MUCH-EFFICIENT AND COST-EFFECTIVE MANUFACTURING OF ANTIBODY BIOTHERAPEUTICS EMPLOYING INTEGRATED NEGATIVE CHROMATOGRAPHY TECHNOLOGY

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Key Words: mixed-modal, integrated-continuous, high-throughput, antibody therapeutics, custom resins

New approaches for fully connected and integrated downstream processes to reduce costs and improve efficiency are being assessed with the implementation of the NCAP Project (negative chromatography antibody purification).

This project aims to resolve the manufacturing bottleneck facing modern antibody bio-therapeutics through exploring the great potential of the negative chromatography technology, i.e. purifying antibodies by binding all the surrounding impurities instead of binding target antibodies. High-throughput, miniaturised technologies have been implemented to enable the screening of multiple novel ligands based on a custom agarose backbone.

The objectives are: (1) replace the conventional expensive and fragile protein-A affinity chromatography medium with inexpensive and more robust small-ligand-based media; (2) investigate novel downstream processes incorporating as many negative chromatography steps as possible to achieve much-efficient and capacity-unlimited manufacturing of biopharmaceuticals; (3) upstream and downstream process integration, and intensification by pushing the boundaries of the negative chromatography technology.

This process is independent of the expression level in the upstream and should bring enormous potential cost benefits; providing a platform for truly continuous and integrated manufacturing processes, reducing hold times, enabling faster throughput and reducing the cost of raw materials.

WITHDRAWN

EFFECT OF OVER EXPRESSING PROTECTIVE ANTIGEN ON GLOBAL GENE TRANSCRIPTION IN BACILLUS ANTHRACIS BH500

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Protective antigen (PA) of *Bacillus anthracis* is being considered as a vaccine candidate against anthrax and its production has been explored in several heterologous host systems. Since the expression approaches tested, introduced adverse issues such as inclusion body formation and endotoxin contamination, the production from *B. anthracis* is presently considered as a preferred method. In this presentation we will report on the effect of protective antigen expression on the metabolism of the producing train *B. anthracis*, BH500, by comparing it with a control strain carrying an empty plasmid. The two strains were grown in a bioreactor and RNA-seq analysis of the producing and non-producing strain was performed. Several differences were observed, especially significant were the following: the strain expressing rPA showed increased transcription of *sigL,* the gene encoding RNA polymerase σ^{54}, *sigB*, the general stress transcription factor gene and its regulators *rsbW and rsbV*, as well as the global regulatory repressor *ctsR.* At the same time there were also decreased expression of intracellular heat stress related genes such as *groL, groES, hslO, dnaJ,* and *dnaK* and increased expression of extracellular chaperons *csaA* and *prsA2.* Additionally, major central metabolism genes belonging to TCA, glycolysis, PPP, and amino acids biosynthesis were up-regulated in the PA-producing strain which was associated with decreased specific growth rates. The information and the observation acquired from this study will be presented together with possible approaches to create a better producing strain.

INHIBITION OF PRODUCTIVE/COMPETITIVE ENDOCYTIC PATHWAYS ENHANCES SIRNA DELIVERY AND CELL SPECIFIC TARGETING

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Keywords: Endocytosis, siRNA Therapeutics, Targeted Delivery, Chemical Inhibitors, Delivery Vehicles

While the use of short interfering RNAs (siRNAs) for laboratory studies is now common practice, development of siRNAs for therapeutic applications has slowed, due in part to a still limited understanding of the endocytosis and intracellular trafficking of siRNA-containing complexes. As a result, it is difficult to design delivery vehicles for specific cell types, resulting in inefficient delivery, cytotoxicity, or immunogenicity when used *in vivo*. Our aim is to identify which endocytosis and intracellular trafficking pathways lead to active silencing by siRNA-containing complexes. Our work explores the preferential mechanism of endocytosis (whether by clathrin, caveolin, Arf6, Graf1, flotillin, or macropinocytosis) across multiple cell types (HeLa (cervical), H1299 (lung), HEK293 (kidney), and HepG2 (liver)).
Using Lipofectamine 2000 (LF2K), fluorescently-labeled siRNAs were delivered to cells stably expressing green fluorescent protein (GFP). Chemical inhibitors (Filipin, Dynasore, Cytochalasin D, Chlorpromazine, Amiloride, and Methyl-β-cyclodextrin) were used to identify the specific endocytic pathway internalizing the complexes. By measuring the effect of inhibitors on both intracellular levels of siRNA and GFP silencing, we were able to categorize pathways as being productive/competitive according to their functional role in facilitating gene silencing. In productive pathways, siRNAs are actively delivered to a cell and silence a target protein, whereas in competitive pathways, siRNAs are endocytosed but do not lead to silencing.

To further validate the findings from our inhibitor assay, we overexpressed the following endocytic proteins and quantified their effect on siRNA uptake: Dynamin, Actin, AP2, Clathrin, Caveolin, Flotillin 1, Flotillin 2, Arf6, and Graf1. Together our findings suggest that LF2K-siRNA complexes are internalized through multiple pathways in all cells but that only one of the pathways is productive, leading to GFP silencing (Table 1). Interestingly, our data suggests that the relative expression of endocytic proteins within a cell line may indicate which endocytotic pathway is productive.

Table 1: Productive/competitive pathways utilized for LF2K-mediated RNAi.

Cell Line	Productive Pathway	Competitive Pathway
HeLa	Arf6	Clathrin
HepG2	Arf6	Clathrin
H1299	Flotillin	Clathrin
HEK293	Graf1	Arf6

Additionally, we explored the use of inhibitors in co-cultured cell populations. Based on our results, we were able to selectively inhibit GFP silencing for a specific cell line by targeting its productive pathway (Figure 1). Furthermore, inhibiting siRNA uptake in one targeted cell type enhanced the overall bioavailability of siRNA complexes and GFP silencing in the other cell population.

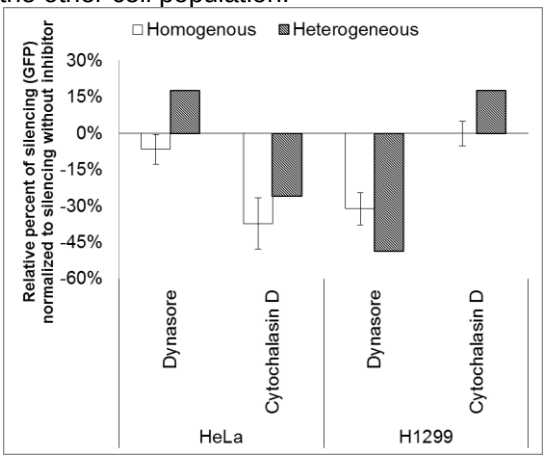

Figure 1: Effect of inhibitors on GFP silencing between single and co-culture (HeLa and H1299) cell populations.

CELL COMMUNICATION NETWORK FACTOR 4 (CCN4/WISP1) SHIFTS MELANOMA CELLS FROM A FRAGILE PROLIFERATIVE TO A RESILIENT METASTATIC STATE AND SUPPRESSES IMMUNE SURVEILLANCE

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Key Words: Immune-oncology, Wnt signaling pathway, metastasis, secreted protein

While deregulated intracellular signaling initiates melanoma, patient survival is limited by progression and metastasis - processes often coordinated by secreted signals. Secreted signals can reinforce cell fate decisions by acting on the same cell and sustain pathology by influencing the stromal and immune cells present within the tumor microenvironment. Understanding how these secreted signals contribute to pathology remains a challenge as the relevance of a secreted signal depends highly on context. Identified by an unbiased phenotypic screen for inhibitors of immune cell crosstalk, Cell Communication Network Factor 4 (CCN4/WISP1) is a secreted matricellular protein that is upregulated in melanoma and breast cancer and correlates with a worse overall outcome. Here, I will discuss our recent in vitro and in vivo results to clarify the functional role that CCN4 plays in melanoma. Interestingly, we found that CCN4 shifts melanoma cells from a fragile proliferative to a resilient metastatic state. CCN4 drives this phenotypic shift by activating AKT Ser/Thr kinase and MEK/ERK signaling pathways that induce snail family transcriptional repressor 1 (SNAI1) expression. SNAI1 then initiates a transcriptional response similar to the epithelial-mesenchymal transition (EMT), including E-cadherin repression and fibronectin and N-cadherin induction. In vivo, knocking out CCN4 represses tumor metastasis of B16F10 and YUMM1.7 melanoma cells in syngeneic C57BL/6Ncrl and immunocompromised NOD-scid IL2Rgamma^{null} (NSG) mice. While CCN4-KO variants of B16F10 and YUMM1.7 cells grow faster that WT cells both in vitro and in NSG mice, tumors initiated by CCN4-KO variants consistently grow slower in immunocompetent hosts. This reduction in tumor growth by CCN4-KO variants also corresponds to an increase in tumor-infiltrating lymphocytes. CCN4-KO variants of B16F0 and YUMM1.7 melanoma cells are also more responsive to immune checkpoint blockade. While some mechanistic details of this heterocellular crosstalk remain unclear, the results suggest an intriguing collateral target to both enhance the efficacy of immune checkpoint blockage and inhibit metastasis.

