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Lipid synthesis and encapsulation by *Cryptococcus curvatus*

by

Diliara R. Iassonova

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Program of Study Committee:
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Chapter 1: Introduction

For humans, diet is the only source of the essential fatty acids. They are precursors of long chain polyunsaturated fatty acids, eicosapentaenoic (EPA) and docosahexaenoic acid (DHA). DHA and EPA consumption is associated with numerous human health benefits including reduced coronary vascular disease, blood pressure, inflammatory and autoimmune disorders. Fish oil and algae are the two major sources of DHA and EPA. Dietary supplementation of fish oil and algae has some serious problems due to fish oil off-flavor and prompt oxidation of polyunsaturated fatty acids. Feeding dairy cows with fish oil and algae to increase the DHA and EPA in milk had limited success because rumen hydrogenation changes the fatty acid profile from what was fed. Also, feeding dairy cows with polyunsaturated fatty acids caused significant decreases in feed intake, milk and milk fat production.

The rapidly growing biodiesel industry generates crude glycerol as a by-product from the transesterification of the oils. These quantities of glycerol have glutted the glycerol market. There is an immediate need for innovative methods for crude glycerol utilization into value-added product to increase biodiesel production efficiency and costs.

The oleaginous yeast *Cryptococcus curvatus*, previously known as *Apiotrichum curvatum* ATCC 20509 and *Candida curvata*, was discovered at Iowa State University in 1978. This yeast was reported as an efficient oil producer and easy to grow with minimal nutritional requirements. An important *C.curvatus* characteristic is ability to utilize a wide range of substrates including glycerol and oils and convert them into lipids that are stored as intracellular triacylglycerols. This ability to encapsulate fed fatty acids may have significant uses as a means to protect long chain polyunsaturated fatty acids, which are prone to oxidation. Thus, microbial encapsulation of polyunsaturated fatty acids into *Cryptococcus curvatus* may be a solution for the off-flavor, oxidation, and biohydrogenation problems. Moreover, there is a potential that crude glycerol could be converted into oil by *Cryptococcus curvatus* and further transesterified into biodiesel.

Objectives

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The goals of this study were to encapsulate polyunsaturated fatty acids into *Cryptococcus curvatus*, understand effect of encapsulation on oxidative stability and rumen biohydrogenation of the oil, investigate the potential of using encapsulated fish oil as a dietary supplement for dairy cows, and to develop a fermentation method for biodiesel industry-derived glycerol utilization. To achieve these goals, *C. curvatus* grown in batch and fed-batch fermentation techniques for oil encapsulation were compared. The protective effect of encapsulation on oxidative stability by forced oxidation test and rumen biohydrogenation by *in vitro* study with rumen fluid system was determined. A pilot feeding trial was conducted with lactating dairy cows. Fed-batch fermentation procedures were developed for fish oil encapsulation and for *de novo* oil synthesis from biodiesel industry-derived glycerol.

Dissertation organization

This dissertation is divided into eight chapters. Chapter 2 contains literature review of the concerning topics of oleaginous microorganisms, health implications of omega-3 fatty acids, rumen biohydrogenation and biodiesel industry-derived glycerol glut problem. The following four chapters (Chapters 3-6) are largely independent, with each discussing the results from different experiments. In particular, Chapter 3 discusses the oxidative stability of encapsulated oils, Chapter 4 focuses on the encapsulation effect on the rumen biohydrogenation, and Chapter 5 reports the results from feeding trial. Chapter 6 suggests the scale up procedure for fish oil encapsulation. Chapter 7 includes the results obtained using the biodiesel industry derived glycerol for single cell oil synthesis by *Cryptococcus curvatus* and Chapter 8 presents general conclusions.

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Chapter 2: Literature Review

Oleaginous Microorganisms

Oleaginous microorganisms have been studied since the mid Nineteen Century (Ratledge and Wynn, 1997). They have been defined as microorganisms that are capable of accumulating at least 20% of their biomass as lipid. Oleaginicity is a relatively rare characteristic; less than 100 species of yeast, mold and algae are oleaginous (Ratledge and Evans, 1989; Ratledge, 1988, 1997, 2006). Each oleaginous microorganism has a different maximal amount of lipid that it is able to accumulate (Table 1a-c). Some organisms can only accumulate 25% on dry weight basis, whereas some have the ability to produce 50% lipids and a few are capable of accumulating 70-80% lipids (Ratledge, 1991). Oleaginous microorganisms produce and accumulate lipids under special conditions: exhaustion of an essential ingredient, and usually a nitrogen source, although mineral depletion has been shown to cause lipid accumulation in some organisms (Ratledge, 1984; 1988). During the *tropophase* or growth phase, all essential nutrients are available for the growth and little lipid accumulates. When nitrogen becomes limiting, but carbon sources are still abundant, the microorganism experiences an unbalanced conditions known as *idiophase.* During *idiophase,* proliferation stops because of lack of nitrogen (critical for nucleic acids and protein synthesis), but cells continue to consume available carbon, and synthesize and accumulate lipids as a reserve storage material (Ratledge, 2006). Cells can use this lipid reserve for energy and as a carbon source when environmental conditions are not favorable and other carbon sources are not available. Oleaginous yeast, mold, and algae store lipid as triacylglycerols (Hammond et al, 1981; Bigogno et al., 2002; Dyal et al., 2005).

Some bacteria are known to store reserve materials such as poly-β-hydroxybutyrate , and poly-β-hydroxyalkanoates (Chien et al., 2007; Catalán et al., 2007; Jiang et al., 2008). Non-oleaginous organisms are not able to store lipids under any conditions (Botham and Ratledge, 1979; Ratledge and Wynn, 2002). Under nitrogen limiting conditions, non-oleaginous organisms cease cell proliferation and division and may store polysaccharides (Sutherland, 1999). Oleaginous organisms have an enzyme, adenosine triphosphate:citrate lyase, and a

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way to produce acetyl-coenzyme A (CoA), which are necessary for fatty acid biosynthesis that non oleaginous organisms do not have (Ratledge, 2006).

Biochemistry of oil synthesis in oleaginous organisms

C. Ratledge intensively studied the biochemistry of oleaginous organisms for 30 years. In 1979, Botham and Ratledge compared the non-oleaginous yeast *Candida utilis* and oleaginous yeast *Candida 107.* They found that the yeasts similarly assimilated glucose, before and after nitrogen limitation, and had equal acetyl-CoA carboxylase activities. But in the oleaginous *Candida 107,* the amount of adenosine monophosphate (AMP) decreased under nitrogen-limiting conditions to 5% of its value under carbon-limited conditions. In non-oleaginous yeast, AMP concentration variation was small in the presence of nitrogen limitation. In 1979, Botham and Ratledge proposed two reasons for oleoginicity:

1) the ability to produce a continuous supply of acetyl-CoA to the cell cytosol;
2) the ability to produce sufficient supply of nicotinamide adenine dinucleotidephosphate (NADPH).

In oleaginous organisms, the cytosolic enzyme adenosine triphosphate (ATP):citrate lyase is responsible for acetyl-CoA formation:

Citric Acid + CoA + ATP→ acetyl-CoA + oxaloacetate + ADP + P_i **(1)**

Citric acid synthesis takes place in the mitochondriaof the cell and acetyl-CoA production (Reaction (1)) occurs in the cytosol, where fatty acid synthesis takes place. Citrate is transported from mitochondrion to the cytosol by citrate/malate translocase (Evans et al., 1983a, b). The difference between non-oleaginous and oleaginous microorganism is that, in the cytosol of the oleaginous microorganisms, the activity of isocitrate dehydrogenase is dependent on the AMP concentration, but, in the nonoleaginous microorganisms, AMP concentration has no effect on the isocitrate dehydrogenase activity. In oleaginous microorganisms, the amount of AMP is controlled by the activity of AMP deaminase:

AMP → inosine 5'-monophosphate + NH_3 **(2)**

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Reaction (2) shows that AMP deaminase is an enzyme that is regulated by the nitrogen concentration. Thus, nitrogen limitation initiates series of reactions (Ratledge, 2004) that start with increased activity of AMP deaminase (Evans and Ratledge, 1985; Wynn et al., 2001). Next, a decreasing amount of AMP occurs in the cytosol and in mitochondria. A low content of AMP in the mitochondria stops isocitrate dehydrogenase, because in oleaginous organisms isocitrate dehydrogenase activity depends on the AMP concentration (Evans et al., 1983ab). The deactivation of the isocitrate dehydrogenase slows down or stops the conversion of isocitrate to α-ketoglutarate. Isocitrate in the mithochondrion rapidly converts into citrate via aconitase, and low concentrations of isocitrate are found in the mitochondria (Evans et al., 1983a). As a result of the citrate concentration increases in mitochondria, citrate enters the cytosol in exchange for malate via citrate/malate translocase. In the cytosol, citrate is cleaved by adenosine triphosphate:citrate lyase to acetyl-CoA and oxaloacetate (Reaction (1)) and acetyl-CoA participates in fatty acid biosynthesis. Oxaloacetate is transformed to malate by malate dehydrogenase, and the malate is used in the citrate transport system (Figure 1).

The reductant NADPH is essential for fatty acid synthesis. The malic enzyme is considered a major NADPH generator:

$$\textbf{Malate} + \textbf{NADP}^+ \rightarrow \textbf{piruvate} + \textbf{CO}_2 + \textbf{NADPH} \qquad \textbf{(3)}$$

Malic enzyme activity is found in a majority of oleaginous organisms, but the possibility of an alternative NADPH-producing enzyme has also been suggested (Ratledge, 2004).

Thus, in oleaginous organisms lipid biosynthesis begins with adenosine triphosphate:citrate lyase that provides a high concentration of acetyl-CoA, and then fatty acid synthesis proceeds as in non-oleaginous yeasts via fatty acid synthetase (Figure 2). Acyl carrier protein (ACP) is a domain of the multifunctional fungal fatty acid synthase system. ACP shuttles between enzymatic centers of the fatty acid synthesis cycle to deliver intermediate reaction products that are covalently attached to its prosthetic group (Leinbundgut et al., 2007). After seven complete cycles, palmitoyl-ACP may be elongated via palmitate elongase or modified by additional enzymatic changes (Ratledge, 1988).

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Synthesized long-chain acyl CoA esters are esterified to glycerol to form various lipids. Oleaginous yeast accumulate 90% of total lipids as triacylglycerols (Hammond et al, 1981; Ratledge, 1988).

The oleaginous yeast, *Cryptococcus curvatus* previously known as *Apiotrichum curvatum* ATCC 20509 and *Candida curvata*, was discovered at Iowa State University (Moon and Hammond, 1978). Four yeast strains were isolated from dairy drain that had ability to ferment lactose from cheese whey permeate and accumulate significant amount of lipids (Hammond et al, 1981). An important *C. curvatus* characteristic is the ability to utilize a wide range of substrates. *C. curvatus* growth was evaluated on various monosaccharides, disaccharides, refined glycerol, ethanol, fatty acids, various oils, and agricultural wastes (Glatz et al., 1984; Glatz et al., 1985; Lee et al., 1992; Meesters et al., 1996). *C. curvatus* grows well while using all the available whey nitrogen and carbon. Then, the yeast begins to convert the carbon into intracellular oil and typically ends with 50-73% oil on a dry weight basis. The oil produced on lactose has a fatty acid composition similar to that of palm oil. Carbon source and its concentration have been shown to affect lipid accumulation. Carbohydrate source does not have significant influence on fatty acid composition of the yeast oil (Ratledge and Evans, 1989), but amount of total lipid accumulated by yeast cell depends on substrate (Evans and Ratledge, 1984).

Omega-3 (ω-3) fatty acids

Eicosapentaenoic acid (EPA, 20:5; Δ5,8,11,14,17; 20:5 n-3), docosahexaenoic acid (DHA, 22:6 ; Δ4, 7,10,13,16,19) and *α*-linolenic acid (ALA, 18:3; Δ9,12,15) are members of the omega-3 polyunsaturated fatty acids (PUFA) family. They are defined by the double bond on the third carbon atom from the methyl end of the molecule. Linoleic acid and ALA are considered essential fatty acids because humans lack the ability to synthesize them and must obtain them from diet. Humans can synthesize DHA and EPA from ALA by a series desaturations and elongations (Figure 3); however, the conversion seems to be very poor. The ALA to DHA conversion efficiency is in the range of 0.1 to 8% (Pawlosky et al., 2001; Burdge and Wootton, 2002; Francois et al., 2003; Hussein et al., 2005). The conversion of ALA to DHA is higher in women and increased during pregnancy (Burdge and Wootton,

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2002). Fish oil, seafood, fungi, and marine algae are the major sources of DHA and EPA (Shahidi and Senanayake, 2006). Canola, soybean, linseed, and walnut oils are the major dietary sources of ALA. Linseed oil contains 45-60% ALA (Shahidi and Senanayake, 2006) whereas the other sources are much poorer.

Omega-3 fatty acids have a wide range of health benefits for humans and animals because of their essential role in several physiological processes; they are part of cell membranes and directly affect their fluidity. They participate in cell signaling, gene expression, and in eicosanoid metabolism (Garg et al., 2006). Eicosanoids include prostanoids (prostaglandins, prostacyclins, tromboxanes), leukotrienes, and hydroxy fatty acids. They are hormone-like compounds that have diverse effects on cardiovascular, reproductive, respiratory, renal, endocrine, skin, nervous, and immune systems. Prostanoids can be formed in the most tissue, but leukotrienes are generated in blood cells. Prostaglandin E_2 and prostacyclin I_2 are produced in the endothelial cells of blood vessels from arachidonic acid. These compounds have a wide range of functions. Prostaglandins participate in aggregation or disaggregation of platelets, the dilation and constriction of smooth muscle cells, controlling blood pressure, and regulating inflammatory mediation. Prostacyclin I_2 is a vasodilator and prevents blood clot formation. In contrast, thromboxane A_2 that is formed in platelets from arachidonic acid promotes the aggregation of blood platelets, the clotting of blood within blood vessels and inflammatory reactions. Leukotrienes participate in inflammatory response, have a strong effect in bronchoconstriction, and increase vascular permeability.

In 1972, Bang and Dyeberg correlated high intake of omega-3 fatty acids with a low incidence of coronary heart disease in Greenland's west-coast Eskimo population. This work inspired a number of epidemiological studies of the health benefits of oily fish consumption (Newman et al., 1993; Hu et al., 2003; He et al., 2004). These studies showed that consumption of DHA and EPA has anti-arrhythmic effects, reduces cardiovascular mortality, decreases ventricular fibrillation, and increases survival after myocardial infarction (Marchioli, 2001; Calder, 2004; Balk et al., 2006). Friedberg et al. (1998) conducted a metaanalysis to test the effect of fish intake on hypertriglyceridemia in diabetes and concluded that fish oil may be useful in treating dislipidemia in diabetes.

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DHA is essential for proper infant brain development and normal adult brain function (Innis, 2005, 2008). Significant amounts of DHA are found in mammalian central nervous systems, especially in brain grey matter and in the visual elements of the retina (Innis, 2008). Martinez (1992) studied the brain tissues of infants whose deaths were not neurologically related and found that the amount of DHA in brain increased during development in the prenatal and postnatal periods up to at least 2 years of age. Innis and Friesen (2008) reported that infant girls whose mothers had a DHA-deficiency during pregnancy were prone to have below average visual acuity. The amount of DHA in brain and retina can be altered by dietary supplementation with DHA (Innis, 2005). Giusto et al. (2002) studied the aging of brains of rats and reported a significant decrease of PUFA arachidonic (20:4 n-6), adrenic (22:4 n-6), and DHA and an increase in the monounsaturated fatty acids oleic and eicosenoic. These changes were correlated with aging, especially in phosphatidylethanolamine and serine.

Recent studies suggested that nutritional supplementation of omega-3 fatty acids from fish oil may have therapeutic benefits for patients with Alzheimer's disease (Calon et al., 2004; Boudrault et al., 2008). Mamalakis et al. (2006) found an inverse relationship between adipose DHA and depression in adults, a result that agreed with previous epidemiological data. This finding suggested that a low dietary DHA intake is associated with an increased risk for depression in adults. Peet and Horrobin (2002) reported that administration of 1 g/d EPA ethyl ester was effective in treating depression. Several epidemiological, animal, and clinical studies support the use of low-fat diets containing omega-3 fatty acid supplements for preventing the development and progression of prostate and breast cancers (Connolly et al., 1999; Aronson et al., 2001; de Deckere, 1999). Dietary omega-3 fatty acids also are associated with decreasing amounts of plasma interleukins and tumor necrosis factor that may act as free radical scavengers (Fisher et al., 1986; Seljeflot et al., 1999). Omega-3 fatty acids have prominent anti-inflammatory effects that may be mediated by a reduction of the arachidonic acid metabolites leukotriene B_4, and thromboxane A_2. Omega-3 fatty acids consumption has been suggested for prevention of the early stages of inflammatory bowel disease (Belluzzi et al., 2000) and rheumatoid arthritis (Volker and Garg, 1996). There is some evidence that linseed (flaxseed) consumption has a positive effect on renal function for

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patients with lupus nephritis (Clark et al, 2001). EPA has positive therapeutic effect for psoriasis vulgaris (Danno and Sugie, 1998).

Official dietary recommendations for omega-3 (ω-3) fatty acids

The first official nutritional recommendation for regular fish consumption, twice a week, with one meal being fatty fish, was made in 1994 in the United Kingdom (Ackman, 2006). In 2002 and 2003, the American Heart Association released a committee report that recommended "patients without documented chronic heart disease should eat a variety of fish, flaxseed, canola, and soybean oils and walnuts at least once a week; patients with documented chronic heart disease should consume 1 g of EPA+DHA per day, preferably from oily fish; EPA+DHA supplements could be considered with the physician. Patients, that have high values of low density lipoprotein fraction in blood, should consume 2-4 g of DHA+EPA per day provided as capsules under a physician care" (Ackman, 2006).

In 2004 the U.S. Food & Drug Administration released a qualified health claim: "Consumption of omega-3 fatty acids may reduce the risk of coronary heart disease." FDA evaluated the data and determined that, although there is scientific evidence supporting the claim, the evidence is not conclusive" (US Food and Drug Administration 2004). In 2005 the United Kingdom, health claim was approved by the Joint Health Claims Initiative that said: "Eating 3 g weekly, or 0.45 g daily, of long-chain n-3 PUFA, as part of a healthy lifestyle, helps maintain heart health" (Joint Health Claims Initiative, 2005).

Encapsulation

Despite numerous health benefits of fish and linseed oils, their food application, production, and transportation entails significant problems because of the oxidative instability of PUFA. Frankel (2005) theorized that DHA oxidation is 50 times faster than that of oleic acid.

Encapsulation of oils rich in omega-3 fatty acids was suggested as a method for protection against oxidation (Garg et al., 2006). Several technologies for the encapsulation of fish oil have been suggested (Klinkersorn et al., 2006). The encapsulation process begins with the formation of fish oil emulsions with a single or a mixture of emulsifiers such as proteins,

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polysaccharides, or lecithin. The emulsions are spray-dried to form microcapsules. Encapsulation by double emulsification followed by enzymic gelation with microbial transglutaminase cross-linked proteins has also been suggested for protein-based microcapsulation of fish oils (Cho et al., 2003). Freezing fish oil emulsions results in better stability of the oil and its flavor (Klinkersorn et al., 2005). These technologies have several disadvantages: 1) the oil portion of the microcapsules is low, from 1-30% (Garg et al., 2006); so, only a small amount of omega-3 fatty acids can be incorporated, 2) oil that leaks out during the spray-drying process oxidizes and affects the flavor; and 3) microencapsulation can affect bioavailability and digestability of fish oil (Beysseriat et al., 2006).

PUFA supplementation and protection from hydrogenation

There have been numerous attempts to feed ruminant animals with fish and linseed oils to alter fatty acid composition of milk fat (Baer et al., 2001; Moussavi et al., 2007; Ueda et al., 2003; Loor et al., 2005). However, small PUFA increases in milk have been reported with feed refusal and a reduction in milk fat and milk production (Baer et al., 2001; Loor et al., 2005; Murphy et al., 2008). Fish and linseed oils are rapidly oxidized and develop unpleasant off-flavors because of PUFA oxidation. These off-flavors can discourage feed intake. Depression of milk fat and milk production may be caused by toxicity of DHA and EPA for rumen microorganisms, which also would depress biohydrogenation and fiber digestion (Palmquist and Jenkins, 1980; Wąsowska et al., 2006).

A variety of methods have been suggested to protect PUFA in the rumen, including encapsulation of the PUFA in protein by formaldehyde treatment (Ashes et al., 1979), formation of calcium soaps of PUFA (Enjalbert et al., 1997; Moussavi et al., 2007), protection of PUFA inside of algae (Franklin et al., 1999), tannin treatment of flaxseeds (Kronberg et al., 2007), and protein encapsulation (Gulati et al., 2002).

Gulati et al. (1999) reported that when fish oil protected by formaldehyde treatment was used as a dietary supplement for lactating goats, EPA and DHA transfer efficiency into milk fat was 7-8% from consumed amount of omega-3 fatty acids; however, they also reported

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prominent negative effects such as depressed food intake and milk fat production. In addition, formaldehyde treatment of animal feeds is not legal in the United States.

Franklin et al. (1999) used the marine algae, *Schizochytrium sp*, which were protected from rumen biohydrogenation by coating the algae with xylose. While there were higher amounts of CLA, DHA and vaccenic acid in the milk of fed animals, these increases were accompanied by significant decreases in feed intake and milk fat output.

Gulati et al. (2002) successfully fed PUFA-rich oils encapsulated in a matrix of rumen protein to lactating cows. They achieved transfer efficiencies of EPA and DHA as high as 24 and 14%, respectively, from the cows' diet to milk (0.86% and 1.41% in milk fat, respectively) without any negative effects on feed intake or milk fat production.

Kronberg et al. (2007) reported that tannin treatments to flaxseeds protected ALA from rumen hydrogenation *in vitro*; however, there were no increases in ALA or EPA in plasma neutral lipids when steers were feed tannin-treated flaxseeds.

The feeding of calcium salts of PUFA has been suggested as a way to bypass rumen hydrogenation. Studies have shown that, despite protective effect from hydrogenation conferred by calcium salts, milk fat and fat yield depression were still serious problems (Giesy et al., 2002; Bernal-Santos et al., 2003). Enjalbert et al. (1997) reported that calcium salts of rapeseed oil were protected from rumen hydrogenation and did not affect milk production or fat and protein yields. However, Carriquiry et al. (2008) reported evidence of hydrogenation of calcium-protected PUFA and suggested that calcium soaps of fatty acids can be partially metabolized in the rumen. Similarly, Castaneda-Gutierrez et al. (2007) concluded that calcium salts of fish oil do not protect DHA and EPA against hydrogenation, but calcium salts of fish oil prevented negative effects of fish oil on food intake and milk fat yield observed with unprotected fish oil.

