FINAL REPORT
September 8, 2017

BIOCATALYTIC CONVERSION OF RENDERED ANIMAL FATS TO VALUE ADDED PRODUCTS INCLUDING OMEGA-3 FATTY ACIDS

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Duration of Project: 26 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 and fatty alcohols. This is the second phase of the proposal funded in 2014 with the same title. We are engineering *Y. lipolytica* for funneling rendered animal fats into pathways for biosynthesis of polyunsaturated fatty acids (PUFA) found in fish oils. We are demonstrating the feasibility of producing omega-3 oils using fatty substrates. Specifically, we are producing lipids rich in the omega-3 fatty acid eicosapentaenoic acid (EPA) – one of the most important omega-3 found in fish oil. We are also demonstrating the feasibility of producing other oleochemicals, such as fatty alcohols.

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 major omega-3 fatty acid found in fish oil. A second overall objective is to demonstrate the production of fatty alcohols from rendered animal fats.

The specific objectives of this proposal are:

1. Introduce the entire EPA producing enzymes into *Y. lipolytica*.
2. Improve direct utilization of rendered fats conversion by *Y. lipolytica*.
3. Engineer fatty alcohol synthesis by *Y. lipolytica*.

The expected outcomes of this proposal include a non-pathogenic yeast strain capable of converting rendered fats into EPA and fatty alcohol production.

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.[1] 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).[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;[2-4] 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[5] and provide a cardiac health benefit to humans.[6]

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.[7] Currently, the aquaculture industry relies almost entirely on extracted fish oil processed from these wild-caught so-called 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).[1] 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.[8] Unfortunately, the composition of beef

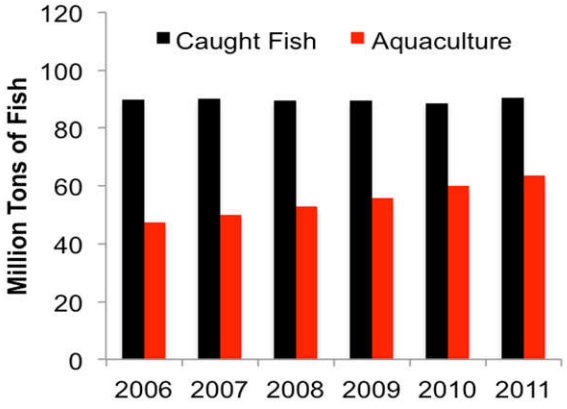

Figure 1. Steady increase in aquaculture as the world's fisheries are being operated at their maximum sustainable production rates.

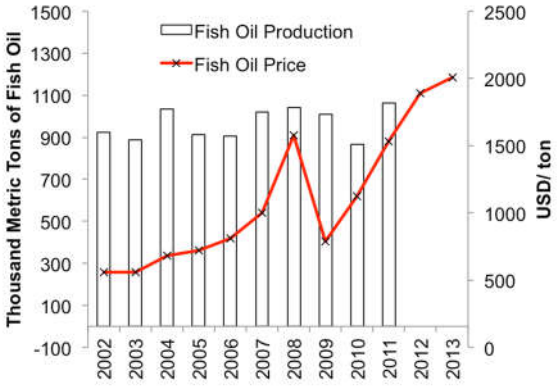

Figure 2. Steady increase in the price of fish as supply is stagnant but demand is increasing.

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.[8] 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.[1] 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.[7] 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*.[9] 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.

