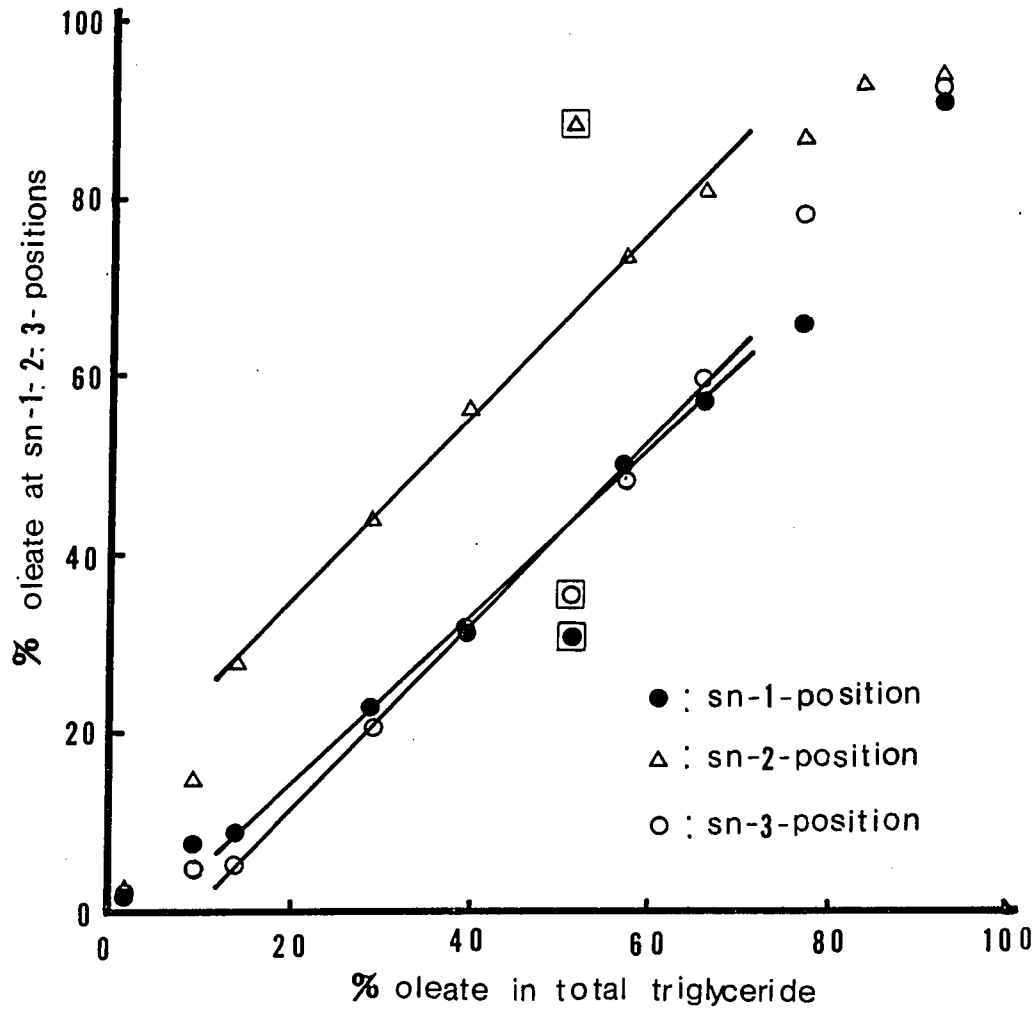

Figure 2. The percentage of oleic acid on the sn-1-, sn-2-, and sn-3-positions of glycerol vs the percentage of oleic acid in the whole triglyceride from the yeast A. curvatum grown on oleic-linoleic mixtures (□: on a palmitic-oleic mixture)

