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Oxidative Stability of Polyunsaturated Triacylglycerols Encapsulated in Oleaginous Yeast

Diliara R. Iassonova · Earl G. Hammond · Samuel E. Beattie

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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 °C 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.

Keywords Oleaginous yeast · Encapsulation · Fish oil · CLA · Oxidative stability

D. R. Iassonova · E. G. Hammond · S. E. Beattie (✉)
Department of Food Science and Human Nutrition,
Iowa State University, 2312 Food Sciences Building,
Ames, IA 50011-1060, USA
e-mail: beatties@iastate.edu

D. R. Iassonova
e-mail: diliara@iastate.edu

E. G. Hammond
e-mail: hammond@iastate.edu

Abbreviations

BHA	Butylated hydroxyanisole
CLA	Conjugated linoleic acid
C. curvatus	*Cryptococcus curvatus*
CLAY	*Cryptococcus curvatus* cells with encapsulated Clarinol^{TM} A-80
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FY	*Cryptococcus curvatus* cells with encapsulated menhaden fish oil
LCPUFA	Long-chain polyunsaturated fatty acids
LY	*Cryptococcus curvatus* cells with de novo synthesized oil from lactose
PV	Peroxide value
TAG	Triacylglycerols

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 [1, 2], reduce the symptoms in rheumatoid arthritis [3] and are essential for the development and function of the brain and retina [4].

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 [5–7].

CLA and LCPUFA are very susceptible to oxidation due to 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 [8] and LCPUFA-containing algae [9]. 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 [10]. *C. curvatus* grows in cheese whey permeate and converts lactose to fatty acids, which it stores in discrete, intercellular droplets as triacylglycerols (TAG). It can accumulate up to 60% of its dry weight as lipids when grown under nitrogen limitation [11]. 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 [12]. In the present study, the oxidative stability of yeast-encapsulated polyunsaturated fatty acids from fish oil and a 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 Difco™ (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 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, $FeCl_3{\cdot}6H_2O$ 0.02 g/L, $MnSO_4{\cdot}H_2O$ 0.002 g/L, $ZnSO_4{\cdot}7H_2O$ 0.0001 g/L, $CuSO_4{\cdot}5H_2O$ 0.0001 g/L, and NaCl 0.6 g/L [13]. The autoclaved basal medium containing 50 g/L lactose was adjusted to pH 5.4 with 3N 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 °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.

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 0.5M sodium hydroxide solution. After 24 h of fermentation, 27 g sterile menhaden fish oil (Omega Protein, Inc., Reedville, VA, USA) containing 27 mg 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 °C. After 24 h of fermentation, 18 g of autoclaved Clarinol™ 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 utilization was determined 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.

These do not have unequal replicates affect because in this paper we evaluated oxidative stability of encapsulated oils (yeast cell protective effect) not a fermentation conditions effect or oil type on encapsulation effect.

Yeast and oil extraction. Oils were fed to the yeast in excess. Thus, it was necessary to remove excess fish oil or Clarinol™ 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 $9{,}000\times g$ 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. [14]. Lipids from the

freeze-dried yeast cells were extracted by the methanol–chloroform method [10].

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 × 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.

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) 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 °C 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 [10] and incubated at 52 °C in the dark. Dead and viable yeast cells were also incubated at 52 °C 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 [15]. 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 using oils of known PV (standards). The AOCS method Cd 8–53 [16] 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 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™ 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™ A-80 in their medium as determined by the residual oil in the hexane extractions. Lee et al. [12] showed 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 [12, 17]. 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 polyunsaturates (Table 2). This may be caused partially by some oil production from lactose before the medium oils

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 + 18 g/L fish oil (FY)	18.4 ± 1.3	49.7 ± 0.8
13.5 g/L lactose + 18 g/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	–
c9, t11-18:2	–	–	27.4	–	38.6
t10, c12-18:2	–	–	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	–

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 °C had mean PV of 2.0 ± 0.4 mequiv/kg for dead yeast and 1.3 ± 0.5 mequiv/kg for viable yeast (Fig. 1). The mean PV of lipid extracted on the starting day from dead and viable LY and stored at 52 °C for 57 days was 174.4 ± 0.6 and 200.0 ± 0.6 mequiv/kg, respectively (Fig. 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 extracted LY oil from dead yeast. Similar to LY oil, extracted FY oil oxidized significantly faster than 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 (Fig. 2). After 28 days of storage at 52 °C, 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.

