FINAL REPORT
September 4, 2019

OPTIMIZATION AND INITIAL BIOPROCESS SCALEUP OF OMEGA-3 PRODUCTION FROM RENDERED FAT

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Project Start Date: **July 1, 2016**

Duration of Project: 39 months

Lay Summary: The primary objective of this proposal is to engineer the yeast, *Yarrowia lipolytica*, to grow on rendered animal fats, and serve as a platform for the production of value-added products, including omega-3 fatty acids. This 3rd phase of the project intends to show improved titers of omega-3 fatty acids and the feasibility of using rendered fat substrates. Omega-3 rich yeast are envisioned as a replacement for fish oil in the aquaculture industry.

Objective (s): The overall objective of this project is to enable a bioprocess for converting saturated and monounsaturated animal fats into polyunsaturated fatty acids (PUFA) rich in eicosapentaenoic acid (EPA), the most important and limited omega-3 fatty acid found in fish oil. The specific objectives of this proposal are:

1. Improve Omega-3 Producing Strain Titer and Yield.
2. Lab Scale-Up of Rendered Fats to Omega-3.

The expected outcomes of this proposal include an optimized non-pathogenic yeast strain capable of converting rendered fats into PUFA and other oleochemicals, demonstrated in a 2L Bioreactor.

Project Overview:

Introduction

As the population and amount of fish eaten per person continues to increase, there has been a greater need to bring more fish to the market place. The annual amount of wild-caught fish has remained constant over the past decade (~90 million tons/year) as the majority of the world's fisheries are fished at or beyond sustainable limits. The need for more fish has largely been met by a growing aquaculture industry (fish farming). In 2012 the global aquaculture industry produced over 66 million tons of fish for human consumption – a 41% increase since 2006 (Figure 1). These fish are fed protein meal and fish oil to fulfill nutritional requirements fish growth and health. Protein meal has been derived from fish, plant and animal sources; however, fish oil, has not been displaced by vegetable and animal fats. The reason is that fish oil contains polyunsaturated fatty acids (PUFA) or omega-3 fatty acids (O3FA) that are needed for fish development and provide a cardiac health benefit to humans.

PUFA required for fish diets is only produced by phytoplankton and algae, which are eaten by and accumulate in fatty fish, such as anchovies and sardines. Currently, the aquaculture industry relies almost entirely on extracted fish oil processed from these wild-caught so-called

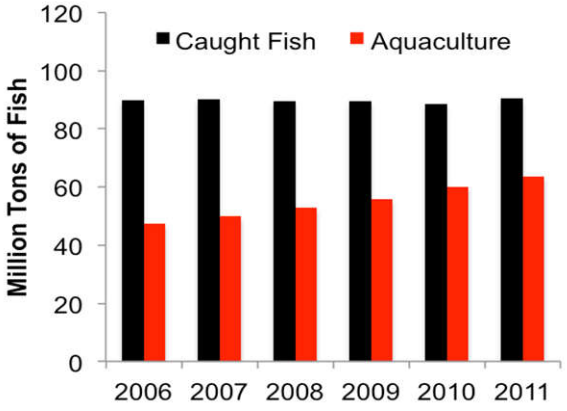

Figure 1. Steady increase in aquaculture as the world's fisheries are being operated at their maximum sustainable production rates.

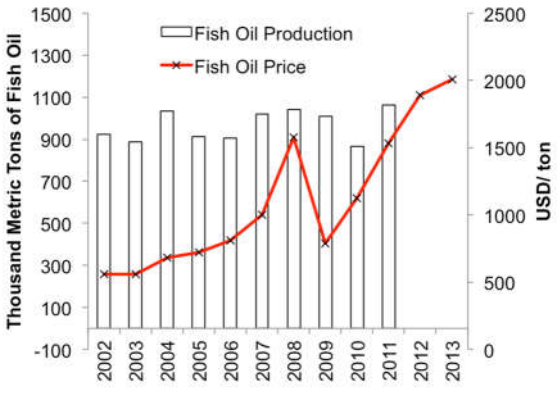

Figure 2. Steady increase in the price of fish as supply is stagnant but demand is increasing.

trash fish. Since the supply of fish oil depends on caught fish, and there is no room to increase the amount of caught fish, the global supply of fish oil has remained unchanged for well over a decade. Concurrently, the demand for fish oil in the aquaculture industry (in addition to the nutritional supplements industry) has exceeded the supply and the price of fish oil has steadily climbed (Figure 2). The simple fact is that **the global demand for omega-3 rich PUFA is growing and the supply can no longer meet that demand**. Therefore, new sources of PUFA are needed for continued growth in the aquaculture and nutritional supplements industry.

Animal and vegetable fats have been considered as replacements for fish oil. Indeed, more of these saturated fats and monounsaturated fats have found their way into fish farms, decreasing the omega-3 content of these farmed fish. Unfortunately, the composition of beef tallow lacks omega-3 PUFA (4%) and does not provide the same value for the fish or the consumer. Similarly, other rendered animal fats likewise do not contain PUFA. By comparison, sardine fish oil has a much larger percentage of EPA and DHA (~40%). A process to convert the saturated and monounsaturated fatty acids in rendered animal fat to PUFAs would open a new and large market for animal co-products and the rendering industry.

Animal and vegetable fats have been considered as replacements for fish oil. Indeed, more of these saturated fats and monounsaturated fats have found their way into fish farms, decreasing the omega-3 content of these farmed fish. Unfortunately, the composition of beef tallow lacks omega-3 PUFA (4%) and does not provide the same value for the fish or the consumer. Similarly, other rendered animal fats likewise do not contain PUFA. By comparison, sardine fish oil has a much larger percentage of EPA and DHA (~40%). A process to convert the saturated and monounsaturated fatty acids in tallow to PUFAs would open a new and large market for animal co-products.

Driven by the stagnation of PUFA supply and growth in demand, the price for fish oil has been approximately $2000 per ton over the past 5 years, and is projected to nearly $3000 per ton by 2020. The rending industry produces an abundant supply of nonedible tallow with a price of $700 per ton. The price of other animal fats is similar in price to tallow. A complete economic evaluation is currently not available, however, ethanol production and other fermentation-based chemical production remains profitable with much smaller margins. Therefore, there is great economic value in developing a process to convert animal fats into omega-3 rich PUFA.

The natural process used by algae and phytoplankton to synthesize PUFA follows the Omega-3 Pathway. Saturated and monounsaturated fatty acids are produced and then sequentially reacted to increase the number of desaturated bonds and elongate the fatty acid chain. These reactions are performed by enzymes exclusively found in algae and phytoplankton. Algae and phytoplankton unfortunately are not good industrial microorganisms and do not produce large quantities PUFA.

Dupont recently published a biochemical process to convert refined glucose into PUFA using a yeast, *Y. lipolytica*. They achieved marked improvements of PUFA yield using the Omega-9 pathway (orange Figure 3). In high-glucose media, yields were approximately 15% EPA by weight. A glucose based process suffers two problems: 1) the conversion of glucose to metabolic building blocks acetyl-CoA results in production of carbon dioxide leading to a 33%

loss of carbon, and 2) the relative high price of glucose makes the economics of such a process more difficult. By comparison, a process utilizing rendered fats (brown) would have two major advantages: 1) Carbon dioxide is not formed, which increases the carbon intensity of the process, and 2) The cost of animal fats is much lower than glucose. This report focuses on specifically on the engineering of *Y. lipolytica* to directly convert animal fats into omega-3 rich PUFA.

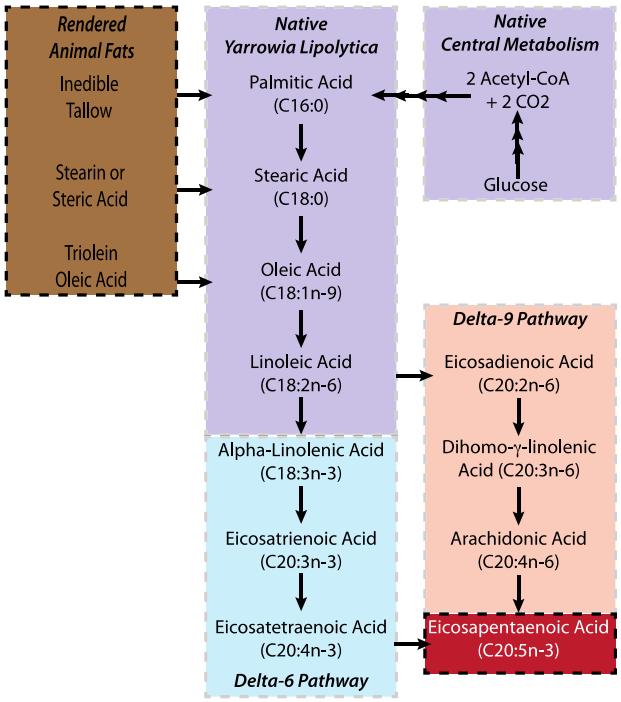

Figure 3. Pathways for Biosynthesis of Polyunsaturated Fatty Acids such as Eicosapentaenoic Acid. The native *Yarrowia* pathway can make linoleic acid from glucose. Engineering the Omega 9 pathway was found more effective at producing EPA than the Omega 6 pathway. This proposal focuses on engineering *Y. lipolytica* to use model fats or fatty acids and inedible tallow instead of glucose for PUFA production. The advantage is that the feedstock is cheaper and is more carbon efficient.

