COVER PAGE

Project Title: Creating multifunctional synthetic lichen platforms for sustainable biosynthesis of biofuel precursors	
Federal Award Identification Number: DE-SC0019388	
Agency Code: 8900	**Organization:** Office of Biological & Environmental Research
Recipient Award Identification Number: Not Provided	**Project Period:** 09/15/2018 - 03/14/2024
Reporting Period: 09/15/2018 - 03/14/2024	**Budget Period:** 09/15/2020 - 03/14/2024
Report Term: Once per Budget Period	**Submission Date and Time:** 06/12/2020 03:27 PM ET
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PRINCIPLE INVESTIGATORS

1. Participant: Michael Betenbaugh		
Project Role: Principal Investigator/Project Director	**Research Organization**: Johns Hopkins University	**Funding Support (if other than this award)**: Not Provided
Contribution to the Project: Dr. Michael Betenbaugh is the lead PI on this project. He organizes and manages the team of collaborators across multiple universities and national labs to ensure this project moves forward, and works directly with his graduate students to advise the project.		
International Collaboration: No		
International Travel: No		

2. Participant: Pavlo Bohutskyi		
Project Role: Co-Investigator	**Research Organization**: Pacific Northwest National Laboratory	**Funding Support (if other than this award)**: Not Provided
Contribution to the Project: Works on co-culturing and expression with fungal hosts.		
International Collaboration: No		
International Travel: No		

3. Participant: Dr. Michael Guarnieri		
Project Role: Co-Investigator	**Research Organization**: National Renewable Energy Laboratory	**Funding Support (if other than this award)**: Not Provided
Contribution to the Project: Leading team at NREL for metabolomics, multi-omics analysis		
International Collaboration: No		
International Travel: No		

4. Participant: Jon Magnuson		
Project Role: Co-Investigator	**Research Organization**: Pacific Northwest National Laboratory	**Funding Support (if other than this award)**: Not Provided
Contribution to the Project: Works at PNNL for fungal engineering.		
International Collaboration: No		
International Travel: No		

5. Participant: Jamey Young		
Project Role: Co-Investigator	**Research Organization**: Vanderbilt University	**Funding Support (if other than this award)**: Not Provided
Contribution to the Project: Co-PI at Vanderbilt University, leading isotope tracing studies.		
International Collaboration: No		
International Travel: No		

6. Participant: Prof. Karsten Zengler		
Project Role: Co-Investigator	**Research Organization**: University of California, San Deigo	**Funding Support (if other than this award)**: Not Provided
Contribution to the Project: Leads team at UCSD for developing community metabolic models		
International Collaboration: No		
International Travel: No		

ABSTRACT

In this project, we were creating a sustainable platform for biofuel production, utilizing carbon-fixing autotrophs to supply oxygen and organic substrates to heterotrophic partners, which in turn produce carbon dioxide to feed the autotrophs. This symbiotic lichen community could lower the input cost, optimize metabolic exchanges and improve the generation of biofuel precursors through multi-omics driven genetic engineering.

The cyanobacteria *Synechococcus elongatus* (*S. elongatus*) was used as the primary autotroph to provide oxygen and organic substrates, especially sucrose, to a co-culture system. The strain with overexpression of sucrose transporter cscB demonstrated a significant increase in sucrose production under salt stress as what we expected. We also implemented 13C metabolic flux analysis on the sucrose secreting strain *S. elongatus* cscB-NaCl. Next, transporters proteins like glutamate exporter mscCG from *Corynebacterium glutamicum* was overexpressed in *S. elongatus* to improve metabolite exchange. We provided sucrose and glutamate to filamentous fungi to enable their growth and production of biochemicals using substrates from the cyanobacterium.

Two fungi (*Aspergillus nidulans* and *Aspergillus niger*) and two yeast strains (*Rhodotorula toruloides* and *Lipomyces starkeyi)* served as heterotrophs in this system. They were co-cultured with *S.elongatus* under different pH condition, and growth on different carbon sources to understand the symbiotic system and optimize the parameters for production. The two fungi strained grew much better at pH 7, 8 and 9 with Yeast Nitrogen Base (YNB) supplementation. Two yeast strains grew well on pH 7 and 8 with YNB supplementation. All four species achieved the highest biomass level when utilizing sucrose as the main carbon sources, which demonstrates a great pairing with *S.elongatus*.

In addition to investigate the metabolite exchange and growth condition of the co-culture system, we incorporated an *Aspergillus* strain expressing three genes (PAND, BAPAT and HPDH). Expression of these three genes enabled production of 3-hydroxypropionic acid (3HP) with a titer of 3-6 g/L regardless of pH and other nutrient conditions. Moreover, we developed a computational model with different heterotrophic fungi symbiosing with *S.elongatus* to evaluate the exchange of hundreds of metabolites. The accuracy and sensitivity had been optimized by high-throughput phenotyping assays.

This project demonstrated ways to enhance a synthetic lichen platform's efficiency and scalability to create a robust system for sustainable bioproduct synthesis. The project has enabled us to explore the technological and commercial potential of sustainable lichen co-culture. The project has also trained the next generation of scientists to address sustainability, carbon fixation, and design solutions to contemporary global challenges. The lichen platform represented a novel approach to sustainably producing bioproducts with a reduced carbon footprint and lower costs.

ACCOMPLISHMENTS

Major goals of the project

The goal of this project was to develop synthetic lichen communities of autotrophic and heterotrophic microbes as a novel sustainable symbiotic platform for the production of biofuels and commodity chemicals. Carbon-fixing autotrophs provided oxygen and organic substrates to their heterotrophic partners, which in turn produced valuable biochemicals and carbon dioxide which could serve as a carbon source for autotrophs. By optimizing and enhancing these interactions, we could create a robust, sustainable synthetic lichen community. Multi-omics driven genetic engineering and mathematical modeling can improve metabolite exchange and product generation capabilities for this microbial co-culture platform.

Our aims were:

1. *Computational Modeling*: Develop computational lichen models for relevant cyanobacteria in partnership with heterotrophic fungal partners (such as *Aspergillus nidulans* and *niger*). These models aimed to elucidate potential shared metabolite pools and specify exchange rates required to maximize generation of biomass and products of interest to the DOE.
2. *Metabolite Exchange Engineering*: Overcome exchange bottlenecks in cyanobacteria like *S. elongatus* by expressing transporters to excrete a wider selection of shared metabolites such as glutamate, aspartate, and others. The capacity to channel these metabolites into specific products of interest such as 3-hydroxypropionic acid (3HP) was investigated with metabolic engineering of heterotrophic production partners including yeast and filamentous fungi. The repertoire of shared metabolites generated by *S. elongatus* were expanded and channeled into target products from heterotrophic partners for improving the performance of synthetic lichens.
3. *'Omics profiling and 13C labeling*: Perform transcriptomic, proteomic, and metabolomic profiling to identify which pathway genes and transporters were expressed and which metabolites were available in the medium when compared to monocultures. These expression profiles and measurements were used to identify potential exchange candidates that accumulate to significant levels in the cyanobacteria. 13C labeling was undertaken to follow the movement of carbon from CO2 into final products and biomass in order to evaluate the impact of exchange engineering modifications on metabolic fluxes and product yields.

Accomplishments under the goals

Yarrowia lipolytica is a promising oleaginous yeast platform currently under development for the bioproduction of an array of fuel and platform chemical intermediates. Wild-type *Y. lipolytica* does not possess sucrose-utilization capacity when cultivated in minimal media, limiting its co-cultivation potential with our team's engineered, sucrose-secreting cyanobacterial host. Thus, in order to enable sucrose utilization in *Y. lipolytica*, we integrated a constitutive promoter element upstream of the strain's native sucrose invertase. The resultant strain displayed the capacity to utilize sucrose as a sole carbon and energy source. Unexpectedly, constitutive overexpression of sucrose invertase conferred additional substrate utilization capacity; the engineered strain displayed the capacity for cultivation on both mannose and glycerol as sole carbon and energy sources (Figure 1).

Concurrently, we have initiated efforts to establish a new phototrophic sucrose secretion host. We have screened >300 unique algal cultivars to identify top-candidate production hosts with rapid growth rate and robustness in varied media conditions that can be well-suited to co-culture applications. Our top-candidate strain, Picocholorum renovo, boasted high biomass productivity (34 g m−2 day−1) and thermal- (growth up to 40 °C) and salinity tolerance (growth at 107.5 g L−1 salinity), suggesting it was uniquely suited for co-culture applications. A series of potential sucrose transport proteins (SWEET transporters) have been identified for overexpression in P. renovo.

We have also tested several heterotrophic partners at PNNL, including *Aspergillus pseudoterreus* (aka *A. terreus*) ATCC 32359, *Aspergillus niger* ATCC 11414, *Rhodotorula glutinis* ATCC 204091, and *Lipomyces starkeyi* NRRL Y-11558 for growth in cyanobacterium medium at high pH. The ultimate biomass density of samples harvested at stationary phase is shown in Figures 2a and 2b. Additionally, we tested potential exchange of metabolites as substrates for heterotroph growth, and potential toxicity of NaCl. We tested cyanobacterium BG11 medium supplemented with 5mM $(NH_4)_2SO_4$ and 20 g/L of specific substrate at pH 8.4. Incubation temperature was 30°C and 200 rpm. The ultimate biomass density of samples harvested at stationary phase is shown in Figures 3a and 3b.

We have also incorporated a three gene (PAND - aspartate decarboxylase, BAPAT - β-alanine-pyruvate aminotransferase, HPDH - 3-hydroxypropionate dehydrogenase) β-alanine-aspartate pathway into A. pseudoterreus ATCC 32359 (Ap) to produce 3-hydroxypropionic acid (3HP) from aspartate. Expression of this pathway under constitutive promoters resulted in production of 3HP with titers in the 3-6 g/L range regardless of pH or other nutrient conditions. Furthermore, we used the same three heterologous gene biosynthetic pathways but with additional genes deleted/added to increase precursor molecules and avoid catabolism of 3HP in Aspergillus niger. (Figure 4)

Additionally, Biolog plate experiments for *Aspergillus pseudoterreus* (aka *A. terreus*) ATCC 32359 were performed to investigate carbon utilization by heterotrophs., with data shown in Figures 5-7. We also established a method for tracking growth rate for *S. elongatus* – yeast co-culture using flow cytometry, and for physical separation (cell sorting) of the *S. elongatus* and yeast cell for specific intracellular metabolite analysis and metabolic flux analysis (Figures 8-10). The ability to determine a specific phototroph:heterotroph cell ratio could be very valuable in understanding metabolic flux. We also increased sucrose production by Synechococcus elongatus PCC 7942 engineered with the sucrose transporter CscB by nearly 95% through immobilization of cells into alginate matrix and through application of engineered by S. elongatus with sucrose-phosphate synthase (SPS) (Figure 11).

We have successfully transformed WT *S. elongatus* with two plasmids received from Addgene, PAM1414 (luxAB gene) and PAM5087 (IPTG-induced expression of YFP). We used the PAM5087 backbone for expression of mscCG (glutamate/aspartate exporter) via Gibson Assembly.

^{13}C-MFA can thereby be applied to decipher metabolic network regulation and hence guide metabolic engineering. *Synechococcus elongatus* PCC 7942 synthesizes sucrose under NaCl stress as a means of coping with osmotic pressure, while overexpression of CscB, a sucrose transporter, confers the ability to secrete sucrose out of cells (Ducat, 2012). We investigated global metabolic fluxes in the sucrose-secreting (*cscB*$^{+}$) strain versus the wild-type PCC 7942 ^{13}C-MFA. Our study was designed to investigate *a*) the metabolic response of the wild-type PCC 7942 to osmotic stress upon NaCl supplementation to the BG11 culture medium, and *b*) the metabolic response to sucrose secretion in the *cscB*-expressing strain upon NaCl supplementation to the BG11 culture medium. We performed ^{13}C-MFA for three cyanobacterial cultures – wild-type grown in BG11 medium (WT), wild-type grown in BG11 medium with additional 100 mM NaCl (WT_NaCl), and cscB^{+} strain grown in BG11 medium with additional 100 mM NaCl (cscB_NaCl) – under photoautotrophic conditions. We added ^{13}C-labelled bicarbonate into the growing cyanobacterial cultures to initiate the labeling experiment, and sampled and rapidly quenched the cells at times 0, 1, 2, 5, and 10 min followed by metabolite extraction and GC-MS analysis to quantify labeling trajectories of more than a dozen intracellular metabolites. We then used the INCA software to estimate pathway fluxes and generate a quantitative flux map of each strain and growth condition.

