1989

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Wan Soo Park
Iowa State University

Order Number 9014938

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Park, Wan Soo, Ph.D.

Iowa State University, 1989

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

Role of peroxisomes in lipid metabolism of the oleaginous yeast *Apiotrichum curvatum*

by

Wan Soo Park

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Major: Food Technology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University

Ames, Iowa

1989

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ABBREVIATIONS USED

Ab:	absorbance
ADP:	adenosine-5'-diphosphate
ATP:	adenosine-5'-triphosphate
CoA:	coenzyme A
C/N ratio:	carbon:nitrogen ratio
DAB:	diaminobenzidine
DCW:	dry cell weight
FAD:	flavin adenine dinucleotide
$FADH_2$:	flavin adenine dinucleotide, reduced
NAD:	nicotinamide adenine dinucleotide
NADH:	nicotinamide adenine dinucleotide, reduced
NADP:	nicotinamide adenine dinucleotide phosphate
NADPH:	nicontinamide adenine dinucleotide phosphate, reduced
TCA cycle:	tricarboxylic acid cycle

INTRODUCTION

Oleaginous yeasts can accumulate lipid when grown on a carbohydrate source under nitrogen limitation (18, 114, 115, 117, 147, 148), and can also utilize the accumulated lipid in the presence of an exogenous nitrogen source if starved for carbon (64). Several yeasts including Apiotrichum curvatum can also grow on an exogenous lipid as a carbon and energy source (6, 55, 65). This physiological flexibility and adaptability can make oleaginous yeasts a good model system to investigate the control of lipid metabolism in eucaryotic cells and to study the role of peroxisomes in that metabolism.

Recently, attention has begun to be paid to peroxisomes in oleaginous yeasts (65). Peroxisomes have been studied mainly in yeasts grown on n-alkanes or methanol, and are known to carry out several important metabolic functions, including peroxisomal beta-oxidation and glyoxylate metabolism (47, 48, 142). Activities of some peroxisomal enzymes were measured in cell-free extracts of oleaginous yeasts, including A. curvatum (65, 118, 125), and the presence of peroxisomes was confirmed in these organisms by electron microscopy (65). The separation of peroxisomes and the description of their role in the metabolic functions of oleaginous yeasts have not been reported.

To investigate the role of peroxisomes in lipid metabolism in the oleaginous yeast A. curvatum, lipid production, changes in cell composition, and catalase activity as a marker enzyme for peroxisomes were compared in cells grown on lactose with different carbon:nitrogen

(C/N) ratios; induction of peroxisomal enzyme activity was studied in cultures prepared under various growth conditions; peroxisomes were separated from other subcellular organelles by sucrose density gradient centrifugation; and the functions of peroxisomes were characterized in this yeast.

Metabolic Functions of Peroxisomes in Eucaryotic Cells

Peroxisomes present in most eucaryotic cells are subcellular respiratory organelles with catabolic pathways. They are characterized biochemically by the presence of hydrogen peroxide-producing oxidases and catalase, and morphologically by the existence of a single limiting membrane, a fine granular matrix, and sometimes a crystalline or tubular core (7, 9, 11, 33-35, 47, 48, 53, 63, 85, 87, 91-93, 102, 112, 123, 127, 136-138, 142). They are thought to contain neither their own genetic material (67) nor energy-coupled electron transport systems (137). The metabolic pathways in peroxisomes vary depending upon tissue, substrate availability, and stage of development (137).

Peroxisomes were first discovered in 1954 by Johannes A. G. Rhodin, an electron microscopist, in mouse kidney cells (34). He saw small organelles that had not been described before and called them "microbodies." After isolation techniques were refined in the mid-1950s, peroxisomes were separated from many types of eucaryotic cells and characterized biochemically.

In 1965, the de Duve group (8) localized several oxidases and

catalase in organelles isolated from rat liver and named these organelles "peroxisomes" on the basis of their functions. In 1967, the Beevers group (20) found microbodies in the germinating castor bean endosperm, located the fatty acid beta-oxidation system and some of the glyoxylate cycle enzymes in these organelles, and named the organelles "glyoxysomes." In 1968, the Tolbert group (138) isolated similar organelles from green leaves, found they participated in photorespiration, and called them "leaf peroxisomes."

Yeast peroxisomes were first reported in cells of Saccharomyces cerevisiae grown on glucose by the Avers group (3) in 1968. They have since been studied mainly in nonoleaginous yeasts grown on n-alkanes (47, 48), fatty acids (47, 48), methanol (47, 48, 142), or amines (94, 152) as a carbon or nitrogen source.

Peroxisomes are ubiquitous in eucaryotic cells and are known to function in various cell types in fatty acid beta-oxidation, cellular respiration, plasmalogen biosynthesis, cholesterol metabolism with bile acid synthesis, gluconeogenesis, alcohol oxidation and peroxidation, glycolate and glycerate pathways, and purine and polyamine catabolism (11, 34, 47, 48, 83, 85, 92, 137, 142). Many peroxisomes in animal, plant seeds, and fungal cells share at least two biochemical capabilities: respiration associated with hydrogen peroxide metabolism and fatty acid beta-oxidation. Although metabolic functions of peroxisomes in eucaryotic cells are not clear yet, peroxisomes appear to play essential roles in lipid metabolism in eucaryotes (11, 34, 47, 48, 83, 85, 137).

Fatty acid beta-oxidation

Fatty acid beta-oxidation, different in several respects from mitochondrial beta-oxidation, is found in peroxisomes (10, 21, 27, 56, 74, 82, 84, 86, 109, 124, 140). It is comprised of a fatty acid activating enzyme, four beta-oxidation enzymes, two auxiliary enzymes (2,4-dienoyl-CoA reductase and an enoyl-CoA isomerase) that are required for the oxidation of some polyunsaturated fatty acids (31, 62, 71), and a short-chain carnitine acyltransferase. In peroxisomal beta-oxidation, carnitine is not required for the entry of fatty acids into peroxisomes, but it probably helps in the removal of end products such as acetyl-CoA and short-chain acyl-CoAs (85, 137). Peroxisomes contain an FAD-linked acyl-CoA oxidase that transfers electrons to oxygen to produce hydrogen peroxide, which is decomposed by catalase (109).

Fatty acids as substrates for peroxisomal beta-oxidation may have either an odd or an even number of carbons, be saturated or unsaturated, and be seven to at least 26 carbons in length (85, 137). Other substrates include the CoA-derivatives of dicarboxylic fatty acids, the side-chain of partially oxidized cholesterol, and glutaric acid (85, 137). This pattern of substrate specificity overlaps extensively with that of mitochondrial beta-oxidation. Carnitine acyltransferase activity to transfer the products out of peroxisomes is substantial for acetyl-CoA, is maximal for octanoyl-CoA (hexanoyl-CoA in rats), and declines with increasing chain length (42, 98).

Peroxisomal beta-oxidation enzymes are unique proteins with molecular properties that are quite different from those of their

mitochondrial counterparts (60, 137), and are encoded by different nuclear genes (51, 97, 113). Many enzymes in the two organelles are isozymes, but not the same proteins. For example, peroxisomes contain a bifunctional enzyme with enoylhydratase and L-3-hydroxy fatty acyl-CoA dehydrogenase activities, in contrast to mitochondria that contain the two separate enzymes. Peroxisomal thiolase is different from thiolases in mitochondria and in the cytoplasm (137).

One role of mammalian peroxisomal beta-oxidation is the metabolism of those fatty acids and other substrates that are poorly handled by mitochondria (83, 85, 126). Peroxisomes contribute to bile acid formation by catalyzing the beta-oxidative shortening of the side chain of cholesterol. They also are involved in the chain-shortening of long unsaturated fatty acids by performing only two or three cycles of beta-oxidation.

Peroxisomal beta-oxidation also generates acetyl-CoA to be used in biosynthetic reactions from fatty acids. This function is shared with mitochondria in mammalian cells (81, 82, 140). In yeasts (47, 74) and plant seeds (27), however, mitochondria are incapable of beta-oxidation, and peroxisomes play an essential role in the use of fat as an energy source. Acetyl-CoA generated in the plant peroxisomes is used to synthesize sugar in a process that also requires the peroxisomal enzymes of the glyoxylate cycle. In mammalian cells, the peroxisomal beta-oxidation system forms acetyl-CoA to be transferred to the mitochondria for ATP production. Also, the mitochondrial beta-oxidation system forms acetyl-CoA to be further oxidized in the Krebs cycle as a source of

energy.

The NADH produced in peroxisomal beta-oxidation may function in providing a pool of nucleotides for other cell processes and in maintaining the cell's redox potential. The $FADH_2$ and the NADH produced in mitochondrial beta-oxidation can be reoxidized to produce ATP through the electron transport system.

Recently, Leighton et al. (88) have shown that the physiological activity of peroxisomal beta-oxidation in mammalian cells increases in parallel with the intracellular ATP concentration. The regulation of peroxisomal fatty acid catabolism in these cells thus appears to be opposite to that of mitochondrial fatty acid oxidation.

Respiration

The first peroxisomal function discovered by the de Duve group (8, 35) was a simple respiratory pathway. The pathway is based on the formation of hydrogen peroxide (H_2O_2) by several oxidases and the decomposition of the H_2O_2 by catalase.

One function of peroxisomal respiration reactions is to dispose of excess reducing equivalents without conserving energy as ATP. Substrates for the oxidases are urate, L-alpha-hydroxy acids, L- and D-amino acids, beta-oxidation substrates, polyamines, glutaryl-CoA, and oxalate (35, 85, 137).

A second function of the compartmentalization of much of the cell's H_2O_2 metabolism within peroxisomes is to protect cells from H_2O_2. Mammalian cells have a second protective system, glutathione peroxidase,

in the cytosol that can decompose H_2O_2 that escapes from peroxisomes or that is generated from cytosolic reactions (23). The existence of such a second protective system in yeasts or plants is not yet clear. Recently, Verduyn et al. (146) have reported that cytochrome c peroxidase serves as a second protective system in yeasts. Cytochrome c peroxidase, located in the intracristate space of mitochondria, is a key enzyme of H_2O_2 detoxification in yeasts like Hansenula polymorpha. Catalase can effectively compete with mitochondrial cytochrome c peroxidase for H_2O_2 only if H_2O_2 is generated in the peroxisomes. Glutathione peroxidase was reported to be absent in H. polymorpha.

Peroxisomal respiration is thermogenic because the energy of the oxidation is not conserved as ATP. Peroxisomes of brown adipose tissue are strikingly induced during cold adaptation (11).

Gluconeogenesis

Gluconeogenesis occurs in different ways in mammalian and nonmammalian cells. In mammalian cells on a high protein diet or during starvation when tissue protein is utilized (66, 105, 106), gluconeogenesis from amino acids occurs. Transamination and oxidation reactions in the peroxisomes play a major role in this system. In yeasts and plants, gluconeogenesis occurs through the glyoxylate cycle that may be closely associated with peroxisomal beta-oxidation (9, 19, 20, 26, 47, 48, 63). Peroxisomes from yeasts (47, 48, 93), Tetrahymena (63), Euglena (56), and various fungi (93) have been reported to contain isocitrate lyase and malate synthase, but not the other enzymes of the glyoxylate

cycle. In contrast, glyoxysomes from germinating castor beans contain, in addition to the two key enzymes, citrate synthase (20) and a small amount of aconitase (26), needed for the glyoxylate pathway to produce succinate from two acetyl-CoAs. The locations of the enzymes common to both the TCA cycle and the glyoxylate cycle have not been studied in other germinating seeds.

Other functions

Other peroxisomal functions include purine and polyamine catabolism, alcohol peroxidation, plasmalogen and cholesterol biosynthesis in mammalian cells (85), alcohol oxidation in yeasts (142), and glycolate and glycerate pathways in leaf peroxisomes (137).

Most eucaryotes catabolize purines via xanthine and urate to allantoin, in which the last step is catalyzed by the peroxisomal enzyme urate oxidase (85, 123). The polyamines spermine and spermidine are both degraded in rat liver by a single peroxisomal enzyme, polyamine oxidase, with the formation of putrescine, 3-aminopropionaldehyde, and H_2O_2. The peroxisomes of some yeasts grown on methylamine, 1-aminoalkanes, or putrescine as sole source of carbon, nitrogen, and energy contained amine oxidase (94, 152).

In mammalian cells, production of alkyl-glycerol-3-phosphate, a key intermediate in the synthesis of plasmalogens and other ether lipids, occurs in the peroxisomes through the action of dihydroxyacetone phosphate acyltransferase (85). The key regulatory enzyme in cholesterol biosynthesis, 3-hydroxy-3-methyl-glutaryl-CoA reductase, is located in

the endoplasmic reticulum. When rats are treated with cholestyramine to induce this enzyme, its level in liver peroxisomes is increased from less than 5% of total activity in normal rats to 20-30% of total activity (77, 78).

Although most ethanol oxidation is catalyzed by the cytosolic enzyme alcohol dehydrogenase, at high ethanol concentrations peroxisomes contribute to ethanol detoxification by means of the peroxidatic reaction of catalase (107).

Leaf peroxisomes play a major role in the conversion of glycolate to glycine and serine in the oxidative photosynthetic carbon cycle of photorespiration, and in the reversible NAD-linked interconversion of glycerate and serine (53, 137-139).

Yeast Peroxisomes

Yeast peroxisomes that contained several enzymes including catalase were first detected by Avers and her group (3) in cells of Saccharomyces cerevisiae grown on glucose. The function of peroxisomes in yeast metabolism has not been clarified and varies depending upon species or substrates used as carbon or nitrogen sources (47, 48, 94, 142, 152). In yeast cells, the synthesis of the organelle seems to be induced only by special carbon or nitrogen sources (47, 48, 142, 152).

Alkane-utilizing yeasts

During studies of the physiology and metabolism of alkane-utilizing yeasts like Candida tropicalis pK 233, the Fukui group (110, 134, 135) first discovered the existence of peroxisomes in these organisms. The appearance of conspicuous numbers of peroxisomes in yeast cells grown on alkanes or higher fatty acids was accompanied by a large increase in cellular catalase activity (75, 108). Such changes were, however, rarely observed in cells grown on ethanol, acetate, or glucose. The occurrence of such organelles has also been demonstrated in other alkane-utilizing yeasts (47, 110, 131). Peroxisomes in alkane-utilizing yeasts participate in the degradation of the fatty acids derived from alkanes to two-carbon units, and in the synthesis of gluconeogenic intermediates from these two-carbon units (104, 128, 129). By contrast, the alkane hydroxylation system seems not to be located in the organelles, but rather in the microsomes and elsewhere in the cell (17, 32, 49, 149).

The fatty acids derived from alkanes enter the peroxisomes and are activated to the corresponding CoA-esters by an acyl-CoA synthetase. The enzyme in the peroxisomes is different from that in microsomes and mitochondria. Distinct subcellular localizations of two long-chain fatty acyl-CoA synthetases were demonstrated in mutant strains of Candida lipolytica grown on higher fatty acids or alkanes (68, 95, 96, 130). One (acyl-CoA synthetase II) was located exclusively in peroxisomes and was linked to the beta-oxidation system in the organelle. The other (acyl-CoA synthetase I) was present outside the organelles and was responsible for the production of acyl-CoA used directly for the synthesis of

cellular lipids.

The distribution of enzymes among peroxisomal, mitochondrial and supernatant fractions from alkane-grown Candida tropicalis was reviewed by Fukui and Tanaka (47). The supernate could contain enzymes from broken organelles as well as cytosolic enzymes. The NAD-linked glutamate dehydrogenase, a marker enzyme for mitochondria in mammalian cells (89), seemed to be a cytosolic enzyme in this yeast strain. Catalase, D-amino acid oxidase, and uricase were marker enzymes for peroxisomes (35), and fatty acid beta-oxidation was detected only in the peroxisomal fraction. Among the five enzymes of the glyoxylate cycle, only two (isocitrate lyase and malate synthase) were detected in peroxisomes; the others were detected in mitochondria as tricarboxylic acid cycle enzymes. The NAD-linked isocitrate dehydrogenase seemed to be mitochondrial, but the NADP-linked enzyme seemed to be peroxisomal.

Methanol-utilizing yeasts

Peroxisomes are found in yeast cells grown on methanol, but are rarely detected in the same cells grown on glucose or ethanol (28, 30, 46, 50, 121, 122, 132, 143, 150). Alcohol oxidase and catalase in the peroxisomes participate in methanol oxidation (29, 72, 73, 120, 141). The peroxisomes in methanol-assimilating yeasts like Candida boidinii (formerly Kloeckera sp. 2201) and Hansenula polymorpha play an indispensable role in the initial oxidation of methanol to form formaldehyde and hydrogen peroxide (47, 48, 142). Oxidation of formaldehyde by a glutathione-dependent dehydrogenase occurs in the

cytoplasm.

Other yeasts

The peroxisomes in methylamine-assimilating yeasts like the methylotrophic (one-carbon compound-utilizing) yeast H. polymorpha and the nonmethylotrophic yeast C. utilis were found to contain amine oxidase when cultures were grown on methylamine as sole nitrogen source (152). Other yeasts, grown on 1-aminoalkanes or putrescine as sole source of carbon, nitrogen, and energy also contained peroxisomal amine oxidase (94).

The peroxisomes of Saccharomyces cerevisiae were reported to contain enzymes of the glyoxylate cycle (malate synthase and isocitrate lyase) in addition to catalase (127). Parish (111) reported that peroxisomes isolated from derepressed S. cerevisiae cells grown on lactose with uric acid contained catalase and urate oxidase, along with low activities of D-amino acid oxidase and L-alpha-hydroxy acid oxidase (glycolate oxidase), but no glyoxylate cycle enzymes.

Peroxisomes in oleaginous yeasts

Yeast peroxisomes have been studied mainly in yeasts grown on n-alkanes or methanol. Recently, activities of peroxisomal enzymes such as carnitine acetyltransferase (65, 118), catalase (65), isocitrate lyase (65), isocitrate dehydrogenase (65), and D-amino acid oxidase (125) were reported in cell-free extracts of some oleaginous yeasts, including A. curvatum. The presence of peroxisomes was confirmed in electron

micrographs of A. curvatum and Lipomyces starkeyi (65).

Lipid Accumulation in Oleaginous Yeasts

Oleaginous microorganisms

Since the early interest in fungal lipids in the 1870's, lipid accumulation in microorganisms has been studied with thoughts of developing alternative sources of lipids for food and industrial uses, producing a valuable product from treatment of waste materials, or biomodifying existing lipids (6, 18, 55, 114, 115, 117, 147, 148).

Fungi have generally been preferred over bacteria and algae as sources of lipids because of the higher yields obtainable with some species, the quality of the lipid produced, the lack of toxic contaminants, and the relative ease of growing the organisms (18, 115, 117). Yeasts have generally been preferred over molds because their single-cell mode of growth has been easier to handle than the mycelial growth of molds and they tend to convert substrate to lipid more efficiently (18, 115, 117).

Oleaginous organisms are defined as those that can accumulate more than 20% of their dry weight as lipid (148). All oleaginous organisms contain ATP:citrate lyase, a key enzyme for lipid biosynthesis; nonoleaginous yeasts do not contain this enzyme (13, 15).

A great deal is known about patterns of lipid accumulation in yeasts (18, 114, 115, 117). Lipid is produced throughout the growth cycle of all yeasts, whether oleaginous or not. But, while nonoleaginous yeasts

cease both growth and lipid synthesis as they enter stationary phase, oleaginous organisms continue to produce lipid after other biosynthetic processes have ceased as long as a usable carbon source remains in the growth medium.