Yeast supplementation

A number of viable and dead yeast products are used as dietary supplements for ruminant animals to enhance dry matter intake and overall animal performance (Cole et al., 1992;

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Robinson, 1997; Stella et al., 2007). Possibly, viable yeast in supplement are able to grow in the rumen and produce nutrients that stimulate the growth of rumen bacteria (Newbold et al., 1996; Robinson, 1997). Oxygen is toxic for many cellulolytic bacteria found in the rumen. Possibly, yeast stimulates the growth of rumen bacteria by consuming dissolved oxygen especially at the interface of the rumen bacteria and fiber. Yeast cultures also provide a mixture of micronutrients that have beneficial effect on rumen bacteria, which improves fiber utilization and prevents fiber accumulation in the rumen (Miranda et al., 1996; Chaucheyras-Durand et al., 2008). Viable and dead strains of *Saccharomyces cerevisiae* are the most intensively studied and the most popular yeast supplement added to the diet of ruminant animals (Robinson, 1997. Dawson et al. (1990) compared viable and inactive *S. cerevisiae* cells supplements and concluded that dead cells did not alter the concentrations of cellulolytic bacteria in rumen cultures. *S. cerevisiae* dietary supplementation significantly increased the dry matter intake and milk and milk fat production of ruminant animals (Giger-Reverdin et al., 1996; Stella et al, 2007; Chaucheyras-Durand et al., 2008). A combination of viable *S. cerevisiae* and *Armillariaheimii* (white rot fungi) was reported to increase forage digestibility (Mpofu and Ndlovu, 1994). Cultures of live brewer's yeasts were successfully used as supplement to ruminants (Robinson, 1997). As there are no known negative effects of feeding dead yeast to ruminants, dietary yeast supplementation has a long history and positive image in dairy and cattle industry. However, *C.curvatus* has not been fed to ruminants before. Poultry feeding has shown that the alive *C.curvatus* organism have no negative effects on poultry (Hussein et al., 1996).

Glycerol, a biodiesel industry by product

The US biodiesel industry will produce close to 0.8 billion kg per year by the end of 2009. This amount will be added to 250 million kg of glycerol derived each year from other industries (Kraus, 2008). Biodiesel is produced when triacylglycerols from animal or vegetable oil are converted into methyl esters of fatty acids (biodiesel) and glycerol. Production of 100 kg of biodiesel results in approximately 9 kg of impure low quality glycerol (Figure 4).

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Because of the recent increase in production of biodiesel with a concomitant increase in the amounts of glycerol on the market, the price of glycerol has dropped dramatically. The glycerol consumption distribution between industries is presented in Table 2 (Johnson and Taconi, 2007).

Crude biodiesel-based glycerol contains a variety of impurities including water, free fatty acids and methanol, which make it unsuitable for use in food or cosmetics without substantial and expensive refining, chemical treatment, and fractional vacuum distillation. There is need for the processes that converts large quantities of crude glycerol into valuable products that have large markets. Glycerol can be converted into numerous compounds (Ashby et al., 2005; Dharmadi et al., 2006; Johnson and Taconi, 2007). Glycerol can be converted to hydroxyl acetone by dehydration reaction, and then by a hydrogenation reaction to propylene glycol (Suppes et al., 2005). Propylene glycol, besides its high value (Table 3), has potential to displace toxic ethylene glycol in antifreeze application (Shelley, 2007). Glycerol can be oxidized to produce glyceric acid or tartronic acid, but the process requires platinum and gold as a catalyst to achieve high selectivity and conversion (Garcia et al., 1995; Demirel-Gulen et al., 2005). Glycerol can be used as a substrate in industrial fermentation processes. The most studied technique is biotechnological conversion of glycerol into 1,3-propanediol (Biebl et al., 1999; Deckwer, 1995; Himmi et al., 1999), a high value product with applications in the textile and chemical industries (Zeng and Biebl, 2002). Prokaryotic cells from *Enterobacteriaceae* family, such as *Citrobacter freundii* and *Klebsiella pneumonia*, are able to metabolize glycerol and synthesize 1,3-propanediol (Zeng et al., 1997). By using a fed-batch fermentations, efficiency of 1,3-propanediol production was reported as high as 70.4 g/L for product-tolerant mutants of *Clostridium butyricum* and 70–78 g/L for *Klebsiella pneumonia* (Zeng and Biebl, 2002). Papanikolaou et al. (2008) used batch fermentation and reported 47.1 g/L of 1,3-propanediol yield that have been produced by *Clostridium butyricum* F2b from crude glycerol as a substrate. Glycerol also can be used in fermentations for ethanol and hydrogen and α-amylase production. Ito et al. (2005) reported the maximal hydrogen production rate of 63 mmol/L*h from diluted crude glycerol and ethanol yield of 0.85 mol/mol of glycerol by using *Enterobacter aerogenes* HU-101. Glycerol was used as a only substrate for *Yarrowia lipolytica* for production of biomass and α-amylase (Kim et al.,

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2000). Microbial dihydroxyacetone synthesis is considered more economical than the chemical process (Hekmat et al., 2003). Biotransformation efficiency of glycerol to dihydroxyacetone was improved by overexpression of glycerol dehydrogenase (ORFs *sldAB*) in *Gluconobacter oxydans* DSM 2343 such that 30 g/L of dihydroxyacetone was produced by the overexpression strains compared with 18–25 g/L in control strains when 50 g/L glycerol was supplied (Gatgens et al., 2007). *Yarrowia lipolytica* was suggested as potential producer of citric acid from glycerol (Aiba and Matsuoka, 1979; Papanikolaou P et al., 2002). Imandi et al. (2007) optimized production of citric acid by *Yarrowia lipolytica* by using crude glycerol from biodiesel production; they achieved 77.4 g/L of the citric acid production when 0.27g/L yeast extract, 54.4 g/L glycerol, and 13.7% salt solution were used (Imandi et al., 2007). When *Yarrowia lipolytica* was cultivated with crude glycerol as a substrate in continuous fermentation process for single cell lipid production the maximal lipid production was 1.2 g/L*h with cell lipid content 43% w/w dry weight (Papanikolaou and Aggelis, 2002). Meesters et al. (1996) used refined glycerol when they cultivated *Cryptococcus curvatus*. They reported significant glycerol concentration effect on *Cryptococcus curvatus* growth, achieved biomass production 118 g/L with lipid cell content 25% and suggested *Cryptococcus curvatus* as a potential oleaginous microorganism for crude glycerol conversion into single cell oil.

Despite relatively high value of products from industrial fermentation, they do not have sufficient markets to utilize entire crude glycerol surplus from the biodiesel industry (Table 3). However, oil synthesized *de novo* in microbial cells from glycerol could be further converted to biodiesel. Thus, single cell oil production from crude glycerol could be a solution for glycerol glut problems.

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Table 1a. Oleaginous yeast.

Oleaginous yeast	*Substrate*	*Fat content (% dry mass)*	*Dry mass (g/L)*	*Fermentation*	*Reference*
Candida guilliermondi	glucose	25.0	-	batch	Ratledge, Boulton,1985
Candida paralipolytica	glucose	32.0	-	batch	Ratledge, 1982
Candida sp107	glucose	42.9	9.8	batch	Moreton, 1985
Candida tropicalis	n-alkanes	32.0	20.3	batch	Andreevskaya et al., 1984
Cryptococcus albidus	ethanol	71.1	14	batch	Krylova et al, 1985
Cryptococcus curvatus	crude glycerol	25.0	118.0	fed-batch	Meesters et al., 1996
Cryptococcus curvatus	banana juce	73.7	10.6	batch	Vega et al, 1988
Cryptococcus terricolus	glucose	65.0	21.0	batch	Ratledge, 1982
Endomyces vernalys IFO O114	glucose	30.0	10.4	batch	Ratledge, 1982
Lipomyces lipoferus	glucose	48.0	15.0	batch	Ratledge, 1982
Lipomyces starkeyi	lactose	31.0	10	batch	Ratledge, 1982
Lipomyces starkeyi	ethanol, glucose	54.2	153.0	fed-batch	Yamauchi et al, 1983
Lipomyces starkeyi	glucose	63.0	-	batch	Ratledge, 1982
Lipomyces tetrasporus	glucose	64.0	-	batch	Ratledge, 1982
Rhodosporidium toruloides	glucose	66.0	19.1	batch	Ratledge, 1982
Rhodosporidium toruloides	glucose	30.1	12.3	batch	Moreton, 1987
Rhodosporidium toruloides	glucose	48.0	151.5	fed-batch	Li et al., 2007
Rhodosporidium toruloides	glucose	51.0	10.9	batch	Evans, Ratledge, 1984
Rodotorula glutinis	glucose	40.0	185.0	fed-batch	Pan et al, 1986
Rodotorula glutinis	sucrose	53.0	21.0	batch	Chahal et al., 1979

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Table 1a. Oleaginous yeast (continued).

Oleaginous yeast	*Substrate*	*Fat content (% dry mass)*	*Dry mass (g/L)*	*Fermentation*	*Reference*
Rodotorula glutinis	glucose	71.0	-	-	Ratledge, 1982
Rodotorula gracilis	molasses	40.0	44	-	Ratledge, 1982
Rodotorula gracilis	glucose	60.7	19.1	batch	Sattur, Karanth, 1989
Rodotorula gracilis	glucose	49.8	34	continuous	Choi et al, 1982
Rodotorula gracilis	glucose	66.0	17	batch	Ratledge, 1982
Rodotorula gracilis	ethanol	62.0	15	batch	Ratledge, 1982
Rodotorula gracilis	alkanes	32.0	-	batch	Ratledge, 1982
Rodotorula graminis	glucose	29.0	9.6	batch	Evans, Ratledge, 1984
Rodotorula graminis	glucose	41.0	15.8	batch	Ratledge, 1982
Rodotorula mucilaginosa	glucose, sucrose	28.0	-	-	Ratledge, 1982
Schwanniomyces occidentalis	glucose	23.0	3.3	batch	Guerzoni et al., 1985
Trichosporon cutaneum	glucose	38.0	10.9	batch	Evans, Ratledge, 1984
Trichosporon cutaneum	lactose	45.0	24	batch	Ratledge, 1982
Trichosporon pullulans	glucose	30.0	10.4	batch	Ratledge, 1982
Trigonopsis variabilis	glucose	40	-	-	Ratledge, 1982
Waltomyces lipofer	peat	39.5	7.7	batch	Zalashko et al., 1976
Waltomyces lipofer	glucose	63.5	21.7	batch	Ratledge, 1982
Willopsis saturnus,	glucose	30.0	10.4	batch	Evans, Ratledge, 1984
Yarrowia lipolytica	crude glycerol	43.0	8.1	continuous	Papanikolaou, Aggelis, 2002

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Table 1b. Oleaginous algae.

Oleaginous yeast	*Fat content (% dry mass)*	*Reference*
Shizochytrium limacinum	50.6	Chi et al., 2007
Botryococcus braunii	53	Ratledge, 1982
Chlorella pyrogenosa	72	Ratledge, 1982
Chlorella vulgaris	30	Ratledge, 1982
Chlorosarcinopsis negevensis	32	Ratledge, 1982
Cilindrotheca closterium	27	Ratledge, 1982
Cylindrotheca fusiformis	28	Ratledge, 1982
Desert isolate no. 103	34	Ratledge, 1982
Dunaliella primolecta	54	Ratledge, 1982
Dunaliella salina	47	Ratledge, 1982
Dunaliella tertiolecta	42.4	Hong et al., 2008
Monodus subterraneus	36	Ratledge, 1982
Nitzschia sp.	47	Ratledge, 1982
Pennate marine diatom	35	Ratledge, 1982
Phaeodactylum tricornutum	31	Ratledge, 1982
Radiosphaera negevensis	43	Ratledge, 1982
Scenedesmus sp. 3	25	Ratledge, 1982
Stichococcus bacillaris	32	Ratledge, 1982
Thalassiosira pseudonana	37	Hong et al., 2008
Nannochloris atomus	38.8	Hong et al., 2008
Neochloris oleoabundans	42	Hong et al., 2008

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Table 1c. Oleaginous fungi.

Oleaginous yeast	*Substrate*	*Fat content (% dry mass)*	*Dry mass (g/L)*	*Reference*
Aspergillus nidulans	sucrose	50.0	9.3	Azeem et al, 1999
Absidia spinosa	glucose	28.0	5.5	Ratledge, 1982
Aspergillus fischeri	sucrose	53.0	19.8	Ratledge, 1982
Aspergillus flavus	glucose	28.0	5.3	Ratledge, 1982
Aspergillus minutus	glucose	35.0	4.4	Ratledge, 1982
Aspergillus ochraceus	sucrose	48.0	13	Ratledge, 1982
Aspergillus sydowii	sucrose	42.1	-	Azeem et al, 1999
Aspergillus terreus	starch	24.0	4.4	Ratledge, 1982
Aspergillus terreus	sucrose	57.0	13	Ratledge, 1982
Aspergillus ustus	lactose	28.0	8.7	Ratledge, 1982
Blastomyces dermatitidis	glucose	41.0	-	Ratledge, 1982
Chaetomium globosum	lactose	54.0	-	Ratledge, 1982
Cladosporium herbarium	glucose	29.0	11	Ratledge, 1982
Claviceps purpurea	glucose	31.0	-	Ratledge, 1982
Conidiobolus nanodes	glucose	25.7	9.8	Kendrick, Ratledge, 1992
Cunninghamella elegans	glucose	56.0	-	Ratledge, 1982
Entomorphthora coronata	glucose	45.0	-	Ratledge, 1982
Entomorphthora exitales	glucose	24.6	11.6	Kendrick, Ratledge, 1992
Entomorphthora obscura	glucose	34.0	-	Ratledge, 1982
Entomorphthora thaxteriana	glucose	32.0	-	Ratledge, 1982

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Table 1c. Oleaginous fungi (continued)

Oleaginous yeast	*Substrate*	*Fat content (% dry mass)*	*Dry mass (g/L)*	*Reference*
Entomorphthora virulenta	glucose	26.0	-	Ratledge, 1982
Epidermophyton floccosum	glucose	28.0	-	Ratledge, 1982
Fusarium bulbigenum	glucose	50.0	15	Ratledge, 1982
Fusarium equisetti	sucrose	36.2	-	Azeem et al, 1999
Fusarium graminearum	glucose	31.0	-	Ratledge, 1982
Fusarium lini	glucose	35.0	5.6	Ratledge, 1982
Fusarium lycopersicum	glucose	40.0	9.2	Ratledge, 1982
Fusarium oxysporum	sucrose	51.0	-	Azeem et al, 1999
Gibberella fujikoroi	glucose	48.0	7.8	Ratledge, 1982
Histoplasma capsulatum	glucose	37.0	-	Ratledge, 1982
Histoplasma duboisii	glucose	42.0	-	Ratledge, 1982
Malbranchea pulchella var sulfurea	glucose	25.0	-	Ratledge, 1982
Microsporum canis	glucose	29.0	-	Ratledge, 1982
Microsporum gypseum	glucose	29.0	-	Ratledge, 1982
Mortierella alpina	malt agar	32.0	-	Totani, Oba, 1987
Mortierella alpina	glucose, olive oil	44.0	22.5	Shinmen et al., 1989
Mortierella ramannia	dextrose	54.2	29.0	Dyal et al., 2005
Mortierella vinacea	various	66.0	20	Ratledge, 1982
Mucor albo-ater	glucose	42.0	3	Ratledge, 1982
Entomorphthora thaxteriana	sucrose	36.2	14	Ratledge, 1982

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Table 1c. Oleaginous fungi (continued).

Oleaginous yeast	*Substrate*	*Fat content (% dry mass)*	*Dry mass (g/L)*	*Reference*
Mucor miehei	glucose	25.0	-	Ratledge, 1982
Mucor mucedo	glucose	51.0	5	Ratledge, 1982
Mucor plumbeus	maltose	23.5	-	Sajbidor et al., 1988
Mucor plumbeus	glucose	28.0	5.5	Ratledge, 1982
Mucor pusillus	glucose	26.0	-	Ratledge, 1982
Mucor ramannianus	glucose	56.0	1	Ratledge, 1982
Mucor spinosus	glucose	47.0	3.3	Ratledge, 1982
Myrothecium sp	carbohydrate	30.0	-	Ratledge, 1982
Parietochloris incica	agarose	27.1	5.4	Bigogno et al, 2002
Penicillium gladioli	sucrose	32.0	5.7	Ratledge, 1982
Penicillium javanicum	mango seed	57.1	0.56	Jambhulkar et al., 1986
Penicillium lilacinum	sucrose	47.0	12.4	Ratledge, 1982
Penicillium lilacinum	glucose	56.0	17	Ratledge, 1982
Penicillium soppi	glucose	40.0	12.5	Ratledge, 1982
Penicillium spinulosum	sucrose	64.0	16	Ratledge, 1982
Pythium irregulare	glucose	42.0	-	Ratledge, 1982
Pythium irregulare	sucrose	31.0	-	Ratledge, 1982
Pythium ultimum	glucose	48.0	-	Ratledge, 1982
Rhizopus arrihizus	glucose	49.0	-	Ratledge, 1982
Rhizopus nigricans	lactose	50.0	0.85	Jambhulkar et al., 1986

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Table 1c. Oleaginous fungi (continued).

Oleaginous yeast	*Substrate*	*Fat content (% dry mass)*	*Dry mass (g/L)*	*Reference*
Rhizopus oryzea	glucose	36.0	2.5	Ratledge, 1982
Sclerotium bataticola	glucose	46.0	5	Ratledge, 1982
Stilbella thermophila	glucose	38.0	-	Ratledge, 1982
Tricholoma nudum	glucose	48.0	5.5	Ratledge, 1982
Trichophyton verrucosum	glucose	44.0	-	Ratledge, 1982
Trycomyces blakesleeanus	glucose	33.0	1	Ratledge, 1982
Ustilago zeae	glucose	59.0	3.3	Ratledge, 1982

"-" indicates that data was not reported.

Table 2. Distribution of glycerol consumption in different products and industries (adapted from Johnson and Taconi, 2007)

Industry	*Consumption (%)*
Cosmetics, soaps, pharmaceuticals	26
Alkyd resins	6
Food and drinks	8
Tobacco	4
Cellulose films	3
Polyglycerol esters	12
Esters	11
Paper	1
Resale	17
Other uses	12

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Table 3.Possible valued products that can be made by glycerol reduction or oxidation (adapted from Johnson and Taconi, 2007)

Name	*Chemical formula*	*Chemical structure*	*Price ($/lb)*	*US capacity (MMlbs)*
Glycerol	$C_3H_8O_3$		0.05-0.45	250
Tartronic acid	$C_3H_3O_5$		N/A	N/A
Dihydroxyacetone	$C_3H_6O_3$		2.00	N/A
Mesoxalic acid (Ketomalonic acid)	$C_3H_2O_5$		Likely high	N/A
Glyceraldehydes	$C_3H_5O_3$		N/A	N/A
Glyceric acid	$C_3H_6O_4$		Likely high	N/A
Malonic acid	$C_3H_4O_4$		14	< 1
Hydroxypyruvic acid	$C_3H_4O_4$		High	N/A
Lactic acid	$C_3H_6O_3$		0.70-0.85	< 5
Pyruvic acid	$C_3H_4O_3$		High	Small
Propylene glycol	$C_3H_8O_2$		0.44-1.00	1410
Propionic acid	$C_3H_6O_2$		0.46-0.62	440
Glycidol	$C_3H_6O_2$		> $11,000	N/A
Acrylic acid	$C_3H_5O_2$		0.45-1.01	2880
Propanol	C_3H_8O		0.52	260
Isopropanol	C_3H_8O		0.28-0.49	1965
Acetone	C_3H_6O		0.1325-0.4225	3441
Propylene oxide	C_3H_6O		0.64-0.795	5190
Propionaldehyde	C_3H_6O		0.40	400
Allyl alcohol	C_3H_5O		1.00	60
Acrolein	C_3H_4O		0.64	>250

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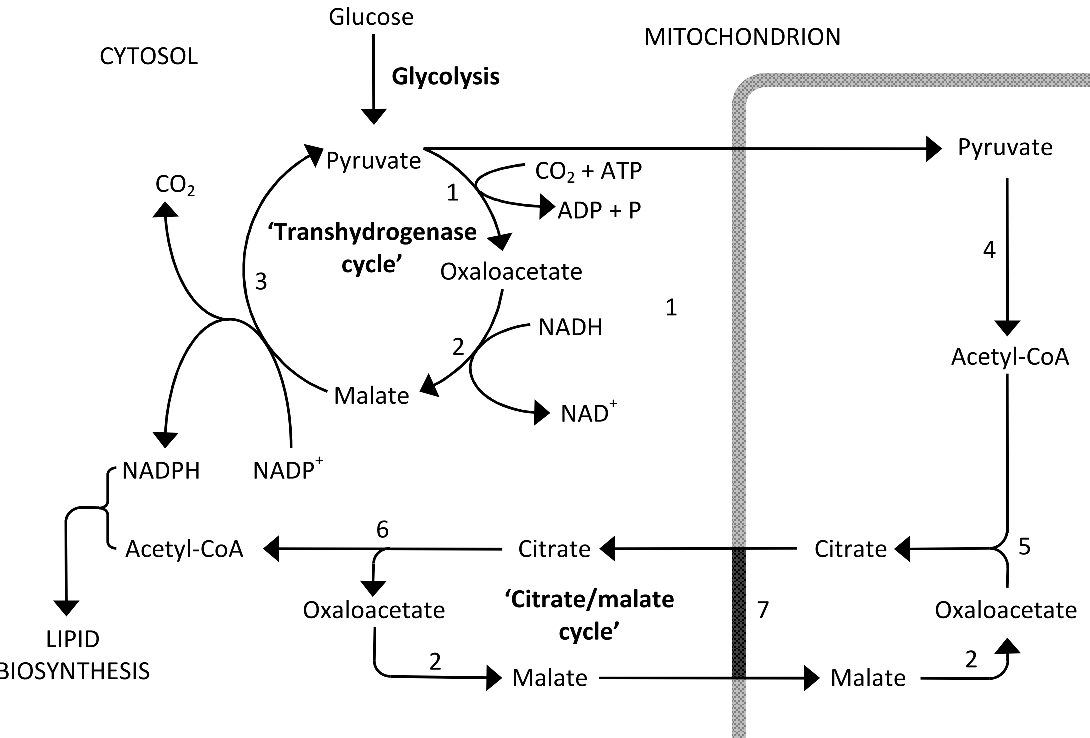

Figure 1. Citrate/malate cycle and the cytosolic 'transhydrogenase' cycle. Enzymes: 1-pyruvate decarboxylase; 2-malate dehydrogenase; 3-malic enzyme; 4-pyruvate dehydrogenase; 5-citrate synthase; 6-ATP:citrate lyase; 7-citrate/malate translocase (Adapted from Wynn et al., 2001).