We have found that the growth rate varied significantly between the three conditions: "WT" > "WT_NaCl" > "cscB_NaCl". While the carbon fixation rate (*Rubisco* flux in Figure 12) in WT_NaCl decreased by 15% compared to that of WT, the *Rubisco* flux in cscB_NaCl increased by 34% compared to that of WT_NaCl, and increased by 15% compared to that of WT (Figure 12). Other examined fluxes, *i.e*., *GAPDH*, *FBA*, *TKT* and *SBA*, in the carbon-fixing CBB cycle of the *cscB*$^{+}$ strain under salt stress also increased compared to the WT and WT_NaCl. It has been reported that introducing a sucrose sink in *S. elongatus* leads to increased photosynthesis rate (Abramson, 2016). Our ^{13}C-MFA results confirm this finding. The increased carbon fixation flux in cscB_NaCl was mostly attributed to sucrose biosynthesis since *PGI*, *PGMT* and *SPP* fluxes were all increased significantly (Figure 12). Conversely, the glycolysis fluxes (*ENO*, *PYK*, *PPC*, *MDH* and *ME*) and the TCA cycle fluxes (*CS* and *PDH*) decreased in the cscB_NaCl case compared to the WT and WT_NaCl cultures (Figure 12, 13).Overall, the cscB_NaCl overexpression strain also showed a substantial metabolic rewiring and a significant increase in sucrose production under salt stress (Figure 14). In addition, the cscB_NaCl strain with overexpressing GADPH (glyceraldehyde dehydrogenase) demonstrated a 20% increase in sucrose productivity compared to regular cscB_NaCl strain.

For metabolic modeling, there have been two major activities: i) reconstruction, curation and refinement of metabolic models for fungi and ii) benchmarking of models using our high-throughput phenotyping assays. We updated and reshaped existing models as well as created new metabolic models. We have done intensive manual curation in *Aspergillus nidulans* and *A. niger* metabolic models. These models were reconstructed in a semi-automatic matter using the latest annotation of the genome. We added new reactions and metabolites as well as updated names and IDs to achieve the maximum standards of quality. The refined version of these models was used as a template to reconstruct the new model of *A. pseudoterreous*. The current version of the models contains: *A. nidulans* 2,373 reactions, 1,884 metabolites and 1,230 genes; *A. niger* 2,241 reactions, 1,821 metabolites and 1,396 genes and *A. pseudoterreus* 2,221 reactions, 1,822 metabolites and 1,380 genes.

Secondly, we ensured the high quality of our new and updated models (3 total). We have benchmarked models for Nostoc and Aspergilli strains using our high-throughput phenotyping assays. We used experimentally determined growth phenotypes under 190 carbon and 95 nitrogen sources collected from BIOLOG plates PM1, PM2 and PM3. Experimental information enabled a second round of manual curation to broaden simulation capabilities for each model, increasing the number of true positive and true negative predicted outcomes. For example, in *A. nidulans* model we included 129 new reactions to fix false negative predicted growth and converted to true positive growth over L-proline, D-alanine, dulcitol, etc. We also curated the model eliminating false positive predictions for formate, acetate, adenosine and inosine. Overall, Nostoc and *Aspergullis nidulans* models achieved over 85% accuracy and sensitivity.

Opportunities provided for training and professional development

This project has provided training for several graduate students. At Johns Hopkins University, two PhD students (Chien- Ting Li, Jackson Jenkins), one visiting PhD scholar (Liqun Jiang), and two MS students (John Del Toro, Kai-Wen Huang) have been trained in bacterial and fungal culturing, microbial coculturing, fluorescence microscope imaging, plasmid design, and genetic engineering of cyanobacteria. At Vanderbilt University, one Post-Doctoral Researcher (Bo Wang) has been developing metabolic flux analysis techniques. Bo also started a tenure-track position at University of Minnesota in Fall 2024. This has also provided professional development opportunities as students presented updates to the extended project team, and present posters at conferences and university poster sessions. Additionally, the project has enabled a training opportunity through the Department of Energy's Science Undergraduate Laboratory Internship (Loretta Lutackas, Colorado State University), providing training in molecular cloning and functional genomic technologies.

Results dissemination to the community of interest

Multiple posters were presented at the DOE 2020 Genomic Sciences Program Annual PI Meeting. One project related to flux modeling was presented by Cristal Zuniga (UCSD) and Bo Wang. A second poster was presented by Jackson Jenkins to summarize successes in cell culture and engineering for this project. A series of manuscripts encompassing development of synthetic co-culture pairings and genome-scale modeling have been published in peer-reviewed journals, establishing a first-in-class foundation for synthetic lichen development.

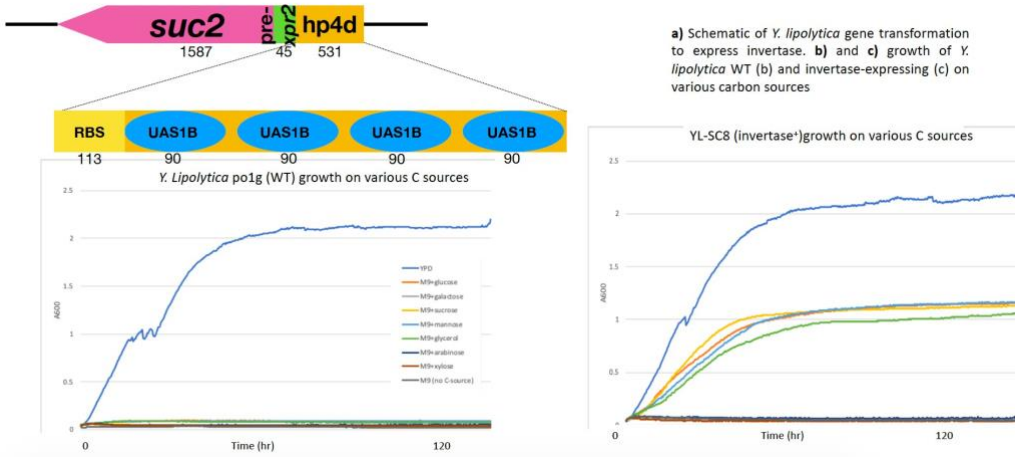


Figure 1. Genetic engineering of *Y. lipolytica* has conferred expanded substrate utilization capacity.

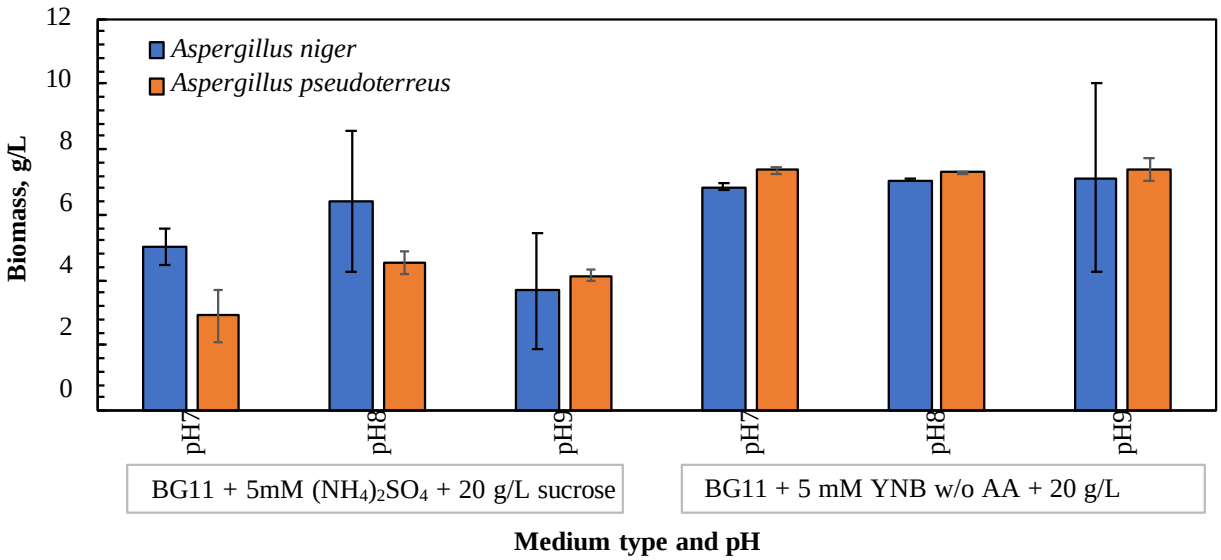


Fig. 2a. Biomass density for heterotroph batch cultures at incubation time 4 days

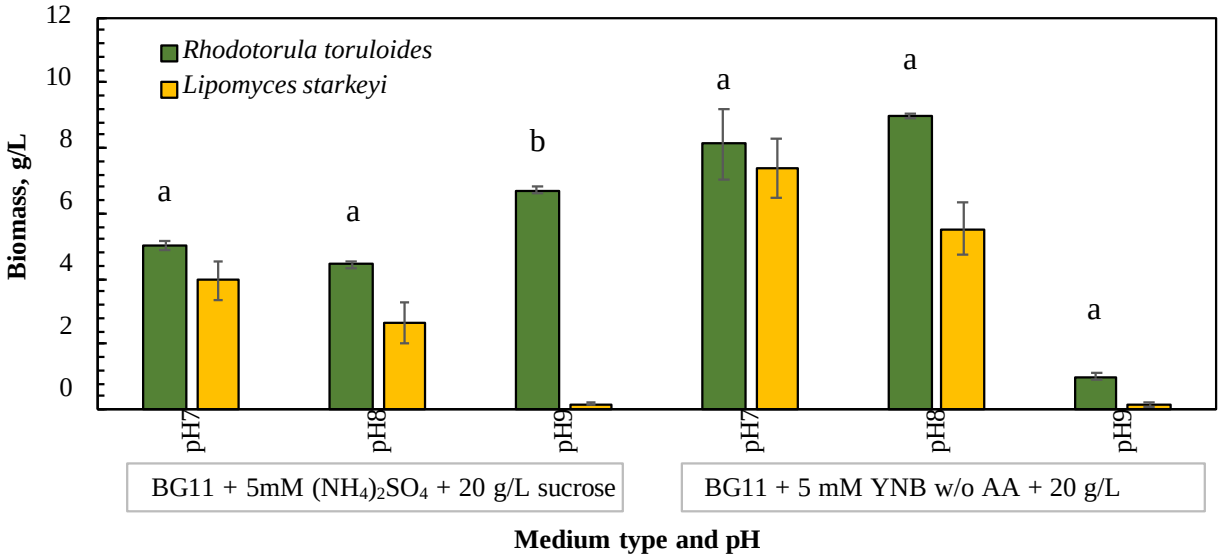


Fig. 2b. Biomass density for heterotroph batch cultures at incubation time for *R. glutinis* and *L. starkeyi* was 4 days for samples "a" and 12 days for sample "b" (BG11 + 5mM $(NH_4)_2SO_4$ + 20 g/L sucrose at pH 9) since no growth was observed at day 4.

