NATURAL WHOLE-CELL OIL MICROCAPSULES AS INNOVATIVE DIETS FOR LIVE FEEDS

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Abstract

Aquaculture plays an increasingly important role in meeting the world's food needs. To meet the demands of an ever growing human population, improvements in finfish larvae feed are needed. Rotifers are a widely used live feed for finfish larvae, but lack certain key nutrients such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). In order to enrich rotifers while avoiding decreased water quality from excess oil, this study investigated the encapsulation of EPA and DHA within the oleaginous yeast, *Yarrowia lipolytica*. A 2^3 factorial experiment was conducted to determine the effects of altering the culture conditions to improve EPA and DHA concentrations within the yeast. The factors investigated were hydrolysis of enrichment oil, temperature, and oxygen presence. Additionally, this research studied the utilization of a waste feedstock, papaya seed oil, in order to culture *Y. lipolytica*. This study demonstrated that the factor of hydrolysis and the interaction between temperature and oxygen to be statistically significant ($P<0.05$), with the best combination of hydrolysis, 25^{o}C, and oxygen presence yielding an enriched yeast with 9.88 mg/g EPA per dry cell weight and 9.71 mg/g DHA per dry cell weight, and 27% total lipid by weight. For utilization of waste papaya seed oil, it was determined that an ethanol pretreatment of the papaya seeds prior to lipid extraction was able to minimize the anti-microbial compound benzyl isothiocyanate concentrations within the oil, thus allowing for *Y. lipolytica* culturing. Taken together the results of this study show the possibility of pretreating papaya seeds to unlock the oil's utilization as a waste feedstock for culturing *Y. lipolytica* and the ability to alter culture conditions to influence EPA and DHA concentrations within this yeast.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations & Terms

BDT: Benzenedithiol

BITC: Benzyl isothiocyanate

DHA: Docosahexaenoic acid

EPA: Eicosapentaenoic acid

FA: Fatty acid

Finfish: Refers to true fish, as opposed to other aquatic organisms like starfish

GC: Gas chromatography (gas-liquid chromatography)

HG: High glucose, referring to a type of media that has high levels of glucose (see materials and methods)

OD/OD_{600}: Optical density measured at 600 nm.

RP-HPLC: Reverse phase high performance liquid chromatography

SD: Synthetic Dextrose (d-glucose), used as a growth media (see materials and methods)

Chapter 1. Introduction

1.1 The Importance of Aquaculture

As the world's wild fisheries reach the limits of maximum sustainability or overexploitation, the total tonnage of fish from wild catch has been reaching a plateau according to reports by the Food and Agriculture Organization of the United Nations (1). This means as the human population continues to increase, more and more of the seafood demand will need to be met by aquaculture.

Indeed, this ever increasing importance of aquaculture is reflected in the fact that back in 1970, only 4% of the world's fish came from aquaculture, fast forward to 2013, FAO statistics showed that fish from aquaculture represented 43.1% of the world's total. With further increases in market share expected in the future. The FAO predicts that soon, more fish will be produced from aquaculture than from wild catch. Moreover, 2013 saw 97.2 million tonnes of live weight aquaculture produced that had an estimated 157 billion USD in value (2).

While these numbers are very impressive, what exactly is aquaculture? In essence, aquaculture is the farming of aquatic flora and fauna, akin to that of traditional land-based agriculture (3). And like agriculture, the potential boons from aquaculture depends on the knowledge, techniques, and research that improves quality and yields.

1.2 The Issue of Feed in Aquaculture

While there have been great strides in aquaculture research, from the industry's modern beginnings to the more recent improvements in high density aquaculture, feed research remains of interest.

Feed remains one of the highest costs for aquaculture farmers, accounting for 50-70% of production costs (4). This cost represents a hindrance for current aquaculture farmers, and also a deterrence for potential new farmers. Of particular importance is the effective large scale raising

of finfish larvae (5). After utilization of the yolk-sac, finfish larvae are susceptible to starvation if not provided with adequate nutrition. Because the larvae are often raised in hatcheries, they are entirely dependent on the diet supplied by farmers. Thus, in order to promote sustainability and economic feasibility of finfish aquaculture, efficacious feed for larvae survival and growth is imperative.

1.2.1 Types of Feed

To meet the need for feed, there are two broad types to consider, live feed and manufactured inert feed. Despite improvements in manufactured feed, such feeds have met with mixed success with regards to finfish larvae (6, 7). Thus, live feed remains an integral part of the larvae diet.

Within the realm of live feeds, there are several candidates for use, namely that of shrimps (*Artemia* sp.), copepods, and rotifers (*Brachionus* sp.). Of these three types of live feed, shrimps, due to their size, may not be suitable for all finfish larvae species, and although copepods are a part of the natural diet of fish larvae in the wild, their relatively higher costs lower yields remains problematic (8). On the other hand, rotifers have not only been shown to be viable feed for finfish larvae, but also have been shown to be easier to grow than copepods (9). Other potential benefits of rotifers include, shape, motility eliciting visual response for feeding, digestibility, and relatively low cost of production (10). Thus, this research was conducted with rotifers as part of the feed type in mind.

1.2.2 Rotifers cultured using yeast

Besides the benefits mentioned above, the popularity of rotifers in aquaculture is due in part to the discovery that rotifers could be cultured on yeast, usually *Saccharomyces cerevisiae* (10). Yeasts are relatively cheap and easy to grow, making it an ideal candidate for rotifer culture. In fact, *S. cerevisiae* has been shown that it, by itself, was sufficient to cultivate rotifers (11). By utilizing these two organisms, both of which are relatively fast, cheap, and easy to

grow, increases in rotifer production were observed and it was believed this would allow for the intensification of aquaculture production.

1.2.3 The drawbacks of rotifers cultured using yeast

Despite early optimism of culturing rotifers using yeast, soon several limitations arose. One of the main drawbacks was that rotifers grown on yeast often lack certain fatty acids (FA), especially highly unsaturated fatty acids (HUFAs) such as eicosapentaenoic acid (EPA C20:5, n-3) and docosahexaenoic acid (DHA C22:6, n-3) (12).

This lack of EPA and DHA in rotifers was problematic because research has shown the importance of EPA and DHA in developing an efficacious feed for larvae (5). EPA and DHA are considered essential fatty acids for many finfish species, because they are required for biological function, but are not synthesized by the finfish, and thus must be acquired through diet. Therefore, to fully actualize the benefits from using yeast, there had to be some way of enriching the rotifer with the needed EPA and DHA.

1.3 Methods for enriching rotifers with EPA and DHA

Several methods for fortifying rotifers with EPA and DHA have been investigated. Literature has demonstrated that the nutritional value of rotifers depended on what the rotifer ingests (5). Thus, many enrichment strategies have revolved around the notion of enriching the diet of the rotifer.

Traditional techniques involved attempts at adding emulsified oils containing HUFAs directly to the rotifer cultures. Despite some success, the major drawback was that this muddled the water with oil, decreasing water quality. Additionally, too much added oil could lead to increased rotifer mortality (13).

Another strategy was to enrich the rotifer by adding a microalgae to the rotifer feed (14, 15). However, this too provided its own drawbacks, mainly the production costs and storage of

such diets (16). Alternatively, manufactured feeds could be used to fortify rotifers, however costs and dependency on imported feeds could be an issue with this method. Furthermore, it has been shown that manufactured oil powders used in attempts to fortify rotifers with DHA met with limited success (17).

1.4 Methods for enriching yeast with EPA and DHA

Most of the above mentioned methods utilized a baseline diet for rotifers, such as yeast, and then attempted to enrich the rotifer independent of the yeast. However, it was hypothesized that if the yeast itself could be enriched, then this would allow for encapsulation of EPA and DHA, forming a dietary capsule of sorts for the rotifer. By placing EPA and DHA within the yeast cell, this would resolve the problem of water quality by preventing excess oil in the culture. Previous attempts at enriching yeast with EPA and DHA resulted in the FA adsorbed to the surface of the yeast cell (18). Further research into using the yeast *Pichia methanolica* HA-32 showed improvements and used methods that attempted to wash away externally absorbed FA, but also required high quality oils with high DHA content (28%) (17).

1.4.1 Identifying the yeast *Yarrowia lipolytica* to use for EPA and DHA encapsulation

One aspect to note was that the previous work, mentioned above, utilized yeast strains that were non-oleaginous. Because the desired products, EPA and DHA, are often found in triglycerides, it was determined that this research would focus on one of the more studied oleaginous microorganisms, *Yarrowia lipolytica*, to leverage the yeast's natural cellular machinery to aid in EPA and DHA encapsulation.

Y. lipolytica has garnered much attention from many different fields due to its natural ability to produce lipases, surfactants, and accumulate lipids intracellularly as lipid bodies. This yeast has been reported to be able to accumulate over 40% lipids by dry weight (19). This yeast is able to accumulate lipids by two main methods, *de novo* and *ex novo* (20). Briefly, *de novo* lipid synthesis involves the synthesis of lipids during conditions of excess glucose and nitrogen

limitation (high C:N ratio). Nitrogen limitation leads to decreased nucleic acid and protein production, thus limiting cellular division, however carbon sources from the media may still be taken up by the cells (21). This continued intake and processing of high levels of glucose via glycolysis leads to excess levels of acetyl-CoA which is converted to citric acid (citrate) and instead of entering the citric acid cycle, the excess citric acid is redirected to the cytosol where it is reverted back to acetyl-CoA and then utilized in lipid synthesis, and subsequent storage as triglycerides in lipid bodies. Thus, excess carbon is converted into lipids under nitrogen limitation.

While naturally occurring strains of *Y. lipolytica* lack the genes coding for the appropriate enzymes to synthesize EPA and DHA from shorter chain FA, there has been research into genetic modification of *Y. lipolytica* to add the genes that would allow it to synthesize EPA (22). While this genetically modified organism (GMO) is capable of accumulating 30.2% EPA (% of total lipids), this GMO has two inherent problems. The first is that the particular strain used was a proprietary strain developed by DuPont, making commercialization and use of this strain restricted. Secondly, because this GMO would be utilized in aquaculture that is often open to the environment, there are issues and controversy that may result from the GMOs use, not to mention possible consumer concerns. Thus, this research aims to provide a method for encapsulating EPA and DHA, but utilizing non-GMOs.

Because EPA and DHA cannot be synthesized in natural strains, this research turned to the process of e*x novo* synthesis, which involves uptake of lipids from the media. Secretion of extracellular lipases by *Y. lipolytica* along with biosurfactants and additional membrane bound lipases help break down lipids for uptake, via both passive diffusion and facilitated by active transport (21). Once in the cell, fatty acids can be directed toward β-oxidation for utilization in energy production, or undergo triacylglycerol synthesis and storage in lipid bodies. The FA stored in lipid bodies can at a later time be mobilized and also undergo β-oxidation.

1.5 Identifying factors for FA accumulation

One of the main goals of this research was to develop an enriching process for *Y. lipolytica* to encapsulate EPA and DHA. Because the current research aimed to avoid GMOs, it was hypothesized that altering culture conditions of the enriching yeast could yield improvements in EPA and DHA levels. In a very broad sense, EPA and DHA levels in *Y. lipolytica* are the net result of FA accumulation and FA degradation.

FA accumulation is due in part to the production, secretion, and action of various enzymes including extracellular lipase and surfactants. One of the determining factors in the production of these secreted products is temperature. The optimal temperature for lipase production is close to the optimal temperature for growth, at 28-30^{o}C (23). Thus, a slightly elevated temperature has potential benefits to lipid accumulation. Likewise, oxygen also has a potential benefit to lipid accumulation. Because *Y. lipolytica* is an aerobic organism, and does not undergo fermentation, oxygen presence is important for cellular functions, including the production and secretion of lipase (24). FA accumulation is also dependent on the hydrolysis of the oil substrate since triglycerides need to be converted into free FA prior to absorption into the cell.

FA degradation can occur in several ways. One possible avenue of degradation is the oxidation. Because EPA and DHA are highly unsaturated FA, they are especially susceptible to oxidation. The process of FA oxidation can be initiated by several means including light and increased temperature, resulting in formation of free radicals, which can then produce peroxyl radicals by reacting with molecular oxygen. These peroxyl radicals can then propagate the oxidation reaction by reacting with a new FA, causing formation of another free radical, continuing the chain reaction (25).

