Science and Technology Group Annual Report FY2020

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1. Introduction

Molecular biology is widely used to create synthetic microorganisms designed to produce high value biochemicals such as polypeptides (insulin precursors, growth hormones, antimicrobial peptides, antibodies), small bioactive molecules (antibiotics, food additives, antimalarials), lipids and other specialty chemicals. With plummeting costs of DNA synthesis and novel forms of genes editing the aspirations of metabolic engineers in companies and academic labs to design, test, and build biological systems are being unleashed.

By entrapping engineered microorganisms in a specialized matrix, we are able to halt growth, arrest the cell cycle and thus stabilize the genomes and extend the life cycle of recombinant microorganisms. This increases production rates and yields and, over a longer period, may decrease overall production cost. This technique can be applied to a variety of microbiology platforms, such as bacterial coliforms, budding and related yeast, molds and even metazoan cells.

2. Activities and Findings

Engineered microorganisms produce a wide range of high-value biochemicals such as peptides, small bioactive molecules, lipids and other specialty chemicals. However, engineered microorganisms are often genetically unstable, particularly those that contain complex heterologous pathways and require long fermentation times. This genetic instability limits production yields, shortens production runtime, increases overall costs, and hampers the conversion of cells into efficient biochemical factories.

We discovered that the entrapment of engineered microorganisms in specialized matrices arrests cell growth. We have also seen a drastic increase in the secondary metabolism rate of entrapped cells. Cell entrapment stabilizes genomes, increases production rates and yields, obviates the need for continuous selection and reduces nutrient requirements and fermenter overpopulation. This can significantly decrease overall production costs. Furthermore, cell entrapment unleashes the aspirations of metabolic engineers by removing the difficult challenge of maintaining the stability of genetic constructs, a requirement in conventional bioproduction.

In this POC project, we propose to use our technology to produce an important antioxidant, astaxanthin. Astaxanthin is a carotenoid widely used in salmonid aquaculture, food, cosmetics, nutraceuticals, and pharmaceuticals as a natural antioxidant and biological dye. Astaxanthin exhibits anti-inflammatory, antioxidant, cardiovascular, ocular, and skin-protective activity. The global market for astaxanthin is projected to be $1.3 billion by 2027. Most astaxanthin on the market today is chemically synthesized from petroleum products because of cost considerations. By leveraging the global trend towards sustainable, bio-based chemical production and a technology that can produce a cost-competitive alternative to petroleum-based products, we aim to transform the astaxanthin market.

We obtained strains of Xanthophyllomyces dendrorhous/Phaffia rhodozyma selected to produce high amounts of the natural (3R, 3′R)-isomer of astaxanthin, the same as in the Antarctic krill. These yeast cells produce an amount of astaxanthin which is currently non-competitive with the chemically synthesized racemate. In collaboration with 2 companies with expertise in the fisheries industry in Japan, and in bioreactor design and extraction methods, we will use our technology to entrap X. dendrorhous and extract astaxanthin using a proprietary blend of food-grade solvents and detergents. We expect that the technique will increase production yield several-fold. At the price of >$1,000 per kilogram, this enhancement will make natural astaxanthin a viable product, particularly for industries aimed at direct human consumption. The POC funding will allow us to arrive at a scalable process that can be marketed and licensed to companies that produce feed or food additives.

Study 1: Determination of astaxanthin yields under aerobic conditions in the mutant P. rhodozyma.

1) Optimization of growth conditions. To optimize growth conditions, we varied three parameters: 1. the amount of glucose in the medium, 2. the amount of light during the outgrowth, and 3. the temperature during

the outgrowth. We measured, as outcomes, the coloration of cells, cell density, and the amount of astaxanthin.

Methods. Wildtype and mutant P. rhodozyma (ATCC 24203, 74219 or 74220) were purchased from ATCC and S. cerevisiae (BY4741) was obtained from collaborators. For **1**, the CSHL protocol was adjusted to increase glucose from 2% to 8% glucose. For **2**, the coiled fluorescent light was mounted approximately 30-35 cm from the top of the plates or liquid culture. For **3,** a chilled incubator was used. A thermogun was used to confirm temperatures for all liquid cultures as agitation generates heat. Comparisons were made at day 7 and at day 14.

Results. On both solid and liquid media, 2% glucose, constant white light, and 20C temperature resulted in larger colonies or higher cell titer. All experiments moving forward used 2% glucose in the rich growth media to promote faster growth and red-orange cultures (Fig. 1).

