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CHEMICAL CHARACTERIZATION OF LIPIDS SYNTHESIZED BY CERTAIN YEAST STRAINS GROWN ON WHEY PERMEATE

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Major professor

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CHEMICAL CHARACTERIZATION OF LIPIDS SYNTHESIZED BY CERTAIN YEAST STRAINS GROWN ON WHEY PERMEATE

BY

Pervaiz Akhtar

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ABSTRACT

CHEMICAL CHARACTERIZATION OF LIPIDS SYNTHESIZED BY CERTAIN YEAST STRAINS GROWN ON WHEY PERMEATE

By

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The study was designed to identify yeast strains capable of utilizing whey permeate for lipid biosynthesis, to study triacylglycerol structure and to investigate the effect of cabon:nitrogen ratio on various parameters.

Lipomyces starkeyi ATCC 12659 was identified as a high producer of lipids. It synthesized 36.9% lipids based on dry cell mass at carbon:nitrogen ratio of 30:1 in shake flask experiments. The significant ($P<0.05$) effect of both carbon:nitrogen ratio and the yeast strain was observed on dry cell mass yield and lipid biosynthesis. Palmitic and oleic acids were found as predominant fatty acids in triacylglycerol fraction, whereas phospholipid fraction was dominated by oleic acid among the yeast strains which synthesized high amount of lipids. The sn-1 and sn-2 positions of triacylglycerol fractions were occupied by unsaturated fatty acids, mainly oleic acid. Position sn-3 showed the high contents of saturated fatty acids especially palmitic acid. The presence of alpha-tocopherol was detected in the lipids synthesized by Lipomyces starkeyi ATCC 12659.

THIS DISSERTATION IS DEDICATED TO THOSE WHO ENDEAVOURED HARD AND DEVOTED THEIR TALENTS AND LIVES FOR THE WELFARE OF HUMANITY

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Finally and for many reasons, I owe the greatest

debt - an immense one - to my loving wife, sweet sons, parents, in-laws, sister and brothers.

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The capability of microorganisms to synthesize substantial amounts of lipids has been known for many years. German scientists pioneered the commercial production of microbial lipids, particularly during World Wars I and II, though their attempts were apparently not successful. These early endeavors were extensively reviewed by Woodbine (1959). Despite the huge increase in the production of edible oils and fats over the past 25 years, there is still an overall shortage in the world supply, especially in developing countries. Interest in the production of lipids by unconventional routes such as fermentation and plant cell culture has blossomed lately as evident from several recent reviews (Ratledge,1984; Ratledge and Boulton,1985; Ratledge,1988).

Reasons for this renewed interest include the development of improved methods for large scale cultivation of microorganisms and some new practical applications of microbial lipids. The use of renewable and inedible plant waste hydrolyzates, industrial waste materials and other cheap substrates as sources of more valuable products is also getting attention. The instabilities experienced in supplies and prices of edible oils and fats is playing a pivotal role

in motivating scientists to explore new sources of edible oils and fats. It has recently been reported that John and E. Sturge Ltd (Selby, North Yorkshire,UK) has started commercial production of lipids rich in gamma linolenic acid by a fungal fermentation process (Sinden,1987). This announcement has increased speculation that substential quantities of lipids may be produced by utilizing cheap and surplus carbohydrate sources through similar fermentation processes.

The economic feasibility of yeast lipid production would be far more favorable if high priced lipids like cocoa butter could be produced. Cocoa butter is the most expensive edible fat in the world. The European price of cocoa butter for the trading year 1984/85 was quoted as US $4,500 per metric tonne. Other vegetable oils such as soybean oil, palm oil and corn oil were sold for $392, $560 and $254, respectively, per metric tonne (Moreton,1988). High price and erratic supply of cocoa butter have forced manufacturers of chocolate products to explore ways of supplementing cocoa butter with other type of fats without altering the quality characteristics of the final product. Recently, it has been demonstrated that some mutants of the yeast, Apiotrichum curvatum ATCC 20509, lacking in Δ^9-desaturase which is responsible for converting stearic acid to oleic acid in vivo, produced high amounts of lipids consisting of more than 90% triacylglycerols. These triacylglycerols contained 60-64% saturated fatty acids and resembled the composition of cocoa butter when grown in the

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presence of oleic acid and Tween 80 in a semi-defined growth medium containing glucose as the carbon source (Ykema et al.,1989). Microbial lipids,therefore, have enough potential to be used for interesterification and as a substitute for certain lipids. However, their acceptance as an edible foodstuff may require Food and Drug Administration approval and, in addition, toxicological evaluation through extensive feeding trials would be necessary to obtain approval for food use.

Microorganisms capable of synthesizing high levels of lipid are defined as oleaginous microbes. Ratledge (1982) reported that an oleaginous organism must accumulate at least 20-25% lipid on the basis of its dry cell mass to be categorized as oleaginous. Not all microorganisms accumulate high quantities of lipids. Relatively few species within a given genera of yeasts and other microorganisms such as bacteria, molds and algae have been identified as potential sources of lipid production (Hammond and Glatz,1989). Even this capability is not shared by all strains of a given species. Yeasts are generally preferred over bacteria and algae as a source of oil production because of the high yield obtainable with some species, the quality of oil produced, the absence of toxic contaminants and the relative ease of growing yeast (Ratledge,1978). Yeasts can also be used for the production of novel lipids which are not readily synthesized by plants or animals. Commonly used oleaginous yeasts belong

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to the genera Candida, Cryptococcus, Endomycopsis, Rhodotorula and Trichosporon. The lipid content as a percentage of the biomass may range from 25% to as high as 70% in these genera (Ratledge,1982; Rattray,1984).

Disposal of whey arising from the manufacturing of cheese or casein presents a major challenge for the world dairy industry. The world-wide production of liquid whey is estimated to be 85 million metric tons per annum (Zall,1984). While approximately 16 billion kilograms of whey are produced in the United States, only about one half of this production is utilized as dried whey powder. In addition, a small portion of liquid whey is used for feeding hogs within an economic radius of 30-40 kilometers of dairy plants. The rest is dumped into sewers or disposed of on land (Ghaly et al.,1988). Because of its high biochemical oxygen demand (BOD, between 40,000 - 60,000 parts per million), whey may disrupt the biological process of sewage disposal plants (Ghaly and Singh,1985). Land disposal of whey may also impose environmental pollution problems. While some attempts have been made to recover protein from whey, little work has been done to utilize the remaining whey permeate which is rich in lactose. However, untreated whey permeate poses a problem as an environmental pollutant (Meyrath and Bayer,1979) and remains a major disposal problem. Lactose could be used as a potential source of carbohydrate for conversion into lipids by microorganisms. The initial carbon:nitrogen ratio of whey

permeate makes it excellent substrate for lipid production. However, whey permeate could also be used for the production of single cell protein. Fleotenmeyer et al. (1985) reported that oleaginous yeasts could reduce the BOD's of whey and whey permeates by 85% and 95%, respectively. The utilization of whey lactose as a fermentation feedstock will thus be of interest to the dairy industry.

Some studies have already been done to identify yeast strains which can efficiently synthesize lipid by utilizing different carbon and nitrogen sources under various experimental conditions (Yoon and Rhee,1983; Evans and Ratledge,1984a; Glatz et al.,1985; Ykema et al.,1989). However, few studies have focused on the triacylglycerol structure of microbial lipids.

Thus, the major objectives of the present study were:

1. To determine the efficiency of synthesizing lipids by some new yeast strains utilizing whey permeate as a carbon source.
2. To study the influence of the carbon : nitrogen ratio of the culture media on lipid production by yeast strains and on the fatty acid composition of lipids ; and
3. To study the triacylglycerol structure of microbial lipids.

REVIEW OF LITERATURE

Several species of oleaginous bacteria, yeast, mold and algae capable of synthesizing lipids in high quantity have been identified. Some of them can accumulate lipid up to 70% of their biomass when grown in a medium containing excess carbon and being deficient in nitrogen (Rattray et al.,1975; Ratledge,1978). Under such conditions, protein and nucleic acid synthesis is curtailed, whereas lipid synthesis is triggered (Kessell, 1968; Gill et al., 1977). On the other hand, non-oleaginous organisms do not accumulate lipids in high amount under these growth conditions. Babij et al. (1969) observed that Candida utilis, a non-oleaginous yeast strain, did not accumulate more than 7 or 8% of its biomass as lipid, even under optimum growth conditions. Similar results were obtained by Thorpe and Ratledge (1972). Many lipid-producing yeasts are recognized as facultative anaerobes. These microorganisms obtain their energy requirement mainly from fermentation under aerobic or anaerobic conditions (Matile et al., 1969).

Mechanism of lipid synthesis in microbes

The basic physiology of lipid accumulation in microorganisms has been understood for many years. Production

of intracellular triacylglycerols is induced when a nutrient limits the growth of oleaginous microorganisms while a carbon source is still available in excess. For instance, nitrogen limitation in the presence of glucose has been found critical for the synthesis of lipid by microorganisms (Roy et al.,1978; Hansson and Dostálek, 1986). The mechanism of lipid accumulation in Lipomyces starkeyi grown in batch culture (Boulton,1982) is depicted in Figure 1. The effect of other limiting nutrients such as phosphorous, magnesium or iron on lipid accumulation has also been investigated. The biomass yields in these cases appeared to be lower than that achieved by nitrogen-limited cultures, hence lower lipid production (Nielsen and Rojowski, 1950; Gill et al., 1977). It is likely that the deficiency of these micro-nutrients poses some metabolic problems to the microbial cells, consequently curtailing their efficiency for lipid synthesis.

In the initial phase of growth, organisms grow and divide normally. However, with the depletion of nitrogen from the growth medium, excessive glucose is dissimilated via the glycolytic pathway and the pentose-phosphate shunt. Both of these pathways eventually lead to the production of intramitochondrial acetyl coenzyme A which is the precursor of fatty acid synthesis and this assists in the accumulation of lipid as a carbon energy storage. The intermediary metabolism as linked to fatty acid biosynthesis in oleaginous yeasts (Ratledge,1987) is shown in Figure 2. The biochemical

Figure 1. Lipid accumulation in the oleaginous yeast, Lipomyces starkeyi, growing in batch culture (Boulton,1982).

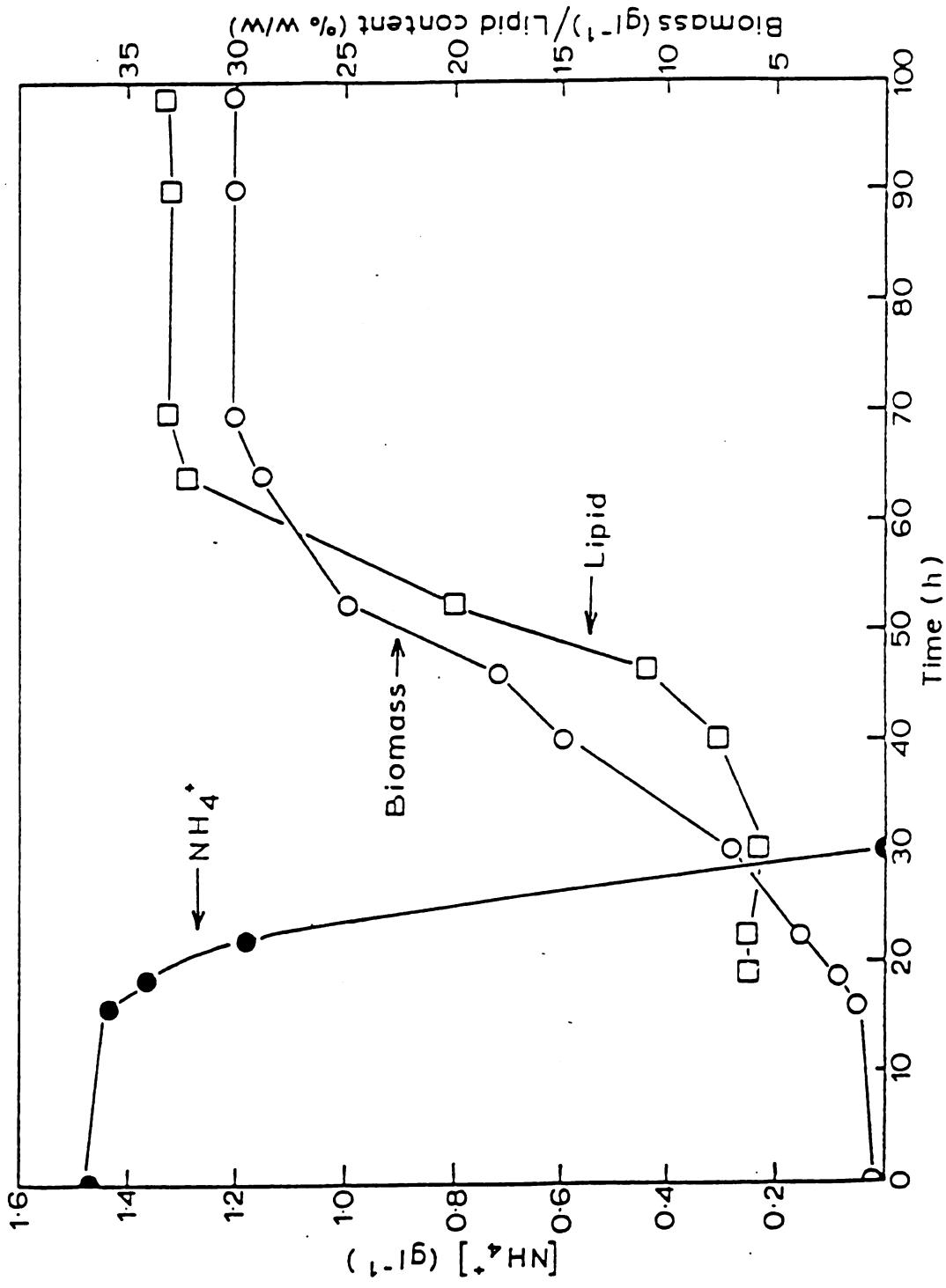
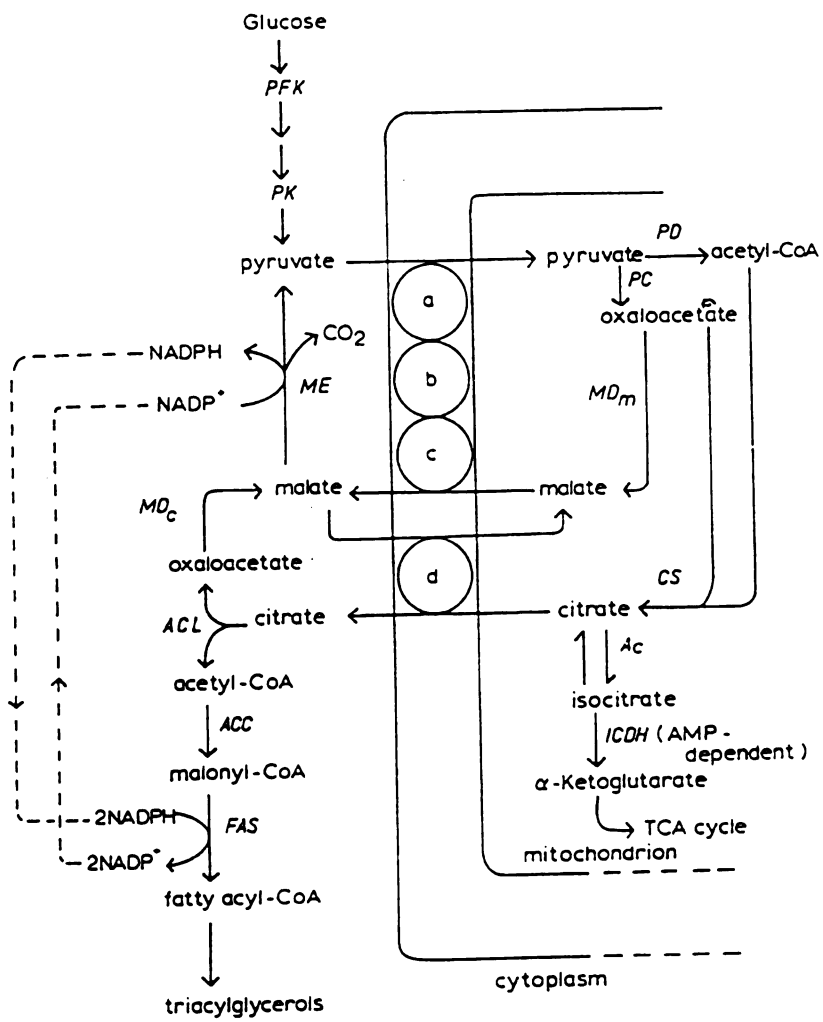

[NH4+] (gl-1)
Biomass (gl-1)/Lipid content (% w/w)
Time (h)
NH4+
Biomass
Lipid

Figure 2. Intermediary metabolism as linked to fatty acid biosynthesis in oleaginous microorganisms (Ratledge,1987).