Additionally, FA degradation can proceed via β-oxidation, which is the catabolic mechanism utilized by cells to mobilize and begin metabolism of FA from lipid stores. Unlike most eukaryotes, β-oxidation in yeasts occurs only in peroxisomes. The mobilized FA enters the peroxisome and is converted into acyl-CoA. Acyl-CoA is then degraded via the β-oxidation pathway utilizing oxygen, H_2O, and NAD^+ to produce H_2O_2, NADH, and 3-ketoacyl-CoA that

can reenter the β-oxidation pathway, or be converted to acetyl-CoA to enter the glyoxylate cycle producing succinate and oxaloacetate. Succinate can enter the citric acid cycle in the mitochondria, while oxaloacetate may enter the cytoplasm and undergo gluconeogenesis (26).

Thus, by analyzing these aspects of FA accumulation and FA degradation, this research identified three culture factors that may affect EPA and DHA levels, while also being relatively easy to control. The factors identified were hydrolysis of enriching lipids, temperature, and oxygen presence. As noted from above, the factors of temperature and oxygen both have confounding effects. These two factors have potential effects on both increasing lipid accumulation and degradation. Thus, this study aims to help elucidate the net effect of these factors. A simplified overview is available with Fig. A1 in the appendix

1.6 Producing *Y. lipolytica* locally in tropical and subtropical regions

After identifying *Y. lipolytica* as the oleaginous organism of interest for encapsulating EPA and DHA, consideration was paid to possible feedstock for growing this yeast. Because this research focuses on impact on the tropical and subtropical regions that require importation of most goods, it would be a boon to these regions to be able to grow *Y. lipolytica* using locally available feedstock, and thus reduce costs and risks of disruption associated with imports. A suitable feedstock that was identified was *Carica papaya*, a major crop cultivated in tropical regions. In fact, in 2014, Hawaii produced 23.5 million pounds of papaya, with an estimated value of $11.3 USD (27). Furthermore, it is estimated that culled papaya represent 35%-50% of produced papaya (28). This represents a large source of cheap, waste feedstock. There have been several studies investigating the use of waste papaya to produce value added products via microbial culture (28, 29), however, these studies avoided the use of papaya seeds, a feedstock this research aims to utilize.

1.6.1 Papaya seeds as waste feedstock

Papaya seeds are a promising untapped feedstock for oleaginous microbial culture. Papaya seeds represent a large volume of the papaya fruit and the seeds contain high lipid content, up to 30% (30), great for oleaginous organisms such as *Y. lipolytica*. Culturing *Y. lipolytica* on oil substrate would have the added benefit of decreased contamination, as a reduced number of microbial species are able to grow on lipids as a sole carbon source, as opposed to glucose. This in turn would facilitate rural growth of *Y. lipolytica* in agricultural regions where papaya are cultivated. However, there is a potential obstacle that may explain the apparent dearth of papaya seed oil culture studies, the fact that papaya seed oil extraction may result in formation of a compound called benzyl isothiocyanate (BITC).

1.6.2 Obstacle of benzyl isothiocyanate (BITC)

BITC is an obstacle to utilization of papaya seed oil because BITC has anti-microbial activity (31, 32). BITC is a compound that is formed when the enzyme myrosinase (thioclucosidase) catalyzes the conversion of the precursor benzyl glucosinolate into BITC. Normally, myrosinase and benzyl glucosinolate are spatially separated with myrosinase largely sequestered in the vacuoles of specialized myrosin cells, and glucosinolate located in the vacuoles of separate but adjacent "s-cells" (33). Specifically for papaya seeds, myrosinase activity has been reported in the sarcotestae (fleshy outer coat) and also in the embryo (34). When tissue damage occurs, myrosinase and benzyl glucosinolate come into contact hydrolyzing benzyl glucosinolate into BITC. In this way, this system acts as a defense against microbes during tissue damage.

Unfortunately, effective oil extraction requires the mechanical crushing of seeds, which generates BITC. It should be noted that while BITC has anti-microbial activity, the literature is unclear as to the effect of BITC on yeast (35, 36) and these studies investigated plated yeast cultures, not liquid yeast cultures.

1.6.3 Ethanol as a pretreatment for deactivating myrosinase

The literature has shown that simple alcohols such as ethanol are able to reduce myrosinase activity (37). However, that study investigated the effect of ethanol on commercial enzyme standard, not *in vivo* plant tissue. Other possible methods for myrosinase deactivation have been investigated and include methanol, biodegradation, and ultraviolet irradiation (38). Despite this, ethanol as a pretreatment remains an attractive option due to the ease of use and low-tech threshold, making it more appealing for application in rural areas.

Ethanol soaking would be a pretreatment for the papaya seeds because myrosinase needs to be deactivated prior to oil extraction in order to avoid BITC formation. Additionally, since ethanol is not consumed in the deactivation of myrosinase, the same batch of ethanol could potentially be used to pretreat several batches of seeds, provided evaporation is kept to a minimum.

1.7 Summary and focus of the current study

A review of the literature has shown that in order to address the issue of producing an efficacious live feed for finfish larvae, EPA and DHA are of importance. While rotifers offer aquaculture farmers several advantages, the lack of EPA and DHA in rotifers is an obstacle to their use. Thus, in order to fortify rotifers with EPA and DHA, the current study investigates the encapsulation of these omega-3 fatty acids within an oleaginous yeast, *Y. lipolytica*. Furthermore, to alleviate the need for importation, locally available waste feedstock for *Y. lipolytica* could be provided by papaya seed oil. However, in order to unlock papaya seed oil, BITC concentrations need to be addressed. To this end, an ethanol pretreatment to deactivate myrosinase prior to oil extraction is a promising option to investigate.

1.7.1 Objectives:

The current study aims to develop a method to encapsulate EPA and DHA within the oleaginous yeast, *Y. lipolytica*, thereby creating an fortified yeast to aid in the production of an enrichment feed for finfish larvae.

The present research also aims to utilize a locally available waste feedstock to culture *Y. lipolytica* to enable production of this yeast and its large repertoire of value added products, helping to reduce waste, and also reduce reliance on imports within the tropical and subtropical regions.

1.7.2 Hypotheses:

It was hypothesized that by utilizing *Y. lipolytica*, capable of uptake and storage of FA from the media, that this yeast would be able to encapsulate EPA and DHA, and provide an enriched feedstock for culturing rotifers destined for finfish larvae feed.

It was also hypothesized that altering culture conditions of hydrolysis, temperature, and oxygen presence, that the levels of EPA and DHA within *Y. lipolytica* could be altered, thus resulting in higher EPA and DHA concentrations without needing to utilize genetic modification.

Furthermore, it was hypothesized that this enriched yeast could be stored in various methods such as a dry storage, lyophilized powder, or a wet, refrigerated paste.

Additionally, it was postulated that *Y. lipolytica* could also be cultured on papaya seed oil, if BITC concentration in the oil is not inhibitory, unlocking papaya oil as a cheap, waste feedstock for oleaginous microbial cultures

Chapter 2. Materials & Methods

All chemicals are reagent grade purchased from commercial vendors such as Sigma and VWR, unless otherwise specified.

2.1 Yeast culture conditions and media

Y. lipolytica strain ACA-DC-10509 was precultured in 5 mL synthetic dextrose (SD) media. SD media consisted of 1.7 g L^{-1} of yeast nitrogen base without nitrogen and without amino acids, 5 g L^{-1} of ammonium sulfate, and 20 g L^{-1} of d-glucose. This preculture was grown for 24 hrs in a shaking incubator at 25^{o}C and 200 rpm.

This preculture was used to inoculate beveled flasks that contained SD media and grown at 25^{o}C and 200 rpm. Growth cultures were inoculated to an initial optical density (OD) of 0.01 AU using GeneQuant Pro spectrophotometer at a wavelength of 600 nm.

During the FA enrichment phase, the yeast undergo a media switch from SD to high glucose (HG) media. Yeast cells grown in SD media were collected via centrifugation at 7500 rpm for 3 minutes, then resuspended into HG media, which consisted of 80 g L^{-1} of d-glucose, 6.3 g L^{-1} of potassium phosphate monobasic and 20.6 g L^{-1} of potassium phosphate dibasic, adjusted to pH of 7.4. After media switch to HG, 4% of menhaden fish oil was added as an extracellular lipid source of EPA and DHA for enrichment.

The yeast culture was enriched with various culture conditions. All cultures where shaken at 100 rpm. Each factor was tested at two levels. Temperature was controlled by either an incubator set to 25^{o}C, or in a cold room set to 4^{o}C. Oxygen presence was either allowed via breathable flask cap or disallowed via nitrogen flushing and rubber stopper. Hydrolysis of the lipids used in enrichment was achieved by incubating menhaden fish oil with a final concentration of 1M NaOH in a capped, nitrogen flushed glass container. This container was placed in a water bath set at 60^{o}C, with constant stirring of the hydrolyzing oil for 1 hr.

2.2 Reducing sugar assay

Glucose in culture was measured via reducing sugar assay. Culture supernatant was collected via centrifugation at 7500 rpm for 3 min. 3,5-dinitrosalicylic acid (DNS) solution is prepared by weighing 5 g of DNS, 5 g of NaOH, 1.115 mL of 90% liquefied phenol, 0.25 g of sodium sulfite, and distilled water to a total volume of 500 mL. The assay is conducted by mixing 500 µL of sample and 500 µL DNS reagent in 1.7 mL tubes. The tubes are boiled for 5 min, after which 250 µL of 40% potassium sodium titrade was immediately added to stop the reaction. 200 µL of each sample was pipetted into a 96 well plate and absorbance was read at 580 nm using a Tecan Safire Microplate Reader.

2.3 Titration to monitor hydrolysis of oils

To quantify the progression of hydrolysis, titration using a graduated burette was conducted. Oil was hydrolyzed as previously described (section 2.1). At set time points during hydrolysis, samples were taken. The samples were titrated using a standardized 0.364 M HCl solution. A pH indicator of 0.5% (w/w) phenolphthalein in a solution of 1:1, ethanol to water, was used.

2.4 Gas Chromatography (GC) for fatty acid analysis

FA samples were analyzed using a method (39) with slight modification. Briefly, lyophilized yeast was homogenized using mortar and pestle. Samples were weighed then placed in glass vials. 1 mL of n-hexane was added to each sample, followed by 1 mL of internal standard (1 mg mL^{-1} of heneicosanoic acid in n-hexane), then 2 mL of 14% boron trifluoride (BF3) in methanol. The sample vials were then flushed with nitrogen and then tightly capped. The samples were then vortexed for 1 min, then placed in an 80^{o}C water bath shaking at 120 rpm for 3 hrs. The samples were hand shaken periodically, every 10 min for the first hour, then every

20 min for the next 2 hrs. After incubation for 3 hrs, the samples were cooled to room temperature. 2 mL of distilled water was then added to each sample, then centrifuged at 4000 rpm for 12 mins. The top organic layer was then collected and injected into the GC.

A Varian 3800 Gas Chromatograph with a flame ionization detector (FID) and Omegawax 320 column was used with helium as the carrier gas at a flow rate of 60 cm s^{-1} and a constant head pressure of 347 kPa. Makeup gases of air and nitrogen were used with flow rates of 450 mL min^{-1} and 10 mL min^{-1} respectively. The FID was set to 275^{o}C, sampling frequency at 50 Hz, with sample volumes of 2 µL. Temperature was initially set to 100^{o}C with a 1 min hold, then ramp at 10^{o}C min^{-1} to 200^{o}C with a 9 min hold, followed by 10^{o}C min^{-1} to 225^{o}C with a 7 min hold. Total runtime was 33 min.

2.5 Fatty acid analysis via Reverse-Phase High Performance Liquid Chromatography (RP-HPLC)

FA samples were prepared by thawing and resuspending enriched yeast pellet, previously stored at -80^{o}C, into 95% ethanol, then lysing the yeast cells using a Qsonica Q125 ultrasonicator (125 W and 20 kHz) and a Qsonica 4435 horn, operated at 75% amplitude for 3 minutes with cycles of 30 secs on and 30 secs off. Samples were kept in an ice water bath during sonication. Then, to hydrolyze polyunsaturated fatty acids for UV detection, a final concentration of 1 M NaOH was then added to the sonicated samples. The samples were flushed with nitrogen, protected from light, and incubated at 25^{o}C overnight while shaking at 200 rpm. The next day samples were centrifuged at 7500 rpm for 3 minutes to collect the soluble fatty acid salts in the supernatant and dispose of the cellular debris. The supernatant was then adjusted to pH 6. The now hydrophobic FA's were extracted by addition of HPLC-grade cyclohexane and mixed at 200 rpm for 1 hr. The samples were then centrifuged at 5000 rpm for 3 minutes for phase separation. The organic layer containing FA's was collected and filtered using a syringe and syringe mounted 0.2 µm PTFE filter.