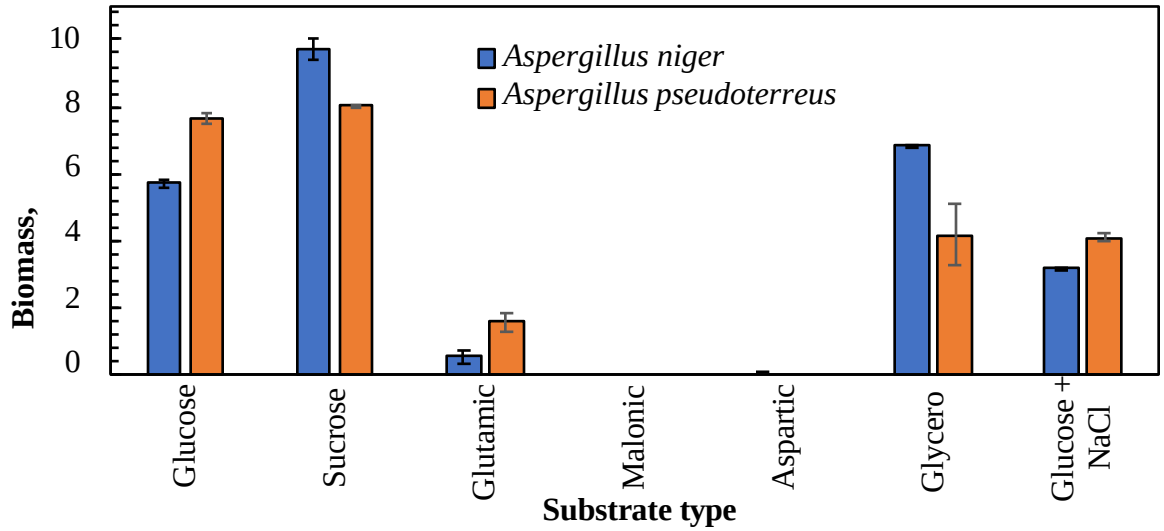


Fig. 3a. Biomass density for fungi batch cultures at incubation time 6 days (culture was inoculated by conidia germinated in Complete Medium)

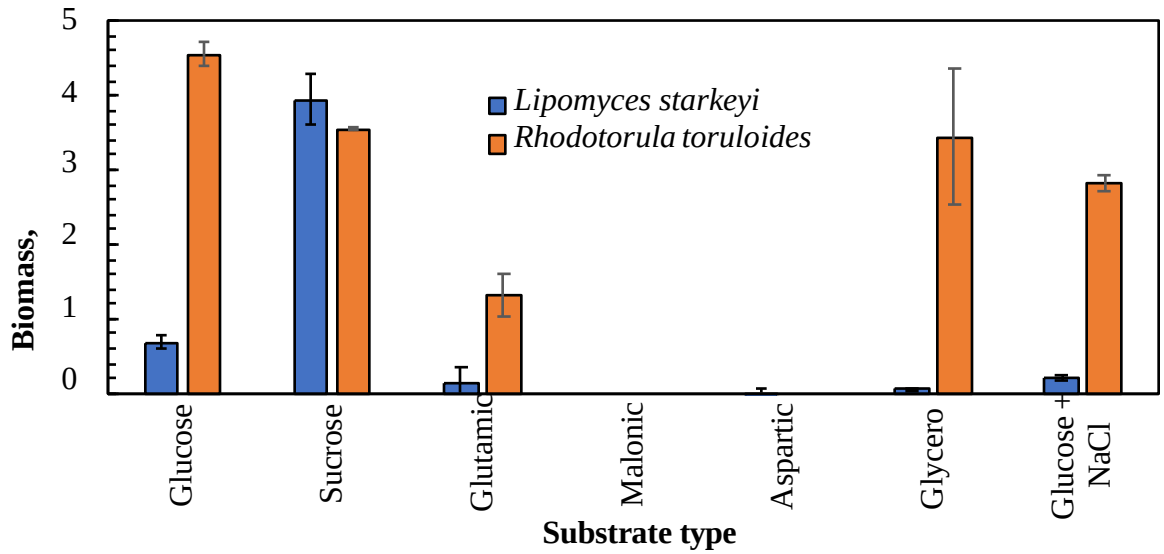


Fig. 3b. Biomass density for yeast batch cultures at incubation time 6 days

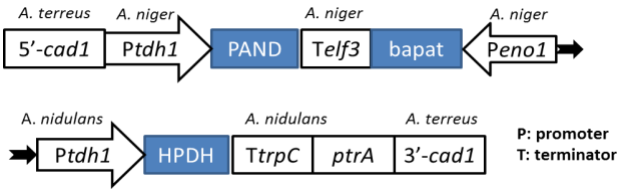


Fig 4. Three gene pathway for the production of 3-hydroxypropionic acid (3HP). Pathway genes in blue are: PAND, BAPAT, HPDH. The *cad1* gene is the target gene for homologous recombination to both knockout this native acid production pathway and insert the 3HP pathway.

Biolog plate experiment for *Aspergillus pseudoterreus* (aka *A. terreus*) ATCC 32359

PM1 MicroPlate™ Carbon Sources

A1 Negative Control	A2 L-Arabinose	A3 N-Acetyl-D-Glucosamine	A4 D-Saccharic Acid	A5 Succinic Acid	A6 D-Galactose	A7 L-Aspartic Acid	A8 L-Proline	A9 D-Alanine	A10 D-Trehalose	A11 D-Mannose	A12 Dulcitol
B1 D-Serine	B2 D-Sorbitol	B3 Glycerol	B4 L-Fucose	B5 D-Glucuronic Acid	B6 D-Gluconic Acid	B7 D,L-α-Glycerol-Phosphate	B8 D-Xylose	B9 L-Lactic Acid	B10 Formic Acid	B11 D-Mannitol	B12 L-Glutamic Acid
C1 D-Glucose-6-Phosphate	C2 D-Galactonic Acid-γ-Lactone	C3 D,L-Malic Acid	C4 D-Ribose	C5 Tween 20	C6 L-Rhamnose	C7 D-Fructose	C8 Acetic Acid	C9 α-D-Glucose	C10 Maltose	C11 D-Melibiose	C12 Thymidine
D-1 L-Asparagine	D2 D-Aspartic Acid	D3 D-Glucosaminic Acid	D4 1,2-Propanediol	D5 Tween 40	D6 α-Keto-Glutaric Acid	D7 α-Keto-Butyric Acid	D8 α-Methyl-D-Galactoside	D9 α-D-Lactose	D10 Lactulose	D11 Sucrose	D12 Uridine
E1 L-Glutamine	E2 m-Tartaric Acid	E3 D-Glucose-1-Phosphate	E4 D-Fructose-6-Phosphate	E5 Tween 80	E6 α-Hydroxy Glutaric Acid-γ-Lactone	E7 α-Hydroxy Butyric Acid	E8 β-Methyl-D-Glucoside	E9 Adonitol	E10 Maltotriose	E11 2-Deoxy Adenosine	E12 Adenosine
F1 Glycyl-L-Aspartic Acid	F2 Citric Acid	F3 m-Inositol	F4 D-Threonine	F5 Fumaric Acid	F6 Bromo Succinic Acid	F7 Propionic Acid	F8 Mucic Acid	F9 Glycolic Acid	F10 Glyoxylic Acid	F11 D-Cellobiose	F12 Inosine
G1 Glycyl-L-Glutamic Acid	G2 Tricarballylic Acid	G3 L-Serine	G4 L-Threonine	G5 L-Alanine	G6 L-Alanyl-Glycine	G7 Acetoacetic Acid	G8 N-Acetyl-β-D-Mannosamine	G9 Mono Methyl Succinate	G10 Methyl Pyruvate	G11 D-Malic Acid	G12 L-Malic Acid
H1 Glycyl-L-Proline	H2 p-Hydroxy Phenyl Acetic Acid	H3 m-Hydroxy Phenyl Acetic Acid	H4 Tyramine	H5 D-Psicose	H6 L-Lyxose	H7 Glucuronamide	H8 Pyruvic Acid	H9 L-Galactonic Acid-γ-Lactone	H10 D-Galacturonic Acid	H11 Phenylethyl-amine	H12 2-Aminoethanol