BIOLOGICALISATION: A NATURE-BASED DIGITAL MANUFACTURING REVOLUTION

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Key Words: Industry 4.0, Biologicalisation, bio-inspired, transformation

Industry 4.0 is changing our manufacturing concepts [*1*]. Biologicalisation is the use of 4.0 principals in concert with biological and bio-inspired materials, chemistries and functions to support an efficient and sustainable manufacturing schema. From product design, to development and manufacturing, biomimetic product designs and bio-integrated manufacturing systems describe this *biological transformation of manufacturing* [*2*].

Many processes and chemistries a well as systems and supply chains are enhanced by the harmonization of digital manufacturing principals with biological structures and chemistries. Thus enabling efficient and robust production supporting a global circular economy.

Progress in our understanding of biological elements, phenomena, materials and chemistries enables this revolution. In fact, a Nobel Prize winner in Chemistry this year, Frances H. Arnold illustrates this. She invented systems directing the evolution of enzymes now routinely used in development of such tools as catalysts in manufacturing. This technology also supports such other 4.0 goals as the more sustainable manufacturing of pharmaceuticals and renewable fuels [*3*].

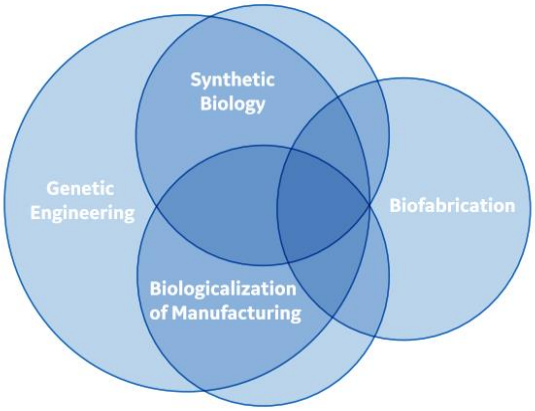

Figure 1 – Arial 10 pt Italics

Figure 1. Relationship of some newer technologies to industrial biologicalisation.

The history of acetic acid manufacturing illustrates our move from biological to synthetic to bio-integrated manufacturing. The *German Method* of acetic acid production percolated an alcoholic solution through wood shavings with *Acetobacter*. We next generated it synthetically from rather inorganic and chlorinated intermediates [*4*]. Today the biologicalisation of the process through genetic and metabolomic engineering, as well as digital manufacturing-based advances in fermentation, are promising more sustainable acetic manufacturing. These include lower energy requirements and the possibility of such low-cost carbohydrate sources as organic wastes and agricultural residues.

1. https://www.automation.com/automation-news/article/industry-40-only-one-tenth-of-germanys-high-tech-strategy
2. https://www.researchgate.net/publication/324486404_Biologicalisation_Biological_transformation_in_manufacturing
3. https://www.nobelprize.org/prizes/chemistry/2018/press-release/
4. https://en.m.wikipedia.org/wiki/Acetic_acid

CONDITIONAL PROTEIN RESCUE (CPR) BY BINDING-INDUCED PROTECTIVE SHIELDING

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Key Words: protein stability, protein sensing, prodrugs, nanobodies, synthetic biology

The rational regulation of protein concentration remains an elusive problem in synthetic biology. Several strategies have been developed at the transcriptional level, but these suffer from suboptimal response kinetics due to slow turnover of any protein already synthesized. The direct fusion of degradation tags to a protein of interest (POI) results in rapid fluctuations in protein concentration, and several degradation tags have been developed that function conditionally on the presence of a small molecule. However, no solutions currently exist that allow the intracellular concentration of a POI to be regulated by the cellular environment.

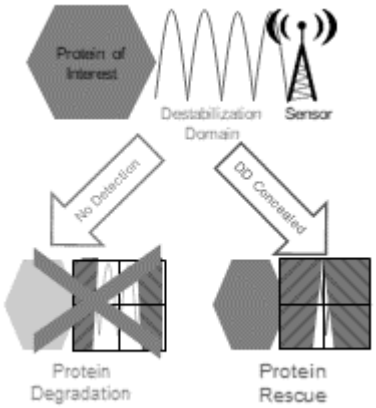

Figure 2 – Protein-based modulator for translating between endogenous cellular information and the concentration of a protein of interest.

To that end, we propose a conditional protein rescue (CPR) system: a protein modulator capable of translating information concerning endogenous cellular proteins to desirable changes in the concentration of a POI. The POI is fused to a destabilizing domain (DD) and a small sensor domain, a product which—by virtue of the DD—is inherently unstable via proteasomal degradation (Figure 1, top). In the absence of the sensor's target, the protein remains unstable and exists at low intracellular concentrations (Figure 1, bottom left with curtain open revealing the DD). When the sensor's target is present, the interaction with the sensor sterically masks the DD and rescues the POI from degradation (Figure 1, bottom right with closed curtain in the windowpane concealing the DD).

As an initial proof-of-concept, we used YFP as our POI and the short peptide SpyTag as the sensor. When SpyTag interacts with its target, SpyCatcher, a covalent bond is formed, providing maximum bond strength and thus optimal conditions for CPR to occur. YFP levels remained low in the absence of SpyCatcher, and upon co-expression with SpyCatcher, YFP levels rose rapidly indicating CPR functions as predicted. Furthermore, adding an ubiquitin-like (UbL) domain to the N-terminus of YFP enhanced the degradation kinetics and reduced background to undetectable levels while still allowing for rescue in the presence of SpyCatcher. This indicates that CPR is modular with tunable sensitivity to meet the needs of the circumstance.

Cancer is often marked by dramatic changes in a cell's proteome. If this information could be accurately translated into a therapeutic output, then that technology opens the door to a next generation of "smart therapeutics". To demonstrate this conceptually, we changed the sensor in CPR to a camel single-domain antibody fragment (nanobody), a small, monomeric protein with high specificity towards its antigen. As an output POI, we chose yeast cytosine deaminase (yCD), a prodrug converting enzyme capable of converting the innocuous prodrug 5-fluorocytosine (5-FC) to the cytotoxic drug 5-fluorouracil (5-FU). GFP served as a visually traceable cancer-protein surrogate and the target of a well characterized nanobody. The nanobody was not large enough to effect protein degradation, and no cytotoxicity was observed in the absence of GFP, even when 5-FC was administered. However, when GFP was co-expressed, cell death was observed on levels similar to when 5-FU was administered directly. This demonstrates that CPR can be used to control a wide variety of proteins.

In summary, CPR represents a crucial first step towards the conditional control of intracellular protein concentration, and it serves as a novel modulator between endogenous proteome information and a desired output protein. CPR could fill a much need niche in synthetic biology as the field begins to tackle personalized medicine, autonomous cell manufacturing, and other bioengineering challenges. This process can be expanded to other proteasomal degradation mechanisms, and the layering of them presents the opportunity for building protein-based Boolean logic gates for more complex decision making applications.

UTILIZING CRISPR/CAS9 TO IDENTIFY CHROMOSOMAL LOCI

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Key Words: FISH, Cas9, CRISPR, DuoLink, cytogenetics

Current methods for imaging transgene integration sites use DNA-FISH, which is a time- and labor-intensive process. Every genomic target requires a costly probe specific to the locus of interest. Through CasFISH, the only customized reagent needed is a target-specific crRNA, while a singular tracrRNA, dCas9, and antibody set can be applied to any newly synthesized crRNA, significantly reducing cost and effort when targeting multiple sites. The CRISPR/Cas9 system provides highly specific targeting of genomic DNA by means of a Cas9 nuclease complexed with guide-RNA which binds a 20 base-pair target sequence within the genome. Introducing two point-mutations to the catalytic region of Cas9 results in a nuclease-inactive enzyme referred to as dCas9. This catalytically “dead” system can be used by leveraging the intact DNA-interrogative properties to direct proteins to areas of interest within the genome. Our group has applied this approach to image telomere-specific repeats on chromosomes in metaphase spreads via immunofluorescence within our host cell line, CHOZN GS-/-. Utilizing the CasFISH method has been successful for us and others in targeting large, repetitive regions of interest, but to our knowledge, there has been no success in targeting smaller, non-repetitive regions using CasFISH alone. Our current work seeks to solve this problem by combining CasFISH with other existing technologies, such as DuoLink PLA (proximity ligation assay) or quantum dots, which are artificial semiconductor nanoparticles. Here, we detail our efforts in exploring these technologies, which promise to reduce the time and effort spent in visualizing transgene integration sites and other genomic loci of interest.