Materials & Methods.

Chemicals and Strains. All chemicals were purchased from Sigma Aldrich unless otherwise noted. Fatty acid and lipid standards were purchased from NuCheck Prep (WI). All molecular cloning enzymes were purchased from New England Biolabs. All cloning and plasmid propagation were carried out in E. coli DH10B. Strains of *Y. lipolytica* were obtained from ATCC, including W29, PO1f strain (MATa leu2-270 ura3-302 xpr2-322 axp1), and the Dupont Y8412 strain. The pSL16 shuttle vector was originally obtained from Dr. Matsuoka at Sojo University. Rendered beef and poultry fats were obtained through ACREC.

Cloning and Episomal Expression. SLIC was used for all cloning works. Codon optimization and gene synthesis of EPA desaturases was provided GenScript. Primers and other oligos were prepared from Eurofins. Expression of all genes were driven by TEF-intron promoter. Terminator for all the genes is CYC1.

Integration. Gene integration was accomplished stepwise into a series of so-called standard sites, where we have previously characterized integration. The integration is encouraged by using a CRISPR-Cas9 system to target single strand breaks to specific genomic loci and using

homologous sequences to the target loci. Colonies are screened and segregated into single clones.

Gene Knockout. Genes were knocked out either by a single indel created by a single CRISPR-Cas9 induced double strand break, or by using flanking gRNA sequences that excise targets of interested. Using two gRNAs creates a whole gene deletion and much simpler screening. Colonies are screened and segregated into single clones.

Microbial Culture. DH10B was grown at 37 °C in Luria-Bertani media supplemented with 100 μg/mL ampicillin with constant shaking at 200 rpm. The pre-culture of *Y. lipolytica* was performed in 2 ml YPD or YSC medium culture consisting of different carbon sources, yeast nitrogen base and dropout CSM. Shake flask fermentation were carried out in base or rich media at 28 °C with constant shaking at 250 rpm. When investigating the growth of *Y. lipolytica* on animal fats, certain amount of beef tallow and chicken fat were used as carbon sources.

Dry Cell Weight Measurements. For dry cell weight measurements, at least 5 ml of cell culture was washed with distilled water twice and with or without hexane once by centrifugation at 6000 g for 5 min in order to remove the residual glucose or fats. The cell pellet was oven dried at 100 °C overnight. Then the dry cell weight was determined gravimetrically.

Lipid Extraction. The separation of lipid species was conducted by thin layer chromatography (TLC). A mixture of hexane/diethyl ether/acetic acid (70/30/1, v/v) was used as solvent system. 5% phosphomolybdic acid or crystal iodine was used for visualization of TLC results.

GC Analysis. To measure the fatty acid profile and the total lipid production, cell pellets from 1 ml culture were first washed using the same approach described above. An adapted method was used for transesterification and GC analysis. Briefly, 500 μL methanol solution containing 1 M NaOH were added to cell pellets. Then 100 μL internal standard solution (2 mg/ml glyceryl triheptadecanoate in hexane) was added to each cell pellet. The samples were vortexed at 2000 rpm at room temperature for 40 min. Then the solution was neutralized with 40 μL sulfuric acid and 850 μl hexane was added to extract the fatty acid methyl esters (FAMEs). All mixtures were separated by centrifuging at 8000 rpm for 2 min, and the top hexane layer was subjected to be analyzed by using GC-FID. Samples were injected with an injection volume of 1 μl, split ratio of 10, and injector temperature of 250 °C. FAME species were separated on an Agilent J&W DB-23 capillary column (30m×0.25mm×0.15μm), with helium carrier gas at a flow rate of 1ml/min. The temperature of the oven starts from 175 °C and then ramped to 200 °C within 6 min. The FID was operated at a temperature of 280 C with a helium make up gas flow of 25 ml/min, hydrogen flow of 30 ml/min, and air flow of 300 ml/min.

Results & Discussion

Objective 1. Optimization of EPA (C20:5) Producing *Y. lipolytica* Strain

1.1. Overexpression of Elongase and Desaturase to Relieve Bottlenecks

Our approach is to determine which of the four enzymes (Δ6 desaturase, Δ6 elongase, Δ5 desaturase, and Δ17 desaturase) is most rate limiting. We have made genetic constructs to express each enzyme separately from strong promoters. The yield of exogenous fatty acid converted to the product fatty acid was measured. We sorted out a problem with the analytical methods – mainly there is a subtle discrepancy in the retention times of the fatty acids; therefore, we performed additional experiments to be certain that we are correctly identifying the fatty acids. We discovered that knocking out the MFE1 leaves the peroxisomal acyl-CoA oxidase (POX) still active. This is tricky since the product of the POX enzymes is a 1,2 desaturated fatty acid, which has a very similar retention time compared to the product of desaturases. We therefore exclusively used the PEX10 knockout to discourage beta-oxidation.

To construct strains more easily, we created a vector/insert system that contains multiple restriction signs and unique hook sequences. This allows sequential or parallel cloning of up to 4 genes, as shown in Figure 4. This was used to generate our strain with the full pathway for EPA expressed.

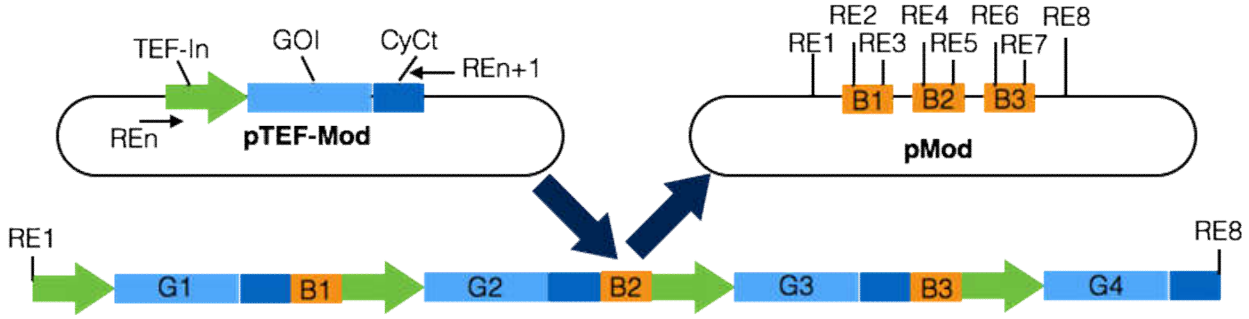

Figure 4. Our cloning system

We identified the bottleneck of the EPA pathway by introducing the entire pathway through episomal expression. We saw the accumulation of LA and ARA, which indicated the first and last step were the rate-limiting step. Therefore, we tried to relieve the bottleneck by adding additional copy numbers of O6D and S17D. The results (Figure 5) below showed this approach significantly increased the EPA production as well as reduced the intermediate's level.

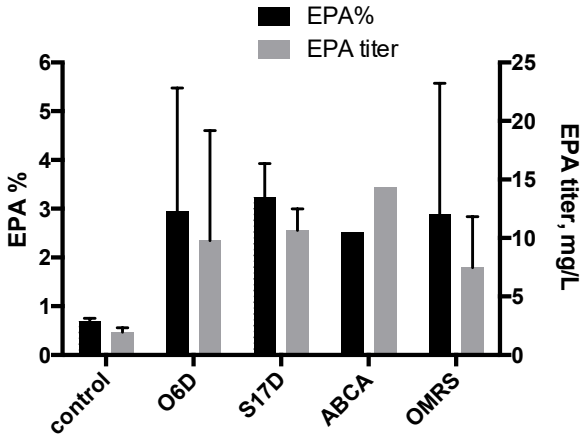

Figure 5. Effect of expressing various genes on EPA production.

We also used CRISPR-Cas9 to randomly integrate the entire EPA pathway using hygromycin selective marker. The resulting strain produced similar level of EPA compared to the episomal expression.