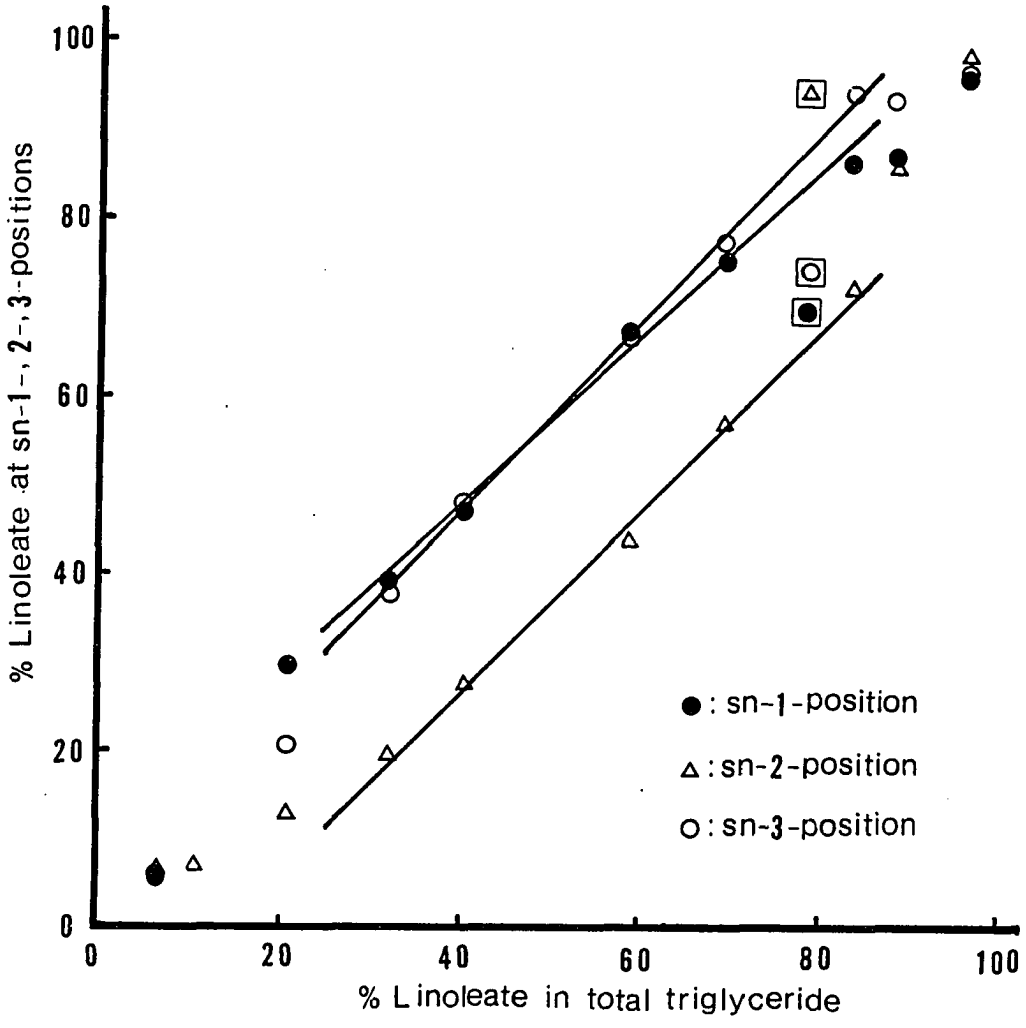

Figure 3. The percentage of linoleic acid on the sn-1-, sn-2-, and sn-3-positions of glycerol vs the percentage of linoleic acid in the whole triglyceride from the yeast A. curvatum grown on oleic-linoleic mixtures (□: on a palmitic-linoleic mixture)

Table 2. Linear regression of the percentage of fatty acids at the 3 positions of glycerol vs the percentage in the whole triglyceride[a]

Fatty acid	Position	Intercept	Slope	r[b]
Oleic acid	sn-1	-4.76	0.94	0.9986
	sn-2	14.05	1.03	0.9992
	sn-3	-9.30	1.03	0.9991
	sum	-0.01	3.00	
Linoleic acid	sn-1	10.02	0.93	0.9966
	sn-2	-14.04	1.01	0.9984
	sn-3	4.02	1.06	0.9994
	sum	0.00	3.00	

[a]Ranges used for this linear regression were 13.8 - 65.7% and 31.9 - 83.6% in the whole triglyceride for oleic and linoleic acids, respectively.