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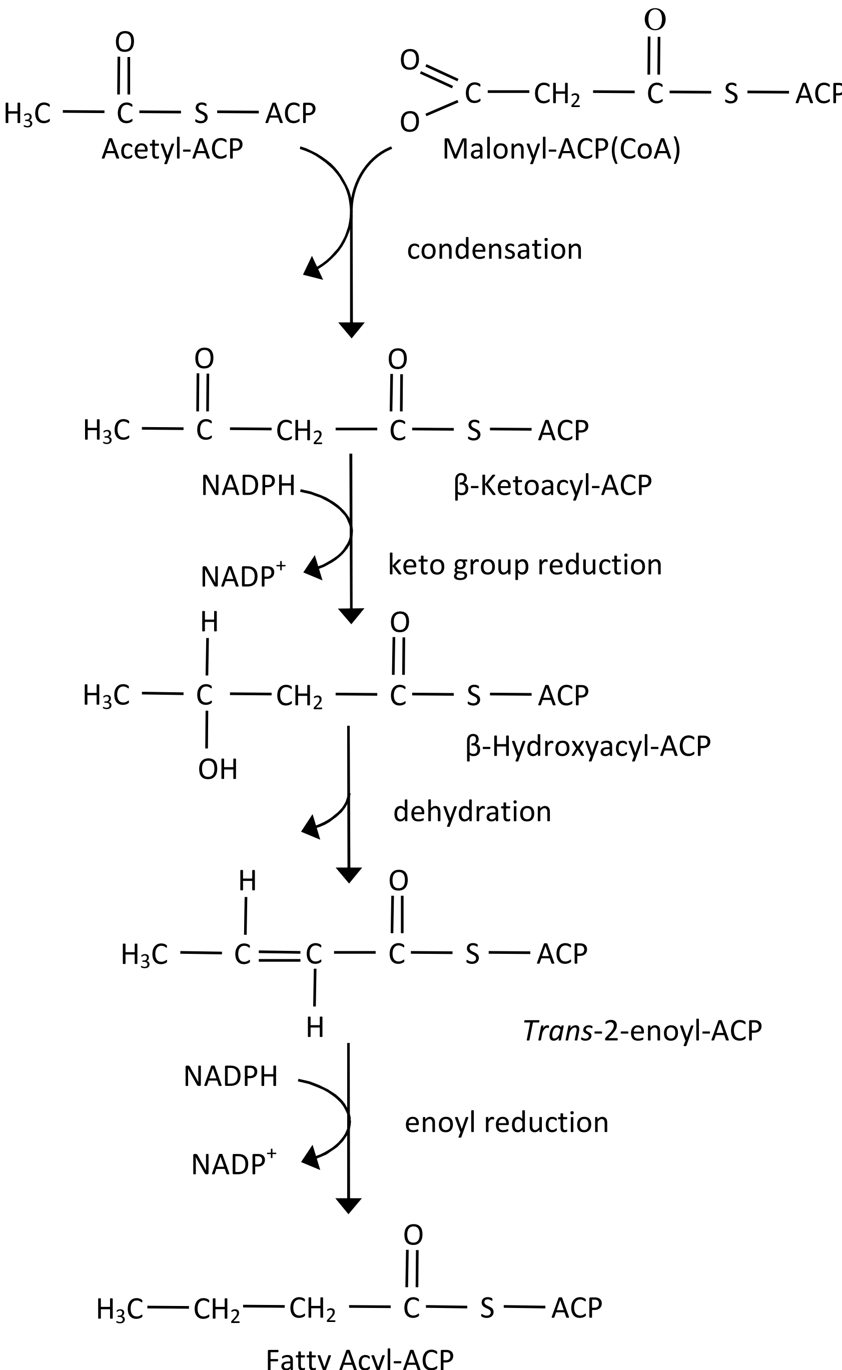

Figure 2. Fatty acid synthesis in animal cells and yeasts (Singh *et al.,* 1985, Chang and Hammes, 1990). ACP - acyl carrier protein.

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H_3C ... COOH
18:3n-3

Δ6-Desaturase → 2H

H_3C ... COOH
18:4n-3

Elongase 2C →

H_3C ... COOH
20:4n-3

Δ5-Desaturase → 2H

H_3C ... COOH
20:5n-3

Elongase 2C →

H_3C ... COOH
22:5n-3

Elongase 2C →

H_3C ... COOH
24:5n-3

Δ6-Desaturase → 2H

H_3C ... COOH
24:6n-3

β-Oxidation → 2C

H_3C ... COOH
22:6n-3

Figure 3. The conversion of ALA into EPA and DHA (adapted from Burdge and Calder, 2005)

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$$\mathrm{CH_2-CH-CH_2}\ (\mathrm{O-C(=O)-R_1},\ \mathrm{O-C(=O)-R_2},\ \mathrm{O-C(=O)-R_3}) + 3CH_3OH \xrightleftharpoons{NaOH} \mathrm{CH_3-O-C(=O)-R_1},\ \mathrm{CH_3-O-C(=O)-R_2},\ \mathrm{CH_3-O-C(=O)-R_3} + \mathrm{CH_2(OH)-CH(OH)-CH_2(OH)}$$

Triglycerides

Methyl esters
(Biodiesel)

Glycerol

Figure 4. Biodiesel production

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Chapter 3: Oxidative Stability of Polyunsaturated Triacylglycerols Encapsulated in Oleaginous Yeast

Modified from a paper published in *The Journal of the American Oil Chemists' Society*

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Abstract

Oleaginous yeast cells have the ability to synthesize oil from carbon sources or to adsorb fatty acids from their growth medium. Fish oil or conjugated linoleic acid (CLA)-rich oils encapsulated in *Cryptococcus curvatus* were protected from oxidation for more than 7 weeks. Oil-containing dead and viable yeast as well as oils extracted from dead or viable yeast were incubated at 52 ºC in the dark. Oils extracted from yeast at the beginning of the experiment began oxidizing almost immediately and exceeded peroxide values (PV) of 20 mequiv/kg within a few days and eventually reaching PV > 100 mequiv/kg. After 56 days of incubation, the PV value of oil from viable cells grown on fish oil was 3.8±0.1 and 5.5±0.8 mequiv/kg from dead cells. After 42 days of incubation, the PV of oil from viable CLA-containing yeast was 1.1±0.2 mequiv/kg and 1.7±0.5 from dead CLA-containing yeast. *C. curvatus* encapsulation significantly improved oxidative stability of long-chain polyunsaturated fatty acids (LCPUFA) and CLA. Yeast cell viability was not critical for oxidative stability of the encapsulated oil.

Introduction

Fish oil is the major dietary source of omega-3 long-chain polyunsaturated fatty acids (LCPUFA) eicosapentaenoic acid (EPA 20:5n-3) and docosahexaenoic acid (DHA 22:6n-3). These fatty acids have important physiological effects in human and animal health. Studies have reported that consumption of EPA and DHA may prevent cardiovascular diseases and some types of cancer (Angerer et al., 2002; Williams and Burdge, 2006), reduce the symptoms in rheumatoid arthritis (Fortin et al., 1995; Hur and Park, 2007) and are essential for the development and function of the brain and retina (Lauritzen et al., 2001).

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Conjugated linoleic acids (CLA) are a family of diene fatty acids that are positional and geometrical-isomers of cis, cis-9,12-octadecadienoic acid (18:2). Numerous health benefits have been associated with CLA, including possible amelioration of carcinogenicity, diabetes, obesity, and atherosclerosis. Moreover, CLA may have stimulating effects on bone formation and the immune system (Chilliard et al., 2000; Belury, 2002; Hur and Park, 2007).

CLA and LCPUFA are very susceptible to oxidation because of their high degree of unsaturation and the positioning of their double bonds. The lack of oxidative stability of LCPUFA is a serious problem that affects their consumption and application in human and animal nutrition. Feed refusal by animals has been noted for fish oils (Donovan et al., 2000) and LCPUFA-containing algae (Franklin et al., 1999). Off-flavors in marine oils, which limit their use, are attributed to both the source and the products of LCPUFA oxidation. Encapsulating LCPUFA helps stabilize these oxidation-prone fatty acids.

Cryptococcus curvatus (*C. curvatus*) is an oleaginous yeast that was first isolated at Iowa State University (Moon and Hammond, 1978). *C. curvatus* grows in cheese whey permeate and converts lactose to fatty acids, which it stores in discrete, inter-cellular droplets as triacylglycerols (TAG). It can accumulate up to 60% of its dry weight as lipids when grown under nitrogen limitation (Park et al, 1991). In addition to converting simple sugars to lipids, *C. curvatus* is able to grow on a variety of fats and oils and deposit them as TAG with approximately the same fatty acid composition found in the substrate (Lee et al, 1992). In the present study, the oxidative stability of yeast-encapsulated polyunsaturated fatty acids from fish oil and CLA-rich oil was determined in dead and viable yeast cells. The hypothesis of this research was that the encapsulation in oleaginous yeast could provide an adequate method for the protection of LCPUFA against oxidation.

Materials and Methods

Culture and culturing conditions

Freeze-dried *C. curvatus* ATCC 20509 (formerly known as *Candida curvata D* and *Apiotrichum curvatum*) was activated by suspension in yeast and mold broth DifcoTM

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(Becton, Dickinson and Company, Sparks, MD, USA) and incubation at 30ºC for 24 h. After plating the culture on Potato Dextrose Agar DifcoTM (Becton, Dickinson and Company, Sparks, MD, USA), a colony was transferred from the plate to a basal medium broth supplemented with carbon and nitrogen sources. The basal medium was KH_2PO_4 2.5 g/L, $MgSO_4 \cdot 7\ H_2O$ 1.0 g/L, $CaCl_2 \cdot 2\ H_2O$ 0.2 g/L, $FeCl_3 \cdot 6\ H_2O$ 0.02 g/L, $MnSO_4 \cdot H_2O$ 0.002 g/L, $ZnSO_4 \cdot 7\ H_2O$ 0.0001 g/L, $CuSO_4 \cdot 5\ H_2O$ 0.0001 g/L, and NaCl 0.6 g/L (Vega et al., 1988). The autoclaved basal medium containing 50 g/L lactose was adjusted to pH 5.4 with 3 N hydrochloric acid solution. Thiamine hydrochloride (0.001 g/L) and asparagine (0.8 gL) were added by sterile filtration. *C. curvatus* was grown in shake flasks at 30 ºC for 24 h with agitation (200 rpm) and transferred three times into fresh medium. The third transfer was used as inoculum for the encapsulation experiments.

Fish oil encapsulation

A 2-L fermenter (Biostat M, B. Braun, Allentown, PA, USA) containing the basal medium and 13.5 g/L lactose was sterilized in the fermenter jar; and sterile asparagine and thiamine hydrochloride were added. A 2% inoculum of a 24-h yeast culture (30 ml/1.5 L) was used. Air flow, dissolved carbon dioxide, temperature, and agitation were monitored. The pH was automatically adjusted to 5.4 by adding 0.5 M sodium hydroxide solution. After 24 h of fermentation, 27 g sterile menhaden fish oil (Omega Protein, Inc., Reedville, VA, USA) containing 27 mg of butylated hydroxyanisole (BHA) was added and the fermentation was continued for an additional 72 h.

CLA encapsulation was done in Fernbach shake flasks containing 1 L of medium similar to that used for fish oil. The flasks were shaken at 200 rpm at 30 ºC. After 24 h of fermentation, 18 g of autoclaved Clarinol TM A-80, containing 38.6% c9, t11-CLA and 35.4% t10, c12-CLA (Loders Croklaan, Wormerveer, The Netherlands) and 18 mg BHA. The fermentation was continued for an additional 72 h.

De novo yeast oil synthesis was achieved under the same conditions except the lactose level initially was 50 g/L, no oil or antioxidants were added and fermentation was continued for 96 h. Growth was monitored by direct microscopic counts using a hemocytometer. Lactose

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utilization was determined by using an assay kit (Boehringer Mannheim/R-Biopharm AG, Darmstadt, Germany).

All fermentations were performed in at least duplicate; de novo synthesis – six fermentations, fish oil encapsulation – two fermentations, CLA encapsulation – two fermentations.

Yeast and oil extraction

Oils were fed to the yeast in excess. Thus, it was necessary to remove excess fish oil or Clarinol TM A-80 from the yeast before the oxidation experiments began. The contents of the fish oil and CLA fermentations were washed with 0.5 L of hexane to remove the unassimilated oil. The hexane layer was recovered and evaporated. Yeast cell were harvested by centrifugation at 9,000 g for 30 min and washed twice with distilled water, and the yeast phase was freeze-dried (Virtis Ultra-35, Gardiner, NY, USA). Lipids were extracted from the wet yeast by sequential ethanol, hexane and benzene extractions according to Hammond et al. (1981). Lipids from the freeze-dried yeast cells were extracted by the methanol-chloroform method (Moon and Hammond, 1978).

Fatty acid composition

Recovered oils were converted to methyl esters with 4% sulfuric acid in methanol at 50 °C overnight. Fatty acid composition was obtained by gas chromatography with a HP 5890 Series II gas chromatograph (Hewlett-Packard Company) with a fused-silica capillary column SP-2423 (60 m x 0.25 mm i.d., 0.20 μm) (Supelco, Inc., Bellefontaine, PA). The carrier gas (helium) flow rate was 1.9 ml/min and the split ratio was 24.8. The column temperature was held at 140 ºC for 6 min, programmed to 220 ºC at 10 ºC/min and held at 220 ºC for 15 min. The injector and detector temperatures were 230 ºC. Quantitative analysis was done using methyl heptadecanoate as an internal standard.

Stability test

To compare the oxidative stability of encapsulated oils in dead and viable yeast, half of the freeze-dried fish oil-encapsulated yeast (FY) and yeast with de novo synthesized oil (LY)

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were killed by autoclaving for 15 min. Half of the CLA-encapsulated yeast cells (CLAY) were killed immediately after fermentation and hexane washing by exposure to 55 ºC for 2 h.

Samples of all the yeast treatments were plated on potato dextrose agar to verify that the thermal treatments had been effective. Oil from the freeze-dried dead and viable yeast was extracted by the methanol–chloroform method (Moon and Hammond, 1978) and incubated at 52 ºC in the dark. Dead and viable yeast cells were also incubated at 52 ºC in the dark, and periodically oil was extracted from the yeast by the methanol–chloroform method. Peroxide values (PV) of oils were determined according to a modified iron oxidation test (Hamm and Hammond, 1967). In this method oil samples are mixed with ammonium thiocyanate and ferrous chloride solution and after 10 min absorbance at 515 nm is measured. PV was calculated based on a standard curve, which was generated by using oils of known PV (standards). The AOCS method Cd 8–53 (Firestone, 1998) was used determine the PV value of the standards used to establish the standard curve.

Statistical analysis

Data from the PV measurements were plotted against time. Data were analyzed by using analysis of variance (ANOVA) with the SAS mixed models procedure. Repeated measures method was used for PV comparisons over time. The level of significance was set at $\alpha = 0.05$.

Results and Discussion

Fermentation results

Cryptococcus curvatus fermentation can be divided into two phases consisting of a 24-h growth phase when all nutrients are abundant followed by a 72-h fattening phase when nitrogen is limiting and the cells biosynthesize or accumulate fat. When *C. curvatus* was grown on media with 50 g/L lactose, yeast-produced biomass until nitrogen was depleted and then residual lactose was converted to oil. Such yeast (LY) had a lipid content of 41.6±3.7% by weight and a dry cell mass of 15.7±0.6 g/L after 4 days of fermentation (Table 1). *C. curvatus* grew faster and produced more biomass when lactose was used as the carbon source compared with fish oil as carbon source. Because of this, lactose was used as the carbon

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source for the growth phase in all experiments. During the growth phase, the biomass production rate was inversely related to the amount of lactose in the media (data not shown). After 24 h of fermentation, dry cell mass was 10.0±1.8 g/L, the yeast oil content was 8.9±0.9% of cell dry weight, and only 0.1±0.3 g/L of the lactose of the initial 13.5 g/L was left in the media. Then, 18 g/L fish oil or Clarinol TM A-80 were added as a carbon source for the lipid accumulation phase where growth ceases while oil content increases. Yeast were able to assimilate or use over 88.8% of the fish oil and 97.2% of Clarinol TM A-80 in their medium as determined by the residual oil in the hexane extractions. Lee et al. (1992) have shown that the yeast changed the positions of the fatty acids on the glycerol backbone suggesting that the yeast hydrolyze and re-esterify the feedstock TAG during oil accumulation. Possibly, some of the fatty acids were used for energy production. The yeast accumulating CLA showed the highest lipid content, 68.9% of cell dry weight. Previous studies reported that *C. curvatus* was able to grow well on TAG and long chain unsaturated fatty acids (Lee et al., 1992, 1993). The fatty acid profile of yeast oils was similar to the substrate oil but with greater percentage of saturated acyl groups and oleate and lower percentage of polyunsaturated fatty acids (Table 2). This change may be caused partially by some oil production from lactose before the medium oils were added. Yeast fermented on lactose only (LY) synthesized oil rich in oleate, palmitate, and stearate (Table 2).

Stability test results

For each of the feedstock oils, there was no difference in the fatty acid profiles of encapsulated oils extracted from dead and viable yeast. Oil extracted from LY on the starting day of the experiment had a PV of 0.7±0.2 mequiv/kg for dead yeast and 0.8±0.1 mequiv/kg for viable yeast. Oil extracted on the 57th day of incubation of LY at 52 ºC had mean PV of 2.0±0.4 mequiv/kg for dead yeast and 1.3±0.5 mequiv/kg for viable yeast (Figure 1). The mean PV of lipid extracted on the starting day from dead and viable LY and stored at 52 ºC for 57 days was 174.4±0.6 and 200.0±0.6 mequiv/kg, respectively (Figure 1). Thus, extracted LY oil oxidized significantly faster than encapsulated LY oil, and viable encapsulated LY oil had slightly lower PV than dead-encapsulated LY oil. Extracted LY oil from viable yeast oxidized faster than did extracted LY oil from dead yeast. Similar to LY oil, extracted FY oil

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oxidized significantly faster than did encapsulated FY oil. Initial PV of oils extracted from FY yeast was 2.1±0.3 mequiv/kg for dead yeast and 1.6±0.2 mequiv/kg for viable yeast (Figure 2). After 28 days of storage at 52 ºC, PV of unprotected oil from viable and dead FY was 180.3±0.6 and 212.7±0.6 mequiv/kg, respectively. Unprotected oils from viable and dead FY had polymerized by the fifth week of the experiment. In contrast to the high oxidation rate of unprotected FY oils, encapsulated FY oils oxidized slowly. After 56 days of incubation at 52 ºC encapsulated viable FY oil had PV equal 3.8±0.1 mequiv/kg and encapsulated dead FY oil had PV equal 5.5±0.8 mequiv/kg (Figure 2).

Because of the limited sample size and initial low peroxide value of oil from CLAY, we measured only the stability of oil encapsulated in the yeast (Figure 3) during a 6-week period. After 42 days of incubation at 52 ºC, the mean PV of oil from viable CLAY did not change significantly compare to its PV at zero time (Figure 3). Oil from dead CLAY had initially a mean PV of 0.9±0.3 mequiv/kg and did not change significantly for 28 days, but at 42 days oil from dead CLAY had a PV of 1.7±0.5 mequiv/kg (Figure 3).

Suzuki et al. (2004) studied oxidative stability of unprotected TAG from bitter gourd oil (61.6% CLA) and CLA oil (69.5% CLA) under conditions similar to ours. They reported that after 4 days of incubation at 50 ºC in the dark bitter gourd oil and CLA oil had a PV over 20 mequiv/kg. CLAY oil (48.7% CLA) contained less CLA than oils in Suzuki et al. (Suzuki et al., 2004) experiments but considering the LY oil stability after 42 days of incubation at 52ºC unprotected CLA oil from yeast likely would have significantly higher PV than 1.7 mequiv/kg.

All encapsulated oils demonstrated impressive oxidative stability during the experimental time. Our result showed that dead *C. curvatus* cells did not lose their ability to protect oil against oxidation compared with live cells. *C. curvatus* has a thick cell wall, and TAGs are stored intracellular in lipid vacuoles that have a membrane (Holdsworth et al., 1988; Park et al., 1991). Cell walls and membranes reduce contact of TAG with oxygen. Also, TAG are stored in lipid bodies that are small droplets separated from each other which helps prevent cascade oxidation. It is unclear whether *C. curvatus* produces an antioxidant or takes up the BHA in the medium. Possibly, oil that leaked out from damaged cells was responsible for

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slow lipid oxidation that took place in encapsulated samples. Heat treatment kills the yeast but cell walls and membranes stayed intact thus their vacuole-stored TAGs continue to be stable to oxidation.

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Table 1. Biomass (g/L) and lipid accumulation (% cell dry weight) of *C. curvatus* grown on various substrates.

Substrate	*Cell dry weight (g/L)*	*Lipid content (% dry wt.)*
50 g/L Lactose (LY)	15.7 ± 0.6	41.6 ± 3.7
13.5 g/L Lactose + 18g/L Fish Oil (FY)	18.4 ± 1.3	49.7 ± 0.8
13.5 g/L Lactose + 18g/L Clarinol™ (CLAY)	16.1 ± 1.0	68.9 ± 0.4

Table 2. Fatty acid composition of feedstock oils and oils extracted from *C. curvatus* grown on various substrates in weight %.