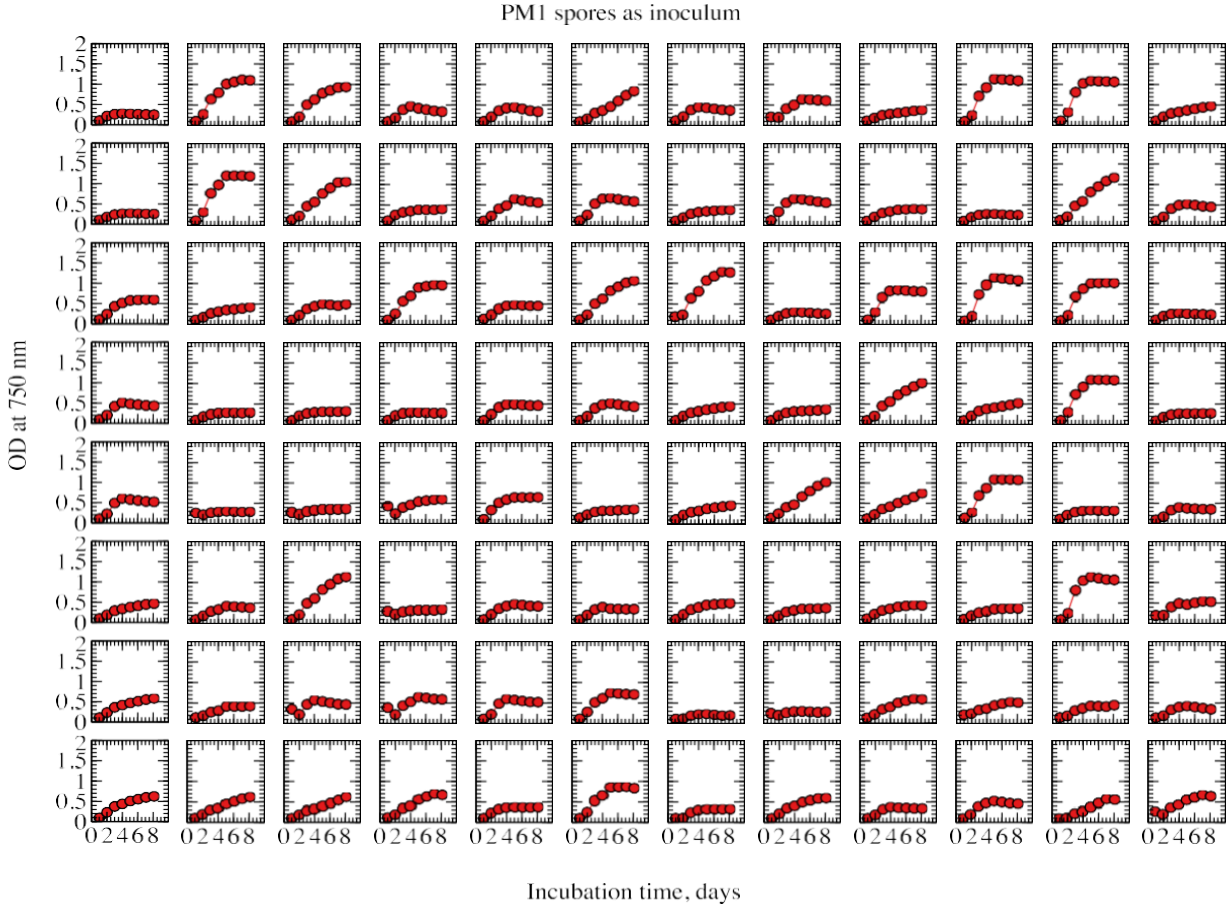


Fig. 5a. Fresh *A. pseudoterreus* conidia were used as inoculum

PM1 MicroPlate™ Carbon Sources

A1 Negative Control	A2 L-Arabinose	A3 N-Acetyl-D-Glucosamine	A4 D-Saccharic Acid	A5 Succinic Acid	A6 D-Galactose	A7 L-Aspartic Acid	A8 L-Proline	A9 D-Alanine	A10 D-Trehalose	A11 D-Mannose	A12 Dulcitol
B1 D-Serine	B2 D-Sorbitol	B3 Glycerol	B4 L-Fucose	B5 D-Glucuronic Acid	B6 D-Gluconic Acid	B7 D,L-α-Glycerol-Phosphate	B8 D-Xylose	B9 L-Lactic Acid	B10 Formic Acid	B11 D-Mannitol	B12 L-Glutamic Acid
C1 D-Glucose-6-Phosphate	C2 D-Galactonic Acid-γ-Lactone	C3 D,L-Malic Acid	C4 D-Ribose	C5 Tween 20	C6 L-Rhamnose	C7 D-Fructose	C8 Acetic Acid	C9 α-D-Glucose	C10 Maltose	C11 D-Melibiose	C12 Thymidine
D-1 L-Asparagine	D2 D-Aspartic Acid	D3 D-Glucosaminic Acid	D4 1,2-Propanediol	D5 Tween 40	D6 α-Keto-Glutaric Acid	D7 α-Keto-Butyric Acid	D8 α-Methyl-D-Galactoside	D9 α-D-Lactose	D10 Lactulose	D11 Sucrose	D12 Uridine
E1 L-Glutamine	E2 m-Tartaric Acid	E3 D-Glucose-1-Phosphate	E4 D-Fructose-6-Phosphate	E5 Tween 80	E6 α-Hydroxy Glutaric Acid-γ-Lactone	E7 α-Hydroxy Butyric Acid	E8 β-Methyl-D-Glucoside	E9 Adonitol	E10 Maltotriose	E11 2-Deoxy Adenosine	E12 Adenosine
F1 Glycyl-L-Aspartic Acid	F2 Citric Acid	F3 m-Inositol	F4 D-Threonine	F5 Fumaric Acid	F6 Bromo Succinic Acid	F7 Propionic Acid	F8 Mucic Acid	F9 Glycolic Acid	F10 Glyoxylic Acid	F11 D-Cellobiose	F12 Inosine
G1 Glycyl-L-Glutamic Acid	G2 Tricarballylic Acid	G3 L-Serine	G4 L-Threonine	G5 L-Alanine	G6 L-Alanyl-Glycine	G7 Acetoacetic Acid	G8 N-Acetyl-β-D-Mannosamine	G9 Mono Methyl Succinate	G10 Methyl Pyruvate	G11 D-Malic Acid	G12 L-Malic Acid
H1 Glycyl-L-Proline	H2 p-Hydroxy Phenyl Acetic Acid	H3 m-Hydroxy Phenyl Acetic Acid	H4 Tyramine	H5 D-Psicose	H6 L-Lyxose	H7 Glucuronamide	H8 Pyruvic Acid	H9 L-Galactonic Acid-γ-Lactone	H10 D-Galacturonic Acid	H11 Phenylethyl-amine	H12 2-Aminoethanol

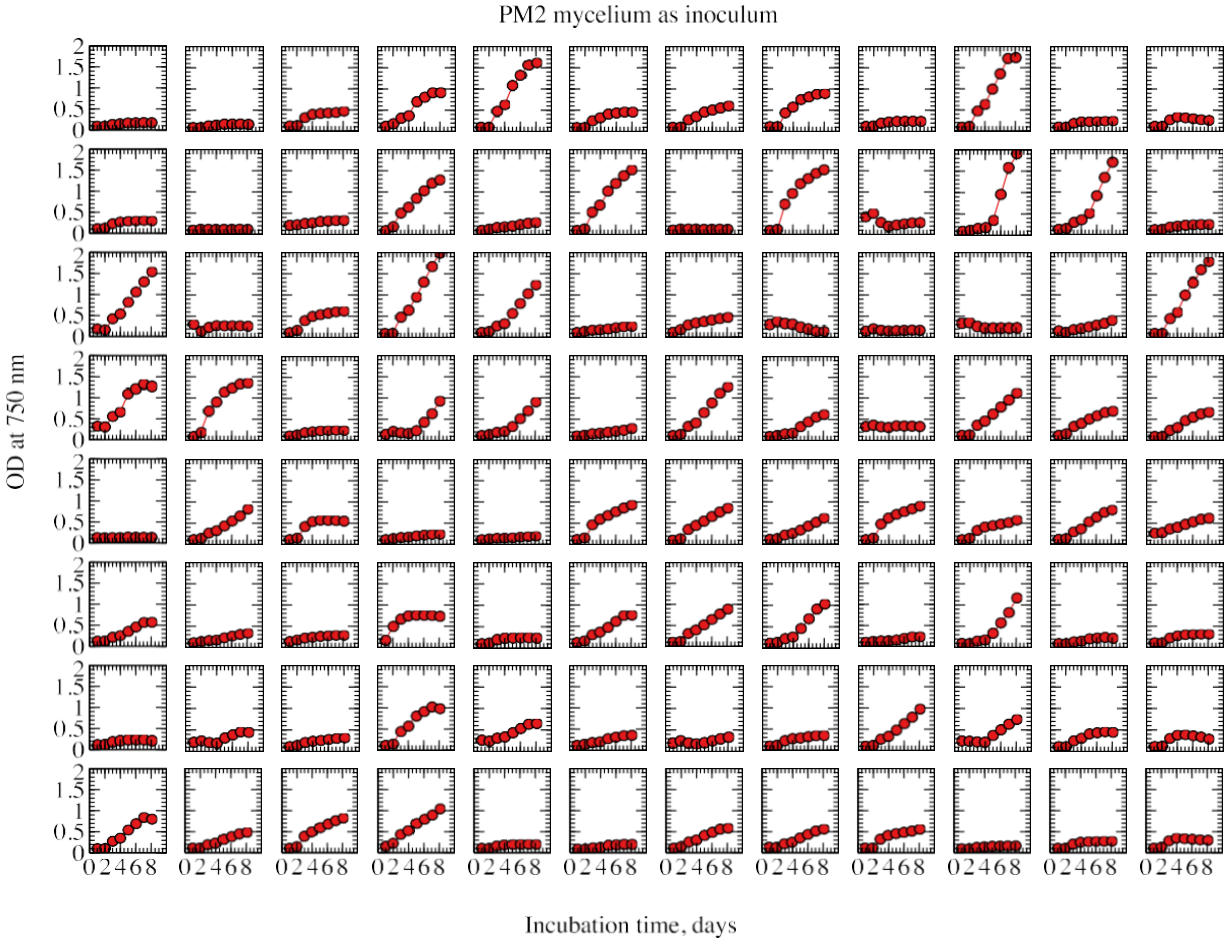


Fig. 5b. Macerated *A. pseudoterreus* mycelium was used as inoculum

PM2A MicroPlate™ Carbon Sources

A1 Negative Control	A2 Chondroitin Sulfate C	A3 α-Cyclodextrin	A4 β-Cyclodextrin	A5 γ-Cyclodextrin	A6 Dextrin	A7 Gelatin	A8 Glycogen	A9 Inulin	A10 Laminarin	A11 Mannan	A12 Pectin
B1 N-Acetyl-D-Galactosamine	B2 N-Acetyl-Neuraminic Acid	B3 β-D-Allose	B4 Amygdalin	B5 D-Arabinose	B6 D-Arabitol	B7 L-Arabitol	B8 Arbutin	B9 2-Deoxy-D-Ribose	B10 i-Erythritol	B11 D-Fucose	B12 3-O-β-D-Galacto-pyranosyl-D-Arabinose
C1 Gentiobiose	C2 L-Glucose	C3 Lactitol	C4 D-Melezitose	C5 Maltitol	C6 α-Methyl-D-Glucoside	C7 β-Methyl-D-Galactoside	C8 3-Methyl Glucose	C9 β-Methyl-D-Glucuronic Acid	C10 α-Methyl-D-Mannoside	C11 β-Methyl-D-Xyloside	C12 Palatinose
D1 D-Raffinose	D2 Salicin	D3 Sedoheptulosan	D4 L-Sorbose	D5 Stachyose	D6 D-Tagatose	D7 Turanose	D8 Xylitol	D9 N-Acetyl-D-Glucosaminitol	D10 γ-Amino Butyric Acid	D11 δ-Amino Valeric Acid	D12 Butyric Acid
E1 Capric Acid	E2 Caproic Acid	E3 Citraconic Acid	E4 Citramalic Acid	E5 D-Glucosamine	E6 2-Hydroxy Benzoic Acid	E7 4-Hydroxy Benzoic Acid	E8 β-Hydroxy Butyric Acid	E9 γ-Hydroxy Butyric Acid	E10 α-Keto-Valeric Acid	E11 Itaconic Acid	E12 5-Keto-D-Gluconic Acid
F1 D-Lactic Acid Methyl Ester	F2 Malonic Acid	F3 Melibionic Acid	F4 Oxalic Acid	F5 Oxalomalic Acid	F6 Quinic Acid	F7 D-Ribono-1,4-Lactone	F8 Sebacic Acid	F9 Sorbic Acid	F10 Succinamic Acid	F11 D-Tartaric Acid	F12 L-Tartaric Acid
G1 Acetamide	G2 L-Alaninamide	G3 N-Acetyl-L-Glutamic Acid	G4 L-Arginine	G5 Glycine	G6 L-Histidine	G7 L-Homoserine	G8 Hydroxy-L-Proline	G9 L-Isoleucine	G10 L-Leucine	G11 L-Lysine	G12 L-Methionine
H1 L-Ornithine	H2 L-Phenylalanine	H3 L-Pyroglutamic Acid	H4 L-Valine	H5 D,L-Carnitine	H6 Sec-Butylamine	H7 D,L-Octopamine	H8 Putrescine	H9 Dihydroxy Acetone	H10 2,3-Butanediol	H11 2,3-Butanedione	H12 3-Hydroxy 2-Butanone