[b]Correlation coefficient.

of the intercepts (positive or negative) indicates that the placement of acyl group on the position is favored or resisted, respectively. Slopes in Table 2 greater than 1 indicate that as the amount of an acyl group in the whole oil increases, there is a tendency to place more of it on that particular glycerol position than is present in the whole oil. A slope less than 1 indicates less is placed on a glycerol position than is found in the whole oil. The pattern of the plots

varied with the components of the mixtures used as substrates. In Figures 2 and 3, the points shown in the squares were from triglycerides in cultures that were grown on palmitic-oleic and palmitic-linoleic mixtures, respectively. In both instances the palmitate, which is accumulated mostly on the sn-1 and sn-3 positions, caused an increase in the unsaturate on the sn-2 position.

Theoretical treatment of glyceride distribution

Figures 2 and 3 reveal that a plot of the amounts of oleate and linoleate on the three positions of glycerol versus the amount of these acyl groups in the whole oil were linear through the middle of their range. Such linear relations were first suggested by Christie and Moore(5), and they have been demonstrated for varieties of soybean (6,7) and its wild relative Glycine soja as well as oat and its wild relative Avena sterilis (7). Fatemi and Hammond (6) made the observation that if the slopes and intercepts of such plots were determined, that for a particular acyl group, the slopes for the three positions totaled 3, and the intercepts 0. This is also true for the slopes and intercepts of Figures 2 and 3. The use of plant varieties for such plots limits the range of the data to the range of fatty acid compositions that are available. Pan and Hammond (7) pointed out that although these plots were linear over the range that could be observed, it was impossible for them to be linear over their entire range and that the lines must bend toward zero and 100% at their extremes. The data obtained with A. curvatum has made it possible to examine a much longer range and verify

this prediction.

Many of the observations about plots like those of Figures 2 and 3 can be explained by a simple mathematical model of triglyceride synthesis. Assume that the fatty acids being assigned to the three positions of glycerol are drawn from a single pool and that the pool is in a steady state so that fatty acids are added at the same rate they are withdrawn. Let the fatty acids be designated a,b,c....n and the steady state concentration of the fatty acids in the pool be A,B,C....N. Assume that the amount of each fatty acid attached to each glycerol position is governed by a rate constant, k, where k(1A) is the rate constant for placing fatty acid a on the sn-1 position, k(2B) is the rate constant at which fatty acid b is placed on position the sn-2 position, etc. Assume that the rate of acyl distribution is governed by the rate constant and the steady state concentration so that a(1), the observed proportion of fatty acid a on sn-1,

$$a(1) = k(1A)A/[k(1A)A + k(1B)B + k(1C)C...k(1N)N]$$

then,

$$k(1A)A + k(1B)B + k(1C)C....k(1N)N = 1$$

because all the acyl groups on a glycerol position, in this instance the sn-1 position, fill it completely and total 1.

Thus,

$$a(1) = k(1A)A$$

We can write a similar equation for each fatty acid and each position of the glycerol, but there is no way to observe the concentration in the pool or the k's. We can conclude that

$$a(1)/a(2) = k(1A)/k(2A)$$

If we let a(t) be the proportion of a in the whole triglyceride,

$$3a(t) = a(1) + a(2) + a(3)$$

and the slope of a line with an ordinate of a(1) and an abscissa a(t) is

$$a(1)/a(t) = 3a(1)/[a(1) + a(2) + a(3)]$$

$$= 3k(1A)A/[k(1A)A + k(2A)A + k(3A)A]$$

$$= 3k(1A)/[k(1A) + k(2A) + k(3A)]$$

Thus, the slope of the line will be constant, and if we sum the three slopes for a on the sn-1, sn-2 and sn-3 positions

$$\text{sum} = 3[k(1A) + k(2A) + k(3A)]/[k(1A) + k(2A) + k(3A)] = 3$$

Thus, this simple model accounts for the linear relation observed in A. curvatum and plant varieties and for the slopes of the three lines for each acyl group adding to 3. The model assumes that the rate of assignment is never influenced by the amount of glycerol, glycerol phosphate, monoglyceride or diglyceride that also are presumed substrates for the reactions. This would be true if these substrates always were present in adequate or steady state concentrations. It also assumes that mono- and di-glyceride are never final products.