These filtered samples were then analyzed using Shimadzu Prominence RP-HPLC system with a DGU-20A3 degasser, LC-20AB pump, SPD-20A UV/VIS detector, CBM-20A communications BUS, and a Kromasil C8 column. Samples injected into the 20 µL loop were run with a mobile phase of 70% HPLC-grade acetonitrile and 30% HPLC-grade water adjusted to pH 3, using in-line mixing. The mobile phase was run isocratically at 1 mL min^{-1} for 30 min, with the detector set to 215 nm.

2.6 Lyophilization

Lyophilization was carried out on centrifuged, enriched yeast samples by freezing the samples in appropriate tubes either by liquid nitrogen or by placing the samples in -80^{o}C for at least 24 hrs. The samples of approximately 2 to 5 grams were lyophilized using a Labconco freeze dryer for 24 hrs.

2.7 Shelf life and FA retention of enriched yeast

To test the storage of enriched yeast in lyophilized form, enriched yeast cells were collected via centrifugation at 7500 rpm for 5 min, rinsed as previously mentioned, and then lyophilized as previously mentioned, then placed into capped glass vials. The vials were stored protected from light at room temperature for 30 days. Samples were analyzed for FA via GC as previously mentioned.

To test the storage of enriched yeast in paste form, enriched yeast cells were collected via centrifugation and washed as previously mentioned. After the final centrifugation of the wash cycle, the yeast paste was transferred to a glass vial. Then 1 mL of a solution containing 15% ascorbic acid and 7.5% citric acid (pH 2.2) was added. The vial was capped with a Teflon lined open-top screw cap, then briefly vortexed. Subsequently, vacuum was pulled. The samples were then stored at 4^{o}C for 30 days. Samples were analyzed for FA via GC as previously mentioned.

To test FA retention, lyophilized enriched yeast at the end of 30 days of storage were incubated in filter sterilized sea water to simulate feeding of the enriched yeast to a rotifer culture. The retained fatty acids in the yeast were analyzed after 2 days via GC. The sea water was obtained from near the shoreline of Makapu'U Beach Park, then filtered through a 0.2 µm PES filter.

2.8 Benzyl Isothiocyanate (BITC) assay

BITC was measured by using a modified 1,2-benzenedithiol (BDT) cyclocondensation assay (40). Briefly, samples containing BITC were pipetted into glass vials and the volume was made up to 1 mL with a 10 mM potassium phosphate buffer solution at pH 8.5. To these samples, 1 mL of 4 mM BDT in methanol was added. The samples were capped, briefly vortexed, then placed into a water bath at 65^{o}C for 3 hrs. Then, the samples were filtered using a syringe mounted 0.2 µm PES filter. The samples were measured for absorbance using a Shimadzu UV160U spectrophotometer set at 365 nm.

2.8.1 RP-HPLC analysis of BDT cyclocondensation assay

Filtered samples from the above BITC assay were injected into a Shimadzu Prominence RP-HPLC system previously mentioned. Samples were run with a C-18 column, with a mobile phase of 80% HPLC methanol and 20% HPLC water at a flow rate of 1 mL min^{-1}. Using a Shimadzu SPD-20A UV/VIS detector, absorbance was measured at a wavelength of 365 nm.

2.8.2 *Y. lipolytica* culture growth in the presence of BITC

Using *Y. lipolytica* strain p69MNG/polyG, cultures were inoculated using the same preculture method as previously mentioned to an OD_{600} of 0.01. Cultures were grown on yeast nitrogen base olate (YNBO) media consisting of 1.7 g L^{-1} of yeast nitrogen base without nitrogen

and without amino acids, 5 g L^{-1} of ammonium sulfate, and 1 g L^{-1} of yeast extract. All cultures were grown at 25^{o}C at 250 rpm.

To determine the effect of BITC on culture growth, 4% cold pressed papaya oil (contained no measurable BITC) was added to the cultures. Pure BITC was added to create various final concentrations of BITC. Growth of cultures were then measured after 3 days of growth via OD at 600nm as previously described.

To determine if pretreated papaya seed oil could sustain culture growth, the same method was applied except 4% of the sample oil was used instead.

2.8.3 Collection and storage of papaya seeds

Carica papaya were purchased from a local Safeway on Oahu. Seeds were harvested from the fruit, lightly rinsed with tap water, then frozen at -20^{o}C.

2.8.4 Ethanol pre-treatment

To test the ethanol pre-treatment of papaya seeds, previously frozen seeds were thawed in a room temperature water bath, rinsed, and placed in a glass flask. Then, 95% ethanol was added to fully submerge the seeds. The flasks were capped with aluminum foil, then placed in a 42^{o}C water bath for various lengths of time, from 0 to 4 days. After ethanol soaking, the seeds were collected, rinsed, and dried in an oven at 65^{o}C overnight to a constant dry weight.

2.9.1 Soxhlet papaya seed oil extraction

Dried papaya seeds were weighed, then homogenized using a mortar and pestle. The resulting ground seed was placed in a cellulose thimble and the oil was extracted via soxhlet extractor using hexanes as a solvent. The hexane within the soxhlet extractor was vaporized using a hotplate and cooled using a condenser supplied with cold water (4^{o}C) from a water

circulator. The samples underwent the continuous extraction for 4 hrs, at which point, there was no visible coloration from oil seen in the extracting hexane.

The hexane solvent was removed by rotary evaporation using a Buchi Rotavapor R-114. The water bath was set to 35^{o}C. With most of the hexane removed, residual hexane removal was done by continuous purging of samples with air in a fume hood for 3 minutes. The oil samples were then allowed to sit in the fume hood overnight to achieve a constant weight.

Chapter 3. Results & Discussion

The main aim of this research is to produce a method for encapsulating EPA and DHA in a natural strain of the yeast *Y. lipolytica* for use in rotifer enrichment that is intended for use in aquaculture rearing of finfish larvae. In developing a non-genetically modified method of enriching the yeast, three phases were identified: growth, enrichment, and harvesting/post-harvesting.

3.1 Biomass Growth of *Y. lipolytica*

For the growth phase of the yeast, the main objective was to develop a method that resulted in the most biomass produced prior to entering the enrichment phase. This study utilized the ACA-DC 10509 strain of *Y. lipolytica* because previous work done by Dr. Papanikolaou has shown this natural strain to be effective in accumulating intracellular lipid. Utilizing SD media, three parameters were measured during this initial growth phase, cell growth, sugar remaining in the media, and nitrogen remaining in the media.

As demonstrated in Fig. 1, after approximately 72 hours, the culture OD reached a peak between 3 and 4. Both sugar and nitrogen content in the media decreased in conjunction with increases in culture OD. The glucose content of the media decreased to near zero, while nitrogen, although depleted, still remained in the media.

The OD growth and corresponding decreases in glucose and nitrogen was expected since increases in OD relates to increases in culture cell number. The changes in slope reflect the lag phase, exponential phase, and stationary phase, during which the rate of cellular division would change, reflected in the consumption of the media nutrients, glucose and nitrogen.

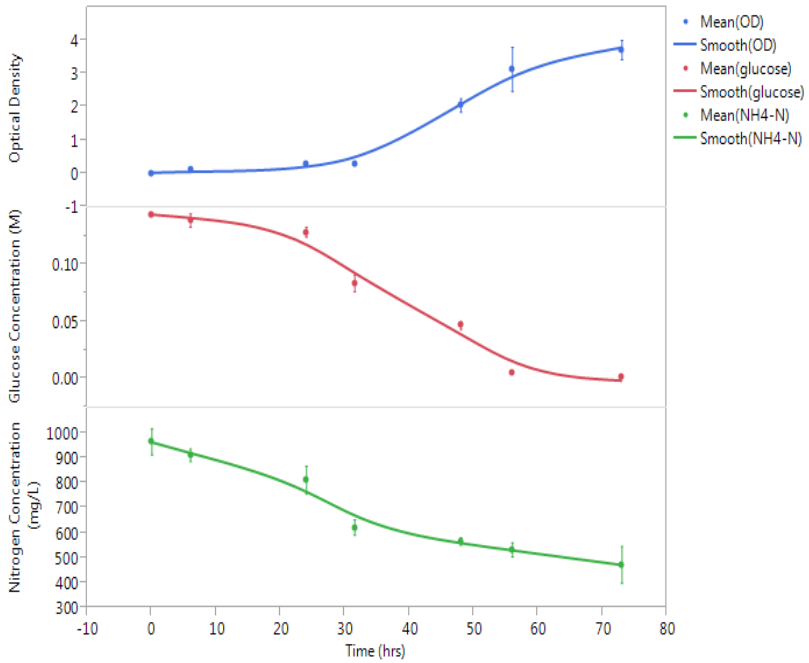

Fig. 1: Time course of *Y. lipolytica* strain ACA-DC 10509 cultured on SD media, at 25°C, and 200 RPM, measuring optical density (top), glucose concentration in media (middle), and nitrogen concentration in media (bottom). Run in triplicate, all cultures inoculated at an OD_{600} of 0.01.

This data provided the length of time required to achieve high biomass grown on SD media at 25°C, at 200 RPM. High biomass growth prior to enrichment was preferred since this would provide the most cells to absorb and encapsulate EPA and DHA. The growth phase data would be used to set a protocol of a growth period of 3 days.

3.2 Enrichment of *Y. lipolytica* with EPA and DHA

After growth, the next phase was enrichment. In this phase *Y. lipolytica* was incubated with an extracellular lipid source, menhaden oil, that provided EPA and DHA to be encapsulated within the yeast. One of the first aspects to investigate was media carbon to nitrogen ratio (C:N). Literature has shown that the C:N is important to FA accumulation in oleaginous organisms. To confirm this was also true for the strain used in this study, EPA and DHA levels were measured

in two enrichment medias with differing C:N. Cultures were either enriched in SD media, with a 7.55 theoretical C:N, or in HG media, with a very high C:N (no nitrogen).

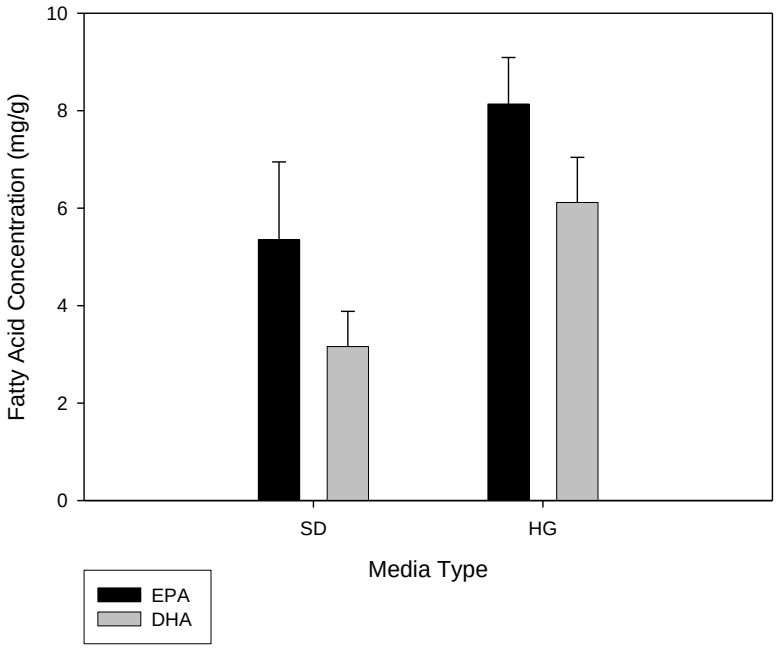

Fig. 2: Comparison of EPA and DHA concentrations in yeast enriched in SD or HG media. Enriched samples were washed, then analyzed for EPA and DHA via GC. Run in duplicate. HG media resulted in a higher mean for both EPA and DHA compared to SD media. SD samples had an EPA mean concentration of 5.35 mg/g and a DHA mean concentration of 3.16 mg/g. HG samples had an EPA mean concentration of 8.135 mg/g and a DHA mean concentration of 6.115 mg/g.

Fig. 2 showed that if the yeast was enriched in HG media, the yeast would have higher concentrations of both EPA and DHA compared to yeast enriched in SD media. This was expected since the HG media had a higher C:N ratio, shown by literature to aid in lipid accumulation. This finding allowed the study to determine that subsequent enrichment experiments would all utilize HG media since it was demonstrated to improve FA accumulation for the specific strain used in this study. Subsequent experiments measuring EPA and DHA levels in the yeast demonstrated that the ideal time of enrichment with HG media was 3 days.