Nitrogen limitation is most frequently used to favor lipid accumulation, and carbon:nitrogen ratios as high as 80:1 are commonly used in the growth medium in oleaginous fermentations (115). Limitations of other nutrients such as phosphate, sulfate, iron, and inositol have been shown to stimulate lipid accumulation in some organisms (18, 147, 148). Factors that affect lipid composition within a cell include growth rate, the nature of the substrate, and environmental conditions such as temperature, pH, and dissolved oxygen concentration (18, 25, 36, 119, 147, 148, 151).

When grown under nitrogen limitation, oleaginous yeasts can accumulate intra- and extracellular carbohydrates in addition to intracellular lipid. Some yeasts, including A. curvatum, have been reported to accumulate some extracellular slime during growth and to metabolize it during the lipid production phase (101). Carbohydrates accumulated intracellularly to 42% of dry cell weight in NH_4-grown cells of Rhodosporidium toruloides and seemed to be mainly glycogen (38). Also, A. curvatum was found to accumulate, in addition to lipid, about 10% of its biomass as glycogen (64).

Studies with Apiotrichum curvatum

Apiotrichum curvatum ATCC 20509 was first reported in our laboratory as an oleaginous yeast capable of efficient conversion of lactose in cheese whey to oil (100, 101). This yeast was originally identified as Candida curvata but was reclassified as A. curvatum based on its types of conidiogenesis and the presence of xylose in cell hydrolysates (2). It can grow on a wide range of carbohydrates (55) and can accumulate up to 60% of its dry weight as intracellular lipid when grown under nitrogen limitation (101). The major lipid that is stored, usually in the form of discrete droplets, is triglyceride with a fatty acid composition similar to that of cocoa butter (59).

Moon in 1977 studied the physical conditions and nutritional requirements for growth and lipid production from whey and whey permeate (99). Subsequently, Choi defined the most suitable method for extraction of the lipid (24). Teasdale studied the effect on oil production of the use of different carbon:nitrogen ratios and different types of whey and milk products as fermentation substrates (133).

Baehman attempted to improve the fermentation through mutagenesis of the yeast and isolation of mutants able to grow at extremes of temperature and pH (4). Floetenmeyer studied oil accumulation by cultures growing on various food processing and agricultural waste products as carbon sources, and also determined the efficiency of oil production in continuous culture fermentation (43). Bati studied biomodification of the fatty acid composition of animal fat and plant oil through microbial fermentation (5). He grew various yeasts on beef

tallow, corn oil, linseed oil, olive oil, and palm oil as carbon sources, and examined the composition of the oil accumulated by the organism.

Brown carried out a kinetic study on this yeast grown in nitrogen-limited broth in batch and single-stage continuous fermentations, and suggested a kinetic model to describe the relationship between growth and lipid accumulation (22). Li isolated mutants of A. curvatum that could grow at high temperature and searched for other yeasts able to produce or modify oil at high temperature (90). Vega optimized growth and lipid production by A. curvatum in banana juice (144).

Most of this work is summarized in several papers (6, 44, 54, 55, 59, 100, 101, 145).

Biochemical mechanism of lipid synthesis

The biochemical mechanisms of lipid synthesis in oleaginous yeasts have been studied extensively by Colin Ratledge and his colleagues at the University of Hull, England (12-16, 37-41, 116).

Generally, when culture growth is limited by the supply of nitrogen or presumably by any other nutrient except carbon, lipid biosynthesis can begin in the cytoplasm. The key to oleaginicity lies in the build-up of ATP and the depletion of AMP. This depletion of AMP in nitrogen-limited cells leads to an inactivation of mitochondrial NAD-linked isocitrate dehydrogenase. As a result, citrate accumulates, is transported out of the mitochondrion, and is converted to acetyl-CoA and oxaloacetate by the enzyme ATP:citrate lyase. Citrate was found to accumulate intra- and extracellularly before lipid production occurred in oleaginous yeasts

(37). The extramitochondrial acetyl-CoA is used for fatty acid biosynthesis; acetyl-CoA carboxylase, the regulatory enzyme of this pathway, is stimulated by citrate.

Lipid Assimilation in Oleaginous Yeasts

Assimilation of exogenous lipid

Biomodification of low-grade lipids to a more desirable form might be accomplished by growing oleaginous yeasts on lipids as carbon sources (6, 55). In general, alkane-utilizing yeasts can utilize fatty acids as carbon sources because fatty acids can be derived from n-alkanes via fatty alcohols and can be shortened to produce acetyl-CoA through the peroxisomal beta-oxidation system (47, 48). To use lipids as carbon sources, oleaginous yeasts require lipase(s) to hydrolyze triglycerides to form glycerol and free fatty acids.

Various oleaginous yeasts including Apiotrichum curvatum have been tested for their ability to produce lipase and to assimilate lipids as carbon sources (6). Candida lipolytica, which is also an alkane-utilizing yeast, seemed most promising for lipid biomodification; its fatty acid composition was quite similar to that of the substrate lipid. Similarly, the fatty acid composition of an alkane-utilizing yeast is usually a reflection of the alkane on which it is cultivated (18, 117).

Metabolism of endogenous lipid

Oleaginous yeasts can utilize accumulated lipid in the presence of an exogenous nitrogen source when starved for carbon (64). The lipid serves as a storage material of carbon and energy to be used by the cells.

This situation is very similar to that in the germinating castor bean endosperm (26, 27, 34, 137). The bean's glyoxysomes, which are special forms of peroxisomes, contain a fatty acid beta-oxidation system and glyoxylate cycle enzymes. The stored triglycerides are hydrolyzed to fatty acids and glycerol by lipases, the fatty acids are activated to acyl-CoAs and then converted to sugars, and the sugars support the growth of the young seedling. Castor bean endosperm contains an alkaline lipase in the glyoxysomes, possibly in their membranes, as well as two lipases in the lipid body with acid and neutral pH optima (61, 103). Very little is known about the control of lipase activity in plants and yeasts, and how fatty acid release from triglyceride is coordinated with fatty acid oxidation to acetyl-CoA.

Explanation of Dissertation Format

This dissertation follows an alternate format. The main portion of this dissertation is divided into three parts, each being a complete paper submitted to a professional journal. Part I is a comparison of Apiotrichum curvatum grown on lactose in media that contained different carbon:nitrogen ratios. Part II discusses induction of peroxisomes in

this yeast. Part III reports on subcellular localization of fatty acid beta-oxidation and key enzymes of the glyoxylate cycle in peroxisomes of A. curvatum. All experiments were performed by the author with helpful advice and discussion from Dr. Bonita A. Glatz and Dr. Patricia A. Murphy. Manuscripts were also prepared by the author with discussion with Drs. Glatz and Murphy.

PART I.

LIPID METABOLISM AND CELL COMPOSITION OF THE OLEAGINOUS YEAST *Apiotrichum curvatum* GROWN AT DIFFERENT CARBON:NITROGEN RATIOS

ABSTRACT

Apiotrichum curvatum ATCC 20509, an oleaginous yeast that can accumulate large quantities of intracellular lipid when grown with excess carbon, was grown in nitrogen-limited, balanced, and carbon-free medium with asparagine as nitrogen source and lactose as carbon source. Biomass and lipid accumulation were measured, cell composition was analyzed, and catalase activity was followed as marker enzyme for peroxisomes. The organism accumulated 54% of its dry weight as intracellular lipid when grown under nitrogen limitation, and accumulated no more than half that amount when grown in balanced medium. When starved for carbon, cells utilized endogenous lipid and carbohydrate as carbon and energy sources. Intracellular carbohydrates also seemed to be used as intermediates for lipid accumulation and lipid turnover. Catalase activity was strongly induced when cells metabolized endogenous lipid. The lipid content of cells was inversely related to catalase activity and to protein or nitrogen content. Lipid content showed no correlation with intracellular carbohydrate content.

INTRODUCTION

Apiotrichum curvatum ATCC 20509 (formerly Candida curvata D) was first reported in our laboratory as an oleaginous yeast capable of efficient conversion of lactose in cheese whey to oil (15, 16). The organism can grow on a wide range of carbohydrates (9) and accumulate up to 60% of its dry weight as intracellular lipid when grown under nitrogen limitation (16). The major lipid that is stored, usually in the form of discrete droplets, is triglyceride with a fatty acid composition similar to that of cocoa butter (11).

Lipid accumulation in oleaginous yeasts, including A. curvatum, has been quite well explained both biochemically and physiologically (2, 3, 5, 6, 18). Citrate, which is believed to be a key intermediate for lipogenesis, was found to accumulate intra- and extracellularly before lipid production in oleaginous yeasts (5).

Carbohydrates can also accumulate intra- and extracellularly in oleaginous yeasts grown under nitrogen limitation. Some yeasts, including A. curvatum, produced extracellular slime during growth and metabolized it during the lipid production phase (16). Carbohydrates, mainly glycogen, accumulated intracellularly to 42% of cell dry weight in NH_4-grown cells of Rhodosporidium toruloides (6). Also, A. curvatum accumulated about 10% of its biomass as glycogen (12).

Oleaginous yeasts can also utilize accumulated lipid in the presence of an exogenous nitrogen source if starved for carbon (12). The lipid apparently serves as an energy reserve, similar to carbohydrate storage

materials.

Recently, some attention has been paid to the possible functions of peroxisomes in oleaginous yeasts (13). Yeast peroxisomes, which have been studied mainly in yeasts grown on n-alkanes or methanol, are known to carry out several important metabolic functions, including peroxisomal fatty acid beta-oxidation and the glyoxylate cycle (7, 8). Activities of some peroxisomal enzymes were reported in cell-free extracts of A. curvatum (13), and the presence of peroxisomes in this organism was confirmed by electron microscopy (13).

In the present investigation, growth of A. curvatum in defined media with lactose as carbon source at different carbon:nitrogen (C/N) ratios was studied. Lipid production, cell composition, and catalase activity as a marker enzyme for peroxisomes were followed during growth.

MATERIALS AND METHODS

Yeast

Apiotrichum curvatum ATCC 20509 (formerly Candida curvata D) was used in all experiments. Stock cultures were maintained on YPD agar slants at 4°C and were transferred monthly. YPD agar contained (g/l): yeast extract, 10; peptone, 20; dextrose, 20; and agar, 20.

Media

The basal medium (20) contained the following constituents (g/l): KH_2PO_4, 2.5; $MgSO_4 \cdot 7H_2O$, 1.0; $FeCl_3 \cdot 6H_2O$, 0.02; $CaCl_2 \cdot 2H_2O$, 0.2; $ZnSO_4 \cdot 7H_2O$, 0.001; $MnSO_4 \cdot H_2O$, 0.002; NaCl, 0.06; $CuSO_4 \cdot 7H_2O$, 0.0001; and thiamine-HCl, 0.001. Lactose and asparagine were added as carbon and nitrogen sources, respectively, at the following concentrations (g/l): lactose 40, asparagine 4.4 (carbon:nitrogen ratio or C/N=18); lactose 40, asparagine 15.9 (C/N=5); lactose 40, asparagine 0.8 (C/N=99); lactose 0, asparagine 5.0 (C/N=0). The amount of carbon in asparagine was not taken into account in calculating C/N ratios. The pH was adjusted to pH 5.5, and all media were sterilized in the autoclave at 121°C for 15 min.

Shake flask studies

Cultures were grown in 250-ml Erlenmeyer flasks containing 100 ml of medium at 32°C with shaking at 200 rpm in a Gyrotory water bath shaker (Model G76, New Brunswick Scientific, New Brunswick, NJ). A 1% inoculum of a cell culture grown in YPD slant for 24 hr was used. A separate

flask was analyzed at each sampling time. After centrifugation at 2000 x g for 10 min, the supernate was used for determination of lactose and nitrogen residues in the medium, and the cell paste from 80 ml of medium was used for determination of total lipid. The cell paste from 10 ml of culture was washed with distilled water twice, resuspended in distilled water, and used for determination of dry cell weight (DCW) and nitrogen content of whole cells. The remaining cell paste from 10 ml of medium was used for preparation of cell-free extracts.

Preparation of cell-free extracts

Cells were harvested by centrifugation at 2000 x g for 10 min, washed twice with distilled water and resuspended in the same volume of 50 mM phosphate buffer (pH 7.0). The cell suspension was sonicated with a disintegrator (Model BP-2, Blackstone Ultrasonics, Inc., Sheffield, PA) for six 1-min intervals in an ice-water bath and cooled between sonications. The cell-free extract was collected as the supernatant fraction after centrifugation of the suspension at 2000 x g for 10 min and was used for determination of catalase activity, protein, and intracellular carbohydrate.

Dry cell weight measurement

Membrane filters (0.2 μ pore size, type ME24, Schleicher & Schuell, Keene, NH) were preweighed after drying for 5 min at full power in a microwave oven (Model No. 56-4474-10, Tappan Appliance Div., Mansfield, OH, frequency 2450 MHz). A 1- or 2-ml sample of the washed cell

suspension was filtered through a preweighed filter under vacuum, and dried in the microwave oven for 4 min at 50% power and then for 5 min at full power. The filter was cooled in a desiccator and weighed. Drying cycles in the microwave oven for 5 min at full power were repeated until a constant weight was obtained. The dry cell weight could be obtained within 2 to 3 hr by this method.

Analyses

Protein content was measured in the cell-free extract by the modified micro-Lowry method (17) with bovine serum albumin (Type A-7030, fatty acid- and globulin-free, Sigma Chemical Co., St. Louis, MO) as standard. Lipid content of the cell mass was measured as total fatty acids by aqueous alkaline hydrolysis of wet cells with 12% alcoholic KOH followed by hexane extraction (15). Nitrogen residue in the culture supernate and nitrogen content of whole cells were determined by the micro-Kjeldahl method by procedure 47.021 (1). Copper selenite was used instead of mercuric oxide. Carbohydrate content was measured in the cell-free extract as total intracellular carbohydrate by the phenol-sulfuric acid method (4) with glucose as standard. Residual lactose in the culture supernate was determined by the same method (4) with lactose as standard. Catalase (EC 1.11.1.6) was assayed in the cell-free extract at 30°C spectrophotometrically by the method of Tolbert (19), and its specific activity was expressed as μmoles of H_2O_2 consumed/min/mg protein. All reported results are the average of two replicate measurements.

RESULTS AND DISCUSSION

Typical growth of Apiotrichum curvatum in nitrogen-limited medium

Apiotrichum curvatum goes through a two-stage growth cycle in nitrogen-limited medium: an initial growth phase with little lipid accumulation followed by a fattening phase in which cell division slows but lipid accumulation continues. This two-stage fermentation occurs when nitrogen is depleted and residual carbohydrates are converted to lipid (15, 16).

Figure 1 shows a typical growth curve for A. curvatum in nitrogen-limited medium (C/N=99). The amount of lactose in the medium decreased slowly until the end of the fermentation (170 hr) at which time 96% of the lactose had been consumed. In contrast, nitrogen in the medium sharply decreased by 94% to 10.5 mg/l during vigorous growth of the organism (35 hr), and then increased slowly to 36.8 mg/l during the fattening phase. The amount of nitrogen that was assimilated as biomass in whole cells sharply increased to 0.173 g/l by 24 hr and then decreased slowly during the remaining incubation time. Possibly there was some cell lysis after 24 hr, or the organism produced some extracellular nitrogen-containing compounds that were not detected by the phenol-sulfuric acid method.

Dry cell weight (DCW) and accumulated lipid reached their maximum at 120 hr and then remained constant. The maximum DCW obtained was 15 g/l. Cell yield on lactose, defined as g of DCW produced per g of lactose consumed, was 0.34 g/g. Lipid production and fat coefficient,

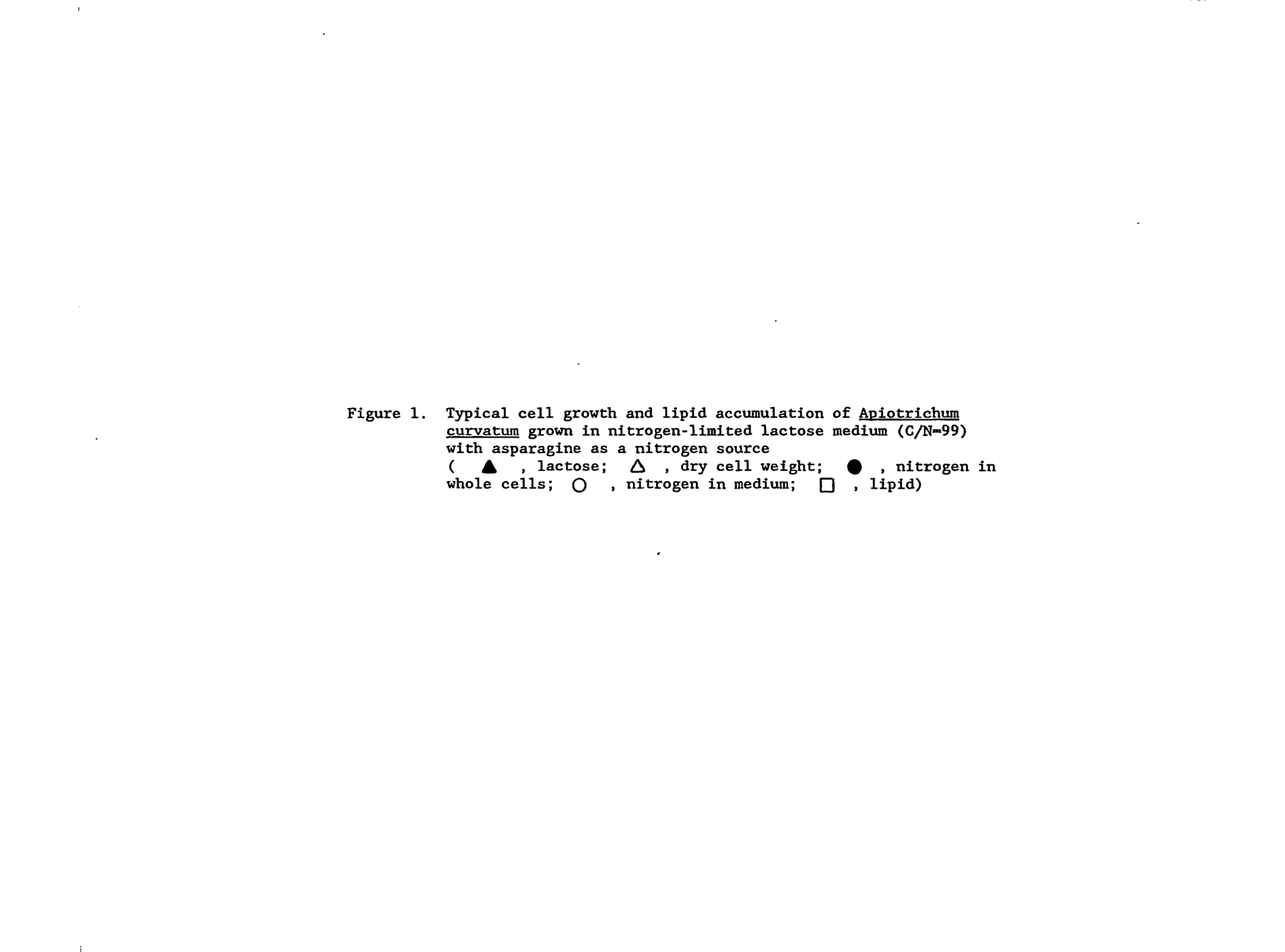
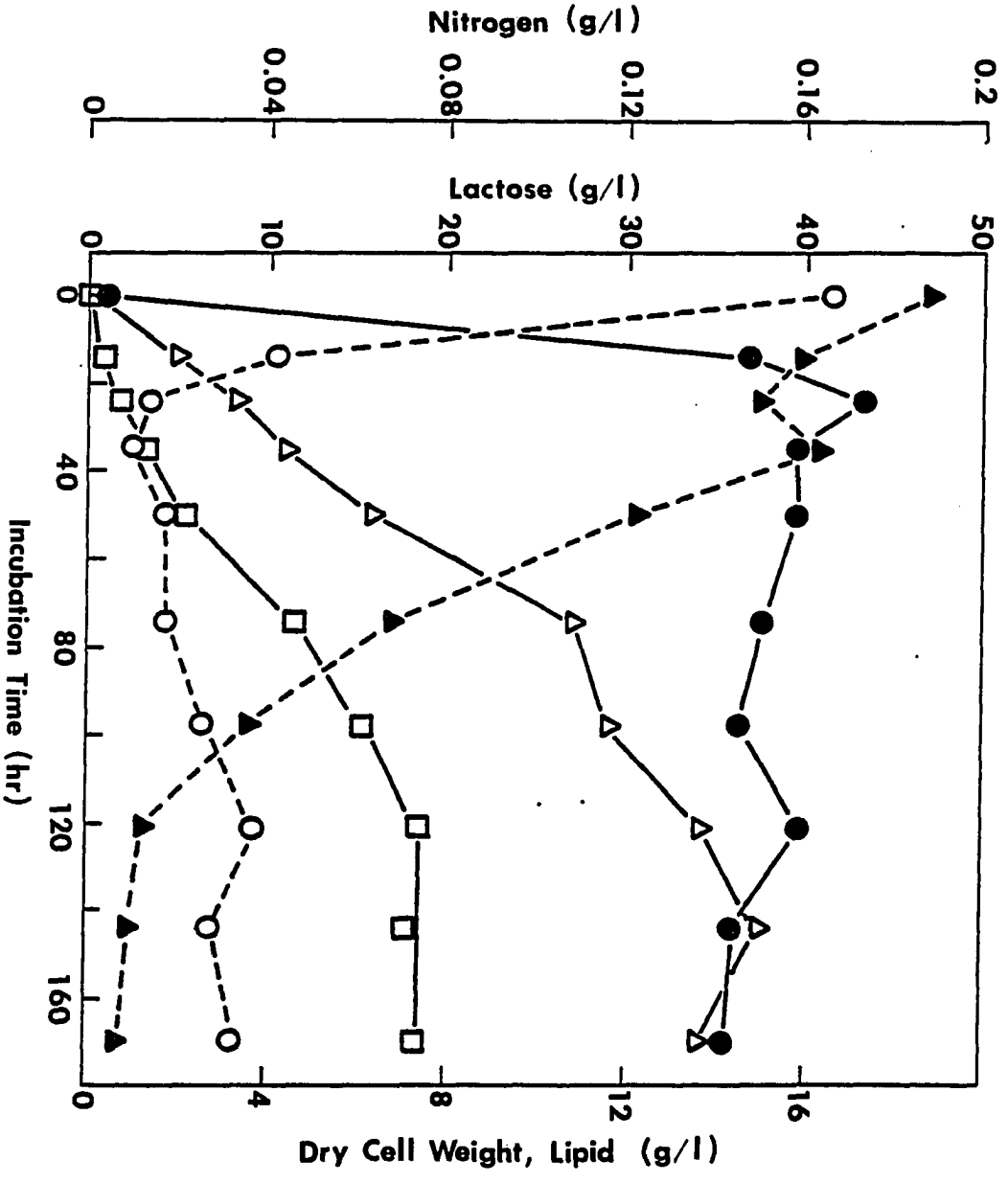