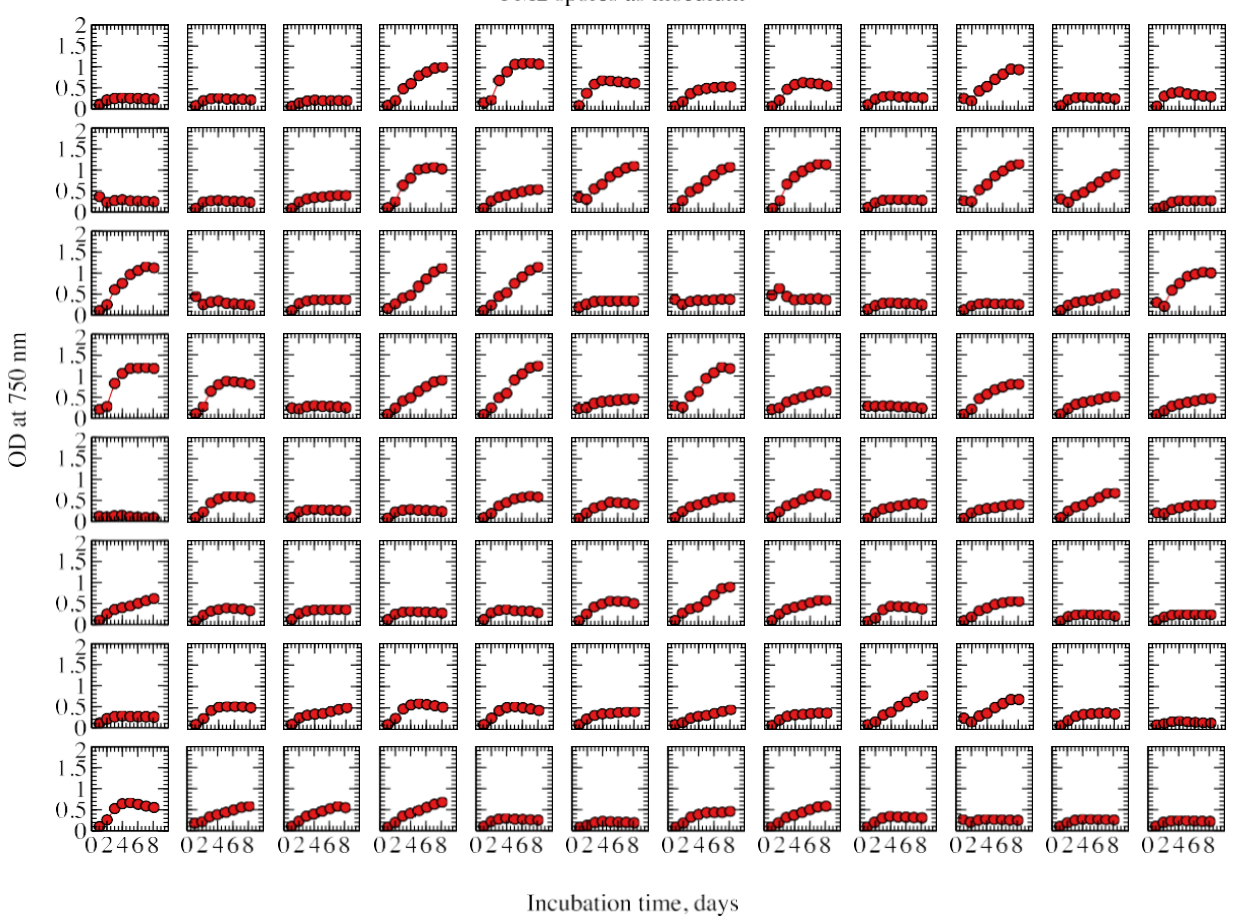


Fig. 6a. Fresh *A. pseudoterreus* conidia were used as inoculum

PM2A MicroPlate™ Carbon Sources

A1 Negative Control	A2 Chondroitin Sulfate C	A3 α-Cyclodextrin	A4 β-Cyclodextrin	A5 γ-Cyclodextrin	A6 Dextrin	A7 Gelatin	A8 Glycogen	A9 Inulin	A10 Laminarin	A11 Mannan	A12 Pectin
B1 N-Acetyl-D-Galactosamine	B2 N-Acetyl-Neuraminic Acid	B3 β-D-Allose	B4 Amygdalin	B5 D-Arabinose	B6 D-Arabitol	B7 L-Arabitol	B8 Arbutin	B9 2-Deoxy-D-Ribose	B10 i-Erythritol	B11 D-Fucose	B12 3-O-β-D-Galacto-pyranosyl-D-Arabinose
C1 Gentiobiose	C2 L-Glucose	C3 Lactitol	C4 D-Melezitose	C5 Maltitol	C6 α-Methyl-D-Glucoside	C7 β-Methyl-D-Galactoside	C8 3-Methyl Glucose	C9 β-Methyl-D-Glucuronic Acid	C10 α-Methyl-D-Mannoside	C11 β-Methyl-D-Xyloside	C12 Palatinose
D1 D-Raffinose	D2 Salicin	D3 Sedoheptulosan	D4 L-Sorbose	D5 Stachyose	D6 D-Tagatose	D7 Turanose	D8 Xylitol	D9 N-Acetyl-D-Glucosaminitol	D10 γ-Amino Butyric Acid	D11 δ-Amino Valeric Acid	D12 Butyric Acid
E1 Capric Acid	E2 Caproic Acid	E3 Citraconic Acid	E4 Citramalic Acid	E5 D-Glucosamine	E6 2-Hydroxy Benzoic Acid	E7 4-Hydroxy Benzoic Acid	E8 β-Hydroxy Butyric Acid	E9 γ-Hydroxy Butyric Acid	E10 a-Keto-Valeric Acid	E11 Itaconic Acid	E12 5-Keto-D-Gluconic Acid
F1 D-Lactic Acid Methyl Ester	F2 Malonic Acid	F3 Melibionic Acid	F4 Oxalic Acid	F5 Oxalomalic Acid	F6 Quinic Acid	F7 D-Ribono-1,4-Lactone	F8 Sebacic Acid	F9 Sorbic Acid	F10 Succinamic Acid	F11 D-Tartaric Acid	F12 L-Tartaric Acid
G1 Acetamide	G2 L-Alaninamide	G3 N-Acetyl-L-Glutamic Acid	G4 L-Arginine	G5 Glycine	G6 L-Histidine	G7 L-Homoserine	G8 Hydroxy-L-Proline	G9 L-Isoleucine	G10 L-Leucine	G11 L-Lysine	G12 L-Methionine
H1 L-Ornithine	H2 L-Phenylalanine	H3 L-Pyroglutamic Acid	H4 L-Valine	H5 D,L-Carnitine	H6 Sec-Butylamine	H7 D,L-Octopamine	H8 Putrescine	H9 Dihydroxy Acetone	H10 2,3-Butanediol	H11 2,3-Butanedione	H12 3-Hydroxy 2-Butanone

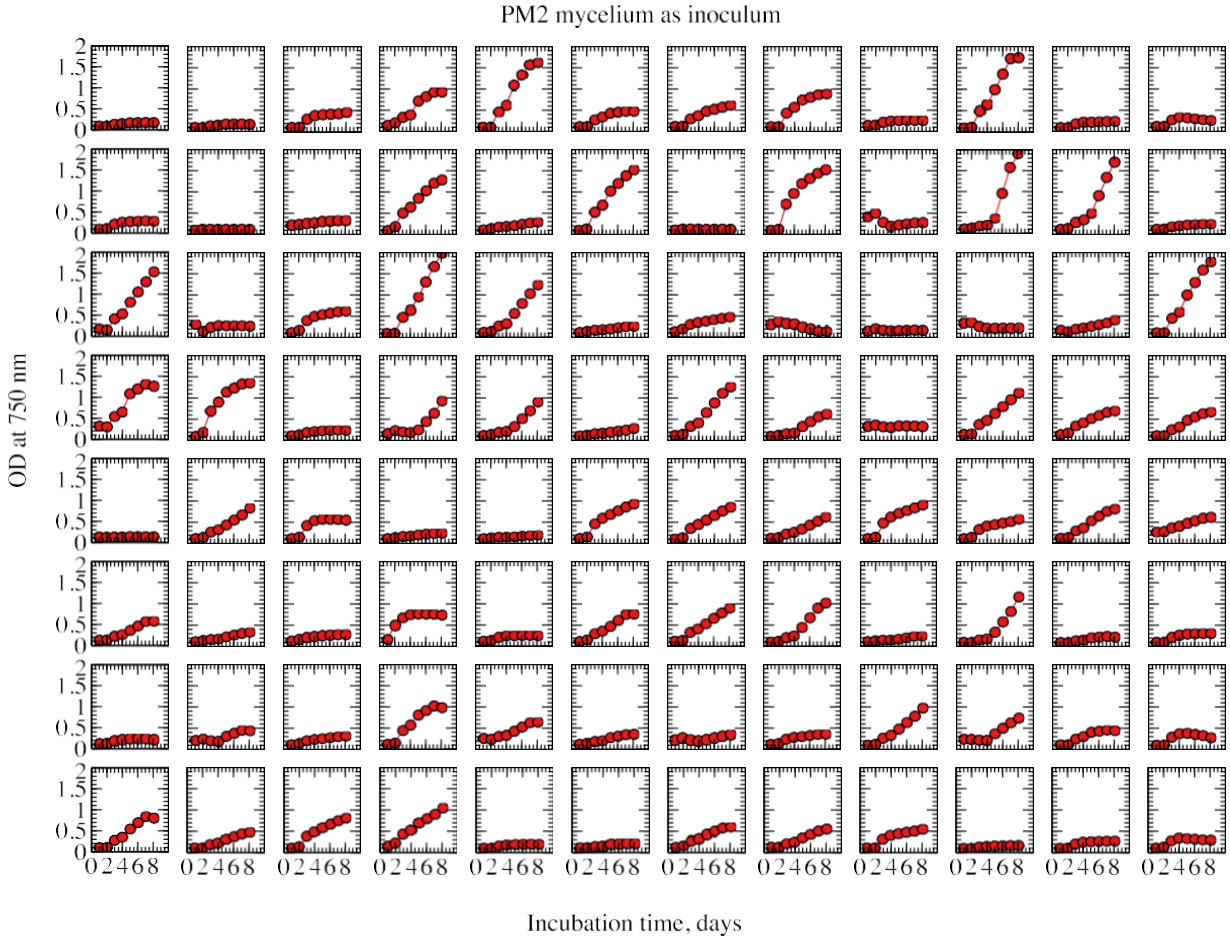


Fig. 6b. Macerated *A. pseudoterreus* mycelium was used as inoculum

PM3B MicroPlate™ Nitrogen Sources

A1 Negative Control	A2 Ammonia	A3 Nitrite	A4 Nitrate	A5 Urea	A6 Biuret	A7 L-Alanine	A8 L-Arginine	A9 L-Asparagine	A10 L-Aspartic Acid	A11 L-Cysteine	A12 L-Glutamic Acid
B1 L-Glutamine	B2 Glycine	B3 L-Histidine	B4 L-Isoleucine	B5 L-Leucine	B6 L-Lysine	B7 L-Methionine	B8 L-Phenylalanine	B9 L-Proline	B10 L-Serine	B11 L-Threonine	B12 L-Tryptophan
C1 L-Tyrosine	C2 L-Valine	C3 D-Alanine	C4 D-Asparagine	C5 D-Aspartic Acid	C6 D-Glutamic Acid	C7 D-Lysine	C8 D-Serine	C9 D-Valine	C10 L-Citrulline	C11 L-Homoserine	C12 L-Ornithine
D-1 N-Acetyl-L-Glutamic Acid	D2 N-Phthaloyl-L-Glutamic Acid	D3 L-Pyroglutamic Acid	D4 Hydroxylamine	D5 Methylamine	D6 N-Amylamine	D7 N-Butylamine	D8 Ethylamine	D9 Ethanolamine	D10 Ethylenediamine	D11 Putrescine	D12 Agmatine
E1 Histamine	E2 β-Phenylethyl-amine	E3 Tyramine	E4 Acetamide	E5 Formamide	E6 Glucuronamide	E7 D,L-Lactamide	E8 D-Glucosamine	E9 D-Galactosamine	E10 D-Mannosamine	E11 N-Acetyl-D-Glucosamine	E12 N-Acetyl-D-Galactosamine
F1 N-Acetyl-D-Mannosamine	F2 Adenine	F3 Adenosine	F4 Cytidine	F5 Cytosine	F6 Guanine	F7 Guanosine	F8 Thymine	F9 Thymidine	F10 Uracil	F11 Uridine	F12 Inosine
G1 Xanthine	G2 Xanthosine	G3 Uric Acid	G4 Alloxan	G5 Allantoin	G6 Parabanic Acid	G7 D,L-α-Amino-N-Butyric Acid	G8 γ-Amino-N-Butyric Acid	G9 ε-Amino-N-Caproic Acid	G10 D,L-α-Amino-Caprylic Acid	G11 β-Amino-N-Valeric Acid	G12 α-Amino-N-Valeric Acid
H1 Ala-Asp	H2 Ala-Gln	H3 Ala-Glu	H4 Ala-Gly	H5 Ala-His	H6 Ala-Leu	H7 Ala-Thr	H8 Gly-Asn	H9 Gly-Gln	H10 Gly-Glu	H11 Gly-Met	H12 Met-Ala