HOF FREEZE-THAW TECHNOLOGY IMPLEMENTATION FOR BIOLOGICS BULK DRUG SUBSANCE AT BRISTOL-MYERS SQUIBB (BMS)

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Key Words: HOF, Freeze-thaw, FFTp, blast freeze

Biologics drug substance is ideally stored and transported in a frozen state to minimize potential temperature related instabilities. Currently, BMS utilizes compressor-based freezers or liquid N2 (LN2) blast freezer units that achieve cooling by convection. These processes are relatively long (up to 3 days), and provide limited capacity (e.g., up to 8 containers per blast-freezer unit).

With the projected improvements in Drug Substance (DS) process yields (for example, a product reaching up to 80 filled containers per batch), the existing blast freezers capacity could easily exceed full utilization. This level of throughput would lead to the freezing process unit operation becoming a cadence limiting step and resulting in increased hold times prior to freezing. This poster describes successful first time implementation of active plate HOF freezing and thawing technology for freezing and thawing a biologics bulk drug substance at DS and Drug Product (DP) manufacturing sites (Figure 1). The poster will describe various challenges identified and resolved before technology implementation in manufacturing. The HOF technology led to faster (up to 2 cycles per day, 10 containers per cycle per unit) and a more robust freezing process (consistent freezing of a wide variety of loads). Furthermore, the HOF technology has application for thawing DS at the DP sites. In this poster, we will also present our roll out plans for implementation of the active-plate as the platform BMS technology for freezing and thawing DS, across both internal network and external partners.

The lessons learned during first implementation are being leveraged across the network. The work outlined in this poster is highly relevant to the central theme of 2019 meeting (The Next Generation of Biochemical and Molecular Engineering: The role of “emerging technologies” in tomorrow’s products and processes) since the plate-based freeze-thaw technology eliminates freezing and thawing processes becoming cadence limiting steps at a time of increasing recombinant protein throughputs.

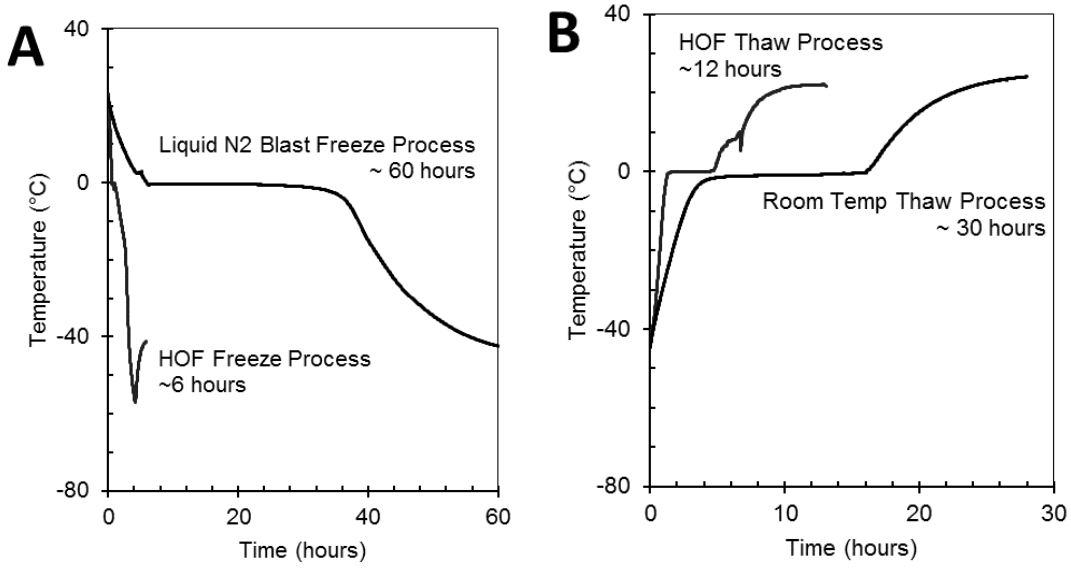

Figure 3 – Improvements in freeze and thaw process durations for 12L Flexible Freeze Thaw Plate (FFTp) containers using HOF technology: (A) Comparison of HOF freezing durations with liquid N2 blast freezing duration; (B) Comparison of HOF thaw duration with room temperature thaw duration

LOW-COST AND USER-FRIENDLY BIOSENSOR TO TEST THE INTEGRITY OF mRNA MOLECULES SUITABLE FOR FIELD APPLICATIONS

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Key Words: RNA integrity; biosensor; low-cost, low-tech; point-of-care diagnostics; RNA degradation

The use of mRNA in biotechnology has expanded with novel applications such as vaccines and therapeutic mRNA delivery recently demonstrated. For mRNA to be used in patients, quality control assays will need to be routinely established. Currently, there is a gap between the highly sophisticated RNA integrity tests available and broader application of mRNA-based products by non-specialist users, e.g. in mass vaccination campaigns. Therefore, the aim of this work was to develop a low-cost biosensor able to test the integrity of a mRNA molecule with low technological requirements and easy end-user application. The biosensor is based on a bi-functional fusion protein, composed by the λN peptide that recognizes its cognate aptamer encoded on the 5' end of the RNA under study and β-lactamase, which is able to produce a colorimetric response through a simple test. We propose two different mechanisms for signal processing adapted to two levels of technological sophistication, one based on spectrophotometric measurements and other on visual inspection. We show that the proposed λN-βLac chimeric protein specifically targets its cognate RNA aptamer, boxB, using both gel shift and biolayer interferometry assays. More importantly, the results presented confirm the biosensor performs reliably, with a wide dynamic range and a proportional response at different percentages of full-length RNA, even when gene-sized mRNAs were used. Thus, the features of the proposed biosensor would allow to end-users of products such as mRNA vaccines to test the integrity of the product before its application in a low-cost fashion, enabling a more reliable application of these products.

ALTERNATIVE TRANSFECTION METHODS FOR SF9 CELLS IN VACCINE DEVELOPMENT

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Key Words: Insect cells; transient; virus-like particles; vaccine; PEI

Current CHO and HEK293 platform processes are suitable for a majority of gene-based and protein-based vaccine candidates, but do not always provide adequate production of virus-like particles (VLPs) as observed by inconsistent and low titers. Thus, alternative production platforms are being considered. One option that we are exploring is the use of insect cells as an alternative host, specifically Sf9, since they are biologically well suited to produce VLP's for mosquito-borne viruses.

We first considered a Baculovirus infection method, and successfully produced all components of two VLP's in our system. However, during initial studies of this process, we encountered issues with the assembly of the VLPs due to the low pH of Sf9 cultures (Figure 1). Thus, we pH adapted Sf9 cells to 7.0 and determined optimal bioreactor parameters to control pH throughout the process. With the capability to maintain cells at this higher pH, the Baculovirus/Sf9 platform looks promising. However, there are additional manufacturing complications to consider. There are concerns with Baculovirus contamination of dual-use equipment and more extensive and costly processing downstream to address viral inactivation and isolation. Thus, we are also considering a transient transfection process.

Several researchers have shown that PEI-based transient transfection of insect cells is a viable and reproduceable option.[1-4] Thus, we are evaluating whether a Sf9 platform process utilizing PEI would be more feasible with the current manufacturing capabilities of our organization. Using pH adapted Sf9 cells, we are presently determining optimal transfection and expression conditions using design of experiment (DOE). However, lower titers are anticipated in comparison to the Baculovirus system. As a result, productivity of transiently transfected Sf9 cells will be further investigated in a perfusion culture.