Figure 1. Typical cell growth and lipid accumulation of Apiotrichum curvatum grown in nitrogen-limited lactose medium (C/N=99) with asparagine as a nitrogen source
(▲ , lactose; △ , dry cell weight; ● , nitrogen in whole cells; ○ , nitrogen in medium; □ , lipid)


Nitrogen (g/l)
0
0.04
0.08
0.12
0.16
0.2
Lactose (g/l)
0
10
20
30
40
50
Incubation Time (hr)
0
40
80
120
160
Dry Cell Weight, Lipid (g/l)
0
4
8
12
16

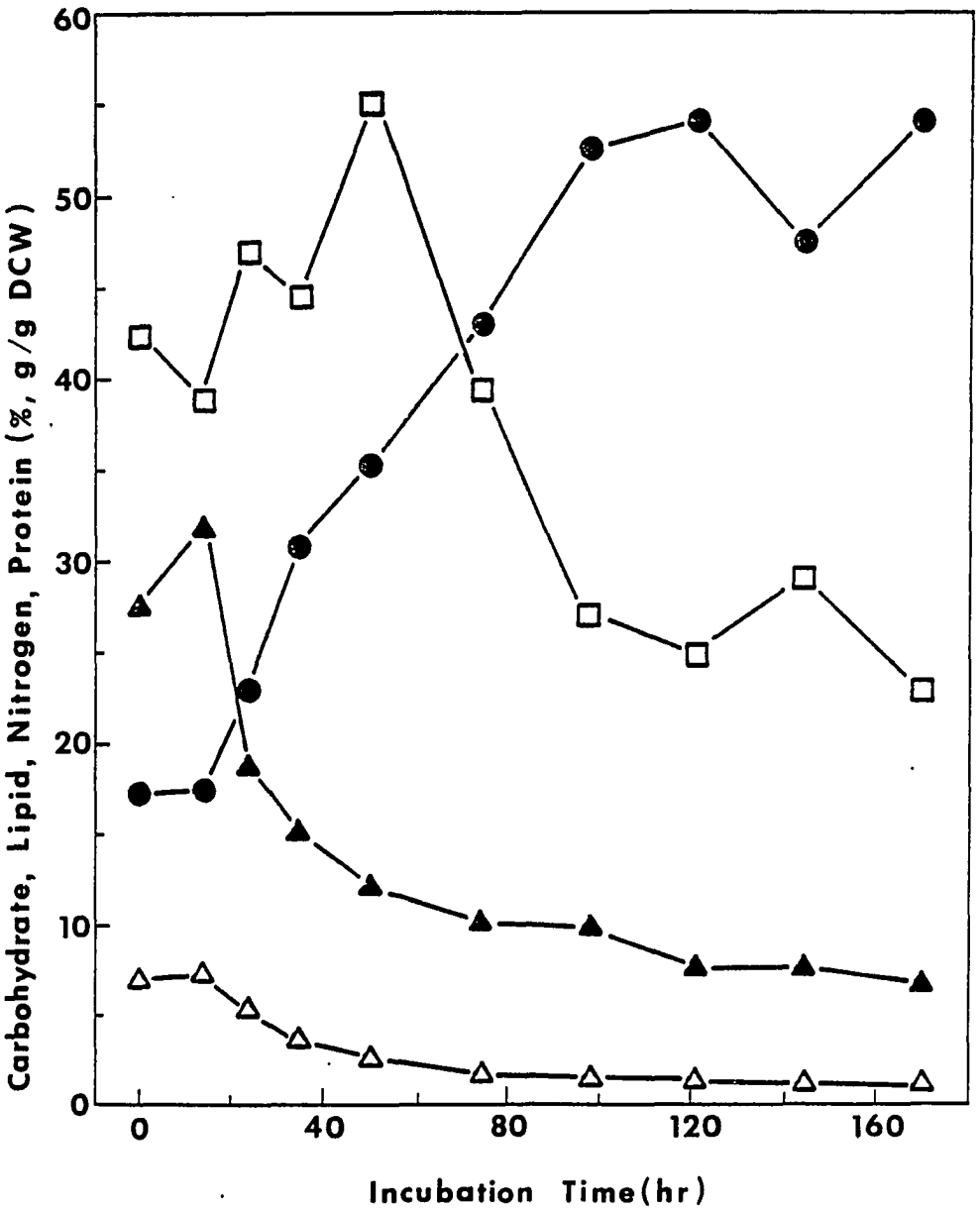
defined as g of lipid produced per 100 g of lactose consumed, were 7.38 g/l and 16.9, respectively.

Changes in cell composition of A. curvatum during this growth cycle are shown in Figure 2. Lipid content was constant for the first 14 hr of growth, then began to increase. The intracellular carbohydrate content increased to 55% of DCW during growth and early lipid accumulation (up to 50 hr), then decreased to 23% as lipid accumulation continued. It is possible that the intracellular carbohydrate that accumulates early is converted to lipid later. Protein and nitrogen contents per cell mass were steady for the first 14 hr of growth, then decreased to very low levels (6.5% and 1.0%, respectively) as incubation continued and lipid accumulated.

Evans and Ratledge (5) suggested that citrate accumulates intracellularly in A. curvatum before lipid production. In the current study, the accumulated carbohydrate must be something other than citrate, because citrate is not detected by the phenol-sulfuric acid method (4).

Glycogen could be the alternative carbohydrate storage material in oleaginous (6, 12) and nonoleaginous yeasts (10, 14). A reciprocal relationship between carbohydrate and lipid contents was reported in Rhodosporidium toruloides (6). Recently, A. curvatum grown in a nitrogen-limited medium with glucose and NH_4Cl was reported to accumulate about 10% (w/w) of its biomass as glycogen (12).

Figure 2. Changes in cell composition of Apiotrichum curvatum grown in nitrogen-limited medium (C/N=99)
(□ , carbohydrate; ● , lipid; ▲ , protein; △ , nitrogen in whole cells)


60
50
40
30
20
10
0
0
40
80
120
160
Carbohydrate, Lipid, Nitrogen, Protein (%, g/g DCW)
Incubation Time(hr)

Comparison of Apiotrichum curvatum grown in nitrogen-limited and balanced media

Two types of metabolism can be observed for A. curvatum, depending on the C/N ratio of the growth medium. Table 1 shows characteristics of growth of A. curvatum at three C/N ratios. As C/N ratio decreased, lactose utilization did not change significantly, but nitrogen utilization did. More total nitrogen was consumed, but this represented a smaller percentage of the available nitrogen. Cell mass production and cell yield on lactose did not change significantly at different C/N ratios, but cell yield on nitrogen was much less at low C/N than at high C/N ratio. Cells grown in the balanced medium contained four times as much nitrogen as those at high C/N ratio when cell yield on nitrogen at C/N=5 (19.8 g DCW/g N) was compared with that at C/N=99 (72.6 g DCW/g N).

Lipid production at high C/N ratio was much more efficient than at low C/N ratio. Lipid content of cells was twice as high, and fat coefficient (g lipid produced / 100 g lactose used) was also high. At low C/N ratio, cells contained a similar proportion of protein, but less carbohydrate.

The cell composition of A. curvatum during growth in balanced medium (C/N=18) changed in a similar fashion as in nitrogen-limited medium, as shown in Figure 3. In this medium, intracellular lipid increased very slowly to only 25% of dry cell weight; intracellular carbohydrate content began to decrease earlier (12 hr) than occurred at C/N=99 (50 hr). Changes in cell composition during growth at C/N=5 (not shown here) are similar to these.

Table 1. Comparison of cell growth of Apiotrichum curvatum in lactose media with different carbon:nitrogen (C/N) ratios

	C/N=99[a]	C/N=18[b]	C/N=5[c]
Lactose used (g/l)	30.3	31.1	27.3
(%)	64.4	61.9	61.5
Nitrogen used (mg/l)	148.8[d]	466.0	581.0
(%)	89.6	44.8	15.7
Cell mass (g/l)	10.8	11.4	11.5
Cell yield on lactose (g/g)	0.36	0.37	0.42
Cell yield on nitrogen (g/g)	72.6	24.5	19.8
Lipid produced (g/l)	4.6	2.9	2.3
Lipid content (%)	42.9	25.4	20.0
Fat coefficient (g lipid/100g lactose used)	15.2	9.3	8.4
Protein produced (g/l)	1.1	1.3	1.4
Protein content (%)	10.1	11.4	12.1
Carbohydrate produced (g/l)	4.2	2.7	2.3
Carbohydrate content (%)	39.3	23.7	23.8
Fermentation time (hr)	74	72	72

[a] C/N=99: 40 g/l of lactose and 0.8 g/l of asparagine.

[b] C/N=18: 40 g/l of lactose and 4.4 g/l of asparagine.

[c] C/N=5: 40 g/l of lactose and 15.9 g/l of asparagine.

[d] The amount of nitrogen used was calculated at 35 hr.

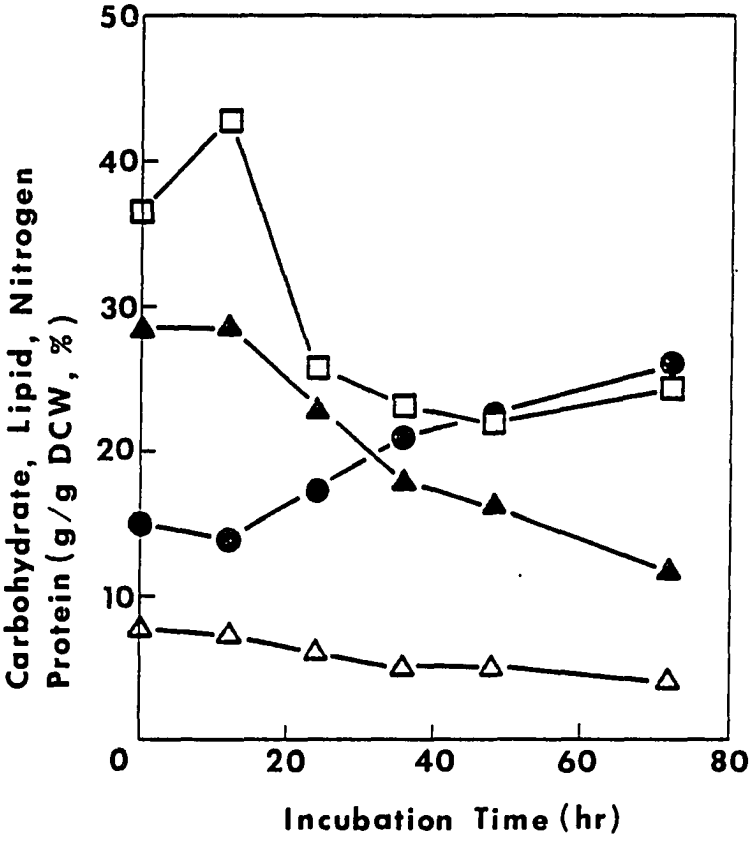


Figure 3. Changes in cell composition of Apiotrichum curvatum grown in balanced medium (C/N=18)
(□ , carbohydrate; ● , lipid; ▲ , protein; △ , nitrogen in whole cells)

Transfer of cultures between media with different carbon:nitrogen ratios

To determine if accumulated lipid could serve as an energy reserve in cells starved for carbon, cultures were grown for 74 hr at two different C/N ratios (99 and 18), washed, and resuspended in fresh medium with three different C/N ratios. Results are summarized in Table 2 and changes of cell composition after transfer are shown in Figure 4.

Cells grown in nitrogen-limited medium and transferred to medium without lactose increased cell mass after transfer by consuming nitrogen in the medium and depleting their endogenous lipid and carbohydrate as carbon sources. Cell yields on endogenous lipid and carbohydrate were calculated to be 2.34 g/g and 3.06 g/g, respectively. Holdsworth and Ratledge (12) reported an approximate theoretical value of 1:1.7 (w/w) for the conversion of triglyceride to biomass, and obtained 1.9 g biomass per g endogenous lipid utilized when A. curvatum was grown in a two-stage chemostat under carbon-starvation. Our higher yield suggests that other carbon sources (asparagine and endogenous carbohydrate) contributed to biomass.

Cells grown under nitrogen limitation and transferred after 84 hr to balanced medium (C/N=18) produced 12.0 g/l cell mass by consuming the lactose and nitrogen in the medium (Table 2). The cell yields on lactose and nitrogen were 0.34 g/g and 22.4 g/g, respectively, and these values were similar to those shown in Table 1 for cultures grown in balanced medium. Endogenous lipid and carbohydrate were not utilized for cell metabolism; the exogenous carbon source was used preferentially. Although the total amount of intracellular lipid and carbohydrate

Table 2. Characteristics of cultures after transfer to a medium with a different carbon:nitrogen (C/N) ratio

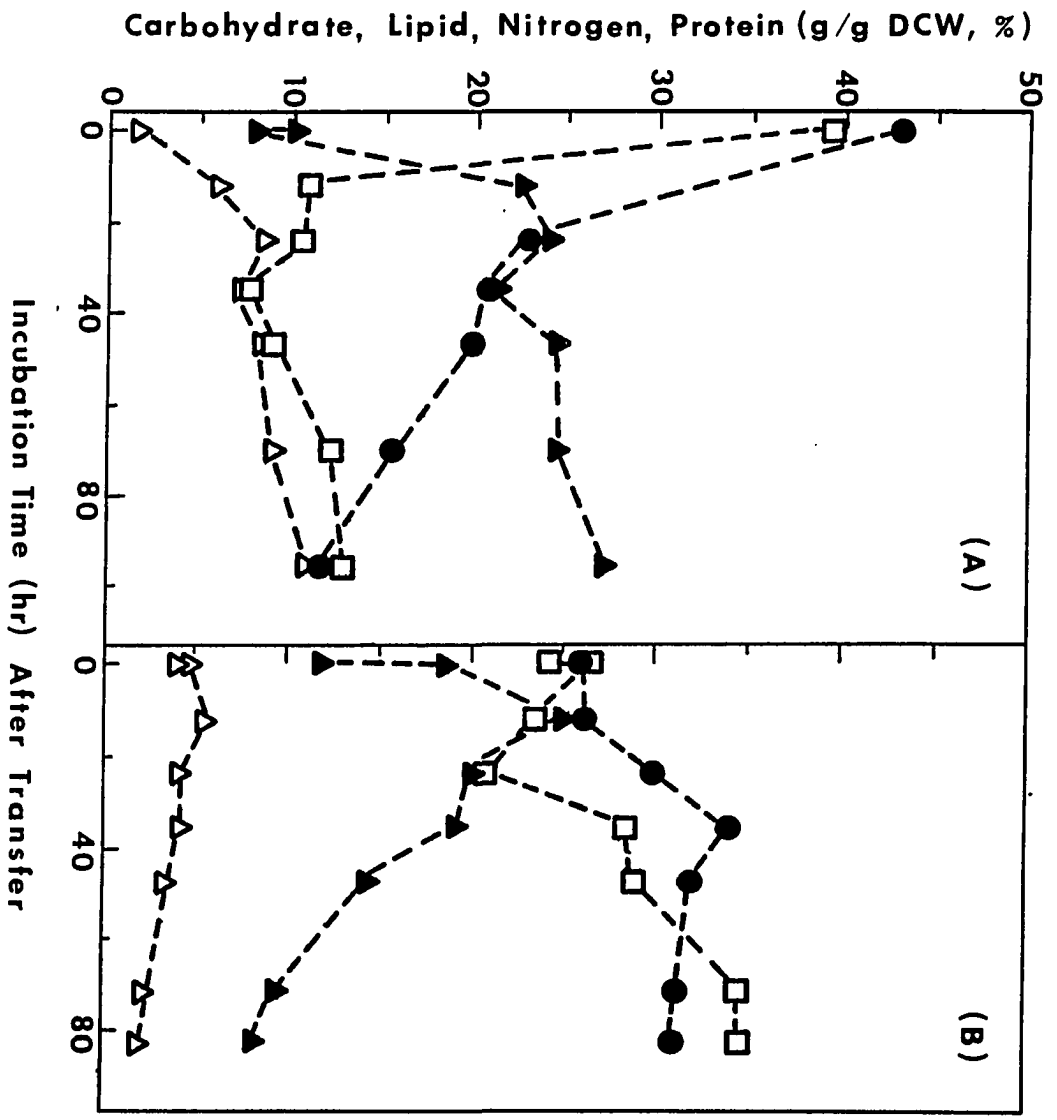
	Transfer from initial to second medium		
	99--0[a]	99--18[a]	18--99[a]
Lactose used (g/l)	nd**	35.3	33.0
(%)	nd**	84.1	66.0
Nitrogen used (mg/l)	375.5	535.0	187.0
(%)	36.9	65.3	93.0
Cell produced (g/l)	1.5	12.0	10.7
Cell yield on lactose (g/g)	nd**	0.34	0.32
Cell yield on nitrogen (g/g)	4.0	22.4	57.2
Lipid produced (g/l)	-0.64*	4.20	3.42
Cell yield on endogenous lipid (g/g)	2.34	nd**	nd**
Change in lipid content (%)	-31.3*	-9.9*	8.2
Protein produced (g/l)	0.89	2.44	0.61
Change in protein content (%)	18.9	10.2	-3.5
Carbohydrate produced (g/l)	-0.49*	1.84	3.90
Cell yield on endogenous carbohydrate (g/g)	3.06	nd**	nd**
Change in carbohydrate content (%)	-26.4*	-8.9*	10.4
Time (hr) after transfer	96	96	83

[a]C/N ratio of medium before transfer--C/N ratio of medium after transfer.