Mitochondrial transport process: a,b,c, interlinked pyruvate-malate translocase systems; d,citrate-malate translocase.
Enzymes: AAC, acetyl-CoA carboxylase; AC, aconitase; CL, ATP:citrate lyase; CS, citrate synthase; FAS, fatty acid synthase complex; ID, isocitrate dehydrogenase; MD_c, malate dehydrogenase (cytosolic); MD_m, malate dehydrogenase (mitochondrial); ME, malic; PC, pyruvate carboxylase; PD, pyruvate dehydrogenase; PFK, phosphofructo kinase; PK pyruvate kinase.


Glucose
PFK
PK
pyruvate
pyruvate
PD
acetyl-CoA
PC
oxaloacetate
a
b
c
d
NADPH
NADP+
CO2
ME
MDm
MDc
malate
malate
oxaloacetate
CS
ACL
citrate
citrate
Ac
acetyl-CoA
isocitrate
ACC
ICDH (AMP-dependent)
malonyl-CoA
α-Ketoglutarate
2NADPH
FAS
TCA cycle
2NADP+
mitochondrion
fatty acyl-CoA
cytoplasm
triacylglycerols

difference between an oleaginous and non-oleaginous microorganism lies in the manner and rate by which they generate acetyl coenzyme A. Oleaginous microorganisms seem to produce acetyl coenzyme A in an effective manner because of the presence of an enzyme adenosine triphosphate:citrate lyase which is completely absent in non-oleaginous microorganisms. As a result of intramitochondrial coenzyme A production, an increase in the intracellular adenylate energy occurs with a concomitant decrease in the intracellular concentration of adenosine monophosphate by the action of adenosine monophosphate deaminase. The decrease in adenosine monophosphate level does not concide with any noticeable appearance of either adenosine diphosphate or adenosine triphosphate. Botham and Ratledge (1979) have shown that the intramitochondrial NAD^+-dependent isocitrate dehydrogenase of oleaginous yeasts is inactivated due to the low level of adenosine monophosphate, thereby restricting the oxidative role of the tricarboxylic acid cycle. This enzyme in oleaginous yeasts has a much higher dependency on the presence of adenosine monophosphate for its activity than it has in non-oleaginous yeasts.

Subsequent studies by Boulton and Ratledge (1980) indicated that the limited supply of nitrogen leads to the activation of citrate synthase in equilibrium with aconitase and results in the synthesis of citrate within the mitochondria. Citrate not only has an inhibitory effect on the

activity of phosphofructokinase, but it also stimulates acetyl-coenzyme A carboxylase (Bothum and Ratledge,1979). Evans and Ratledge (1984b) reported that at low intracellular concentration of ammonium ions, citrate inhibited the activity of phosphofructokinase, thus channelling glucose into high molecular weight carbohydrate production. However, this inhibition can be relieved under high intracellular concentrations of ammonium ion, thereby enabling the conversion of glucose to lipids. As adenosine triphosphate:citrate lyase is a rate limiting step in fatty acid biosynthesis (Boulton and Ratledge,1981), citrate should accumulate to a high steady state concentration before lipid accumulation begins. Citrate is then transported into the cytosol in exchange for malate (Evans et al.,1983) where it is converted into acetyl-coenzyme A and oxaloacetate by the action of adenosine triphosphate:citrate lyase. This reaction provides the precursor of the two carbon unit for lipogenesis.

The cytoplasmic malate does not originate from oxaloacetate, the second product of the reaction catalyzed by adenosine triphosphate:citrate lyase. Instead, it is produced by the action of malate dehydrogenase in mitochondria due to the inactivation of iso-citrate dehydrogenase.

$$\text{Oxaloacetate} + \text{NADH} \longrightarrow \text{Malate} + \text{NAD}^+$$

The oxaloacetate, as the precursor of cytoplasmic malate, itself arises from pyruvate and the uptake of pyruvate into the mitochondria is an event which can be coupled to the

transport of malate out of the mitochondria, though not as a simple single translocase event as happens in the case of the citrate/malate translocase system (Prebble, 1981). The final key step involved is the operation of the pyruvate malate cycle in which pyruvate carboxylase, malate dehydrogenase and malic enzyme catalyze their individual reactions. However, the reaction catalysed by malic enzyme is the key step which provides NADPH, which serves as reducing power for fatty acid synthesis.

$$\text{Malate} + \text{NADP}^{+} \longrightarrow \text{Pyruvate} + CO_2 + \text{NADPH}$$

Carbon sources for oleaginous organisms

Several carbon sources have sucessfully been used in the production of lipids by microorganisms. They include by-products of the agro-industries such as molasses, whey, whey permeate, potato starch, rice straw and banana (Kaur and Morgan,1982; Yoon *et al.*,1982; Moon *et al*,1983; Floetenmeyer *et al.*,1985; Ykema *et al.*,1989). In addition glycerol, ethanol and a full range of common pentoses, hexoses and disaccharides have also been evaluated (Moreton,1988). Of these substrates, glucose appears to be assimilated most efficiently by oleaginous microorganisms and is readily converted into lipids. Methanol is not utilized by oleaginous yeasts (Moreton,1988).

Results obtained by growing *Rhodotorula gracilis* indicated that glucose was only marginally better than ethanol

as a substrate (Krumphazl et al.,1973). Cryptococcus albidus grown on ethanol produced lipid contents up to 31% (Eroshin and Krylova,1983; Zhelifonova et al.,1983). However, Yamauchi et al. (1983) obtained only 21% conversion of ethanol to lipid by growing Lipomyces starkeyi.

Effect of carbon source on fatty acid composition

Moon and Hammond (1978) reported that significantly more saturated lipids were produced by Candida curvata D when whey permeate was used as a carbon source instead of whey. This may be related to the increased yield of oil by the yeast grown on whey permeate. However, no such effect was observed when Trichosporon cutaneum 24 was grown on the same substrates.

Nitrogen sources for oleaginous organisms

Various nitrogen sources consisting of organic and inorganic compounds have been utilized to grow yeasts for the production of lipids (Yoon et al.,1982; Farag et al.,1983; Evans and Ratledge,1984a). There is no firm evidence to indicate that oleaginous organisms have a preference for any particular type of nitrogen source when grown under controlled conditions. However, Evans and Ratledge (1984a) found that some organisms accumulate more lipid in shake flask experiments when asparagine or L-glutamate is used as the nitrogen source as compared to using ammonium chloride under the same experimental conditions. In one case involving

Rhodosporidium toruloides CBS 14, the lipid content increased from 18% to 51% of the dry biomass on replacing ammonium chloride with a nitrogen source of organic origin. The addition of 1% sodium chloride or potassium chloride to the medium also increased lipid production by this organism. A recent study by Moreton (1988) substantiated some of these conclusions using shake flask experiments. However, he found no systematic difference with either glutamate as nitrogen source or with the addition of 1% potassium chloride when cultures were grown under controlled conditions in a fermenter. He obtained the highest yield of lipids using glucose and ammonium chloride as sources of carbon and nitrogen, respectively, during fermenter studies.

Effect of C:N ratio of growth medium on lipid synthesis

The relative carbon to nitrogen ratio of the growth medium seems to be critical for the production of lipids by yeast (Yoon et al.,1982; Evans and Ratledge,1984a,b; Turcotte and Kosaric, 1989). Oleaginous organisms usually do not express their potential capability for synthesizing lipids to any great extent in media with a C:N ratio of less than 20. The optimum ratio for different organisms appears to vary between 30 and 80 (Moreton, 1988).

Yoon et al (1982) reported that both the C:N ratio and nitrogen sources affected the lipid content of Rhodotorula gracilis NRRL Y-1091. Goulet (1975) and Turcotte and Kosaric

(1989) who used Rhodotorula glutinis and Rhodosporidium toruloides ATCC 10788, respectively, reported that the individual fatty acid composition of the lipids changed significantly only prior to nitrogen depletion and remained constant thereafter. This steady state was maintained even though lipids were still being synthesized. Similar changes were reported by Kates and Paradis (1973) who used Candida lipolytica. Evans and Ratledge (1984a) studied the effect of various nitrogen sources on lipid production by Rhodosporidium toruloides and observed that the type of nitrogen supplied to the cells at an initial C:N molar ratio of 108 greatly influenced the lipid content. However, Turcotte and Kosaric (1989) observed an optimum initial C:N molar ratio of about 77 for maximum lipid production by Rhodosporidium toruloides ATCC 10788 using glucose or fructose as carbon source and ammonium sulfate, ammoniun nitrate or urea as nitrogen sources. They reported that the concentration of individual fatty acids varied as a function of both C:N ratio and type of carbon source used. No significant change was observed due to the type of nitrogen source used. At a C:N ratio of 55, the oleic acid (C18:1), stearic acid (C18:0) and palmitic acid (C16:0) concentrations were 60%, 3% and 10%, respectively. Oleic acid decreased to 40% when the C:N ratio was increased to 99, and no further decreases occured thereafter, although palmitic acid and stearic acid increased to 25% and 10%, respectively. However, both linoleic acid (C18:2) and

linolenic acid (C18:3) changed less than 5%. The sugar source also produced similar variations in the oleic acid, stearic acid and palmitic acid contents as those observed in the case of changing C:N ratio. However, linoleic acid varied from 5% to 28% due to the effect of the sugar source and no linolenic acid was found when the cells were grown in the presence of fructose.

Cryptococcus albidus var. albidus is regarded as an exception among lipid synthesizing yeast strains, since it is reported to be able to accumulate up to 70% of lipids regardless of the C/N ratio of the growth medium (Pedersen,1961). Hansson and Dostálek (1986) reported that Cryptococcus albidus var. albidus CBS 4517 was able to synthesize lipid under nitrogen-limited as well as excess-nitrogen conditions. However, nitrogen-limited conditions enhanced lipid-synthesizing capacity.

Effect of other nutrients

Gill et al. (1977) grew Candida sp.107 (NCYC 911) in continuous culture limited by nitrogen, phosphorus and magnesium. Carbon-limited cultures had a lipid content of approximately 10% and a biomass yield of 50%. In contrast, nitrogen-limited cultures contained a maximum of 37.1% lipid at a dilution rate of $0.06h^{-1}$, with a high biomass yield of 60.2%, equivalent to a lipid yield of 22.4% of the substrate. Phosphorus limitation produced biomass levels less than 50%

of those observed under nitrogen limitation and lipid yields of only 10-15%. Magnesium limitation gave an even lower yield of biomass and lipid content. A C:P ratio close to 200:1 was found to be the optimum for lipid production. Earlier, Nielsen and Rojowski (1950) found similar trends with iron limitation in the media of Rhodotorula gracilis utilizing glucose as had Gill et al. (1977) in the case of nitrogen, phosphorus and magnesium limitation.

Effect of temperature on lipid accumulation

The fatty acid composition of most microorganisms and plants is substantially affected by environmental temperature as lipid composition plays an important role in maintaining appropriate membrane fluidity. Higher temperatures favor the synthesis of saturated fatty acids, while lower temperatures enhance the production of unsaturated fatty acids. For instance, Moon and Hammond (1978) found that fatty acids produced by Candida curvata D at 15°C were slightly more unsaturated than those produced at the optimum temperature (30°C). However, they did not observe a similar trend in the case of Trichosporon cutaneum 40.

Ferrente et al. (1983) found an increase in linoleic acid content at 10°C as compared to fermentation at 25°C. Similarly, Ervin et al. (1984) reported that Acinetobacter H01-N grown at 17°C had higher concentrations of polyunsaturated wax esters than had the yeast grown at 30°C.

On the other hand, Hasson and Dostálak (1986) observed no difference in the lipid composition of Cryptococcus albidus var. albidus over the temperature range 20°C to 25°C.

Effect of medium pH on lipid accumulation

Various studies have shown that oleaginous yeasts can grow over a wide range of pH. The pH profile of the growth medium does not appear to be critical for most of the oleaginous yeasts, except at low pH values. For instance, Kessell (1968) grew Rhodotorula gracilis over a pH range of 3.0 to 6.0 and found a reduction in palmitic acid and linoleic acid at pH 4.5 as compared to pH 3.0 and 6.0. Ratledge et al. (1984) observed that Candida 107 grew over the pH range of 3.5 to 7.5 without showing significant difference in biomass, lipid synthesis and fatty acid composition. Davies (1988) obtained similar results while growing Candida curvata in the pH range of 3.45 to 5.70. However, he observed a substantial effect in biomass and lipid composition at pH 2.75.

Effect of growth rate and oxygen tension on lipid synthesis and composition

Kleinzeller (1944) reported that aeration did not influence significantly the lipid content in most lipid-producing microorganisms. Brown and Rose (1969) observed only a slight increase in cell biomass of the facultative anaerobe Candida utilis when oxygen tension was increased under

nitrogen-limiting conditions. This variation in behavior may be due to differences in the mitochondrial systems of obligate aerobes and facultative anaerobes (Matile et al.,1969).

Gill et al. (1977) observed variation in the fatty acid composition due to growth rate of Candida grown with a restricted supply of carbon or nitrogen in the medium. However, Ratledge (1979) observed no significant change in the fatty acid composition due to growth rate when Rhodotorula glutinis NCYC 154G was grown under nitrogen-limiting conditions. This suggests that the effect may be strain specific. Variation in fatty acid composition was also reported by Choi et al. (1982) with increase in growth rate of the obligate aerobe Rhodotorula gracilis NRRL Y-1091 while grown in continuous culture under nitrogen limitation. They found an increase in the protein content of the biomass but cell biomass, lipid content and lipid productivity were decreased. The specific lipid production rate remained constant at about 0.012 g lipid/g dry biomass/h. Maximum lipid contents, 49.8% (w/w), were obtained at a growth rate of $0.02h^{-1}$. The growth rate also affected fatty acid composition. Linoleic acid and linolenic acid increased with increasing growth rate, while other fatty acids such as palmatic acid, stearic acid and oleic acid decreased with increasing growth rate. However, increasing the oxygen concentration from 45 to 234 μM increased the lipid content without affecting the degree of unsaturation.

Synthesis of modified lipid by yeast

Extensive studies have been carried out to modify the composition of lipids synthesized by oleaginous yeasts. Modification of yeast lipids is likely to be restricted to the production of high priced fats for economic reasons. A number of exogenous lipid sources and fatty acid esters are used to modify the composition because of capability of oleaginous organisms to utilize lipids and fatty acid esters.

Glatz et al. (1984) grew Candida lipolytica on corn oil, linseed oil, olive oil and palm oil. They found identical lipid compositions when the organism was grown on palm oil and olive oil. When linseed oil was used in the growth medium, the linolenic acid present in the linseed oil was exchanged with oleic acid in the lipid synthesized by the organism. Similar exchanges occurred with linoleic acid and oleic acid in the case of the corn oil substrate. Scragg and Moreton (as cited by Moreton,1988) showed that addition of antibiotic cerulenin (2,3-epoxy-4-oxo-7,10 dodecadienamide) from Cephalosporium caerulens to the growth medium at a concentration of 10 μg ml^{-1} inhibited de novo synthesis of fatty acids in the yeast Candida sp.107. Addition of the antibiotic to the growth medium containing 1.4 mg ml^{-1} of methyl esters of oleic acid, palmitic acid and stearic acid allowed the growth nearly 50% of the control without the antibiotic. They also observed that variation in the composition of the methyl esters in the growth medium was

reflected in the fatty acid composition of extracted lipids.

Ykema et al.(1989) attempted to isolate mutants of Apiotrichum curvatum which synthesize lipids resembling cocoa butter. Cocoa butter mainly consists 1,3-disaturated-2-unsaturated triacylglycerols, with 60-64% saturated fatty acids (Fineke,1965). They observed that isolated mutants, grown in the presence of relatively small amounts of oleic acid, synthesized high amounts of lipids containing more than 90% triacylglycerols. These triacylglycerols had saturated fatty acids comparable to those in cocoa butter.

In an other effort to produce lipids resembling the composition of cocoa butter, Verwoert et al. (1989) recently used intraspecific spheroplast fusion, between a methionine auxotrophic mutant and an unsaturated fatty acid mutant, to modify the fatty acid composition of lipids synthesized by Apiotrichum curvatum ATCC 20905. Protoplasts of an unsaturated fatty acid mutant and a methionine auxotrophic mutant suspended in buffer were mixed in a 1:2 ratio following the generation of protoplasts through enzymic treatment. Prototrophic colonies were incubated on a selective regeneration medium after polyethylene glycol (PEG) treatment. Fusion products resulted from nuclear hybridization. Fusion frequencies (10^{-4} to 10^{-5}) were 10^{3} to 10^{6} fold higher than the back mutation frequencies for each parent. Furthermore, no prototrophic colonies were observed in the fusion control plates when PEG treatment was omitted. In order to verify that

the recovered prototrophs were indeed hybrids and not reverted mutants, flow-cytometric DNA-content analysis was performed. The DNA levels of the investigated hybrids ranged from 1.3 to 2.0 times the values obtained in case of parental strains. Lipids isolated from the hybrids contain a higher level of saturated fatty acids compared to wild-type Apiotrichum curvatum indicating a partially decreased Δ^9-desaturase activity in hybrid strains. The authors considered the intraspecific spheroplast fusion technique as a promising approach for the production of cocoa butter equivalents. Other methods that have been described in the literature for the microbial production of cocoa butter equivalents include supplementation of growth medium with saturated fatty acids (Gierhart, 1984) and the addition of sterculic acid (an inhibitor of Δ^9-desaturase) as reported by Moreton (1985).