We tested EPA production on some of our best performing strains using beef tallow, chicken fat, and model triglyceride triolein as substrates. We introduced an additional copy of the first desaturase O6D, the last desaturase S17D, and the whole EPA pathway cassette through episomal expression into a strain that already contained a fully integrated EPA pathway. Results (Figure 6) indicate that additional expression of S17D produced the highest EPA when beef tallow was used, probably due to the relief of product inhibition by pulling acyl flux towards the end product. Beef tallow is slightly better than chicken fat for S17D and OMRS. O6D strain performed similarly for beef tallow and chicken fat. The conversion rate of linoleic acid to EPA peaked around 25% when beef tallow was used for S17D strain.

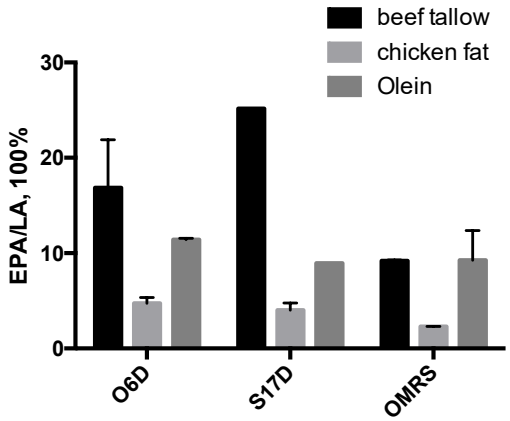

Figure 6. EPA production from beef tallow and chicken fat.

1.2. Overexpression of Fatty Acid Activation and TAG Forming Enzymes.

We have tested a large set of fatty acid activation and TAG forming or cycling enzymes that we will overexpress. We have successfully cloned 14 genes listed in Table 1. We can investigate the impact of overexpressing these different genes in a strain of *Y. lipolytica* that has O6D and M6E integrated (and later O6D, M6E, E5D, and S17D). These genes cluster around key nodes important to omega-3 production: acyl-CoA binding proteins; fatty acids / acyl-CoA transporters, PC-acyl-CoA cycling.

Table 1. Genes hypothesized to influence omega-3 accumulation.

	PCR	SLIC	digest verified	sequencing verified	Pathway
ACBP-ER	Yes	Yes	Yes	Yes	Binding protein
ACBP	Yes	Yes	Yes	Yes	Binding protein
AAL1-ΔPST1	Yes	Yes	Yes	Yes	Activation
FAT1-ΔPST1	Yes	Yes	Yes	Yes	Activation
FAT2-ΔPST1	Yes	Yes	Yes	Yes	Activation
FAT3	Yes	Yes	Yes	Yes	Activation

FAT4	Yes	Yes	Yes	Yes	Activation
CPT	Yes	Yes	Yes	Yes	PC-CoA cycling
LPCAT	Yes	Yes	Yes	Yes	PC-CoA cycling
GPCAT	Yes	Yes	Yes	Yes	PC-CoA cycling
LPAAT	Yes	Yes	Yes	Yes	PC-CoA cycling
LPAET	Yes	Yes	Yes	Yes	PC-CoA cycling
ABCA	Yes	Yes	Yes	Yes	Transport

We episomally overexpressed individual acyl-CoA regulation genes in EPA producing strain. We found 13 out of 14 genes considerably improved the EPA production. We grouped these 14 genes in 4 categories.

For category 1: we overexpressed two genes, FAA1 and AAL1. Both of them encode fatty acid synthetases that activate the free fatty acid to be acyl-CoA. FAA1 encoding enzyme is present in cytoplasm while AAL1 encoding enzyme is peroxisomal. Figure 1A showed that the FAA1 increased the EPA percentage by almost two folds. The AAL1 seems has no significant effects on EPA production. We also looked at the effects of these 2 genes on the accumulation of LA (Figure 1B). Both of them decreased the LA accumulation, probably due to generating more LA-CoA from LA.

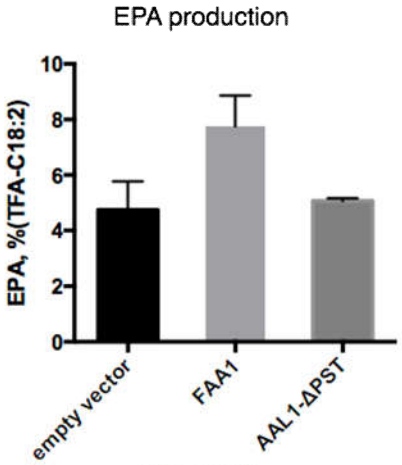

Figure 1A

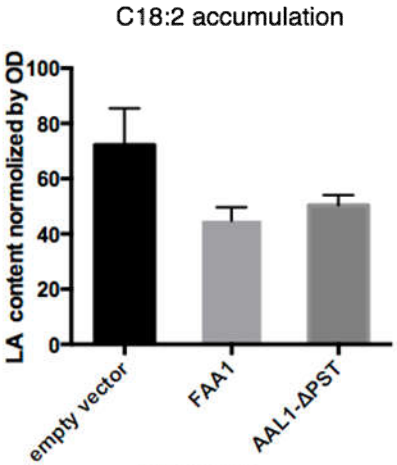

Figure 1B

For category 2: There were genes encoding two acyl-CoA binding proteins ACBP and ACBP-ER we overexpressed. ACBP is a cytoplasm enzyme that can act as buffering system for Acyl-CoAs, while ACBP-ER is an ER membrane bonding protein. Overexpressing those two enzymes increased the EPA percentage from 4.7 to 6.9 and 7.1% respectively (Figure 2A). The two ACBPs increased the LA accumulation possibly by providing more buffering capabilities of LA-CoA that allows more LA getting into the cells (Figure 2B).

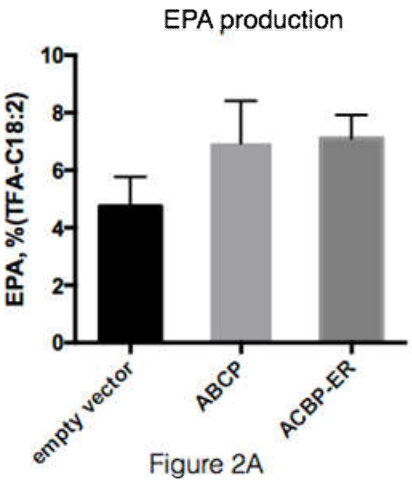

Figure 2A

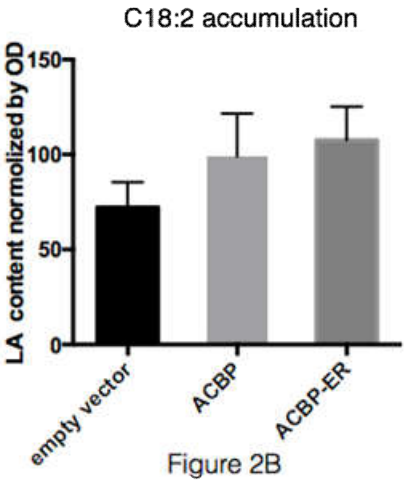

Figure 2B

For category 3: FAT1 to 4 encodes putative enzymes that may enhance the flux of fatty acids or acyl-CoA among ER, lipid body and peroxisome [6]. All the four genes overexpressed increased the EPA percentage with the highest improvement from FAT1 (Figure 3A). FAT1 to 3 slightly increased the LA accumulation, but FAT4 reduced the level of LA (Figure 3B).

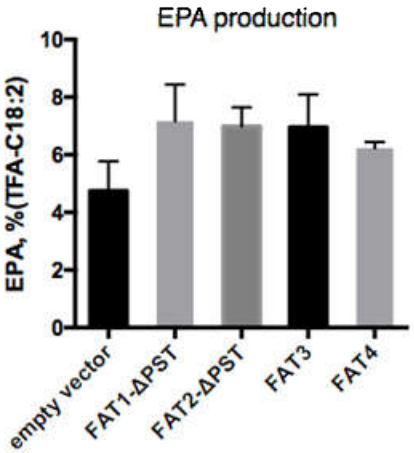

Figure 3A

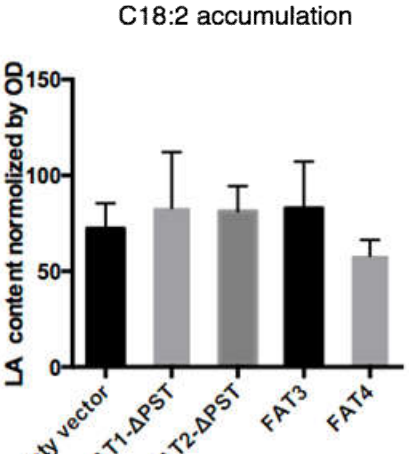

Figure 3B

For category 4: There are 5 genes we targeted to use to increase the acyl exchange among DAG, PC and Acyl-CoA pools. By doing that, we expected to redirect more acyl-group to desaturation and elongation process before they end up in TAG. All 5 genes overexpression led to increase of the EPA percentage. (Figure 4A). They were showed to have different effects on LA accumulation, with CPT decreasing the level while LPAAT significantly increased the LA level (Figure 4B).