*Negative signs mean consumption or decrease.

**Not determined.

Figure 4. Changes in cell composition in Apiotrichum curvatum transferred (a) to carbon-free medium after growth in nitrogen-limited medium (C/N=99) and (b) to nitrogen-limited medium (C/N=99) after growth in balanced medium (C/N=18) for 72 hr
(● , lipid; □ , carbohydrate; ▲ , protein; △ , nitrogen in whole cells)


Carbohydrate, Lipid, Nitrogen, Protein (g/g DCW, %)
0
10
20
30
40
50
Incubation Time (hr) After Transfer
0
40
80
(A)
(B)

increased by 4.2 g/l and 1.84 g/l, respectively, after transfer, the relative amounts of these components per total biomass actually decreased by 9.9% and 8.9%, respectively. The increase in protein content of cells during vigorous growth diluted the lipid and carbohydrate.

Cells grown in nitrogen-rich medium (C/N=18) and transferred after 72 hr to nitrogen-limited medium (C/N=99) increased cell mass by consuming lactose and nitrogen in the medium (Table 2). The cell yield on lactose (0.32 g/g) was similar to that normally obtained at C/N=99 (Table 1), but the cell yield on nitrogen (57.2 g/g) was much lower.

Changes in cell composition after transfer from high-carbon to no-carbon medium and after transfer from nitrogen-rich to nitrogen-limited medium are shown in Figure 4. Lipid content of cells transferred from high-carbon (C/N=99) to no-carbon (C/N=0) medium decreased sharply immediately after transfer, and intracellular carbohydrate was consumed even more rapidly (Figure 4(a)). Protein and nitrogen contents increased after transfer probably because more enzymes were needed for cell growth. Nitrogen may have been taken up from the medium as well. In contrast, lipid and carbohydrate content of cells transferred from nitrogen-rich (C/N=18) to nitrogen-limited (C/N=99) medium increased after transfer (Figure 4(b)). Protein was produced, but the percentage of protein in cell mass decreased.

Catalase activity and metabolism of Apiotrichum curvatum

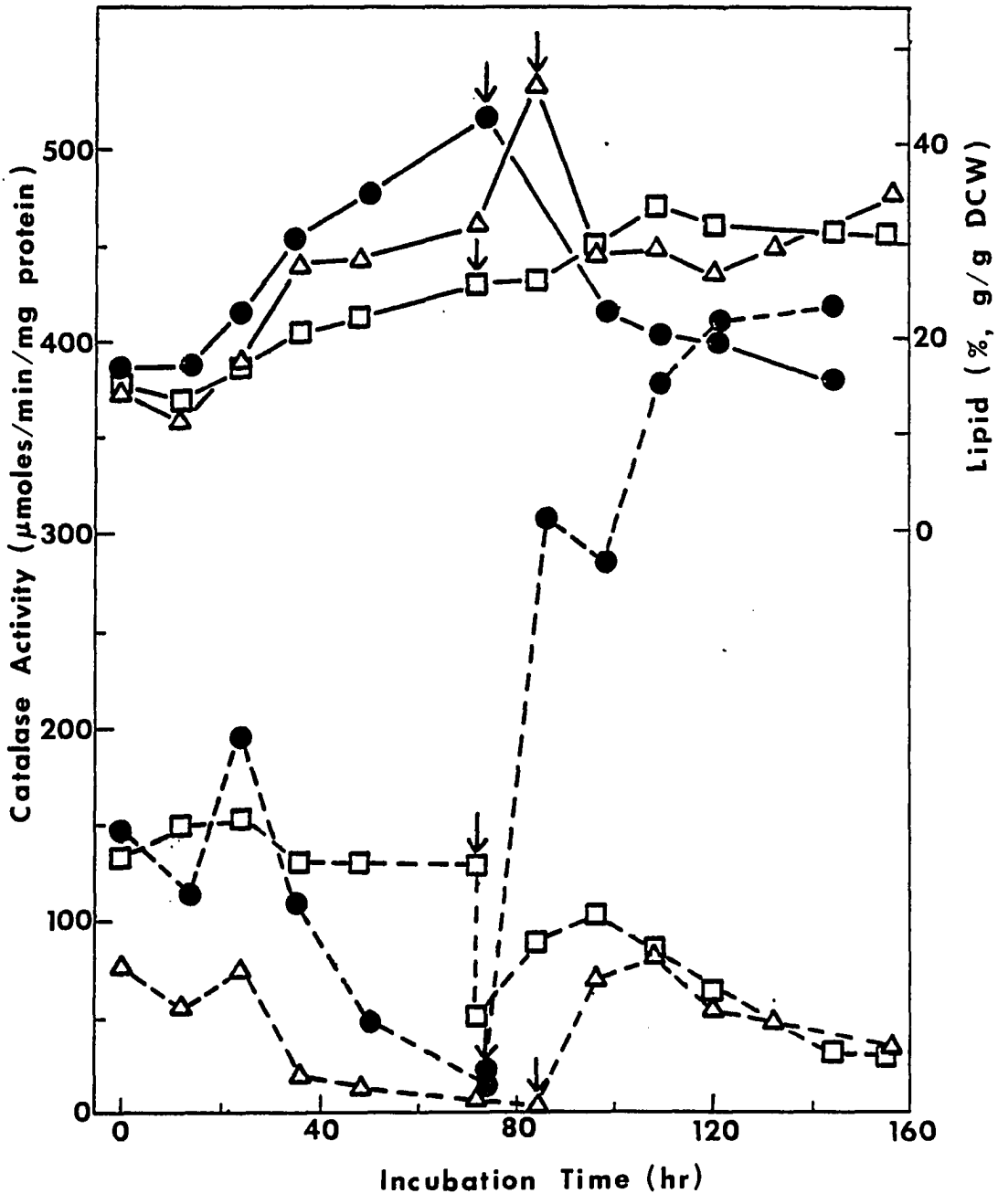
Catalase activity as a marker enzyme of peroxisomes was measured in the cells of A. curvatum transferred between media with different C/N

ratios. These data along with the changes in lipid content of cells are shown in Figure 5. Specific catalase activity in the cells grown in balanced or nitrogen-limited medium was constant or increased during growth, then decreased as lipid accumulated, to about 6.3 μmoles/min/mg protein after 98 hr. Maximum specific catalase activity in the cells grown in nitrogen-limited medium was 195 μmoles/min/mg protein at 24 hr.

Specific catalase activity increased sharply after transfer in the cells transferred from nitrogen-limited to carbon-free medium. In contrast, specific catalase activity in the cells transferred from nitrogen-limited to nitrogen-rich or from nitrogen-rich to nitrogen-limited medium increased slightly, then decreased. These results suggest that when cells were forced to metabolize endogenous lipid and carbohydrate to generate cell mass, specific catalase activity increased dramatically. If a carbon source was present in the growth medium, strong catalase induction did not occur even if the cell had accumulated lipid. When accumulated lipid was used as carbon source, strong catalase induction occurred. These results agree with the suggestions of Fukui and Tanaka (7, 8) that peroxisomes play a role in fatty acid beta-oxidation in yeasts. Recently, Holdsworth et al. (13) showed that peroxisome proliferation and activities of catalase and other peroxisomal enzymes were greater in A. curvatum cells utilizing endogenous or exogenous lipids than in cells grown on exogenous carbohydrate.

Changes in specific catalase activity could be correlated with changes in lipid content in A. curvatum. Data from transfer studies and other growth studies on lipid content as percentage of nonlipid biomass

Figure 5. Relationship of catalase activity to lipid content in cells of Apiotrichum curvatum transferred between media with different C/N ratios.
Arrows indicate the time when cultures were transferred (□ , C/N=18 to 99; △ , C/N=99 to 18; ● , C/N=99 to 0;– – – –, specific catalase activity; ———, lipid content)


Catalase Activity (μmoles/min/mg protein)
Lipid (%, g/g DCW)
Incubation Time (hr)
0
100
200
300
400
500
0
20
40
0
40
80
120
160

and specific catalase activity are plotted in Figure 6. These values are reciprocally related: the greater the lipid content, the lower the specific catalase activity.

Relationship between lipid content and other cellular components in Apiotrichum curvatum

Lipid accumulation in oleaginous yeasts can be affected by many environmental factors, including the carbon and nitrogen content of the growth medium (18). In the current study, cellular lipid content as percentage of nonlipid biomass was also reciprocally related to cellular nitrogen (or protein) content, regardless of C/N ratio of the growth medium (Figure 7). Cellular lipid content was not related to intracellular carbohydrate content (data not shown). Catalase activity in the cells increased with increasing cellular nitrogen (or protein) content.

Conversion of carbon in the growth medium to intracellular lipids or carbohydrates seems to depend on the substrates used as carbon or nitrogen sources (6, 14). A reciprocal relation between carbohydrate and lipid content was reported in Rhodosporidium toruloides cells grown on various nitrogen sources (6). A similar relation was observed in Candida tropicalis cells grown on glucose and hydrocarbons as carbon sources (14). In the current study such a reciprocal relation was not seen. The intracellular carbohydrates measured in this study may be intermediates for lipid production and turnover as well as final storage materials such as glycogen. Perhaps the lipid content of A. curvatum might be

Figure 6. Relationship between catalase activity and lipid content in cells of Apiotrichum curvatum in lactose media with various C/N ratios
(● , C/N=0; ○ , C/N=99; △ , C/N=18; ▲ , C/N=5)

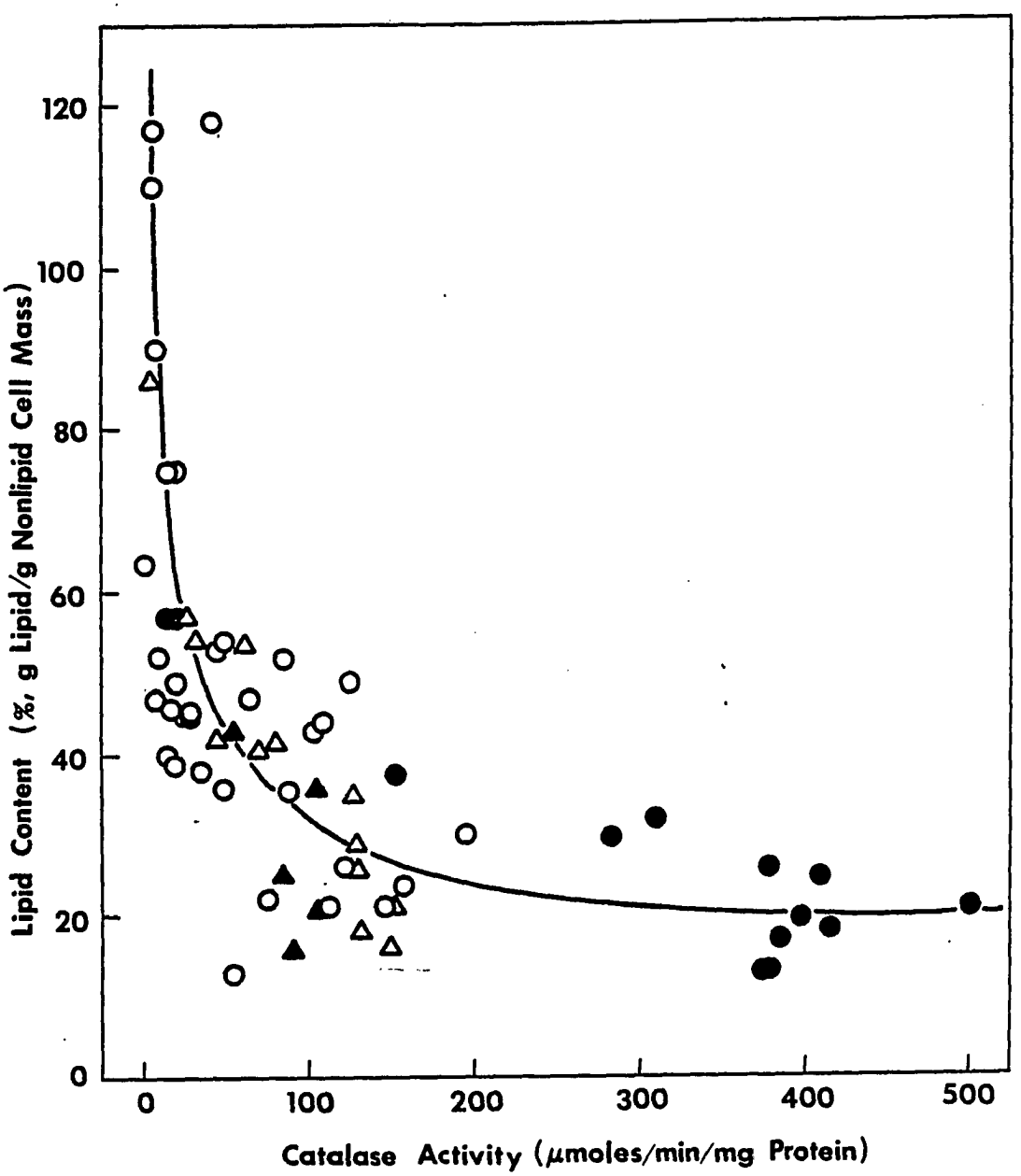
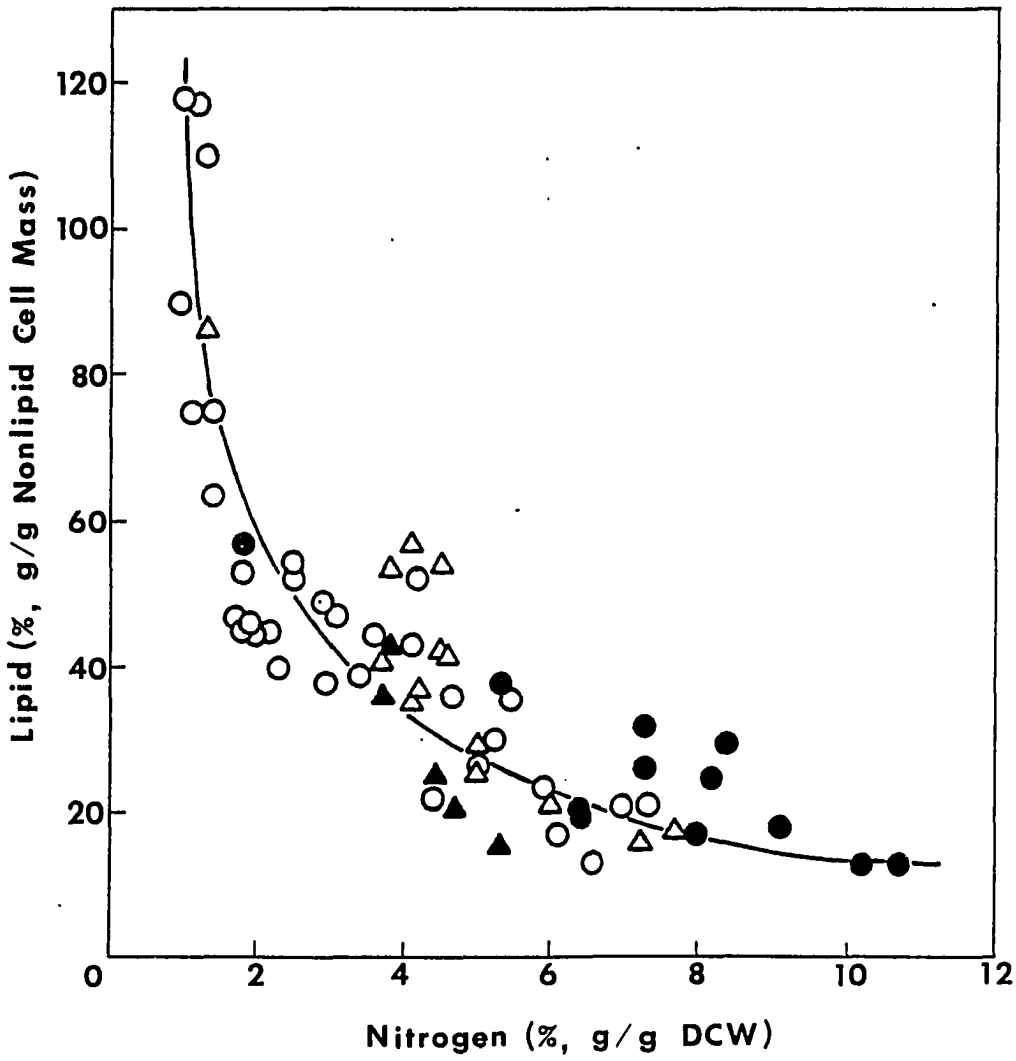

Lipid Content (%, g Lipid/g Nonlipid Cell Mass)
120
100
80
60
40
20
0
0
100
200
300
400
500
Catalase Activity (μmoles/min/mg Protein)

Figure 7. Relationship between lipid content and nitrogen content in cells of Apiotrichum curvatum in lactose media with various C/N ratios
(● , C/N=0; ○ , C/N=99; △ , C/N=18; ▲ , C/N=5)


Lipid (%, g/g Nonlipid Cell Mass)
120
100
80
60
40
20
0
2
4
6
8
10
12
Nitrogen (%, g/g DCW)

reciprocally related to carbohydrate content if different carbon or nitrogen sources were used.

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PART II.

CATALASE ACTIVITY AND EVIDENCE OF PEROXISOMES IN THE OLEAGINOUS YEAST *Apiotrichum curvatum*

ABSTRACT

The presence and induction of peroxisomes were investigated in the oleaginous yeast Apiotrichum curvatum ATCC 20509 (formerly Candida curvata D). Catalase, a marker enzyme for peroxisomes, was measured in cell-free extracts prepared by sonication. The nature of the carbon and nitrogen sources in the growth medium greatly affected catalase activity. Cells grown on corn oil had very high specific activity of catalase, but those grown on glucose, sucrose, or maltose had low activity. Specific activity of catalase was generally greater in exponential-phase cells than in stationary-phase cells. High specific activity of catalase was measured in cultures grown in media that supported poor growth (for example, with soluble starch as carbon source or with methylamine, urea, or asparagine as nitrogen source). High specific activity of catalase was induced in cultures transferred from a carbon-rich medium to a medium without a carbon source. Peroxisomal fatty acid beta-oxidation was detected only in cells grown on corn oil. Peroxisomes with a homogeneous matrix and core surrounded by a single-layer membrane were observed with an electron microscope in corn oil-grown cells. Staining with 3,3'-diaminobenzidine revealed that catalase activity was located in peroxisomes. These results suggest that peroxisomes play a role in lipid degradation in this oleaginous yeast.