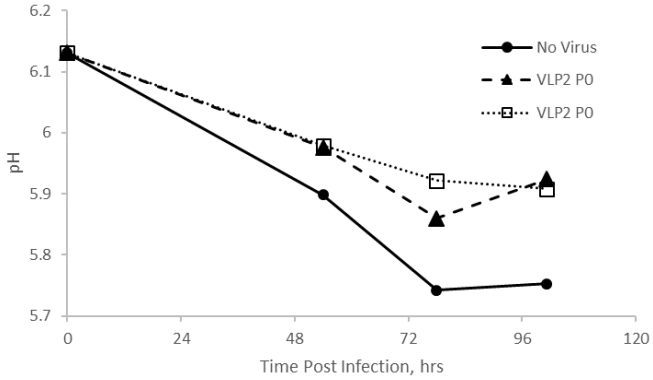

Figure 4 – pH of Sf9 cells post-infection.

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IMPROVING THE EFFICIENCY OF HUMAN NEURAL STEM CELL DIFFERENTIATION BY TARGETING TRANSCRIPTION FACTORS TBR1 AND TBR2 WITH CRISPR-CAS9 GENOME EDITING

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Cell-based therapies are attractive for treating neurodegenerative diseases. Various neuronal cell lineages can be generated through stem cell differentiation to restore impaired functions. The process of differentiation depends on the presence or absence of specific transcription factors, which are cell lineage- dependent. For example, the transition between an intermediate progenitor to a postmitotic glutamatergic neuron has been correlated to the downregulation and subsequent upregulation of (T-Box, Brain) TBR2 and TBR1, respectively. This suggests that both TBR1 and TBR2 are important for the development of the glutamatergic neurons. Although different types of neuronal cells have been generated in vitro, these methods often lead to heterogeneous cell populations due to the generation of unintended/off-target cell types. Potential health risks have been associated with these off-target effects in human studies which impede the advancement of cell-based therapies. Thus, there is a need to more precisely control the lineage commitment of stem cells.

To address this challenge, we created knockout of specific transcription factors in a human embryonic H9-derived neural stem cell (hNSCs) line, to restrict their lineages and reduce the heterogeneity of the cell population. We used CRISPR-Cas9 to restrict the differentiation of NSC lineages by targeting TBR1 and TBR2. Fluorescence-activated cell sorting, used to quantify the neuronal population after differentiation, showed that NSCs lacking TBR1 or TBR2 reduced the percentage of glutamatergic neurons as compared to wild-type control under the same differentiation condition. Thus, this study indicate thatthe absence of specific transcription factors can restrict lineage commitment and thereby improvethe efficacy of cell-based therapy.

HIGH-THROUGHPUT APPLICATION OF METABOLIC FLUX ANALYSIS FOR INVESTIGATION OF MAMMALIAN CELL CULTURE PERFORMANCE

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Key Words: metabolic flux analysis, AMBR15, high-throughput

An important consideration in bringing a new molecule through development is speed to clinic. Increased utilization of high throughput tools, process platforms, and *in silico* modelling can allow for screening of more conditions in a shorter period of time, enabling either a faster development time or increased optimization capability.

Here we discuss the application of a basic metabolic flux analysis model to better understand and manipulate mammalian cell culture performance. With this tool, we aim to improve hypothesis-generation and design experiments more rationally. Studies were performed across multiple CHO cell lines producing various recombinant enzyme products in both 10L benchtop bioreactors and an AMBR15 small-scale system. Based on predictions from the metabolic flux analysis model, certain amino acids were supplemented in the media to modulate growth rate, titer, and metabolic byproducts. Effects on these parameters were then assessed and compared across process scales and cell lines.

This case study demonstrates the utility of high throughput process development tools in conjunction with metabolic modelling for better understanding of existing processes and cell lines. This method of screening provides the comprehensive data sets that are necessary to elucidate effects of amino acid supplementation on mammalian cell culture performance. Additionally, application of *in silico* modelling techniques such as this may lead to fewer required experiments, shortening development timelines.

CHARACTERIZATION OF CATALYTIC *α*-1,3-GLUCANASE ISOZYMES FROM *PAENIBACILLUS GLYCANILYTICUS* FH11 BY USING *BREVIBACILLUS* SYSTEM; ESSENTIAL FOR SUPPRESSION OF *STREPTOCOCCUS MUTANS* BIOFILMS

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Key Words: *Streptococcus mutans*, alpha-1,3-glucanase, catalytic domains, *Bacillus circulans*, *Paenibacillus glycanilyticus*, biofilm.

S. mutans has been implicated in the etiology of dental caries by facilizing the colonization of tooth surfaces and playing a key role in the development of the virulent dental plaque. *α*-1,3-Glucan, which is a key structural constituent of the biofilm matrix (dental plaque), synthesized by glucosyltransferase type B (gtfB) in the presence of ingested sucrose. *α*-1,3-Glucanases also called mutanases, which hydrolyze *α*-1,3-glucan, are classified into two families of glycoside hydrolases, fungal (type 71) and bacterial (type 87). Because of being considered to degrade *α*-1,3-glucan, *α*-1,3-glucanases have been purified and characterized from various microbial sources. However, there are few reports on *S. mutans* biofilm study. For the host cell expression, *Brevibacillus* system is an effective bacterial expression system for secretory proteins. *B. choshinensis* is a gram-positive bacterium and easy to handle non-sporulating bacterium, lacking extracellular protease, that has been already shown to provide a high level of recombinant protein expression. Recently, many proteins are produced from this expression system and use for medical treatment, research study (1). Therefore, in this study we attempted to use *Brevibacillus* expression system to express, purify, and characterize of *α*-1,3-glucanase. In addition, we aimed to investigate the effect of recombinant enzyme on *α*-1,3-glucan biofilm produced by *S. mutans* from the viewpoints of formation and the effect of toothpaste agent on enzyme activity. Two novel catalytic domains of *α*-1,3-glucanase isozyme genes were cloned from *P. glycanilyticus* strain FH11 and heterologously expressed in *Brevibacillus* system. The recombinant isozymes, in termed CatAgl-FH1 and CatAgl-FH2, were purified to homogeneity with specific activity 0.70 U/mg and 0.77 U/mg respectively. The molecular mass of catalytic domain was estimated 62 kDa by SDS-PAGE. Both recombinant enzymes exhibited the different properties. The optimal pH of CatAgl-FH1 and CatAgl-FH2 were 5.5 and 6.0, respectively. The pH stability of CatAgl-FH1 and CatAgl-FH2 were in a range of pH 4.0-11.0 and 4.5-9.0, respectively. The optimal temperature of CatAgl-FH1 and CatAgl-FH2 were 60°C and 55°C, respectively and they were stable until 60°C. Thin Layer chromatography revealed their mode of hydrolysis towards *α*-1,3-glucan was endo-cleavage pattern. The major products of CatAgl-FH1 were di- and trisaccharide but mainly trisaccharide was for CatAgl-FH2. Both enzymes showed high tolerance against high concentration of sodium fluoride. However, each enzyme activity on surfactants were stepped down when sodium dodecyl sulfate and benzethonium concentration were increased.

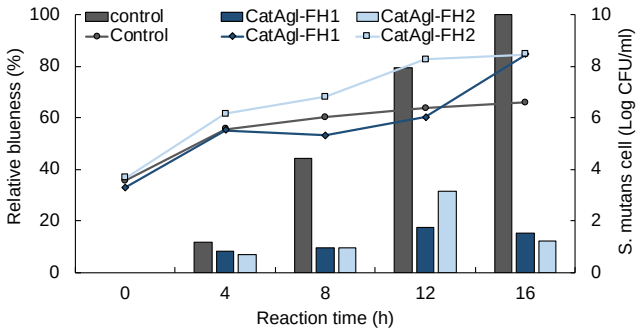

Figure 5 – The formation of S. mutans biofilm adhered to glass plate.

For *S. mutans* biofilms studies, artificial models were developed to verify the possibility of suppressing *α*-1,3-glucan biofilm that adhered to glass plate. Both of recombinant enzymes inhibited biofilm formation via hydrolyzing *α*-1,3-glucan more than 80% compared to untreated biofilm at 16 h reaction time without affecting bacterial growth. Therefore, the recombinant enzymes could be applied in oral hygiene products such as toothpaste, mouthwash and chewing gums for support mechanical cleaning of tooth surfaces.