But this simple model fails in that the plots should all have intercepts of zero, and they obviously do not. The presence of these intercepts also requires the lines to deviate from linearity at small values for the concentration of the acyl group in the oil. The presence of these intercepts and the fact that they sum approximately to zero suggests that there must be a mechanism that effectively takes a fixed amount of an acyl group from glycerol positions with negative intercepts

and adds them to those with positive intercepts. The amount of this transfer is equal to the sum of the positive and negative intercepts. The amount of such a transfer evidently is not affected by the amount of the acyl group present in the whole oil. This insensitivity to acyl concentration cannot be true when the amount to be transferred is less than the amount of an acyl group present. This may explain why this model breaks down at small concentrations of an acyl group in the oil so that the model applies only to the middle of the observed lines. The presence of intercepts suggests that there may be two pathways leading to triglycerides, one accounting for slopes and one for intercepts.

Obviously the actual mechanisms by which various acyl groups end up on various positions of glycerol may be much more complex than the simple model we have proposed. If the net effect of a complex pathway is such that rates are limited and the final distribution is controlled by the factors assumed in the model, then the model may correctly predict the observed distribution.

Effect of substrate mixtures of fatty acid on the composition of yeast triglycerides

The other binary mixtures of fatty acids were not capable of being studied over the wide range used for oleic-linoleic mixtures, because the acyl group composition of A. curvatum triglycerides varied over a relatively narrow range, regardless of the ratios of fatty acids in the medium. Also the emulsions of solid saturated high-melting acids used as substrates made it difficult to isolate the yeast triglyceride for

stereospecific analysis. Table 3 shows the fatty acid composition of triglycerides isolated from A. curvatum when it was grown on various binary combinations of fatty acids as carbon sources. When large proportions of stearic acid were present in the medium, the amount of accumulated yeast oil decreased (data not shown). This agrees with our previous observation (Part I in this dissertation). Less than 10% stearic acid was observed in the yeast triglycerides even from a medium with 75% stearic acid. Seemingly, the yeast reluctantly used and incorporated stearic acid into its triglycerides. In a study using cell-free extracts and spheroplasts, Holdsworth and Ratledge (13) reported that the activity of fatty acyl coenzyme A (CoA) synthetase in A. curvatum was some 6- to 8-fold lower with stearate than with palmitate, oleate and linoleate. The acyl CoAs that are produced in this reaction can be used either for ß-oxidation or for synthesis of cellular lipids (14). The poor substrate activity of this enzyme with stearate might account for the very limited utilization of stearate and its low incorporation into the triglyceride. In the stearic-oleic and palmitic-oleic mixtures, the presence of saturated acids seemed to significantly decrease the linoleate found in the yeast triglyceride compared with that found with oleate alone.

Holdsworth and Ratledge reported that the activities of fatty acyl CoA synthetase for palmitic and linoleic acids were similar. However, as the proportion of palmitic acid increased in the medium, the yeast accumulated less triglyceride. The yeast seemed to prefer palmitate over stearate since the yeast oil resembled that from palmitic more than

Table 3. Fatty acid composition of triglyceride from A. curvatum grown on various combinations of saturated and unsaturated fatty acids as carbon sources. The compositions observed for single fatty acids used in the mixtures are included for comparison