INTRODUCTION

Peroxisomes are ubiquitous in eucaryotic cells and are known to function in various cell types in fatty acid beta-oxidation, cellular respiration, gluconeogenesis, plasmalogen biosynthesis, cholesterol metabolism with bile acid synthesis, alcohol oxidation and peroxidation, glycolate and glycerate pathways, and purine and polyamine catabolism (4, 8, 9, 21, 24, 43). Many peroxisomes in animal, plant, and fungal cells share at least two biochemical capabilities: respiration associated with hydrogen peroxide metabolism and fatty acid beta-oxidation. Yeast cells seem to be an excellent system for investigating the function and development of peroxisomes because the synthesis of the organelles is induced only by special carbon or nitrogen sources (12, 13, 45).

Although yeast peroxisomes were first described in Saccharomyces cerevisiae grown on glucose (1), they have been studied mainly in yeasts grown on n-alkanes (12, 13), fatty acids (12, 13), methanol (12, 45), or amines (26, 47) as carbon or nitrogen sources. Peroxisomes in alkane-utilizing yeasts such as Candida tropicalis pK 233 participate in the degradation of the fatty acids derived from alkanes to two-carbon units and in the synthesis of gluconeogenic intermediates from these two-carbon units. Peroxisomal enzyme activity is induced by the alkane or by fatty acids (12, 13). The occurrence of such organelles has also been demonstrated in other alkane-utilizing yeasts (12, 33, 38). Distinct subcellular localization in peroxisomes of one of two long-chain fatty acyl-CoA synthetases was reported for mutant strains of Candida

lipolytica grown on higher fatty acids or alkanes (18, 27, 28).

The peroxisomes in methanol-assimilating yeasts such as Candida boidinii (formerly Kloeckera sp. 2201) and Hansenula polymorpha play an indispensable role in the initial oxidation of methanol to form formaldehyde and hydrogen peroxide, and are inducible by methanol (12, 45). The peroxisomes in methylamine-assimilating yeasts such as the nonmethylotrophic yeast Candida utilis and the methylotrophic yeast H. polymorpha were found to contain amine oxidase when cultures were grown on methylamine as sole nitrogen source (47). Other yeasts, grown on 1-aminoalkanes or putrescine as sole source of carbon, nitrogen, and energy also contained peroxisomal amine oxidase (26).

Recent publications (17, 35, 37) have reported activities of a few peroxisomal enzymes in cell-free extracts of some oleaginous yeasts including Apiotrichum curvatum, and have included electron micrographs that confirmed the presence of peroxisomes in these cells (17). The oleaginous yeast A. curvatum ATCC 20509 (formerly Candida curvata D) was isolated several years ago as an organism that could grow on lactose and accumulate lipid (29, 30). This organism can grow on a wide range of carbohydrates as well as exogenous and endogenous lipids as carbon source and can accumulate up to 60% of its dry weight as lipid when grown under nitrogen limitation (10, 14, 16). It could be a good model system for investigating the lipid metabolism of eucaryotic cells and the role of peroxisomes in this metabolism.

The purpose of this study was to determine if A. curvatum contained peroxisomes and to measure peroxisomal enzyme activity in cultures prepared under various growth conditions.

MATERIALS AND METHODS

Microorganisms

Oleaginous yeasts used in this study included Apiotrichum curvatum ATCC 20509, Candida lipolytica 1094 and 1095, Lipomyces starkeyi CBS 1809, Rhodosporidium toruloides CBS 14, Rhodotorula glutinis ATCC 32765 and BC 12-21. Nonoleaginous yeasts were Sporobolomyces holsaticus ATCC 34889, Candida utilis ATCC 9226, Kluyveromyces fragilis ATCC 36534, Saccharomyces cerevisiae ATCC 4098 and ATCC 2341, Saccharomyces diastaticus ATCC 36902, and Schizosaccharomyces pombe ATCC 26189. All yeasts were maintained on YPD agar slants that contained (g/l): yeast extract, 10; peptone, 20; dextrose, 20; agar, 20.

Media and cultivation of yeasts

The defined medium used for shake flask studies contained the following constituents (g/l): lactose, 40; L-asparagine, 0.8; KH_2PO_4, 2.5; $MgSO_4 \cdot 7H_2O$, 1.0; $FeCl_3 \cdot 6H_2O$, 0.02; $CaCl_2 \cdot 2H_2O$, 0.2; $ZnSO_4 \cdot 7H_2O$, 0.001; $MnSO_4 \cdot H_2O$, 0.002; NaCl, 0.06; $CuSO_4 \cdot 7H_2O$, 0.0001; and thiamine-HCl, 0.001. Other carbon sources were added when needed to give the equivalent amount of carbon that was present in lactose. Corn oil was purchased from a grocery store and was added when needed at 18 g/l. Banana juice, which contained 5.2% glucose, 3.9% fructose, 15.3% sucrose and 0.9% lipid (46), was diluted 1:5 with distilled water and used without further supplementation. Other nitrogen sources were added when needed to give the equivalent amount of nitrogen that was present in L-

asparagine. If two nitrogen sources were used together, each contributed half of the total nitrogen. The amino nitrogen content of yeast extract was assumed to be 4.1% (5). Thiamine-HCl was eliminated when yeast extract was used as nitrogen source. The amounts of lactose and asparagine in the defined medium, whose carbon:nitrogen (C/N) ratio was 99, were changed if other C/N ratios were required. The amount of carbon in asparagine was not taken into account in calculating C/N ratios. The pH was adjusted to pH 5.5, and all media were sterilized in the autoclave at 121°C for 15 min.

Cultures were grown in 250-ml Erlenmeyer flasks containing 100 ml of medium at 32°C with shaking at 200 rpm in a Gyrotory water bath shaker (Model G76, New Brunswick Scientific, New Brunswick, NJ). The sterilized media were inoculated with 1.0 ml of a cell suspension (5 ml) harvested in the same medium from a fresh YPD slant.

Preparation of cell-free extracts

After cultivation, cells were harvested by centrifugation at 2000 x g for 10 min, washed twice with distilled water and resuspended in the same volume of 50 mM phosphate buffer (pH 7.0). The cell suspension thus obtained was sonicated with a disintegrator (Model BP-2, Blackstone Ultrasonics, Inc., Sheffield, PA, USA) for six 1-min intervals in an ice-water bath and cooled between sonications. The cell-free extract was collected as the supernatant fraction after centrifugation of the suspension at 2000 x g for 10 min, and used for catalase assays or other analyses.

Analyses

Culture growth was measured as absorbance (Ab) at 540 nm, which was converted to dry cell weight (DCW) according to a standard curve prepared with A. curvatum. Catalase (EC 1.11.1.6) was assayed at 30°C spectrophotometrically by the method of Tolbert (42), and its activity was expressed as μmoles of H_2O_2 consumed/min/ml of medium or mmoles of H_2O_2 consumed/min/g DCW. When DCW data were not available, catalase activity was also expressed as μmoles of H_2O_2 consumed/min/Ab_{540}. Peroxisomal beta-oxidation of fatty acids was assayed as palmitoyl-CoA-dependent NADH production (23), and its activity was expressed as nmoles of NADH produced/min/Ab_{540}. Antimycin A was added at 0.22 μg/ml of reaction mixture in place of KCN (20). All reported results are the average of two replicate measurements.

Electron microscopy

The method of Veenhuis et al. (44) was essentially followed. Yeast cells were harvested and washed once with distilled water. Cells were fixed with 6% (w/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 30 min at 0-2°C and washed five times with the buffer. They were then immersed in 0.1% $CuSO_4$ in 0.09% NaCl and washed once with 0.1 M Tris-HCl buffer (pH 8.5). A portion of the cells was stained with 3,3'-diaminobenzidine (DAB) by incubating them in a 5-ml reaction mixture containing 0.06% H_2O_2 and 10 mg DAB in 0.1 M Tris-HCl buffer (pH 8.5) for 3.5 hr at 32°C. In the presence of catalase and H_2O_2, DAB undergoes oxidative polymerization and yields manganese or osmium black when fixed

with $KMnO_4$ or osmium tetraoxide. After incubation, cells were post-fixed with 1.5% (w/v) $KMnO_4$ for 30 min at room temperature. After fixation, cells were washed twice with water and embedded in 1.5% (w/v) agar. After dehydration in a graded alcohol series, the agar blocks were embedded in Epon/Araldite resin. Ultrathin sections were made with an LKB III Ultramicrotome using a diamond knife. Electron micrographs were taken with a JEOL JEM-100CX II electron microscope at 80 kV.

RESULTS AND DISCUSSION

Preparation of cell-free extracts by sonication

The optimal sonication treatment for preparation of cell-free extracts and recovery of enzyme activity was determined by measuring catalase activity in the extract from a mixture of cells of Apiotrichum curvatum grown on lactose and corn oil. Catalase activity detected in the cell-free extract was much greater than that detected in intact cells, possibly because of a permeability barrier of the intact cells to hydrogen peroxide as was reported in n-alkane-grown Candida tropicalis (40). Measured catalase activity increased with sonication time to six 1-min treatments and did not change with increased sonication. Catalase activity was unstable in the cell-free extract and decreased by 50% after 48 hr at $4^{o}C$. No catalase activity was detected in extracts prepared by continuous sonication for 6 min, probably because the enzyme was inactivated by the heat produced. Sodium azide added to the cell-free extract inhibited catalase activity completely.

Catalase activity of Apiotrichum curvatum with different carbon sources

Once catalase, a marker enzyme for peroxisomes, was detected in Apiotrichum curvatum, its activity in cultures grown on various carbon sources was measured. Ten carbon sources were used in defined medium with yeast extract as nitrogen source. As shown in Table 1, the best growth was supported by sucrose and maltose, and poor growth was obtained only on soluble starch. The greatest total activity of catalase was

Table 1. Effect of carbon sources on catalase activity in Apiotrichum curvatum

Carbon sources	Biomass[a] (g/l)	Catalase Activity (μmoles/min/ml)	(mmoles/min/g DCW)
Fructose	7.0	174.5	24.9
Galactose	3.7	164.1	44.4
Glucose	6.0	119.0	19.8
Lactose	5.4	129.9	24.1
Maltose	10.7	152.4	14.2
Sucrose	11.6	176.3	15.2
Soluble starch	1.2	191.8	159.8
Banana juice	6.1	207.8	34.1
Glycerin	5.1	253.7	49.7
Corn oil	8.0	425.2	53.2

[a]Cultures grown in defined medium containing yeast extract as nitrogen source for 48 hr.

measured in the culture grown on corn oil, but the greatest activity per g of cell mass was measured in the culture grown on soluble starch. The least activity per g of biomass was found in cultures grown on glucose, sucrose and maltose.

Recently, Holdsworth et al. (17) reported that activities of a few peroxisomal enzymes such as catalase, isocitrate lyase, or $NADP^+$-dependent isocitrate dehydrogenase increased more in A. curvatum cells growing on exogenous lipid than in cells utilizing their endogenous lipid when transferred from nitrogen-limited medium. Teranishi et al. (39) reported that catalase activity of C. tropicalis pK 233 was induced by hydrocarbons but not by glucose, galactose, ethanol, acetate, or lauryl alcohol and that catalase induction on hydrocarbons was sensitive to

cycloheximide but not to chloramphenicol.

Catalase activity was measured during growth of A. curvatum on corn oil, soluble starch, galactose, glucose, glycerin, or lactose as carbon source. As shown in Figure 1, catalase activity increased in all cultures in exponential phase (through 36 hr). Catalase activity of cultures grown on glucose or lactose was much lower than that of other cultures during this growth phase. As cells reached stationary phase, catalase activity decreased sharply for all other cultures except that grown on soluble starch. The catalase activity of C. tropicalis pK 233 was reported to be greater during exponential growth than in stationary phase (40). In contrast, catalase activity of C. tropicalis grown on glucose was low throughout exponential growth but increased strikingly in stationary phase (11).

Catalase activity of Apiotrichum curvatum with different nitrogen sources

To investigate the effects of nitrogen sources on catalase activity in Apiotrichum curvatum, five nitrogen sources were used in defined medium with lactose as carbon source. As shown in Table 2, ammonium sulfate, methylamine, urea, and asparagine did not support good growth. Very little catalase activity was detected in the culture grown on ammonium sulfate, but the other nitrogen sources supported good catalase activity. When growth was improved by supplementing asparagine with thiamine or by mixing yeast extract with ammonium sulfate or urea, catalase activity per cell mass was quite low. There are few papers that show the effects of nitrogen sources other than amines on peroxisome

Figure 1. Effect of carbon sources on catalase activity of Apiotrichum curvatum grown in defined medium with yeast extract as nitrogen source
(● , galactose; ○ , lactose; △ , corn oil; ■ , glycerin; ▲ , soluble starch; □ , glucose)

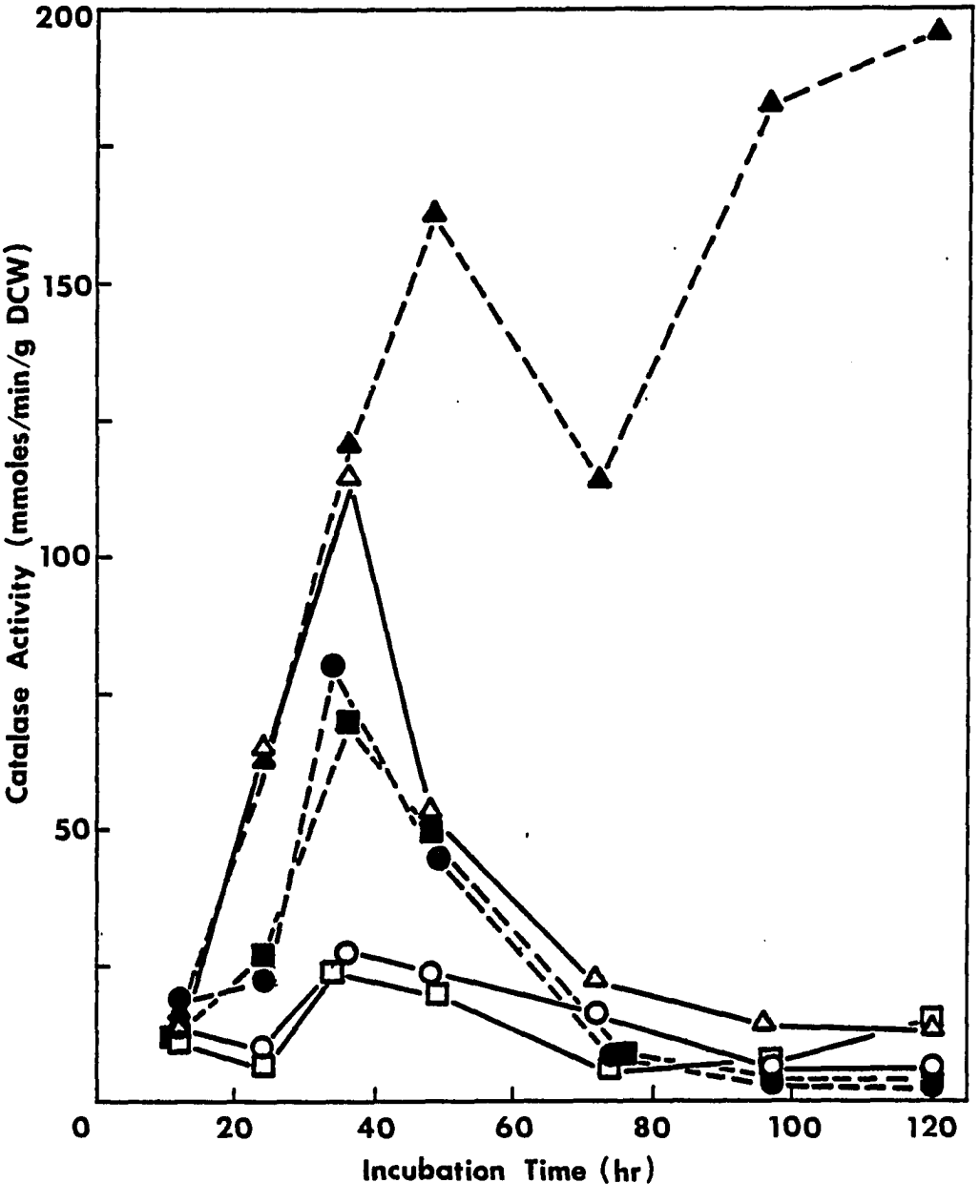

200
150
100
50
0
20
40
60
80
100
120
Catalase Activity (mmoles/min/g DCW)
Incubation Time (hr)

Table 2. Effect of nitrogen sources on catalase activity in Apiotrichum curvatum

Nitrogen sources	Biomass[a] (g/l)	Catalase activity (μmoles/min/ml)	(mmoles/min/g DCW)
Ammonium sulfate	0.8	0.4	0.5
Asparagine	0.4	66.5	166.3
Methylamine	0.1	8.2	82.0
Urea	0.9	145.8	162.0
Yeast extract	5.4	129.9	24.1
Asparagine + thiamine-HCl	5.1	30.9	6.1
Yeast extract + ammonium sulfate	7.4	36.0	4.9
Yeast extract + urea	6.8	95.5	14.0

[a]Cultures grown in defined medium containing lactose as carbon source for 48 hr.

activity in yeasts.

According to Zwart et al. (47), peroxisomes that contained amine oxidase and catalase were induced in Hansenula polymorpha and Candida utilis after growth on glucose and methylamine. Cells grown on glucose and ammonium sulfate did not contain these enzymes. Recently, Simonetta et al. (37) reported that D-alanine could induce the synthesis of D-amino acid oxidase in the oleaginous yeast Rhodotorula gracilis. The highest specific activity of D-amino acid oxidase was detected in cells grown in a medium containing glucose as carbon source and D-alanine as nitrogen source. In contrast, this enzyme activity was negligible when both glucose and ammonium were present in the growth medium, even in the presence of D-alanine.

Catalase activity was measured during incubation of cultures with lactose and various nitrogen sources. Catalase activity per cell mass was low throughout the incubation period for cultures grown with yeast extract (see Figure 1) or with asparagine plus thiamine (Figure 2). Cells grown on urea or asparagine as sole nitrogen source showed increasing enzyme activity throughout the incubation time. Final enzyme activities were much higher in these cultures than in the cultures grown with different carbon sources.

Effect of unfavorable carbon or nitrogen sources on catalase activity

The results presented here suggest a relationship between catalase activity and the ability of carbon or nitrogen sources to support good growth. This is illustrated in Figure 3, in which the values obtained for maximum growth and maximum catalase activity in the previous experiments are plotted against one another. With four exceptions, growth and catalase activity are negatively correlated, regardless of carbon or nitrogen source. The exceptions are corn oil, galactose, and glycerin, which supported good growth and high catalase activity, and ammonium sulfate, which supported neither. These three carbon sources could be good inducers of peroxisomes in Apiotrichum curvatum. Ammonium sulfate may inhibit peroxisome induction.