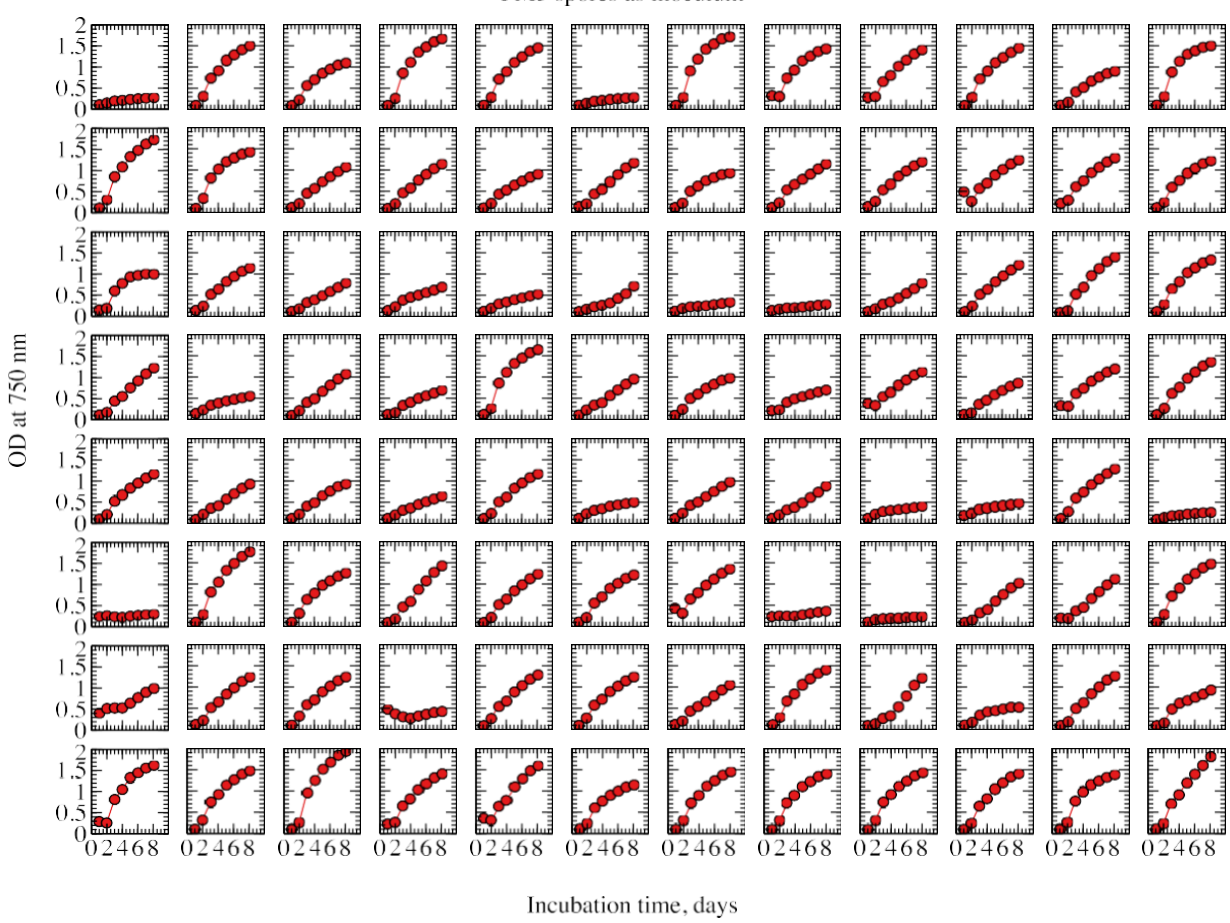


Fig. 7a. Fresh *A. pseudoterreus* conidia were used as inoculum

PM3B MicroPlate™ Nitrogen Sources

A1 Negative Control	A2 Ammonia	A3 Nitrite	A4 Nitrate	A5 Urea	A6 Biuret	A7 L-Alanine	A8 L-Arginine	A9 L-Asparagine	A10 L-Aspartic Acid	A11 L-Cysteine	A12 L-Glutamic Acid
B1 L-Glutamine	B2 Glycine	B3 L-Histidine	B4 L-Isoleucine	B5 L-Leucine	B6 L-Lysine	B7 L-Methionine	B8 L-Phenylalanine	B9 L-Proline	B10 L-Serine	B11 L-Threonine	B12 L-Tryptophan
C1 L-Tyrosine	C2 L-Valine	C3 D-Alanine	C4 D-Asparagine	C5 D-Aspartic Acid	C6 D-Glutamic Acid	C7 D-Lysine	C8 D-Serine	C9 D-Valine	C10 L-Citrulline	C11 L-Homoserine	C12 L-Ornithine
D-1 N-Acetyl-L-Glutamic Acid	D2 N-Phthaloyl-L-Glutamic Acid	D3 L-Pyroglutamic Acid	D4 Hydroxylamine	D5 Methylamine	D6 N-Amylamine	D7 N-Butylamine	D8 Ethylamine	D9 Ethanolamine	D10 Ethylenediamine	D11 Putrescine	D12 Agmatine
E1 Histamine	E2 β-Phenylethyl-amine	E3 Tyramine	E4 Acetamide	E5 Formamide	E6 Glucuronamide	E7 D,L-Lactamide	E8 D-Glucosamine	E9 D-Galactosamine	E10 D-Mannosamine	E11 N-Acetyl-D-Glucosamine	E12 N-Acetyl-D-Galactosamine
F1 N-Acetyl-D-Mannosamine	F2 Adenine	F3 Adenosine	F4 Cytidine	F5 Cytosine	F6 Guanine	F7 Guanosine	F8 Thymine	F9 Thymidine	F10 Uracil	F11 Uridine	F12 Inosine
G1 Xanthine	G2 Xanthosine	G3 Uric Acid	G4 Alloxan	G5 Allantoin	G6 Parabanic Acid	G7 D,L-α-Amino-N-Butyric Acid	G8 γ-Amino-N-Butyric Acid	G9 ε-Amino-N-Caproic Acid	G10 D,L-α-Amino-Caprylic Acid	G11 β-Amino-N-Valeric Acid	G12 α-Amino-N-Valeric Acid
H1 Ala-Asp	H2 Ala-Gln	H3 Ala-Glu	H4 Ala-Gly	H5 Ala-His	H6 Ala-Leu	H7 Ala-Thr	H8 Gly-Asn	H9 Gly-Gln	H10 Gly-Glu	H11 Gly-Met	H12 Met-Ala