Fatty acids	*Encapsulated oil source*			*Feedstock oil*	
	Lactose de novo synthesis	*Fish oil*	*Clarinol™ A-80*	*Fish oil*	*Clarinol™ A-80*
14:0	0.5	5.5	9.6	6.7	4.4
16:0	27.6	21.3	2.3	16.9	1.5
16:1	0.6	11.0	-	9.6	-
18:0	15.2	3.4	32.1	6.2	17.3
18:1	48.1	27.9	5.9	13.7	2.7
18:2	5.0	4.7	-	3.2	-
18:3	1.0	2.4	-	3.2	-
20:0	0.3	1.2	-	1.4	-
*c*9, *t*11-18:2	-	-	27.4	-	38.6
*t*10, *c*12-18:2	-	-	21.3	-	35.4
20:4	-	3.4	-	4.0	-
20:5	-	10.8	-	16.2	-
22:5	-	1.3	-	3.1	-
22:6	-	5.4	-	14.0	-
minor compounds <1%	1.5	1.4	-	1.3	-

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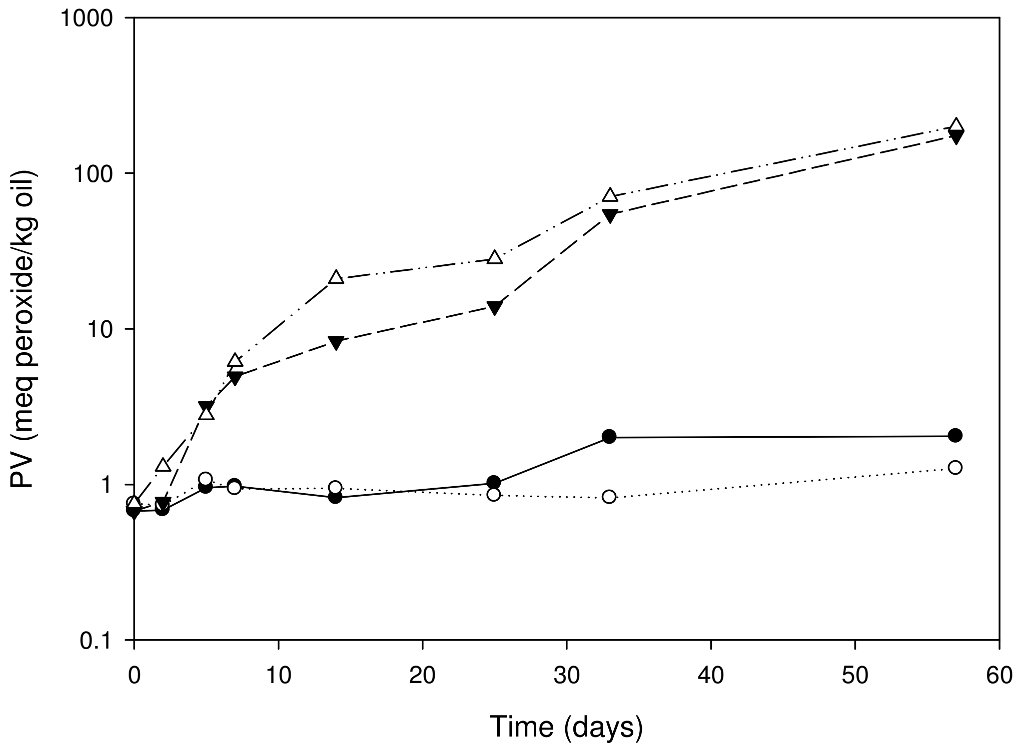

Figure 1. Peroxide values of oils extracted on day 0 and stored at 52 ºC for 57 days from dead (filled triangles) (standard error of the mean (SEM) ± 21.20) and viable (open triangles) (SEM ± 24.12) yeast fermented with 50 g/L lactose (LY), and oils extracted from dead (filled circles) (SEM ± 0.20) and viable (open circles) (SEM ± 0.06) lactose-fermented yeast (LY) on 0, 2, 7, 14, 25, 33, and 57 days of incubation at 52 ºC

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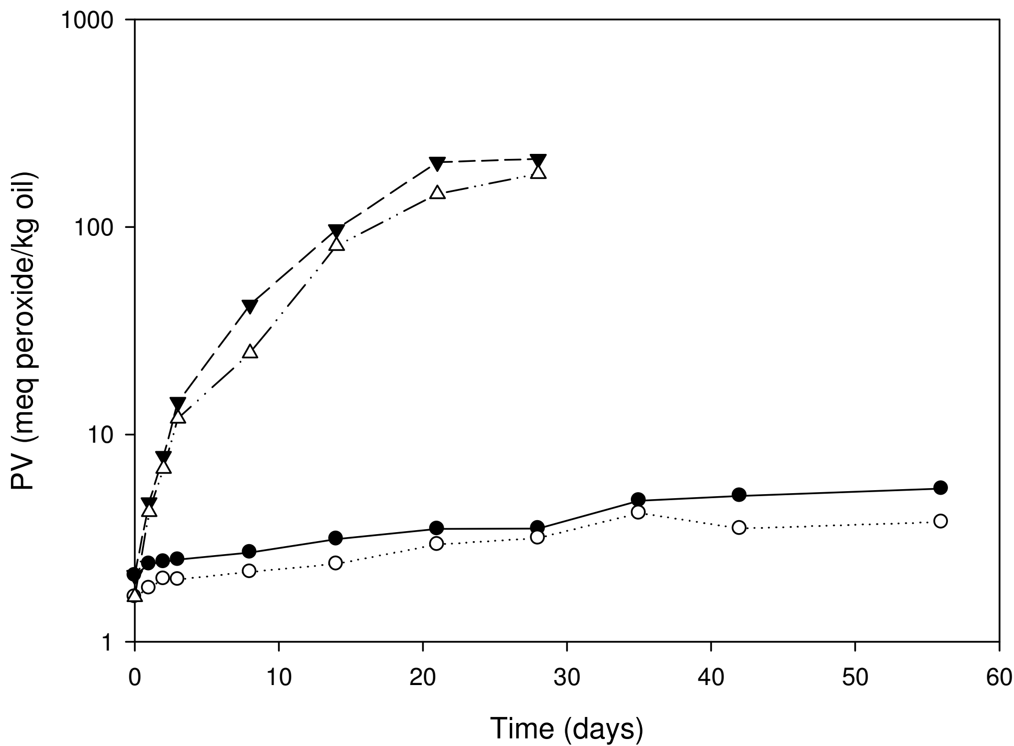

Figure 2. Peroxide values of oils extracted from dead (filled triangles) (SEM ± 25.95) and viable (open triangles) (SEM ± 21.25) yeast with fish oil (FY) on day 0 and stored at 52 ºC for 56 days, and peroxide values of oils extracted from dead (filled circles) (SEM ± 0.36) and viable (open circles) (SEM ± 0.26) yeast with fish oil (FY) on 0, 1, 2, 3, 8, 14, 21, 28, 35, 42, and 56 days of incubation at 52 ºC

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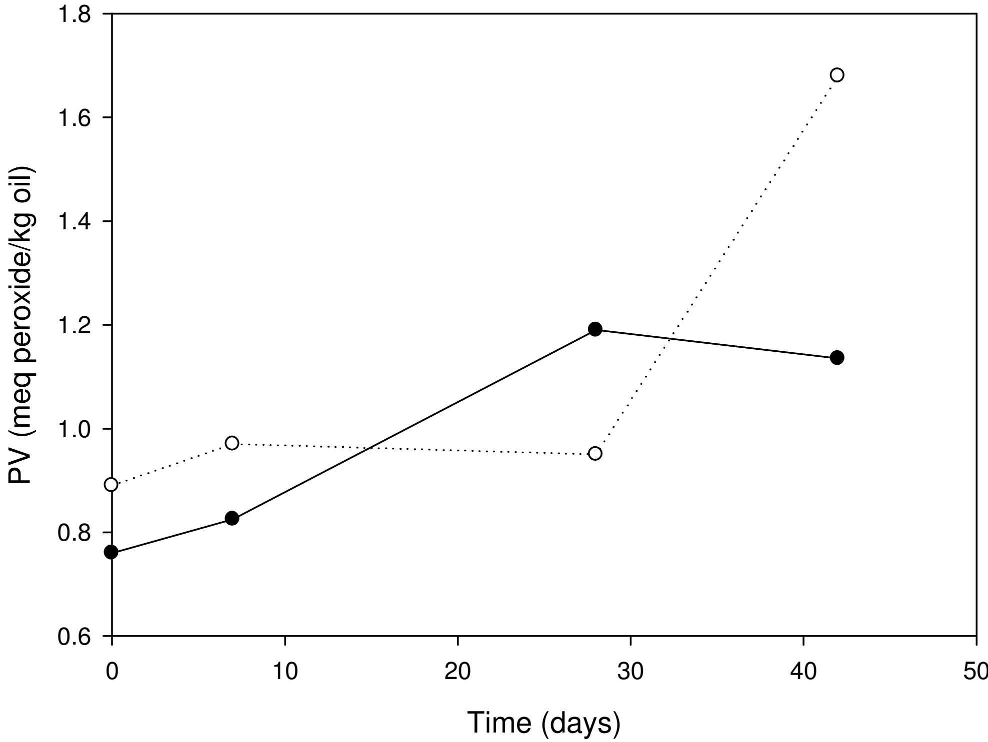

Figure 3. Peroxide values of oils extracted on 0, 7, 28, and 42 days of incubation at 52 ºC from dead (open circles) (SEM ± 0.19) and viable (filled circles) (SEM ± 0.11) yeast encapsulated with Clarinol TM A-80 oil (CLAY)

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Chapter 4: Protection of Polyunsaturated Fatty Acids from Microbial Hydrogenation by Encapsulation in *Cryptoccoccus Curvatus*

A paper to be sumbitted to *The Journal of Food Science*

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Abstract

Dietary unsaturated triacylglycerols are extensively hydrolyzed by rumen lipases and the resulting fatty acids are hydrogenated by rumen bacteria to yield more saturated fatty acids, which are delivered to the small intestine. The objective of this experiment was to compare the survival of various polyunsatured fatty acids (PUFA) that were either unprotected or encapsulated in viable or dead *Cryptococcus curvatus* yeast cells and incubated with anaerobic rumen fluid. Linseed and fish oils were encapsulated separately in yeast and some of the yeast containing encapsulated fish oil were killed with a heat treatment. These treatments along with unprotected linseed and fish oils were incubated with rumen fluid using *in vitro* anaerobic digestions. Lipid was added to the digestions at 50, 100 or 200 mg. Samples were taken at 0, 2, 4, 8, 16 and 24 h, and the fat was extracted, converted to methyl esters and analyzed by gas chromatography. Unsaturated fatty acid concentrations were greater for the lipids protected by encapsulation during exposure to the rumen fluid than for unprotected linseed and fish oils. After 24 h of incubation, 54-82% of protected PUFA remained compared with less than 13% for unprotected fish or linseed oils. Yeast cell viability was not necessary for the stability of the encapsulated fish oil. Encapsulation in yeast was an effective method to partially protect PUFA from rumen hydrogenation.

Introduction

Dietary long-chain omega-3 fatty acids, specifically eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, can contribute various health benefits to humans and animals (Nestel, 1987; Stone, 1996; Ruxton et al., 2004; Wang et al., 2006; Bilby et al., 2006). α-Linolenic acid (ALA) can be elongated to long-chain omega-3 fatty acids by humans (Vermunt et al., 2000; Barcelo-Coblijn et al., 2008). Fish oils and linseed oil are good

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sources of these fatty acids (Kris-Etherton et al., 2003). Many feel that it would be advantageous to raise the amounts of omega-3- fatty acids in milk (Pennington and Davis, 1975; Grummer, 1991; Franklin et al., 1999). Direct supplementation of the diets of ruminant animal with fish and linseed seed oils have resulted in some PUFA increases in milk, but these desirable results are associated with feed refusal and a reduction in milk fat and milk production (Baer et al., 2001; Loor et al., 2005; Murphy et al., 2008). *In vitro* experiments have demonstrated that milk fat from cows supplemented with omega-3 fatty acids was low in PUFA because the PUFA were extensively hydrogenated in the rumen (Enjalbert et al., 2003; Dohme et al., 2003; Troegeler-Meynadier et al., 2003; Carriquiry et al., 2008). As a result, the fatty acids in the milk are predominantly saturated. Dietary linoleic and linolenic acids are hydrogenated almost completely to stearic acid with conjugated linoleic acid (CLA) and trans-vaccenic acid (TVA) as intermediates (Wide and Dawson, 1966; Vossenberg and Joblin, 2003; Jenkins et al., 2008). CLA, which has demonstrated anticarcinogenic and antiatherogenic effects in animals, could be potentially beneficial to human health (Belury, 2002, 2003**).** The desaturation of TVA by the Δ9-desaturase enzyme in animal tissues is the predominant pathway for *cis*-9, *trans*-11 CLA synthesis in ruminant animals **(**Jenkins et al., 2008).

Cryptococcus curvatus is an oleaginous yeast that was first isolated at Iowa State University (Moon et al., 1978). *C. curvatus* grows well in cheese whey permeate and converts lactose into triacylglycerols*,* which the yeast stores as intercellular droplets. *C. curvatus* can accumulate up to 60% of its dry weight as lipids when grown under nitrogen limitation (Park et al., 1991). *C. curvatus* also can grow on fat, which the yeast deposits as triacylglycerols with little modification of the fats unsaturation or chain length of the constituent fatty acids (Lee et al., 1992).

Our hypothesis was that PUFA from fish and linseed oils and encapsulated in *Cryptococcus curvatus* cells would be protected from rumen hydrogenation. To test this hypothesis, we compared the survival of various PUFA encapsulated into viable and dead *C. curvatus* cells with unprotected oil when added to anaerobic rumen fluid at various concentrations. *In vitro*

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methods were used because they have been widely used for the rumen metabolism studies and they are considerably less expensive and time consuming than *in vivo* methods.

Materials and Methods

Culture

Freeze dried *C. curvatus* ATCC 20509 was activated by growing on Difco™ Yeast and Mold Broth (Becton, Dickinson and Company, Sparks, MD, USA) at 30˚C for 24 h. The culture was plated on Difco™ Potato Dextrose Agar. A colony was transferred from the plate to a basal medium supplemented with 50g/L lactose. The basal medium was 2.5 g/L KH_2PO_4, 1.0 g/L $MgSO_4 \cdot 7H_2O$, 0.2 g/L $CaCl_2 \cdot 2H_2O$, 20.0 mg/L $FeCl_3 \cdot 6H_2O$, 2.0 mg/L $MnSO_4 \cdot H_2O$, 1.0 mg/L $ZnSO_4 \cdot 7H_2O$, 0.1 mg/L $CuSO_4 \cdot 5H_2O$, and 60 mg/L NaCl (Vega et al, 1988). The autoclaved synthetic medium was adjusted to pH 5.4 with 3 N hydrochloric acid solution. Thiamine hydrochloride (1.0 mg/L) and asparagine (0.8 g/L) were added to the synthetic medium by sterile filtration. *C. curvatus* was grown in shake flasks at 30˚C for 24 h with agitation (200 rpm) and transferred three times into fresh medium. The third transfer was used as inoculum for the encapsulation experiments.

Fermenter

A 20-L Bioflo 4500 Fermenter/Bioreactor *(New Brunswick Scientific,* Edison, NJ*)* equipped with air flow, temperature, and agitation control was used. The pH was controlled with 0.5 M sodium hydroxide and 0.5 N hydrochloric acid solutions. Dissolved oxygen was monitored, and 1-2 mL silicone antifoam 204 (Sigma-Aldrich, Saint Louis, MI) was added as needed.

The synthetic medium with 1.35% lactose and without asparagine and thiamine was sterilized in the fermenter jar. Asparagine and thiamine were added by sterile filtration to the sterilized, cooled basal media. The medium was adjusted to pH 5.4 and 30.0°C, and saturated with air at 20 L/min, and 400 mL of *C. curvatus* inoculum was added. After 20 h of fermentation the air rate was decreased to 5 L/min, and 360 g sterile menhaden fish oil (Omega Protein, Inc, Reedville, VA, USA) or linseed oil (Rexall, Inc., Boca Raton, FL) was

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added at 0.06 ml/min. Both oils were supplemented with 0.36 g butylated hydroxy anisole (BHA). The oil accumulation continued for 216 h. Sterile samples were taken at 0, 1, 2, 4, 5, 6, 12, 24 h and then daily in duplicates. Lactose utilization was measured by lactose assay (Boehringer Mannheim/R-Biopharm AG, Darmstadt, Germany), and direct microscopic cell counts were performed with a hemocytometer. Two fermentations with linseed oil encapsulation and four fermentations with fish oil encapsulation were performed for the *in vitro* experiments.

Yeast and oil extraction

For the oil fermentations, the content of the fermenter jar was washed with 2 L hexane to remove the unassimilated oil. The upper, hexane layer was recovered and evaporated. Yeast cell were harvested by centrifugation at 9000 g for 30 min and washed twice with distilled water. Lipid was extracted by successive ethanol, hexane, and benzene extractions according to Hammond et al. (1981). Recovered oils were converted to methyl esters with 4% sulfuric acid in methanol at 90°C for 90 min for fatty acid composition analysis (Selivonchik, 1977). Fatty acid composition was obtained by gas chromatography with a HP 5890 Series II gas chromatograph (Hewlett-Packer Company, Avondale, PA) with a fused-silica capillary SP-2423 column (60 m x 0.25 mm i.d., 0.20 μm) (Supelco, Inc., Bellefonter, PA). The carrier gas helium at a flow rate of 1.9 mL/min, and the split ratio was 24.8. The column temperature was held at 140°C for 6 min, programmed to 220°C at 10°C/min and held at 220°C for 26 min. The injector and detector temperatures were 230°C. Quantitative analysis was done using heptadecanoic acid as an internal standard.

In vitro experiments

Cows (n=2) with a rumen fistulas supplied rumen fluid. Rumen fluids were collected before the morning meal, and 1 L was filtered through 4 layers of cheese cloth and mixed with 37.5 g of ground hay and 1 L of buffer solution containing 9.24 g/L $NaHCO_3$, 19.5 g/L $Na_2HPO_4.2H_2O$, 0.705 g/L NaCl, 0.675 g/L KCl, 0.108 g/L $CaCl_2.2H_2O$, and 0.18 g/L $MgSO_4.7H_2O$ (Enjalbert et al., 2003) pre warmed to 39°C, and saturated with CO_2 (pH 6.9). The 50:50 mixture of rumen fluid and buffer solution (RFB) was brought to the laboratory

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within 1 h after fluid collection in a thermos bottle. The RFB was incubated with linseed oil encapsulated in viable yeast (LY), fish oil (FO) encapsulated in both viable (FY) and dead yeast (dFY), and unprotected linseed oil (LO) and FO and at three concentrations of oil for the each source 50, 100 and 200 mg/20 mL RFB. The fatty acid composition of the oil sources are presented in Table 1. Wet yeast encapsulated with FO from two fermentations were killed after harvesting and hexane washing by exposure to 55˚C for 2 h. Samples of the dead yeast were plated on potato dextrose agar to verify that the thermal treatments had been effective. A control RFB without any added oil source was also prepared. Tubes with 20 mL of RFB and oil source were gassed with N_2 before closing and placed in a shaker at 39°C and 130 rpm. Six tubes for each variable were prepared. Incubation was stopped by placing the tubes in freezer after 0, 2, 4, 8, 16 and 24 h of incubation. The frozen samples were freeze-dried (Virtis Ultra-35, Gardiner, NY, USA), and the lipid was extracted by consecutive treatment with ethanol, hexane and benzene according to Hammond et al. (1981). Recovered oils were converted to methyl esters with 4% sulfuric acid in methanol at 90°C for 90 min for fatty acid composition analysis. Methyl esters retention times, including 11-trans-vaccenic acid and *cis*-9, *trans*-11-CLA, were identified by commercial standards (Nu-Check Prep, Inc, MN, USA).

Statistical analysis

Data were analyzed by a analysis of variance (ANOVA) with the SAS 9.1 (SAS Institute, Cary, NC) general linear model procedure for randomized block design. The statistical model included fixed effects encapsulation, fat content, hr of incubation and their interactions, where vial effect was considered as a block. Differences were considered significant when $P<0.05$.

Results

PUFA encapsulation

C. curvatus was grown in a 20-L fermenter in two stages. First, to achieve maximum biomass, *C. curvatus* was grown in a media with 1.35% (w/w) lactose and 0.8 g/L asparagine

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until the biomass reached a maximum and the lactose was completely utilized, but little fat was deposited. The amount of lactose as well as dissolved oxygen sharply decreased during the first 14 h to 20.5% for lactose and 2.8% for dissolved oxygen. Subsequently, the amount of lactose slowly decreased to 1.3% and dissolved oxygen increased to 80% by 24 h. Cell number reached a maximum at 13 h and remained constant. Next a lipid substrate was added, and lipid accumulation was allowed for 9 days. During the "lipid accumulation" stage, *C. curvatus* accumulated lipids slowly. The final dry yeast yield and lipid production are reported in Table 1. Yeast with encapsulated linseed seed oil (LY) and fish oil (FY) accumulated 33.1 and 34.8% of oil, respectively. The fatty acid profile of the yeast oils was similar to that of the substrate oil but the yeast oil was higher in saturated and oleic acid and lower in polyunsaturated fatty acids (Table 1).

Experiments with linseed oil and encapsulated linseed oil in viable yeast.

Table 2 shows experiments with LO and with LY after 24 h of incubation in RFB. All the fatty acids were regarded as 100 at zero time; so, values greater than 100 indicate an increase while those less than 100 indicate disappearance. Saturated fatty acids increase and polyunsaturated fatty acid decrease because of hydrogenation. Monounsaturated fatty acids can increase or decrease depending on circumstances. The amount of palmitate increased during the 24-hour incubation. The biggest changes occurred during the first 4 h. Samples with LO demonstrated a significantly higher palmitate increase than samples with LY ($p < 0.05$). There also was a significant effect of the amount of added oil. For LO, there was an increase of palmitate with the amount of oil added to the incubation. For LY there were smaller differences with fat load, and the lowest oil level yielded the most palmitate at 24 h.

The increase in amount of stearate in samples with LO was significantly greater than in LY samples at 24 h (Table 2) ($p < 0.05$). There was no effect for stearate for the amounts of oil added at zero time ($p > 0.05$). Similar to stearate, an increase in the amount of oleate in samples with LO was greater than in LY samples at 24 h (Table 2) ($p < 0.05$). There also was effect of the amounts of fat added (Table 2) ($p < 0.05$).

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LO and LY samples differed significantly in the amount of linoleate remaining after 24-h incubation (Table 2). Samples with 50 mg and 200 mg LO had less than 27% of the initial amount of linoleate remained after 8 h and less than 26% from initial amount at 24 h of incubation (Figure 1). In the experiment with 100 mg LO added only 52.1% remained at 8 h and 35.4% at 24 h. In the experiments with LY, the amount of linoleate for all these added fat concentrations decreased gradually during incubation. After 8 h of incubation 73-79% from initial amounts of linoleate were recovered, and after 24 h of incubation 46-81% linoleate remained in the samples (Table 2).

The amounts of ALA remaining in the samples were significantly different for LO compared with LY (Figure 1) ($p< 0.05$). There was no significant effect of the amount of added fat on ALA amounts, and all fat levels behaved similarly ($p > 0.05$). In LO experiments, only 11-15% of the initial amount of ALA remained after 8 h of incubation and at 24 h less than 14.3% remained. In experiments with LY the amount ALA decreased gradually during incubation. After 8 h of incubation 54-79% of the initial amount of ALA remained, and after 24 h of incubation 44-51% of the initial remained (Figure 1).

The amount of TVA increased over time from 0 at zero time to several mg/g lipid at 24 h (Figure 2). The highest amount of TVA was found in samples with 200 mg LY and 200 mg LO. The amount of CLA increased significantly with time of incubation and the amount increased significantly with added oil load. Samples with LY produced less CLA formation compared to samples with LO as might be expected if encapsulation protects the oil from hydrogenation (Figure 2).

Experiments with fish oil and encapsulated fish oil in viable and dead yeast

In experiments with FO, the amount of palmitate produced during 24-h incubation was higher than in samples with FY (Table 3). After 24-h incubation 50, 100, and 200 mg FO samples produced 117, 146, and 143% of palmitate. Correspondingly, with similar amount of oil added as FY, the yields of palmitate were 108%, 104%, and 139%.

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All FO and FY samples demonstrate decreases in palmitoleate during the first 8 h of incubation, but, in later hours of incubation, the amounts of palmitoleate in 100 and 200 mg samples the palmitate increased to 96 and 104% at 24 h of incubation (Table 3) while the amount of the 50 mg FO and all the FY samples continued to decrease.

Similar to the results with LO and LY, the amount of stearate produced during incubation was much greater for FO than for FY samples (Table 3). FY samples demonstrated slow and gradual changes in amount of stearate.

In contrast to the LO and LY experiments, the amounts of oleate in FO and FY samples significantly decreased during incubation. The two higher concentrations of FO samples decrease rapidly in oleate during the first 8 h of incubation. FY samples gradually decreased in their amount of oleate during incubation and remained at 77-99% at 24 h of incubation (Table 3).

During incubation FO samples lost significantly more linoleate than did FY samples (Table 3) ($p < 0.05$). Amounts of linoleate decreased significantly during the first 8 h of incubation for all samples. At 24 h of incubation FO samples remained less than 13% of the original linoleate while FY samples retained more than 46%.