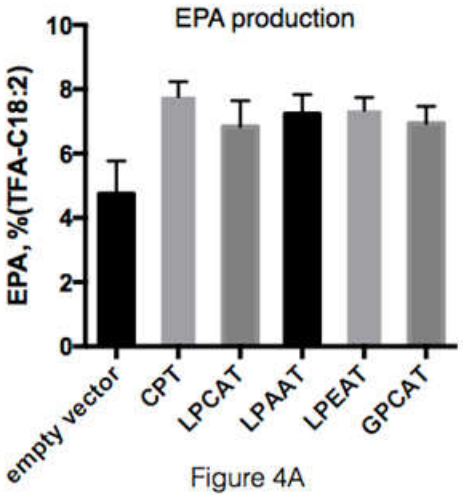

Figure 4A

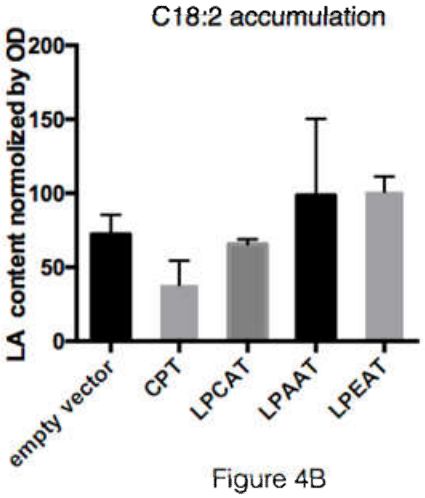

Figure 4B

Integration of the second OMRS into D17 site improved EPA production by 3 fold (Figure 7). Overexpression of the acyl-editing gene *CPT* increased EPA production regardless the copy number of OMRS. In fact, OMRSx2-CPT showed the highest EPA titer (~25 mg/L) among all previously tested high production strains (OMRS-O6D, OMRS-S17D or OMRS-OMRS episomal expression). The ratio of EPA to ALA also reached its highest value. The calculated conversion rate (EPA/(EPA+LA)) is 21.5%.

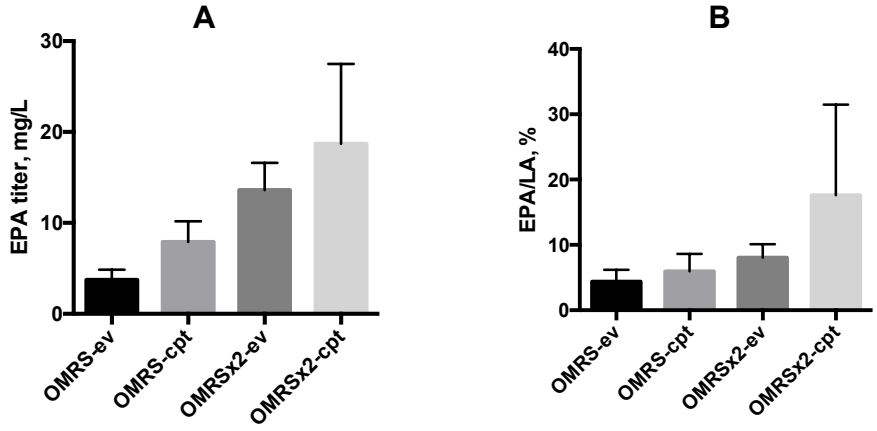

Figure 7. Testing additional copies of the pathway

We tested different combination of FAA1, ACBP-er, ABCA for EPA production (Figure 8). We found that FAA1 itself improved EPA more than 30%. ACBPer alone didn't increase the EPA titer. ABCA alone led to about 2.5 folds more EPA. By combining FAA1 and ACBPer, we got more EPA than ACBPer alone. FAA1 offset the increase of EPA from ABCA when expressed together with ABCA. Combining three genes together produced about the same level of EPA than ABCA alone. We also found that combination of CPT, LPCAT and GPCAT increased the EPA production. Even though GPCAT alone also significantly improve EPA production, it's beneficial to put CPT and LPCAT together with GPCAT. This combination may show better result when higher acyl-coA flux is provided.

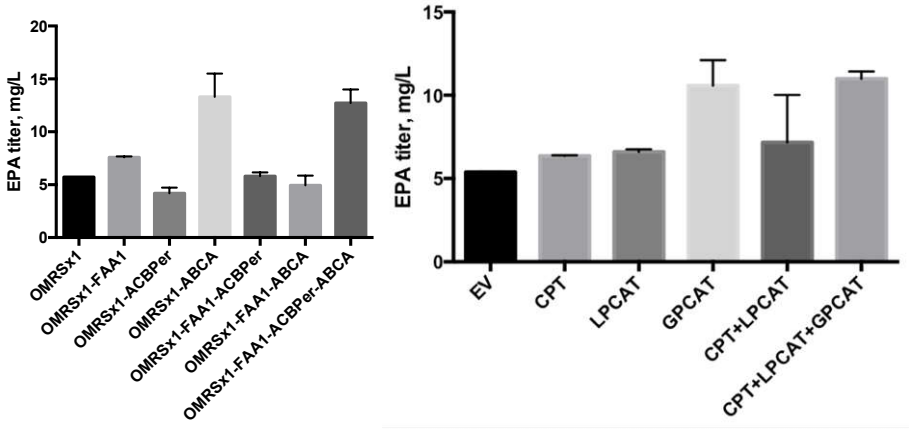

Figure 8. Combinations of EPA Flux Enhancing Targets.

We have identified low nitrogen (LN) media conditions used to generate biomass that significantly improve the EPA production (Figure 1). We believe that effectively the substrates are primed for EPA production. (Figure 9).

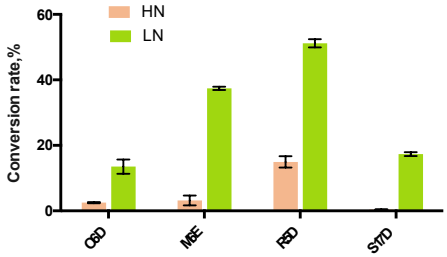

Figure 9. Low nitrogen increases EPA production significantly.

Reassessment of various strains we previously made, and combinations of new strains show that 2 sets of genes plus an additional O6D copy resulted in the highest titer, similar to 2 sets of genes and a novel putative transporter, ABCA (Figure 10). Interestingly, an additional copy of S17D did not improve titer; however, we expect it still be a rate limiting step. These titers, it should be noted, are based on 1 g/L linoleic acid feed, of which ~50% is consumed.

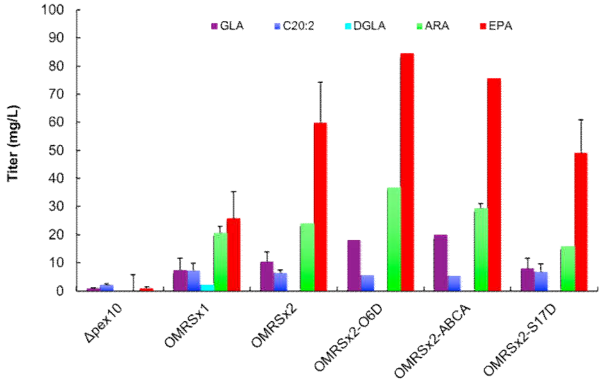

Figure 10. Reassessment of various strains with low nitrogen condition.

Assessment of a great number of gene targets (Figure 11) intended to increase fatty acyl-CoA flux showed marginal improvements for any given target, with FAT4 having some benefit. The combination of CPT and LPCAT from the acyl-editing pathway here showed the best ratio of EPA:ARA. **Importantly, the best titer achieved here is over 100 mg/L, from 1 g/L of linoleic acid that is 50% consumed, resulting in a 10-20% overall conversion.**

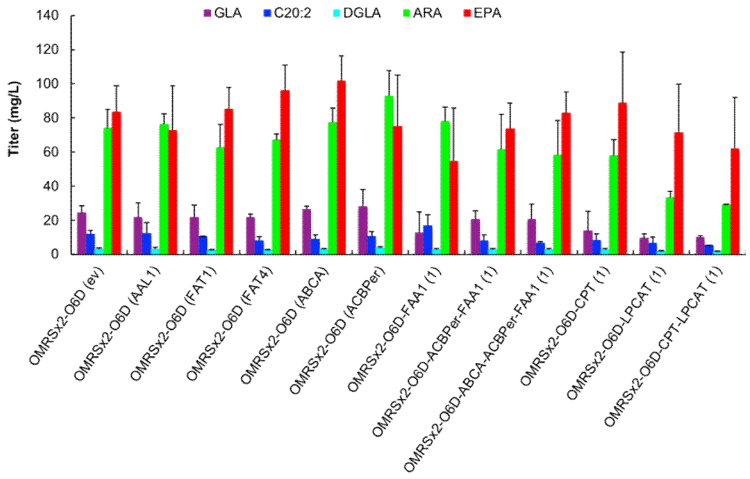

Figure 11. Reassessment of acyl-CoA flux targets with low nitrogen.