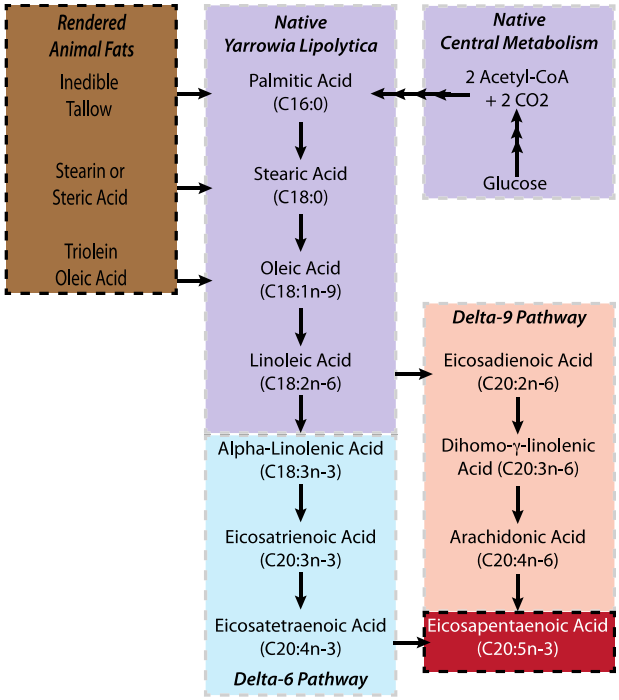

Figure 3. Pathways for Biosynthesis of Polyunsaturated Fatty Acids such as Eicosapentaenoic Acid. The native *Yarrowia* pathway can make linoleic acid from glucose. Engineering the Omega 9 pathway was found more effective at producing EPA than the Omega 6 pathway. This proposal focuses on engineering *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.

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[7]. 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[8] 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

Introduction of Novel EPA (C20:5) Pathway into *Y. lipolytica.* In order to avoid conflict with the existing Dupont patent, and to avoid bottlenecks in "acyl editing" that requires shuttling fatty acids between acyl-CoA and phospholipid pools, we decided to pursue an exclusively acyl-CoA pathway instead. We have identified known acyl-CoA dependent desaturases, which are quite rare in nature, and used a bioinformatics approach to identify additional potential acyl-CoA dependent desaturases. A list of these enzymes in provided below (Table 1). The highlighted

	Microorganism	**substrate specificity**	**conversion rate (%)**	**ID**
Acyl-CoA Δ6D	*Micromonas pusilla*	ω3	ω3 (63), ω6 (4.9)	EEH58637
	Ostreococcus tauri	**ω3, ω6**	**ω3 (82) ω6 (69)**	**AY746357**
	Mantoniella squamata	?	ω3 (34)	CAQ30479
Δ6E	*Ostreococcus tauri*	delta 6, C18	C18:3 (55), C18:4(75)	AY591335
	Thalassiosira pseudonana	delta 6, C18	C18:3 (70), C18:4(85)	AY591337
	Mortierella alpina	**delta 6, C18**	**C18:3(62),C18:4(73)**	**ADE06662.1**
Acyl-CoA Δ5D	***Emiliania huxleyi***		**ω6 (60)**	**XP_001778179.1**
	Leishmania major		ω3 (5), ω6 (6)	XP_001681021.1
	Rebecca salina			**A4KDP0**
	Mantoniella squamata		ω3 (8.2), ω6 (9.5)	CAQ30478.1
Δ17D Acyl-CoA&PC	*Pythium aphanidermatum*	ω6-C20, ω6-C18	C20:4(55), C20:3(28), C18:3(5)	FW362186.1,
	Phytophthora sojae	ω6-C20, ω6-C18	C20:4(47), C20:3(35), C18:3(6)	XM_009526057.1
	Phytophthora ramorum	ω6-C20, ω6-C18	C20:4(37), C20:3(30), C18:3(4)	FW362214.1
Acyl-CoA Δ17D	***Saprolegnia diclina***	**ω6-C20**	**C20:4(26), C20:3(5)**	**AAR20444.1**

Table 1. The characterized and putative acyl-CoA dependent desaturases that based on literature and sequence similarity.

and putative enzymes have been characterized or investigated in literature. These enzymes were used first for constructing EPA pathway. Δ6 desaturase from *Ostreococcus tauri* and Δ5 desaturase from *Emiliania huxleyi* have been characterized as acyl-CoA dependent desaturase. Δ17 desaturase from *Saprolegnia diclina* was suggested to be acyl-CoA dependent desaturase from indirect evidences. Δ 5 desaturase from *Rebecca salina* was a putative acyl-CoA dependent desaturase based on sequence similarity with Δ 5 desaturase from *E. huxleyi*. These desaturases and elongase have been codon optimized to increase the expression level in *Y. lipolytica*. Firstly, the enzymes were expressed in *pex10* strain individually under TEF-intron strong promoter. The conversion efficiencies of these enzymes are summarized in Table 2. O6D, M6E and S17D showed high conversion rate when supplying 1 g/L exogenous fatty acid and glycerol as corresponding substrates. Only E5D showed relatively low conversion efficiency. We then used another delta-5 desaturase from *R. salina*, which was more efficient than delta-5 desaturase from *E. huxleyi*.