ALA in FO samples behaved similarly to linoleate (Table 3) ($p < 0.05$). At 24 h of incubation, FO samples remained less than 15% and FY samples retained more than 42% of the initial amounts of ALA.

The amount of arachidonate decreased over time (Table 3). FO samples retained significantly less arachidonate than did the FY as the higher oil loading values. For both FO and FY, retention at the 50 mg loading was significantly lower that at higher loadings.

In experiments with dFY, only changes in the amounts of EPA and DHA with incubation time were statistically significant. Amounts of EPA significantly decreased over time and depended on fat source (Figure 3). Samples with FY and dFY demonstrated significantly less hydrogenation of EPA than did samples with FO. For FY samples, the most rapid loss happened during first 4 h of the incubation, and 49-83% of the initial EPA remained at 24 h.

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In experiments with FO, EPA sharply decreased during first 8 h of incubation then decreased slowly or remained constant. At 24 h of incubation samples with FO contained 20-37% of initial EPA.

All FO and FY samples demonstrate similar changes in DHA amounts with time. Except for 50 mg FY samples, 40-60% of the initial amount was hydrogenated during the first 8 h of incubation and then remained stable or slowly decreased (Figure 3 and Table 3). DHA was significantly less hydrogenated in FY and dFY samples than in FO samples over time (Figure 3). In the experiments with FY and dFY, the amount of DHA gradually decreased with incubation time, and 47-84% of initial DHA remained after 24 h of incubation. With FO there was a sharp decrease in DHA during first 8 h of incubation and then a slow DHA decrease. After 24 h of incubation only 17-27% from initial amount of DHA was left.

As in LO experiments significant TVA and CLA formation was detected in FO experiments with time (Figure 4). More TVA and CLA were formed when the oil was not encapsulated.

Discussion

Our results demonstrated significant protection of PUFA encapsulated in *C. curvatus* from rumen hydrogenation compared with unprotected oils. In this study, more than 70% of the linoleate and 80% of the ALA from unprotected linseed oil were hydrogenated at 8 h. With unprotected FO, hydrogenation of linoleic acid and ALA was gradual, but after 24 h less than 15% remained. This difference in rate between LO and FO may be attributed to the greater proportion of the total unsaturation that these acids comprise in LO than FO. Previous studies have reported wide ranges in hydrogenation rate for linoleic and linolenic acids (Enjalbert et al., 2003; Van Nevel and Demeyer, 1996; Ribeiro et al., 2007), and the variation seem to depend on the experiment design and the amount and source of the added oil. The hydrolysis of triacylglycerol does not seem to be rate limiting step (Beam et al., 2000; Enjalbert et al, 2003),

EPA and DHA in unprotected fish oil were hydrogenated in smaller proportions than linoleic acid and ALA, which agrees with previous studies. AbuGhazaleh and Jenkins in 2004

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reported higher hydrogenation of EPA compared with DHA, but our results demonstrated very similar rates at 24 h in unprotected FO. Gulati et al (1999) reported only 15% of EPA and DHA hydrogenated after 24 h of incubation, which is considerably less than in our unprotected FO.

Encapsulation allowed more than 47% of EPA and DHA to escape hydrogenation after 24 h. The effect of yeast viability on protection from hydrogenation was tested by the dFY experiments. Significantly more stearate and palmitate were formed with unprotected oils than encapsulated during incubation. Oleate was hydrogenated in samples with unprotected FO, and the lowest added oil amount showed the least hydrogenation. With unprotected LO, significant increases in oleate were observed. Other studies have not reported such an increase (Van De Vossenberg and Joblin, 2003; Proell et al., 2002), but Wilde and Dawson (1966) proposed an ALA hydrogenation pathway with oleic acid as one of intermediate products. Thus, it is possible that LO oleic acid was hydrogenated from ALA, which is the most abundant fatty acid in LO. In agreement with previous studies (Duckett et al., 2002), PUFA were hydrogenated to other intermediates products, CLA and TV. The CLA and TV were formed more quickly from unprotected fats.

Our results suggested that encapsulation in *C. curvatus* cell was an effective method to protect PUFA and slow their rumen hydrogenation. In spite of incomplete protection, encapsulation in *C. curvatus* may be a way to increase the PUFA content in milk and tissues. Previous work from our laboratory has shown that encapsulation protects PUFA from oxidation and that the encapsulated oil has much less fishy aroma than the fish oil that was fed to the yeast (Iassonova et al., 2008)

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Table 1. Yield (g/L), fat as dry weight percentage and fatty acid composition of *C. curvatus* fattened with fish and linseed oils along with the fatty acid composition of the oils that were fed. Values are means (n=2) within the same types of oils.

	Fish oil	*Encapsulated ish oil*	*Linseed oil*	*Encapsulated linseed oil*
Dry yeast yield g/L	-	12.36	-	14.94
Fat content (% w/w)	-	34.82	-	33.11
Myristic	7.2	11.3	-	-
Pentadecanoic	0.4	0.3		
Palmitic	17.8	19.2	6.2	11.3
Palmitoleic	11.1	11.0	-	-
Stearic	5.5	8.5	3.2	6.6
Oleic	17.9	22.9	14.4	26.1
Linoleic	3.3	2.3	12.3	13.7
Linolenic	2.5	2.1	64.0	42.3
Arachdonic	1.9	1.2	-	-
EPA	13.5	8.8	-	-
Docosapentenoic	3.0	1.9	-	-
DHA	14.1	9.3	-	-
Others	1.8	1.2	-	-

Table 2. Relative percentage of various fatty acids after 24 h of *in vitro* incubation of linseed oil (50, 100, or 200 mg per sample added at 0 time) and linseed oil encapsulated (50mg, 100mg, or 200 mg oil in the yeast per sample added at 0 time) in viable *C. curvatus*. The percentages of each fatty acid was arbitrarily set at 100% at 0 h. Values are means of relative amounts (%) of two independent experiments.

Fatty acids	***Linseed oil added***			***Encapsulated linseed oil added***		
	50 mg	**100 mg**	**200mg**	**50 mg**	**100 mg**	**200 mg**
Palmitic	205.7	279.3	411.8	144.0	123.9	128.0
Stearic	366.9	314.7	372.4	137.5	124.5	143.1
Oleic	159.8	213.4	308.8	114.1	89.2	91.7
Linoleic	25.5	35.4	25.7	81.4	65.7	46.1
Linolenic	8.9	12.3	14.3	54.5	44.2	55.1

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Table 3. The relative percentage of various fatty acids after 24 h of *in vitro* incubation with fish oil and fish oil encapsulated in either dead or viable *C. curvatus*. The weight percentage of each fatty acid was arbitrarily set at 100% at 0 h. Values are means of relative amounts (%) of two independent experiments.

Fatty acids	***Fish oil***			***Encapsulated fish oil (viable)***			***Encapsulated fish oil (dead)***
	50 mg	**100 mg**	**200 mg**	**50 mg**	**100 mg**	**200 mg**	
Myristic	132.2	164.3	179.4	55.2	49.4	69.8	95.3
Palmitic	116.7	145.6	143.4	107.9	103.7	139.3	98.8
Palmitoleic	64.0	103.8	95.6	40.3	43.9	54.4	89.2
Stearic	381.6	284.8	299.0	153.5	107.2	143.8	112.7
Oleic	73.9	51.6	53.8	85.3	77.0	99.8	103.8
Linoleic	12.4	6.7	6.9	53.4	46.4	68.3	65.3
Linolenic	12.7	14.5	14.9	60.6	42.0	71.1	73.2
Arachidonic	29.1	51.6	53.2	36.5	81.9	106.1	58.3
EPA	37.4	20.9	20.4	53.8	48.8	62.5	82.0
Docosapentanoic	45.8	39.9	41.1	33.6	35.2	45.6	45.6
DHA	26.4	22.5	16.9	57.1	47.4	65.4	79.6

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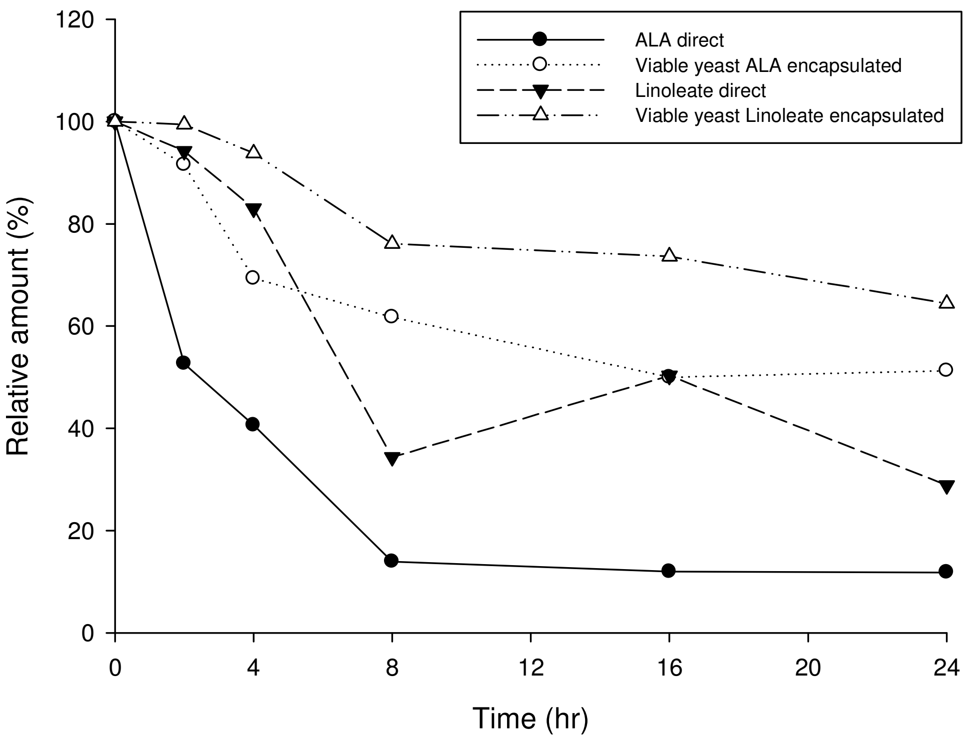

Figure 1. Changes in ALA and linoleate amounts during *in vitro* incubation with linseed oil (direct) and linseed oil encapsulated in viable yeast. Amount of each fatty acid was arbitrarily set at 100% at 0 h. Values are the means of relative amounts (%) of two independent experiments averaged over three added fat concentrations.

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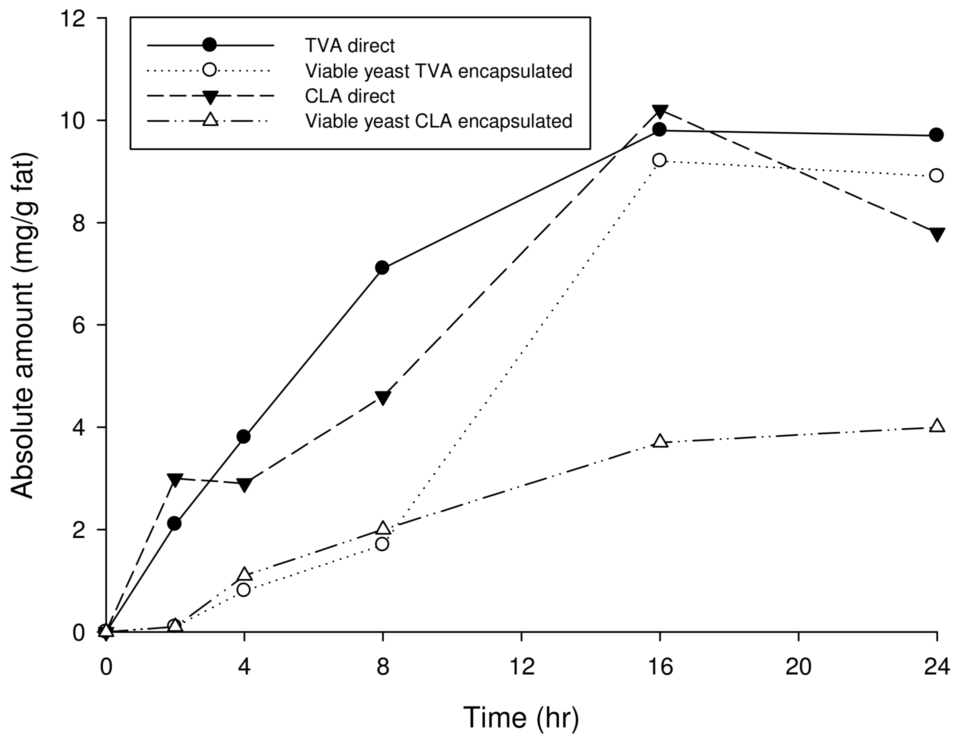

Figure 2. Change in TVA and CLA amounts during *in vitro* experiments with linseed oil (direct) and linseed oil encapsulated in viable *C. curvatus*. Values are the means of absolute amounts (mg/g fat) of two independent experiments averaged over three added fat concentration (50, 100, and 200 mg).

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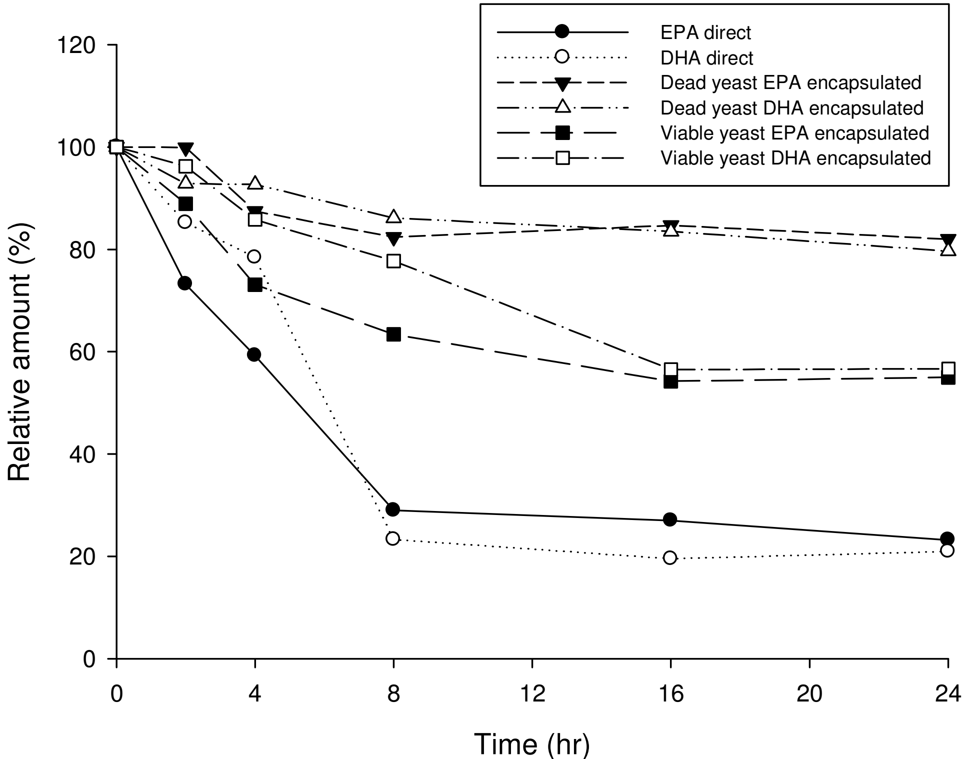

Figure 3. Change in DHA and EPA percentages during *in vitro* experiments with fish oil (direct) and fish oil encapsulated in dead or viable *C curvatus*. Amount of each fatty acid was arbitrarily set at 100% at 0 h. Values are means of the relative amounts (%) of two independent experiments averaged over fat concentration (50, 100, and 200 mg).

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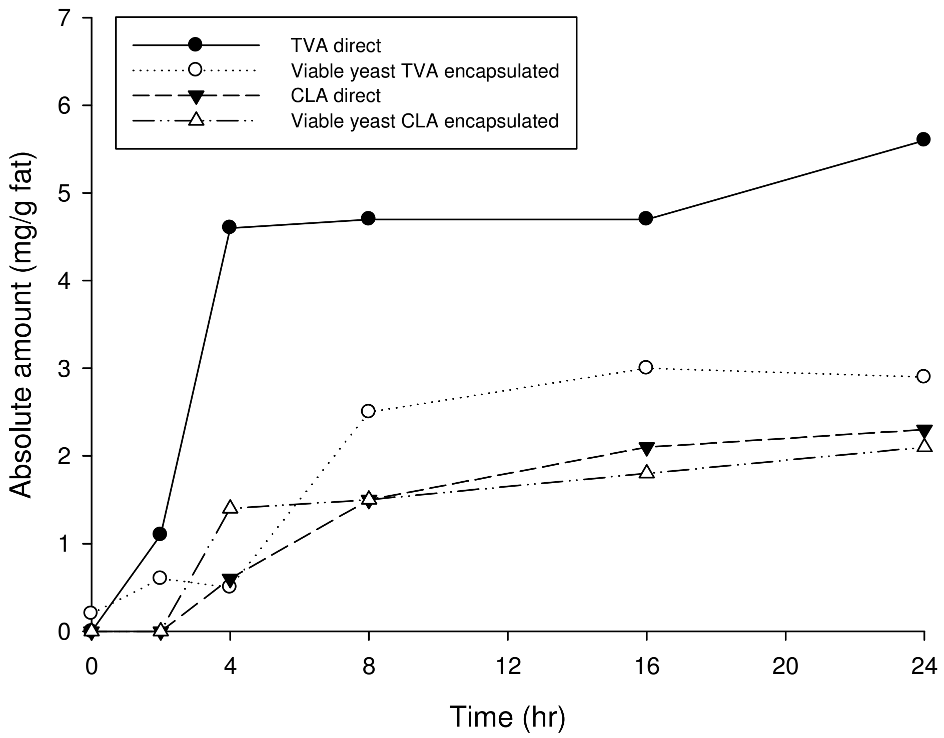

Figure 4. Change in TVA and CLA during *in vitro* experiments with fish oil (direct) and fish oil encapsulated in viable yeast. Values are means of absolute amounts (mg/g fat) of two independent experiments averaged over fat concentration (50, 100, and 200 mg).

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Chapter 5: Effects of Fish Oil Encapsulated in Yeast on Bovine Milk Production and Composition

A paper to be sumbitted to *The Journal of Food Science*

Diliara R. Iassonova, Donald C. Beitz, Earl G. Hammond, Samuel E. Beattie

Abstract

Slurries of the yeast *Cryptococcus curvatus* containing encapsulated long chain polyunsaturated omega-3 fatty acids (LCPUFA) from fish oil were incorporated into the total mixed ration for lactating dairy cows to determine the effect of feeding the encapsulated fatty acids had on milk composition and production. The palatability of the fed LCPUFA yeast supplement also was monitored. After a two-week pre-supplement adjustment period, the encapsulated LCPUFA supplement was added to the total mixed ration in increasing amounts over 5 days to a maximal amount of 5.2 L of yeast /day (10.1% DM). Animals were fed the encapsulated oil for a total of 25 days. The treatment diet consisted of a regular feed with 48.6 g encapsulated LCPUFA/day for 20 days. Dietary yeast with encapsulated oil had no evidence of an effect on milk yield, milk fat content or food intake. Dietary treatment also had no no evidence of an effect on milk fat composition. No omega-3 fatty acids were detected in milk fat after 20 days of feeding the treatment diet. Results from this experiment demonstrate that wet dead yeast containing encapsulated oil can be used as a dietary supplement for lactating cows; but, the yeast did not contribute fatty acids to milk fat synthesis.

Introduction

Dairy milk fat contains a relatively high percentage of saturated fatty acids (66%) including laurate (12:0), myristate (14:0), and palmitate (16:0) and therefore has a relatively high atherogenicity index, which is associated with a high risk of cardiovascular disease, weight gain, and obesity (Jensen et al., 1990; Ulbricht and Southgate,1991; Sacks and Katan, 2002).

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There have been efforts to alter the fatty acid profile of milkfat, but these have resulted in little commercial success.

For humans, consumption of the long-chain polyunsaturated omega-3 fatty acids (LCPUFA), eicosopentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA 22:6 n-6), has numerous health benefits including improved neurological development in infants and decreased risk of atherosclerosis and coronary heart disease (Mozaffarian et al., 2006; Kidd, 2007; Elvevoll et al., 2008). The atherogenic index of milk fat can be decreased by feeding polyunsaturated fatty acids (PUFA) (Bauman et al., 2001, 2003). Direct diet supplementation of lactating cow diets with LCPUFA from fish oil (Baer et al., 2001; Moussavi et al., 2007) or linseed oil (Ueda et al., 2003; Loor et al., 2005) resulted in an increase of these fatty acids in the milk, but there was also feed refusal and decreases in milk fat and milk production (Baer et al., 2001; Loor et al., 2005; Murphy et al, 2008). Milk fat is low in omega 3 fatty acids because dietary PUFA are extensively biohydrogenated in the rumen. Dietary PUFA might be protected from rumen biohydrogenation by encapsulation with oleaginous yeast (Iassonova et al., 2007). Results of our research have demonstrated that the oleaginous yeast *Cryptococcus curvatus* is able to accumulate LCPUFA (Iassonova et al., 2008), and *in vitro* rumen digestion studies have shown that encapsulation by yeast protects LCPUFA from rumen biohydrogenation during 24 h of incubation (Iassonova et al., 2008, Chapter 4). Encapsulated PUFA were also significantly more oxidatively stable compared with unprotected PUFA (Iassonova et al., 2008).

The objective of this *in vivo* study was to determine the acceptability of encapsulated PUFA by cows and the effect of encapsulated fat on the composition and yield of milk.

Materials and Methods

Yeast encapsulated fish oil

The encapsulated fish oil yeast (FY) supplement (10.1% DM) was produced in a 100-L fed-batch fermentations (Iassonova et al., 2008; Chapter 6). Yeast were killed immediately after fermentation by heating to 55˚C and holding for 2 h. Samples from each fermentation were

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plated on potato dextrose agar to verify that yeast were killed. The dead FY were stored at 4°C in 50-L plastic containers. The top layer containing yeast cells with fat content 59.2±2.2% was skimmed from each container and used as the FY supplement in the feeding experiment.

Feeding experiment

Three multiparous (average lactations of 2.3±0.6) Holstein cows (average day in milk 124±10.8) from the Iowa State University Dairy Farm were used in the feeding experiment. Cows consumed a control diet (Table 1) for two weeks and the treatment diet with FY for 25 days, followed by a control diet for 12 days to observe possible post treatment effects. Animal care was according to the Guide for Care and Use of Agricultural Animals in Agricultural Research and Teaching and the experiment procedure was approved by the ISU Office of Research Assurances. Cows were placed to the free-stall barn 5 days before starting experiment for acclimation and training for use of the Calan Broadbent (American Calan, Inc., Northwood, NH) feeding gates. Animals were fed twice a day *ad libitum*; feed intake and refusal was recorded daily during the feeding trial. Animal weight was recorded weekly. After an acclimation period, all cows were fed a control diet for two weeks (weeks -2 and -1). The yeast supplement was added to the diet during week 0, and the amount of yeast in the diet was increased gradually during 5 days: from 1.0 L on first day, 2.0 L on the second day, 3.0 L on day 3, 4.0 L on day 4, and 5.2 L on day 5. Cows received 5.2 L/day (10.1% DM) FY as a treatment for 20 days. Animals were fed the control diet for the next 12 days. Total feeding trial time was 57 days. The chemical composition of the fats in the control and treatment diets is presented in Table 2. The yeast encapsulated oil increased the amount of fat in the treatment diet compared to the control by 1.03% DM. Cows were milked twice a day at approximately 10:00 and 22:00, milk production was recorded electronically at each milking.