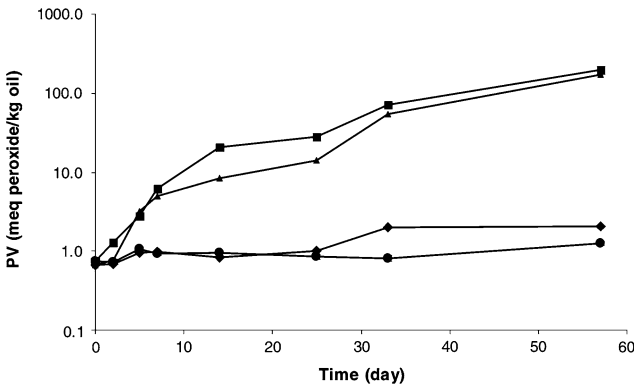

Fig. 1 Peroxide values of oils extracted on day 0 and stored at 52 °C for 57 days from dead (*filled triangles*) (standard error of the mean (SEM) ± 21.20) and viable (*filled squares*) (SEM ± 24.12) yeast fermented with 50 g/L lactose (LY), and oils extracted from dead (*filled diamonds*) (SEM ± 0.20)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

After 56 days of incubation at 52 °C 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 (Fig. 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 (Fig. 3) during a 6-week period. After 42 days of incubation at 52 °C, the mean PV of oil from viable CLAY did not change significantly compare to its PV at zero time (Fig. 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

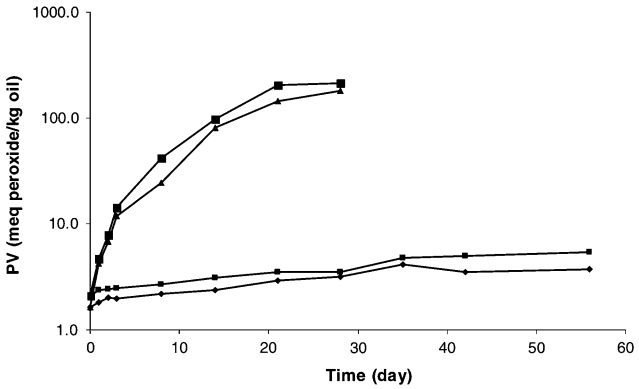

Fig. 2 Peroxide values of oils extracted from dead (*filled triangles*) (SEM ± 25.95) and viable (*filled squares*) (SEM ± 21.25) yeast with fish oil (FY) on day 0 and stored at 52 °C for 56 days, and peroxide values of oils extracted from dead (*filled diamonds*) (SEM ± 0.36) and viable (*filled circles*) (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 °C

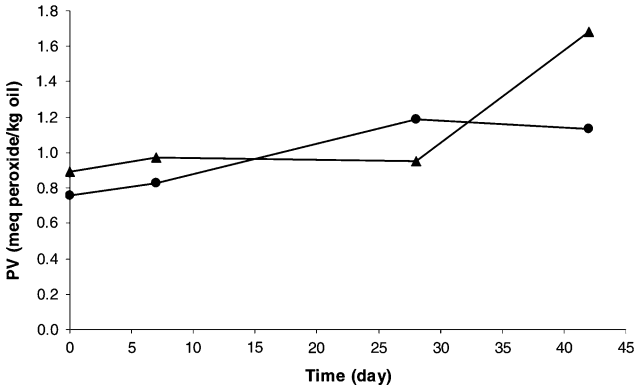

Fig. 3 Peroxide values of oils extracted on 0, 7, 28, and 42 days of incubation at 52 °C from dead (*filled triangles*) (SEM ± 0.19) and viable (*filled circles*) (SEM ± 0.11) yeast encapsulated with Clarinol™ A-80 oil (CLAY)

42 days oil from dead CLAY had a PV of 1.7 ± 0.5 mequiv/kg (Fig. 3).

Suzuki et al. [18] 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 °C 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. [18] experiments but considering the LY oil stability after 42 days of incubation at 52 °C 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 [11, 19]. 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 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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