	C16:0	**C18:1**	**C18:2**	**C18:3**	**DGLA**	**ARA**	**EPA**	**Conv. Eff.**
O6D	43.51	77.65	135.54	26.33	0.00	0.00	0.00	**16.27**
M6E	25.51	6.15	3.43	219.76	81.00	0.00	0.00	**26.93**
E5D	42.12	98.33	34.99	1.67	13.65	1.09	0.00	**7.41**
R5D	41.30	79.74	84.18	0.00	1.24	0.94	0.00	**43.11**
S17D	69.81	120.35	10.73	12.27	0.89	120.29	33.31	**21.69**

Table 2. Fatty acid composition (mg/L) and enzyme conversion efficiency (%) of strains overexpressing codon-optimized desaturases and elongase. O6D, M6E, E5D, S17D were individually overexpressed in *pex10* strain under low nitrogen condition.

After testing individual genes, we put together O6D, M6E, R5D and S17D stepwise in a single plasmid driven by TEF intron promoters. The strain overexpressing the plasmid that containing all four cassettes was cultivated using a 2-stage strategy. The first stage used rich nitrogen media to promote the biomass generation and the second stage with nitrogen limited media induced nitrogen accumulation. The EPA production is showed in Table 3. GLA and DGLA were not determined indicating there is no accumulation of intermediates by using acyl-CoA dependent pathway to produce EPA.

	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	20:2	DGLA	ARA	EPA
OMRS	93.6±35.4	10.2±0.7	13.1±1.8	185.3±30.9	136.9±45.5	nd	7.4±2.9	nd	2.9±0.4	2.9±0.6

Table 3. Fatty acid composition (mg/L) of pex10 strain co-expressing O6D, M6E, R5D and S17D. Data represent biological triplicates and standard deviation. C18:2 is supplemented at 1 g/L.

Improve Direct Utilization of Rendered Fats Conversion by *Y. lipolytica*. We first generate several knockout strains by targeting peroxisome related genes in order to partially or completely

disrupt beta-oxidation. Table 4 shows a list of genes that have been knocked out using CRISPR-Cas9 system to prevent the degradation of fatty acid in peroxisome.

Name	ID	protein description
Pex10	YALI0C01023g	peroxisomal assembly protein 10
Mfe	YALI0E15378g	multifunctional beta- oxidation enzyme
Pot1	YALI0E18568g	peroxisomal 3-ketoacyl-CoA thiolase
Pox2	YALI0F10857g	peroxisomal Acyl-CoA oxidase 2
Pxa1	YALI0A06655g	peroxisomal fatty acyl CoA transporter 1
Pxa2	YALI0D04246g	peroxisomal fatty acyl CoA transporter 1

Table 4. A list of target genes for knockout using CRISPR/Cas9 system.

Both Δpex10 and Δmfe knockout strains could not grow on oleic acid, which indicated that the beta-oxidation is completely or partially disrupted. Knocking out the rest genes didn't affect the growth on oleic acid, suggesting that there are redundant genes to these knockout ones. We then determined the lipid accumulation for Δpex10, Δmfe and parent Po1f strains by supplementing 1 g/L exogenous linoleic acid (Table 5). Results indicated that Δpex10 and Δmfe significantly increase lipid accumulation by 49% and 88% compared with Po1f, respectively. We observed that the Δmfe strain produced 2 byproducts that resulted from partial beta-oxidation.

	C16:0	C16:1	C18:0	C18:1	C18:2	bp1	bp2	total lipid
Po1f-ev	22	4	13	30	379	0	0	448
mfe-ev	18	3	7	18	586	33	8	673
pex10-ev	30	7	14	17	783	0	0	851

Table 5. Fatty acid composition and total lipid production (mg/L).

Fatty Alcohol Production within the Peroxisome from Animal Fats. In order to observe fatty alcohol production, we had to create a PEX10 knockout. The resulting strain cannot perform beta-oxidation, so we grew cells on glycerol instead. However, it allowed us to identify the MAACR CoA reductase as highly active, resulting in 250 mg/L of fatty alcohols (Figure 4). Combining this with overexpression of FAA1 to increase carbon flux to fatty acids results in between 450 mg/L and 900 mg/L of fatty alcohol (Figure 5). Additional expression of ACC1 did not improve the fatty alcohol production.