Nutrients that supported high catalase activity allowed A. curvatum to continue to grow very slowly throughout the incubation period. Perhaps cultures able to grow only poorly on a particular substrate carry out metabolic activities that are specific to such stress conditions and

Figure 2. Effect of nitrogen sources on catalase activity of Apiotrichum curvatum grown in defined medium with lactose as carbon source (△ , asparagine; ■ , asparagine with thiamine; ● , urea; □ , yeast extract; ▲ , yeast extract + ammonium sulfate; ○ , yeast extract + urea)

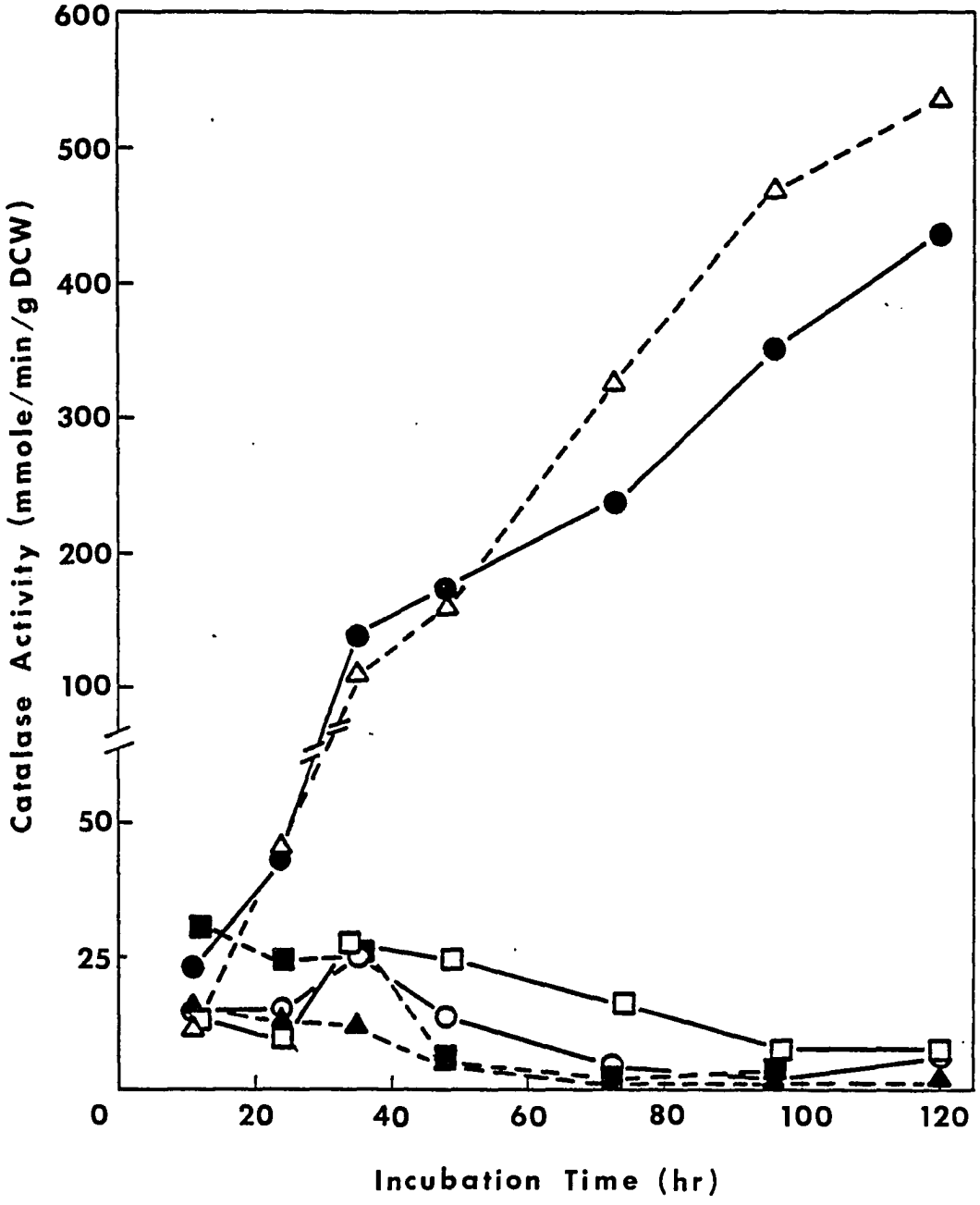
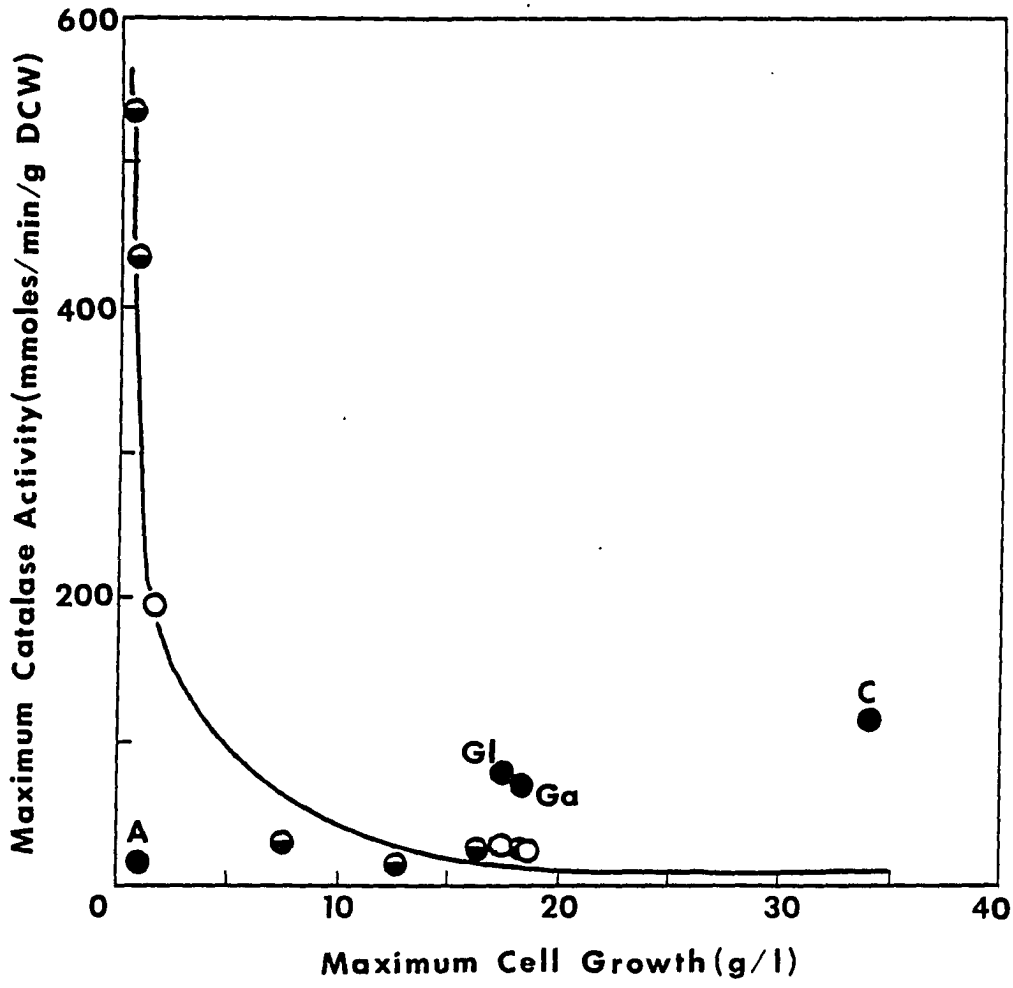

600
500
400
300
200
100
50
25
0
20
40
60
80
100
120
Catalase Activity (mmole/min/g DCW)
Incubation Time (hr)

Figure 3. Relationship between catalase activity and growth of Apiotrichum curvatum on various carbon (○) and nitrogen (◒) sources for 120 hr.
Data points for certain substrates: A, ammonium sulfate; C, corn oil; Ga, galactose; Gl, glycerin


600
400
200
0
10
20
30
40
Maximum Catalase Activity(mmoles/min/g DCW)
Maximum Cell Growth(g/l)
A
Gl
Ga
C

involve peroxisomal enzymes.

Catalase activity and metabolism of endogenous lipid in Apiotrichum curvatum

A possible advantage of oleaginous yeasts over nonoleaginous strains could be their ability to metabolize accumulated intracellular lipid when carbon becomes limiting in the environment. To measure catalase activity under such different metabolic conditions, cultures were grown in defined media with lactose and asparagine supplemented with thiamine-HCl at two different carbon:nitrogen (C/N) ratios. A high C/N ratio (C/N=99) favored lipid accumulation, and a moderate ratio (C/N=18) supported good growth but less lipid accumulation. The cultures were then washed and resuspended in fresh medium that contained a high or moderate C/N ratio or no carbon (C/N=0). Catalase activity was measured in the cultures before and after transfer.

As shown in Table 3, catalase activity was moderate in cultures before transfer. If a culture was transferred from a high to moderate C/N ratio or from a moderate to high C/N ratio, catalase activity decreased slightly after transfer. However, when cells grown at a high C/N ratio were transferred to a medium without carbon, catalase activity was observed to increase sharply after transfer.

If the accumulated lipid in the cells is to be used as a carbon and energy source, it must begin to be metabolized. The increase in catalase activity suggests that peroxisomes play a role in this lipid metabolism. Recently, Holdsworth et al. (17) reported that activitites of a few

Table 3. Comparison of catalase activity in Apiotrichum curvatum cultures before and after transfer between media with various carbon:nitrogen (C/N) ratios

C/N ratios of media[a]		Maximum catalase activity (mmoles/min/g DCW)	
1st	2nd	Before transfer	After transfer
99	18	24.2	16.3
18	99	47.4	21.8
99	0	39.4	148.2

[a]C/N=99: 40 g/l lactose and 0.8 g/l asparagine supplemented with thiamine. C/N=18: 40 g/l lactose and 4.4 g/l asparagine supplemented with thiamine. C/N=0: no lactose and 4.4 g/l asparagine supplemented with thiamine. The amount of carbon in asparagine was not taken into account in calculating C/N ratios.

peroxisomal enzymes such as catalase were greater in A. curvatum cells utilizing endogenous lipids than in cells grown in nitrogen-limited medium containing glucose and ammonium sulfate.

Beta-oxidation of fatty acids was first described in glyoxysomes from germinating castor bean endosperm (2, 7) and has since been found in peroxisomes from Euglena (15), Tetrahymena (3), rat liver (22, 25, 32), mouse liver (31), human liver (6), and n-alkane-grown Candida tropicalis (19, 36). Therefore, a peroxisomal fatty acid beta-oxidation pathway seems to be a general function of peroxisomes. As shown in Table 4, peroxisomal beta-oxidation was detected in A. curvatum cells grown on corn oil, but not in cells grown on starch or glucose. Beta-oxidation activity was also detected in Candida lipolytica cultures, but not in

Table 4. Detection of peroxisomal fatty acid beta-oxidation activity in Apiotrichum curvatum and other yeasts

Yeast strains[a] and carbon source	Catalase activity (umoles/min/Ab)	beta-Oxidation activity (nmoles/min/Ab)
A. curvatum		
Corn oil	46.7	0.61
Glucose	17.5	0.0
Starch	142.1	0.0
C. lipolytica 1094		
Corn oil	51.5	1.77
Glucose	46.6	0.94
S. cerevisiae		
Glucose	5.0	0.0

[a]Cultures were incubated for 48 hr in defined medium containing 3.64 g/l yeast extract as a nitrogen source.

Lipomyces starkeyi or Saccharomyces cerevisiae cells. Peroxisomal beta-oxidation may be induced by the lipid substrate in A. curvatum.

Electron microscopy

Apiotrichum curvatum cultures grown on glucose or corn oil were observed with the electron microscope. As shown in Figure 4, cell walls, cell membranes, nuclei and mitochondria were clearly recognized in all preparations. A few granules with a homogeneous matrix and core surrounded by a single-layer membrane were observed in glucose-grown cells (Figure 4(a)). Many more such granules were observed in corn oil-grown cells (Figure 4(b)), and were stained black in DAB-treated preparations (Figure 4(c)). These granules closely resembled microbodies

Figure 4. Electron micrographs of Apiotrichum curvatum:.
(a) DAB-treated cells grown on glucose for 24 hr.
Abbreviations used: CM, cell membrane; CW, cell wall; F, fat granule; M, mitochondrion; N, nucleus


(A)
F
CW
M
F

Figure 4. (continued)
(b) untreated cells grown on corn oil for 24 hr.
Abbreviations used: CM, cell membrane; CW, cell wall; F, fat granule; M, mitochondrion; N, nucleus; P, peroxisomes

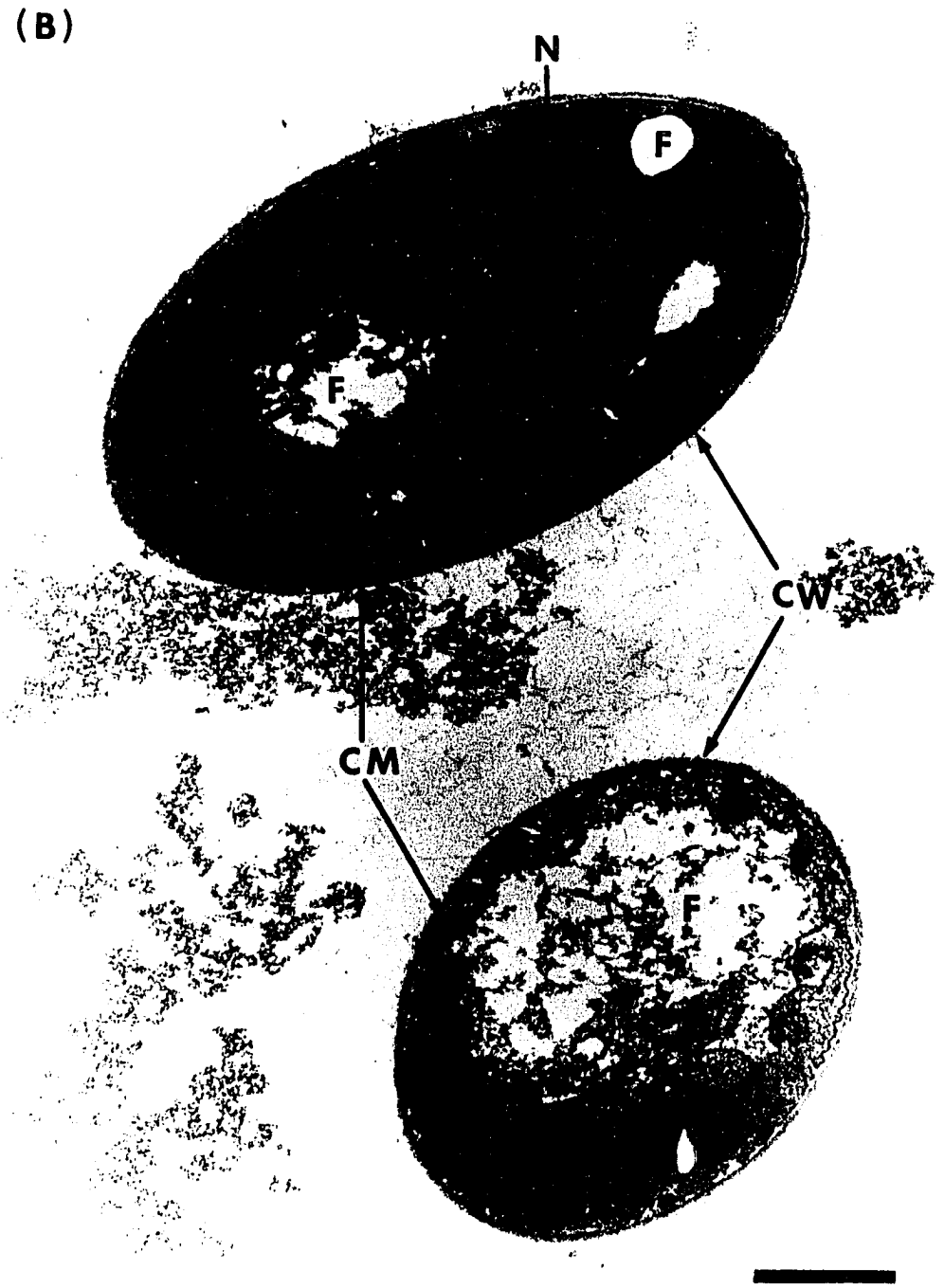
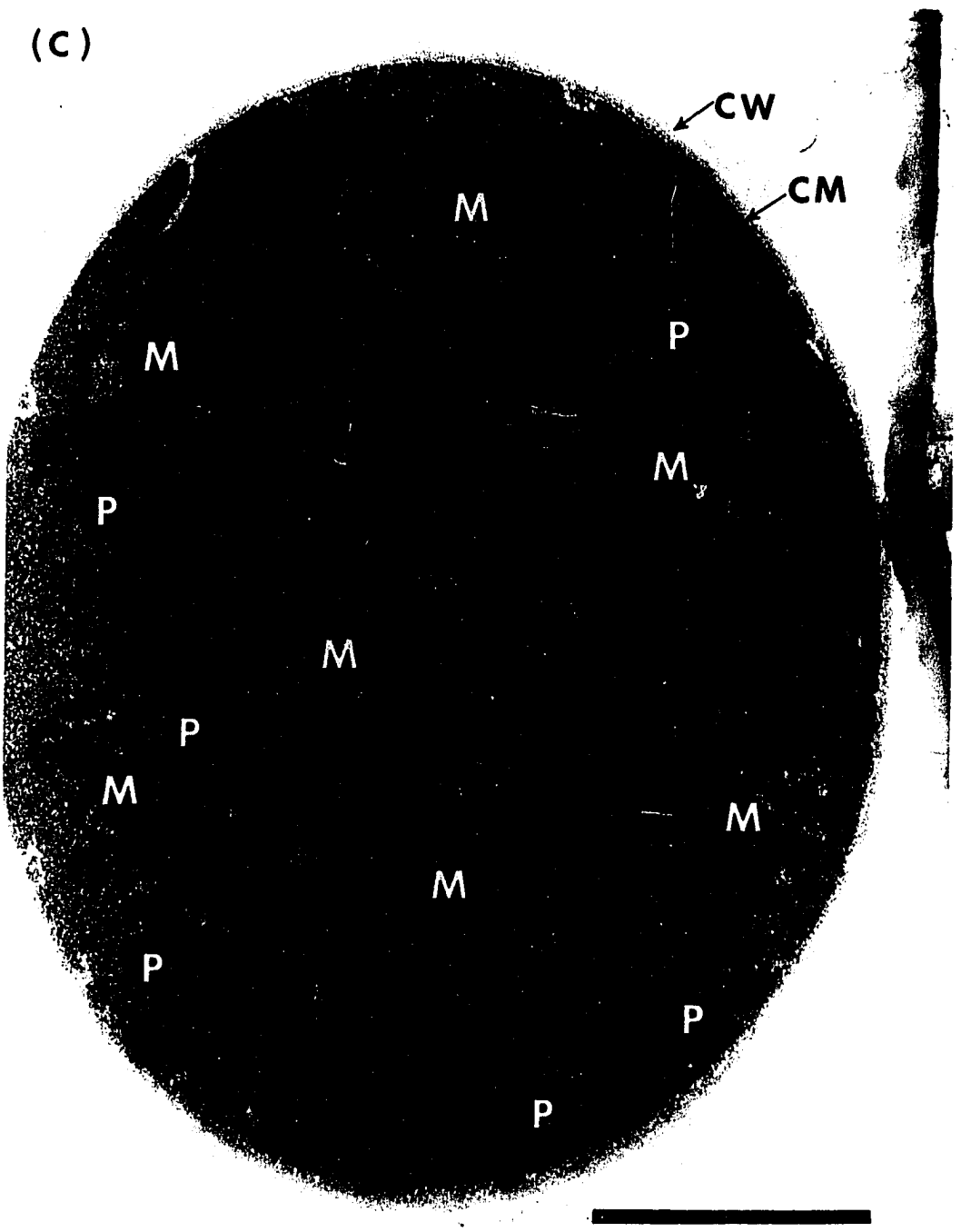

(B)
N
F
F
CW
CM
F

Figure 4. (continued)
(c) DAB-treated cells grown on corn oil for 24 hr (bar = 1 um).
Abbreviations used: CM, cell membrane; CW, cell wall; M, mitochondrion; N, nucleus; P, peroxisomes


(C)
CW
CM
M
P
M
M
P
M
P
M
M
P
M
M
P
P
P

or peroxisomes observed in higher plant and animal cells (9). The correlation of catalase activity with proliferation of these granules and staining of the granules with DAB indicate that these granules contain catalase and are, in fact, peroxisomes (44). Staining of the mitochondrial cristae or membrane with DAB may be due to the localization of flavin-dependent oxidase or some peroxidase, such as cytochrome c peroxidase, in these regions (34, 41). Recently, Holdsworth et al. (17) observed proliferation of peroxisomes in A. curvatum cells that were utilizing their endogenous lipids or that had been grown on exogenous lipid, but not in cells grown in nitrogen-limited medium containing glucose and ammonium sulfate.