We identified a new target for knockout - the ACOT3 thiolase of unknown function results in doubling of EPA titer in an unoptimized strain (OMRSx1) – see Figure 12.

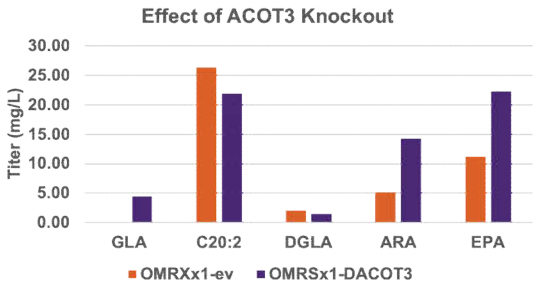

Figure 12. Effect of ACOT3 knockout double EPA titer in unoptimized strains.

We recently (Figure 13) tested ACOT3 and ACOT1 knockout in the more highly optimized strains. Unfortunately, the ACOT3 grew very poorly, leading to low titers.

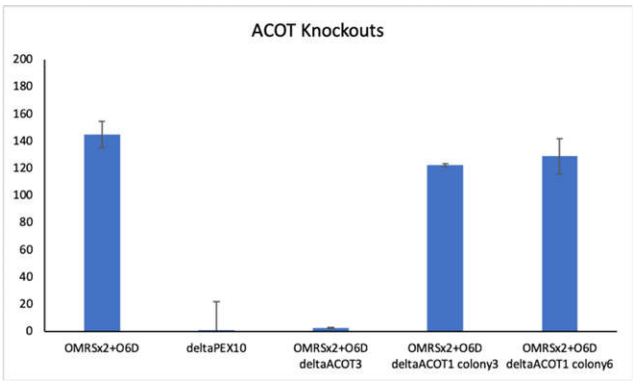

Figure 13. Knockout of ACOT1 and ACOT3 in the OMRSx2+O6D did not result in improved EPA titer.

Summary Figures

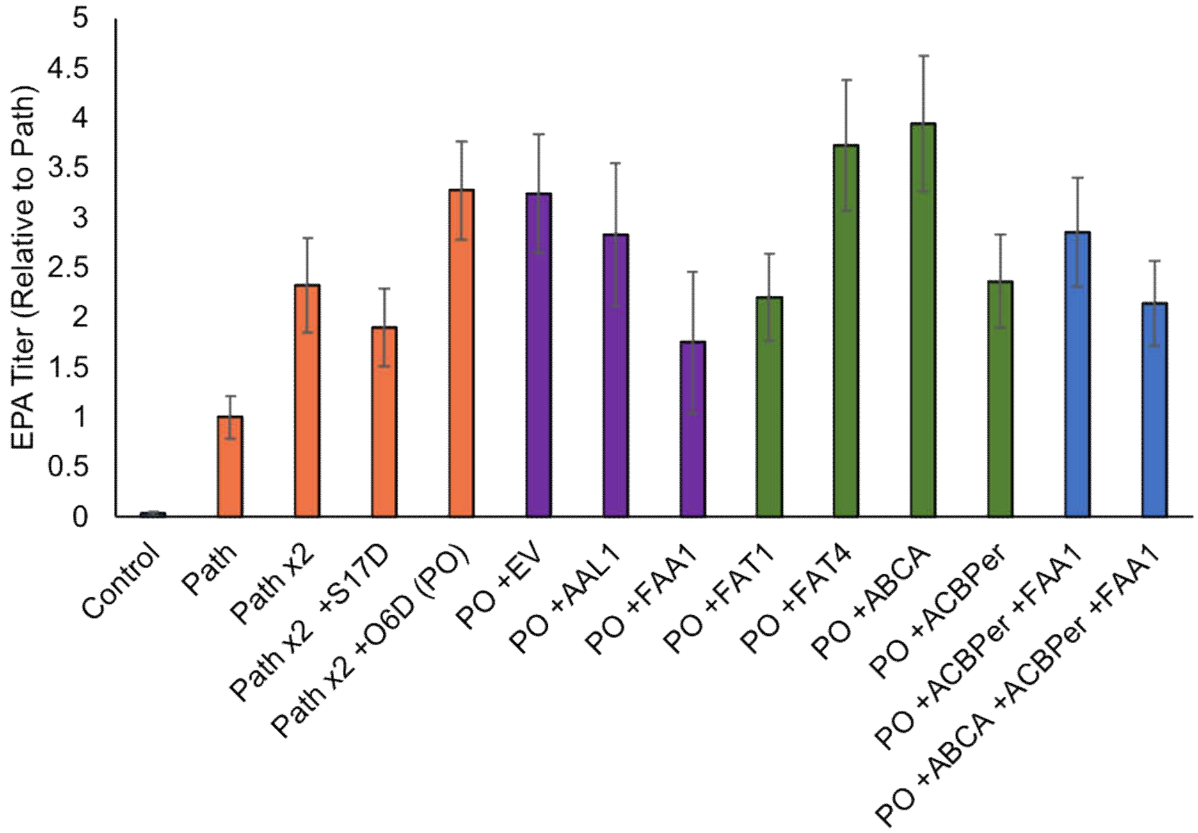

Figure 14. Comparison of titers in low nitrogen conditions, relative to the 1 pathway strain.

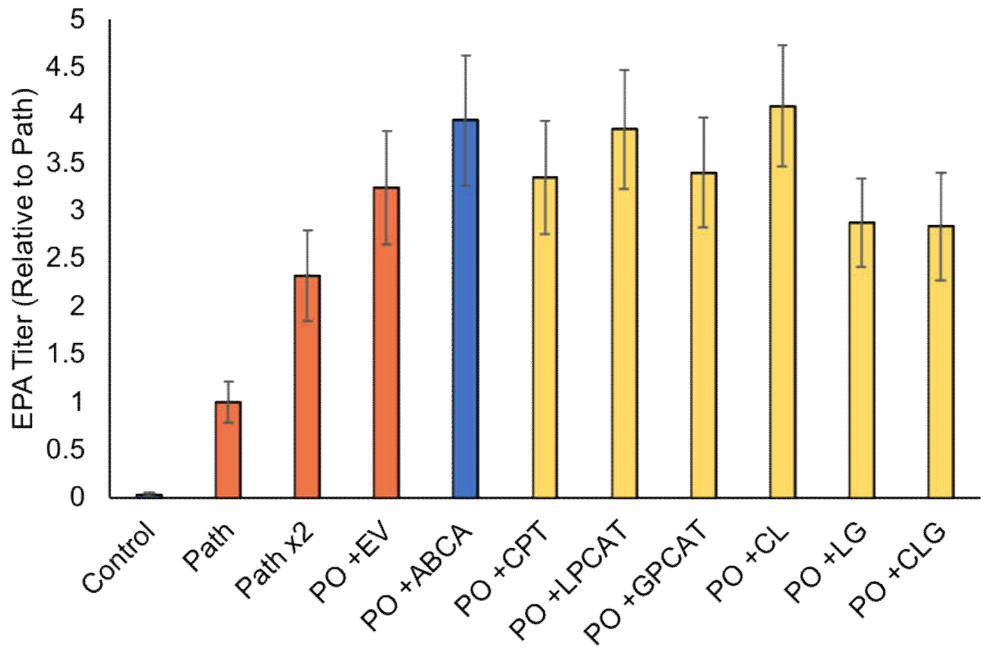

Figure 15. Comparison of titers in low nitrogen conditions, relative to the 1 pathway strain.