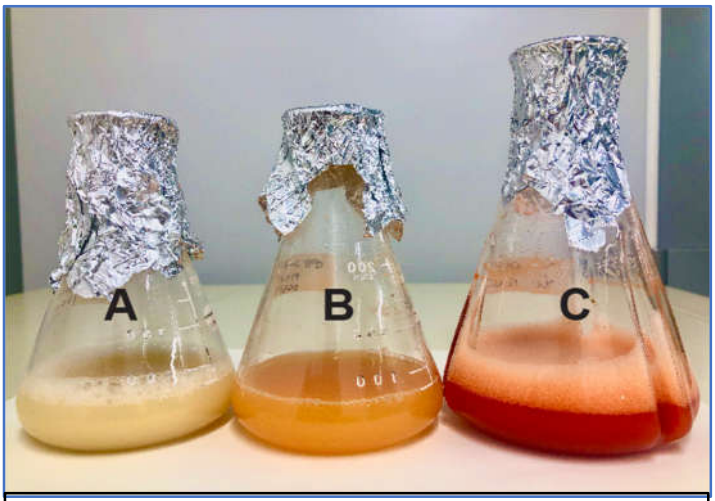


Figure 1. Cultures of S. cerevisiae (A), P. rhodozyma (B) and mutant P. rhodozyma (C) grown at optimal conditions. Note the deep red color in C, caused by the accumulation of astaxanthin.

2) Disruptive Extraction Of Astaxanthin

Methods. Cell wall disruption: All cells were grown for 2 weeks, followed by cell isolation and acid or detergent treatment followed by vortexing and 30 min of sonication. Solvent extraction of astaxanthin: Solvents used HCl acid (1.0M, 2M, 4M) or acetic acid (1.0M, 2M, 4M) or 10% and 20% of TritionX100, SDS, Tween20, acetone, hexane, dichloromethane, isopropanol, 2 isopropanol: 1 hexane, ethanol, mineral oil, rice oil, MCT oil, cotton seed oil. Treated and control pellets were then resuspended in a solvent, vortexed vigorously for 2 min, and then put in a heated incubator (30C) with back-and-forth agitation for 12 hrs. Afterwards, samples were pelleted and the supernatant was assayed for the presence of astaxanthin indirectly using 480 nm absorbance.

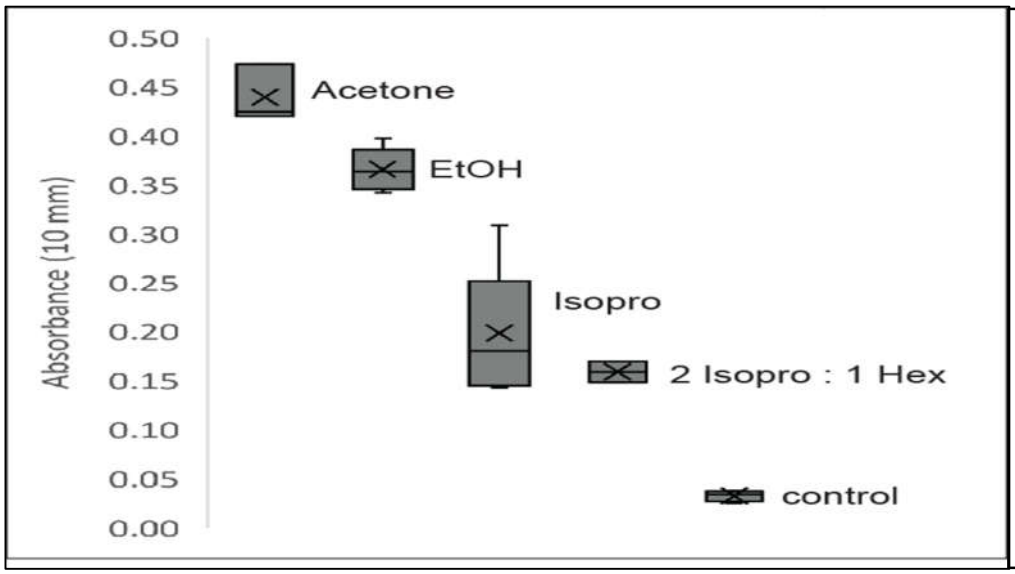


Figure 2. Acetone and ethanol are best extractors of astaxanthin. Boxplot of absorbance at 480 nm of astaxanthin extracted with different solvents as indicated. Crosses are medians. Horizontal lines are means. Boxes indicate 50% of the data. Whiskers indicate 100% of the data.