In general, peroxisomes of alkane- and methanol-utilizing yeasts have a homogeneous matrix surrounded by a single-layer membrane, but morphological features can vary significantly (12). The morphological features of peroxisomes in A. curvatum were similar to those of the organelles in alkane-grown yeasts (12).

Comparison of catalase activity in Apiotrichum curvatum and other yeast strains

Six oleaginous yeasts and five nonoleaginous yeasts were grown on various carbon sources and monitored for catalase activity. As shown in Table 5, catalase activity was detected in all strains on most of the carbon sources. Rhodosporidium toruloides CBS 14 and Schizosaccharomyces pombe produced very little catalase; similar results have been reported in Rhodosporidium toruloides CBS 14 and ATCC 26217 (17). Most yeast

Table 5. Comparison of catalase activity among oleaginous and nonoleaginous yeast strains grown on various carbon sources

Yeast strains[a]	Catalase activity (μmoles/min/Ab) on various carbon sources				
	Galactose	Glucose	Starch	Corn oil	Lactose
(a) Oleaginous yeasts					
A. curvatum					
ATCC 20509	39.1	17.5	142.1	46.7	21.3
C. lipolytica					
1094	100.3	46.6		51.5	
1095	61.0	26.7			
L. starkeyi					
CBS 1809	2.5	17.1			
R. toruloides					
CBS 14	0.3	0.0			
R. glutinis					
ATCC 32765	14.2	9.5	18.7		
BC 12-21	6.6	7.3	16.8		
S. holsaticus					
ATCC 34889		0.0	8.1		
(b) Nonoleaginous yeasts					
C. utilis					
ATCC 9226	81.3	40.8			
K. fragilis					
ATCC 36534	5.5	9.5			9.5
S. cerevisiae					
ATCC 4098	12.5	10.1			
ATCC 2341	9.7	6.5			
S. diastaticus					
ATCC 36902			39.1		
S. pombe					
ATCC 26189	0.0	1.6			

[a]Cultures were incubated for 48 hr in defined medium containing 3.64 g/l yeast extract as nitrogen source.

strains that showed a high specific catalase activity per g of cell mass did not grow well on that carbon source. Catalase seems to be widespread in yeast strains, regardless of their oleaginicity. It is likely that peroxisomes are common in yeasts other than the alkane-utilizing (12, 13) and methanol-utilizing (12, 45) yeasts in which they have been studied most.

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PART III.

SUBCELLULAR LOCALIZATION OF FATTY ACID beta-OXIDATION AND KEY ENZYMES OF THE GLYOXYLATE CYCLE IN PEROXISOMES OF THE OLEAGINOUS YEAST *Apiotrichum curvatum*

ABSTRACT

Peroxisomes from Apiotrichum curvatum ATCC 20509 (formerly Candida curvata D) grown on corn oil were separated from other subcellular fractions in a discontinuous sucrose gradient. Major peaks of activity of fatty acid beta-oxidation and of two key enzymes in the glyoxylate cycle were found in fractions containing peroxisomes, but not in fractions corresponding to the mitochondria. Peroxisomal beta-oxidation showed equivalent activity with palmitoyl-CoA or n-octanoyl-CoA as substrate. Mitochondria did not seem to contain NAD-linked glutamate dehydrogenase.

INTRODUCTION

Oleaginous yeasts can accumulate lipid when grown on a carbohydrate source under nitrogen limitation (3, 16, 20), and can also utilize the accumulated lipid in the presence of an exogenous nitrogen source if starved for carbon (10). Several yeasts including Apiotrichum curvatum can also grow on an exogenous source of lipid (1, 9). This physiological flexibility and adaptability can make oleaginous yeasts a good model system to investigate the control of lipid metabolism in eucaryotic cells.

Yeast peroxisomes, which have been studied mainly in yeasts grown on n-alkanes or methanol, carry out several important metabolic functions including fatty acid beta-oxidation and gluconeogenesis (7, 8). Recently, activities of some peroxisomal enzymes such as carnitine acetyltransferase (11, 21), catalase (11), isocitrate lyase (11), isocitrate dehydrogenase (11) and D-amino acid oxidase (22), were reported in cell-free extracts of some oleaginous yeasts, including A. curvatum. The presence of peroxisomes was confirmed in electron micrographs of A. curvatum (11) and Lipomyces starkeyi (8). However, few publications have reported the separation of peroxisomes or described in detail their role in the metabolism of oleaginous yeasts.

In the present study, peroxisomes were recovered from cells that had been broken by a combination of homogenization, sonication, and treatment with Zymolyase, and were separated by sucrose gradient centrifugation.

Activities of fatty acid beta-oxidation and key enzymes of the glyoxylate cycle were found in peroxisomes, but not in mitochondria.

MATERIALS AND METHODS

Microorganism and cultivation

Apiotrichum curvatum ATCC 20509 (formerly *Candida curvata* D) was grown with rotary shaking (200 rpm) at 32°C in a defined medium (26) containing 18 g/l corn oil as carbon source, 3.64 g/l yeast extract and 10 g/l Brij 58.

Purification of peroxisomes

The method for peroxisome isolation described by Kamiryo et al. (12) was modified as follows. Cells were harvested in late-logarithmic phase by centrifugation at 3800 x g for 10 min, washed with water, suspended in 50 mM potassium phosphate buffer (pH 7.5) containing 0.6 M KCl, 10 mM Na_2SO_3 and 10 mM 2-mercaptoethanol, and incubated at room temperature for 15 min. Zymolyase-100T (Kirin Brewery Co., Tokyo, Japan) was added to the cell suspension at a concentration of 2 mg per g wet cells, and incubated at 32°C for 3 hr. All subsequent operations including the separation of peroxisomes were conducted at 0-4°C.

The cells were collected by centrifugation at 3800 x g for 10 min, washed once with the same phosphate buffer containing 0.6 M KCl, and broken in F buffer (5% Ficoll 400, 0.6 M sorbitol, 2.5 mM potassium 3-(N-morpholino) propane sulfonate (MOPS) at pH 7.2, 0.1% ethanol and 1 mM EDTA) with 20 strokes in a loosely fitting Potter-Elvejhem Teflon homogenizer. The homogenate was centrifuged at 1200 x g for 10 min. The supernate was saved and the precipitate was sonicated at maximum power

with a disintegrator (Model BP-2, Blackstone Ultrasonics, Inc., Sheffield, PA, USA) in F buffer for 1 min. This mixture was centrifuged as above, the supernate was saved, and the precipitate was sonicated again in F buffer for 1 min. After centrifugation, the supernates from these treatments were combined and centrifuged at 20,000 x g for 20 min. The precipitate from this step, suspended in 6 ml F buffer, was labelled the particulate fraction, and 5 ml was applied to a discontinuous sucrose density gradient consisting of 4-, 6-, 12-, and 6-ml sucrose solutions of 25, 35, 42, and 53% (w/w), respectively; all sucrose solutions contained 2.5 mM MOPS (pH 7.2), 0.1 % ethanol and 0.5 mM EDTA. Centrifugation was at 28,000 rpm for 1.5 hours in a SW 29 rotor (Beckman Instruments Inc., Palo Alto, CA, USA) and 2-ml fractions were obtained from the bottom of the tube.

Analyses

Organelles were identified by employing the established procedures for determination of the following marker enzymes: catalase (EC 1.11.1.6) (23) for peroxisomes, cytochrome c oxidase (EC 1.9.3.1) (5) and NAD-linked glutamate dehydrogenase (EC 1.4.1.2) (17) for mitochondria. Mitochondrial beta-oxidation (25) was assayed as palmitoyl-CoA-dependent oxygen consumption, which was measured with a Clark-type electrode at 32°C. The oxygen concentration of air-saturated water was taken to be 246.6 µM. The procedure was modified to include 100 µM ADP, 0.5 mM DL-(+)-carnitine and 60 µM palmitoyl-CoA in the assay mixture. Peroxisomal beta-oxidation (15) was also measured as oxygen consumption rate, with 60

µM palmitoyl-CoA as substrate in the presence of 22 ng/ml antimycin A. If required, 60 µM n-octanoyl-CoA in both beta-oxidation assays replaced palmitoyl-CoA. Isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2) were assayed by the method of Nabeshima et al. (18). Protein was measured by the modified micro-Lowry method (19). Sucrose density was measured by refractometry.

RESULTS AND DISCUSSION

Proliferation of peroxisomes was induced in cells grown on corn oil as carbon source as reported in Part II. Purification of peroxisomes from cells grown for 18-20 hr in corn oil-containing medium was successful if Brij 58 or Brij 78 was added to the growth medium. Apiotrichum curvatum was found to be resistant to several common lytic enzymes used to break yeast cells (not shown), but a combination of sonication, homogenization, and treatment with Zymolyase was very effective in releasing cellular contents.

Figure 1 illustrates the results of a typical purification experiment. Catalase, the marker enzyme for peroxisomes, and cytochrome c oxidase, a marker for mitochondria appeared as well separated peaks of activity. However, NAD-linked glutamate dehydrogenase, which is another mitochondrial marker enzyme in animal (17, 25) and plant systems (3), was barely detectable throughout the entire gradient (Figure 1(a)). This enzyme seems to have a cytosolic rather than mitochondrial location. It was also reported as a cytosolic enzyme in Candida tropicalis (7). Peroxisomes sedimented to a sharp peak with an equilibrium density of 1.22 g/ml at 20°C, whereas mitochondria had a density of 1.18 g/ml (Figure 1(a) and (b)). These isopycnic densities were very similar to those reported in C. tropicalis (6).

The particulate fraction that was loaded onto the gradient contained 28% of the catalase activity, 61% of the cytochrome c oxidase activity and 13% of the protein from the homogenate (Table 1). About 84% of the

Figure 1. Distribution of enzymatic activity between peroxisomes and mitochondria from a representative sucrose density gradient prepared from Apiotrichum curvatum grown on corn oil. (a) The marker enzymes; catalase (———) for peroxisomes and cytochrome c oxidase (– – –) or glutamate dehydrogenase (–·–·–) for mitochondria; (b) protein (———) and density (– – –); (c) fatty acid beta-oxidation in peroxisomes (———) and mitochondria (– – –); (d) isocitrate lyase (———) and malate synthase (– – –)

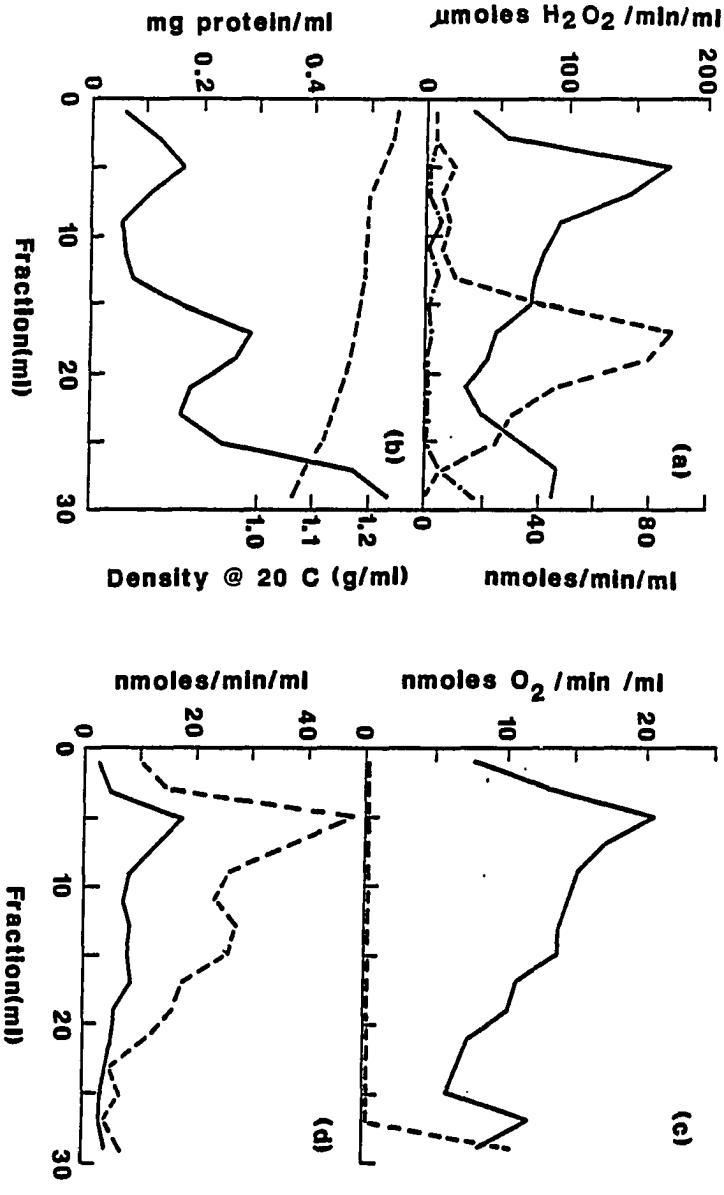
mg protein/ml
0
0.2
0.4
µmoles H_2O_2 /min/ml
0
100
200
Fraction(ml)
0
10
20
30
(b)
(a)
1.0
1.1
1.2
0
40
80
Density @ 20 C (g/ml)
nmoles/min/ml
nmoles/min/ml
0
20
40
nmoles O_2 /min /ml
0
10
20
Fraction(ml)
0
10
20
30
(d)
(c)

Table 1: Purification of peroxisomes from Apiotrichum curvatum cells[a] grown on corn oil

Fractions	Protein (mg)	Catalase (μmoles/min)	Cyt. oxidase (μmoles/min)	Ratio of catalase to cyt. oxidase
Homogenate[b]	91.7	9827.4	1.65	5956
Particulate[c]	11.6	2784.0	1.01	2756
Peroxisomes[d]	0.5	633.0	0.03	21100
Mitochondria[d]	1.1	181.8	0.33	551

[a]4.72 g (wet weight) of freshly grown cells were used.

[b]The homogenate was defined as the cell suspension broken with 20 strokes in a Teflon-homogenizer.

[c]The particulate was defined as the fraction suspended in F buffer after centrifugation of combined supernates at 20,000 x g for 20 min.

[d]Peroxisomes were pooled from fractions 3 and 4 (4-8 ml), and mitochondria were pooled from fractions 9 and 10 (16-20 ml) of the sucrose gradient.

catalase activity and 71% of the cytochrome c oxidase activity from the particulate were recovered from the gradient. The peroxisomal fraction contained 27% of the catalase activity and 4% of the cytochrome c oxidase activity, and the mitochondrial fraction contained 8% of the catalase activity and 47% of the cytochrome c oxidase activity, on the basis of the recovered activity from the gradient. The ratio of catalase to cytochrome c oxidase activity was high in the peroxisomal fraction. The specific catalase activity (1266 μmoles/min/mg protein) in this fraction was 11-fold higher than that of the cell homogenate and 5-fold higher

than that of the particulate fraction.

The activities of fatty acid beta-oxidation and of two key glyoxylate cycle enzymes were assayed in the entire gradient. Peroxisomal beta-oxidation activity with palmitoyl-CoA as substrate corresponded with high-density particulate catalase activity (Figure 1(c)). Mitochondrial beta-oxidation activity was not detected in the mitochondrial peak or in any other fraction. These results are similar to those reported for glyoxysomes from germinating castor bean endosperm (4) or peroxisomes from alkane-utilizing yeasts (7, 8, 13).

When n-octanoyl-CoA was used as substrate, the same results were obtained. The specific activity of peroxisomal beta-oxidation was approximately the same with both substrates (Table 2), which suggests that this system is equally active with both long-chain and short-chain

Table 2: Effect of chain length of fatty acids on beta-oxidation in Apiotrichum curvatum grown on corn oil

	Beta-oxidation (nmoles O_2/min/mg protein)	
Substrates	Peroxisomal[a]	Mitochondrial[b]
Palmitoyl-CoA	61.7	0.0
Octanoyl-CoA	58.1	0.0

[a]Peroxisomal beta-oxidation was assayed in fraction 2 of the sucrose gradient.

[b]Mitochondrial beta-oxidation was assayed in fraction 9 of the sucrose gradient.

fatty acyl-CoA molecules.

Fatty acid beta-oxidation seems to occur only in peroxisomes of A. curvatum, as is the case in the glyoxysomes from germinating castor bean endosperm (4). This contrasts with fatty acid beta-oxidation in animal systems (14, 25), which occurs in both peroxisomes and mitochondria, and is most active with long-chain (C_{10}-C_{22}) fatty acyl-CoA substrates (24).

Activities of isocitrate lyase and malate synthase, two key enzymes of the glyoxylate cycle, also corresponded with particulate catalase activity but not with cytochrome c oxidase activity (Figure 1(c)). These results suggest that these enzymes are located in the peroxisomes, and agree with those reported for glyoxysomes from germinating castor bean endosperm (2, 3) and peroxisomes from alkane-utilizing yeasts (7, 8, 18). The locations of the other enzymes in the glyoxylate cycle are yet to be determined in A. curvatum. These enzymes are not peroxisomal in alkane-utilizing yeasts (7), but are present in the glyoxysomes and in the mitochondria of germinating castor bean endosperm (3).

We suggest that when A. curvatum is grown on corn oil as sole carbon source, it converts triglycerides to fatty acids and glycerol by the action of lipase(s), whose cellular location is presently unknown. These fatty acids are converted by the peroxisomal beta-oxidation system to acetyl-CoA, which is combined with glyoxylate in the peroxisomes to form malate. Each turn of the glyoxylate cycle generates one extra C_4 acid, which is converted to phospho-enolpyruvate for gluconeogenesis elsewhere in the cells. This tight coupling between beta-oxidation and the glyoxylate cycle in the peroxisomes of A. curvatum can account for a very

high conservation of carbon in converting lipid into carbohydrates and other cellular metabolites during growth on corn oil.

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SUMMARY AND DISCUSSION

The studies in this dissertation explored the role of peroxisomes in lipid metabolism of the oleaginous yeast Apiotrichum curvatum.

Oleaginous yeasts can accumulate a large amount of intracellular lipid when grown with excess carbon under nitrogen limitation, and this lipid accumulation has been quite well explained both biochemically and physiologically (18, 114, 115, 117, 147, 148). Oleaginous yeasts have also been reported to utilize their accumulated lipid in the presence of an exogenous nitrogen source if starved for carbon (64), and to utilize an exogenous lipid as a carbon and energy source (6, 55, 65). Such assimilation of exogenous and endogenous lipids in oleaginous yeasts has not been clearly explained yet, but could involve the metabolic functions of peroxisomes (65).

Yeast peroxisomes, which have been studied mainly in cells grown on n-alkanes or methanol, are known to carry out several important metabolic functions, including peroxisomal fatty acid beta-oxidation and the glyoxylate cycle (47, 48, 142). Although little attention has been paid to peroxisomes in the oleaginous yeasts, recent publications (65, 118, 125) reported activities of a few peroxisomal enzymes in cell-free extracts of some oleaginous yeasts including A. curvatum, and confirmed the presence of peroxisomes in these cells by electron microscopy.