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IMPLEMENTING DYNAMIC FORMALDEHYDE REGULATION IN ESCHERICHIA COLI FOR SYNTHETIC METHYLOTROPHY

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Key Words: refactoring, dynamic regulation, transcriptional balancing

Genetic engineering of microbes frequently focuses on over-expression of heterologous enzymes to increase product titers and yields. However, recent trends suggest that more balanced approaches are capable of achieving desired cellular outcomes while minimizing the metabolic burden and cost associated with extremely high expression. These approaches include aligning core cell interests with the researcher through precise regulatory changes, and balancing expression levels directly in response to cell needs. Implementing dynamic regulation is particularly appropriate for engineering non-native substrate utilization, due to the complexity of entrenched regulatory networks necessary for existing substrate pathways. Here, we apply a dynamic regulation approach to the utilization of non-native substrate methanol by the model organism *Escherichia coli*. Methanol is an attractive non-food feedstock option due to its high degree of reduction, increasing supply via chemical and bioconversion processes from natural gas, and decreasing cost. As a model organism, *E. coli* has been engineered to produce a wide array of products which could in turn be produced from methanol. Attempts to generate a strain of *E. coli* capable of efficiently utilizing methanol as a substrate have been met with various bottlenecks. Formaldehyde is a cytotoxic compound and the product of the first step of methanol assimilation, catalyzed by methanol dehydrogenase (Mdh). Improper pathway balancing and gene regulation can easily lead to formaldehyde accumulation, limiting the efficient assimilation of methanol in engineered methylotrophic *E. coli* strains. An *E. coli* formaldehyde-inducible promoter was used to drive expression of key methanol assimilation genes in the ribulose monophosphate (RuMP) pathway, emulating native methylotrophic regulation mechanisms and avoiding the need to add costly inducers. Additional regulatory targets were identified with RNA-seq and regulated with formaldehyde. Expressing accessory genes to aid with the regeneration of intermediates and placing key genes under formaldehyde control led to 34% higher average carbon labeling when grown with ^{13}C-labeled methanol, and 10% higher methanol growth benefit. Strains were further improved with adaptive laboratory evolution, and high performing strains were isolated and analyzed. This work was supported by the US DOE ARPA-E agency through contract no. DE-AR0000432.

MODULAR DESIGN OF HETEROLOGOUS PATHWAYS FOR PORTABILITY ACROSS DIVERSE MICROORGANISMS

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Key Words: metabolic architecture, microbial process, technology platform, feedstock engineering

We utilized a method for the high-throughput assembly of heterologous biochemical pathways, and an integration platform for these pathways into the genomes of diverse, non-model microorganisms. Carbon catabolic pathways have been chosen as the first test cases for the pathway design and assembly method. Catabolic pathway optimization has the inherent advantage that growth selections can be used to separate pathways with differing productivities. As such, it provides a tractable testbed for the development of modular engineering approaches. First, we selected pathway enzymes from a group of evolutionarily-divergent host organisms, to capture a wide diversity of possible enzyme structures and pathway configurations. The coding sequences of each pathway have been synthesized and assembled into individual plasmids with unique barcodes, to be combinatorially constructed into pathways using the CombiGEM DNA assembly method [1]. These pathways will be assembled in pooled reactions, resulting in the final generation of ~1M pathways. These pathways will be integrated into the recipient microbial host genome using a phage serine recombinase system [2]. A “Landing Pad” comprised of three attB sites will be integrated into the host genome using the Tn7 transposase [3]. Finally, the designed pathways will be integrated into the recipient genome, through recombination of the Landing Pad attB site, with the respective attP site on the target pathway’s destination vector. Modified hosts will be grown under the selection of the target carbohydrate substrate. The identification and fitness of each pathway will be measured by sequencing the barcodes of the final versus initial pooled populations. In-depth analysis including metabolomics, transcriptomics and proteomics can illuminate regulatory and metabolic changes that result from introduction of the heterologous pathway. Experimental evolution can also be used with poorly-functioning pathways to select for improved variants. Characterizing the resulting mutants can help to identify factors that were initially limiting activity. Comparing successful, unsuccessful, and evolved pathways will help to explain why species differ in their ability to functionally express various heterologous pathways of interest. Ultimately, the final pathway analysis can be used to design modular metabolic units that are highly active and portable across dissimilar microbes. Understanding the requirements for effective use of various heterologous pathways will allow selection of the best pathway for a particular host, based on its unique genetics and physiology.

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A COLD-RESISTANT RUBISCO WITHOUT SMALL SUBUNIT EXHIBITS THE HIGHEST TURNOVER NUMBER TOWARDS CO_2

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Key Words: Rubisco, photosynthesis rate, high turnover number, cold-resistant

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) has long been a primary engineering target to improve photosynthesis efficiency due to its slow catalytic rate towards CO_2. However, the difficulty in finding and engineering a fast Rubisco over the past decades bring about the confusion that whether a constraint exists in Rubisco's catalytic potential. Here, we screened 29 Rubiscos with different forms, which were originated from microbes from different genera in different living environments. A highly active form II Rubisco was found. It showed 4.2- and 2.8-fold increased specific carboxylation activity and turnover number compared with those of Rubisco from *Synechococcus* PCC7002, the known fastest Rubisco in nature. Interestingly, even in ice-cold water (0°C), it was able to fix CO_2 at a rate which was 63% of that of 7002 Rubisco at 37°C. Integration of this highly active Rubisco into *S. elongatus* PCC7942 improved its specific growth rate and photosynthetic rate by 50% and 100%, respectively. Structural analysis revealed that it was a hexamer with three pairs of large subunit homodimers around a central 3-fold symmetry axis. The loop 6 and C terminus were crucial for its high carboxylation activity. The lack of small subunit and no need for any chaperon for its heterologous expression/assembly making its further manipulating in plant and molecular engineering in *E. coli* much easier.

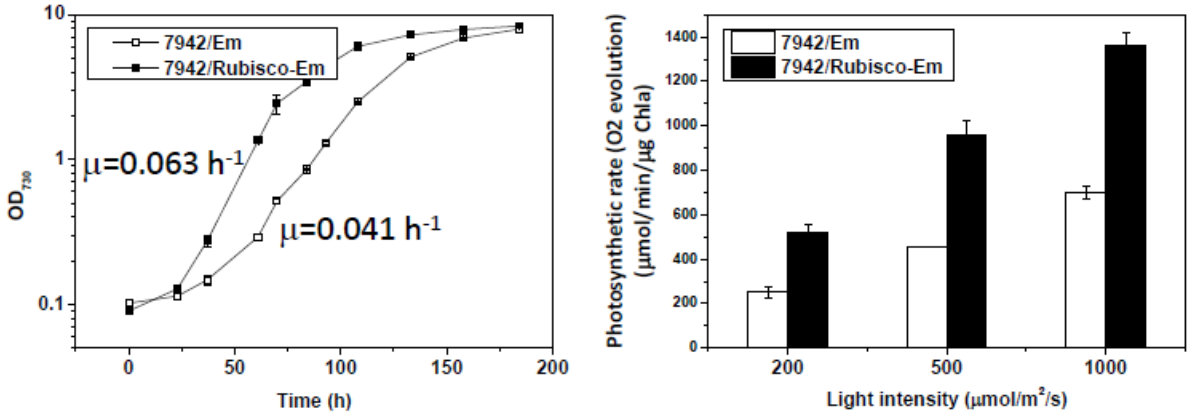

Figure 6 – Integration of this highly active Rubisco significantly improved the growth rate and photosynthetic rate of S. elongatus PCC7942.

IDENTIFYING FUNCTIONAL ROLES OF SNPS USING METABOLIC NETWORKS FOR IMPROVED PLANT BREEDING

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Key Words: *GWAS, Arabidopsis, Poplar, functional SNPs, metabolic engineering*