Stereospecific analysis of microbial lipid

Progress in understanding the metabolism and function of microbial lipids has required, with increasing frequency, knowledge of how the fatty acids are distributed at position 1, 2, and 3 of sn-glycerol. Extensive studies conducted on the distribution of fatty acids in the triacylglycerols obtained from vegetable oils and animal fats are found in the literature (Brockerhoff and Yurkowski,1966; Akesson,1969; Christie and More,1969; Fatemi and Hammond,1977; Parodi,1979; Pan and Hammond,1983). However, only limited work has been

done on the composition of triacylglycerols derived from microorganisms.

Haley and Jack (1974) determined the stereospecific distribution of fatty acids in triacyglycerols, phosphotidylcholine and phosphotidylethanolamines of Lipomyces lipoferus. They reported that triacylglycerols had predominantly unsaturated fatty acids at position sn-1 and sn-2, with oleic acid (C18:1) being the major component. The percentage of unsaturated fatty acids found at sn-1 and sn-2 positions of triacylglycerols was observed as 61% and 88%, respectively. Position sn-3 had equal proportions of unsaturated and saturated fatty acids. In both classes of phospholipids, positions sn-1 and sn-2 had a quantitatively distinctive fatty acid distribution and C 18 unsaturates were predominant at position sn-2. Position sn-2 of phosphatidylcholine was 26% more unsaturated than was position sn-1. In the case of phosphatidylethanolamine, position sn-2 was 10.8% more unsaturated than position sn-1. Position sn-1 and sn-2 of these phosphoglycerides had different fatty acid profiles than positions sn-1 and sn-2 of the triacylglycerols.

Thorpe and Ratledge (1972) studied the fatty acid distribution in triacylglycerols of seven different yeasts (Candida lipolytica, Candida tropicalis, Candida utilis, Candida 107, Hansenula anomala, Rhodotorula glutinis and Rhodotorula graminis) grown on glucose or n-alkanes. In each

yeast approximately 80% of the total lipid consisted of triacylglycerols. Five yeasts, from three genera, when grown on glucose produced triacylglycerols containing almost entirely unsaturated fatty acids in position sn-2. These results were similar to those obtained from triacylglycerols of plant sources (Vander Wal,1964). Rhodotorula graminis and Candida 107 produced an unusually high proportion of saturated fatty acids in the lipid (52% and 46%, respectively) which, therefore, led to a high content of 1,3-disaturated-2-monounsaturated triacylglycerol in the neutral lipid fraction (53% and 50%, respectively). When Candida 107 and Candida tropicalis were grown on individual n-alkanes, from C_{12} to C_{16}, the fatty acid composition varied according to the chain length of the substrate, although with n-tridecane neither yeast produced tridecanoic acid in the triacylglycerol. With n-dodecane, only Candida tropicalis contained an appreciable amount of dodecanoic acid in the triacylglycerol (32% of the fatty acids). Saturated fatty acids were found at the sn-2 position of the triacylglycerols on each alkane substrate. Candida 107 grown on n-tetradecane produced 46% of its triacylglycerols with a saturated fatty acid at position sn-2.

DeBell and Jack (1975) determined the positional distribution of fatty acids in triacylglycerols, phosphatidylcholine and phosphatidylethanolamine from the mycelium and sporangiophores of the fungus Phycomyces

blakeslecanus. At the sn-1 positions of the triacylglycerols from both regions of the fungus, greater than 65% of the fatty acids were palmitic acid and oleic acid. At the sn-2 position oleic acid, linoleic acid and linolenic acid comprised greater than 85% of the sporangial fatty acids and more than 90% of the mycelial fatty acids. Position sn-3 of both the sporangial and mycelial triacylglycerols contained approximately 40% palmitic acid, approximately 30% linoleic acid and 21% linolenic acid. Phosphatidylcholine and phosphatidylethanolamine both contained approximately 98% unsaturated fatty acids at position sn-2. Palmitic acid, linoleic acid and linolenic acid contributed more than 85% of the fatty acids at position sn-1.

Synthesis of tocoperols by microorganisms

It is well established that eukaryotes contain tocopherols, an antioxidant, found abundantly in oil from plant sources. Tocopherols act as inhibitors of free radical formation and as physico-chemical quenchers of the superoxide anion radical and singlet oxygen. They, therefore, protect biological membranes by stabilizing the lipid layer against peroxidation (Tappel,1972; Foote,1976; Fukuzawe et al.,1985). On the other hand, contradictory data are found in the literature concerning the presence of lipid antioxidants in prokaryotes (Zarubina et al.,1990). It has been previously

reported by Hidetoshi et al. (1983) that the photosynthetic microorganisms are the only group of microorganisms that can produce tocopherols. However, recent studies do indicate that Escherichia coli strains also possess antiradical activity in order to protect their biological membranes (Bogoslovskaya et al.,1985). Bacilli have been extensively studied as organisms which produce many biologically active substances. Zarubina et al. (1990) studied certain features of lipid metabolism in natural variants of Bacillus brevis var.G.-B and Bacillus brevis mutant 101. They found that under submerged cultivation conditions variants P^+ and Bacillus brevis mutant 101, which produce the antibiotic gramicidin S, also synthesize tocopherols during the logarithmic phase of growth at higher levels than cells of other colony and morphological variants. The greater levels of tocopherols were found in the P^+-variant. It is noteworthy that the cells of P^+-variant contained somewhat higher levels of total lipids than cells of other gramicidin S-producing variants.

MATERIALS AND METHODS

Materials

Whey Permeate

Sweet whey permeate produced at the Dairy Pilot Plant, Michigan State University was used as a substrate for biosynthesis of lipids.

Yeast Strains and Growth Media

Four yeast strains, Apiotrichum curvatum ATCC 10567, Cryptococcus albidus ATCC 56297, Lipomyces starkeyi ATCC 12659, and Rhodosporidium toruloides ATCC 10788, were obtained from the American Type Culture Collection (Washington, D.C). YM broth, sabouraud dextrose broth and agar were purchased from Difco Laboratories (Detroit, MI). YM broth was used for the subculturing of Apiotrichum curvatum ATCC 10567, Cryptococcus albidus ATCC 56297 and Rhodosporidium toruloides ATCC 10788. YM broth contained yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L and dextrose 10 g/L and its pH was 6.2. Sabouraud dextrose broth containing neopeptone 10 g/L and dextrose 20 g/L was used for the subculturing of Lipomyces starkeyi ATCC 12659. pH of sabouraud dextrose broth was 5.6.

Reagents, Solvents and Chemicals

All reagents, solvents and chemicals utilized in this study were reagent grade.

Experimental

Storage and Analysis of Whey Permeate

Whey permeate was packed in twenty 10 liter polyethylene bags (Cryovac Division, WR Grace and Co., Duncan, SC) and stored at -15°C in a walk-in freezer until used for study. The whey permeate was thawed at room temperature before using as a substrate for the production of lipids by yeast. The composition of whey permeate was determined as 3.64% lactose, 0.08% protein, 0.003% fat, 0.34% ash and 4.96% total solids. Its pH value was 6.29.

Preservation and Subculturing of Yeast Strains

Lyophilized cultures of all four yeast strains were thawed at room temperature and transferred to broths as described above. These strains were grown at 25°C for 48 hours before subculturing in fresh media. After their activation, 0.5 mL of 24 hour old cultures of all strains were transferred to cryogenic tubes along with 0.5 mL of 40% sterilized glycerol. Six cryogenic tubes were prepared for each strain and immediately frozen and stored at -80°C until required. Stock cultures were transferred periodically to freshly prepared broth or slants and grown for 48 hours before use or storage.

Preparation of Culture Media

Whey permeate was thawed at room temperature and blended by stirring with a nitrogen source (asparagine) and other nutrients to make up the composition as shown in Table 1. Culture media with different carbon:nitrogen ratios were prepared by varying the amount of added asparagine, assuming 10.5% nitrogen and 26% carbon in the yeast extract (Ykema et al.,1988). All other nutrients except manganese sulfate and cupric sulfate were added in the ratios described by Ykema et al. (1988). Manganese sulfate and cupric sulfate were incorporated at the levels used by Vega et al. (1988).

Fermentation Conditions

Twenty-five mL of prepared culture medium were used as a seed culture and transferred to 100 mL Erlenmeyer flasks with baffles at the bottom. These flasks were specially designed by Bellco Glass Inc. (Vineland, NJ) for biotechnological studies. The culture medium was sterilized at 121°C for 15 minutes in an autoclave after adjusting the pH to 5.5, with an Orion pH meter (model 601A/digital ionilyzer, Orion Research Inc. Cambridge, MA). Cotton plugs were used to close the flasks. The seed culture was inoculated with 1.0 mL of 48 hour old yeast cells grown in broth. The suspension of seed cultures for all yeast strains were incubated at 30°C and kept shaking at 200 rpm in a water bath for 24 hours. The yeast strain Cryptococcus albidus ATCC 56297 was incubated at 20°C as suggested by Hansson and Dostálak

Table 1. Composition of the culture media at different carbon : nitrogen ratios

Ingredient (g/L)[1]	Carbon:Nitrogen ratios 30:1	45:1	60:1	75:1
Asparagine	1.486	0.655	0.252	0.013
Yeast extract	0.650	0.650	0.650	0.650
Potassium phosphate	3.500	3.500	3.500	3.500
Sodium phosphate	1.000	1.000	1.000	1.000
Magnesium sulfate	0.750	0.750	0.750	0.750
Calcium chloride	0.050	0.050	0.050	0.050
Ferric chloride	0.005	0.005	0.005	0.005
Zinc sulfate	0.005	0.005	0.005	0.005
Manganese sulfate	0.020	0.020	0.020	0.020
Cupric sulfate	0.0001	0.0001	0.0001	0.0001

[1] Composition of all ingredients is given on the basis of one litre of whey permeate.

(1986).

A volume of 250 mL of the same culture medium was transferred to each Erlenmeyer flask (1 liter) having baffles and sterilized at 121°C for 15 minutes after adjusting the pH of the culture medium to 5.5. The seed cultures were then aseptically transferred to one liter Erlenmeyer flasks containing 250 mL of culture medium and closed with cotton plugs. The strains were incubated for varying times depending upon their growth behavior and consumption of carbon and nitrogen sources. At 24 hour intervals, 3 mL of sample were drawn aseptically from each flask and used for determining optical density, lactose concentration and protein content.

Harvesting of Yeast Cells

After the completion of fermentation, cells were harvested by centrifugation and by removing the supernatant. Cells were washed two times with 0.1 M phosphate buffer, centrifuged and the supernatant removed each time. Cells were weighed and a portion was taken to determine dry cell mass. Lipids were extracted from the remaining cells and stored for further studies.

Methods of Analysis

Analysis of Whey Permeate

Ash and total solids were determined according to proceedures 14.006 and 22.018, respectively, of AOAC (1984).

Lactose was determined by a method described by Teles et

al. (1978). The lactose was quantitated by reading the absorbance at a wavelength of 520 nm against a similarly treated reagent blank using a double beam spectophotometer (Bausch and Lomb Inc., Rochester, NY). Color development was based on the combined action of phenol, sodium hydroxide, picric acid, and sodium bisulfite with lactose. Results were compared with a standard solution of lactose after reacting with reagents as described for the test samples.

Protein content was determined by the method of Waddel (1956). This method is based on differential measurements at wavelegths of 215 nm and 225 nm. The reading obtained at 225 nm was substracted from that at 215 nm and the difference multiplied by 144 which gave the protein concentration in the sample in μg/mL.

The lipid content was determined by the solvent extraction method of Folch et al. (1957).

pH determination was made by using a pH meter, model 601A/digital ionilyzer (Orion Research Inc., Cambridge, MA).

Analysis of Fermentation Medium

Growth of the cells in the fermentation medium was monitored by recording the optical density of an aliquot of the culture at a wavelength of 610 nm (Adler et al.,1985) using a double beam spectrophotometer (Bausch and Lomb Inc., Rochester, NY).

Residual lactose and protein contents were determined every 24 hours during the fermentation process by the methods

described previously.

Dry cell mass was determined after harvesting the cells by the method of Vega et al. (1988), except that drying was carried out at 100°C in an oven for 24 hours instead of drying in a vacuum oven at 65°C.

Extraction of Lipids from Yeast Cells

Lipids from yeast cells were extracted by a method of Bligh and Dyer (1959) using methanol and chloroform. Extracted lipids were transferred to tared vials, weighed and dissolved in chloroform. One to two drops of methanol were also added before flushing the vials with nitrogen. Samples were stored at -20°C for subsequent analysis.

Separation of Lipid Classes

Thin-layer chromatography was used to separate triacylglycerols and phospholipids as described by Engeseth (1990). A small amount of lipid sample dissolved in chloroform for each yeast strain grown at different carbon:nitrogen ratios was dried under a stream of nitrogen gas. A volume of 400μL of a mixture of dichloromethane and methanol (9:1, v/v) was added to each of the samples and 200μL of it streaked onto thin-layer chromatographic 20cm x 20cm glass plates coated with silica gel type G (Sigma Chemical Co., St. Louis,MN), (particle size 10-40μ) with a thickness of 250nm. The plates were activated in an oven at 105°C for two hours and then cooled in a desiccator before use. The plates were developed

in a closed glass chamber using a mixture of petroleum ether, diethyl ether and glacial acetic acid (80:20:1, v/v). The developed plates were removed from the tanks, air-dried and sprayed with rhodamine G (0.05% in 95% ethanol) prior to viewing under ultraviolet light. The bands of triacylglycerols and phospholipids were identified by using pure soybean oil and phosphatidylethanolamine (Sigma Chemical Co., St. Louis, MO) as standards, marked, scraped and collected separately in a disposable pipette plugged with glass wool at the tip using a vacuum pump. The triacylglycerol and phospholipid fractions were eluted with dichloromethane/ methanol solvent (9:1,v/v) into tared vials. These vials were flushed with nitrogen and subsequently stored in a freezer until used for fatty acid analysis.

Quantitation of Lipid Classes

Triacylglycerol and phospholipid fractions from each lipid source were separated on silica gel G plates as described above. The lipid classes on the developed plates were visualized after spraying with sulfuric acid:chromic acid (95:5, v/v) and subsequently charring at 150°C for 20 minutes. Quantitation of each fraction was achieved by using a Shimazu densitometer (Shimadzu Dual-Wavelength Thin layer Chromato Scanner Model CS-930). The individual triacylglycerol and phospholipid data were expressed as a percentage of the total lipid content.

Fatty Acid Analysis

Methyl esters of triacyglycerols were prepared following the boron trifluoride-methanol method of Morrison and Smith (1964). Methyl esters of phospholipids were prepared according to the alkaline esterification method described by Maxwell and Marmer (1983) to avoid the possible formation of dimethylacetals which are derived from plasmalogens under acidic esterification conditions (crackel et al.,1988). Plasmalogens containing aldehydes bound as enol ethers are found in greater concentrations in the phospholipid fraction (Maxwell and Marmer,1983). Acidic esterification conditions yield a stable dimethylacetal derivative of the aldehyde which, under certain conditions can interfere with the analysis of fatty acid methyl esters. Fatty acid analysis was performed by gas chromatography.

Gas Chromatographic Analysis of Fatty Acid Methyl Esters

Fatty acid methyl esters were separated and quantitated using a Hewlett Packard gas chromatograph, (Model 5890A, Hewlett Packard, Avondale, PA) equipped with a flame ionization detector. A fused silica capillary column (DB-225, J & W Scientific, Folsom, California) with a length of 30 meters and an inside diameter of 0.25 mm was used for separation of the fatty acid methyl esters. Helium was used as a carrier gas and a split ratio of 20:1 was maintained. The GC oven temperature was initially held at 175°C for 10 minutes, then increased at a rate of 1.5°C /min to a final

temperature of 200°C and held for 47 minutes. The injector and detector temperatures were maintained at 275°C and 300°C, respectively.

Identification of the fatty acid methyl esters was based on comparison of retention times of samples to those of standard fatty acid methyl esters (Supelco, Bellefonte, Pennsylvania). Peaks area of each fatty acid was computed by an integrator (Model 3392A, Hewlett Packard, Avondale, PA) and reported as weight percent of total fatty acid methyl esters.