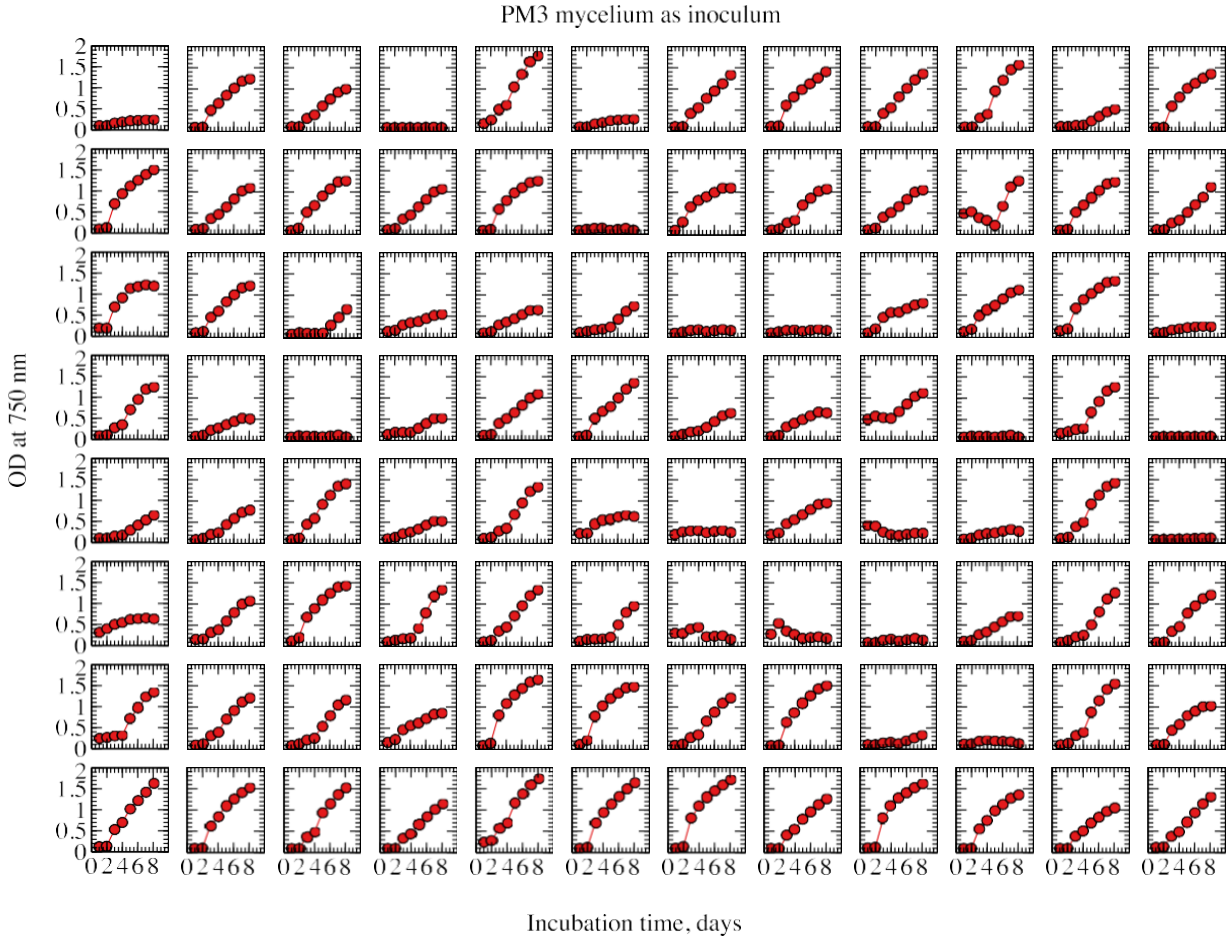


Fig. 7b. Macerated *A. pseudoterreus* mycelium was used as inoculum

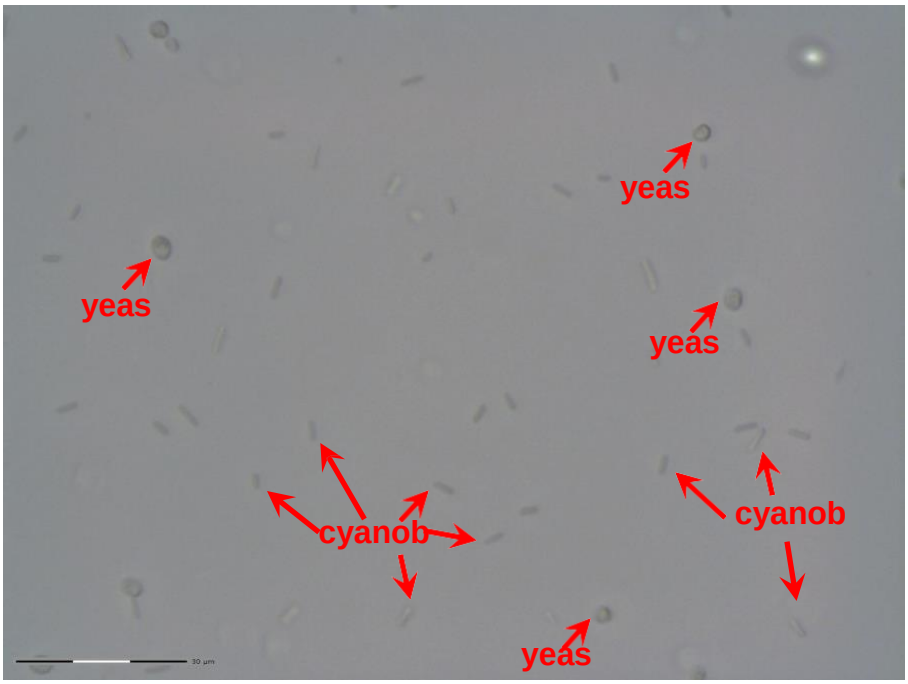


Fig. 8. Microscopy image of *S. elongatus* – *R. glutinis* mixed culture

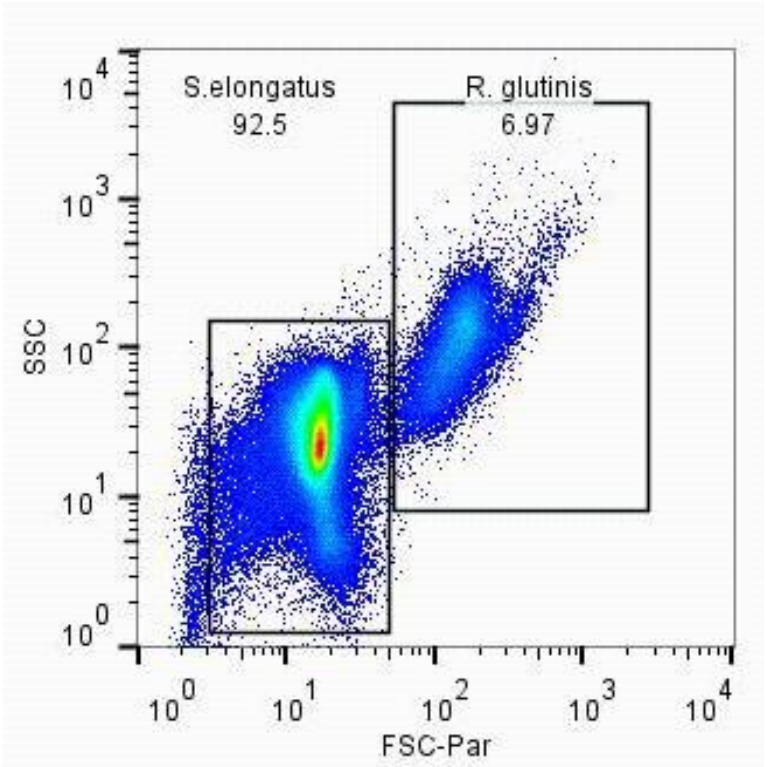


Fig. 9. *S. elongatus* – *R. glutinis* flow cytometry

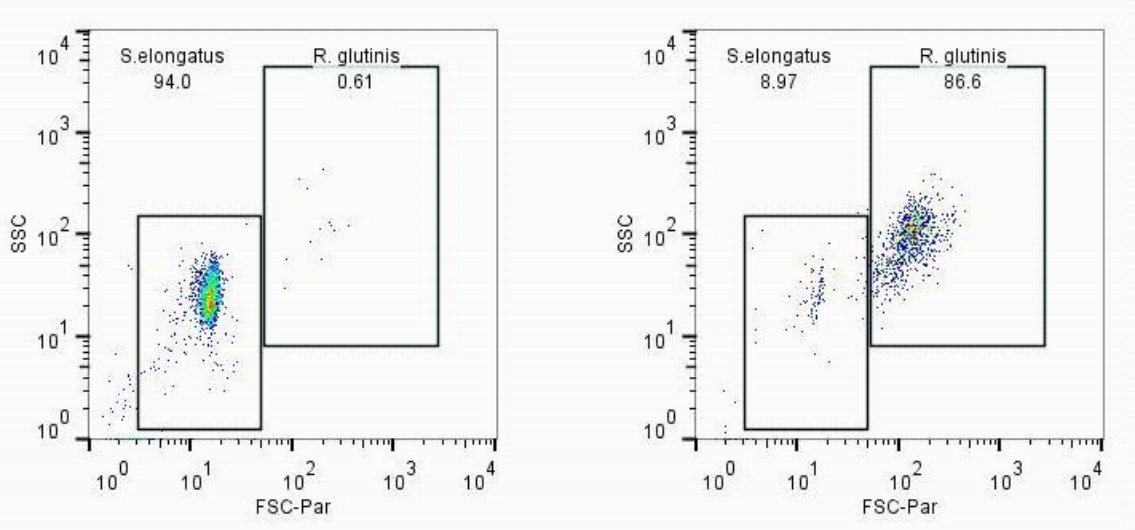


Fig. 10. Results of cell sorting of *S. elongatus* and *R. glutinis* based on the differences in cell size and shape

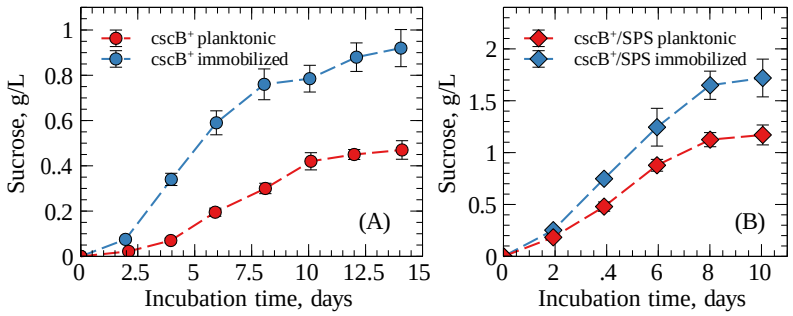
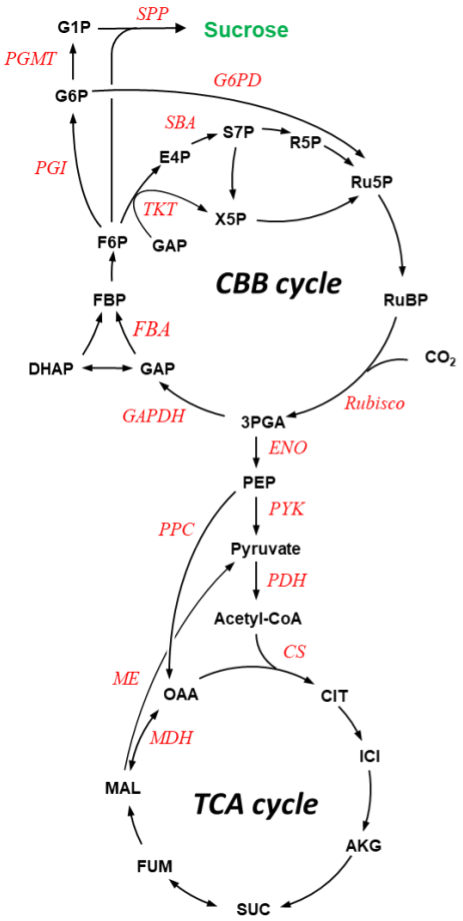
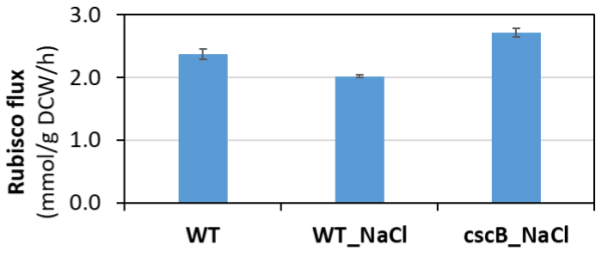


Fig. 11. Sucrose production by planktonic and immobilized S. *elongatus cscB*$^{+}$ and S. *elongatus cscB*$^{+}$*/SPS*





		WT	WT_NaCl	cscB_NaCl
CBB cycle	*Rubisco*	2.3741	2.0182	2.7160
	GAPDH	4.1856	3.5646	5.0406
	FBA	0.8848	0.7935	1.2348
	TKT	0.8204	0.6707	0.8675
	SBA	0.7944	0.6508	0.8494
Sucrose synthesis	*PGI*	0.0644	0.1228	0.2930
	PGMT	0.0000	0.0000	0.0743
	SPP	0.0000	0.0000	0.0743
	G6PD	0.0026	0.0753	0.1758
Glycolysis	*ENO*	0.5008	0.4251	0.3482
	PYK	0.1511	0.1034	0.0928
	PPC	0.2976	0.2817	0.2193
	MDH	0.1273	0.1107	0.1011
	ME	0.1632	0.1785	0.1258
TCA cycle	*PDH*	0.2522	0.2342	0.1753
	CS	0.0641	0.0897	0.0445

Figure 12. Metabolic fluxes in the wild-type and *cscB*-expressing *S. elongatus* 7942 strains with or without salt stress elucidated by ^{13}C-MFA. 100 mM NaCl was added into the BG11 culture medium to induce salt stress. "WT", the wild-type strain grown in BG11 medium; "WT_NaCl", the wild-type grown in BG11 medium with additional 100 mM NaCl; "cscB_NaCl", the cscB^{+} strain grown in BG11 medium with additional 100 mM NaCl. Error bars indicate standard errors.

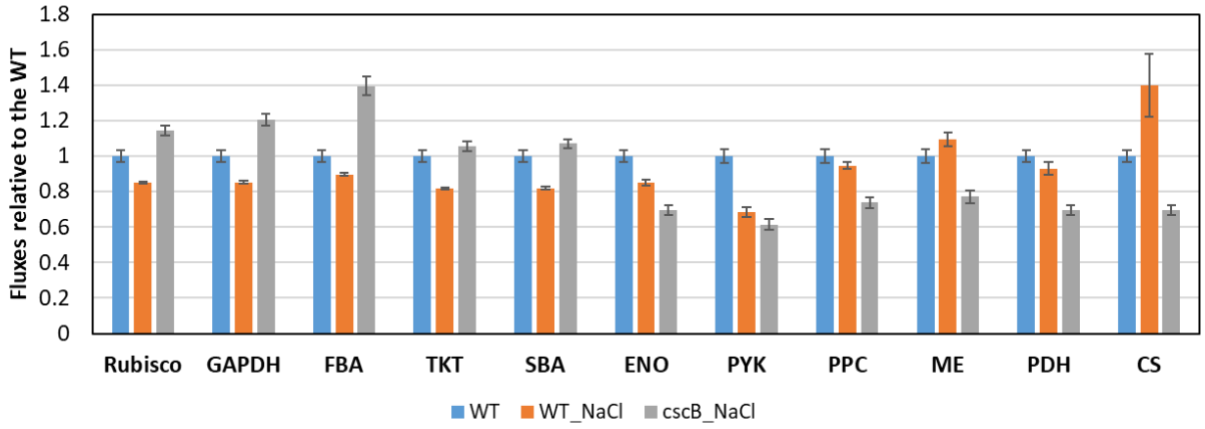


Figure 13. Comparison of metabolic fluxes in three cyanobacterial cultures – WT, WT_NaCl, and cscB_NaCl. The flux for each enzyme is normalized to that of the wild-type strain according to the data in Figure 1. Error bars indicate standard errors.