Genetic sources of phenotypic variation have been a major focus of studies in plants aimed at improving agricultural yield and understanding adaptive processes. Genome-wide association studies (GWAS) aim to identify the genetic background behind a trait by examining the associations between specific phenotypes and single-nucleotide polymorphisms (SNPs). Although such studies are now commonly performed, biological interpretation of the results remains a challenge; especially due to the confounding nature of population structure and the systematic biases it introduces. Here, we propose a complementary analysis referred to as SNPeffect that sifts out functional SNPs from the tens of thousands typically identified during a genome sequencing study by integrating biochemical knowledge encoded in metabolic models, superimposed with phenotypic measurements. By design, SNPeffect can handle both monogenic and polygenic traits while offering mechanistic interpretations of the deciphered genotype-to-phenotype relations. SNPeffect was used to explain phenotypic variations such as differential growth rate and metabolite accumulation in *A. thaliana* and *P. trichocarpa* accessions as the outcome of activating and inactivating SNPs present in the enzyme-coding regions of the genotypes. To this end, we also constructed a non-compartmentalized genome-scale metabolic model for *Populus trichocarpa*, the first for a perennial woody tree. As expected, our results indicate that plant growth is a complex polygenic trait which is primarily governed by carbon and energy partitioning. Growth-affecting SNPs in coding regions were found to primarily be in amino-acid metabolism, glycolysis, TCA cycle, and energy metabolism. Faster-growing *Arabidopsis* genotypes were predicted to have higher fluxes through the protein metabolism pathways, indicating that increase in amino acid levels has a positive growth effect. Faster genotypes were also seen to preferentially employ the energy-efficient purine salvage pathway as opposed to *de novo* purine biosynthesis for generating energy metabolites AMP and GMP. We also found putative causal SNPs to be distributed among genes belonging to glycolysis, pyrimidine metabolism, folate biosynthesis, and shikimate metabolism, which can serve as candidate genes for further experimental characterization and/or targeted plant breeding. For both *Arabidopsis* and poplar, a number of deactivating SNPs were predicted to be in genes belonging to the lignin biosynthetic pathway, indicating that the energetics of producing lignin is a major growth determinant. To further decipher the underlying genetic landscape, we calculated all possible epistatic interactions using flux-balance analysis. Interestingly, we detected a significant positive correlation between the number of negative epistatic interactions in a genotype and its replicative fitness, indicating that functional genetic redundancies are beneficial for growth in *Arabidopsis*. This possibly serves to increase robustness to mutational and/or environmental perturbations as these can then be buffered by shuttling metabolic flux through unaffected parts of the network. We anticipate that putative causal roles for many more SNPs can be gleaned if this analysis is repeated with additional genotypes, phenotypes (such as genotype-specific rates of photosynthetic oxygen evolution or nutrient exchange fluxes) and/or omics datasets (such as proteomics or transcriptomics). Hence, as genome sequencing and plant phenotyping technologies are rapidly decreasing in cost, undertaking large-scale studies that incorporate diverse datasets is also becoming more feasible. As more such data is made available, the need for complex analytical tools will also rise. We envision SNPeffect to pave the way for more tools that can mechanistically elucidate the genetic landscape underlying the wide phenotypic variations that is characteristic of plants.

CHEMICALLY INDUCED DIMERIZATION MODULES AS A PLATFORM FOR PLANT BIOSENSOR ENGINEERING

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Key Words: Protein engineering, biosensors, plant biotechnology

Protein biosensors for small molecules have important applications in agriculture, medicine, and security, but it remains difficult to rapidly produce a high-affinity sensor for a given ligand. This is partly due to two major challenges. First, most small molecule ligands have only a small number of residues with which a protein can make energetically favorable contacts, making it difficult to engineer high-affinity binding. Second, even if a high-affinity binding protein is engineered, it is difficult to transduce the binding event into an output.

The majority of plant hormone perception occurs by chemically induced dimerization, where binding of the hormone to a soluble receptor causes a conformational change that allows the receptor to form a heterodimer with an interaction partner. These CID modules make an ideal platform for engineering small molecule biosensors because they naturally address the two primary challenges above: their unique architecture allows sensitive biosensors to be constructed from low-affinity receptors and protein dimerization provides a natural method of ligand binding transduction. The ability to engineer CID modules would lead directly to *in planta* biosensors and would also have broader applications to biosensor design in other biological systems.

Here we describe the development of a general biosensor engineering platform using the abscisic acid receptor PYR1 of *Arabidopsis thaliana*, which was previously engineered to sense the agrochemical mandipropamid.[1] We combine comprehensive mutagenesis[2,3], high-throughput screening, deep sequencing, and machine learning to rapidly construct a model of the fitness landscape for binding of PYR1 to a specific ligand. We then use this model to design a targeted library to screen for higher affinity sensors. For high-throughput screening, we use both an established yeast two-hybrid (Y2H) screen and a novel yeast surface display (YSD) system. These techniques offer complementary advantages: Y2H is straightforward to implement and requires no purified protein, while YSD offers higher throughput and more stringent quantification of protein-protein interactions. Finally, we describe early development of two additional CID modules from the gibberellin and strigolactone sensing networks of *A. thaliana*.

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OPTIMIZATION OF BENZYLISOQUINOLINE ALKALOID PRODUCTION IN YEAST

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Key Words: benzylisoquinoline alkaloid, plant secondary metabolite, synthetic biology, metabolic engineering, *Saccharomyces cerevisiae*

Benzylisoquinoline alkaloids (BIAs) are a large family of plant secondary metabolites. Several members of the BIA family are commercially available as probiotics and pharmaceuticals. Still more BIAs have been demonstrated to have pharmaceutically relevant properties. Unfortunately, most BIAs do not accumulate in sufficient quantities *in planta* to merit further study. Microbial synthesis of BIAs could open the door to further commercial development of BIAs.

Both *Escherichia coli* and *Saccharomyces cerevisiae* have been developed into microbial platforms for BIA synthesis. Currently, *E. coli* produces significantly higher *de novo* titers of BIAs (160 mg/L in *E. coli* vs. 2 mg/L in yeast). However, yeast remain an attractive target for BIA synthesis, as BIA derivatization often requires endomembrane-associated cytochrome P450s that tend to be more easily expressed in eukaryotic hosts. In this work we demonstrate a general improvement of BIA synthesis in yeast by manipulation of multiple areas of yeast metabolism, including increase of precursor synthesis, reduction of precursor catabolism, and balance of flux through pathway branchpoints. This improvement brings yeast titers in line with *E. coli* titers, establishing yeast as a competitive platform for BIA synthesis.

TOWARDS THE DEVELOPMENT OF A YEAST-BASED OPIOID BIOSENSOR

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Biosensors can be applied as powerful screening tools to quickly and efficiently optimize synthetic metabolic pathways, an essential process for the development of strains with commercially viable yields. In particular, an opioid biosensor is needed to accelerate the development of opioid-producing yeast strains and the search for non-euphoric, analgesic compounds that could replace opioids as pain management tools. We are constructing such a biosensor by linking opioid receptors to the pheromone response pathway of the yeast *S. cerevisiae*. Opioid receptors are plasma membrane localized G protein-coupled receptors (GPCRs), some of which have successfully been linked to the pheromone response pathway through chimeric alpha G protein subunits. Adding complexity, functional opioid receptors require the presence of the animal sterol cholesterol as opposed to the fungal sterol ergosterol. We have successfully engineered a cholesterol-producing yeast with alpha subunit chimeras and optimized GFP-based reporters but have not yet detected activity upon introduction of opioid receptors.

Now, using microscopy-based approaches, we are highlighting proper localization of exogenous receptors as an under-reported road block in yeast biosensor development. By tagging a subset of opioid receptors with green fluorescent protein we demonstrate that while expressed, opioid receptors are restricted to the endoplasmic reticulum (ER) independent of which sterol is present. They are either dispersed throughout the ER or in puncta that colocalize with the early Golgi marker Cop1. In an effort to induce ER-export, we have systematically generated mutations in the human mu-opioid receptor and tested mutations in yeast quality control machinery. Our ongoing efforts highlight novel strategies to properly localize exogenous membrane proteins in yeast for biosensor development.

UTILISATION OF DIELECTRIC SPECTROSCOPY TO MEASURE LIVE BIOMASS AS A PAT TOOL FOR CONTINUOUS MANUFACTURING AND OTHER APPLICATIONS

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Following FDA's PAT initiative, it is imperative that critical process parameters (CPP) that significantly impact critical quality attributes (CQA's) of a process need to be monitored and controlled strictly. It is also well known that viable biomass is one such CPP, which can impact CQA's in a biopharmaceutical process. In addition, for reasons already established, the present drive is to move towards process intensification and continuous biomanufacturing. The need for controlling biomass at a specific high cell concentration at high viability also spells the need for a technology that allows implementation of automatic cell bleeding strategies. Dielectric spectroscopy, often referred to as capacitance measurement, has been used for monitoring biomass in bioprocesses in real time routinely over the past two decades. Based on the ability of cells to get polarised under the influence of an infinitesimal electric field, it is only sensitive to the presence of live cells. This presentation will not only cover the need and benefit of this technology as well as the detailed theory behind it, but it will also cover the various applications it has been successfully used for. Applications surrounding monitoring biomass in suspension, microcarrier and 3D tissue cultures will be presented. Controlling complex nutrient feed and cell concentration automatically based on capacitance measurement will be discussed in detail. In this section, the strategy of automatic cell bleeding in perfusion cultures will be explained in detail. Other applications involving the use of capacitance measurement for identification and optimisation of feed timing, harvest point detection, scale up success and outlier detection will be looked into. Finally the concept of frequency scanning with dielectric spectroscopy and its application and perceived benefits will be explored.