Results. Acid treatment, but not detergent treatment, extracted the most astaxanthin. Acetone and ethanol proved to be the best solvents among those tested (Fig 2).

3) Production Of Astaxanthin In Entrapped Cells

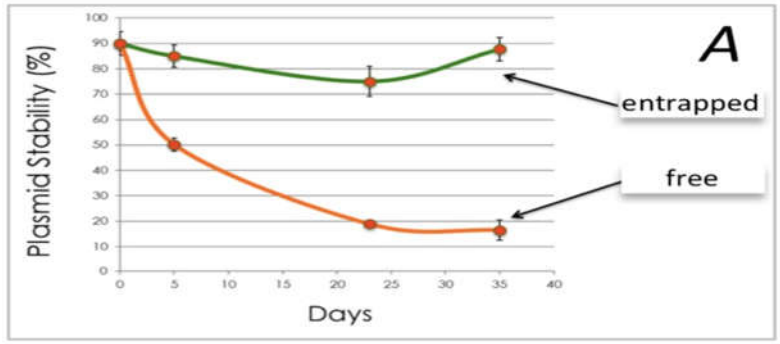
Methods. Entrapping cells to create beads: Colonies collected from liquid culture were mixed with sterile sodium alginate, creating a viscous solution that was pipetted into sterile 0.2M $CaCl_2$. This created spheres (beads) within the 0.2M CaCl2. The beads were left in the 0.2M $CaCl_2$ overnight to allow them to harden and crosslink. Following this, the cells were resuspended in YPD and grown the same as normal liquid cultures on a shaker.

Results. Astaxanthin was extracted from entrapped and free cells. The amount of astaxanthin was 2x higher in entrapped cells compared to the same number of free cells (Fig. 3).

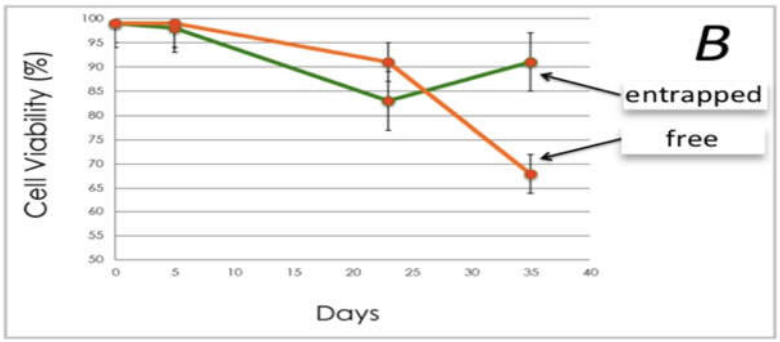
Figure 3. Entrapped cells produce more astaxanthin per cell than free cells. Boxplot of absorbance at 480 nm of astaxanthin in entrapped cells and free cells. Crosses are medians of several measurements. Horizontal lines indicate means. Boxes indicate 50% of the data. Whiskers indicate 100% of the data.

Study 2: Benefits of cell entrapment – increased stress tolerance, stabilization of hybrid genomic structure, high metabolism rates and little increase in cell biomass.

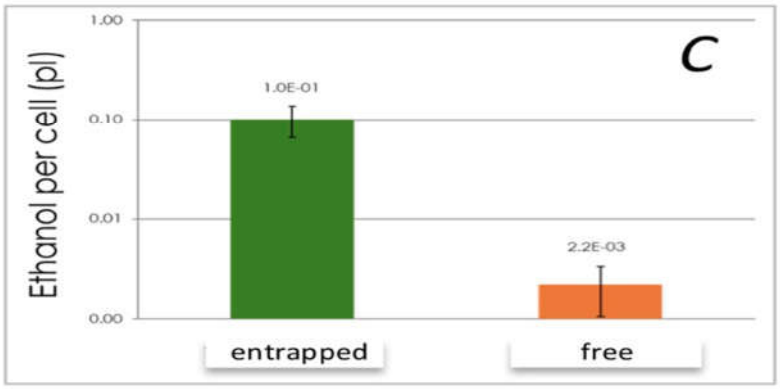
At present, most industrial-scale fermentations directed toward biofuel production use variants of Saccharomyces cerevisiae yeast, and are carried out in batch mode, where a portion of the fermentable carbon in these systems is necessarily diverted into microbial biomass. Entrapping cells increases stress tolerance and supports high secondary metabolism rates combined with little increase in biomass (because the cells do not divide). Entrapped cells are run for several months with little apparent loss in cell viability and anabolic (synthetic) capacity (2,23–25). Moreover, in these systems virtually all substrate (sugars) is directed towards the desired end-product, rather than to increase yeast biomass.