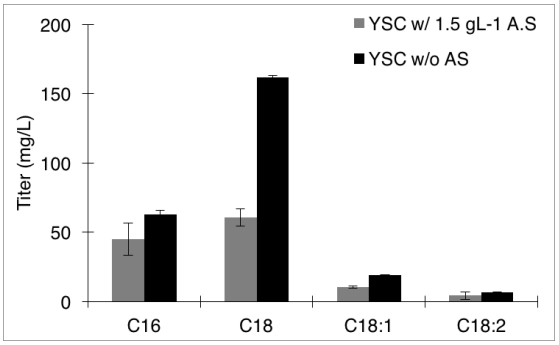

Figure 4. Fatty alcohol production under high nitrogen (1.5 g/L ammonia sulfate) and low nitrogen conditions (without ammonia sulfate).

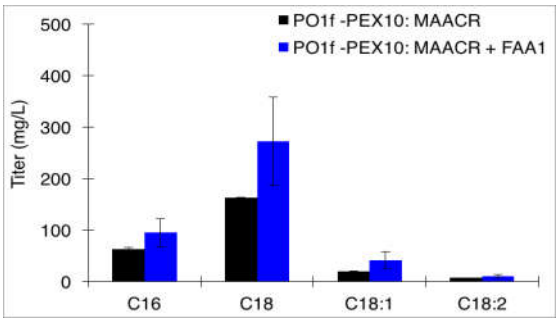

Figure 4. Fatty alcohol production of pex10 knockout strains overexpressing MAACR and combination of MAACR and FAA1.

We created a ΔFAO knockout to delete the major fatty alcohol oxidase gene responsible for fatty alcohol degradation. Data indicate this knockout leads to significantly higher accumulation of fatty alcohol (Figure 5). This was a precursor to making a POX3 knockout that might allow longer chain fatty acids to be processed into shorter chain fatty acids of higher value when converted to fatty alcohols. This data was collected using poultry fat and urea as a nitrogen source.

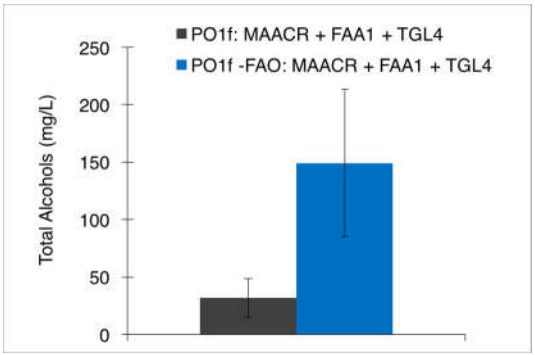

Figure 4. Fatty alcohol production of FAO knockout strains overexpressing MAACR, FAA1 and TGL4.

Conclusion. We have successfully introduced the complete EPA pathway into *Y. lipolytica*. The acyl-CoA desaturases demonstrated high efficiency towards utilizing exogenous fatty acids and avoiding the accumulation of intermediates. We also increased the exogenous fatty acid uptake and lipid accumulation within the cells by disrupting the beta-oxidation pathway. We demonstrated the capability of *Y. lipolytica* to produce fatty alcohols from animal fats. These data are encouraging for success in our year 3 project that will further enhance EPA production from animal fats.

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.

Publications: At this point, we've made a conscious decision to avoid publication of data related to our novel omega-3 production process; however, this work has advanced far enough where we finally plan to reveal it. We have presented the omega-3 work several times in the past few months. Our work with alternative substrates, including animal fats, will be included in two invited reviews that will be submitted this year.

1. Shabbir Hussain, Gambill L., Blenner, M.*, "Unlocking Alternative Substrate Metabolism in *Yarrowia lipolytica*", *Frontiers in Microbiology* (Invited Review – In Preparation).

2. Yaguchi, A., Spagnuolo, M., Blenner, M*. "Engineering Utilization of Non-conventional Feedstocks" *Current Opinion in Biotechnology* (Invited Review – In Preparation).

Our related work on *Y. lipolytica* genetic engineering tools has been published:
1. 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* (In Press). DOI: 10.1002/biot.201700248

2. 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).

3. 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).

4. 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).

5. 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**.

6. 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**.