Thus, a protocol for enrichment was developed that involved 3 days of growth, followed by a media switch to HG and another 3 days of enrichment.

3.3 Factors of Temperature, Oxygen, and Hydrolysis on Fatty Acid Accumulation

With a method for enrichment established, it was hypothesized that other alterations to the culture conditions could also have an effect on FA accumulation. As mentioned in the introduction, the levels of EPA and DHA are due to the effects of FA uptake/storage verses the effects of FA degradation. Thus, the factors of temperature, oxygen, and hydrolysis of source lipids were investigated.

In order to test these factors and the interactions between the factors, a 2^3 factorial design was used. Each factor was given two levels. Temperature was tested at 4^oC verses 25^oC. Oxygen was tested with atmospheric oxygen available to the culture verses a nitrogen flushed and sealed culture. Hydrolysis was tested with hydrolyzed menhaden oil used to enrich the culture verses non-hydrolyzed oil to enrich the culture. The experiment was run in triplicate as a randomized complete block with replicates as blocks. The factorial design allowed the current study to not only consider the three main effects of hydrolysis, oxygen, and temperature, but also the 2-way and 3-way interactions that may affect EPA and DHA concentrations. Samples from the cultures of the various combinations of factors was analyzed via RP-HPLC.

3.3.1 Identification and Quantification of EPA and DHA

To identify EPA and DHA, samples of pure EPA diluted into cyclohexane, pure DHA in cyclohexane, and a blank where analyzed via RP-HPLC. The retention times were recorded. EPA eluded at approximately 8.8 min while DHA eluded at approximately 10.9 min. A typical combined EPA and DHA standard was as follows:

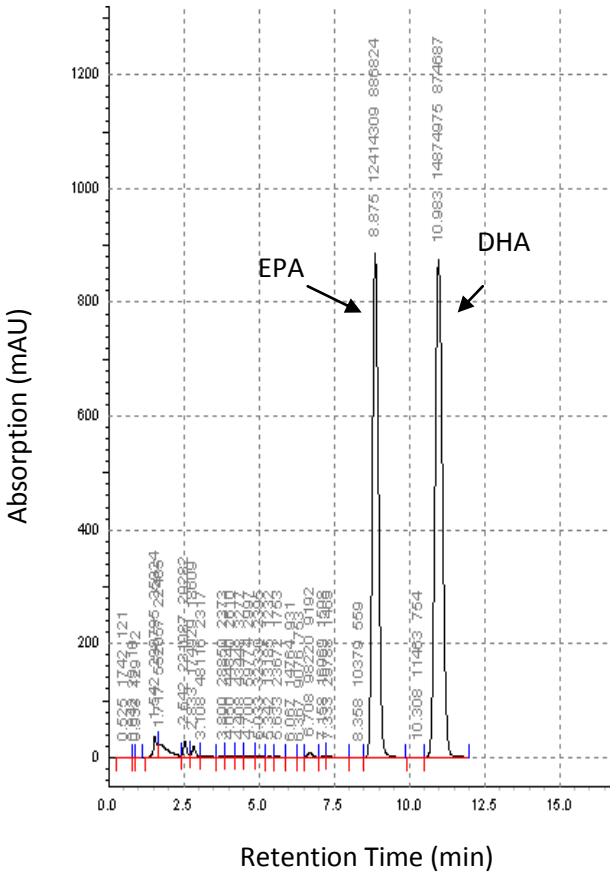

Fig. 3: RP-HPLC chromatogram of 0.5 mM EPA and DHA standards. EPA eluded at approximately 8.9 min while DHA eluded at approximately 11.0 min. Absorbance was measured at 215 nm.

The RP-HPLC chromatograms of the standards (Fig. 3) showed sharp peaks, demonstrating the effectiveness of UV/VIS at detecting the compounds of interest. With identifying retention times determined, to enable quantification of samples with unknown concentrations of EPA and DHA, standard curves were constructed (Fig. 4 and Fig. 5).

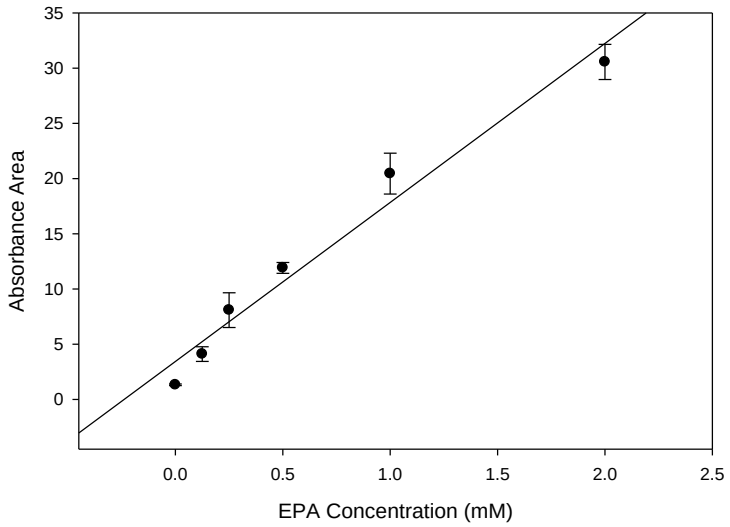

Fig. 4: Standard curve for EPA. Pure EPA was diluted to several known concentrations ranging from 0 mM to 2 mM. Absorbance was measured via RP-HPLC at 215 nm. Peak area plotted against the corresponding standard EPA concentration. $R^2 = 0.97$

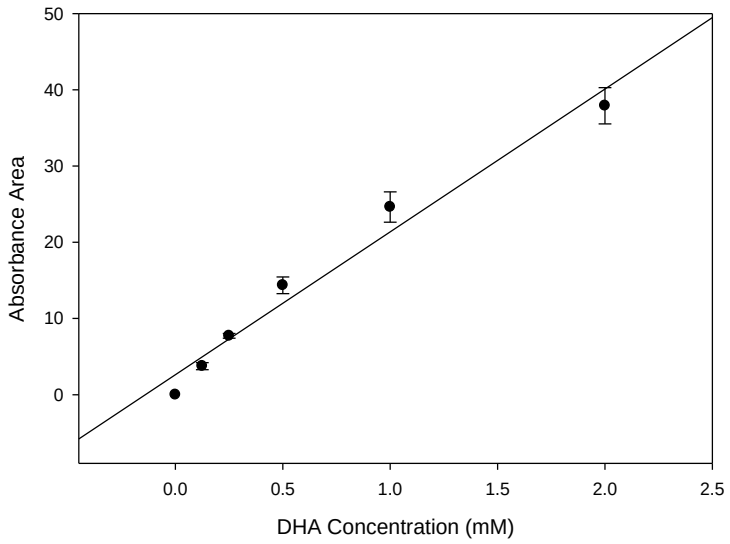

Fig. 5: Standard curve for DHA. Pure DHA was diluted to several known concentrations ranging from 0 mM to 2 mM. Absorbance was measured via RP-HPLC at 215 nm. Peak area plotted against the corresponding standard DHA concentration. $R^2 = 0.971$

In order to identify and quantify EPA and DHA in unknown samples, one issue needed to be resolved. To detect EPA and DHA using a UV/Vis detector, the two FA of interest would need to be hydrolyzed. Normally, a UV/Vis detector would require a chromophore to be attached to fatty acids for detection. However, due to the multiple double bonds (unsaturations) present in EPA and DHA, detection was possible at low wavelengths. Additionally, EPA and DHA must be in the form of a free fatty acid (opposed to in the form of a triglyceride). While the pure samples of EPA and DHA previous tested were already in free fatty acid form, the EPA and DHA stored in enriched yeast would be stored in lipid bodies as triglycerides, thus requiring the hydrolysis of these triglycerides prior to RP-HPLC analysis. Thus, an experiment was setup to determine the required time for hydrolysis.

3.3.2 Determination of treatment time for hydrolysis

To determine the treatment time for oil hydrolysis that would allow for full hydrolysis, a time course experiment was conducted. At set time points, samples were taken from the hydrolysis reaction. The menhaden fish oil was hydrolyzed by adding a known volume and concentration of NaOH. As hydrolysis occurs, NaOH was consumed creating glycerol and fatty acid salts, decreasing the amount of NaOH left in solution. By titrating these samples with a HCl solution with a known, standardized concentration, the amount of NaOH that was consumed at each time point was quantified.

The results from in Fig. 6 show that the amount of NaOH consumed increased until approximately 30 minutes, after which it plateaued. Based on this data, a standard of 60 minutes was utilized to hydrolyze in subsequent experiments. 60 minutes was chosen as opposed to 30 to help ensure hydrolysis had gone to completion. Since, 60 minutes had a much smaller standard deviation than 30 minutes, it is possible that at 30 minutes, some replicate samples did not reach completion yet, resulting in the increased variation at that time point. This helped to solidify 60 minutes as a more reliable time to ensure that hydrolysis reached completion.

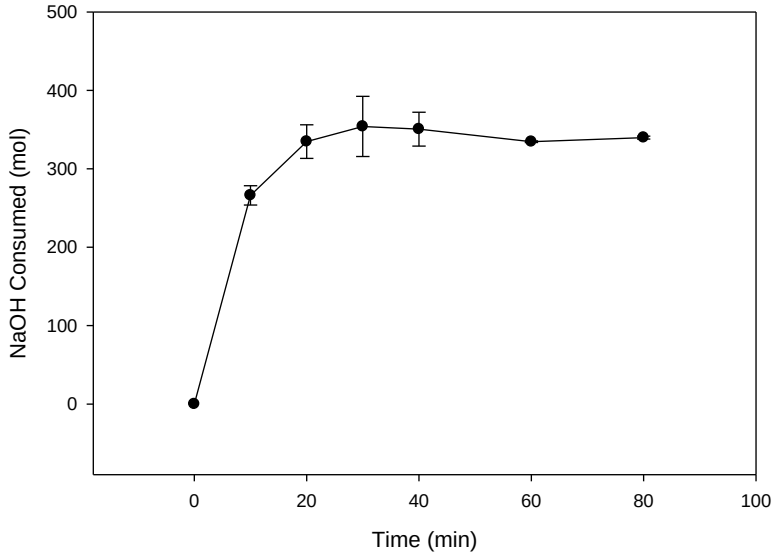

Fig. 6: NaOH consumed at different time points during hydrolysis. Menhaden fish oil was hydrolyzed using known volumes of 1M NaOH at 60^{o}C. Samples were taken at different time points during hydrolysis and titrated with standardized 0.364M HCl to determine how much NaOH was utilized in the reaction. Phenolphthalein was used as the titration indicator.

3.3.3 The effect of hydrolysis, temperature, and oxygen on EPA and DHA

Now, with the ability to identify and quantify EPA and DHA in unknown samples, the 2^3 factorial experiment investigating the factors of hydrolysis, temperature, and oxygen was run. The F-tests for the factors showed that for both EPA and DHA, the factors of hydrolysis of source oil was very statistically significant ($P<0.001$) and the interaction of temperature and oxygen were statistically significant ($P<0.05$).

After determining that hydrolysis of the source oil was a statistically significant factor, for both EPA and DHA, the least squares (LS) means plots were used to determine the effect of hydrolysis on EPA and DHA concentrations. Because our experiment was balanced (no missing data), LS means is the same as the mean. As seen in Fig. 7 and Fig. 8, hydrolysis improved both EPA and DHA concentrations found in the enriched yeast samples, compared to no hydrolysis.

Because this study aimed to develop a method for maximizing EPA and DHA encapsulation, it was determined from Fig. 7 and Fig. 8 that hydrolysis should be utilized.

The difference between samples with hydrolyzed oil and non-hydrolyzed oil could be due to the fact that hydrolysis of the source oil would generate free FA from the triglycerides. The free FA would be easier to accumulate because there would be a reduced need for *Y. lipolytica* to produce and secrete extracellular lipases and biosurfactants. Additionally, chemical hydrolysis of the source oil may be more effective in fully hydrolyzing the triglycerides compared to the bioactivity of the produced lipases, which could provide more access to EPA and DHA.

As mentioned previously, besides hydrolysis, the interaction of temperature and oxygen was also found to be statistically significant. Again, the effect of the interaction can be determined from the LS means plots.

The LS means plot for the significant interaction between the factors of oxygen and temperature show similar trends for both EPA and DHA (Fig. 9 and Fig. 10 respectively). If there was no interaction, the effect of one factor would have a constant effect and not be influenced by another factor, and hence would result in parallel lines. Because the LS means plots are not parallel, we know that an interaction between the factors exists. In both cases, when cultures were grown at 4^{o}C, the presence or absence of oxygen had little effect. However, when the cultures were grown at 25^{o}C, cultures with oxygen showed significantly higher concentrations of both EPA and DHA. Thus, the results suggested that improved EPA and DHA levels could be achieved with cultures grown with oxygen present at 25^{o}C.