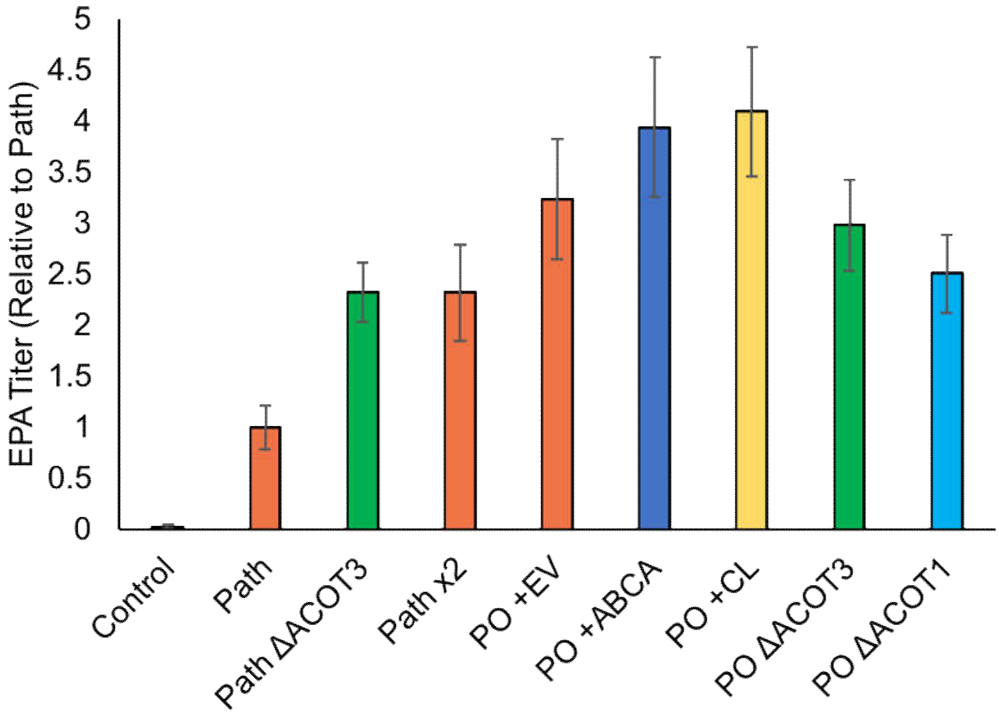

Figure 16. Comparison of titers in low nitrogen conditions, relative to the 1 pathway strain.

Objective 2. Lab Scale-Up of Rendered Fats to Omega-3.

2.1 Screening Media Conditions in 50 mL Shake Flasks and Scale Up to 1L Shake Flask

The effect of initial fat concentration, and carbon-to-nitrogen ratio on the cell growth were established, showing optimal conditions of 10g/L with high C:N ratios, ~200:1 using either beef tallow or poultry fat.

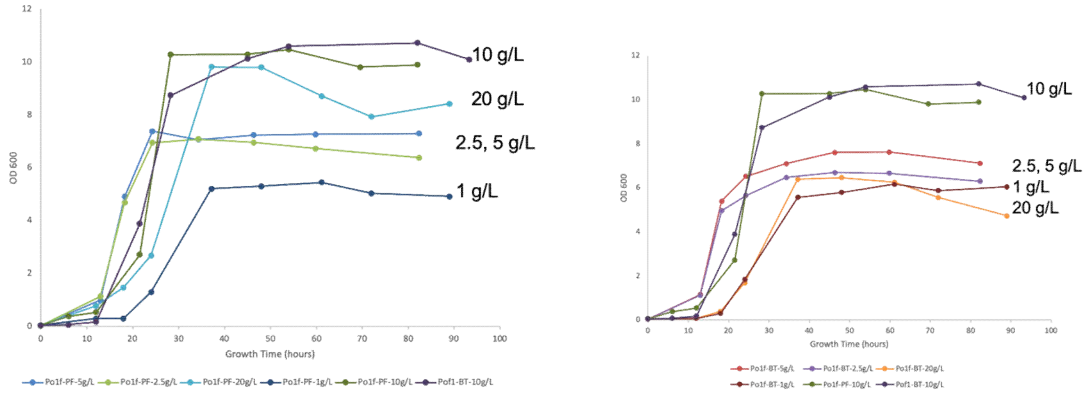

Figure 17. Yarrowia growth curves using beef tallow and poultry fat with varying loading.

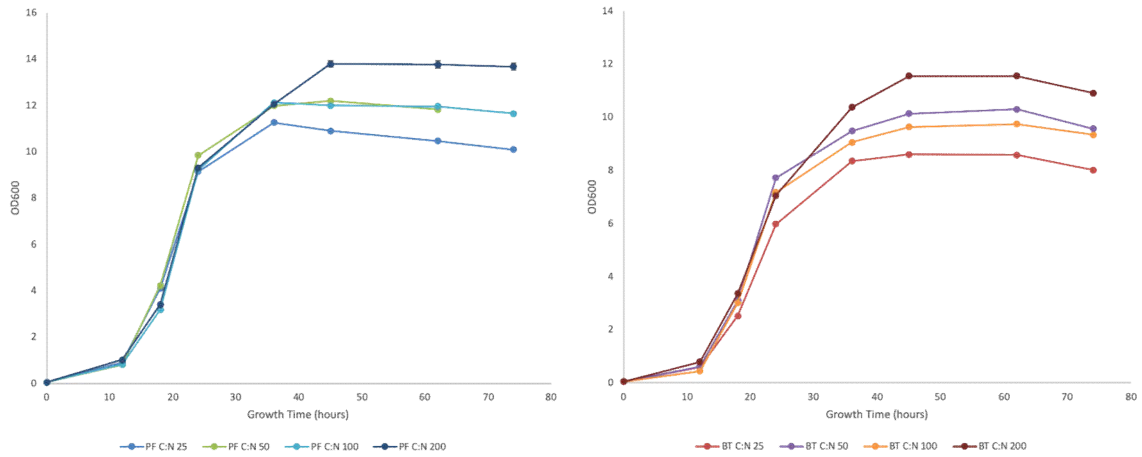

Figure 18. Yarrowia growth curves using beef tallow and poultry fat with varying C:N ratio.

2.2 Scale Up Omega-3 Process to 2L Bioreactor

Based on discussions with the ACREC renderers, we did not fully address this task as improving the strain first was a priority. As a result, more effort was spent on these tasks.

We did send out some samples to a collaborator, Dr. Dongming Xie, at UMass Lowell. We recently performed a bioreactor experiment using our current omega-3 producing strain (OMRSx2-O6D) and used 10g/L of beef tallow. Two runs were performed that used different types of baffles to create different mixing regimes. Our results indicate a peak titer of 200 mg/L (about 2x the flask titer in flasks. It should be noted that the process was very unoptimized to use beef tallow as it contains a high amount of stearic acid, and the conditions used likely contributed to lower than expected titers.

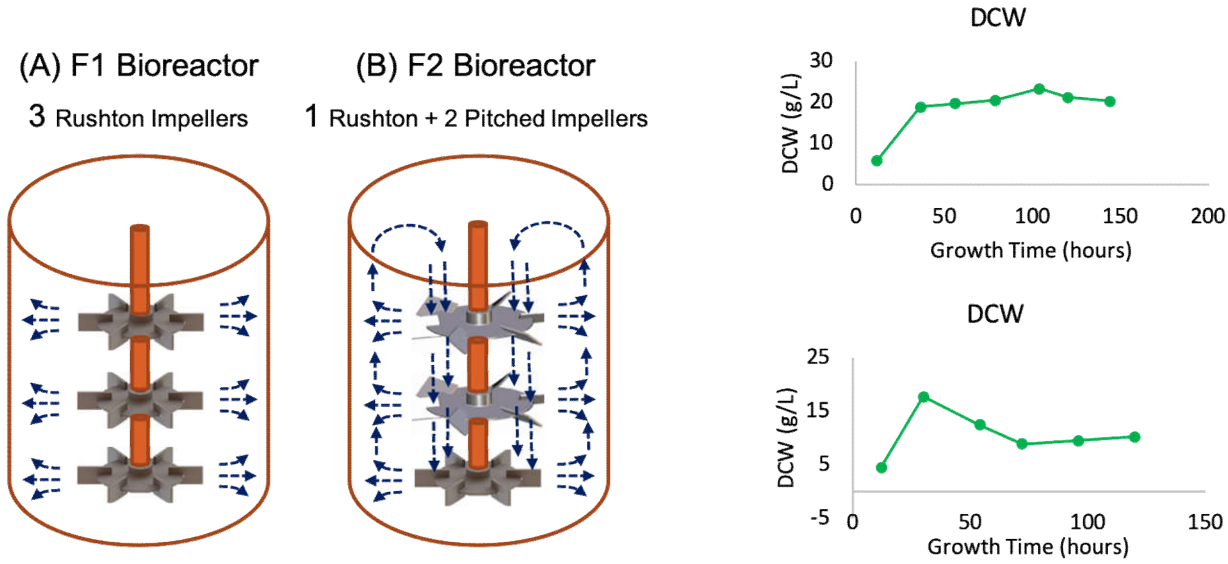

Figure 20. Bioreactor production of omega-3 fatty acids using beef tallow.