Carbon sources	16:0	16:1	18:0	18:1	18:2
P[a]:S[b] = 75:25	60.7	11.6	1.3	23.9	2.4
50:50	56.5	8.7	2.7	28.3	3.8
25:75	50.8	4.5	6.5	33.6	4.5
P:O[c] = 75:25	39.9	3.1	1.6	50.7	4.8
50:50	17.7	-	1.5	78.3	2.5
25:75	16.9	-	1.6	78.8	2.7
P:L[d] = 75:25	25.3	1.7	1.9	6.3	64.8
50:50	15.0	-	1.7	2.3	81.1
25:75	14.9	-	2.0	4.0	79.1
S:O = 75:25	0.9	-	9.7	86.4	3.1
50:50	0.5	-	2.5	95.1	1.2
25:75	0.5	-	3.1	94.7	1.7
S:L = 75:25	-	-	6.0	2.1	91.9
50:50	0.5	-	5.7	1.5	92.3
25:75	0.5	-	3.7	1.3	94.5
Palmitic 100%	61.3	13.9	0.8	21.5	2.6
Stearic 100%	0.9	-	48.0	46.1	5.1
Oleic 100%	0.8	-	0.7	92.2	6.3
Linoleic 100%	0.9	-	1.5	1.3	96.3

[a]Palmitic acid.

[b]Stearic acid.

[c]Oleic acid.

[d]Linoleic acid.

that from stearic acid when the yeast was grown on palmitic-stearic mixtures. When the substrates were 50:50 or 25:75 palmitic-oleic acid mixtures, the acyl composition of the yeast oil was similar regardless of the proportion of palmitic acid in the substrate oil. Similar results were obtained for palmitic-linoleic acid mixtures. It is suggestive that 15 - 18% palmitic acid found in these oils corresponded to the solubility of palmitic acid in oleic acid at 33°C, namely 15.7% (15). Probably the access of the yeast to solid lipid substrate is less than for liquid fatty acids. When the proportion of palmitate in palmitic-oleic and palmitic-linoleic acid mixtures was 75%, greater amounts of palmitate were found in the yeast oil.

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GENERAL SUMMARY

A. curvatum was grown on various lipids as carbon sources. When commercial animal and vegetable oils were used as substrate, the yeast grew well, and the yeast triglyceride had a fatty acid profile similar to that of the substrate oil, but the acyl groups of the substrate oil were redistributed according to the specificity of the yeast's enzymes for triglyceride biosynthesis. The yeast favored placing oleoyl groups on sn-2, and saturated acyl groups on sn-1 and sn-3 positions. The yeast seemed to utilize corn oil and lactose equally well in a mixed-substrate medium.

Saturated short-chain free fatty acids less than 14 carbons were not utilized in A. curvatum's oil depots; however, the yeast grew on glycerol tributyrate and tricaprate as a carbon source. With a tricaprin substrate some capryl groups were detected in the yeast triglyceride, but butyryl groups were not deposited when tributyrin was the substrate. Lauric acid did not support the growth of the yeast. The yeast showed very limited growth on myristic acid as a carbon source but deposited some triglyceride composed of more than 90% myristic acid.

An emulsion of palmitic acid was well utilized by A. curvatum. On this substrate the yeast oil contained large amounts of palmitate on the sn-1 and -3 positions, and 11 to 16% at the sn-2-position. About 20% oleic acid was produced from palmitic acid presumably through elongation and desaturation. When binary mixtures of palmitic-oleic and palmitic-linoleic acids were used as carbon sources, the yeast accumulated less oil with greater proportions of palmitic acid in the substrate. Also,

the triglycerides from yeast grown on binary mixtures containing 25 and 50% palmitic acid contained similar acyl compositions.

Stearic acid gave very limited growth of A. curvatum. The substrate stearic acid was extensively desaturated to oleic and linoleic acids before being deposited in the yeast triglyceride. Much less saturated acid was found in the yeast triglyceride and its sn-2-position than when palmitic acid was the substrate. When binary mixtures containing up to 75% stearic acid were used as carbon sources, the stearic acid had little effect on the acyl composition of the yeast triglycerides. Arachidic acid did not support the yeast growth.