Stereospecific Analysis of Triacylglycerols

Stereospecific analysis of the triacylglycerol fraction was carried out by employing the following procedures:

i) Lipolysis of the triacylglycerol fraction with pancreatic lipase (Sigma Chemical Co., St. Louis, Missouri) was carried out on 40-50 mg samples by the procedure of Luddy et al. (1964) with certain modifications. The original procedure recommended the use of 0.5 mL 6 N HCl to stop lipolysis, but Synder and Piantadosi (1968) demonstrated that such acidic conditions promoted acyl migration in monoacylglycerols and diacylglycerols obtained as a result of lipolytic reaction. Therefore, 5 mL of ethanol were added at the end of the reaction period to stop the enzymic reaction (Synder and Piantadosi,1968). Other modifications included the incorporation of hexane (0.25 ml) in the lipolysis medium before the addition of enzyme to ensure homogeneous dispersion of the lipid (Christie and Moore,1969).

Hydrolysed lipid fractions were isolated by preparative thin-layer chromatography by the method described by Luddy et at. (1964). The components separated into four well-defined layers representing monoacylglycerols, diacylglycerols, free fatty acids and unreacted triacylglycerols. To ensure that truly representative diacylglycerols are obtained as a result of lipolysis, the composition of the isolated diacylglycerols was compared with those calculated from the composition of original triacylglycerols and the 2-monoacylglycerols obtained from pancreatic lipase hydrolysis using the computation procedure described by Litchfield (1972).

ii) The next step in stereospecific analysis was the conversion of the isolated diacylglycerols into phospholipids. Phosphorylation of the diacylglycerol fraction was achieved following the method of Brockerhoff (1966a).

iii) The final step, hydrolysis of the phospholipid, was carried out by using ophiophagus hannah venom (Sigma Chemical Co., St. Louis, Missouri) containing phospholipase A according to the procedure described by Christie and Moore (1969). The fractions containing lysophosphatide, unhydrolysed phosphotide and free fatty acid were isolated using thin-layer chromatography as described by Christie and Moore (1969). All fractions were methylated and analyzed for fatty acid composition by using the same procedure and conditions as described earlier.

Fatty acid composition of the monoacylglycerols, obtained after lipolysis, represented the nature of the fatty acids at the sn-2 position. Fatty acid analysis of the lysophosphatide fraction indicate the nature of the fatty acids present at the sn-1 position. The nature of the fatty acids at the sn-3 position was determined by two methods (Brockerhoff and Yurkowski,1966; Fatemi and Hammond,1977). First, the fatty acid composition of the sn-1 and sn-2 positions was subtracted from the composition of the whole triacylglycerols. Second, composition of the sn-2 position was subtracted from the composition of sn-2,3 diacylphosphatides that remained unhydrolyed by the snake venom enzymes. The former method is regarded as the most accurate, but agreement between the values calculated by both methods is considered as a general check on the reliability of the method (Christie and Moore,1969). The average of these two values is taken to represent the fatty acid composition at the sn-3 position.

Alpha-Tocopherol Determination

A known quantity of lipid was dissolved in 200 μL of ethanol, centrifuged and filtered. Quantitation of alpha-tocopherol was achieved by using a high performance liquid chromatograph (Model U6K, Water Associates, Milford,MA) equipped with an ultra-violet detector, model 440 (Water Associates, Milford, MA). A reverse phase column (ultrasphere OSD, Beckman Instruments Ins., Fullerton, CA) with a length of 15 cm, internal diameter of 4.6 mm and 5 μ particle size

was used for separation of alpha-tocopherol. HPLC-grade methanol (100%) was used as the eluting solvent at a flow rate of 0.8 μL/minute. Alpha-tocopherol was identified by comparing the retention time with that of an alpha-tocopherol standard (Henkel Corp., Minneapolis, Minnesota).

The presence of alpha-tocopherol in microbial lipids was further verified by derivatizing the samples and then analyzing by gas chromatography (Slover et al.,1983). Lipids were saponified with alcoholic KOH (50%) containing 5% ascorbic acid. The unsaponifiable fraction was extracted two times with hexane. The combined hexane extracts were washed with a 2% sodium chloride solution, passed through anhydrous sodium sulfate and then dried under a stream of nitrogen. The unsaponifiable fraction and an alpha-tocopherol standard were derivatized by adding 50 μL bis(trimethylsilyl)-trifluoroacetamide (BSTFA) plus 1% trimethylchlorosilane (TMCS) and 100 μL silylation-grade pyridine (Pierce Chemical Co., Rockford, IL) and leaving for 30 minutes at room temperature. The trimethylsilyl derivative of alpha-tocopherol was separated and quantitated using gas chromatography. A methyl silicone fluid capillary column (Hewlett Packard, Avondale, PA) with a length of 25 m, an inside diameter of 0.2 mm and film thickness of 0.2 μm was used. Helium was used as the carrier gas with a split ratio of 20:1. The GC oven temperature was initially held at 180°C for 10 minutes, then increased at a rate of 5°C/minute to a final temperature of

260°C and held for 30 minutes. Alpha-tocopherol was tentatively identified by comparing with the relative retention time of a silylated alpha-tocopherol standard. Peak area was computed by integration (Hewlett Packard, Model 3392A).

Statistical Analysis

The experimental designs used for this study were a split block design and a replicated two factor design. The data were analyzed statistically by using the procedures outlined by Gill (1988). Tukey's test was used to determine the level of significance of difference between the mean values of various parameters and performed by using the MSTAT-C microcomputer statistical program (Michigan State University,1989).

RESULTS AND DISCUSSION

Fermentation characteristics of yeast strains

Four yeast strains, Apiotrichum curvatum ATCC 10567, Cryptococcus albidus ATCC 56297, Lipomyces starkeyi ATCC 12659 and Rhodosporidium toruloides ATCC 10788 were grown on culture media containing whey permeate as the substrate. The initial high carbon:nitrogen ratio of whey permeate makes it a good substrate for lipid production (Moon et al .,1978). As the relative carbon to nitrogen ratio of the growth medium seems to be critical for the production of lipids by yeast (Yoon et al.,1982; Evans and Ratledge,1984a; Turcotte and Kosaric,1989), four different carbon:nitrogen ratios were employed in the culture medium by incorporating yeast extract and asparagine as the nitrogen source. It has been observed that a high nitrogen concentration relative to carbon in the culture medium enhances biomass production, but results in the synthesis of very low amounts of lipid (Moreton,1988). During the fermentation process, different parameters such as growth rate, lactose utilization and protein consumption were investigated to monitor the point at which the fermentation process could be curtailed for lipid extraction. Fermentation process was curtailed when the carbon source in the culture medium was almost completely exhausted and the cells were in

the stationary phase.

Growth rate

Growth rate was monitored by recording the optical density of the culture media at 610 nm. All the yeast strains under study behaved differently with regard to their growth on whey permeate (Tables 2-5). Statistical analysis of the data (Appendices B-E) revealed that carbon:nitrogen ratios and cultivation time both have a significant ($p<0.01$) effect on the growth rate of Apiotrichum curvatum ATCC 10567 and Cryptococcus albidus ATCC 56297. The significant first-order interaction ($p<0.01$) between carbon:nitrogen ratios and cultivation time suggests that the effects of these variables are highly dependent upon each other. The carbon:nitrogen ratio and cultivation time also showed a significant ($p<0.01$) effect on the growth rate of Rhodosporidium toruloides ATCC 10788, but the non-significant first-order interaction between these variables suggests that the effects of these variables are independent of each other in the case of this strain. However, only cultivation time had a significant ($p<0.01$) influence on the growth rate of Lipomyces starkeyi ATCC 12659. Apiotrichum curvatum ATCC 10567 continued growing exponentially up to 48 hours, whereafter its growth started declining gradually. However, maximum optical density of the culture medium with a carbon:nitrogen ratio of 30:1 was recorded at the end of 96 hours of fermentation.

On the other hand, Cryptococcus albidus ATCC 56297 and

Table 2. The growth rate of Apiotrichum curvatum ATCC 10567 at different C:N ratios as monitored by recording the optical density of the culture media at 610 nm[1]

Cultivation time (hr)	Optical density at C:N ratios[2]			
	30:1	45:1	60:1	75:1
0	1.70±0.10[a]	1.68±0.08[a]	1.49±0.29[a]	1.79±0.09[a]
24	24.50±0.10[a]	26.30±1.00[a]	32.55±2.65[a]	30.65±0.15[a]
48	57.50±2.50[ab]	59.20±2.50[a]	54.35±1.85[ab]	46.40±0.50[b]
72	82.45±1.75[a]	74.25±0.05[a]	62.70±1.90[b]	54.95±2.85[b]
96	90.63±0.38[a]	81.25±2.35[a]	69.05±2.05[b]	59.35±4.25[b]
120	86.90±8.20[a]	80.50±5.00[a]	68.35±5.55[b]	60.30±6.30[b]

1 All values represent the average of two replicates along with the standard deviation.

2 C:N ratio, carbon to nitrogen ratio.

a,b Values in the same row bearing the same superscript are not significantly different from each other at $P<0.05$.

Table 3. The growth rate of Cryptococcus albidus ATCC 56297 at different C:N ratios as monitored by recording the optical density of the culture media at 610 nm[1]

Cultivation time (hr)	Optical density at C:N ratios[2]			
	30:1	45:1	60:1	75:1
0	1.39±0.06[a]	1.43±0.08[a]	1.47±0.01[a]	1.50±0.02[a]
24	11.80±0.20[a]	10.55±0.45[a]	9.75±0.45[a]	9.60±0.20[a]
48	18.20±0.20[a]	15.80±0.20[a]	15.15±0.15[a]	14.00±0.30[a]
72	23.75±2.35[a]	20.60±2.10[ab]	20.20±2.00[ab]	18.85±2.45[b]
96	31.25±3.95[a]	28.70±3.60[a]	27.05±2.35[ab]	23.90±2.50[b]
120	39.25±2.75[a]	35.15±1.95[a]	30.60±1.80[b]	26.85±1.85[b]
144	45.45±1.75[a]	38.00±1.10[b]	32.50±1.80[c]	27.97±0.55[d]
168	49.30±1.00[a]	38.75±1.15[b]	34.05±0.85[c]	29.70±0.00[c]
192	51.15±0.35[a]	41.80±0.60[b]	35.45±0.35[c]	29.15±0.35[d]

1 All values represent the average of two replicates along with the standard deviation.

2 C:N ratio, carbon to nitrogen ratio.

a,b,c,d Values in the same row bearing the same superscript are not significantly different from each other at $P<0.05$.

Table 4. The growth rate of Lipomyces starkeyi ATCC 12659 at different C:N ratios as monitored by recording the optical density of the culture media at 610 nm

Cultivation time (hr)	Optical density at C:N ratios[2]			
	30:1	45:1	60:1	75:1
0	1.18±0.10[a]	1.20±0.08[a]	1.24±0.05[a]	1.18±0.06[a]
24	1.14±0.04[a]	1.17±0.05[a]	1.19±0.02[a]	1.21±0.01[a]
48	1.10±0.30[a]	2.10±0.50[a]	1.10±0.30[a]	1.15±0.35[a]
72	3.20±0.30[a]	3.90±0.30[a]	3.05±0.55[a]	3.10±0.40[a]
96	4.20±0.00[a]	4.40±0.40[a]	3.70±0.30[a]	3.65±0.85[a]
120	4.75±0.15[a]	5.60±0.60[a]	5.15±0.05[a]	5.80±0.00[a]
144	6.90±0.50[a]	7.20±0.90[a]	6.15±0.05[a]	6.50±0.90[a]
168	8.70±1.20[a]	8.00±1.20[a]	8.85±0.85[a]	11.75±1.45[a]
192	11.65±3.35[a]	11.10±1.70[a]	10.70±1.80[a]	13.70±0.00[a]
216	14.10±2.80[a]	12.75±0.35[a]	12.95±0.35[a]	15.80±0.00[a]
240	19.00±4.40[a]	16.30±0.10[b]	20.10±0.00[ab]	23.70±0.00[a]

1 All values represent the average of two replicates along with the standard deviation.

2 C:N ratio, carbon to nitrogen ratio.

a,b Values in the same row bearing the same superscript are not significantly different from each other at $P<0.05$.

Table 5. The growth rate of Rhodosporidium toruloides ATCC 10788 at different C:N ratios as monitored by recording the optical density of the culture media at 610 nm[1]

Cultivation time (hr)	Optical density at C:N ratios[2]			
	30:1	45:1	60:1	75:1
0	1.73±0.07[a]	1.55±0.05[a]	1.49±0.09[a]	1.61±0.01[a]
24	7.70±0.40[a]	6.80±0.50[ab]	6.25±0.55[b]	6.10±0.50[b]
48	7.20±0.40[a]	6.65±0.45[a]	6.40±0.30[a]	6.40±0.70[a]

1 All values represent the average of two replicates along with the standard deviation.

2 C:N ratio, carbon to nitrogen ratio.

a,b Values in the same row bearing the same superscript are not significantly different from each other at $P<0.05$.

Rhodosporidium toruloides ATCC 10788 reached their maximum exponential growth in about 24 hours. However, Lipomyces starkeyi ATCC 12659 showed no signs of growth during the first 48 hours of the fermentation period. This period was possibly used by this yeast strain to adopt its metabolic pathways to the available conditions of the fermentation. Within the next 24 hours, this yeast strain reached the plateau of its exponential growth phase. Turcotte and Kosaric (1989) observed depression in growth rate around 20 hours, although it took 70 hours for the nitrogen source to be reduced to a zero level while growing Rhodosporidium toruloides ATCC 10788 in a culture medium containing glucose as the carbon source.

Lactose utilization

A number of yeast strains have been identified as capable of utilizing lactose which is a major constituent of whey permeate. At present, Apiotrichum curvatum ATCC 20509 has been identified as the most effecient yeast strain capable of utilizing whey permeate for the synthesis of lipids and is capable of accumulating up to 60% of its dry weight as intracellular oil droplets (Moon et al.,1978). Among all the substrates used for growing different yeast strains to synthesize microbial lipids, glucose appeared to be assimilated most efficiently by oleaginous microorganisms and is readily converted into lipids (Moreton,1988).

Lactose utilization by the yeast strains is summarized in Tables 6-9. Results indicate that Apiotrichum curvatum ATCC

Table 6. Lactose utilization[1] by *Apiotrichum curvatum* ATCC 10567 during fermentation of culture media having different C:N ratios

Cultivation time (hr)	Lactose content (g/l) at C:N ratios[2]			
	30:1	45:1	60:1	75:1
0	45.82±0.44[a]	41.91±1.40[a]	42.77±1.59[a]	44.33±4.07[a]
24	33.50±4.78[a]	33.95±4.09[a]	37.26±2.33[a]	36.11±0.98[a]
48	23.55±2.02[a]	21.43±3.00[a]	25.58±0.22[a]	28.09±2.30[a]
72	8.78±4.57[b]	12.43±0.21[ab]	16.61±1.72[ab]	20.71±2.61[a]
96	0.00±0.00[c]	6.35±1.57[bc]	14.88±0.28[ab]	21.25±0.66[a]
120	0.00±0.00[c]	1.84±0.00[bc]	10.69±0.40[ab]	17.87±2.72[a]

1 All values represent the average of two replicates along with the standard deviation.

2 C:N ratio, carbon to nitrogen ratio.

a,b,c Values in the same row bearing the same superscript are not significantly different from each other at $P<0.05$.

Table 7. Lactose utilization[1] by Cryptococcus albidus ATCC 56297 during fermentation of culture media having different C:N ratios

Cultivation time (hr)	Lactose content (g/l) at C:N ratios[2]			
	30:1	45:1	60:1	75:1
0	37.60±5.75[a]	37.29±4.92[a]	39.24±5.03[a]	40.55±3.71[a]
24	34.67±5.46[a]	29.61±0.66[a]	35.09±3.77[a]	37.25±0.14[a]
48	31.02±3.81[a]	28.67±0.48[a]	32.80±2.41[a]	36.30±5.17[a]
72	25.21±1.51[a]	26.90±2.15[a]	30.64±2.21[a]	31.83±3.89[a]
96	23.61±4.01[ab]	18.31±0.41[b]	23.46±2.13[ab]	30.32±2.38[a]
120	15.66±5.29[b]	16.97±3.27[b]	22.29±4.14[ab]	28.02±3.57[a]
144	12.61±8.54[c]	17.63±7.99[bc]	22.14±6.22[ab]	26.60±1.62[a]
168	6.58±5.47[b]	10.53±4.60[b]	19.60±5.53[a]	25.13±5.13[a]
192	5.14±0.00[b]	10.05±5.03[b]	19.63±5.01[a]	24.82±4.82[a]

1 All values represent the average of two replicates along with the standard deviation.

2 C:N ratio, carbon to nitrogen ratio.

a,b,c Values in the same row bearing the same superscript are not significantlu different from each other at P<0.05.