The observed difference could be due to improved cellular functions involved with the uptake and storage of FA at 25^{o}C with oxygen. As mentioned previously, the literature has stated that FA uptake is likely a combination of passive and active transport. Because *Y. lipolytica* is an aerobic organism and has been shown to have no growth under anaerobic conditions (41), removal of oxygen could result in decreased active transport resulting in the lower observed FA levels in cultures with no oxygen. Additionally, the cellular processes involved in FA assimilation into the lipid body may also be curtailed, resulting in the observed lower concentrations of EPA and DHA. Furthermore, although there was a downward trend for

cultures grown at 25^{o}C compared to 4^{o}C when the cultures did not have oxygen (dashed line in Fig. 9 and Fig. 10), this decrease was not statistically significant in either case.

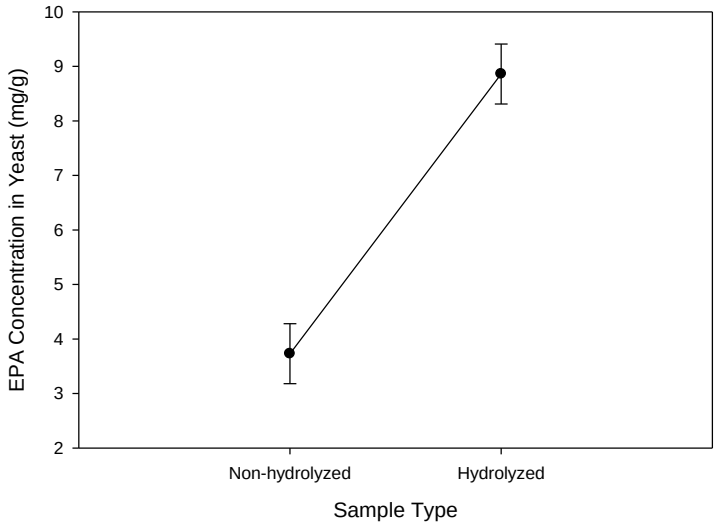

Fig. 7: Least square (LS) means plot for the factor of hydrolysis on EPA. Enriched yeast samples were analyzed for EPA via GC. The non-hydrolysis samples LS mean was 3.73 mg/g. The hydrolyzed samples LS mean was 8.86 mg/g.

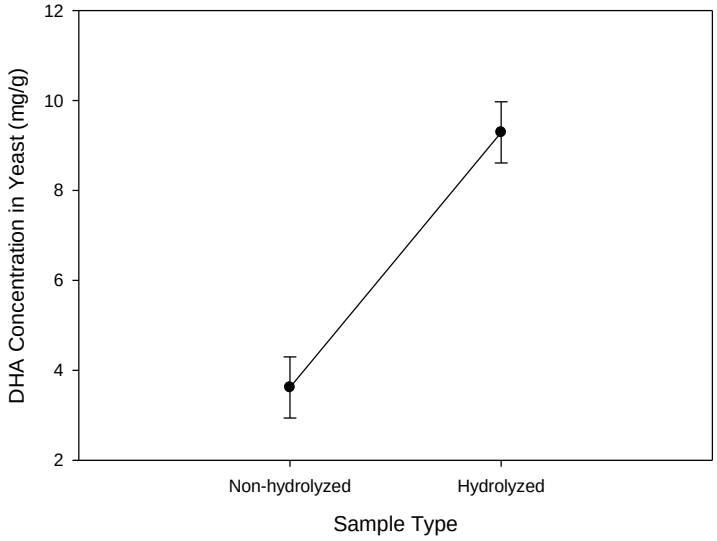

Fig. 8: Least square (LS) means plot for the factor of hydrolysis on DHA. Enriched yeast samples were analyzed for DHA via GC. The non-hydrolyzed samples LS mean was 3.62 mg/g. The hydrolyzed samples LS mean was 9.29 mg/g.

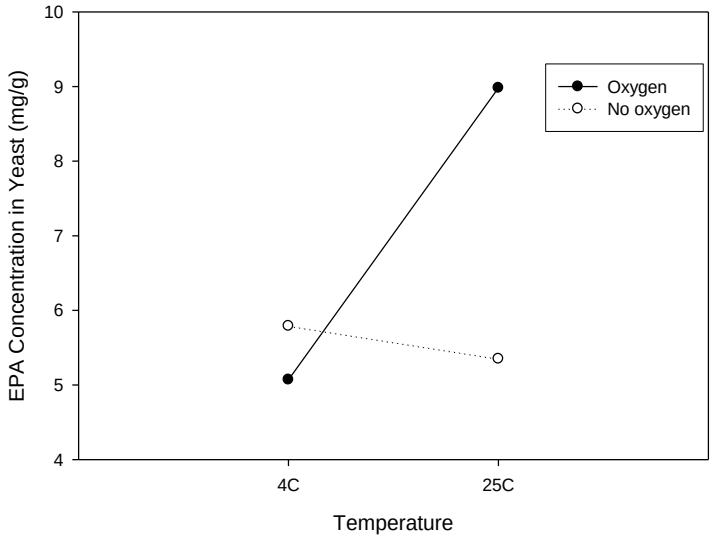

Fig. 9: Least square (LS) means plot for the interaction of temperature and oxygen on EPA. Enriched yeast samples were analyzed for EPA via GC. The LS mean was 5.79 mg/g for samples with no oxygen at 4°C, 5.35 mg/g for samples with no oxygen at 25°C, 5.07 mg/g for samples with oxygen at 4°C, and 8.98 mg/g for samples with oxygen at 25°C.

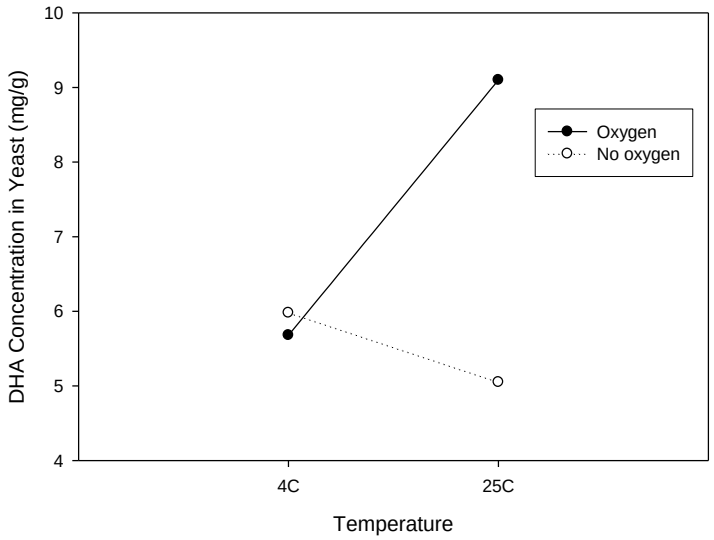

Fig. 10: Least square (LS) means plot for the interaction of temperature and oxygen on DHA. Enriched yeast samples were analyzed for DHA via GC. The LS mean was 5.98 mg/g for samples with no oxygen at 4°C, 5.05 mg/g for samples with no oxygen at 25°C, 5.07 mg/g for samples with oxygen at 4°C, and 9.10 mg/g for samples with oxygen at 25°C.

This result was unexpected. It was previously believed that the lower temperature and removal of oxygen would decrease EPA and DHA degradation by avoiding oxidation of the source lipids and by avoiding β-oxidation within the cell, and that these would be the driving factors in altering EPA and DHA accumulation. While these degradation effects may be occurring, the results clearly show that the positive effects of oxygen and 25^{o}C on FA accumulation outweigh the negative effects of FA degradation for the factors and levels tested.

Taking the results of the factorial experiment as a whole, considering the factors and interactions that were significant, with the trends in the LS means plots, the factorial experiments have indicated that the best combination that was tested was to enrich *Y. lipolytica* with hydrolyzed oil with oxygen at 25^{o}C. This combination resulted in enriched yeast with EPA concentrations of 9.88 mg/g, and DHA concentrations of 9.71 mg/g. Additionally, gravimetric measurements of total lipids put the enriched yeast at 27% lipid by dry weight.

3.4 Media switching verses simple addition of glucose

The method, utilized in the current study, to enrich *Y. lipolytica* with EPA and DHA had up to this point involved growth in SD media with a low carbon to nitrogen ratio (C:N), followed by centrifugation of cells to replace the SD media with HG media (high C:N) for the enrichment phase, due to the higher concentrations of EPA and DHA that HG media showed (Fig. 2). However, practical considerations suggested that centrifugation in order to change to HG media would be energy intensive and less practical in large scale operations. Thus, this research investigated simply adding more glucose to the SD media at the end of the growth period.

It was shown in Fig. 1 that nitrogen levels dropped during the 3 day growth period. Therefore, by adding more glucose (carbon) to the media, the C:N could be easily increased to support lipid accumulation, without the need for centrifugation. GC analysis of EPA and DHA from yeast enriched in this manner showed that there was no significant difference compared to the centrifugation method. Moreover, it was found that adding glucose instead of switching media had the added benefit of resulting in a 2 to 5 times increase in the dry cell weight of

cultures. This benefit is likely the result of the yeast using the nitrogen that remained at the end of the growth period (Fig. 1), along with the added glucose, to continue cellular division. Thus, simply adding more glucose to the media was an improvement since there was no statistical difference seen in EPA and DHA concentrations per cell, but the method resulted in more cells. Therefore, the overall amount of EPA and DHA encapsulated was increased.

3.4.1 Analysis of FA profiles

To glean more information on the FA encapsulated within the yeast cells, this study also looked at the FA profiles that resulted from the enrichment process. The FA profiles from GC chromatograms (example in appendix Fig. A2) were analyzed. Three sample types were examined, these included: menhaden oil (source of FA), enriched yeast (encapsulated FA), and "spent oil," which was the lipid remaining in the media after the enriching process (remaining FA). By investigating these profiles this study aimed to not only determine the efficacy of EPA and DHA encapsulation, but also elucidate any changes in the FA profiles between the enriching oil and the encapsulated oil. The relative compositions of the major fatty acids within each of the sample types are listed in Table 1.

Table 1

Relative composition of the predominant fatty acids common to menhaden oil, enriched yeast, and spent oil. Percentages were based on weight of the specific FA to the total fatty acid weight in respective samples. The yeast culture was grown using best combination of factors previously tested, hydrolysis, oxygen presence, at 25^{o}C, with glucose addition during enrichment.

	Palmitic C16:0	Stearic C18:0	Oleic C18:1 n-9	Linolenate C18:3 n-3	EPA C20:5 n-3	DHA C22:6 n-3
Menhaden oil	14.68 %	2.64 %	5.26 %	12.48 %	15.27 %	11.62 %
Enriched yeast	3.45 %	10.27 %	7.82 %	14.06 %	16.33 %	12.16 %
Spent oil	2.34 %	15.84 %	12.88 %	14.19 %	8.72 %	7.59 %

Table 1 shows that the enriched yeast was able to accumulate slightly higher of EPA and DHA as a percentage of total lipids compared to the beginning menhaden oil. This is reflected in the lower percentages of EPA and DHA in the spent oil. This is expected because the literature has shown that *Y. lipolytica* preferentially uptakes long-chain unsaturated FA like EPA and DHA(42). The presence of EPA and DHA in the remaining spent oil indicates that the 4% menhaden oil added to the culture is likely in excess and could be decreased to utilize less menhaden oil. Alternatively, it might be possible to culture more yeast, then add it to the same amount of oil. The results in Table 1 also agreed with the literature in that C18:0 was uptaken slower than C18:1 n-9 since the enriching oil started with a relatively higher percentage of C18:1 n-9 than C18:0 (5.26% versus 2.64% respectively), but ended up with a lower percentage of C18:1 n-9 than C18:0 in the spent oil (12.88% versus 15.84%). The results also concurred with the literature in noting that C16:0 is preferentially consumed (42). While C16:0 was high in the enriching oil, it was low in both the enriched yeast and the remaining spent oil. This suggests that C16:0 was taken up and metabolized. Alternatively, C16:0 could have been degraded by spontaneous oxidation. However, due to the saturated nature of C16:0, spontaneous oxidation of C16:0 was less likely than for other unsaturated FA. It should be noted that while previous studies have shown C16:0 to be preferentially metabolized, that study showed little change in the spent oil levels of C16:0, while the current study's results show a large decrease in C16:0 percentage. This could be explained by the fact that the previous study only enriched for about 2 days while the current study enriched for 3 days with a larger biomass, leading to more C16:0 degraded and the resulting uptake from the media.