COMPARTMENT-SPECIFIC METABOLOME ANALYSIS REVEALS THE TIGHT LINK BETWEEN IgG1 FORMATION AND NECESSARILY HIGH MITOCHONDRIAL SHUTTLE ACTIVITIES IN CHINESE HAMSTER OVARY CELLS

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Chinese hamster ovary (CHO) cells are the dominating host for the production of pharmaceutical proteins, in particular monoclonal antibodies (mABs). Although production titers improved more than 100 fold during the last 2 decades, similar enhancements of cell specific productivities are less pronounced. They demand for detailed subcellular studies to identify promising metabolic engineering targets. In this context, our study focused on compartment specific metabolome analysis to measure metabolic patterns in the cytosol and in the mitochondrion during cell cultivation. Thereof, in vivo shuttle activities were calculated and correlated with cell specific IgG1 formation rates.

The compartment-specific metabolome and labelling analysis (13C) distinguishes between cytosol and mitochondrion. Metabolomics and instationary 13C metabolic flux analysis build on preliminary own studies of 13C analytics (Teleki et al., *Anal Biochem* 2015; Teleki et al. *Metab Eng* 2017) and compartment-specific metabolomics (Matuszczyk et al., *Biotechnol J* 2015; Pfitzenmaier et al., *Biotechnol J* 2016). Further development and optimization has been performed finally reaching the current status that allows monitoring compartment-specific flux distributions and shuttle activities during the course of cell cultivation.

Studying multiple periods of an IgG1 production process the crucial role of the mitochondrion not only as a provider of ATP but also as an essential part of metabolism was unraveled. 13C flux analysis disclosed the time-variant activities of the mitochondrial shuttles that are tightly linked to mitochondrial and cytosolic metabolism. Clear evidence was found that mAB production strongly depends on sufficient NADPH supply provided by cytosolic malic enzyme activity and malate export from the mitochondrion.

ADVANCED TECHNOLOGIES AND COMPUTATION MODELING IN CONTINUOUS BIOPROCESSING

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Biotech companies have been emphasizing the implementation and utilization of process analytical technology (PAT) and chemometrics in biomanufacturing. Advanced technologies and computation modeling have contributed significantly in making this capability come to life. Although there are still gaps in the area of advanced analytics for the process, computation modeling has started to contribute towards process characterization, which will lead to real-time process control.

PAT and modeling infrastructure can be utilized in integrated continuous bioprocessing to gain better process understating, reduce production cycle time, improve yield, advance efficiency by converting from a batch process into a continuous process, and reduce cost by the reduction of waste and energy consumption resulting from the real-time release of a batch with improved quality. Computational models give a tighter operating range and allow for feedback controls.

An overview of PAT and computation models for biotech processes with emphasis on integrated continuous bioprocessing and production of monoclonal antibodies will be presented.

ENGINEERING CLPS FOR ENHANCED N-TERMINAL AMINO ACID BINDING AND USE IN PEPTIDE SEQUENCING

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Key Words: protein engineering, peptide sequencing, biosensor, N-end Rule Pathway

As different single-molecule protein sequencing technologies emerge, the need for reagents that can selectively recognized and detect amino acids with high affinity has become apparent. Naturally occurring proteins that function through recognition of amino (N)-terminal amino acids (NAAs), such as the N-end rule pathway adaptor protein ClpS can be engineered for enhanced affinity and specificity to meet this requirement. The native ClpS protein has a high specificity albeit modest affinity for the amino acid Phe at the N-terminus but also recognizes other residues at the N-terminal position. We employed directed evolution methods to select for ClpS variants with enhanced affinity and selectivity for NAAs. In addition, we combined these mutations with rationally designed mutations to improve the thermal stability of the protein. The results and their possible implication to peptide sequencing will be presented.

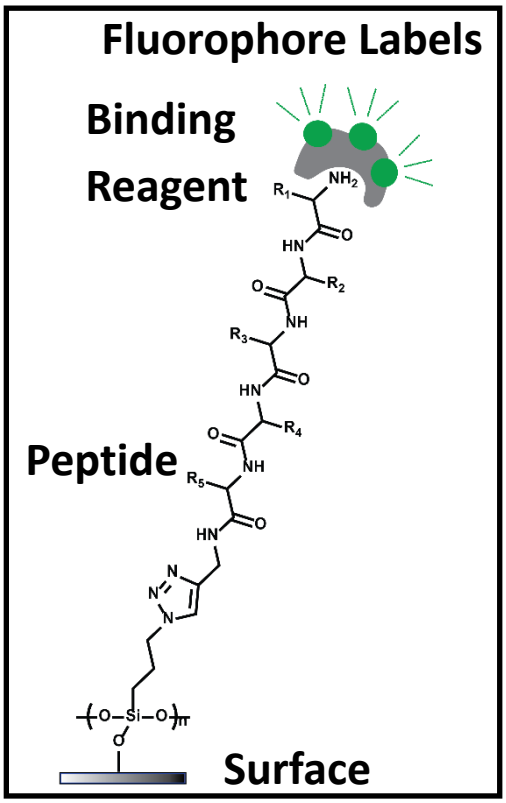

Figure 7 – Example of a binding reagent employed in detection of a surface adhered peptide

WITHDRAWN

POREDESIGNER: A COMPUTATIONAL TOOL FOR THE DESIGN OF MEMBRANE PORES FOR SEPARATIONS

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Key Words: aquaporin, computational protein design, OmpF, water purification, optimization

Monodispersed angstrom-size pores embedded in a suitable matrix are promising for highly selective membrane-based separations. They can provide substantial energy savings in water treatment and small molecule bioseparations. Such membrane proteins (primarily aquaporins) are commonplace in biological membranes but difficult to implement in synthetic industrial membranes due to their modest and non-tunable selectivity. Here we describe PoreDesigner, a computational design workflow for the redesign of the robust beta-barrel Outer Membrane Protein F as a scaffold targeting of any specified pore diameter (spanning 3–10 Å), internal geometry and chemistry. PoreDesigner uses a mixed-integer linear program to optimally place long side -chain hydrophobic amino acids at the pore constriction region that yield a smaller and more hydrophobic pore by maximizing the interaction energy between the pore wall and the permeating water wire. We appended a design assessment step in each iteration by accepting only those designs that fit the user-fed pore dimensions. We first ran PoreDesigner to obtain pore sizes lesser than 4 Å that would exhibit aquaporin-like single file water transport yet maintaining high water permeation rates. 40 accepted OmpF redesigns were obtained and were classified as off-center (OCD), uniform closure (UCD), and cork-screw designs (CSD) dictated by their internal pore architecture. The narrowest pore design from each category was chosen and set in a membrane-patch and an all-atom 200ns molecular dynamics forward-osmosis simulation was performed to corroborate the PoreDesigner-predicted pore sizes. Subsequently, stopped-flow light scattering experiments on these three designs revealed complete salt rejection by the UCD mutant and an order of magnitude higher single-channel water permeabilities than any reported aquaporin till date (for all three designs). Follow-up efforts are being made to tune the membrane-pore interactions for various biomimetic membrane materials, by systematic alteration of the hydrophobicity of the membrane-facing residues without altering their pore size. This would enable easier incorporation of these redesigned proteins in 2D planar membrane sheets and serve as viable filtration assemblies for performing precise angstrom-scale separations. PoreDesigner has been made freely downloadable from http://www.maranasgroup.com/software.htm.

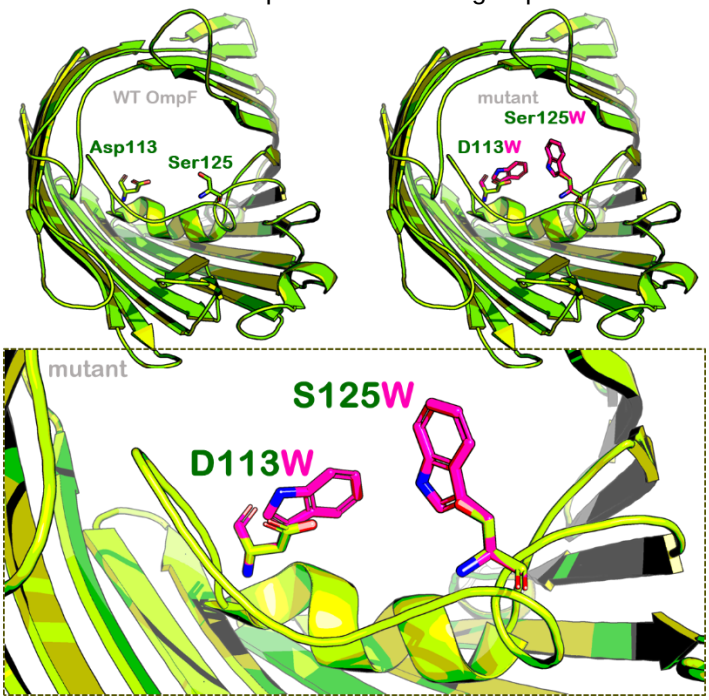

Figure 8. Two mutations from PoreDesigner predicted in silico redesign of OmpF. The D113W and S125W mutations resulted in a smaller pore lumen and a more hydrophobic inner pore wall. The wild type residues have been shown in green and the altered residues have been shown as pink sticks.