Table 8. Lactose utilization[1] by Lipomyces starkeyi ATCC 12659 during fermentation of culture media having different C:N ratios

Cultivation time (hr)	Lactose content (g/l) at C:N ratios[2]			
	30:1	45:1	60:1	75:1
0	40.14±5.83	37.50±5.39	41.70±3.71	42.33±2.85
24	38.45±5.12	36.89±4.78	39.20±4.40	41.15±3.64
48	36.79±4.95	35.81±4.67	37.93±3.37	40.56±3.71
72	36.49±5.09	36.09±5.70	37.46±3.25	40.29±3.53
96	35.29±4.23	34.97±4.71	36.80±2.74	38.06±3.99
120	33.42±3.05	33.67±4.41	33.77±1.55	37.74±4.16
144	30.55±5.36	32.29±5.62	31.57±3.05	36.44±3.85
168	27.33±5.85	30.20±7.24	28.98±4.91	31.37±6.17
192	26.11±7.04	27.38±4.70	27.68±6.02	25.03±0.00
216	23.23±8.64	23.43±7.45	19.96±4.21	18.07±0.00
240	18.60±10.1	18.79±9.47	16.19±6.09	14.45±0.00

1 All values represent the average of two replicates along with the standard deviation.

2 C:N ratio, carbon to nitrogen ratio.

Table 9. Lactose utilization[1] by Rhodosporidium toruloides ATCC 10788 during fermentation of culture media having different C:N ratios

Cultivation time (hr)	Lactose content (g/l) at C:N ratios[2]			
	30:1	45:1	60:1	75:1
0	46.40±0.49	48.54±0.95	48.26±0.06	49.07±1.70
24	44.38±1.56	45.41±1.96	44.19±0.07	45.38±1.27
48	41.97±0.79	44.62±2.75	39.48±4.10	43.90±0.22

1 All values represent the average of two replicates along with the standard deviation.

2 C:N ratio, carbon to nitrogen ratio.

10567 utilized lactose more efficiently than the other yeast strains. Statistical analysis of the data (Appendix F) showed that both carbon:nitrogen ratios and cultivation time had significant ($p<0.01$) effects on lactose utilization in the case of Apiotrichum curvatum ATCC 10567. However, the significant ($p<0.01$) first-order interaction between these variables indicates that the effect of these variables was not independent of each other. The results for lactose utilization also correspond to the optical desity measurements. Apiotrichum curvatum ATCC 10567 showed the highest optical density after 120 hours of fermentation when compared with Cryptococcus albidus ATCC 56297 and Lipomyces starkeyi ATCC 12659 at the same cultivation period. At the carbon:nitrogen ratio of 30:1, Apiotrichum curvatum ATCC 10567 consumed all the lactose in 96 hours. These results closely agree with those obtained by Ykema et al (1989), who showed that yeast strain, Apiotrichum curvatum ATCC 20509, consumed all the lactose in 100 hours at an effective carbon:nitrogen ratio of 30. However, in a previous study, they had reported that the same yeast strain (Apiotrichum curvatum ATCC 20509) took 40 hours to consume all the lactose at a carbon:nitrogen ratio of 30:1 (Ykema et al.,1988). It required 65 hours in the case of sweet whey with a carbon:nitrogen ratio even higher than 30 (Davies,1988). These differences in consumption times could be due to the nature and composition of whey permeates utilized in the studies.

Crytococcus albidus ATCC 56297 and Lipomyces starkeyi ATCC 12659 were also capable of utilizing lactose efficiently during fermentation. Satistical analysis of the data (Appendices G-I) showed that both carbon:nitrogen ratios and cultivation time had significant ($p<0.01$) effects on lactose utilization by Cryptococcus albidus ATCC 56297, but the significant ($p<0.01$) first-order interaction between carbon:nitrogen ratios and cultivation time suggests that the effect of these variables was not independent of each other. In the case of Lipomyces starkeyi ATCC 12659, only cultivation time had a significant ($p<0.01$) influence on lactose utilization.

Results indicated that Rhodosporidium toruloides ATCC 10788 was not able to assimilate lactose efficiently as indicated by the decrease in optical density between 24 and 48 hours of fermentation (Table 5). Lactose utilization by this strain did not reveal any effect of either cultivation time or carbon:nitrogen ratio (Appendix 8). Botham and Ratledge (1979) reported that the rate at which cells take up glucose does not limit their rate of growth or can it be the means of regulating lipid synthesis in oleaginous and non-oleaginous yeast strains. Vega et al. (1988) showed that the addition of mineral salts and asparagine to the growth medium enhanced carbon utilization. In the present study, it was observed that the carbon:nitrogen ratio significantly affected lactose utilization. More efficient lactose utilization was

observed at the carbon:nitrogen ratio of 30:1 in the case of Apiotrichum curvatum ATCC 10567 and Cryptococcus albidus ATCC 56297. On the other hand, carbon:nitrogen ratios of 75:1 and 60:1 enhanced lactose utilization in the case of Lipomyces starkeyi ATCC 12659 and Rhodosporidium toruloides ATCC 10788, respectively.

Protein consumption

Evans and Ratledge (1984a) proposed that the rate of lipid production by microorganisms is influenced by the products of catabolism of the nitrogen source present in the growth medium, rather than by the result of direct stimulation by the nitrogen compound itself. Subsequent studies by these investigators (1984b) supported the hypothesis by showing that intracellular ammonium ion (NH_4^+) is probably the key regulatory metabolite whose concentration is increased prior to the accumulation of citrate and lipid during the initial stages of lipid biosynthesis. They suggested that intracellular ammonium ions begin to accumulate to a significant concentration in the cell during the early stages of growth before it could induce the biosynthesis of lipids.

In the present study, yeast extract and asparagine were the main nitrogen sources in the growth medium. Asparagine is known to release ammonia directly during assimilation. However, fundamental differences in nitrogen metabolism between different species of oleaginous yeasts influence the ability of various organic nitrogen compounds to stimulate

lipid synthesis (Evans and Ratledge,1984b).

The trend of protein consumption by the four yeast strains is shown in Tables 10-13. It is obvious from the results that protein consumption was significantly stimulated during the initial 24 hours of growth by all the yeast strains except Lipomyces starkeyi ATCC 12659 which showed increased protein consumption between 72 and 96 hours.

Statistical analysis of the data (Appendices J-M) showed a highly significant ($p<0.01$) effect of cultivation time on protein consumption. A significant ($p<0.05$) effect of carbon:nitrogen ratios was observed only in case of Apiotrichum curvatum ATCC 10567 and Cryptococcus albidus ATCC 56297. Carbon:nitrogen ratios did not exert any significant ($p>0.05$) influence on protein consumption in the case of Lipomyces starkeyi ATCC 12659 and Rhodosporidium toruloides ATCC 10788.

Dry cell mass of yeast strains

The weights of dry cell mass for all the yeast strains under study are given in Table 14. Results indicate that Apiotrichum curvatum ATCC 10567 produced the greatest dry cell mass out of the four yeast strains evaluated.. A yield of 18.6 g/L dry cell mass was obtained at the carbon:nitrogen ratio of 45:1. Vege et al. (1988) obtained dry cell mass yield of 11.85 g/l while growing Apiotrichum curvatum ATCC 20509 On 20% banana juice, supplemented with asparagine and mineral

Table 10. The change in protein content[1] during fermentation of culture media inoculated with Apiotrichum curvatum ATCC 10567 at different C:N ratios

Cultivation time (hr)	Protein content (g/l) at C:N ratios[2]			
	30:1	45:1	60:1	75:1
0	1.84±0.01	1.77±0.07	1.60±0.01	1.61±0.03
24	1.40±0.02	1.22±0.00	1.21±0.06	1.27±0.06
48	1.39±0.03	1.25±0.02	1.17±0.05	1.18±0.06
72	1.31±0.05	1.25±0.01	1.10±0.03	1.18±0.00
96	1.30±0.07	1.23±0.18	1.10±0.01	1.18±0.07
120	1.18±0.00	1.12±0.02	1.05±0.35	1.07±0.13

1 All values represent the average of two replicates along with the standard deviation.

2 C:N ratios, carbon to nitrogen ratio.

Table 11. The change in protein content[1] during fermentation of culture media inoculated with Cryptococcus albidus ATCC 56297 at different C:N ratios

Cultivation time (hr)	Protein content (g/l) at C:N ratios[2]			
	30:1	45:1	60:1	75:1
0	1.45±0.15	1.40±0.10	1.27±0.13	1.44±0.09
24	0.96±0.08	0.95±0.04	0.82±0.06	0.78±0.04
48	0.86±0.02	0.84±0.01	0.74±0.08	0.72±0.00
72	0.79±0.13	0.73±0.02	0.72±0.01	0.65±0.02
96	0.74±0.14	0.63±0.03	0.55±0.03	0.58±0.06
120	0.61±0.02	0.55±0.06	0.45±0.05	0.41±0.01
144	0.55±0.02	0.48±0.08	0.44±0.01	0.38±0.01
168	0.52±0.00	0.48±0.00	0.43±0.06	0.36±0.01
192	0.50±0.02	0.48±0.05	0.38±0.01	0.34±0.01

1 All values represent the average of two replicates along with the standard deviation.

2 C:N ratio, carbon to nitrogen ratio.

Table 12. The change in protein content[1] during fermentation of culture media inoculated with Lipomyces starkeyi ATCC 12659 at different C:N ratios

Cultivation time (hr)	Protein content (g/l) at C:N ratios[2]			
	30:1	45:1	60:1	75:1
0	1.65±0.11	1.63±0.16	1.59±0.03	1.51±0.04
24	1.60±0.10	1.62±0.18	1.53±0.02	1.48±0.03
48	1.53±0.04	1.40±0.23	1.45±0.05	1.46±0.02
72	1.42±0.08	1.29±0.09	1.44±0.02	1.45±0.00
96	1.31±0.04	1.21±0.08	1.45±0.13	1.39±0.06
120	1.19±0.05	1.20±0.29	1.30±0.05	1.28±0.03
144	1.14±0.01	1.15±0.24	1.19±0.14	1.19±0.09
168	0.96±0.18	1.15±0.25	1.03±0.21	1.07±0.08
192	0.93±0.03	1.14±0.12	1.05±0.03	1.04±0.00
216	0.89±0.04	1.02±0.23	1.03±0.02	0.86±0.00
240	0.89±0.01	0.95±0.16	1.01±0.00	0.86±0.01

1 All values represent the average of two replicates along with the standard deviation.

2 C:N ratio, carbon to nitrogen ratio.

Table 13. The change in protein content[1] during fermentation of culture media inoculated with Rhodosporidium toruloides ATCC 10788 at different C:N ratios

Cultivation time (hr)	Protein content (g/l) at C:N ratios[2]			
	30:1	45:1	60:1	75:1
0	1.74±0.02[a]	1.63±0.03[a]	1.56±0.03[a]	1.59±0.03[a]
24	1.32±0.06[a]	1.17±0.01[a]	1.04±0.00[a]	1.10±0.05[a]
48	1.21±0.00[a]	1.09±0.14[ab]	0.89±0.17[b]	0.85±0.05[b]

1 All values represent the average of two replicates along with the standard deviation.

2 C:N ratio, carbon to nitrogen ratio.

a,b Values in the same row bearing the same superscript are not significantly different from each other at $P<0.05$.

Table 14. The yield of dry cell mass[1] of yeast strains grown on culture media having different C:N ratios

Yeast strains	Dry cell mass (g/l) at C:N ratios[2]			
	30:1	45:1	60:1	75:1
A. curvatum	18.0±0.1[a]	18.6±0.8[a]	14.9±0.6[ab]	12.6±0.6[b]
C. albidus	13.8±3.2[a]	10.8±2.3[ab]	8.6±1.6[b]	7.5±1.5[b]
L. starkeyi	7.0±0.0[a]	6.5±0.5[a]	9.9±1.5[a]	8.7±0.0[a]
R. toruloides	0.8±0.1[a]	0.8±0.1[a]	0.9±0.0[a]	0.8±0.0[a]

1 All values represent the average of two replicates along with the standard deviation.

2 C:N ratio, carbon to nitrogen ratio.

a,b Values in the same row bearing the same superscript are not significantly different from each other at $P<0.05$.

salts after 96 hours of fermentation. On the other hand, Rhodosporidium toruloides ATCC 10788 produced the smallest dry cell mass and this may be due to its inability to utilize lactose efficiently. The maximum dry cell mass yield (0.9 g/L) by this strain was achieved at carbon:nitrogen ratio of 60:1. Cryptococcus albidus ATCC 56297 produced its maximum dry cell mass (13.8 g/L) at the carbon:nitrogen ratio of 30:1, while Lipomyces starkeyi ATCC 12659 yielded its greatest amount (9.9 g/L) at a carbon:nitrogen ratio of 60:1.It is evident from these results that dry cell mass yield was highly dependent upon yeast strain and carbon:nitrogen ratio.

Satistical analysis of the data (Appendix N) also showed that both yeast strains and carbon:nitrogen ratios had a significant ($p<0.05$) effect on the dry cell mass yield. However, the significant ($p<0.01$) first-order interaction indicates that the effects of both these variables were not independent of each other.

Lipid yield of yeast strains

Lipid accumulation in yeast calls can be viewed as a two stage process. In the first stage, the multiplication of cells take place in the presence of an abundance of the nitrogen source. In the second stage, growth limitation results from the exhaustion of the nitrogen source, while excess carbon is converted into lipid. Lipid yield by the yeast strains is presented in Table 15 as a percentage of dry cell mass. Of the

yeast strains investigated, Lipomyces starkeyi ATCC 12659 synthesized the greatest quantity of lipids (36.9% lipid) at the carbon:nitrogen ratio of 30:1. Apiotrichum curvatum ATCC 10567 was the second highest producer of lipid (16.6% at carbon:nitrogen ratio of 60:1). In contrast, Rhodosporidium toruloides ATCC 10788 synthesized 9.1% lipid at a carbon:nitrogen ratio of 45:1, whereas Cryptococcus albidus ATCC 56297 produced only 3.52% lipid at a carbon:nitrogen ratio of 75:1. Ykema et al. (1986) cultivated Apiotrichum curvatum ATCC 20509 in a semi-defined growth medium and reported 30% intracellular lipids on the basis of dry cell mass.

It is apparent from these results that yeast strain and carbon:nitrogen ratio influence the synthesis of lipids. Statistical analysis of the data (Appendix O) showed that yeast strain had a major effect on lipid yield (significant at $P<0.01$). However, the significant ($P<0.05$) first-order interaction indicates that effect of yeast strain is dependant on the carbon:nitrogen ratios. A number of studies performed on other species of yeast also showed that the carbon:nitrogen ratio influenced lipid yield (Yoon et al.,1982; Ykema et al.,1988; Turcotte and Kosaric,1989). Yoon et al (1982) reported that the carbon:nitrogen molar ratio of approximately 41 affected the lipid content of Rhodotorula gracilis NRRL Y-1091. Ykema et al. (1988) studied lipid production by the oleaginous yeast strain, Apiotrichum

Table 15. The percentage of lipid yield[1] obtained from different yeast strains grown on culture media having different C:N ratios

Yeast strains	Percent lipid yield at C:N ratios[2]			
	30:1	45:1	60:1	75:1
A. curvatum	6.0±0.0[b]	7.4±0.0[b]	16.6±0.0[a]	9.0±0.0[ab]
C. albidus	2.1±0.3[a]	1.9±0.3[a]	2.8±1.3[a]	3.5±0.7[a]
L. starkeyi	36.9±1.1[a]	30.4±5.5[ab]	28.7±4.2[ab]	23.4±0.0[b]
R. toruloides	8.6±0.1[a]	9.1±1.2[a]	8.6±0.5[a]	8.3±0.4[a]

1 All values represent the average of two replicates along with the standard deviation.

2 C:N ratio, carbon to nitrogen ratio.

a,b Values in the same row bearing the same superscript are not significantly different from each other at $P<0.05$.

curvatum ATCC 20509, in whey permeate and reported that maximum lipid production rates were obtained at carbon:nitrogen ratios of 30-35. Turcotte and Kosaric (1989) found that an initial molar carbon:nitrogen ratio of about 77 was appropriate for maximum lipid production when using glucose or fructose as the carbon source, and ammonium sulfate, ammonium nitrate and urea as the nitrogen source.

Quantitation of lipids synthesized by yeast strains

Major components identified in yeast lipids include triacylglycerols, free fatty acids. steryl esters and sterols (Litchfield,1972; Yoon and Rhee,1983). Triacylglycerols are the major lipid class in microbial storage lipids (Uzuka et al.,1975). Suzuki and Hasegawa (1974a) reported 78.3% triacylglycerols in the lipids extracted from Lipomyces starkeyi. Phospholipids are generally the second largest component of the lipid. Lipomyces starkeyi contained 10.2% phospholipids (Suzuki and Hasegawa,1974a). The major phospholipids are generally phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol (Erwin,1973; Rattray et al.,1975; Kaneko et al.,1976). Sterols, both free and esterfied to fatty acids, also occur to an appreciable extent in many yeast lipids and can contribute between 3-10% of the total lipid in an oleaginous organism (Ratledge,1982). The major sterol component is ergosterol, with small amounts of zymosterol, lanosterol, fecosterol and episterol (Aries and

Kirsop,1978).