A : Plasmid stability in 35-day course of unselected growth. Treated – cells entrapped in the alginate matrix, untreated – free cells. Error bars are SEM from three independent exp's.



B: Cell viability. Yeast cells were incubated in same medium for 35 days. Treated – cells entrapped in the alginate matrix, untreated – free cells. sampled for viability counts at 5th, 22d and 35th day. Error bars are same as in 1.



C: Ethanol yield per cell. Yeast cells were incubated in 10% glucose for 3 days and ethanol concentration was assessed by gas chromatography. Note log scale on the ordinate. Error bars are same as in 1.

Figure 4. Preliminary results from entrapping yeast cells and checking for their genomic (plasmid) stability, cell viability and ethanol production per cell.

The advantages of entrapped cells are numerous. First, entrapped cells do not divide and maintain extremely high stability of their genomes ((2) and Fig. 4A). Second, entrapped cells exhibit greater tolerance to extremes in acidity, basicity and temperature than their non-entrapped counterparts (23), which results in their viability lasting up to three months (Fig. 4B). Third, entrapment of yeast and other biocatalysts restricts formation of microbial biomass while ensuring high diffusion-rates and therefore high metabolic efficiency. Indeed, entrapment brings about very high cell densities needed to rapidly convert fermentable substrates to the product. Finally, because entrapped cells largely cease to divide, substrate flux to microbial biomass is diminished, shifting the balance of fermentative activity to ethanol production. In our experiments, the Ethanol Red strain of S. cerevisiae yeast being entrapped, fermented glucose to ethanol 50 times faster than free cells on the per cell basis (Fig. 4C).

When yeast is entrapped within a semi-solid matrix (gel) such as Ca alginate and packed into a continuously-fed bioreactor, experimental conditions can be devised where nutrients are non-limiting, even though cell density is extremely high (~10^9 cells mL^{-1} (26)). These conditions consist of rapidly exchanging the reactor void volume with concentrated growth substrate, to which fresh substrate is added and spent medium/product removed.

There are practical advantages to using alginate as the encapsulating matrix: bead size, hardness and porosity can be chemically- and mechanically-controlled so as to optimize for a matrix that is not diffusion-limited, i.e. that the efficiency of ethanol fermentation is not limited by the availability of incoming feedstock sugars. After three to five rounds of cell division, an immobilized yeast population consists largely of arrested, unbudded virgin cells and ceases to divide further (26) Indeed, rates of up to 46 g $l^{-1}h^{-1}$ were achieved with entrapped cells (27), which matches or exceeds the rate observed during mid-log-phase planktonic (non-entrapped) batch culture (28).
In our previous experiments, we estimated ethanol production yields in entrapped vs. free cell cultures. While ethanol yield per gram glucose was estimated to be similar in the entrapped vs. free cells (showing that the entrapment matrix does not slow down cell metabolism, we and other workers have observed that cell-normalized ethanol production rate in ICR can be an order of magnitude greater than that of the free cell culture (2,23,29). Furthermore, in marked contrast to free cells, no evidence of DNA damage after day 17 (our data) and cell viability remains high (>95%), even past 2 weeks of continuous operation (2) and up to 90 days of entrapment (our unpublished data and (30)).
Lastly, our experiments in global expression profiling using DNA microarrays reveal the absence of many transcripts for genes that regulate, or are directly regulated, by the cell cycle, further supporting the view that entrapped cells exist in a state of metabolically-active cell-cycle arrest (31). The result of this is that ethanol production capacity in immobilized cells greatly exceeds that of the free cells.

3. Collaborations

Prof. Frank Rosenzweig, Georgia Institute of Technology, Atlanta GA USA
Dr. Pamela Broussard, University of Montana, Missoula MT USA
Prof. Leonid Kalachev, University of Montana, Missoula MT USA
Dr. Lesley Ellies, UC San Diego, San Diego, CA USA
Dr. Zach Bell, Shoreditch, Onna-son, Kunigami-gun, Okinawa, Japan

4. Publications and other output

Encapsulation enhances protoplast fusant stability
J Gulli, E Kroll, F Rosenzweig
Biotechnology and Bioengineering 2020

5. External Funding

"Enhanced production of astaxanthin specialized entrapped yeast " has been selected to participate in the Proof-of-Concept (POC) Program as a Phase I project.