Lipid metabolism and cell composition of A. curvatum grown at different carbon:nitrogen ratios (C/N) were investigated in Part I. The organism was incubated in nitrogen-limited (C/N=99), balanced (C/N=18),

and carbon-free (C/N=0) media with asparagine as nitrogen source and lactose as carbon source. The organism went through a two-stage growth cycle in nitrogen-limited medium: an initial growth phase with little lipid accumulation followed by a fattening phase in which cell division slowed but lipid accumulation continued. Such a two-stage fermentation is typical of this organism under conditions in which nitrogen is depleted and residual carbohydrates are converted to lipid (100, 101).

When grown in balanced medium, the organism produced lipid much less efficiently. With a plentiful supply of nitrogen in the growth medium, cells could continue to synthesize proteins and nucleic acids for cell division and growth, and converted much of the carbon to nonlipid rather than lipid biomass. The organism accumulated up to 54% of its dry weight as intracellular lipid when grown under nitrogen limitation, but accumulated no more than half that amount when grown in balanced medium.

When the cells grown in nitrogen-limited medium were transferred to a medium without lactose, they utilized not only endogenous lipid but also endogenous carbohydrate as carbon and energy sources. Catalase activity was strongly induced when cells metabolized endogenous lipid. The lipid content of cells was inversely related to catalase activity and to protein or nitrogen content, but showed no correlation with intracellular carbohydrate content. These results suggest that peroxisomes play a role in lipid degradation (i.e., fatty acid beta-oxidation) in this oleaginous yeast.

Intracellular carbohydrates seemed to be used as intermediates for lipid accumulation and lipid turnover in this organism. The

intracellular carbohydrate measured in the current study must be something other than citrate, which was reported to accumulate intracellularly in A. curvatum before lipid production (37), but which was not detected by the carbohydrate assay used in the current study. Accumulation of intracellular carbohydrate in oleaginous yeasts has not received a lot of attention and has been discussed in only a few publications. Apiotrichum curvatum was reported to accumulate some extracellular slime during growth and to metabolize it during the lipid accumulation phase (101). Glycogen was suggested as an alternative carbohydrate storage material in oleaginous yeasts (38, 64). A reciprocal relation between carbohydrate and lipid content was reported in Rhodosporidium toruloides (38), and A. curvatum grown on glucose and ammonium chloride was reported to accumulate about 10% (w/w) of its biomass as glycogen (64).

Glycogen may be a principal storage material in nonoleaginous yeasts (57, 58, 70). Candida tropicalis could accumulate glycogen when grown on glucose in a nitrogen-free medium, but accumulated lipids rather than glycogen when grown on hydrocarbon (70).

The presence and induction of peroxisomes in cultures prepared under various growth conditions were investigated in Part II. Yeast peroxisomes were reported to be induced only by special carbon or nitrogen sources that varied with yeast species (47, 48, 94, 142, 152). In the current study, catalase activity in A. curvatum was greatly affected by the nature of the carbon and nitrogen sources in the medium. Cells grown on corn oil as exogenous carbon source had very high catalase

activity, but those grown on glucose, sucrose, or maltose had low activity. These results suggest that the corn oil is assimilated through fatty acid beta-oxidation and glyoxylate cycle reactions in the peroxisomes rather than through other nonperoxisomal pathways such as glycolysis. Catalase activity detected in cells grown on carbohydrates might be associated with other peroxisomal functions such as disposal of toxic products formed from nucleic acid or protein turnover.

Holdsworth et al. (65) compared A. curvatum cells utilizing exogenous lipid and cells utilizing their endogenous lipids with cells grown in nitrogen-limited medium containing glucose as carbon source. The cells utilizing exogenous lipid showed greater increases in peroxisomal enzyme activities such as catalase than the cells utilizing their endogenous lipids. Catalase activity of Candida tropicalis pK 233 was reported to be induced by hydrocarbons but not by glucose, galactose, ethanol, acetate, or lauryl alcohol, and catalase induction by hydrocarbons was sensitive to cycloheximide but not to chloramphenicol (134, 135).

Specific catalase activity usually reached a maximum during exponential growth phase, but was very high and continued to increase throughout incubation under conditions in which the cells grew poorly (e.g., soluble starch as carbon source; methylamine, urea, or asparagine as nitrogen source). Ammonium sulfate did not support culture growth or catalase induction. The current results suggest a reciprocal relationship between catalase activity and the ability of carbon or nitrogen sources to support good growth. Perhaps cultures able to grow

only poorly on a particular substrate carry out metabolic activities that are specific to such stress conditions and involve peroxisomal enzymes.

Proliferation of peroxisomes with a homogeneous matrix and core surrounded by a single limiting membrane were observed with an electron microscope in corn oil-grown cells, but rarely in glucose-grown cells. Staining with 3,3-diaminobenzidine revealed that catalase activity was located in peroxisomes. Few attempts were made to ascertain whether or not catalase activity existed in subcellular locations other than in peroxisomes. The proliferation of peroxisomes was also observed in yeast cells grown on alkanes (75, 108, 110, 131) or methanol (30, 46, 50, 122, 132, 141, 143), along with large increases of catalase activity.

The subcellular distribution of catalase can be a controversial subject. In general, catalase is well known as a marker enzyme for peroxisomes (35, 89). However, catalase activity can also be measured in the cytosolic fraction of broken cells. It is possible that this activity was released from ruptured peroxisomes. The central importance of catalase as the primary agent of H_2O_2 disposal is accepted in explaining the functions of peroxisome, whatever the nature of the oxidases that have produced the H_2O_2 (35).

Peroxisomes are certainly not the only site of H_2O_2 formation and degradation. There are oxidases that yield H_2O_2 in the cytosol, in the outer membrane of the mitochondrion, and perhaps elsewhere in the cell (34). Most cells have at least one peroxidase in addition to catalase that can break down H_2O_2 as a secondary defense: mammalian cells have cytosolic glutathione peroxidase (23), and Hansenula polymorpha, a

methanol-utilizing yeast, was recently reported to have mitochondrial cytochrome c peroxidase but not glutathione peroxidase (146). With these alternative protective systems in other locations in the cell, it is likely that peroxisomes are the principal sites for catalase. Improved techniques for separation of subcellular organelles are needed to resolve the question of catalase location.

Separation and characterization of peroxisomes in A. curvatum were investigated in Part III. Purification of peroxisomes from cells grown in corn oil-containing medium was successful if Brij 58 or Brij 78 was added to the growth medium. The use of detergents similarly improved peroxisome purification in n-alkane-utilizing yeasts grown on oleic acid (45), but their function is unknown at present. Those detergents might affect cell wall composition or loosen the cell wall structure to ease attack by lytic enzymes. Also, they might affect the densities of subcellular organelles in sucrose gradient centrifugation, as was reported for Triton WR-1339 (89).

Apiotrichum curvatum was resistant to several common lytic enzymes used to degrade yeast cell walls, but a combination of homogenization, sonication, and treatment with lytic enzymes such as Zymolyase was very effective in releasing cellular contents. The resistance of the organism to common lytic enzymes may be due to a difference in cell wall composition, as is seen with Candida species (69, 79). In the current study, the particulate fraction that was loaded onto the sucrose gradient contained 28% of the catalase activity and 13% of the protein from the homogenate. A more efficient but gentle system for lysis of the cells

needs to be developed to increase recovery of peroxisomes, to reduce the percentage of broken peroxisomes, and to separate peroxisomes more efficiently from other subcellular fractions.

Peroxisomes from cells grown on corn oil had an equilibrium density of 1.22 g/ml at 20°C in a discontinuous sucrose gradient, and were well separated from other subcellular fractions. Major peaks of activity of fatty acid beta-oxidation and of two key enzymes in the glyoxylate cycle were found in fractions containing peroxisomes, but not in fractions corresponding to the mitochondria. Peroxisomal beta-oxidation showed equivalent activity with palmitoyl-CoA or n-octanoyl-CoA as substrate. Mitochondria did not seem to contain NAD-linked glutamate dehydrogenase, an enzyme used as a marker for mitochondria in mammalian systems (89). The glutamate dehydrogenase seems to be a cytosolic enzyme in this organism, as it has been found to be in alkane-utilizing yeasts (47).

Results obtained in Part III are similar to those reported for glyoxysomes from germinating castor bean endosperm (20, 26, 27, 137) or peroxisomes from alkane-utilizing yeasts (47, 48, 74, 137). Cells of A. curvatum utilizing endogenous lipids as carbon and energy source seem to be an excellent model system for study of lipid metabolism in germinating seeds. As reported in Part II, peroxisomal fatty acid beta-oxidation activity was detected in cell-free extracts of cells grown on corn oil, and mitochondrial beta-oxidation was not observed. It is likely that the same situation will be found in cells utilizing endogenous lipid as carbon and energy source. Localization of fatty acid beta-oxidation in peroxisomes but not in mitochondria has been reported in alkane-utilizing

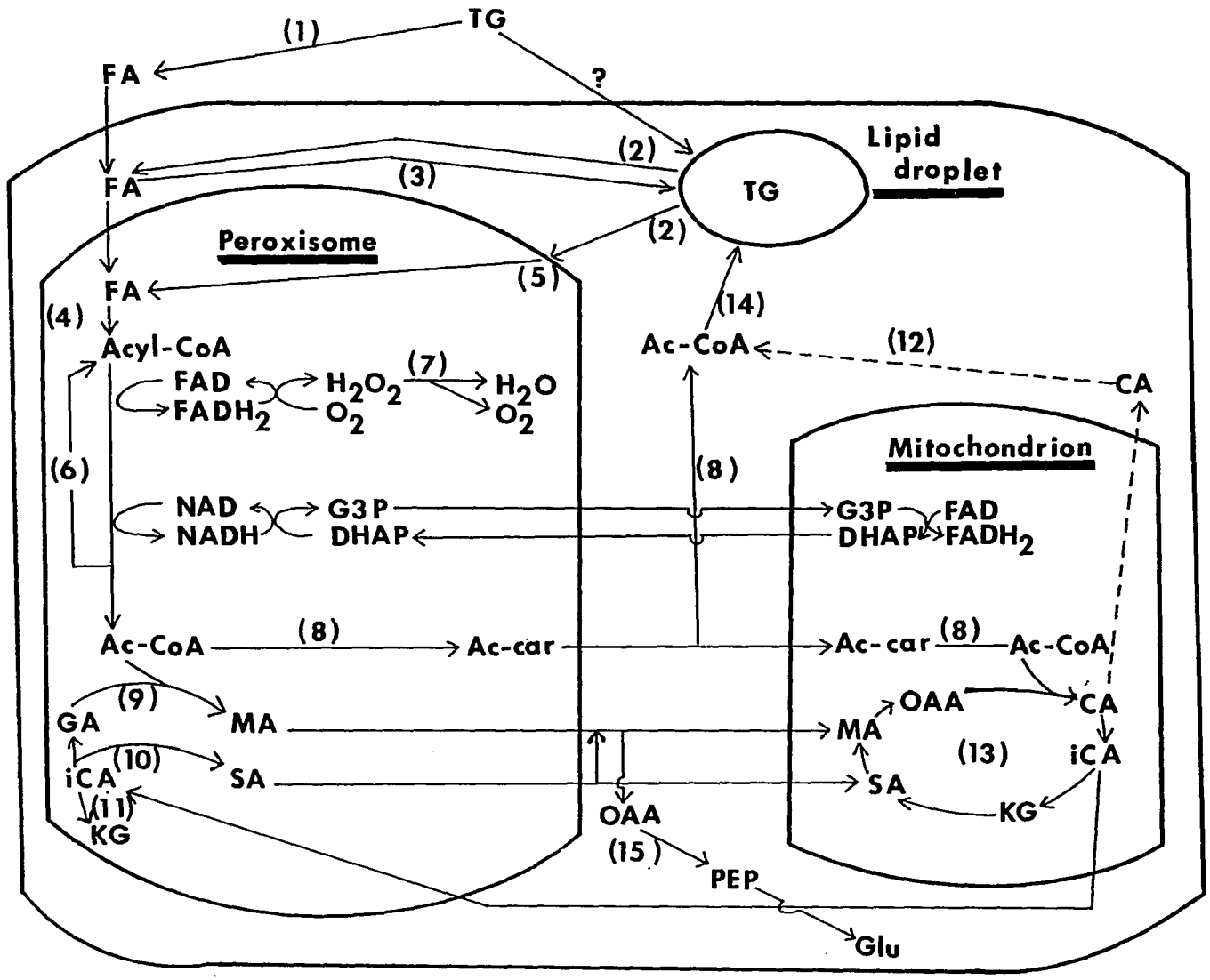
yeasts (47, 74) and in germinating castor bean endosperm (27). Peroxisomes in yeasts and plants thus seem to play an essential role in the use of fat as an energy source.

Two key enzymes, isocitrate lyase and malate synthase, in the glyoxylate cycle seem to exist only in the peroxisomes, too. Although the locations of the other enzymes in the glyoxylate cycle are yet to be determined, it is likely that they will be found only in mitochondria of this organism. This is the case in alkane-utilizing yeasts (47, 48, 93), Tetrahymena (63), Euglena (56), and various fungi (93). In contrast, these enzymes are located in the glyoxysomes and in the mitochondria of germinating castor bean endosperm (20, 26). The glyoxylate cycle may be closely associated with the mitochondrial TCA cycle to share enzymes and with peroxisomal fatty acid beta-oxidation that can supply acetyl-CoA.

Based on results obtained in this dissertation, I suggest that peroxisomes in the oleaginous yeast A. curvatum play an essential role in lipid metabolism including fatty acid beta-oxidation and gluconeogenesis. Various reactions of lipid metabolism are illustrated in Figure 1. When A. curvatum is grown on exogenous or endogenous lipids as carbon and energy source, it converts triglycerides to fatty acids and glycerol by the action of lipase(s), whose cellular location is presently unknown. It is possible that intracellular lipases are located in lipid bodies and in peroxisomes, as is the case in the germinating castor bean endosperm (61, 103). Also, the organism seems to have an extracellular lipase (116).

The fatty acids are converted by the peroxisomal beta-oxidation

Figure 1. Illustration of a proposed lipid metabolism in the oleaginous yeast Apiotrichum curvatum.
Abbreviations used: Ac-car, acetyl-carnitine; Ac-CoA, Acetyl-CoA; G3P, Glycerol-3-phosphate; DHAP, dihydroxyacetone phosphate; CA, citric acid; FA, fatty acids; GA, glyoxylic acid; Glu, glucose; iCA, isocitric acid; KG, alpha-ketoglutaric acid; MA, malic acid; OAA, oxalacetic acid; PEP, phosphoenolpyruvic acid; SA, succinic acid; TG, triglycerides.
Enzymes: 1, extracellular lipase; 2, lipase(s) in lipid droplets; 3, acyl-CoA synthetase; 4, peroxisomal acyl-CoA synthetase; 5, peroxisomal lipase; 6, peroxisomal fatty acid beta-oxidation system; 7, catalase; 8, carnitine acetyltransferases in peroxisomes, mitochondria, and cytoplasm; 9, malate synthase; 10, isocitrate lyase; 11, $NADP^+$-dependent isocitrate dehydrogenase; 12, ATP:citrate lyase; 13, mitochondrial tricarboxylic acid cycle; 14, lipid synthesis system; 15, gluconeogenic system


TG
(1)
FA
?
Lipid
droplet
(2)
FA
(3)
TG
(2)
Peroxisome
FA
(5)
(4)
Acyl-CoA
(14)
Ac-CoA
(12)
CA
FAD
H_2O_2
(7)
H_2O
$FADH_2$
O_2
O_2
Mitochondrion
(6)
(8)
NAD
G3P
G3P
FAD
NADH
DHAP
DHAP
$FADH_2$
Ac-CoA
(8)
Ac-car
Ac-car
(8)
Ac-CoA
OAA
(9)
GA
MA
MA
CA
(13)
(10)
iCA
SA
SA
iCA
(11)
KG
KG
OAA
(15)
PEP
Glu

system to acetyl-CoA, which is combined with glyoxylate by the action of malate synthase in the peroxisomes to form malate. The acetyl-CoA also can be transferred by the peroxisomal and mitochondrial carnitine acyltransferases (80) to mitochondria, where it enters the TCA cycle to generate ATP. Alternatively, acetyl-CoA can be used for lipid biosynthesis in the cytoplasm.

The glyoxylate is formed by isocitrate lyase from isocitrate, which can be supplied from the mitochondrial TCA cycle. Isocitrate within peroxisomes can be degraded by NADP-dependent isocitrate dehydrogenase to alpha-ketoglutarate, which can be used for protein biosynthesis. Malate and succinate formed from the glyoxylate cycle can be transferred to mitochondria and used as intermediates of the TCA cycle. The NADH produced from peroxisomal beta-oxidation can be transferred through a peroxisomal glycerol-phosphate shuttle (52, 76) to the mitochondrial electron transport system to generate ATP.

Each turn of the glyoxylate cycle generates one extra C_4 acid, which is converted to phosphoenolpyruvate for gluconeogenesis elsewhere in the cell. This tight coupling between beta-oxidation and the glyoxylate cycle in the peroxisomes of A. curvatum can account for a very high conservation of carbon in converting lipid into carbohydrates and other cellular metabolites during growth on corn oil.

In the future, to establish the complete role of peroxisomes in the oleaginous yeast A. curvatum, peroxisomes must be separated and characterized from cells grown in carbon-starved, nitrogen-limited, and balanced media that contain several different carbon and nitrogen

sources. Characterization of lipase(s) and development of mutants defective in peroxisomal enzymes are also needed.

CONCLUSIONS

1. Cells grown in nitrogen-limited medium could accumulate lipid much more efficiently than those grown in balanced medium.
2. Cells starved for carbon after growth in nitrogen-limited medium utilized endogenous lipid and carbohydrate as carbon and energy source.
3. Intracellular carbohydrates seemed to be used as intermediates for lipid accumulation and turnover.
4. Catalase activity was strongly induced in cultures to utilize the endogenous lipid.
5. Nature of carbon and nitrogen sources greatly affected induction of catalase activity.
6. Catalase activity was generally greater in exponential-phase cells than in stationary-phase cells
7. Presence of peroxisomes was confirmed in electron micrographs and catalase activity was located in peroxisomes.
8. Peroxisomes from corn oil-grown cells were separated from other subcellular fractions in a discontinuous sucrose gradient.
6. Fatty acid beta-oxidation and key enzymes of the glyoxylate cycle were detected only in peroxisomes, but not in mitochondria.

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ACKNOWLEDGMENTS

I am greatly indebted to my major professor, Dr. Bonita A. Glatz and one of my advisory committee, Dr. Patricia A. Murphy, for their helpful guidance and discussions throughout my graduate studies.

I would like to thank Dr. Charles E. Glatz, Dr. Earl G. Hammond, and Dr. Carl L. Tipton for serving on my advisory committee.

My thanks are extended to my lab colleagues, Dale Grinstead, Anand Rao, Tom Rehberger, Anthony Weier, Steve Woskow, and others for help and encouragement.

I am also indebted to Dr. Marvin H. Stromer (Department of Animal Science, ISU) and Ms. Martha Bushlow for electron micrographs.

I am deeply grateful to my wife, Eun Mi, and my son, Jung Ho, for their love, support, and encouragement. I am also deeply grateful to my grandmother, my mother, my sisters, and my brother in Korea, for their love and encouragement.

Finally, this work is dedicated to my grandfather, Han-Sik Park, and my father, Chang-Kil Park, who died while I have been studying in U.S.A., for showing me the great spirit of sacrifice and the value of education.