A. curvatum grew well and accumulated over 50% triglyceride on hexadecenoate and octadecenoate, regardless of the position of the double bond. Limited grow was observed when the cis-11-eicosenoate, gondoic acid, was used as a carbon source; almost no growth was observed with erucic acid as a substrate. For palmitoleic and oleic acids, the yeast triglyceride contained about 90% of the substrate fatty acid; for Petroselinic acid, 84.2% of acyl groups of the yeast triglyceride was petroselinate. Oleoyl groups were favored more than palmitoyl or petroselinoyl groups on the sn-2-position. When gondoic acid was the substrate, only 67.5% was recovered unchanged in the yeast triglyceride. Undecylenic acid (10-undecenoic acid) did not support yeast growth.

Although A. curvatum grew well on fatty acid mixtures isolated from soybean or corn oil, the yeast did not grow on mixtures of purified fatty acids containing more than 20% linoleic acid or more than 15% linolenic acid. However, supplementation with 1000 ppm butylated

hydroxyanisole (BHA) allowed polyunsaturated fatty acids to support yeast growth and oil accumulation. Polyunsaturated fatty acids yielded more yeast biomass and lipid content than did oleic acid. When oleic-linoleic mixtures in various combinations were used as carbon sources, the yeast grew well, and produced triglycerides with an extensive range of acyl composition. The yeast showed a definite preference in incorporating linoleate over oleate into its triglycerides; however, oleate was favored at the sn-2-position. Yeast triglycerides produced on oleic-linoleic mixtures contained less than 3% saturates. When oleic-linoleic acid mixtures were used as carbon sources, linear relations were observed in the plots of percentages of acyl groups at the three glycerol positions versus those of whole yeast triglyceride. These plots were similar to those reported earlier in vegetable oils. A simple mathematical model of triglyceride biosynthesis was proposed to account for these observations. The theory suggests a mechanism which can take and add acyl groups from and to glycerol positions. It also suggests that there may be two pathways leading to triglycerides.

When crambe oil was used as a carbon source, A. curvatum discriminated against the incorporation of C_{20}, C_{22} and C_{24} fatty acids, and these were accumulated in the residual medium oil. However, the incorporation of these fatty acids was not completely avoided. The yeast also discriminated against the deposition of cholesterol from its medium into its fat depots, but it is difficult to define internal and external cholesterol exactly.

The ability of A. curvatum to tolerate and metabolize some unusual

fatty acids of economical importance was tested. The yeast grew on tung oil, castor oil and oil extracted from Vernonia anthelmintica seed, and deposited eleostearate, ricinoleate and vernolate in its triglyceride, respectively. In all three instances, the yeast triglyceride contained significantly less of these fatty acids than was found in the substrate oils.

Following are the major accomplishments of this study:

1. Demonstrated that A. curvatum can and will desaturate and elongate substrates under some conditions, and not simply deposit the same fatty acids as in the medium.
2. Demonstrated only palmitic, oleic, linoleic, linolenic and other unsaturated fatty acids with 16 and 18 carbons were well used. This suggests that it will not be easy to transfer short-chain acyl groups across species boundaries. But, ricinoleate, vernolate, eleostearate, petroselinate biosynthesis should be capable of transfer across species boundaries because these acyl groups were well used by the A. curvatum which does not synthesize them.
3. Found linear segments in the plots of percentages of acyl groups at the three glycerol positions versus those of whole yeast triglyceride when oleic-linoleic acid mixtures were used as carbon sources. These were similar to those reported in vegetable oil varieties. A simple mathematical model of triglyceride assembly was proposed to explain these observations.
4. Determined that it may be possible to use A. curvatum to remove the cholesterol in animal fats by letting the yeast grow on animal fats

and recovering the triglyceride deposited in the yeast.

5. Determined that it may be possible to concentrate the erucic acid in rapeseed and crambe oils by letting the yeast preferentially accumulate the 16- and 18-carbon acyl groups in the oil.

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