Conclusions

We have constructed an omega-3 producing strain that utilizes exogenous fats from rendered products to make omega-3 fatty acids. We have also identified several targets to improve EPA titers; however, we suspect additional bottlenecks limit the production and will require a substantial effort and a new project to overcome. Fortunately, funding from NOAA Sea Grant will support these studies, using plant or animal oils as substrates. Bottlenecks include cellular ER stress response due to the production desaturase enzymes or the failure to funnel stearic and oleic acid effectively into linoleic acids for EPA production. Overall, this is a promising start to creating a bioprocess centered on rendered animal products.

Impacts and Significance: Over 50% of the seafood consumed each year is provided by the aquaculture industry. This number continues to grow as the global population grows, and the per capita consumption of fish increases. As caught fish production is stagnant due to sustainability and overfishing, aquaculture will have to meet this growing demand. The major limitation for aquaculture expansion is the lack of omega-3 containing fish oil. Microbial processes for omega-3 production are being commercialized now that use glucose as a feedstock, however, rendered animal fats have higher theoretical yield (more carbon-efficient) and cheaper feedstock. Omega-3 fatty acids are required for sound aquaculture practices, which currently rely on a limited supply of increasingly expensive omega-3 from small caught fish. We believe that microbial synthesis of omega-3 from animal fats can provide a reliable and economic source to either replace fish oil or to enable further expansion of aquaculture – which may in turn also increase the use of other rendered products in aquaculture. Importantly, this project represents a movement towards using rendered products as a feedstock for a variety of bioprocess products. This opens new markets for rendered fats and de-risks potential fluctuation in price and regulations for use in animal feeds

Publications:

Peer-Review Publications (Bold is directly related)

1. Schwartz, C., Cheng, JF., Evans, R., Schwartz, CA., Wagner, JM., Anglin, S., Beitz, A., Pan, W., Lonardi, S., Blenner, M., Alper, H., Yoshikuni, Y., Wheeldon, I.* "Validating genome-wide CRISPR-Cas9 function in the non-conventional yeast *Yarrowia lipolytica*" *Metabolic Engineering* 55:9 102-110 (2019).

2. **Spagnuolo, M., Yaguchi, A., Blenner, M.* "Engineering of oleaginous yeast for biofuels production: recent advances and future directions." *Current Opinion in Biotechnology* 57:73-81 (2019).**

3. **Gao, D., Smith, S., Rodriguez, G., Shabbir Hussain, M., Blenner, M.* "Dual CRISPR-Cas9 Cleavage in *Yarrowia lipolytica* Leads to Efficient Gene Excision and Targeted Integration", *Biotechnology Journal* 13:9 1-8 (2018) doi: 10.1002/biot.201700590.**

4. **Spagnuolo, M., Shabbir Hussain M., Gambill L., Blenner, M.*, "Alternative Substrate Metabolism in *Yarrowia lipolytica*", *Frontiers in Microbiology* 9:1077 (2018).**

5. Brabender, M., Shabbir Hussain, M., Rodriguez, G., Blenner, M.* "Urea and Urine are Efficient Nitrogen Sources for Growth of *Yarrowia lipolytica*", *Applied Microbiology and Biotechnology* 102(5):2313-2322 (2018) DOI: 10.1007/s00253-018-8769-z

6. **Yaguchi, A., Spagnuolo, M., Blenner, M*. "Engineering Yeast for Utilization of Alternative Feedstocks" *Current Opinion in Biotechnology* 53:122-129 (2018).**

7. Shabbir Hussain, M., Wheeldon, I., Blenner, M.* "A Strong Hybrid Fatty Acid Inducible Transcriptional Sensor Built from *Yarrowia lipolytica* Upstream Activating and Regulatory Sequences", *Biotechnology Journal* 12(1):1-11 (2017). DOI: 10.1002/biot.201700248. **Research is featured on the inside cover.**

8. Schwartz, C., Shabbir-Hussain, M., Frogue, K., Blenner, M., Wheeldon, I.* "Standardized markerless gene integration for pathway engineering in *Yarrowia lipolytica*", *ACS Synthetic Biology* 6(3):402-409 (2017). **ISI Highly Cited.**

9. Shabbir Hussain, M.‡, Rodriguez, G.‡, Gao. D., Spagnuolo M., Gambill, L., Blenner, M.* "Recent Advances in Bioengineering of the Oleaginous Yeast *Yarrowia lipolytica*", *AIMS Bioengineering* 3(4):493-514 (2016).

10. Rodriguez, G.‡, Shabbir Hussain, M.‡, Gambill L., Gao, D., Yaguchi, A., Blenner, M.* "Engineering Xylose Utilization in *Yarrowia lipolytica* by Understanding its Cryptic Xylose Pathway", *Biotechnology for Biofuels* 9:149 (2016).

11. Schwartz, C., Shabbir-Hussain, M., Blenner, M., Wheeldon, I.* "Synthetic RNA Polymerase III Promoters Facilitate High Efficiency CRISPR-Cas9 Mediated Genome Editing in *Yarrowia lipolytica*", *ACS Synthetic Biology* 5(4):356-359. (2016). **Paper is most read during**

past 12 months in 2016-2017. **ISI Highly Cited.**

12. Shabbir Hussain, M., Gambill L., Smith, S., Blenner, M.* "Engineering Promoter Architecture in Oleaginous Yeast Yarrowia lipolytica", *ACS Synthetic Biology*, 5(3):213-223. (2016). **Research is featured on the cover of the March 2016 issue**.

Under Review & In Prep

1. Smith, S.‡, Gao., D.‡, Ganesen, V., Spagnuolo, M., Blenner, M.* "An expanding toolkit for metabolic engineering of *Yarrowia lipolytica*" *Microbial Cell Factories* (In Review).

2. Blenner, M.* "Gene excision by dual-guide CRISPR-Cas9" *Methods in Molecular Biology, Yarrowia lipolytica* (In Review).

3. Blenner, M.* "Simultaneous gene excision and integration with dual-guide CRISPR-Cas9" *Methods in Molecular Biology, Yarrowia lipolytica* (In Review).

4. Smith, S.‡, Shabbir Hussain, M., Blenner, M.* "Understanding the role of hybrid promoter architecture in *Yarrowia lipolytica*" *Biochemical Engineering Journal* (In Preparation)

5. Bailey, M., Spagnuolo, M., Blenner, M.* "Engineering more stable episomal vectors for *Yarrowia lipolytica*". *Engineering in Life Sciences* (In Preparation).

Presentations

1. Blenner, M.* "Engineering non-conventional yeast systems for the production of oleochemicals" Annual Meeting of the American Institute of Chemical Engineers, Orlando, FL (November 2019)#

2. Gao, D., Ganesen, V., Spagnuolo, M., Beitz, A., Blenner, M.* "Host onboarding for Yarrowia lipolytica - trials and tribulations in the development of genomic and genetic engineering tools" Annual Meeting of the Society of Industrial Microbiology and Biotechnology, Washington, DC (July 2019)#

3. Blenner, M.*, "Synthetic Biology – A Critical Technology for Enabling Space Exploration" American Chemical Society Spring Meeting, Committee of Science Symposium Orlando, FL (April 2019).#

4. Blenner, M.*, "A Roadmap for the Use of Biotechnology in Space Exploration" American Chemical Society Spring Meeting, Orlando, FL (April 2019).

5. Blenner, M.* "Untapping the potential for using non-conventional yeast for biochemical production" Energy, Environmental, and Chemical Engineering Departmental Seminar, Washington University in St. Louis, St. Louis MO. (March 2019). #

6. Blenner, M.* "Untapping the potential for using non-conventional yeast for biochemical production" Chemical & Biomolecular Engineering Departmental Seminar, University of Tennessee, Knoxville, TN. (February 2019). #

7. Blenner, M.* "Enhancing Oleochemical Production in Yarrowia Lipolytica through Pathway and Cell Physiology Engineering" International Conference on Biomolecular Engineering, Newport Beach, CA (January 2019).

8. Blenner, M.* "Synthetic Biology – A Critical Technology for Enabling Space Exploration" STAR Tech Conference, Houston, TX (November 2018). #

9. Gao, D., Smith, S., Spagnuolo, M.*, Blenner, M. "Discovery of Novel Genes Regulating Acyl-CoA Availability in *Yarrowia Lipolytica",* American Institute of Chemical Engineers, Pittsburgh, PA (November 2018).

10. Gao, D., Smith, S., Spagnuolo, M., Blenner, M*. "Rewiring *Yarrowia Lipolytica* Lipid Metabolism for the Production of Omega-3 Fatty Acid Using Alternative Substrates", American Institute of Chemical Engineers, Pittsburgh, PA (November 2018).

11. Smith, S., Gambill, L., Shabbir-Hussain, M., Rodriguez, G., Blenner, M.* "Engineering efficient xylose metabolism in oleaginous yeast *Yarrowia lipolytica*". American Society of Agricultural & Biological Engineers Annual Meeting, Detroit, MI (August 2018).