The concentrations of the major classes of lipids synthesized by the four yeast strains are presented in Table 16. Results show that all yeast strains except Rhodosporidium toruloides ATCC 10788 produced greater amounts of triacylglycerols than phospholipids. Lipids from Apiotrichum curvatum ATCC 10567 contained 58.5% to 76.8% triacyglycerols and 6.9% to 11.0% phospholipids depending upon carbon:nitrogen ratio. Cryptococcus albidus ATCC 56297 synthesized 34.4% to 46.8% triacylglycerols and 19.4% to 23.6% phospholipids. Lipomyces starkeyi ATCC 12659 accumulated 40.5% to 66.8% triacylglycerols and 4.5% to 7.7% phospholipids. In contrast, Rhodosporidium toruloides ATCC 10788, produced phospholipids in the range of 31.7% to 38.7% and triacylglycerols varied from 7.7% to 14.9% depending upon carbon:nitrogen ratios. The rest of the components in case of Apiotrichum curvatum ATCC 10567, Cryptococcus albidus ATCC 56297, Lipomyces starkeyi ATCC 12659 and Rhodosporidium toruloides ATCC 10788 (12.8%-33.9%, 33.4%-33.7%, 25.5%-53.0% and 9.8%-52.1%, respectively), which were not identified, may contain monoacylglycerols, diacylglycerols, free fatty acids, sterol esters and sterols.

Kessel (1968) reported 14% triacylglycerols and 26% phospholipids in Rhodotorula gracilis NCYC 59. Thorpe and Ratledge (1972) quantitated the lipid classes of the lipids isolated from Candida sp. no. 107 and reported 72-83% Triacylglycerols and 5% phospholipids. The limits observed for

Table 16. Quantitation of triacylglycerol and phospholipid fractions of lipids obtained from different yeast strains grown on the culture media having different C:N ratios

Yeast	Fraction	Carbon:Nitrogen ratio			
		30:1	45:1	60:1	75:1
A.curvatum					
	TAG	58.5	72.0	67.3	76.8
	PL	7.6	11.0	6.9	10.4
C.albidus					
	TAG	43.0	34.4	38.4	46.8
	PL	23.6	20.9	19.4	19.5
L.starkeyi					
	TAG	50.8	40.5	46.5	66.8
	PL	5.3	6.5	4.5	7.7
R.toruloides					
	TAG	11.8	14.9	8.5	7.7
	PL	35.1	33.0	31.7	38.7

[1] All values represent the average of two replicates.

[2] TAG, triacylglycerol

[3] PL, phospholipid

triacylglycerols and phospholipids were slightly lower and higher, respectively compared to the limits reported in a review by Ratledge (1982).

Fatty acid composition of lipids

(a). Triacylglycerols

Lipids extracted from yeast strains were separated into triacylglycerol and phospholipid fractions using thin-layer chromatography. Fatty acid methyl esters were analyzed by gas chromatography and reported as the percent of the total methyl esters. In this study, lipids were extracted from the yeast strains when they reached the stationary phase of growth in order to get a stable fatty acid composition. This was done because the fatty acid composition of yeast lipids is known to vary with the growth phase (Kaneko et al.,1976; Moon and Hammond,1978; Hansson and Dostálek,1986; Turcotte and Kosaric,1989).

Turcotte and Kosaric (1989) reported a change in the fatty acid profile of lipids before depletion of nitrogen and it became relatively constant thereafter. This steady state remained unchanged, even though lipids were still being synthesized. Similar changes were reported earlier by Kaneko et al (1976) and Hansson and Dostálek (1986). Moon and Hammond (1978) also demonstrated that the lipid composition of Candida curvata R and D strains grown on whey permeate varied considerably during the active growth phase. The degree of

unsaturation and/or the content of the shorter fatty acids was greatest during this phase. The cells probably were richer in phospholipids than triacylglycerols during the growth phase. The fatty acid composition is stabilized when the cells reached the stationary phase. At this time, triacylglycerols are the principal lipids, with palmitic and oleic acids being the predominant fatty acids.

The fatty acid profiles of the triacylglycerol fraction of the four yeast strains are presented in Tables 17-20. Oleic and palmitic acids were the predominant fatty acids in the triacylglycerol fractions of Apiotrichum curvatum ATCC 10567 and Lipomyces starkeyi ATCC 12659. Apiotrichum curvatum ATCC 10567 synthsized 58.8% oleic acid and 17.2% palmitic acid at the carbon:nitrogen ratio of 60:1. In the case of Lipomyces starkeyi ATCC 12659, the triacylglycerols contained 47.8% oleic acid and 36.6% palmitic acid when synthesized at the carbon:nitrogen ratio of 30:1. The stearic acid content was higher than linoleic acid in both the yeast strains.

Similar results regarding the oleic acid and palmitic acid level in oleaginous yeast strains were obtained by other researchers (Moon and Hammond,1978; Misra et al.,1984; Yamauchi et al.,1983; Verwort et al.,1989) while growing different yeast strains on different substrates. Yamauchi et al. (1983) found palmitic and oleic acids as the major constituents of lipids synthesized by the yeast strain, Lipomyces starkeyi IAM 4753, when ethanol was used as a

Table 17. Fatty acid composition[1] of triacylglycerols synthesized by Apiotrichum curvatum ATCC 10567 at different C:N ratios in the culture media

Fatty acid	Percent area at C:N ratios[2]			
	30:1	45:1	60:1	75:1
14:0	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1
14:1	0.1±0.1	-	-	-
UN[4]	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1
16:0	17.5±1.1	17.1±1.4	17.2±1.6	17.8±2.0
16:1	0.2±0.2	0.2±0.2	0.2±0.2	0.2±0.2
UN	0.1±0.1	-	0.1±0.1	0.1±0.1
18:0	15.3±0.6	12.8±0.8	12.2±0.2	12.4±0.2
18:1	52.2±0.5	57.4±0.9	58.8±0.5	58.1±0.4
18:2	11.6±0.3	9.2±0.1	8.6±0.3	8.1±0.1
18:3	2.0±0.1	1.8±0.1	1.8±0.1	1.7±0.0
20:0	0.3±0.3	0.3±0.3	0.3±0.3	0.3±0.3
UN	-	-	0.1±0.1	-
22:0	-	-	-	0.2±0.2
UN	0.5±0.5	1.0±1.0	0.5±0.5	0.9±0.9
Saturated	33.2	30.3	29.8	30.8
Unsaturated	66.1	68.6	69.4	68.1
Unknown	0.7	1.1	0.8	1.1
DBI[3]	81.7	81.4	81.6	79.6

1 All values represent the average of two replicates along with the standard deviation.

2 C:N ratio, carbon to nitrogen ratio.

3 DBI, double bond index.

4 UN, unidentified.

Table 18. Fatty acid composition[1] of triacylglycerols synthesized by Cryptococcus albidus ATCC 56297 at different C:N ratios in the culture media

Fatty acid	Percent area at C:N ratios[2]			
	30:1	45:1	60:1	75:1
14:0	0.4±0.4	0.4±0.4	0.3±0.3	0.7±0.0
UN[4]	0.1±0.1	-	-	-
16:0	29.4±0.2	31.3±1.3	26.7±3.0	30.5±0.4
16:1	1.0±0.1	0.5±0.5	0.8±0.2	0.8±0.0
18:0	1.4±1.4	1.5±1.5	2.6±0.8	2.6±0.4
18:1 W9	29.6±2.2	33.0±0.6	31.0±2.7	30.2±1.7
18:1 W7	0.2±0.2	0.2±0.2	0.3±0.3	-
18:2	36.2±4.1	31.9±0.5	37.1±7.0	33.4±2.1
18:3	1.5±0.0	1.3±0.2	1.3±0.2	1.3±0.2
UN	0.1±0.1	-	-	-
UN	0.1±0.1	-	-	-
UN	-	-	-	0.6±0.6
Saturated	31.2	33.2	29.6	33.8
Unsaturated	68.5	66.9	70.5	65.7
Unknown	0.3	-	-	0.6
DBI[3]	107.7	101.4	110.2	101.7

1 All values represent the average of two replicates along with the standard deviation.

2 C:N ratio, carbon to nitrogen ratio.

3 DBI, double bond index.

4 UN, unidentified.

Table 19. Fatty acid composition[1] of triacylglycerols synthesized by Lipomyces starkeyi ATCC 12659 at different C:N ratios in the culture media

Fatty acid	Percent area at C:N ratios[2]			
	30:1	45:1	60:1	75:1
16:0	36.6±3.1	37.7±3.4	36.3±2.9	40.1±0.0
16:1	2.6±0.2	3.1±1.0	2.3±0.1	2.5±0.0
18:0	7.6±0.0	6.7±0.5	8.1±0.5	8.7±0.0
18:1	47.8±3.9	47.0±5.0	48.1±4.1	43.6±0.0
18:2	5.4±0.6	5.5±1.2	5.3±0.6	5.1±0.0
Saturated	44.2	44.4	44.4	48.8
Unsaturated	55.8	55.6	55.7	51.2
DBI[3]	61.2	61.1	61.0	56.3

1 All values represent the average of two replicates except the values given for C:N ratio 75:1 which represent the values from one experiment along with the standard deviation.

2 C:N ratio, carbon to nitrogen ratio.

3 DBI, double bond index.

Table 20. Fatty acid composition[1] of triacyglycerols synthesized by Rhodosporidium toruloides ATCC 10788 at different C:N ratios in the culture media

Fatty acid	Percent area at C:N ratios[2]			
	30:1	45:1	60:1	75:1
16:0	16.0±0.0	17.3±2.2	14.4±1.2	13.4±2.9
16:1	0.9±0.9	1.1±1.1	1.3±1.3	2.0±2.0
UN[4]	-	-	-	1.4±1.4
18:0	5.5±2.3	6.9±4.2	3.2±3.2	4.3±2.5
18:1 W9	34.8±7.7	32.4±9.9	32.6±9.3	28.2±7.6
18:1 W7	-	1.6±1.6	-	-
18:2	34.6±6.3	30.8±9.8	40.1±8.4	40.2±4.8
18:3	8.2±2.8	7.5±5.1	8.4±4.1	8.5±2.9
UN	-	-	-	1.1±1.1
UN	-	-	-	1.0±1.0
UN	-	2.4±2.4	-	-
Saturated	21.5	24.2	17.6	17.7
Unsaturated	78.5	73.4	82.4	78.9
Unknown	-	2.4	-	3.5
DBI[3]	129.5	119.2	139.3	136.1

1 All values represent the average of two replicates along with the standard deviation.

2 C:N ratio, carbon to nitrogen ratio.

3 DBI, double bond index.

4 UN, unidentified.

substrate. Misra et al. (1984) grew Rhodotorula glutinis isolated from soil on molasses and reported oleic acid (47%) as the major constituent in isolated lipids, followed by palmitic acid (37%) and linoleic acid (8%). Ykema et al.(1989) reported 55.4% oleic acid and 16.9% palmitic acid in the triacylglycerol fraction of lipids isolated from oleaginous yeast Apiotrichum curvatum ATCC 20509.

Cryptococcus albidus ATCC 56297 and Rhodosporidium toruloides ATCC 10788 did not synthesize sufficient quantities of lipids to be classified as oleaginous yeast strains while grown on whey permeate. However, in one study Rhodosporidium toruloides ATCC 10788 when grown in a growth medium containing glucose as carbon source and urea as the nitrogen source synthesized 38.6% lipids (Turcotte and Kosaric,1989).

The fatty acid profile of Cryptococcus albidus ATCC 56297 and Rhodosporidium toruloides ATCC 10788 indicated that both these strains produced palmitic acid, oleic acid and linoleic acid as the predominant fatty acids. The former strain produced 30.5% stearic acid, 30.2% oleic acid and 33.4% linoleic acid at a carbon:nitrogen ratio of 75:1. The fatty acid profile of the triacylglycerol fraction of Rhodosporidium toruloides ATCC 10788 showed that this strain synthesized 17.3% stearic acid, 32.4% oleic acid and 30.8% linoleic acid. The concentrations of individual fatty acids varied as a function of the carbon:nitrogen ratio in all yeast strains and these results were in close agreement with those

previously obtained by Turcotte and Kosaric (1989).

All the yeast strains tested in this study had greater quantities of unsaturated fatty acids than saturated fatty acids in the triacylglycerol fraction irrespective of the carbon:nitrogen ratio. Double bond index is known to indicate the degree of unsaturation. It was calculated by multiplying the percentage of the individual unsaturated fatty acid with the number of bonds it possesses. The highest double bond index (119.2-139.3) was observed in case of neutral lipids from Rhodosporidium toruloides ATCC 10788, whereas those from Lipomyces starkeyi ATCC 12659 had the lowest double bond index (56.3-61.2). Double bond index also varied as a fuction of carbon:nitrogen ratio.

(b) Phospholipids

The composition of the fatty acid of the phospholipid fractions of the yeast strains is given in Tables 21-24. The phospholipid fractions of Apiotrichum curvatum ATCC 10567 and Lipomyces starkeyi ATCC 12659 contained oleic acid (28.0% - 39.8% and 24.7% - 42.4%, respectively) as the predominant fatty acid as it was in the triacylglycerol fraction. In contrast, the phospholipid fractions of Cryptococcus albidus ATCC 56297 and Rhodosporidium toruloides ATCC 10788 contained linoleic acid (47.9 - 62.6% and 21.6% -35.3%, respectively) as the predominant fatty acid. The level of palmitic acid in Apiotrichum curvatum ATCC 10567 and Lipomyces starkeyi ATCC 12659 was lower in the phospholipid fraction and that of

Table 21. Fatty acid composition[1] of phospholipids synthesized by Apiotrichum curvatum ATCC 10567 at different C:N ratios in the culture media

Fatty acid	Percent area at C:N ratios[2]			
	30:1	45:1	60:1	75:1
14:0	-	-	-	0.3±0.3
UN[3]	0.4±0.4	-	0.4±0.4	0.7±0.7
UN	0.2±0.2	0.5±0.5	0.2±0.2	0.4±0.4
16:0	7.9±0.7	4.9±0.7	6.5±0.8	5.1±3.7
16:1	1.1±1.1	1.1±1.1	1.6±1.6	2.3±2.3
17:0	2.1±2.1	2.3±2.3	3.0±3.0	4.4±4.4
18:0	4.0±3.0	1.4±0.0	1.7±0.1	1.2±1.2
18:1	29.4±8.5	28.0±5.6	39.8±8.3	31.5±9.9
UN	-	0.4±0.4	-	0.4±0.4
18:2	22.4±5.2	20.0±5.7	28.0±8.3	20.6±9.9
UN	-	-	-	0.5±0.5
18:3 W6	-	-	-	0.2±0.2
18:3 W3	3.6±1.2	3.7±0.9	5.3±1.3	3.9±2.9
UN	2.1±2.1	2.9±2.9	3.7±3.7	5.3±5.3
19:0	3.7±1.0	4.8±3.4	1.9±1.9	3.2±3.2
UN	0.3±0.3	2.3±1.3	0.8±0.8	1.5±1.5
UN	-	0.5±0.5	-	0.4±0.4
UN	-	0.6±0.6	-	-
UN	-	0.5±0.5	-	0.5±0.5
UN	-	5.5±5.5	-	4.2±4.2
20:0	19.6±19.6	-	-	-
20:1	1.5±1.5	2.4±2.4	3.0±3.0	4.2±4.2
20:2	-	13.3±9.9	-	-
UN	-	0.9±0.9	-	0.7±0.7
UN	1.4±1.4	1.4±1.4	2.4±2.4	3.2±3.2
UN	-	1.5±1.5	-	1.1±1.1
UN	0.3±0.3	1.1±1.1	-	0.6±0.6
22:1	-	-	1.7±1.7	2.2±2.2
22:5 W3	-	-	-	1.4±1.4
Saturated	37.3	13.4	11.2	14.2
Unsaturated	58.0	68.5	74.7	66.3
Unknown	4.7	18.1	14.2	19.5

1 All values represent the average of two replicates along with the standard deviation.

2 C:N ratio, carbon to nitrogen ratio.

3 UN, unidentified.

Table 22. Fatty acid composition[1] of phospholipids synthesized by Cryptococcus albidus ATCC 56297 at different C:N ratios in the culture media