While percentages are useful in determining the relative changes in composition, they do not tell the whole picture. The relative percentages will be determined by changes in the other FA of a sample, thus the columns in Table 1 will not add up to 100%. And this is why C18:0 can have a higher percentage in spent oil than the original menhaden oil, if other FA in the spent oil were decreased in absolute amount, e.g. the decrease in C16:0 could result in C18:0 percentage increasing, even if the absolute amount of C18:0 remained unchanged. Thus, to get a better perspective, the absolute amounts of FA as measured in mg are shown in Table 2.

Table 2

Absolute amounts, in mg, of the predominant fatty acids common to menhaden oil and enriched yeast. The yeast culture was grown using best combination of factors previously tested, hydrolysis, oxygen presence, at 25^{o}C, with glucose addition during enrichment. Total weight of menhaden oil was 10g while total dry cell weight for the enriched yeast was 0.707g. Culture volume of 250 mL.

	Palmitic C16:0	Stearic C18:0	Oleic C18:1 n-9	Linolenate C18:3 n-3	EPA C20:5 n-3	DHA C22:6 n-3
Menhaden oil	1141.139	205.380	409.159	124.774	1187.044	903.558
Enriched yeast	1.586	4.721	3.598	6.466	7.507	5.592

Table 2 showed the absolute amounts in mg of the same fatty acids, from the same samples shown in Table 1. Unfortunately, data on the absolute amounts of FA in the spent oil were unavailable. However, the available results were still insightful. As shown in Table 2, the amount of EPA and DHA within the enriched yeast represents only a small portion of the total EPA and DHA added to the culture. This finding, along with Table 1, point to the fact that the 4% menhaden oil added to the culture was in excess and that less menhaden oil should be added. This is important to know from a cost perspective. Alternatively, it could be possible to add more yeast, having multiple growth phase yeast cultures feeding into a combined batch of enrichment without needing to increase the amount of menhaden oil used. While this information is useful in developing future studies, the remainder of the study still utilized 4% oil in order to maintain consistency and comparable results.

Combining the results from Table 1 and Table 2, and comparing it to data from the literature, which also investigated lipid accumulation in a different yeast species (43), the results from this study compare similarly in EPA and DHA percentages. This study's results, seen in Table 1, showed slightly higher percentages on both EPA and DHA with 16.33% EPA and 12.16% DHA, while the literature cited 10.8% and 5.4% for EPA and DHA respectively. Additionally, this study's enriching feedstock oil had a slightly lower initial EPA and DHA percentage, 15.27 % EPA and 11.62 % DHA, while the literature's oil initially had 16.2% EPA and 14.0 DHA. This means this study's was able to achieve slightly better percentages of EPA and DHA of total fatty acids despite utilizing an enriching feedstock oil that contained less EPA

and DHA initially. However, it should be noted that this study achieved a much lower cell weight and lipid content per cell. This study obtained 2.8g/L of enriched yeast with 27% lipids, while the literature achieved 18.4g/L of enriched yeast with 49.7% lipids. Thus while this study's EPA and DHA percentages compare similarly to the literature, this study's culture produced less total dry cell weight with lower total lipid. It should be noted that the yeast strain used in the current study has been reported to be able to obtain up to 40% lipid (19), which points to the possibility of improvement in this area.

3.5 Best method for enrichment with the conditions tested

To recap, the accumulation of knowledge from this study's experiments demonstrated that the best method for encapsulation of EPA and DHA in *Y. lipolytica*, for the parameters tested, was a 3 day growth in SD media to achieve high biomass, followed by simple addition of glucose to achieve high C:N, thereby promoting lipid encapsulation within the yeast, with an enrichment time of 3 days on hydrolyzed source oil with oxygen at 25^{o}C.

3.6 Shelf life and FA retention

With a developed method and tested factors in place, the current study looked to test the viability of the enriched yeast in different storage options. Taking a cue from algae enrichment feeds and instant algae products, one storage method tested was a paste form.

To mimic commercial algae paste products, a small amount of natural preservatives was added to paste samples, along with pulling vacuum to simulate vacuum sealed packaging. As can be seen in Fig. 11, the paste form of storage was effective in retaining EPA and DHA concentrations. There was no significant difference seen in either EPA or DHA between zero and 30 days of storage ($P > 0.05$). Despite the requirement of refrigeration, the paste form, like many of the other aquaculture-related packaged goods, remains a viable option for storage.

In hopes of circumventing the need for refrigeration, the current study also investigated the use of lyophilization to produce a dry storage akin to the way yeasts like *Saccharomyces cerevisiae* can be stored. Samples of enriched yeast were lyophilized and stored in a capped vial. Unlike the paste form, the lyophilized form did not have preservatives added, nor did it have a vacuum pulled.

The results of the lyophilized enriched yeast shelf life (Fig. 12) showed that after 30 days there was a detectable decrease in the mean concentration of both EPA and DHA. However, due to the relatively high variation, the decrease was not quite statistically significant. This minimal decrease is a promising sign as no preservatives nor vacuum was utilized. Unfortunately due to limit samples a follow-up experiment could not be conducted.

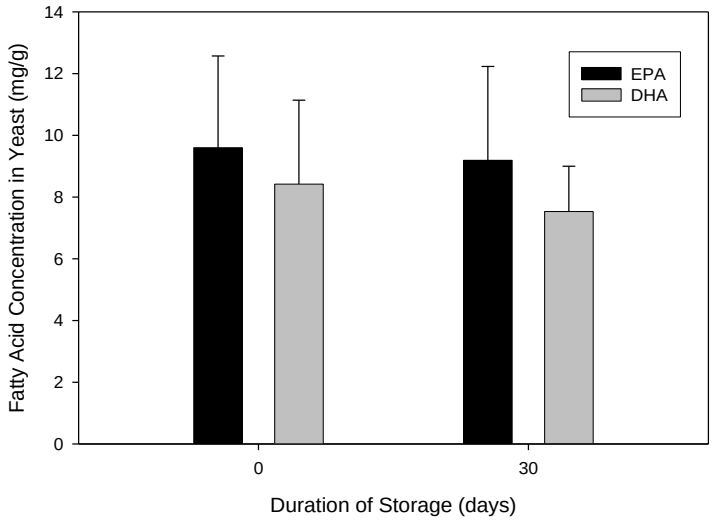

Fig. 11: Shelf life of enriched yeast in paste form stored under vacuum at 4^{o}C for 30 days. Samples were analyzed via GC for EPA and DHA. Samples run in triplicate.

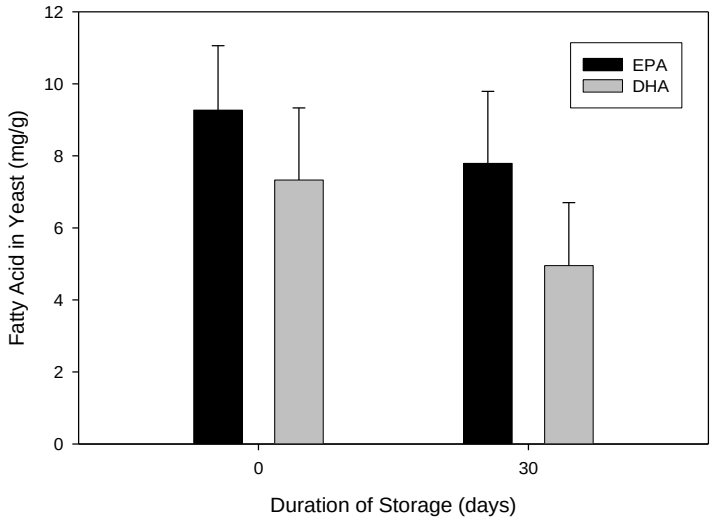

Fig. 12: Shelf life of enriched yeast stored in lyophilized form at room temperature. EPA and DHA was determined via GC. Samples run in triplicate.

In addition to the shelf life experiments, the current study also investigated EPA and DHA retention when the lyophilized yeast, after being stored for 30 days, was resuspended into filter sterilized sea water to mimic feeding to rotifer cultures. After 2 days, the cell mass was collected again, then analyzed for EPA and DHA via GC.

After 2 days of exposure in sea water, the remaining EPA and DHA in the samples was lower than initial readings. There was a statistically significant drop in DHA concentrations, while EPA fared better. These results suggest that batch feeding rotifers daily with this enriched yeast could be beneficial to mitigate possible degradation. The decrease seen during the 2 days could have be exacerbated by the slight degradation already occurring by the end of the 30 day lyophilized shelf life experiment, since as auto-oxidation occurs, it can gain momentum as peroxyl radicals propagate the oxidation reaction.

After 2 days of exposure in sea water, the remaining EPA and DHA in the samples was lower than initial readings. There was a statistically significant drop in DHA concentrations, while EPA fared better. These results suggest that batch feeding rotifers daily with this enriched

yeast could be beneficial to mitigate possible degradation. Fortunately, traditionally, most rotifer feedings for enrichment involved one day or less.

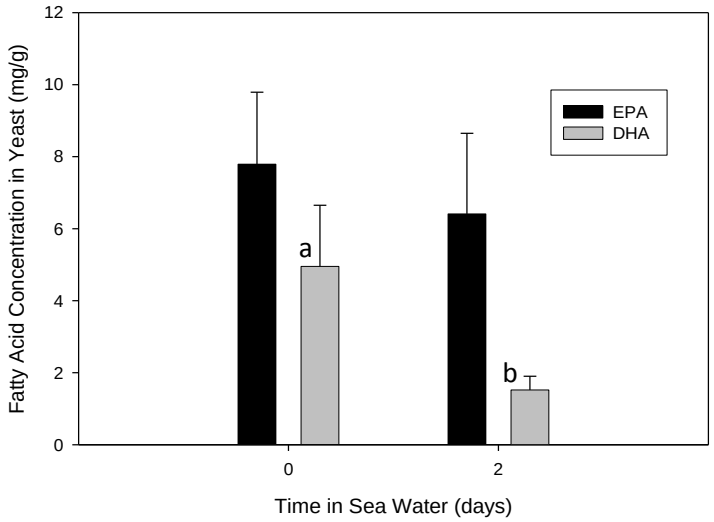

Fig. 13: Fatty acid retention in lyophilized yeast (stored for 30 days prior) in sea water. The lyophilized yeast was incubated in sea water for 2 days to simulate feeding the enriched yeast to a rotifer culture. FA was measured via GC. Samples run in triplicate. There was no statistical difference in initial and final measurements of EPA, however, there was a statistically significant difference between the initial and final DHA concentrations, as indicated by the letters (a, b).

The decrease seen during the 2 days could have be exacerbated by the slight degradation already occurring by the end of the 30 day lyophilized shelf life experiment, since as auto-oxidation occurs, it can gain momentum as peroxyl radicals propagate the oxidation reaction.

It was interesting that DHA was impacted to a much greater degree than EPA. This could due to the fact that DHA has more unsaturations than EPA. Since unsaturations are particularly vulnerable to oxidation, DHA has potentially more opportunity for degradation. The difference could also be a due to a difference in stabilization during storage or perhaps preferential metabolism of DHA over EPA.

Also of note was that most of the shelf life samples had rather high standard deviation. This was partly to do with the fact that the triplicate was done with three separate cultures from

separate flasks. Thus, the biological variation could have caused the wider variation observed. This could have also been due to differences in hydrolysis completion. If some cultures received enrichment lipids that were less hydrolyzed than other cultures, the enriched yeast used as the starting material for shelf life experiments could have also varied more in EPA and DHA concentrations.

3.7 Papaya seed oil as a locally available waste feedstock

After investigating the method, culture factors, and storage conditions to encapsulate the highest concentration of EPA and DHA in *Y. lipolytica*, the current research considers improvements in how *Y. lipolytica* is grown in the first place. While glucose is a standard carbon source utilized to grow biomass for many microorganisms, *Y. lipolytica* has the ability to be grown on, and even pre-cultured on, lipids. This presents a unique opportunity to capitalize on papaya seed oil as a waste feedstock locally available in tropical and subtropical regions.

3.7.1 BITC effect on Y. lipolytica growth

As mentioned in the first chapter, BITC is an obstacle that may prevent use of papaya oil due to its antimicrobial activity. Because the literature was limited as to BITC's effect on *Y. lipolytica* in liquid cultures, in order to assess the viability of using extracted oil from papaya seeds as a feedstock for *Y. lipolytica* culture, first, the effect of BITC on *Y. lipolytica* was investigated.