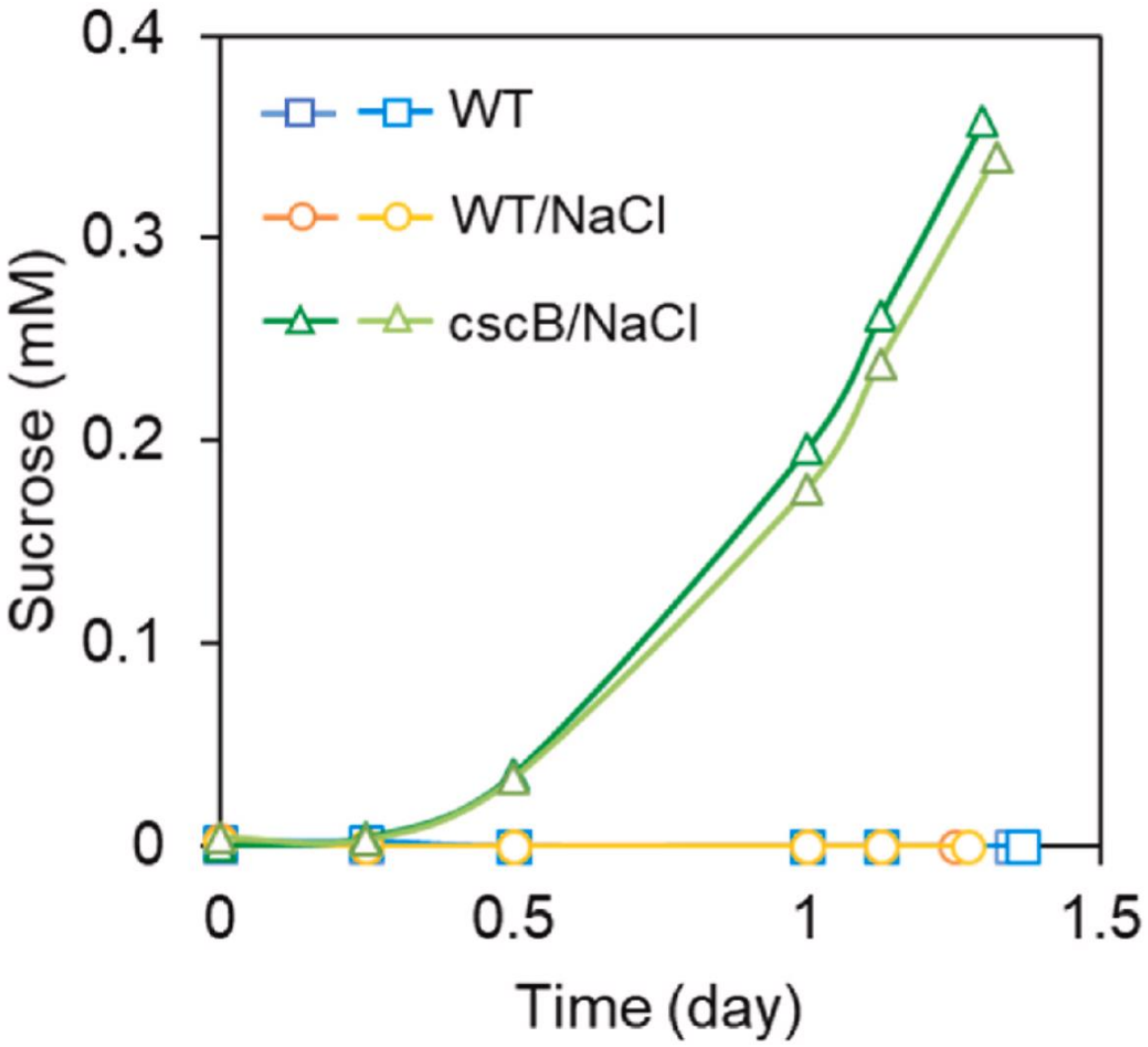


Figure 14. Sucrose accumulated in the culture supernatants.

PRODUCT – DETAILS

All the accepted manuscripts paper has been submitted through OSTI

PUBLICATIONS DETAIL

1. Journal Article: Environmental stimuli drive a transition from cooperation to competition in synthetic phototrophic communities	
Journal: Nature Microbiology	
Publication Date: 2019	**Publication Status:** Published
Volume: 4	**First Page Number or eLocation ID:** 2184-2191
Issue: Not Provided	**Publication Location:** Not Provided
Author(s): Zuniga, C., Li, C.-T., Yu, G., Al-Bassam, M.M., Li, T.-T., Jiang, L., Zaramela, L.S., Guarnieri, M., Betenbaugh, M.J., Zengler, K.	
Publication Identifier Type: DOI	**Publication Identifier:** https://doi.org/10.1038/s41564-019-0567-6
Acknowledgement of DOE Support: Yes	**Peer Reviewed:** Yes

2. Journal Article: Modeling of nitrogen fixation and polymer production in the heterotrophic diazotroph *Azotobacter vinelandii* DJ	
Journal: Metabolic Engineering Communications	
Publication Date: December 2020	**Publication Status:** Published
Volume: 11	**First Page Number or eLocation ID:** e00132
Issue: Not Provided	**Publication Location:** Not Provided
Author(s): Diego Tec Campos, Cristal Zuñiga, Anurag Passi, John Del Toro, Juan D. Tibocha-Bonilla, Alejandro Zepeda, Michael J.Betenbaugh, KarstenZengler	
Publication Identifier Type: DOI	**Publication Identifier:** https://doi.org/10.1016/j.mec.2020.e00132
Acknowledgement of DOE Support: Yes	**Peer Reviewed:** Yes

3**. Journal Article:** Metabolic engineering of *Synechococcus elongatus* 7942 for enhanced sucrose biosynthesis	
Journal: Metabolic Engineering	
Publication Date: November 2023	**Publication Status:** Published
Volume: 80	**First Page Number or eLocation ID:** 12-24
Issue: Not Provided	**Publication Location:** Not Provided
Author(s): Bo Wang, Cristal Zuniga, Michael T Guarnieri, Karsten Zengler, Michael Betenbaugh, Jamey D Young	
Publication Identifier Type: DOI	**Publication Identifier:** 10.1016/j.ymben.2023.09.002
Acknowledgement of DOE Support: Yes	**Peer Reviewed:** Yes

4**. Journal Article:** ^{13}C-Isotope-Assisted Assessment of Metabolic Quenching During Sample Collection from Suspension Cell Cultures	
Journal: Analytical Chemistry	
Publication Date: May 25, 2022	**Publication Status:** Published
Volume: 94	**First Page Number or eLocation ID:** 7731-8084
Issue: 22	**Publication Location:** Not Provided
Author(s): Bo Wang and Jamey D. Young	
Publication Identifier Type: DOI	**Publication Identifier:** 10.1021/acs.analchem.1c05338
Acknowledgement of DOE Support: Yes	**Peer Reviewed:** Yes

5**. Journal Article:** A guanidine-degrading enzyme controls genomic stability of ethylene-producing cyanobacteria	
Journal: Nature Communications	
Publication Date: 26 August 2021	**Publication Status:** Published
Volume: 12	**First Page Number or eLocation ID:** Not Provided
Issue: Not Provided	**Publication Location:** Not Provided
Author(s): Bo Wang, Yao Xu, Xin Wang, Joshua S. Yuan, Carl H. Johnson, Jamey D. Young & Jianping Yu	
Publication Identifier Type: DOI	**Publication Identifier:** 10.1038/s41467-021-25369-x
Acknowledgement of DOE Support: Yes	**Peer Reviewed:** Yes

6**. Journal Article:** Kinetic, metabolic, and statistical analytics: addressing metabolic transport limitations among organelles and microbial communities	
Journal: Current Opinion in Biotechnology	
Publication Date: October 2021	**Publication Status:** Published
Volume: 71	**First Page Number or eLocation ID:** 91-97
Issue: Not Provided	**Publication Location:** Not Provided
Author(s): Cristal Zuniga, Juan D Tibocha-Bonilla, Michael J Betenbaugh	
Publication Identifier Type: DOI	**Publication Identifier:** 10.1016/j.copbio.2021.06.024
Acknowledgement of DOE Support: Yes	**Peer Reviewed:** Yes

7**. Journal Article:** The sum is greater than the parts: exploiting microbial communities to achieve complex functions	
Journal: Current Opinion in Biotechnology	
Publication Date: February 2021	**Publication Status:** Published
Volume: 67	**First Page Number or eLocation ID:** 149-157
Issue: Not Provided	**Publication Location:** Not Provided
Author(s): Livia S Zaramela, Oriane Moyne, Manish Kumar, Cristal Zuniga, Juan D Tibocha-Bonilla, Karsten Zengler	
Publication Identifier Type: DOI	**Publication Identifier:** 10.1016/j.copbio.2021.01.013
Acknowledgement of DOE Support: Yes	**Peer Reviewed:** Yes

8**. Journal Article:** Synthetic microbial communities of heterotrophs and phototrophs facilitate sustainable growth	
Journal: Nature Communications	
Publication Date: July 30 2020	**Publication Status:** Published
Volume: 11	**First Page Number or eLocation ID:** Not Provided
Issue: Not Provided	**Publication Location:** Not Provided
Author(s): Cristal Zuñiga, Tingting Li, Michael T. Guarnieri, Jackson P. Jenkins, Chien-Ting Li, Kerem Bingol, Young-Mo Kim, Michael J. Betenbaugh & Karsten Zengler	
Publication Identifier Type: DOI	**Publication Identifier:** 10.1038/s41467-020-17612-8
Acknowledgement of DOE Support: Yes	**Peer Reviewed:** Yes

9. Journal Article: Mixed and membrane-separated culturing of synthetic cyanobacteria-yeast consortia reveals metabolic cross-talk mimicking natural cyanolichens	
Journal: Scientific Reports	
Publication Date: October 2024	**Publication Status:** Published
Volume: 14	**First Page Number or eLocation ID:** Not Provided
Issue: Not Provided	**Publication Location:** Not Provided
Author(s): Pavlo Bohutskyi, Kyle R. Pomraning, Jack Jenkins, Young-Mo Kim, Brenton C. Poirier, Michael Betenbaugh, Jon Magnuson	
Publication Identifier Type: DOI	**Publication Identifier:** 10.1038/s41598-024-74743-4
Acknowledgement of DOE Support: Yes	**Peer Reviewed:** Yes

INTELLECTUAL PROPERTIES DETAIL
There are no intellectual properties to report.

TECHNOLOGIES AND TECHNIQUES DETAIL
There are no technologies or techniques to report.

OTHER PRODUCTS DETAIL
There are no other products to report

IMPACT

1. Impact on the development of the principal discipline(s) of the project

Developing a co-culture platform for the production of biofuels and bioproducts aims to address several key challenges being faced by the bioenergy community. First, this platform allows for the generation of bioproducts without the need for external carbon feeding. Cyanobacteria like S. elongatus fix carbon dioxide into high energy substrates like sucrose (and glutamate, lysine, and other metabolic engineering targets). This not only removes carbon dioxide from the atmosphere, but also reduces the need for additional substrate feeding to fungal partners that are pumping out bioproducts of interest. From an academic perspective, this project will provide new insights into metabolic interactions in coculture systems. Community metabolic models informed by isotope tracing studies and multi-omics co-culture analysis will suggest changes in metabolic flux from axenic to co-culture settings, and should suggest new analytical methods to understand coculture interactions. As these skills are developed, co-cultures can be more widely used by the scientific community for creation of biofuels, biochemicals, and other sustainably generated products.

2. Impact on other disciplines

The project offers a series of basic and applied science across a range of additional disciplines including environmental science, photobiology, marine science, biochemistry, and genomics. Lichens are widely dispersed in the environment and this project will help to understand the interactions. Understanding the ways in which different phototrophs harvest light will impact photobiology. Many of these organisms live in aquatic environments and thus knowledge is obtained about organismal behavior in these settings. Furthermore, a series of reactions are under way and the group is elucidating biocatalysis of product generation. The study will also elucidate aspects of analytical biochemistry and tools are developed to follow the movement of labeled molecules between species.

3. Impact on the development of human resources

Trainees are being educated in the bioenergy field at levels ranging from undergraduate through graduate and postgraduate level. This training will be important as these students graduate and go onto jobs in the STEM fields.

4. Impact on physical, institutional, and information resources that form infrastructure

Nothing to Report

5. Impact on technology transfer

Commercial technologies involving lichens are still in their infancy. This project represents one of the initial efforts to evaluate the technological and commercial potential of sustainable lichen cocultures.

6. Impact on society beyond science and technology

This project is training the next generation of scientists to tackle issues on sustainability, carbon fixation, and designing solutions to the challenges our world is facing today. This platform will be a new method to sustainably create a variety of bioproducts with a reduced carbon footprint and reduced cost.

7. Foreign Spending

Not Provided

CHANGES - PROBLEMS

1. **Changes in approach and reasons for change**
   Nothing to Report

2. **Actual or anticipated problems or delays and actions or plans to resolve them**
   Most of the participating laboratories were unable to perform experimental research projects between March 2020 and early June 2020 due to COVID-19 pandemic shut down. Computation efforts continued unabated during this period.

3. **Changes that have a significant impact on expenditures**
   Nothing to Report

4. **Significant changes in use or care of human subjects, vertebrate animals, and/or biohazards**
   Nothing to Report

5. **Change of primary performance site location from that originally proposed**
   Nothing to Report

6. **Carryover Amount**
   Nothing to Report