Milk analysis

A milk sample was collected from each cow every third day at the evening and the following morning milking. The samples were stored at -20°C and were analyzed for fatty acid

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composition by gas chromatography as butyl esters according to Chen et al.(2004). Lipids were extracted from the wet yeast and from the feed by sequential ethanol, hexane and benzene extractions according to Hammond et al. (1981).

Fatty acids were analyzed by gas chromatography. The gas chromatograph was a HP 5890 Series II instrument (Hewlett-Packer Company, Avondale, PA) with a flame ionization detector and with a fused-silica capillary column SP-2423 (60 m x 0.25 mm i.d., 0.20 µm) (Supelco, Inc., Bellefontaine, PA, USA). The carrier gas (helium) flow rate was 1.9 mL/min, and the split ratio was 24.8. The column temperature was held at 70°C for 4 min, programmed to 230°C at 20°C/min and held at 230°C for 5 min. The injector and detector temperatures were 250°C. Quantitative analysis was done using valeric acid (5:0) as an internal standard for butyric, caprylic and caproic acids; undecanoic acid (11:0) as an internal standard for capric, lauric and myristic acids; and nonadecanoic (19:0) as an internal standard for fatty acids longer than 14 carbons. Peaks were identified by external standards and verified by GC-MS. Internal and other standards were purchased from Sigma Chemical Co. (St. Louis, MO) and Nu-Check Prep, Inc. (Elysian, MN).

Mass spectrometry

Electron impact GC/MS experiments were conducted using Micromass GCT mass spectrometer (Premier, Waters, Sollentuna, Sweden) coupled to the Agilent 6890 GC System. The mass spectra were recorded in the mass range of 35-650. The carrier gas was helium, and the flow rate was kept at 1.1 ml/min throughout the run. The head pressure was maintained constant at 10 psi throughout the run. The injector port and the interface were set at 230 and 250°C, respectively. The same sample preparation method and GC conditions were used, except that the GC columns were an HP-5 (polydimethylsiloxane with 5% phenyl groups, fused silica open tubular column, 30 m ×0.25 mm i.d., 0.25 µm film thickness) and SP-2423 (60 m x 0.25 mm i.d., 0.20 µm) (Supelco, Inc., Bellefontaine, PA).

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<u>*Statistical analysis.*</u>

Data are presented as means (n=3) with standard error (SE). The values for each subject (cow) averaged within treatment period (control, treatment, post), because there was no evidence of trend over days within period, and then were analyzed by analysis of variance (ANOVA) with the SAS 9.1 (SAS Institute, Cary, NC) general linear model procedure where cows were blocks. The difference was considered significant at $P < 0.05$.

Results and Discussion

The treatment diet consisted of a standard feed with 310.8 g/day encapsulated oil where 48.6 g were encapsulated EPA+DHA/day. Dry matter intake and milk production were not affected by the dietary FY treatment (Table 3). Dietary fish oil supplementation, both direct and protected, is reported to have negative effects on dry matter intake with 200 to 400 g/d of fish oil (Doreau and Chilliard 1997; Franklin et al., 1999). The yeast product used in this study did not have an unpleasant oxidized fish oil aroma because fish oil was completely encapsulated, and when it is encapsulated it is protected from oxidation by the yeast (Iassonova et al., 2008). Baer et al.(2001) reported significant reduction in milk fat when 2% (DM) fish oil added to the cows' diet. In this study, no significant negative effect on milk fat content was found (Table 3). Considerable research has reported that dietary fish oil significantly affects the fatty acid composition of milk by increasing the amounts of long chain PUFA, decreasing the saturation of milk fat and increasing the concentration of conjugated linoleic acids (CLA) and trans vaccenic acid (TVA) (Baer et al, 2001; Osborne et al., 2008; Moussavi et al., 2007). In this study, no omega-3 fatty acids were detected in milk fat after feeding the treatment diet (Table 4) and no significant differences in the amounts of CLA and TVA were found in the milk taken during the control and treatment diets. Effects of fish oil supplementation on CLA, TVA, EPA and DHA concentrations are shown in Table 5. AbuGhazaleh (2008) reported significant elevated CLA, TVA, and DHA concentrations when 100 g fish oil was added to the bovine diet with 300 g sunflower oil for 21 day; however, Murphy et al. (2008) reported increase in CLA and TVA in milk fat but they did not find DHA and EPA in milk fat after 100 g/day fish oil in diet for 54 days. Palmquist and Griinari (2006) reported increase in CLA, TVA, EPA and DHA concentrations after 21 days

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of treatment diet; however, supplementation was two times higher in amount of DHA and EPA than in present study (Table 5). Gulati et al. (2003) found that dietary 1100 g/day of xylose-protected fish oil increased amount of DHA and EPA in milk fat after five days of treatment. It is possible that amount of supplement was insufficient to detect significant increase in PUFA in milk fat. On another hand, the fact that there was no significant increase in CLA and TVA during treatment suggested that possibly the FY survived not only rumen hydrogenation but also conditions in the cows' intestines and thereby by-pass digestive tract. The concentrations of milk CLA decreased after FY treatment; that finding agreed with previous studies results with LCPUFA supplementation (Bauman et al., 2000; AbuGhazaleh et al., 2004; Shingfield et al., 2006). It may be possible to maintain resistance of FY to rumen digestion while encouraging intestinal digestion by treatments such as sonication, enzymes and/or chemical treatments that would target yeast cell wall.

Conclusions

Feeding dairy cows yeast containing encapsulated fish oil had no effect on their milk yield, milk fat content or food intake. This treatment also had no significant effect on milk fat composition. No omega-3 fatty acids from the fish oil were detected in the milk fat after 20 days of the treatment diet. These results demonstrated that liquid dead yeast containing encapsulated fish oil can be used as a dietary supplement for lactating cows; however, further experimentation is needed to improve transfer of the omega-3 fatty acids to the milk fat.

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Table 1. Amounts of the ingredients of the control diet.

Ingredient	*Dry matter (wt%)*
Alfalfa hay	6.1
Cottonseed with linseed	5.1
Soybean meal	4.0
Corn silage	62.1
Protein/Mineral/Vitamin premix	22.7

Table 2. Fatty acid composition of control diet and oil extracted from the yeast.

Fatty Acid	*Control (%)*	*Encapsulated fish oil (%)*
14:0	0.98	6.63
16:0	18.7	20.3
16:1	1.38	12.8
18:0	4.90	3.25
18:1	26.8	29.4
18:2	44.0	4.21
18:3	3.29	3.58
20:4	-	2.55
20:5	-	12.7
22:5	-	1.55
22:6	-	2.98

Table 3. Food intake, fat intake, body weight, milk production and milk fat content of animals fed control diet and the treatment diet with yeast-encapsulated fish oil[a].

	Control	*Treatment*	*Post*	*SE* [b]
DMI, kg/da	29.3	30.5	30.3	1.03
Fat intake (kg/day)[c]	1.65	1.96	1.65	-
Fat intake (% DM)[c]	5.62	6.65	5.62	-
Body weight (kg)	744.8	742.8	742.5	4.6
Milk fat, (%)	2.80	3.00	2.99	0.2
Milk production (kg/day)	56.1	54.6	54.1	0.9

[a] Means (n=3)
[b] *SE*-standard error
[c] Calculated values

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Table 4. Fatty acid compositions in weight percent of milk[a].

Fatty acid	*Control*	*Treatment*	*Post*	*SE*
4:0	3.26	2.95	2.92	0.26
6:0	1.55	1.39	1.44	0.19
8:0	2.23	1.33	1.43	0.31
10:0	2.43	2.49	2.58	0.30
12:0	3.02	3.15	3.20	0.18
14:0	12.7	11.3	14.6	0.49
16:0	31.1	30.7	31.7	0.81
16:1	2.07	2.84	2.34	0.31
18:0	10.0	9.8	9.7	0.12
vaccenic	5.06	6.31	4.49	0.68
18:1	20.9	21.7	20.5	0.56
18:2	3.48	3.73	3.42	0.16
CLA	1.49ab	1.56a	1.01b	0.10
18:3+20:0	0.72	1.01	0.70	0.10

[a]Means (n=3) with different letters within a row differ at $P < 0.05$. *SE*-standard error

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Table 5. Effects of fish oil supplementation on CLA, TVA, EPA and DHA contents in milk fat.

Supplement	*Amount* (per day)	*DHA+EPA* (g/day)	*Duration* (day)	*Fatty Acids in Milk (g/100g total fat) control / treatment*				*Reference*
				TVA	CLA	EPA	DHA	
Fish Oil	100 g	23.9	21	2.3 / 5.3	0.9 / 1.8	0.10 / 0.1	0.05 / 0.07	AbuGhazaleh, 2008
Protected Marine Algae	910 g	37.6	42	1.2 / /7.5	0.4 / 2.6	nd / nd	nd / 0.46	Franklin et al.,1999
Fish Oil	2% DM	NR	35	1.4 / 6.3	0.7 / 2.6	0.09 / 0.38	nd / 0.09	Baer et al., 2001
Fish Oil	214g	45.8	28	NR / 10.2	NR / 1.0	NR / 0.16	NR / 0.05	Osborne et al., 2008
Fish Oil	2% DM	NR	28	0.9 / 4.1	0.6 / 2.3	0.14 / 0.39	0.06 / 0.15	Ramaswamy et al., 2001
Fish Oil	100 g	NR	54	4.4/ 7.1	1.8 / 2.2	nd / nd	nd / nd	Murphy et al., 2008
Fish Oil	52.5 g	NR	54	4.4 / 6.98	1.8 / 2.4	nd / nd	nd / nd	Murphy et al., 2008
Rumen protected Fish Oil	1100 g	200	5	4.1 / 13.7	1.8 / 2.9	nd / 1.3	nd / 2.2	Gulati et al., 2003
Fish Oil	420 g	92.4	21	7.2 / 7.9	4.0 / 3.4	0.06 / 0.3	nd / 0.09	Palmquist, Griinari, 2006
Protected Fish Oil	311 g	48.6	20	5.1 / 6.3	1.5 / 1.6	nd / nd	nd / nd	This study

NR – not reported
nd – not detected

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Chapter 6: Scale-up Process for a Fed-batch Microbial Encapsulation of Fish Oil in *Crypotoccus Curvatus*

A paper to be submitted to *The Journal of Agricultural and Food Chemistry*

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Abstract

A concentrated liquid yeast product containing fish oil encapsulated in *Cryptococcus curvatus* was produced in fed-batch fermentations. Yeast biomass production was 54.9 g/L and yeast fat content was 59.2% on a cell dry weight basis. Fatty acid composition and phospholipids analysis of the encapsulated oil were evaluated. In the yeast, all long chain polyunsaturated omega-3 fatty acids were stored in intracellular lipid droplets rather than as membrane lipids. Phosphatidylcholine was the major *Cryptococcus curvatus* phospholipid.

Introduction

Previously, fish oil was encapsulated into *Cryptococcus curvatus* by batch and fed-batch fermentations on scales of 100 mL to 4 L (Iassonova et al., 2008). The objective of this study was to scale-up the fed-batch fermentation procedure to 100 L to achieve high biomass production and lipid content of *C. curvatus w*hen grown on biodiesel grade glycerol followed by fat accumulation on fish oil. Analysis results are reported on the fatty acid composition of the phospholipids and the triacylglycerols in the encapsulated oil.

Materials and Methods

Culture and culturing conditions

Freeze dried *C. curvatus* ATCC 20509 (formerly known as *Candida curvata* D and *Apiotrichum curvatum*) was activated by suspension in Difco™ Yeast and Mold broth (Becton, Dickinson and Company, Sparks, MD, USA) and incubation at 30˚C for 24 h. After plating the culture on Potato Dextrose Agar Difco™ (Becton, Dickinson and Company, Sparks, MD, USA), a colony was transferred from the plate to a basal medium broth

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supplemented with glycerol and urea. The basal medium was KH_2PO_4 2.5 g/L, $MgSO_4 \cdot 7H_2O$ 1.0 g/L, $CaCl_2 \cdot 2H_2O$ 0.2 g/L, $FeCl_3 \cdot 6H_2O$ 20.0 mg/L, $MnSO_4 \cdot H_2O$ 2.0 mg/L, $ZnSO_4 \cdot 7H_2O$ 1.0 mg/L, $CuSO_4 \cdot 5H_2O$ 0.1 mg/L, and NaCl 60 mg/L (Vega et al., 1988). The autoclaved basal medium containing 16 g/L of glycerol and 0.8 g/L of urea was adjusted to pH 5.4 with 3 N hydrochloric acid solution. Thiamine hydrochloride (4.0 mg/L) was added by sterile filtration. *C. curvatus* was grown in shake flasks at 30˚C for 24 h with agitation (200 rpm) and transferred three times into fresh medium. The third transfer was used as inoculum for the 100-L encapsulation fermentations.

Fish oil encapsulation

A 100-L bioreactor was used in these experiments (Biostat M, B.Braun, Allentown, PA, USA). The growth medium was sterilized *in situ* and contained the basal medium with 0.8 g/L urea and 2000 g biodiesel glycerol (BGL) (16g/L glycerol); 4 mg/L thiamine hydrochloride was added by sterile filtration. A 2-L inoculum of a 24-h yeast culture was used. Air flow, dissolved carbon dioxide, temperature, and agitation were monitored. Fermentations were performed at 30°C, the aeration rate was 1 vol/min during growth phase and 0.1 vol/min during the lipid accumulation stage, and agitation was 200 rpm. The pH was automatically adjusted to 5.4 by adding 0.5 M sodium hydroxide solution and 3 N hydrochloric acid solution. After 30 h of fermentation, 2000 g (16 g/L glycerol) sterile BGL and 0.8 g/L urea were added. After 42 h of fermentation, 1000 g (8 g/L glycerol) sterile BGL and 0.8 g/L urea were added. Sterile menhaden fish oil (Omega Protein, Inc, Reedville, VA, USA) 1800 g, containing 1000 ppm butylated hydroxyanisole was added at 51 and 72 h of fermentation. Glycerol concentration was measured by test kit (Boehringer Mannheim/R-Biopharm AG, Darmstadt, Germany) at 50 h of fermentation before menhaden oil addition. Total fermentation time was 96 h. *C.curvatus* was killed in the fermentation vessel by heat treatment at 55°C for 2 h. Total fermentation product was stored at 4°C until all fermentations were completed. Biomass from six 100-L fermentations was concentrated by collecting top fat yeast layer that separated during storage in refrigerator. The total fermentation product was approximately 340 L with 10.1% (DM) and was used in a dairy cow feeding trial.

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Yeast and oil extraction

Samples (40 mL) from the fermentations were washed with 20 mL hexane to remove the unassimilated fish oil. The top hexane layer was recovered and evaporated. The bottom layer was centrifuged at 9000 g for 30 min and washed twice with distilled water, and the wet yeast phase was collected. Lipids were extracted from the wet yeast sample by sequential ethanol, hexane and benzene extractions according to Hammond et al. (1981). For phospholipid analysis, oils extracted from three randomly selected 100-L fermentations were used. Also, yeast oils from 5-L glycerol fermentations were analyzed.

Fatty acid composition

Oil samples (20 mg) were converted to methyl esters with 2 mL 1 N methanolic sodium methoxide for 1hr at room temperature. Water (1 mL) and hexane (1mL) were added to the methyl esters mixture and then the sample was injected in the gas chromatograph. Fatty acid composition was analyzed by gas chromatography with a HP 5890 Series II gas chromatograph (Hewlett-Packard Company) equipped with a fused-silica capillary column SP-2423 (60 m x 0.25 mm i.d., 0.20 μm) (Supelco, Inc., Bellefontaine, PA, USA). The carrier gas (helium) flow rate was 1.9 mL/min and the split ratio was 24.8. The column temperature was held at 140°C for 6 min, programmed to 220°C at 10°C/min and held at 220°C for 15 min. The injector and detector temperatures were 230°C.

Phospholipid separation from total lipids

The total lipid sample (100 mg) diluted in 2 mL chloroform was transferred to a 1000-mg silica cartridge (Alltech Associate, Inc., Deerfield, IL, USA); an additional 10 mL of chloroform was passed through the cartridge eluting the fraction of neutral lipids components to the first tube. Next, 10 mL of acetone was passed through the cartridge eluting the fraction of glycolipids to the second tube. Then 10 mL of methanol was passed through the cartridge, collecting the phospholipids fraction to the third tube. Solvents were removed with a steam nitrogen and then samples were dissolved in 0.5 mL chloroform, methylated and

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analyzed by gas chromatography. Lipid fractions were confirmed by thin-layer chromatography with appropriate standards. To separate phospholipid classes, 100 µl of the total polar solution was streaked on a 20 x 20, 200-µm Selecto Flexible-Backed TLC silica gel plates (Selecto Scientific, Inc., Suwanee, GA, USA). Plates were developed with chloroform:methanol:acetic acid:water (v:v:v:v, 100:45:5:2). Phospholipid classes were visualized by spraying with 0.1% 2'7'-dichlorofluorescein in methanol and viewing under UV light. Phosphatidylcholine (PC) (R_f=0.33), phosphatidylinositol (PI) (R_f=0.64) and phosphatidylethanolamine (PE) (R_f=0.80) were identified by comparison with PC, PE and PI standards (Avanti Polar Lipids, Inc, Alabaster, AL, USA). The bands were scraped from the plate and extracted three times with 10 mL chloroform:methanol:water (v:v:v, 1:2:08) and then methylated and analyzed by gas chromatography.

Results and Discussion

100-L fermentation

The average biomass production and yeast cell lipid accumulation results for the six fed-batch fermentations are shown in Table 1. The fed-batch fermentation system noticeably improved the dry yeast yield (54.9 g/L) compared to batch fish oil fermentation (18.4 g/L). However, we achieved higher yields when *C.curvatus* was fed-batch fermented on BGL as a sole carbon source (64.8 g/L), but these fermentations were performed on 25 times smaller scale. Lipid accumulation was in fed-batch fermentations (59.2%) also was higher than the batch fish fermentation (49.7%) and slightly lower than fed-batch fermented on BGL fermentations (62.7%). Fish oil assimilation was very efficient: only 200 g (5.6% from added amount) of fish oil remained in the fermentation medium after 96 h fermentation and 2 h heat treatment. The unassimilated oil portion was very small; however, there was a pronounced DHA and EPA concentration effect in the unassimilated oil (Table 2). Lee et al. (1992) found that *C. curvatus* discriminated erucic, docosanoic, docosenoic acids but not eighteen carbon fatty acids from substrate oil. From 875 g DHA+EPA fed only 634 g was encapsulated, 70 g was recovered from unassimilated residual and 171 g was lost. DHA and EPA from the lost portion could have been oxidized, or metabolized by *C. curvatus* for energy. Physical losses and analyses accuracy contribute to this portion of DHA and EPA

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loss. The amount of encapsulated DHA and EPA was also affected by the residual glycerol from the growth stage of fermentation (Table 2). During the growth phase, the feeding of BDG and urea was signaled by measurements of the dissolved oxygen. Dissolved oxygen decreased rapidly during first hours of fermentation and remained low because of cellular respiration. When the dissolved oxygen began to increase, glycerol and/or urea concentrations are/is limited in the medium. Before the lipid accumulation stage, the concentration of glycerol was measured in previous fermentations, and the results showed that residual glycerol affected the fatty acid composition of encapsulated oil. Yeast oil produced *de novo* contained less DHA and EPA and more palmitic and oleic acids (Table 1) when 5 g/L glycerol from the growth stage remained in the medium at the beginning of the fish oil accumulation stage. When the nitrogen source was used up, *C.curvatus* converted residual glycerol to the lipids with fatty acid composition similar to palm oil. Fish oil was then introduced to the medium and fish oil encapsulation process started. The resulting yeast cell fat represented a mixture of two encapsulation processes: *de novo* oil synthesis and accumulation and fish oil encapsulation, possibly via hydrolysis, intracellular transport and esterification.

Phospholipids found in the yeast oil from glycerol and fish oil stages of the fermentations were analyzed separately and characterized. The phospholipid fraction contained PI, PC and PE and amounted to 0.56-1.22% of the total lipids at 96 h. The most abundant fraction was PC (92-100%). PI (0-3%) and PE (0-5%) were less abandant and were not detected in all samples. These results agreed with previous results (Hammond et al., 1981). The phospholipid fraction was similar for both stages of fermentations. Table 3 shows fatty acid composition of phospholipid fraction at various fermentation times. No DHA or EPA were detected in the phospholipids at 72 and 96 h of fermentation. These results suggested that despite expected DHA and EPA participation in the yeast cell membranes, all the DHA and EPA was stored in triacylglycerols. The phospholipid fraction contained only six different fatty acids and unsaturated fatty acids were predominant (Table 3). During the lipid accumulation stage, the amounts of palmitic, palmitoleic, oleic and linolenic fatty acids increased in the phospholipid fraction compared with growth stage, but amount of linoleic and stearic acids decreased (Table 3). Akhtar et al. (1998) studied phospholipid composition

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of *C.curvatus* and reported oleic and linoleic acids as a major fatty acids in phospholipid fraction. In this study oleic and linoleic were also the predominant fatty acids in the phospholipids during fermentation. However, except for oleic and linoleic acids, the amounts and kinds of fatty acids were different from those reported by Akhtar et al. (1998). Similar to the previous report (Hammond et al., 1981), no tocopherol in the yeast oil was detected.

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Table 1. The amounts of ingredients used and the composition of the yeast obtained in the 100L fermentations of *C. curvatus*.

Component (measure)	***Amount***
Glycerol added (kg)	4.0
Fish oil added (kg)	3.6
Residual unassimilated fat (kg)	0.2±1.3
Lipid accumulation (% cell dry wt.)	59.2±2.2
Biomass (g/L)	54.9±1.7

Mean (n=7) ± stdev

Table 2. Fatty acid composition(weight %) of the substrate fish oil, the unassimilated residual oil extracted from fermentation medium at 96 h, and the yeast oil formed at 51 h in the presence and absence of glycerol.

Fatty acid	***Fish oil+BHA autoclaved***	***Residual unassimilated oil***	***Yeast oil no glycerol at 51 hr***	***Yeast oil (+) glycerol at 51 hr***
14:0	8.4	6.0	6.7	5.2
16:0	18.3	15.2	17.9	22.2
16:1	11.6	7.6	10.6	10.4
17:0	1.4	1.2	1.2	1.3
18:0	3.3	4.5	5.5	4.6
18:1	13.6	14.4	28.5	34.2
18:2	2.4	2.8	3.2	5.5
18:3	3.1	3.3	3.6	2.7
20:4	2.8	3.3	3.3	3.1
20:5	14.8	20.9	13.4	6.9
22:6	9.5	14.1	6.1	2.6

Table 3. Fatty acid composition (weight %) of phospholipids extracted from *C. curvatus* fattened with fish oil at various fermentation times (n=3).