To this end, several liquid cultures of *Y. lipolytica* were inoculated at the same OD_{600}. Each of these cultures contained differing final concentrations of pure BITC. By measuring the OD_{600} at the end of a 3 day growth period, the effect of BITC on culture growth was determined. The results are summarized in Fig. 14.

As shown by Fig. 14, it was determined there was a correlation between BITC concentration and culture inhibition, with higher concentrations of BITC showing higher levels

of growth inhibition (shown by lower OD), while lower concentrations of BITC showed lower inhibition. This suggested that BITC was indeed inhibitory to *Y. lipolytica* cultures at certain concentrations. The results also provide a target concentration of BITC which any treatment much achieve in order to avoid culture inhibition, which was around 0.038 mM.

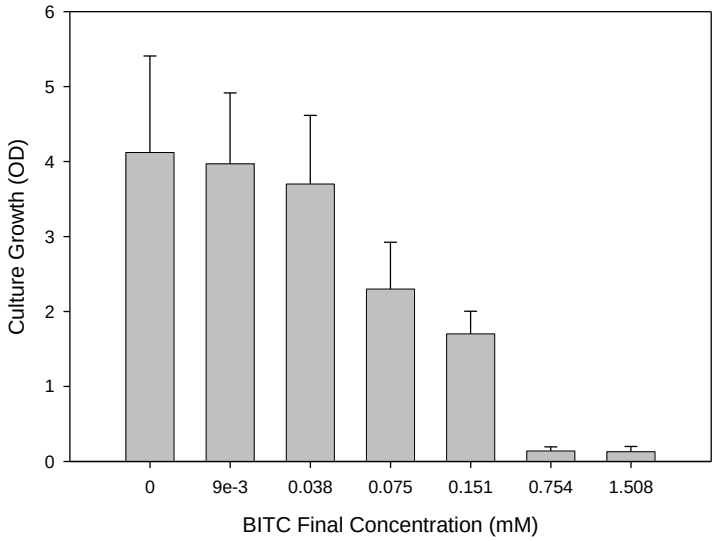

Fig. 14: Inhibition effect of pure BITC on *Y. lipolytica* cultures. Pure BITC was added to 50 mL cultures to produce differing final concentrations of BITC. Culture growth was measured after 3 days via OD_{600}. All cultures were inoculated an OD_{600} of 0.010

With BITC confirmed to have inhibitory effects on *Y. lipolytica*, the next objective was to determine was whether BITC levels in raw, extracted papaya seed oil were above or below the inhibitory level previously determined in Fig. 14. BITC was able to be identified and quantified (Fig. 15 and Fig. 16) utilizing the cyclocondensation reaction previously described coupled with RP-HPLC detection of the reaction product 1,3-benzodithiole-2-thione.

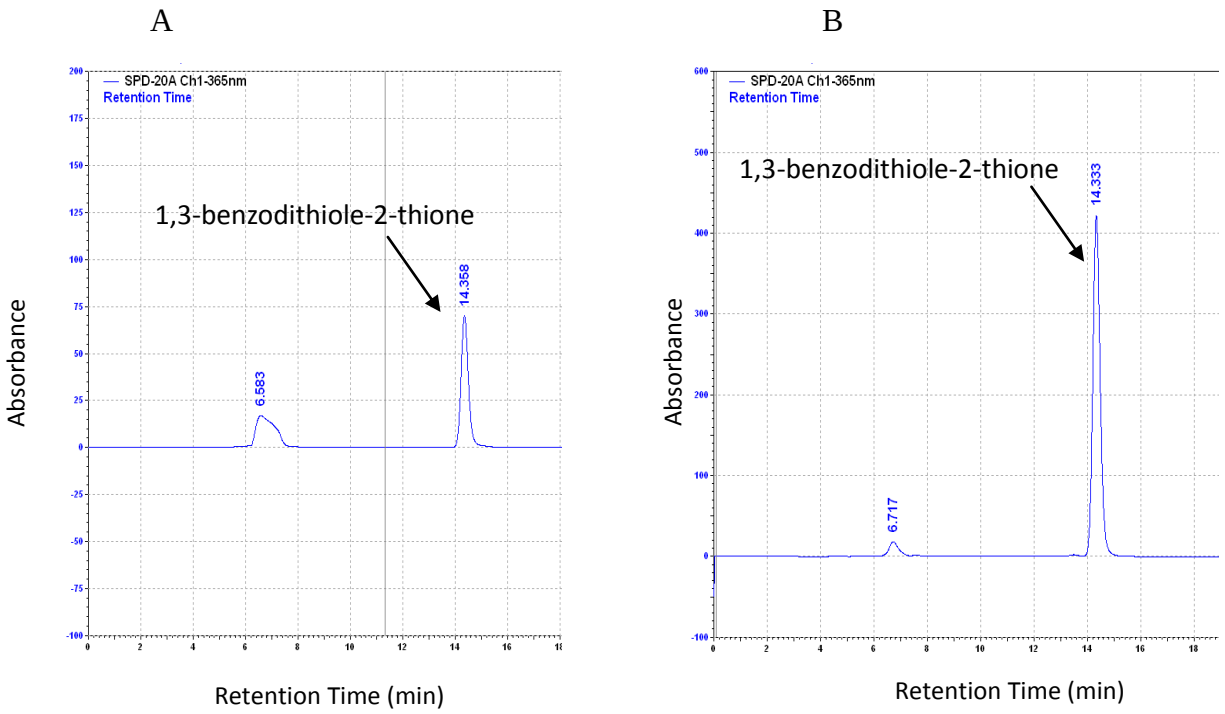

Fig. 15: (A) RP-HPLC chromatogram of 0.151 mM BITC standard after cyclocondensation reaction with BDT. (B) RP-HPLC chromatogram of 4% papaya seed oil, after cyclocondensation reaction with BDT. Retention time of 14.3 minutes was observed in both standard and oil sample.

Utilizing the standard curve (Fig. 16), quantification of BITC in raw, extracted papaya seed oil showed that 4% oil (amount of oil used in cultures) had a BITC concentration of 2.7 mM. Comparing this concentration to Fig. 14 showed that the papaya oil had relatively high levels of BITC, well above the inhibitory range (> 0.038 mM). Indeed, culture attempts using this raw, untreated papaya seed oil failed to show any growth. Thus, in order to unlock papaya oil as a viable feedstock for *Y. lipolytica*, ethanol pretreatment of papaya seeds was investigated.

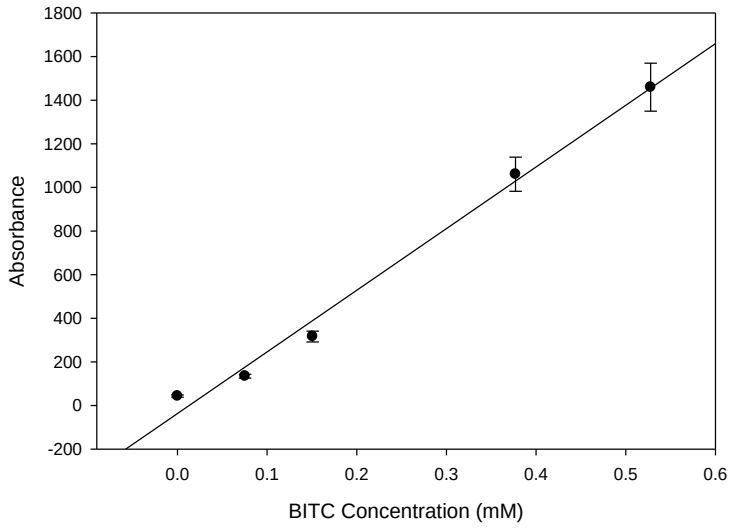

Fig. 16: Standard curve for BITC. Various concentrations of pure BITC were prepared reacted with BDT. The reaction product was detected via RP-HPLC measuring at 365 nm. Regression equation: $y = 2825.9x - 36.612$. $R^2 = 0.99$

3.7.2 Ethanol pretreatment of papaya seeds

As previously stated, ethanol soaking of papaya seeds is an attractive pretreatment due to the low technological barrier and ease of use. By inactivating myrosinase via ethanol it was hypothesized that BITC concentration could be lowered below inhibitory levels. Previous studies pointed to ethanol treatment time as an important factor, thus in order to develop an efficacious pretreatment, an experiment investigating the effect ethanol pretreatment time on papaya seeds was conducted.

The results of various pretreatment times on the efficacy of ethanol to reduce BITC concentrations were shown in Fig. 17. Pretreatment durations of 0 to 2 days all showed BITC concentrations above inhibitory levels previously determined (0.038 mM). The pretreatment times of 3 days and 4 days were near and bellow inhibitory levels respectively.

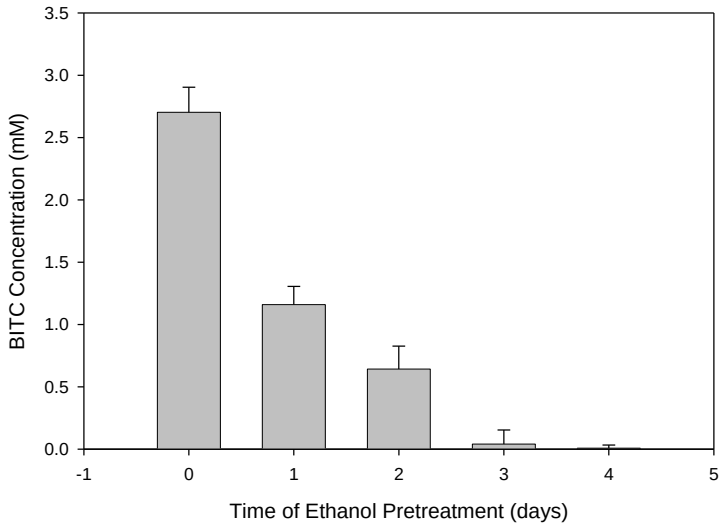

Fig. 17: BITC concentrations in papaya seed oil after various periods of ethanol pretreatment. Papaya seeds were soaked in 95% ethanol for different time intervals as a pre-treatment to oil extraction. These pre-treated seeds were then extracted via soxhlet extraction, the oil was diluted to 4%, and assayed for BITC concentration.

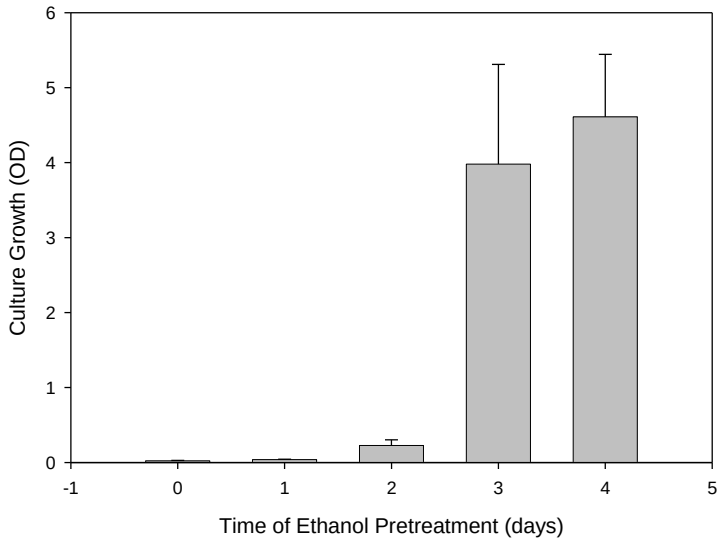

Fig. 18: Growth of *Y. lipolytica* cultures utilizing the extracted oils after various periods of ethanol pretreatment, the same oils that were tested for BITC in Fig. 17. Growth was measured by OD_{600} after 3 days. All cultures were inoculated at an OD_{600} of 0.010.

The results from Fig. 17 showed that the 3 day pretreated oil had a mean BITC concentration of 0.040 mM while the 4 day oil showed a BITC concentration of 0.007 mM. In order to rule out natural myrosinase decay over time as the reason for lower BITC concentrations in day 3 and day 4 pretreatments, untreated papaya seeds were also allowed to sit for 4 days as a control. The oil from these seeds that underwent the same conditions, except with the absence of ethanol, showed the same concentrations of BITC as day 0 pretreated oil. This meant that any natural decay in myrosinase activity during those 4 days was very minimal, and unlikely to cause the large decrease in BITC concentrations seen comparing 0 day pretreated samples to 4 day pretreated samples.