And directly related work was presented at the following conferences or seminars:
1. Blenner, M.* "Yarrowia lipolytica as a robust platform for biochemical production from complex substrates" Departmental Seminar, Ohio University (November 2015). #

2. Blenner, M.*, Plenary Panel on "Outer Space: The Next Biotech Frontier". Biotechnology Industrial Organization (BIO) World Congress on Industrial Biotechnology, San Diego, CA (April 2016).#

3. Blenner, M.* "Synthetic Biology: From Benchtop to Mars." Microbiology Club, Clemson, SC (November 2016). #

4. Blenner, M.* "Critical Challenges for Synthetic Biology Applications in Space Life Support Systems". NASA Ames Research Center, Mountain View, CA (November 2016). #

5. Spagnuolo, M.*, Gao, D., Blenner, M. "PHA Production Using Yarrowia Lipolytica and Alternative Feedstocks" American Institute of Chemical Engineers Annual Meeting, San Francisco, CA (November 2016).

6. 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).

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

8. Blenner, M.* "Expanding Capabilities for Engineering Yeast for Biochemical Production" Chemical & Materials Engineering Departmental Seminar, University of California - Irvine (May 2017).#

9. 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).#

10. 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).

11. 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).#

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

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

Outside funding: Our work with *Y. lipolytica* has become the central research direction in the lab. This focus is reflected in the number of scope of proposals we write focused on this yeast. Listed below are grant proposals written that focus on metabolism of lipids to form omega-3 fatty acids and other products. Preliminary data from ACREC funding has proven critical in several proposals, include the NASA proposal described below.

Projects Funded:

1. NASA Early Career Faculty Award (2015). This project is focused on engineering *Y. lipolytica* for the production of omega-3 and polyesters from waste streams generated during long-term space travel. The principle technology we propose is the conversion of

lipids from algae by an engineered yeast. The preliminary data supporting this proposal were obtained through ACREC funding. This Early Career Award was picked as one of the 8 funded proposals and has received extensive press coverage (Houston Chronicle, Popular Mechanics, SciShow Space Channel, etc.). The project is funded for 3 years and a total of $600,000. Of note, is that NASA has nominated PI Blenner for the Presidential Early Career Award in Science and Engineering – the highest honor for an early career faculty.

2. "Omega-3 and Fatty Acid Production from Sweet Sorghum" USDA/DOE Southeastern Regional Sun Grant, Principal Investigator, $150,000 ($150,000), (2016-2018). 2014-38502-22588. This project uses a different oleaginous yeast, but was supported by preliminary data funded by ACREC.
3. "Collaborative Research: Controlling Cellular Physiology and Enzyme Localization for Enhanced Oleochemical Biosynthesis in Yeast", NSF CBET Cellular & Biochemical Engineering, Principal Investigator, $347,277 ($347,277), (2017-2020). Award CBET:1706134. This project was supported by preliminary data on Fatty Alcohol production funded by ACREC.

Pending:

1. Department of Agriculture – "Biosynthesis of Omega-3 Fatty Acids from Rendred Animal Byproducts". This was proposed as a 4 year project for $497,421. It is a direct extension of ACREC funded work.
2. National Science Foundation – CAREER Award – "Promoter Dynamics as a New Dimension of Synthetic Biology". This was proposed as a 5 year project for a total of $500,000. Has same preliminary data originating from ACREC funding.

Submitted, but Not Funded:

1. $15MM NASA Institute Proposal called "Institute for Sustainable Space Biomanufacturing". We were competitively invited to submit a full proposal. The topic involves finding ways to convert what could be considered wastes into feedstocks for biomanufacturing processes. This will be a 5 year project for a total of $15,000,000 – spread across 6 institutions (Clemson will lead). Several projects proposed are synergistic with the ACREC effort.
2. National Science Foundation – CAREER Award – "Mutli-Scale Design Rules for Engineering Gene Regulation in Non-Conventional Yeast". This was proposed as a 5 year project for a total of $656,423.

Future Work: We requested and received an additional year of ACREC funding to engineer the rendered fat utilizing strain of *Y. lipolytica* into an efficient omega-3 fatty acid producer. The rendering industry will realize income as a result of our project if we can establish animal fats as a cost-effective source for the growth of *Y. lipolytica* and production of its diverse natural and engineered products. Future tasks include improve omega-3 producing strain titer and yield and lab scale-up of the bioprocess of rendered Fats to Omega-3.

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