12. Gao, D., Smith, S., Blenner, M.* "Rewiring Yarrowia Lipolytica Lipid Metabolism for the Production of Omega-3 Fatty Acid Using Alternative Substrates", Metabolic Engineering 12, Munich, Germany (June 2018).

13. Spagnuolo, M., Shabbir Hussain, M., Blenner, M.* "Engineering *Yarrowia lipolytica* for the production of fatty alcohols from sugars and fats", American Oil Chemists Society Annual Meeting & Expo, Minneapolis, MN (May 2018).

14. Bailey, M.*, Spagnuolo, M., Blenner, M. "Engineering plasmid performance in *Yarrowia lipolytica*", SC EPSCoR State Conference, Columbia, SC (April 2018). **2nd Place Undergrad Poster Competition.**

15. Spagnuolo, M.*, Shabbir Hussain, M., Edgecomb, S., Blenner, M. "Transcriptional-sensor based increase in peroxisomal fatty acyl-CoA flux improves fatty alcohol production in *Yarrowia lipolytica*", American Chemical Society Spring Meeting, New Orleans, LA (March 2018).

16. Blenner, M.* "Expanding Capabilities for Engineering Yeast for Biochemical Production" Environmental Engineering & Earth Sciences Departmental Seminar, Clemson University (January 2018). #

17. Shabbir Hussain, M., Blenner, M.* "Understanding the Role of Epigenetics in Fatty Acid Regulated Promoters in *Yarrowia lipolytica*", International Conference on Epigenetics and Bioengineering, Miami Beach, FL (December 2017).

18. Gao, D., Smith, S., Rodriguez, G., Shabbir Hussain, M., Blenner, M.* "Gene Excision by Dual-Cutting CRISPR-Cas9 in *Yarrowia lipolytica*", International Conference on CRISPR Technologies, Raleigh, NC (December 2017).

19. Blenner, M.* "Using Astronaut Waste and in situ Resources to Produce Nutrients and Materials" Space Technology Mission Directorate Day on Capitol Hill, Washington, DC (November 2017). #

20. Blenner, M.* "Tapping into the Potential for Biochemical Production in Non-Conventional Yeast" Chemical Engineering Departmental Seminar, City College of New York (November 2017). #

21. Smith, S.*, Shabbir Hussain, M., Blenner, M. "Devising a Rational Way to Construct an Inducible Expression System in *Yarrowia lipolytica*" Southeastern Regional American Chemical Society Fall Meeting, Charlotte, NC (November 2017).

22. Gao, D.*, Blenner, M. "Engineering a Novel Omega-3 Fatty Acid Biosynthesis Pathway in *Yarrowia lipolytica*" American Institute of Chemical Engineers Annual Meeting, Minneapolis, MN (November 2017).

23. Shabbir Hussain, M.*, Blenner, M. "Using Promoter Architecture to Guide Engineering of Strong Fatty Acid Inducible Hybrid Promoters in *Yarrowia lipolytica*" American Institute of Chemical Engineers Annual Meeting, Minneapolis, MN (November 2017).

24. Blenner, M.* "Synthetic Biology: Enabling Nutraceutical and Materials Production from Mission Waste" Annual Meeting of the American Society for Gravitational and Space Research, Seattle, WA (October 2017).

25. Blenner, M.* "Defining a Role for Synthetic Biology in Providing Nutrients and Vitamins During Deep Space Exploration" Johnson Space Center, Houston, TX (September 2017).#

26. Blenner, M.* "Tapping into the Potential for Biochemical Production in Non-Conventional Yeast" Chemical Engineering Departmental Seminar, University of Virginia (September 2017). #

27. Blenner, M.* "Tapping into the Potential for Biochemical Production in Non-Conventional Yeast" Chemical & Biological Engineering Departmental Seminar, University of Alabama (September 2017).#

28. Blenner, M.* "Biosynthesis of Materials and Nutraceuticals from Astronaut Waste: Towards Closing the Loop" Journey to Mars Symposium at the American Chemical Society Fall Meeting, Washington DC (August 2017).#

29. Blenner, M.* "Synthetic Biology as an Enabling Technology for Nutraceuticals and Materials from Astronaut Waste: Towards Closing the Loop" NASA ECF Continuation Review, Washington, DC (August 2017). ‡

30. Gao, D.*, Blenner, M. “Metabolic engineering of oleaginous yeast *Yarrowia lipolytica* for omega-3 long chain fatty acid production” Annual Meeting of the Society of Industrial Microbiology and Biotechnology, Denver, CO (August 2017).

31. Shabbir Hussain, M.*, Wheeldon, I., Blenner, M., “Using promoter architecture to guide engineering the strongest known fatty acid inducible hybrid promoter in *Yarrowia lipolytica*” Annual Meeting of the Society of Industrial Microbiology and Biotechnology, Denver, CO (August 2017).

32. Blenner, M.* “Engineering oleaginous yeast for biochemical production using non-conventional feedstocks” 2017 Biocatalysis, Bioconversion and Green Manufacturing US-China bilateral Symposium, Beijing, China. (July 2017).#

33. Shabbir Hussain, M., Schwartz, C., Wheeldon, I., Blenner, M.*, “Discovery of a fatty acid response element in *Yarrowia lipolytica* and its use to construct finely tuned fatty acid responsive promoters to enable strain engineering and dynamic regulation” Symposium for Biotechnology for Fuels and Chemicals Meeting, San Francisco, CA (May 2017).

34. Schwartz, C*, Frogue, K., Shabbir Hussain, M., Blenner, M., Wheeldon, I. “CRISPR-Cas9 genome editing and gene regulation tools for rapid engineering of *Yarrowia lipolytica*” Symposium for Biotechnology for Fuels and Chemicals Meeting, San Francisco, CA (May 2017).

35. Blenner, M.* “Expanding Capabilities for Engineering Yeast for Biochemical Production” Chemical Engineering Departmental Seminar, Columbia University (April 2017).#

36. Smith, S.*, Blenner, M., “Developing a genetic tool for metabolic engineering in Yarrowia lipolytica”, ACC Meeting of the Minds, Durham, NC (April 2017). #

37. Frogue, K.*, Schwartz, C.*, Shabbir Hussain, M., Blenner, M., Wheeldon, I. “Development of a synthetic metabolic engineering tool for the yeast Yarrowia lipolytica.”, American Chemical Society Meeting, San Francisco, CA (April 2017).

38. Gao, D., Spagnuolo, M., Rodriguez, G., Brabender, M., Scola, K., Blenner, M.* “Synthetic Biology for Recycling Human Waste into Nutraceuticals and Materials”. American Chemical Society Meeting, San Francisco, CA (April 2017).

39. Rodriguez, G., Gambill, L., Shabbir-Hussain, M., Blenner, M.* “Engineering Robust Xylose Utilization in *Yarrowia Lipolytica* Using Cryptic Metabolic Pathways”. International Conference on Biomolecular Engineering, San Diego, CA (January 2017).

40. Shabbir-Hussain, M., Wiseman, W., Blenner, M.* “Development of Fine-Tuned and Responsive Genetic Engineering Tools for *Yarrowia Lipolytica*”. International Conference on Biomolecular Engineering, San Diego, CA (January 2017).

Outside funding:

Funded

1. "Synthetic Biology for Recycling Human Waste into Nutraceuticals & Materials: Closing the Loop for Long-Term Space Explorations" NASA, $804,144 (4.5 years)

2. "Synthetic Biology for Recycling Human Waste into Nutraceuticals & Materials: Closing the Loop for Long-Term Space Explorations" NASA, $200,000 (1.5 years) – From PECASE

3. "Controlling Cellular Physiology and Enzyme Localization for an Enhanced Oleochemical Biosynthesis in Yeast" NSF $347,277 (3-year)
4. "Enabling Terrestrial Omega-3 Production for Aquaculture from Local Natural Resources" NOAA Sea Grant $150,000 (2-year)

Not Funded

1. "Engineering Oleaginous Microbes for Conversion of Animal Waste into High Value Fats and Oleochemicals" USDA-AFRI $497,291 (4 years)

2. "Biosynthesis of Omega-3 Fatty Acids from Rendered Animal Fats and Plant Oils" USDA $499,974 (4-year) w/ Dongming Xie from Umass Lowell. *Note this was ranked as highly competitive.

Future Work: Future work will focus on continuing to address pathway bottlenecks, and potentially to understanding and mitigating the effect of ER-stress induced by high level expression of desaturase enzymes. Related to this is ER expansion to carry more desaturase enzymes. Another remaining bottleneck is the flux from C18:1 to C18:2, which was not a focus of this proposal.

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