Fatty acid	***Time of fermentation***			
	24 h	***48 h***	***72 h***	***96 h***
16:0	3.5	9.2	18.9	17.1
16:1	-	-	8.2	8.8
18:0	11.2	12.1	6.0	8.4
18:1	27.1	23.8	39.1	33.2
18:2	54.1	48.3	16.4	20.3
18:3	4.5	6.6	10.9	12.4

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Chapter 7: Single Cell Oil Production from Industrial Grade Glycerol

A paper to be submitted to *The Journal of Agricultural and Food Chemistry*

Diliara R. Iassonova, Anthony L. Pometto III, Earl G. Hammond, Samuel E. Beattie

Abstract

The oleaginous yeast *Cryptococcus curvatus* was able to grow, as well as synthesize and accumulate triacylglycerols when cultivated on industrial-grade glycerol that was a byproduct of biodiesel production. Glycerol and urea were the sole carbon and nitrogen sources, respectively. The amount of oil accumulated and its fatty acid composition depended on the glycerol concentration. Under batch fermentation conditions, the yeast accumulated over 50% lipids on a yeast dry weight basis in 48 h when grown on 6.4% glycerol. Fed-batch fermentations increased biomass production to 62.7±2.3% on dry weight basis and lipid accumulation up to 64.8±2.8 g/L. The fatty acid profile of the yeast oil was palmitate (16:0, 28.6%), stearate (18:0, 11.1%), oleate (18:1, 45%) and linoleate (18:2, 11.7%). Results from this study supported the hypothesis that industrial biodiesel-based glycerol can be converted into yeast and single cell lipid. This single cell oil could then be further transformed into biodiesel and potentially increase the yield of biodiesel per feedstock unit.

Introduction

Biodiesel is a relatively new and rapidly growing industry. In the United States, biodiesel production capacity has grown more than 6-fold over the last 2 years. Glycerol is a co-product of the biodiesel industry and is produced when triacyglycerols react with methanol to produce fatty acid methyl esters (Johnson and Taconi, 2007). Production of 9 kg of biodiesel results in approximately 1 kg of impure, low-quality glycerol. Along with the recent increase in production of biodiesel, there has been a corresponding increase in the amounts of glycerol on the market causing the price of glycerol to drop precipitously. Crude biodiesel glycerol contains a variety of impurities, chiefly water, free fatty acids and methanol, which make it unsuitable, without substantial and expensive clean up, for the traditional applications of

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refined glycerol – mainly food and cosmetics. An economically feasible use for crude glycerol is needed.

Glycerol can be used as a carbon source in industrial fermentations. Oleaginous yeasts can convert simple carbon compounds into triacylglycerols. The yeast, *Cryptococcus curvatus* previously known as *Apiotrichum curvatum* and *Candida curvata*, was discovered at Iowa State University (Moon and Hammond, 1978). *C. curvatus* grows well on cheese whey permeate to a high cell density, while using all the available whey nitrogen. Then, the yeast begins to convert the lactose in the whey permeate into intracellular oil and typically ends with 57% oil on a dry weight basis. The oil that is produced on lactose has a fatty acid composition similar to that of palm oil (Lee et al., 1992). The yeast produced about 17 g/L of dry weight on permeate and leave about 5% of the chemical oxygen demand (COD) in the medium after 72 h (Moon and Hammond, 1978). *C. curvatus* also is able to digest oil in its growth medium and deposit it directly as intracellular oil (Lee et al., 1992). Additionally, when fed a variety of oil types, the yeast lipid mirrored the fed fatty acid profile (Lee et al., 1992).

Meesters and Huijberts (1996) reported that when *C. curvatus* was cultured using refined glycerol as the carbon source in a fed-batch fermentation system, cell density reached 118 g/L and accumulated oil that contained 50% oleate, 16% linolenate and stearate, and 18% palmitate. When this fermentation was switched to nitrogen deficiency, oil productivity reached 0.59 g/L*h. This work was done using refined glycerol and not the material produced from biodiesel. A concern is that the glycerol from biodiesel plants contains significant impurities that might limit *C. curvatus*' growth. The objective of this work was to explore the possibility of using *C. curvatus* to convert biodiesel grade glycerol (BDG) into single cell oil, which could be transformed into additional biodiesel.

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Materials and Methods

C. curvatus culturing conditions

In all experiments the oleaginous yeast *C. curvatus* ATCC 20509 was used. Freeze dried *C. curvatus* ATCC 20509 was activated by suspension in Difco^{TM} Yeast and Mold broth (Becton, Dickinson and Company, Sparks, MD, USA) and incubated at 30˚C for 24 h. Culture was plated on Potato Dextrose Agar Difco^{TM} (Becton, Dickinson and Company, Sparks, MD, USA). Then a colony was transferred from the plate to a basal medium broth supplemented with carbon and nitrogen sources. The basal medium was KH_2PO_4 2.5 g/L, $MgSO_4 \cdot 7H_2O$ 1.0 g/L, $CaCl_2 \cdot 2H_2O$ 0.2 g/L, $FeCl_3 \cdot 6H_2O$ 20.0 mg/L, $MnSO_4 \cdot H_2O$ 2.0 mg/L, $ZnSO_4 \cdot 7H_2O$ 1.0 mg/L, $CuSO_4 \cdot 5H_2O$ 0.1 mg/L, NaCl 60 mg/L (Vega et al., 1988), and urea 0.36 g/L or asparagine, 0.8 g/L. Biodiesel grade glycerol (80%) was provided by the Renewable Energy Group, Inc. (Ames, IA, USA) and was obtained directly from the biodiesel manufacturing plant. The autoclaved basal medium containing 64 g/L BDG was adjusted to pH 5.4 with 3N hydrochloric acid solution. Thiamine hydrochloride (1.0 mg/L) was added by sterile filtration. *C. curvatus* was grown in shake flasks at 30˚C for 24 h with agitation (200 rpm) and transferred three times into fresh medium. The third transfer was used as inoculum for the experiments.

Growth and lipid accumulation experiments on BDG and various nitrogen sources

Batch experiments were performed in 250-mL Erlenmeyer flasks containing 100 mL of basal medium at 30°C with shaking at 200 rpm for 72 h. *C. curvatus* was grown on a basal synthetic medium supplemented with 1.0 mg/L thiamine hydrochloride, BDG and a nitrogen source. Three BDG concentrations: 64, 120, or 200g/L; and the three nitrogen sources: 0.8 g/L asparagine, 0.27 g/L ammonium chloride + 0.1 g/L yeast extract or 0.36 g/L urea were used. Growth was monitored by direct microscopic counts using a haemocytometer.

Growth at different urea, BDG and refined glycerol concentrations

A Microbiology Reader Bioscreen C (Oy Growth Curves, AB Ltd., Helsinki, Finland) was used to evaluate carbon to nitrogen ratios using different nitrogen sources and concentrations

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of glycerols. Cells were harvested aseptically from a 24-h *C. curvatus* culture by centrifugation at 9000 x *g* at 4°C. The cells were re-suspended and washed in 25 mL of sterile saline and diluted 1:25 with sterile basal medium without a carbon source. The Bioscreen plate wells were filled with sterile basal medium with a carbon source (BDG or refined glycerol [RG]), urea and *C. curvatus* inoculum. The total sample size was 350 µL. Control wells contained basal medium without inoculum and sterile basal medium without carbon source plus *C. curvatus* inoculum. A complete 5x5 factorial design was used for refined glycerol concentrations (1, 5, 10, 15 and 25%) with urea concentrations (0.1, 0.2, 0.4, 0.6 and 0.8 g/L); a 12x6 complete factorial design for BDG concentrations (1, 2, 3, 4, 5, 6, 8, 10, 12, 15, 20 and 25%) with urea concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 g/L). Plates were incubated in the Bioscreen at 30°C with continuous (low speed) shaking for 70 h. Absorbance was measured hourly at 580 nm. Each sample was run in duplicate, and the experiment was also replicated.

Fed-batch fermentation in Fernbach flasks

Fed batch fermentations were performed in Fernbach flasks containing 1L of basal medium supplemented with 1.0 mg/L thiamine hydrochloride at 30°C with shaking at 200 rpm for 144 h. At 0, 24, 48 and 72 h of fermentation, 16 g BDG and 0.36 g urea were added, and 16 g of BDG were added at 96, 108, 120 and 132 h of fermentation. Glycerol and urea utilizations were measured by glycerol and urea assay kits (Boehringer Mannheim/R-Biopharm AG, Darmstadt, Germany).

Fed-batch fermentation in 5-L fermenters

Two 5-L fermenters were used (model and make). Various fed-batch trials were tested and are given in Table 2. Each fermentation started with 4 L of basal medium with various amounts of BDG and 0.8 g/L urea. The medium was sterilized in the fermenter jar, and afterwards 3 mg/L thiamine hydrochloride was added by sterile filtration. A 2% inoculum of a 24-h yeast culture (80 ml/4L) was used. Urea and BDG were added periodically according to Table 2. Air flow, dissolved carbon dioxide, temperature, and agitation were monitored and recorded. The pH was automatically adjusted to 5.4 by adding 0.5 M sodium hydroxide

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or 3 N hydrochloric acid solutions. Glycerol and urea utilizations were measured by glycerol and urea assay kits (Boehringer Mannheim/R-Biopharm AG, Darmstadt, Germany).

Oil extraction and analysis

Yeast cell were harvested by centrifugation at 9000 g for 30 min and washed twice with distilled water. Dry weight of the washed cells was determined by drying a 10 g sample with 100 mL absolute ethanol, evaporating the ethanol or by freeze drying the yeast phase overnight (Virtis Ultra-35, Gardiner, NY, USA) and weighing. Lipids were extracted from the yeast phase by sequential ethanol, hexane and benzene extractions according to Hammond et al. (1981).

Extracted lipids were methylated with sodium methoxide according to Hammond (1991) and analyzed by gas chromatography with a HP 5890 Series II gas chromatograph (Hewlett-Packard Company, Avondale, PA,USA) with a fused-silica capillary column SP-2423 (60 m x 0.25 mm i.d., 0.20 µm) (Supelco, Inc., Bellefontaine, PA). The carrier gas (helium) flow rate was 1.9 mL/min and the split ratio was 24.8. The column temperature was held at 140°C for 6 min, programmed to 220°C at 10°C /min and held at 220°C for 15 min. The injector and detector temperatures were 230°C. Quantitative analysis was done using methyl heptadecanoate as an internal standard.

Statistical analysis

For the bioscreen experiments a 5x5 and 12x6 complete factorial designs were used. Data from the absorbance measurements was transformed to the logarithm of the cell count and was plotted against time. Bioscreen experimental data were analyzed by using an analysis of variance (ANOVA) by the SAS mixed models procedure and the repeated measures method. Data from other experiments were analyzed by analysis of variance (ANOVA) with the SAS 9.1 (SAS Institute, Cary, NC) general linear model procedure. The difference was considered significant when $P < 0.05$.

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Results

C.curvatus growth on refined glycerol and biodiesel industry-derived crude glycerol

C.curvatus growth rate was evaluated by fermentations in synthetic medium supplemented with either BDG or RG in various amounts (10, 20, 30, 40, 50, 60, 80, 100, 150, 200, and 250 g/L). Six urea concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 g/L) were with BDG and five urea concentrations (0.1, 0.2, 0.4, 0.6, and 0.8g/L) were used in the fermentations. Figures 1-3 show the resulting *C. curvatus* growth curves. *C. curvatus* was able to use glycerol from BDG as a source of carbon. There was no significant difference between *C. curvatus* growth on BDG and RG with 10 g/L glycerol (Figure 1), but there was a significant difference in growth on BDG and RG at 50, 100, 150 and 250 g/L glycerol in the medium. *C. curvatus* grew better on media with RG at 50 and 100 g/L glycerol concentration; however, BDG was the better substrate at 150 and 250 g/L of glycerol (Figure 1). The concentration of glycerol also had a significant effect on the *C. curvatus* growth rate. Figure 1 shows the growth curves of *C. curvatus* cultivated on BDG or RG at various glycerol concentrations. Figure 2 shows that as glycerol concentration increases, the decrease in growth rate accelerates and growth repressed at glycerol concentrations greater than 150 g/L.

The effect of urea concentration on *C. curvatus* growth was evaluated for each glycerol type and concentration. There was no significant effect of urea concentration on *C. curvatus* growth over a wide range of carbon/nitrogen ratios (C/N) (from 8.8 to 2097.1). Interaction between urea concentration and glycerol concentration, represented by C:N ratio in the statistical model, did not significantly effect yeast growth. Selected results are shown for various BDG and urea concentrations in Figures 3-5.

C. curvatus growth and lipid accumulation on media with various nitrogen sources

The purpose of shake flask studies was to compare nitrogen sources at various BDG concentrations. Lipid production and cell counts were measured to determine the success of the fermentations. *C. curvatus* was grown at three concentrations of BDG: 64, 120 and 200 g/L and with 0.8 g/L asparagine or 0.27 g/L ammonium chloride plus 0.1 g/L yeast extract.

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After 24 h the cell masses were constant. In agreement with the Bioscreen experiment, there was negative correlation between *C. curvatus* growth and BDG concentration (Figure 6). The best yeast growth rate was at 64 g/L BDG for both nitrogen sources. There was not a significant difference between 0.8 g/L asparagine and 0.27 g/L ammonium chloride plus 0.1 g/L yeast extract at 120 and 200 g/L BDG. However, *C. curvatus* grew faster on medium with asparagine than on medium with ammonium chloride and yeast extract at 64 g/L BDG (Figure 6). *C. curvatus* grew equally well on urea or asparagine at 64 g/L BDG (Table 3). The BDG concentration significantly affected not only biomass but lipid accumulation. Lipid accumulation was greatest at 64 g/L BDG.   concentration supported the best lipid accumulation. Yeast cells grown on the 120 and 200 g/L BDG had significantly lower fat content. The saturated fatty acids/unsaturated fatty acids ratio was inversely related to the medium BDG concentration (Table 3). The relative amount of palmitic, steric and oleic acids decreased as the BDG concentration of the medium increased. Conversely, the relative amounts of linoleic and linolenic acids were positively correlated with the BDG concentration of medium (Table 3).

Fed-batch fermentations in shake flask

Four fed-batch fermentations were performed in Fernbach flasks in an effort to increase the yield of dry yeast. During the growth phase, BDG and urea were added to the flasks, using urea and glycerol concentration as signals for addition of these substances. The lipid accumulation phase started at 96 h of fermentation when the urea concentration of the medium was zero. No additional nitrogen was added, but BDG for *de novo* lipid synthesis was added, resulting in significant lipid accumulation. The dry yeast yield was 64.8±2.8 g/L and the lipid content of the dry yeast was 62.7±2.3%. The fatty acid profile of the yeast oil was 28.6% palmitate, 11.1% stearate, 45% oleate and 11.7% linoleate (Table 4).

Fed-batch fermentations in 5L- fermenters

The cultivation of *C. curvatus* by fed-batch in shake flasks significantly increased biomass production compared with batch fermentations, so four fed-batch fermentation trials were performed in the 5L fermenters to optimize conditions and reach high cell densities and lipid

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accumulations. Fermentations were performed in two stages: In the first 48 h, cells were grown to a high density. In the second stage, BDG and urea were added according to Table 2. The feeding of BDG and urea was signaled by measurements of the dissolved oxygen, urea and glycerol concentrations. When amounts of glycerol and urea in the medium were sufficient, dissolved oxygen decreased rapidly and remained low because of cell respiration during biomass production. When dissolved oxygen began to decrease, one or both of the nutrients glycerol and/or urea had dropped to concentrations that limited yeast growth, signaling that feeding should occur. During the lipid accumulation stage 30 g/L BDG was added at 51 and 72 h in each of the fermentation trials given in Table 2. Total fed-batch fermentation time was 96 h. The biomass production and yeast cell lipid accumulation results for the four fed-batch fermentations are shown in Figures 7 and 8. The best biomass production of 61.7±2.1 was achieved during fermentation #3 in which 100 g/L glycerol were used. The lipid accumulation was above 50% on dry weight basis, which was not significantly different from fermentations #1 and #2. Yeast from fermentation #4, however, had significantly lower fat content 31.0±4.0. After 48 h of fermentation, the cell density remained constant because nitrogen was limited. Fermentation #4 had the highest yeast cell dry weight at 48 h fermentation, but the lipid accumulation phase was significantly slower between 48 and 72 h compared with the other fermentations. In fermentation #4, yeast cells did not accumulate any fat during last 24 h of fermentation despite the presence of available glycerol in the medium, which resulted in a low fat content and yeast cell dry weight.

Discussion

Meesters et al. (1996) described the growth and lipid accumulation of *C. curvatus* on a refined glycerol substrate. In the present study we found that *C.curvatus* was able to use BDG as effectively as refined glycerol if the glycerol concentration was between 10 and 50 g/L. There is no difference in *C. curvatus*' growth on RG or BDG at 10 g/L glycerol concentration, but *C. curvatus* grows significantly better on RG than on BDG at 50 and 100 g/L glycerol concentrations. The difference in growth rate for the two types of glycerol increases with their concentration. The BDG has some significant impurities, such as salts, free fatty acids, methanol that could limit *C. curvatus*' growth. At 10 g/L glycerol their

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concentrations of these impurities was probably too low to inhibit growth, but as BDG levels increased so did the putative inhibitors. At 150 and 250 g/L glycerol concentrations their osmotic value probably became the limiting factor for *C. curvatus* growth. Biomass production was inhibited almost completely at these high glycerol concentrations. Meesters et al. (1996) also reported significant growth repression when *C. curvatus* was grown on 128 and 256 g/L glycerol.

Over a wide range of C:N ratios and urea concentrations *C. curvatus*' growth rate depended on, and was negatively correlated to, the BDG concentration. Variation of the urea concentrations from 0.1 to 1.0 g/L did not affect *C. curvatus*' growth rate.

Several nitrogen sources, asparagine, urea, yeast extract and ammonium chloride, were compared. Biomass and fat content were higher when asparagine or urea was used as the sole nitrogen source. The composition of yeast oil was correlated with the fat content. The higher the fat content the greater the saturation of the oil. When *C. curvatus* was grown in ways that produce very little fat, a greater proportion of its lipid was in cell membranes, which typically are rich in unsaturated fatty acids compared with the triacylglycerols found in its fat globules. This probably explains the increase in saturation with oil percentage in the yeast. Meesters et al. (1996) characterized the fatty acid composition of yeast oil at various fermentation times; in agreement with our results, they reported that the amount of linoleate decreased while oleate and stearate increased with fermentation time. An earlier study also reported higher degree of unsaturation of yeast oil during growth phase followed by dramatic increase of oleic acid content during lipid accumulation phase (Moon and Hammond, 1978).

High glycerol concentrations inhibited *de novo* synthesis and accumulation of storage lipids. When cultivated in batch fermentations, *C. curvatus* was able to grow well and accumulate up to 60% lipids on media with 64g/L BDG and with urea as the sole nitrogen source. However, yeast dry weight yield was only 13-17 g/L, but as reported by Meesters et al. (1996), fed-batch fermentation provided a way to increase biomass production.

Previously, single cell oil production was reported to be marginally economical because of taxes, substrate and production costs (Moon and Hammond, 1978; Ratledge, 1988). Very

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low substrate costs were insufficient to make the process profitable. In this study we not only used the low-valued substrate BLG but also showed that *C. curvatus* could grow on urea, an inexpensive substrate, which might lead to lower production costs. In addition, using a fed-batch fermentation system significantly increased the yield of oil/liter and greatly improved the efficiency of single oil production. Fermentation time and aeration rate optimization may also improve the economic parameters of the process. In addition, competing vegetable oils are commanding much higher prices now than when previous calculations were made. Industrial biodiesel-based glycerol can be converted into single cell lipid and into biodiesel economically. Yeast triacylglycerol production from industrial grade glycerol potentially can increase the yield of biodiesel per feedstock unit in biodiesel plants and decrease production costs. Further work to optimize fed-batch fermentation conditions may further increase production efficiency.

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Table 1. Carbon:Nitrogen ratios used in bioscreen experiments.

Glycerol concentration (g/L)	*Urea concentration (g/L)*					
	0.1	**0.2**	**0.4**	**0.6**	**0.8**	**1**
10	84.3	42.4	21.4	14.4	10.9	8.8
20	168.2	84.3	42.4	28.4	21.4	17.2
30	252.0	126.2	63.3	42.4	31.9	25.6
40	335.9	168.2	84.3	56.3	42.4	34.0
50	419.8	210.1	105.3	70.3	52.8	42.4
60	503.6	252.0	126.2	84.3	63.3	50.7
80	671.4	335.9	168.2	112.3	84.3	67.5
100	839.1	419.8	210.1	140.2	105.3	84.3
120	1006.8	503.6	252.0	168.2	126.2	101.1
150	1258.4	629.4	314.9	210.1	157.7	126.2
200	1677.8	839.1	419.8	280.0	210.1	168.2
250	2097.1	1048.8	524.6	349.9	262.5	210.1

Table 2. The amounts of BDG (g/L) added to the 5-L fermenters at various times. Urea (0.8 g/L) was added at 0, 30 and 42 h for each fermentations.

Fermentation type	*Fermentation time (h)*				
	0	**30**	**42**	**51**	**72**
1	16	16	16	30	30
2	18	18	18	30	30
3	20	20	20	30	30
4	22	22	22	30	30

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Table 3. Average percentage lipid/yeast dry weight and fatty acid composition of *C. curvatus* grown on media with various BDG concentrations (64, 120, 200 g/L) and different nitrogen sources (0.8 g/L asparagine,0.27 g/L ammonium chloride + 0.1 g/L yeast extract (Yeast Extract) or 0.36 g/L urea.

Nitrogen source:	***Yeast Extract***			***Asparagine***			***Urea***
BDG concentration, g/L:	**64**	**120**	**200**	**64**	**120**	**200**	**64**
14:0	-	-	-	0.6	-	-	-
16:0	24.8	14.4	9.1	27.8	22.14	7.2	21.8
16:1	-	-	-	1.2	-	-	0.8
18:0	11.3	14.2	9.7	8.8	-	3.4	12.1
18:1	42.4	31.8	25.3	50.4	42.7	29.8	43.0
18:2	18.6	31.5	48.4	9.3	35.1	50.6	19.2
18:3	2.9	7.9	7.4	1.3	-	8.8	2.8
Lipid content (%)	29.9	23.2	15.2	54.6	24.4	13.1	47.1
%Unsaturated	63.9	71.3	81.1	61.0	77.9	89.3	65.0
%Saturated	36.1	28.7	18.8	37.1	22.1	10.5	34.0
Saturated/unsaturated	0.6	0.4	0.2	0.6	0.3	0.1	0.5

Table 4. Average percentage of fatty acid composition in oil extracted from *C.curvatus* grown in shake flasks on BDG in a fed-batch fermentations mean±stdev (n=4).