Fatty acid	Percent area at C:N ratios[2]			
	30:1	45:1	60:1	75:1
12:0	-	-	-	0.8±0.8
14:0	-	-	-	0.5±0.5
UN[3]	-	-	0.4±0.4	0.7±0.7
UN	-	-	0.3±0.3	0.3±0.3
16:0	9.5±3.6	13.5±1.3	12.3±1.3	10.8±2.2
16:1	0.9±0.6	0.7±0.7	1.3±1.3	1.9±1.9
17:0	1.1±1.1	0.9±0.9	2.2±2.2	3.3±3.3
18:0	-	1.1±1.1	0.8±0.3	0.5±0.5
18:1	19.8±5.0	15.0±1.1	15.1±0.4	15.5±0.5
18:2	49.4±6.3	62.6±4.8	56.3±9.0	47.9±9.6
18.3 W3	1.7±0.1	2.1±0.1	1.8±0.3	1.8±0.8
UN	1.4±1.4	1.1±1.1	2.5±2.5	3.5±3.5
19:0	0.8±0.8	0.5±0.5	1.6±1.6	2.0±2.0
UN	0.2±0.2	-	0.7±0.7	0.9±0.9
UN	12.2±9.8	-	-	-
20:0	1.2±1.2	-	-	5.0±5.0
20:1	1.0±1.0	-	2.0±2.0	2.6±2.6
UN	0.8±0.8	-	1.4±1.4	2.0±2.0
UN	-	-	0.5±0.5	-
22:1	-	-	0.8±0.8	-
UN	-	2.5±2.5	-	-
Saturated	12.6	16.0	16.9	22.9
Unsaturated	72.8	80.4	77.3	69.7
Unknown	14.6	3.6	5.8	7.4

1 All values represent the average of two replicates along with the standard deviation.

2 C:N ratio, carbon to nitrogen ratio.

3 UN, unidentified.

Table 23. Fatty acid composition[1] of phospholipids synthesized by Lipomyces starkeyi ATCC 12659 at different C:N ratios in the culture media

Fatty acid	Percent area at C:N ratios[2]			
	30:1	45:1	60:1	75:1
UN[3]	-	-	0.4±0.4	-
UN	-	1.5±1.5	1.7±1.7	3.2±0.0
UN	1.0±1.0	0.7±0.7	0.7±0.7	-
UN	0.6±0.6	1.6±0.3	1.6±0.8	-
16:0	4.3±3.1	6.9±1.7	5.3±0.5	11.6±0.0
16:1	5.1±0.7	3.3±1.1	2.7±1.3	1.7±0.0
UN	0.6±0.6	-	0.6±0.1	-
UN	-	0.8±0.8	-	-
17:0	8.5±0.9	5.1±0.8	4.1±1.7	-
UN	0.4±0.4	-	0.2±0.2	-
UN	0.3±0.3	-	-	-
UN	-	-	-	-
18:0	-	1.9±0.3	0.9±0.3	2.2±0.0
18:1	24.7±9.9	27.7±4.1	24.7±6.0	42.4±0.0
UN	0.9±0.0	0.8±0.0	0.4±0.0	-
18:2 W6	7.4±7.4	6.8±1.2	7.5±2.9	17.1±0.0
UN	1.1±1.1	1.1±1.1	0.6±0.6	-
18:3 W6	0.6±0.6	-	0.6±0.2	-
UN	-	3.0±3.0	-	-
UN	-	-	2.6±2.6	-
UN	1.0±1.0	-	-	-
18:3 W3	0.5±0.5	-	0.2±0.2	-
UN	4.4±0.5	6.0±1.1	3.9±0.2	-
19:0	12.9±1.2	9.2±2.0	7.6±0.3	-
UN	3.7±3.7	3.9±3.9	1.9±1.9	-
UN	1.0±1.0	-	0.5±0.5	-
UN	1.1±1.1	-	0.5±0.5	-
UN	-	-	2.8±2.8	-
20:0	-	7.8±7.8	5.0±5.0	-
20:1	4.5±1.1	2.5±2.5	3.4±0.1	-
20:3 W6	1.6±1.6	1.6±1.6	0.8±0.8	-
20:4 W6	0.9±0.9	-	-	-
UN	0.9±0.9	-	0.5±0.5	-
UN	0.8±0.8	-	-	-
UN	-	-	0.6±0.6	-
UN	1.1±1.1	-	0.7±0.7	-
20:5 W3	2.2±2.2	2.1±2.1	3.4±0.5	-
UN	1.8±1.8	-	-	-
UN	-	-	-	21.8±0.0
UN	1.6±1.6	0.9±0.9	1.6±0.2	-
UN	4.5±0.1	4.6±0.4	2.9±0.4	-
UN	-	-	0.5±0.5	-
22:1	-	-	1.5±1.5	-

"Table 23 (cont'd)"

Fatty acid	Percent area at C:N ratios[2]			
	30:1	45:1	60:1	75:1
UN	-	-	2.9±2.9	-
UN	-	-	3.1±3.1	-
22:5 W3	-	-	1.1±1.1	-
Saturated	25.7	30.9	22.9	13.8
Unsaturated	47.5	44.0	45.9	61.2
Unknown	26.8	25.1	31.2	25.0

1 ALL values represent the average of two replicates except the values given for C:N ratio 75:1 which represent the values from one experiment along with the standard deviation.

2 C:N ratio, carbon to nitrogen ratio.

3 UN, unidentified.

Table 24. Fatty acid composition[1] of phospholipids synthesized by Rhodosporidium toruloides ATCC 10788 at different C:N ratios in the culture media

Fatty acid	Percent area at C:N ratios[2]			
	30:1	45:1	60:1	75:1
12:0	-	0.4±0.4	0.7±0.7	1.0±1.0
14:0	-	0.8±0.3	0.7±0.7	0.9±0.9
UN[3]	-	-	-	0.3±0.3
UN	1.3±1.3	-	-	0.7±0.1
UN	-	0.8±0.8	0.5±0.5	0.8±0.8
UN	-	0.4±0.4	0.8±0.8	0.8±0.8
16:0	7.0±1.2	6.7±2.3	8.0±0.0	8.5±4.7
16:1	2.2±1.1	3.0±1.8	2.8±0.7	2.8±1.5
UN	0.7±0.7	-	-	-
17:0	3.1±1.5	4.2±4.2	3.2±1.7	4.3±4.3
18:0	1.9±0.5	2.2±1.2	2.2±2.2	2.9±2.9
18:1	22.9±3.1	21.6±0.1	20.3±1.4	20.3±1.0
UN	-	0.3±0.3	-	0.4±0.4
18:2	35.3±9.6	31.9±9.5	27.3±4.5	21.6±8.4
UN	-	-	2.1±2.1	5.9±5.9
18:3 W3	16.5±2.1	9.6±5.6	6.7±2.3	5.1±2.4
UN	2.9±2.9	4.4±4.4	4.2±1.4	4.6±4.6
19:0	2.2±0.7	3.5±2.1	3.7±0.5	3.5±3.5
UN	0.6±0.6	1.1±1.1	0.8±0.8	1.6±1.6
UN	-	0.2±0.2	-	-
UN	-	-	-	2.7±2.7
20:0	-	-	8.7±8.7	-
20:1	1.8±1.8	3.5±3.5	2.3±2.3	3.7±3.7
20:3 W6	-	0.4±0.4	-	0.6±0.6
UN	1.6±1.6	2.6±2.6	1.7±1.7	2.8±2.8
UN	-	0.7±0.7	-	0.7±0.7
UN	-	0.5±0.5	-	-
UN	-	-	1.7±1.7	-
22:1	-	1.2±1.2	-	1.1±1.1
22:4	-	-	1.6±1.6	-
UN	-	-	-	2.4±2.4
Saturated	14.2	17.8	27.2	21.1
Unsaturated	78.7	71.2	61.0	55.3
Unknown	7.1	11.0	11.8	23.7

1 All values represent the average of two replicates along with the standard deviation.

2 C:N ratio, carbon to nitrogen ratio.

3 UN, unidentified.

linoleic acid was higher than was found in the triacylglycerol fraction of these strains.

In the case of Cryptococcus albidus ATCC 56297 and Rhodosporidium toruloidus ATCC 10788 the levels of palmitic acid and oleic acid were lower in phospholipid fraction in comparison to triacylglycerol fraction.The individual fatty acid composition of phospholipids from the yeast strains varied significantly as a result of variation in carbon:nitrogen ratio. The phospholipid fraction also showed high proportion of unsaturated fatty acids.

Gill et al. (1977) observed palmitic and oleic acids as predominant fatty acids in the phospholipid fraction of the lipids isolated from yeast strain Candida 107 which was grown on glucose in a chemostat under nitrogen-limited conditions. Palmitic and oleic acids were found in the range of 29.5% to 45.9% and 27.6% to 47.3%, respectively depending upon the different dilution rates. Yoon and Rhee (1983) also observed the similar type of results while analyzing phospholipid fraction of the lipids extracted from Rhodotorula glutinis NRRL Y-1091 while growing it on synthetic media in continuous culture under nitrogen-limited conditions by employing different dilution rates. Palmitic acid varied from 31.6% to 36.2% depending upon dilution rate, whereas oleic acid was observed between 20.3% to 27.8%. However, high proportions of myristic acid were also found and it ranged from 13.4% to 24.9%. They further reported that the effect of culture

conditions on fatty acid composition appeared to be strain specific.

(c) Similarities between yeast lipids and lipids from plant and animal sources.

The major fatty acids of animal and plant origin are all saturated or unsaturated monocarboxylic acids with a straight even-numbered carbon chain. The saturated fatty acids such as lauric, myristic, palmitic and stearic acids all occur in plants, but even more abundant are the unsaturated fatty acids like oleic, linoleic and linolenic acids. Olive oil contains 62-83% oleic acid. Davies (1988) reported that palm olein, a low melting point fraction from palm oil contains 39.8% palmitic acid and 42.5% oleic acid. Rape seed oil contains erucic acid in addition to the above mentioned fatty acids. Beef tallow is rich in saturates (35-45%) as compared to unsaturated fatty acids. However, oleic acid (35-45%) is reported as the predominant unsaturated fatty acid in beef tallow. Lard also contain an appreciable amounts of oleic acid (47-83%).

All the fatty acids which have been identified in yeast lipid have also been found in the lipids of plant and animal cells. Oleic and palmitic acids are usually the predominant fatty acids in all oleaginous yeasts (Moon and Hammond,1978; Misra et al.,1983; Yamauchi et al.,1983; Verwort et al.,1989). Linoleic acid tends to be the third most abundant acid. olenic acid as the α-isomer can usually be detected in small amounts.

Results obtained in this study regarding the fatty acid composition of triacylglycerol and phospholipid fractions of lipids isolated from all the four yeast strains showed almost similar trends. Suzuki and Hasagawa (1974b) confirmed the structures of palmitoleic and oleic acids isolated from the lipids of Lipomyces starkeyi using gas chromatography and mass spectroscopy in combination. Short chain fatty acids do not occur above a few percent in yeasts, though Watanabe (1975) found C12 at 6% of the fatty acids in a Lipomyces sp. grown on xylose. Long chain fatty acids (C20:0 and C22:0) together with some monounsaturated acids of similar carbon length can also be detected in small quantities in most cases (Ratledge,1982).

Vernolic acid has been suggested as a minor component of the fatty acids of Lipomyces sp. no. 33 grown on acetate, though not when grown on glucose or xylose (Watanabe,1975). Vernolic acid, although not a common constituent of plant seed oil, is nevertheless present as a minor component in more than just a few plant lipids. Hilditch and Williams (1964) reported that vernolic acid is a major component of Vernonia anthelmintica (Ironweed). The similarity between yeast lipids and those from plant and animal sources is also confirmed by the detection of small amounts of diol lipids, diacyl esters of butane-1,3 diol, butane-1,4 diol and a C_5 diol in a Lipomyces sp. as well as in corn seed oil and rat liver fat (Bergelson et al.,1966).

Bati et al. (1984) reported that Candida lipolytica 1094 synthesized lipids having almost identical fatty acid composition to that of corn oil while grown under optimum fermentation conditions. The only difference observed was in the amounts of palmitic and palmitoleic acids. Yeast lipids had slightly less palmitic acid and a small amount of palmitoleic acid. These results were in contrast to the results obtained during shake flask experiments conducted by Glatz et al.(1984) while growing Candida lipolytica in which significantly less saturated fatty acids were observed. On the other hand, Davies (1988) reported that yeast lipid isolated from Apiotrichum curvatum ATCC 20509 was almost similar in composition and properties to palm olein, a lower melting point fraction from palm oil.

Stereospecific analysis of yeast lipids

The most critical step in stereospecific analysis is the preparation of diacylglycerols representative of the original triacylglycerol fraction of the lipid. The diacylglycerol fraction obtained by hydrolysis with pancreatic lipase may or may not be representative of the original triacylglycerol fraction. Brockerhoff and his co-workers (Brockerhoff,1965a,b; Brockerhoff and Yurkowski,1966; Brockerhoff et al.,1966b;) obtained representative diacylglycerols from corn oil, olive oil, cocoa butter, peanut oil, dog, cat, horse, turkey and frog triacylglycerols. However, they did not obtain

representative diacylglycerols from seal blubber or cod liver triacylglycerols and only occasionally with lard.

As Apiotrichum curvatum ATCC 10567 and Lipomyces starkeyi ATCC 12659 were the only yeast strains among the four yeast strains which synthesized sufficient amounts of lipids, therefore, sterespecific analysis was conducted only for these two yeast strains. The stereospecific distribution of fatty acids obtained by stereospecific analysis of triacylglycerols synthesized by Apiotrichum curvatum ATCC 10567 and Lipomyces starkeyi ATCC 12659 are presented in Tables 25 and 26. The fatty acid composition of diacylglycerols obtained after lipolysis of triacylglycerols from both the yeast strains show close agreement with the fatty acid composition of the original fractions of triacylglycerols used for this study. The monoacylglycerol fraction obtained during lipolysis represents the sn-1 position. The sn-2 position is represented by the lysophosphatide fraction obtained during hydrolysis of phosphatides with phospholipase A enzyme from snake venom. The sn-3 position was computed from the results of other fractions by employing two methods recommended by Brockerhoff and Yurkowski (1966) and Fatemi and Hammond (1977).

Table 27 shows the distribution of fatty acids among the three positions of the triacylglycerol fractions of Apiotrichum curvatum ATCC 10567 and Lipomyces starkeyi ATCC 12659. The triacylglycerol fraction of these yeast strains contained unsaturated fatty acids in amounts greater than the

Table 25. Fatty acid composition different fractions obtained during stereospecific analysis of triacylglycerols synthesized by Apiotrichum curvatum ATCC 10567

Compound	Position	Fatty acid (mole %)			
		16:0	18:0	18:1	18:2
Triacylglycerol	1,2,3	21.6	12.1	57.1	9.2
Diacylglycerol	1,2:2,3	21.0	13.5	57.9	7.6
Diacylglycerol calc.	1.2:2,3	17.5	9.9	63.1	9.5
Monoacylglycerol	2	5.0	3.1	81.4	10.5
Triacylglycerol recovered	1,2,3	22.0	15.1	54.2	8.7
Lysophosphatide	1	24.3	14.2	57.8	3.7
ß-Fatty acid	2	23.0	11.8	52.6	12.6
D-phosphatide	2,3	20.6	12.1	53.4	13.9
By computation[1]	3	35.5	19.0	32.1	13.4
By computation[2]	3	36.2	21.1	25.4	17.3

1 Calculated from 3xTG minus monoacylglycerol and lysophosphatide.

2 Calculated from 2xD-phosphstide minus monoacylglycerol.

Table 26. Fatty acid composition of different fractions obtained during stereospecific analysis of triacylglycerols synthesized by Lipomyces starkeyi ATCC 12659

Compound	Position	Fatty acid (mole %)				
		16:0	16:1	18:0	18:1	18:2
Triacylglycerol	1,2,3	41.9	2.7	8.4	41.4	5.6
Diacylglycerol	1,2:2,3	36.8	2.1	6.9	48.7	5.5
Diacylglycerol calc.	1,2:2,3	35.2	2.9	7.2	48.1	6.6
Monoacylglycerol	2	13.8	3.7	3.4	69.4	9.7
Triacylglycerol recovered	1,2,3	42.9	2.7	8.7	40.0	5.7
Lysophosphatide	1	20.0	5.1	7.7	60.4	6.8
β-Fatty acid	2	37.6	12.1	14.8	30.5	5.0
D-phosphatide	2,3	36.7	1.7	15.1	41.7	4.8
By computation[1]	3	91.9	-0.7	14.1	-5.6	0.3
By computation[2]	3	59.6	-0.3	26.8	14.0	-0.1

1 Calculated from 3xTG minus monoacylglycerol and lysophosphatide.

2 Calculated from 2xD-phosphatide minus monoacylglycerol.

Table 27. Fatty acid distribution in triacylglycerols synthesized by *Apiotrichum curvatum* ATCC 10567 and *Lipomyces starkeyi* ATCC 12659

Yeast strain	Compound or position	Fatty Acid (mole %) 16:0	16:1	18:0	18:1	18:2
A. curvatum	TG	21.6	-	12.1	57.1	9.2
	1	24.3	-	14.2	57.8	3.7
	2	5.0	-	3.1	81.4	10.5
	3	35.9	-	20.1	28.7	15.3
L. starkeyi	TG	41.9	2.7	8.4	41.4	5.6
	1	20.0	5.1	7.7	60.4	6.8
	2	13.8	3.7	3.4	69.4	9.7
	3	75.8	-	20.5	4.2	0.1

contents of saturated fatty acids. Both yeast strains produced triacylglycerols containing almost entirely unsaturated fatty acids at the sn-2 position. This observation agrees with the results obtained from triacylglycerol fractions from plant sources (Vander Wal,1964), but differs from the results obtained in case of animal fat where saturated fatty acids occur on the sn-2 position. Thorpe and Ratledge (1972) invariably found unsaturated fatty acids at the sn-2 position of triacylglycerols synthesized by five different yeast strains. Oleic acid was the predominant fatty acid at position sn-2. Apiotrichum curvatum ATCC 10567 contained 82% oleic acid at the sn-2 position. whereas oleic acid at the sn-2 position of the triacyglycerols synthesized by Lipomyces starkeyi ATCC 12659 was 70.7%. Position sn-1 also contained unsaturated fatty acids in both the yeast strains.