To further support this finding, aliquots of the same oils that were assayed for BITC in Fig. 17 were also used to culture *Y. lipolytica* and were summarized in Fig. 18. The growth experiment showed that the oil from pretreatment of day 0 to 2 showed very little to no growth. While oil from seeds that underwent pretreatment for 3 and 4 days were able to sustain culture growth.

The pretreatment time may seem relatively long, however, it should be noted that because of the simplicity and low input energy required with ethanol soaking, it remains a viable option to decrease BITC concentrations. This long soaking time may be due to ethanol needing to penetrate all the way through the seed to deactivate not only the myrosinase in the sarcotestae, but also the myrosinase in the embryo. Additionally, it is interesting to note that because 3 days of pretreatment resulted in values near the inhibitory level, and due to variation, it is likely that some batches may have higher BITC levels. And since the mean for 3 days was already right at the inhibitory level (0.040 mM vs 0.038 mM), this may help explain why growth experiments (Fig. 18) showed lower growth and larger standard deviation for 3 day pretreated samples compared to the 4 day pretreated samples. Thus, the results point to 4 days as being the better option for pretreatment for the conditions that were tested. The results of the papaya seed experiments as a whole demonstrate that it is possible to lower BITC concentrations in extracted papaya seed oil for utilization in culturing *Y. lipolytica*.

Chapter 4. Conclusion & Future Work

The current research has developed a method for growing, enriching, and storing *Y. lipolytica* to encapsulate EPA and DHA for utilization in improving finfish larvae feed for the tropical and subtropical regions.

For growth of *Y. lipolytica*, it was demonstrated that the locally available papaya waste was able to provide a cheap potential feedstock by utilizing papaya seed oil and addressing the issue of BITC. The low-tech and simple method of ethanol pretreatment unlocks papaya seed oil as an alternative carbon source not only for *Y. lipolytica*, but also for any number of other oleaginous organisms.

For enriching *Y. lipolytica*, this study has shown that it is possible to influence EPA and DHA accumulation by just altering culture conditions, and that for the conditions tested, the best combination of factors was oxygen presence, hydrolysis of source lipids, and 25^{o}C. It was also shown that to promote lipid accumulation, simple addition of carbon to an existing growth media could elevate C:N without the need for switching entire medias.

For storage of enriched *Y. lipolytica* it was demonstrated that even un-optimized storage in the form of a paste could help minimize EPA and DHA degradation for at least 30 days, while noting that rotifer feedings would likely benefit from daily batch feeding.

It is evident that the current research has made an impact in several areas, however, there are always more questioned to be answered in future works. One such future work would be to improve upon the EPA and DHA concentrations. When compared to commercial feed additive or enrichments, the results obtained in this study were on the low end. However, if a response surface study on the factors found to be significant was conducted, this may yield higher results. The current study only looked at two levels for each factor and may have missed the true peak. Utilizing the enriching oil to also serve as a carbon source during growth may also yield higher FA levels. EPA and DHA levels may be improved also by growing the yeast on the fish oil, or by improving the overall lipid accumulation. This study achieved 27% lipid by weight, while

literature suggests lipid content could go as high as 40% (19). Thus if the overall lipid accumulation could be improved, the total amount of EPA and DHA may also be improved.

Additionally, a "multi-function" role of *Y. lipolytica* could be explored by proximate analysis on the enriched yeast, as *Y. lipolytica* is likely to offer other nutritional benefits such as protein, with favorable lysine and threonine profiles (44). Further nutritional benefits could be incorporated, such as the already established selenium yeasts. And investigation into *Y. lipolytica* as a possible probiotic (45, 46). In fact, preliminary results by the current study's author have shown the ACA-DC-50109 capable of growth in certain concentrations of salt water.

In terms of future work involving papaya seed oil, better understanding on the mechanism behind ethanol's effect of decreasing BITC produced from papaya seed tissue. As stated previously in chapter 1, ethanol is likely deactivating the myrosinase activity in the embryo, however, in doing so, it may in fact be permeabilizing the myrosin cells and S-cells, allowing the "leaking" of myrosinase and benzyl glucosinolate, and allowing the formation of BITC, followed by possible degradation of BITC by ethanol (47), or by simply "leeching" BITC into ethanol.

Additionally, the 4% papaya oil used in culture experiments is likely to be in excess. Thus, experiments could be conducted to test if using less oil or feeding the oil batch-wise would allow for higher tolerance of BITC concentrations in the oil. This would allow for perhaps shorter pretreatment times, or just safer tolerance levels.

It is evident that the possible uses of *Y. lipolytica* in the field of aquaculture are numerous. The current research has demonstrated the viability of this unique yeast to utilize different carbon sources such as pretreated papaya seed oil, while also being able to encapsulate and store EPA and DHA intracellularly, thereby providing a possible feed additive that increases EPA and DHA content in finfish larvae feed.

Chapter 5. References

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Appendix

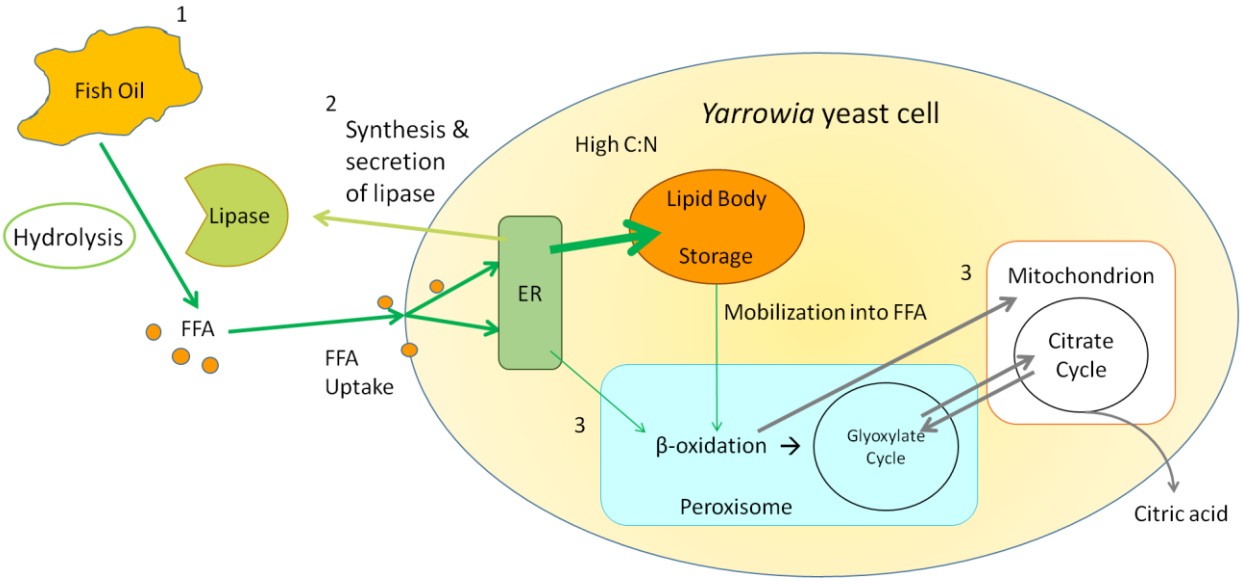

Fig. A1: Simplified fatty acid metabolism for *Y. lipolytica*, adapted from Flickers et al EMS Yeast Research 5 (2005) 527-5430. In the presence of a lipid substrate, *Y. lipolytica* may synthesize and secrete lipase and biosurfactants to hydrolyze extracellular lipids such as fish oil into free fatty acids (FFA). The FFA can then be taken up via active or passive transport and onward to the endoplasmic reticulum (ER). From the ER the lipid may be stored in the lipid body in the form of triglycerides, which is promoted under high carbon to nitrogen ratios (C:N). Alternatively, FFA from the ER may enter the peroxisome instead of being stored, and may undergo β-oxidation, to metabolize the FFA. Temperature and oxygen may alter lipid accumulation in the three areas labeled (1-3). 1) Higher temperatures and oxygen presence may degrade the extracellular lipids via oxidation, thus potentially decreasing the total lipid accumulated. 2) Higher temperatures and oxygen presence may aid in synthesis of lipase and biosurfactants, thus potentially increasing the total lipid accumulated. 3) Higher temperatures and oxygen presence may promote cellular metabolism, leading to higher rates of β-oxidation, thus potentially decreasing the total lipid accumulated.

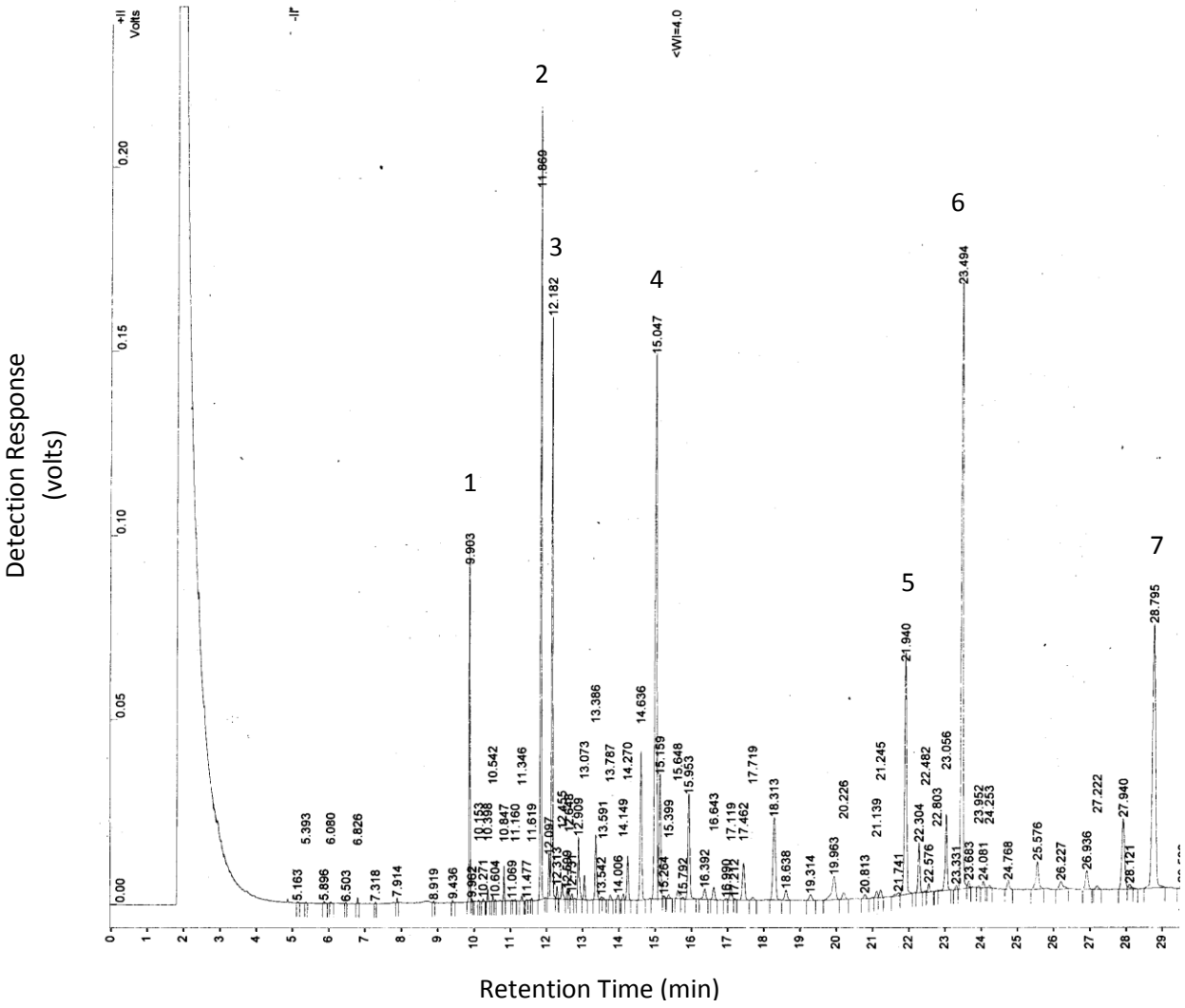

Fig. A2: GC chromatogram of enriched *Y. lipolytica*. The culture was enriched under the best combination of factors previously tested, hydrolysis, oxygen presence, and 25^{o}C. Major peaks: (1) Hexadecanoic acid, C16:0. (2) Octadecanoic acid, C18:0. (3) Oleic acid, C18:1 n-9. (4) Linolenate, C18:3 n-3. (5) Internal standard of heneicosanoic acid, C21:0. (6) EPA, C20:5 n-3. (7) DHA, C22:6 n-3