Fatty acid	***Weigh %***
14:0	0.50 ± 0.1
16:0	28.6 ± 0.7
16:1	1.50 ± 0.1
18:0	11.1 ± 1.4
18:1	45.0 ± 1.3
18:2	11.7 ± 2.1
18:3	1.20 ± 0.2

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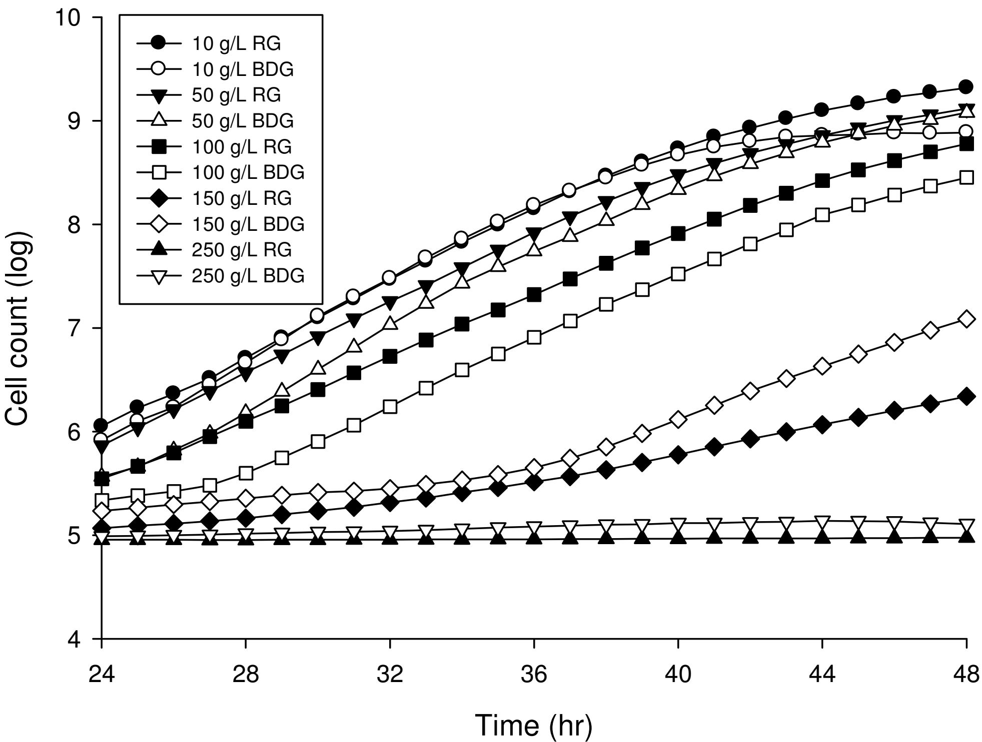

Figure 1. Log cell count versus time of *C. curvatus* grown on media with various concentrations of either RG or BDG with urea as the nitrogen source. Each point is the average over all urea concentrations. Batch fermentation.

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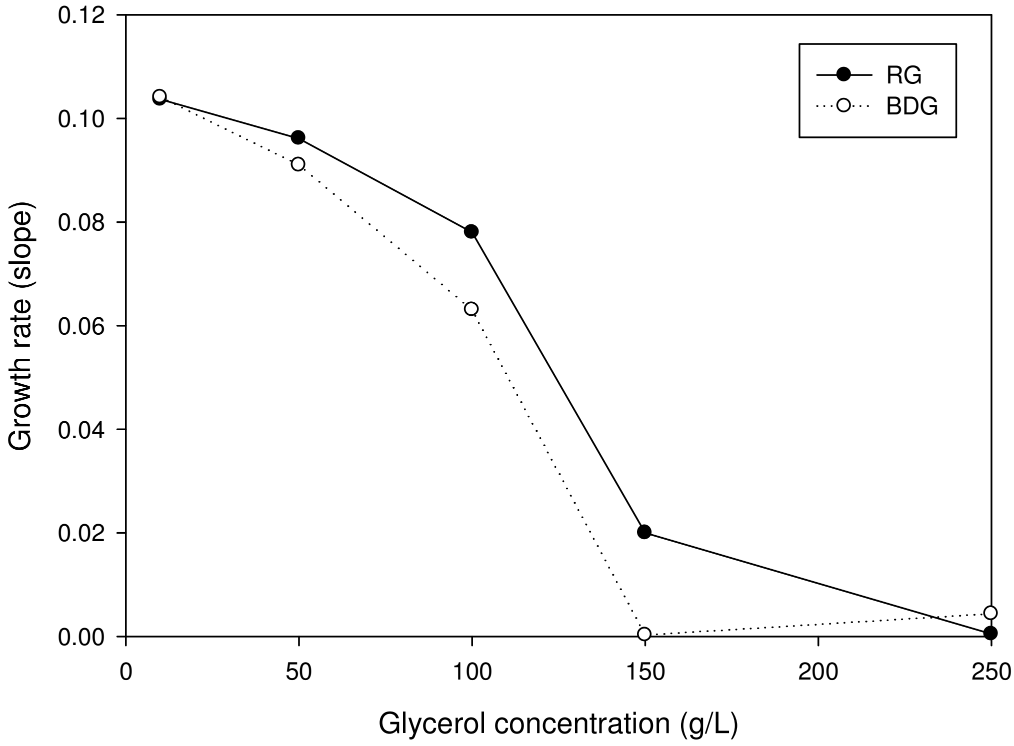

Figure 2. Growth curve slopes of *C. curvatus* grown at different concentrations of pure glycerol (RG) or biodiesel industry-derived crude glycerol (BDG). Batch fermentation.

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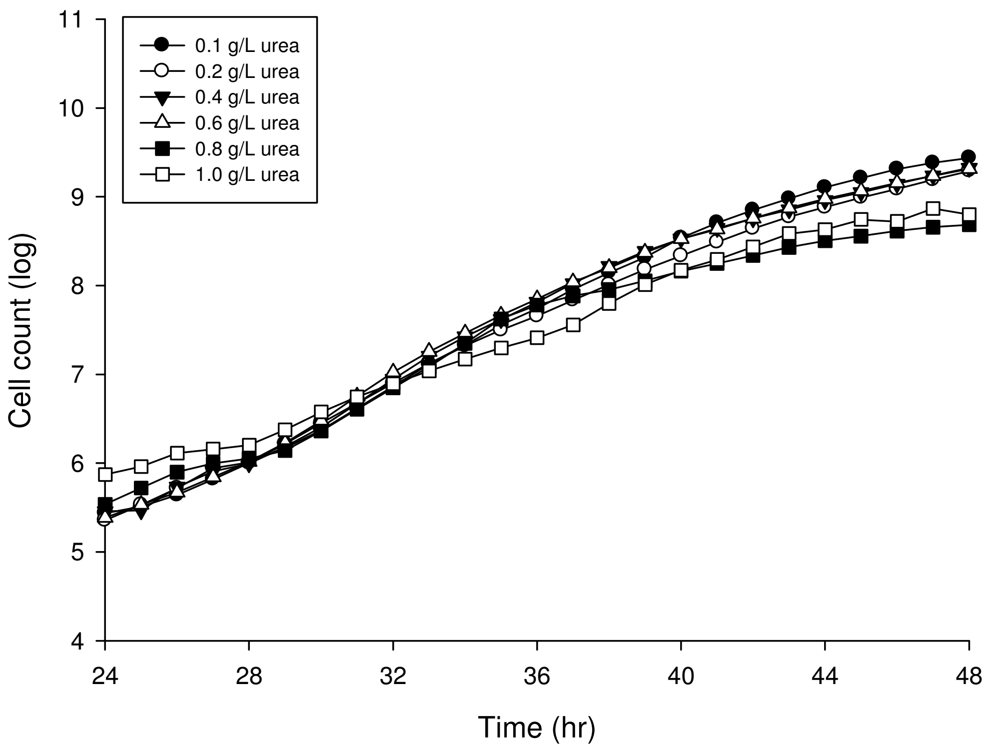

Figure 3 Urea concentration effect on *C. curvatus* growth at 20 g/L glycerol concentration in medium. *C. curvatus* was fermented with different concentrations of urea (0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 g/L) with biodiesel industry-derived crude glycerol (BDG). Each line is average of four lines that corresponds to replicated independent experiments. Batch fermentation.

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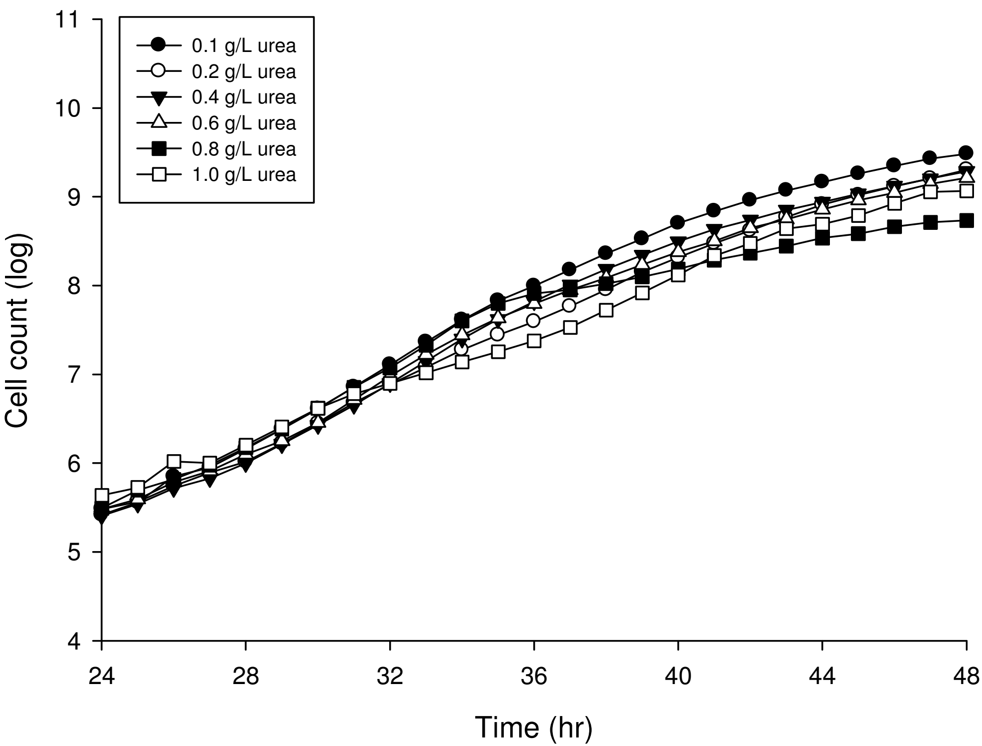

Figure 4. Urea concentration effect on *C. curvatus* growth at 30 g/L glycerol concentration in medium. *C. curvatus* was fermented with different concentrations of urea (0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 g/L) with biodiesel industry-derived crude glycerol (BDG). Each line is average of four lines that corresponds to replicated independent experiments. Batch fermentation.

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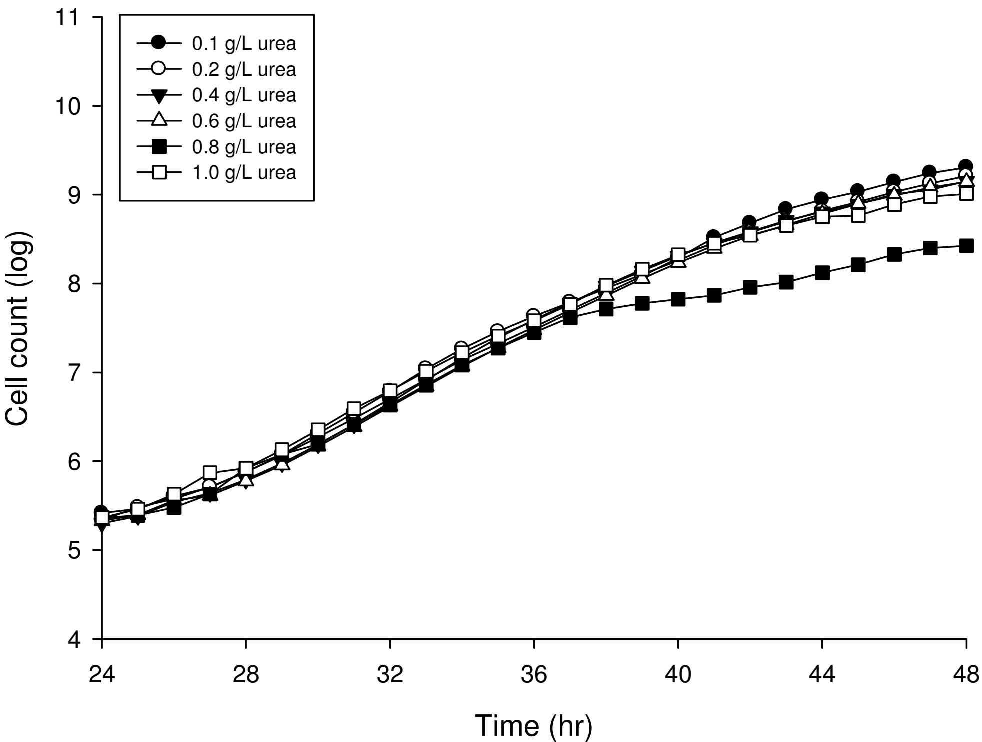

Figure 5. Urea concentration effect on *C. curvatus* growth at 40 g/L glycerol concentration in medium. *C. curvatus* was fermented with different concentrations of urea (0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 g/L) with biodiesel industry-derived crude glycerol (BDG). Each line is average of four lines that corresponds to replicated independent experiments. Batch fermentation.

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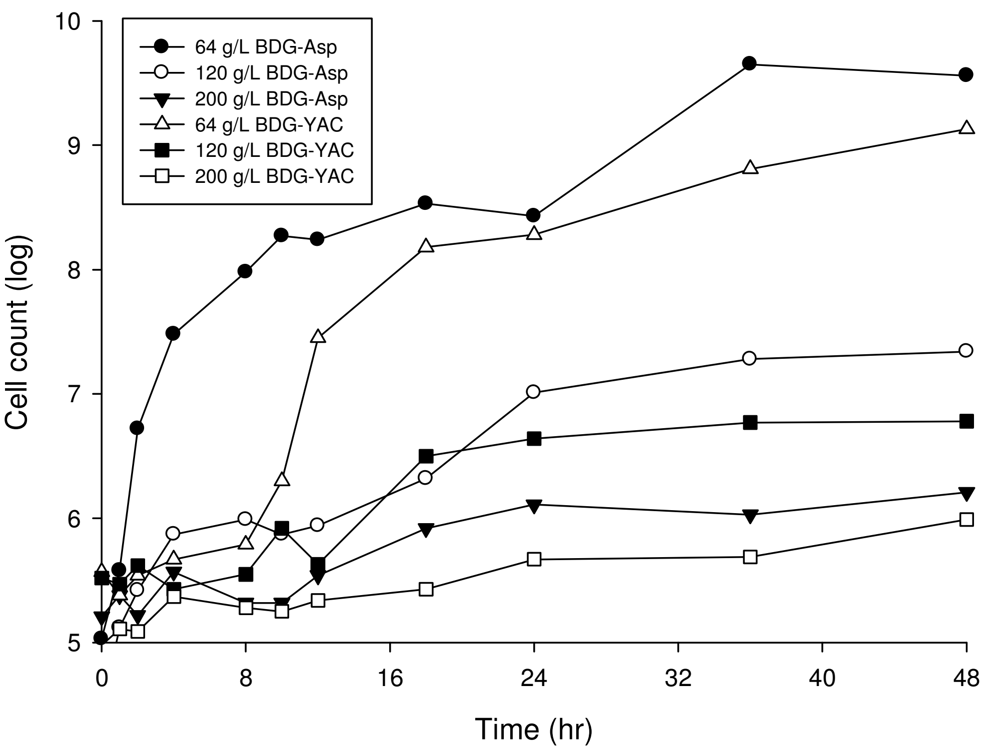

Figure 6. *C. curvatus* growth on medium with different BDG concentrations (64 g/L, 120 g/L, 200 g/L) with 0.8 g/L asparagine (Asp) or 0.27 g/L ammonium chloride with 0.1 g/L yeast extract (YAC). Cell count(log) was plotted over time (hr). Each line is average of replicated independent experiments (n=2). Batch fermentation.

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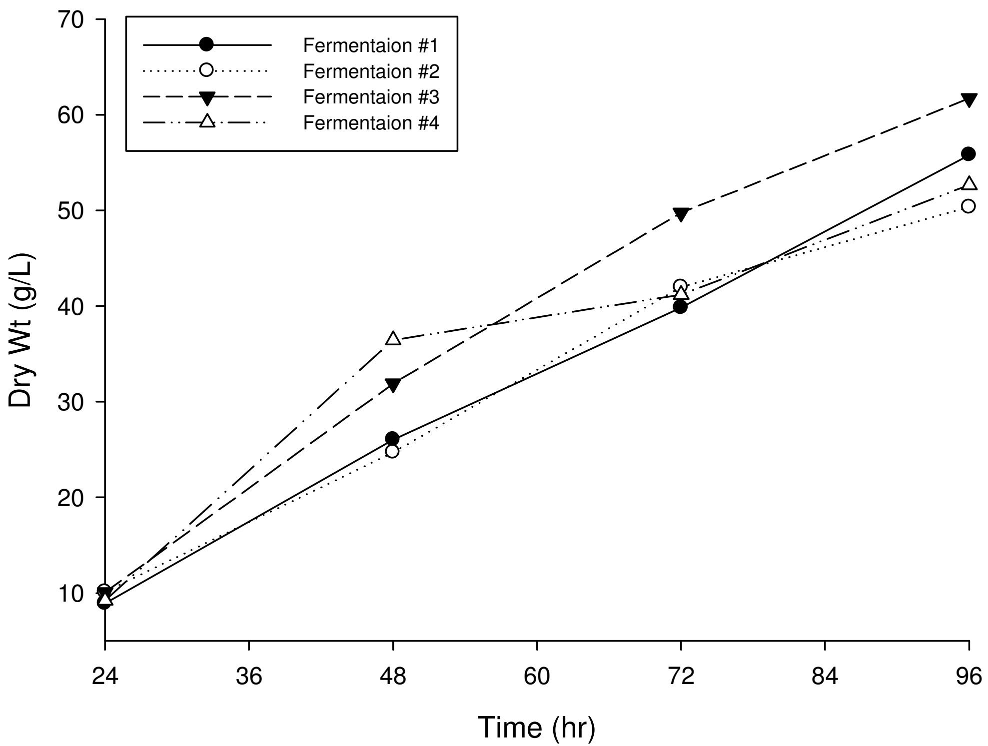

Figure 7. Biomass production. Fed-batch fermentations in 5-L fermenters. Four fermentation trials(table 2): See Table 2 for conditions of fed batching;
BDG was added at 0, 30, 42, 51 and 72 h. Urea (0.8 g/L) was added at 0, 30 and 42 h of fermentation for each trial.

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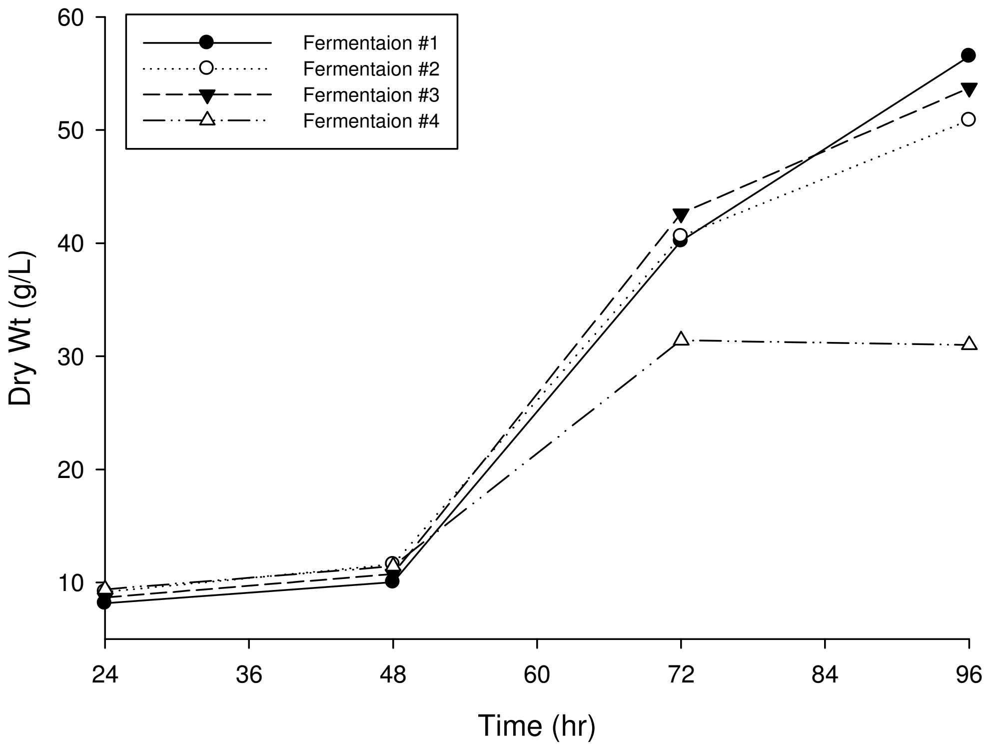

Figure 8. Lipid accumulation. Fed-batch fermentations in 5-L fermenters. Four fermentation trials (table 2): BDG was added at 0, 30, 42, 51 and 72 h. Urea (0.8 g/L) was added at 0, 30 and 42 h of fermentation for each trial.

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Chapter 8: General Conclusions

Oleaginous yeast cells have the ability to synthesize oil from carbon sources or to adsorb fatty acids from their growth medium when grown on restricted medium. Fish oil, linseed or conjugated linoleic acid (CLA)-rich oils were encapsulated in *Cryptococcus curvatus*.

C. curvatus encapsulation improved oxidative stability of long-chain polyunsaturated fatty acids and CLA. In vitro results suggested that encapsulation in yeast was an effective method to protect polyunsaturated fatty acids from rumen hydrogenation. Yeast cell viability was not critical for the oxidative and rumen stability of the encapsulated fish oil. A 100-L fed-batch fermentation procedure was developed for fish oil encapsulation. About 340L (10.1% DM) concentrated liquid yeast product with encapsulated fish oil was produced by six 100L fed-batch fermentations. Biomass production was 54.9 g/L and fat content 59.2% on cell dry weight basis. All DHA and EPA have been stored in intracellular lipid droplets because they have not been found in phospholipid fraction. Phosphatidylcholine was major *Cryptococcus curvatus* phospholipid.

Three lactating cows were fed with wet dead yeast containing encapsulated fish oil. Dietary treatment did not have significant effect on milk fat composition. No increased feed refusal or milk fat production was detected; however, no omega-3 fatty acids from fish oil were detected in milk fat after 20 days on the treatment diet.

The oleaginous yeast *Cryptococcus curvatus* was able to grow as well as synthesize and accumulate triacylglycerols in lipid bodies when cultivated on industrial-grade glycerol that was a byproduct of biodiesel production. Fed-batch fermentations suggested for maximal biomass production up to 64.8±2.8 g/L and lipid accumulation up to 62.7±2.3% on dry weight basis. Thus, industrial biodiesel-based glycerol can be converted by yeast into single cell lipid, which could be further transformed into biodiesel and potentially increase the yield of biodiesel per feedstock unit.

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