The triacylglycerols from Apiotrichum curvatum ATCC 10567 had 38.5% saturated fatty acids and 61.5% unsaturated fatty acids at the sn-1 position. In contrast, the triacylglycerols from Lipomyces starkeyi ATCC 12659 had 27.7% saturated fatty acids and 72.3% unsaturated fatty acids at sn-1 position. Oleic acid was the predominant fatty acid at position sn-1 in the case of both yeast strains. Position sn-3 showed the greatest contents of saturated fatty acids in both yeast strains. For instance, Apiotrichum curvatum ATCC 10567 had 56% saturated fatty acids and 44% unsaturated fatty acids. On the other hand, Lipomyces starkeyi ATCC 12659 had 96.3% saturated

other hand, Lipomyces starkeyi ATCC 12659 had 96.3% saturated fatty acids at the sn-3 position. The predominant fatty acid in both yeast strains was palmitic acid. Haley and Jack (1974) reported similar results for the triacylglycerols of Lipomyces lipoferus ATCC 10742. However, they found equal amounts of saturated and unsaturated fatty acids at position sn-3.

Alpha-tocopherol content

The presence of alpha-tocopherol was studied in the case of Lipomyces starkeyi ATCC 12659 because it was the only yeast strain which synthesized a sufficient amount of lipids to be qualified as an oleaginous yeast. The results in Table 28 show that the levels of alpha-tocopherol ranged from 498.7 μg/g to 966.0 μg/g lipid depending upon the carbon:nitrogen ratio. The presence of alpha-tocopherol in Lipomyces starkeyi ATCC 12659 was also confirmed by analyzing derivatized samples of lipids by gas chromatography. The fatty acid profile of lipids synthesized by this strain indicated the predominance of unsaturated fatty acids. The high degree of unsaturation of the fatty acids in the cells necessitates some protective mechanism against lipid peroxidation.

Alpha-tocopherol is abundantly found in oil from plant sources. However, there are contradictory reports in the literature regarding the occurrence of natural antioxidants in microorganisms. The presence of tocopherols in certain species of bacteria and protozoa was investigated by Green et

Table 28. Alpha-tocopherol contents in lipids synthesized by Lipomyces starkeyi ATCC 12659 grown on culture medium having different carbon:nitrogen ratios

C:N ratio	Weight (μg/g)[1]
30:1	489.7 ± 141.2
45:1	678.3 ± 391.0
60:1	862.3 ± 95.6
75:1	966.0 ± 000.0

[1] All values except value for C:N ratio, 75:1, represent the average of two replicates along with standard deviation.

al. (1959). They found only the presence of alpha-tocopherol in the lipids isolated from some of the photosynthetic bacteria (i.e., containing chlorophyll). However, all of the chlorophyll-containing bacteria did not synthesize alpha-tocopherol. In another study, Vance and Bently (1971) examined the lipid fractions of Euglena gracilis strain Z. In addition to alpha-tocopherol, they also detected the presence of a tocopherol isomer having several similarities to alpha-tocopherol. However, Hidetoshi et al. (1983) found no tocopherols in bacteria, yeast and molds while screening a large number of microorganisms for the presence of tocopherols. Instead, they detected some unidentified reducible substances, especially in photosynthetic bacteria. Similarly, Yoon and Rhee (1983) reported that no tocopherols were detected in the neutral lipid fraction of lipids isolated from Rhodotorula glutinis NRRL Y-1091.

SUMMARY AND CONCLUSIONS

The present study was designed to identify yeast strains capable of utilizing whey permeate for lipid biosynthesis, to study triacylglycerol structure and to investigate the effect of carbon:nitrogen ratio on various parameters such as dry cell mass, lipid synthesis and fatty acid composition.

Apiotrichum curvatum ATCC 10567, Cryptococcus albidus ATCC 56297 and Lipomyces starkeyi ATCC 12659 were able to utilize whey permeate efficiently. Rhodosporidium toruloides ATCC 10788 however, did not use whey permeate efficiently. Lipomyces starkeyi ATCC 12659 produced the highest amount of lipids. It synthesized 23.4% to 36.9% lipids, depending upon the carbon:nitrogen ratio of the growth medium and could be classified as an oleaginous yeast strain. The significant ($P<0.05$) effect of both the yeast strain and carbon:nitrogen ratio was observed on dry cell mass yield and lipid biosynthesis. However, a highly significant ($P<0.01$) first-order interaction indicate that the effect of both these variables were not independent of each other.

The lipids synthesized by yeast strains mainly contained triacylglycerol. The triacylglycerol fraction of Apiotrichum curvatum ATCC 10567 and Lipomyces starkeyi ATCC 12659 contained palmitic acid (17.1%-17.8% and 36.3%-40.1%,

respectively) and oleic acid (52.2%-58.8% and 43.6%-48.1%, respectively) as predominant fatty acids, whereas the fatty acid profile of Cryptococcus albidus ATCC 56297 and Rhodosporidium ATCC 10788 indicated the presence of palmitic, oleic and linoleic fatty acids as predominant fatty acids. The phospholipid fractions of Apiotrichum curvatum ATCC 10567 and Lipomyces starkeyi ATCC 12659 also contained oleic acid (28.0%-39.8% and 24.7%-42.4%, respectively) as the predominant fatty acids, as was observed in the triacylglycerol fraction. In contrast, the phospholipid fractions of Cryptococcus albidus ATCC 56297 and Rhodosporidium toruloides ATCC 10788 contained linoleic acid (47.9%-62.6% and 21.6%-35.3%, respectively) as the predominant fatty acids.

Stereospecific analysis of the triacylglycerol fractions of Apiotrichum curvatum ATCC 10567 and Lipomyces starkeyi ATCC 12659 indicated the presence of unsaturated fatty acids at the sn-1 and sn-2 positions. Oleic acid was the predominant fatty acid at both positions. Position sn-3 showed the greatest contents of saturated fatty acids in both the yeast strains. For instance, Apiotrichum curvatum ATCC 10567 had 56% saturated and 44% unsaturated fatty acids. On the other hand, Lipomyces starkeyi ATCC had 96.3% saturated fatty acids at this position.The predominant fatty acid at sn-3 position in both the yeast strains was palmitic acid.

The presence of alpha-tocopherol was also detected in lipids synthesized by Lipomyces starkeyi ATCC 12659. The level

of alpha-tocopherol ranged from 498.7 μg/g to 966.0 μg/g lipid depenging upon carbon:nitrogen ratio of growth medium.

In conclusion, results from this study indicated that the cell mass and lipid biosynthesis are dependant upon the carbon:nitrogen ratio of the culture medium. Fatty acid composition of lipids synthesized by yeast strains largely depend upon the genera and species of yeast and also on carbon:nitrogen ratio. Optimization of cultivation conditions in the chemostat will possibily increase the synthesis of lipids by Lipomyces starkeyi ATCC 12659. The presence of alpha-tocopherol is linked with the protective mechanism against peroxidation of lipids which was required due to high degree of unsaturation of the fatty acids. As the presence of other isomers of tocopherol may also be possible, therefore, further studies need to be carried out. However, mass spectrometric studies will be helpful in verification.

Lipids synthesized by oleaginous yeasts have a fatty acid composition similar to plant seed oils such as corn oil or the palm olein fraction of palm oil. To become economically feasible, lipid production by oleaginous yeasts has to compete sucessfully with oil production by oil seed crops. Changing the fatty acid composition of Lipomyces starkeyi ATCC 12659 by intraspecific spheroplast fusion or by other genetic techniques may help to produce fatty acid composition similar to that of high priced oils such as cocoa butter.

APPENDICES

Appendix A

Parameters used for carrying out quantitation of lipids classes on shimadzu densitometer[1] model CS-930.

Key	Parameter contents	
Photo Mode	Photo mode	ABS Reflection
Signal Proc	Linearizer	SX7
	Accum no.	1
	Background correct	Off
	Signal average	8
Output Format	Ordinate	X1
	Abscissa	X1
	Output select	Curve and print
	Drift line	On
Peak Detect	Mode	2
	Drift line	0.03
	Single	Height
	Sensitivity	Medium
	Min. area	100
	Min. wedth	10
Scan Width	"X" width	8
	Delt "Y"	0.20
Prog Param	Wavelength 1	580
	Wavelength 2	600
	"X" start pos.	25
	"Y" start pos.	10
	"Y" end pos.	100
	Lane dist.	15
	Total lane no.	8

[1] select dual wavelength normal scan

Appendix B

Analysis of variance for growth rate observed in case of Apiotrichum curvatum ATCC 10567.

Source		Degree of freedom	Sum of square	Mean square	F[1] value
Replication	(A)	1	78.98	78.98	
C : N ratios	(B)	3	1575.28	525.09	64.59*
AB		3	24.38	8.13	
Cultivation time	(C)	5	34938.30	6987.66	123.54*
AC		5	282.81	56.56	
BC		15	1575.15	105.01	21.47*
ABC		15	73.42	4.89	
Total		47	38548.32		

[1] P<0.01

Appendix C

Analysis of variance for growth rate observed in case of Cryptococcus albidus ATCC 56297.

Source		Degree of freedom	Sum of square	Mean square	F[1] value
Replication	(A)	1	89.78	89.78	
C : N ratios	(B)	3	982.91	327.64	697.11*
AB		3	1.42	0.47	
Cultivation time	(C)	8	11466.20	1433.28	142.61*
AC		8	80.38	10.05	
BC		24	595.33	24.81	63.62*
ABC		24	9.36	0.39	
Total		71	13225.38		

1 P<0.01

Appendix D

Analysis of variance for growth rate observed in case of Lipomyces starkeyi ATCC 12659.

Source		Degree of freedom	Sum of square	Mean square	F[1] value
Replication	(A)	1	12.60	12.60	
C : N ratios	(B)	3	23.14	7.71	1.27
AB		3	18.20	6.07	
Cultivation time	(C)	10	2912.01	291.20	92.74*
AC		10	31.39	3.14	
BC		30	77.98	2.60	1.64
ABC		30	47.74	1.59	
Total		87	3123.06		

[1] P<0.01

Appendix E

Analysis of variance for growth rate observed in case of Rhodosporidium toruloides ATCC 10788.

Source		Degree of freedom	Sum of square	Mean square	F[1] value
Replication	(A)	1	2.54	2.54	
C : N ratios	(B)	3	2.84	0.95	47.50*
AB		3	0.06	0.02	
Cultivation time	(C)	2	138.05	19.03	35.24*
AC		2	1.08	0.54	
BC		6	1.23	0.21	7.78
ABC		6	0.16	0.027	
Total		23	145.96		

1 P<0.01

Appendix F

Analysis of variance for lactose utilization by *Apiotrichum curvatum* ATCC 10567.

Source		Degree of freedom	Sum of square	Mean square	F[1] value
Replication	(A)	1	5.81	5.81	
C : N ratios	(B)	3	700.23	233.41	9.87*
AB		3	70.96	23.65	
Cultivation time	(C)	5	8330.25	1666.05	162.70*
AC		5	51.20	10.24	
BC		15	480.52	32.03	3.65*
ABC		15	131.63	8.78	
Total		47	9770.60		

[1] $P<0.01$

Appendix G

Analysis of variance for lactose utilization by *Cryptococcus albidus* ATCC 56297.

Source		Degree of freedom	Sum of square	Mean square	F[1] value
Replication	(A)	1	1092.44	1092.44	
C : N ratios	(B)	3	1185.16	395.05	61.15*
AB		3	19.38	6.46	
Cultivation time	(C)	8	4606.35	575.79	27.06*
AC		8	170.25	21.28	
BC		24	456.29	19.01	3.53*
ABC		24	129.16	5.38	
Total		71	7659.03		

[1] $P<0.01$

Appendix H

Analysis of variance for lactose utilization by *Lipomyces starkeyi* ATCC 12659.

Source		Degree of freedom	Sum of square	Mean square	F[1] value
Replication	(A)	1	1903.34	1903.34	
C : N ratios	(B)	3	43.20	14.40	0.30
AB		3	145.81	48.60	
Cultivation time	(C)	10	4696.62	469.66	72.26*
AC		10	64.97	6.50	
BC		30	221.60	7.39	0.38
ABC		30	586.51	19.55	
Total		87	7662.05		

[1] $P<0.01$

Appendix I

Analysis of variance for lactose utilization by *Rhodosporidium toruloides* ATCC 10788.

Source		Degree of freedom	Sum of square	Mean square	F[1] value
Replication	(A)	1	13.68	13.68	
C : N ratios	(B)	3	25.16	8.39	2.85
AB		3	8.81	2.94	
Cultivation time	(C)	2	125.42	62.71	6.23
AC		2	20.13	10.07	
BC		6	17.09	2.85	0.54
ABC		6	31.44	5.24	
Total		23	241.73		

[1] $P<0.01$

Appendix J

Analysis of variance for protein contents of culture medium in case of *Apiotrichum curvatum* ATCC 10567.

Source		Degree of freedom	Sum of square	Mean square	F[1] value
Replication	(A)	1	0.01	0.01	
C : N ratios	(B)	3	0.27	0.09	12.86
AB		3	0.02	0.007	
Cultivation time	(C)	5	1.76	0.352	29.33
AC		5	0.06	0.012	
BC		15	0.04	0.003	0.20
ABC		15	0.23	0.015	
Total		47	2.48		

[1] $P<0.01$

Appendix K

Analysis of variance for pritein contents of culture medium in case of Cryptococcus albidus ATCC 56297.

Source		Degree of freedom	Sum of square	Mean square	F[1] value
Replication	(A)	1	0.09	0.09	
C : N ratios	(B)	3	0.29	0.097	13.86*
AB		3	0.02	0.007	
Cultivation time	(C)	8	6.24	0.78	55.71*
AC		8	0.11	0.014	
BC		24	0.07	0.003	0.72
ABC		24	0.09	0.004	
Total		71	6.91		

1 P<0.01

Appendix L

Analysis of variance for protein contents of culture medium in case of Lipomyces starkeyi ATCC 12659.

Source		Degree of freedom	Sum of square	Mean square	F[1] value
Replication	(A)	1	0.22	0.22	
C : N ratios	(B)	3	0.03	0.01	0.05
AB		3	0.59	0.197	
Cultivation time	(C)	10	4.62	0.462	154.00*
AC		10	0.03	0.003	
BC		30	0.13	0.004	0.29
ABC		30	0.42	0.014	

1 P<0.01

Appendix M

Analysis of variance for protein contents of culture medium in case of Rhodosporidiun toruloides ATCC 10788.

Source		Degree of freedom	Sum of square	Mean square	F[1] value
Replication	(A)	1	0.00	0.00	
C : N ratios	(B)	3	0.27	0.09	9.00
AB		3	0.03	0.01	
Cultivation time	(C)	2	1.67	0.84	168.00*
AC		2	0.01	0.005	
BC		6	0.03	0.005	0.39
ABC		6	0.08	0.013	
Total		23	2.09		

1 $P<0.01$

Appendix N

Analysis of variance of dry call mass of yeast strains

Source		Degree of freedom	Sum of square	Mean square	F value
Strains	(A)	3	943.54	314.51	50.24*
C : N ratios	(B)	3	26.84	8.95	27.12*
AB		9	82.45	9.16	14.31*
Replications random	(C)	1	20.00	20.00	
AC		3	18.77	6.26	
BC		3	1.00	0.33	
ABC		9	5.76	0.64	
Total		31	1098.36		

1 P<0.01

Appendix O

Analysis of variance for lipid yield of yeast strains

Source		Degree of freedom	Sum of square	Mean square	F[1] value
Strains	(A)	3	3315.21	1105.07	177.95**
C : N ratios	(B)	3	23.79	7.93	0.82
AB		9	260.53	28.95	3.11*
Replication random	(C)	1	2.26	2.26	
AC		3	18.63	6.21	
BC		3	28.99	9.66	
ABC		9	83.80	9.31	
Total		31	3733.21		

[1] P<0.01 and P<0.05

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