FERMENTATIVE OXIDATION OF BUTANE IN BUBBLE COLUMN REACTORS

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Key Words: Oxidation, Butane, Bubble Column, Fermentation, Transport Limitation

To date the use of alkanes as starting materials for selective activation in chemical industry is very challenging. For this task the biocatalytic selective activation offers a number of advantages. The activation starts with C-H functionalization by a sequence of oxidation steps via alcohols, aldehydes/ ketones and carboxylic acids. All these derivatives are bulk-scale products, which are produced with standard chemical methods using high pressures and temperatures. In contrast, microorganisms are able to convert alkanes to various organic compounds at ambient pressure and temperature.[1] For the selective and efficient functionalization of alkanes appropriate fermentation of cells is required. Process engineering is required for a high yielding butane oxidation as well as reactor design. In this context it is essential to investigate the parameters of cell growth and to establish control of the fermentation conditions for production of the hydroxylated target compounds.

At first a suitable reactor set up in accordance to the safety regulations required for handling a flammable gas like butane had to be installed to enable reaction engineering studies of the cell and reactor system. Specialized bubble column reactors are developed on lab scale and characterized in view of the implementation at industrial scale.[2] Additionally, a suitable control system was designed to monitor as well as control standard parameters and to simplify the implementation of further equipment. The mass transfer of the gaseous starting materials into the fermentation media is the key limiting factor for reaching sufficient productivities. The process window is mainly restricted by the requirements of the microorganisms and the flammability region of the substrates.

Process parameters such as gassing rate or butane content are varied to characterize the system and provide understanding of the optimal stoichiometry and resulting volumetric productivities.

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Figure 9: Experimental setup with stirred tank reactor and two 2 liter bubble column reactors for fermentative butane oxidation

HIGH THROUGHPUT INVESTIGATION OF VANILLIN TOXICITY TO YEAST USING AMBR15 MICROBIOREACTORS AND FLOW CYTOMETRY

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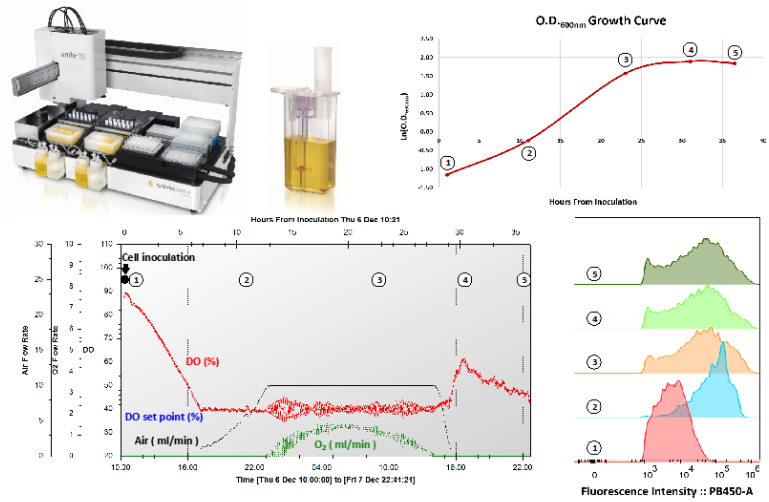
Key Words: cellulosic ethanol; cell division; vanillyl alcohol; fluorescent protein; *Saccharomyces cerevisiae*

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the main flavoring component of the vanilla bean and synthetic vanilla flavoring agent and biotechnological production in metabolically engineered yeast has been attempted. However, vanillin is also one of the most toxic of the aromatic aldehydes to yeast, which became known both through the metabolic engineering attempts and in its effects on yeast during cellulosic ethanol fermentation. Cellulosic sugars are derived from lignocellulosic biomass following pretreatment; this process generates a range of yeast toxicants and inhibitors of which vanillin is among the most potent. Several reports utilizing transcriptomics and gene knockouts have been undertaken in yeast to determine the mechanism(s) of toxicity of vanillin and these have concluded protein production effects or increased radical oxygen species (ROS) and among others.

Here we investigated the mechanism and potency of vanillin toxicity to *Saccharomyces cerevisiae* yeast using a parallel microbioreactor system and by assessing cell physiology impacts via flow cytometry. An initial screen of vanillin (0.5-10 mM) toward *S. cerevisiae* BY4171 indicated potent inhibition of yeast growth at concentrations >2 mM. Control yeast strains (empty vector) or those expressing modified Turquoise Fluorescence Protein (mTFP), were used thereafter. Yeast innocula (OD_{600} ~0.4) were cultured in the Sartorius/TAP Biosystems ambr15 microbioreactor system, 11 ml final volume at 30°C for up to 99 h in the presence of vanillin at concentrations of 2, 4, 6, 8 and 10 mM in batch mode. Microbioreactor dissolved oxygen (DO) and stirring rates were maintained by the control system and automated sampling of the reactors was conducted at specified time intervals. Biomass production (OD_{600nm}) and supernatant vanillin and vanillyl alcohol concentrations were determined. Single cell physiology indicators such as: membrane integrity, ROS generation and mitochondrial membrane potential were measured with the aid of specific fluorescent dyes and TFP levels were measured using a Beckman Coulter Cytoflex flow cytometer.

Yeast incubated with vanillin at all concentrations tested were metabolically active with intact membranes and active mitochondria and were able to produce mTFP at levels similar to yeast in absence of vanillin. In fact, no toxic effects of vanillin to yeast, no elevated ROS and no inhibition of protein production was observed other than an inability of the cells to divide and increase biomass. Once vanillin concentration in the microbioreactor was reduced to ~ 2 mM by the action of yeast converting it to vanillyl alcohol, cell division was reestablished and yeast growth rate (μ_{max}) was similar to that of control yeast except there has been an extended lag phase at high vanillin concentrations.



***Cultivation of Saccharomyces cerevisiae* BY4741 strain expressing mTurquoise2 fluorescent proteins under the stress of 4 mM Vanillin in ambr 15 system**

Oxygen usage increased in yeast incubated with vanillin in a concentration-dependent manner showing vanillin conversion requires additional metabolism. Yeast incubated in the presence of te metabolite vanillyl alcohol (8 mM) showed no change in cell growth or toxicity but consumed additional oxygen, suggesting this product is further metabolized by yeast. The microbioreactor system coupled with flow cytometry are a powerful combination for examining substrates, toxicants and inhibitors of microbes in a high throughput though accurate and reproducible manner and here determining vanillin to be a cell cycle inhibitor of yeast.

USING A MULTI-OMICS SYSTEMS BIOLOGY APPROACH TO ENHANCE CHO PLATFORM PROCESS UNDERSTANDING

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Key Words: systems biology, multi-omics, CHO, transcriptomics, metabolomics

Employing a platform strategy for clone screening and early stage process development can be beneficial not only for shortening process timelines but also for increasing process understanding. By applying the same platform process parameters to different programs with the same host cell line, there are often insights and lessons that can be shared across programs despite differences in the therapeutic protein being produced. This work aims to test and extend this cross-program understanding using a multi-omics systems biology approach, initially incorporating transcriptomics and untargeted metabolomics. By collecting omics samples for the top clones from multiple programs selected in the same platform process, a platform database can be produced to compare the gene expression and metabolite profiles and identify biological pathways that are intrinsically similar, that are clone dependent, and those that are molecule dependent. As a case study, transcriptomic and metabolomic data were collected at multiple time-points from small-scale fed-batch bioreactors for multiple clones producing the same monoclonal antibody. Among these clones, a range of titers, specific productivities, and product quality attributes were observed. By applying the multi-omics approach, there is a potential to identify biological pathways that correlate with unique phenotypes and to then extend these learnings to improve cell culture performance for on-going and future programs.

METABOLOMICS APPROACH FOR INCREASING CHO CELL SPECIFIC PRODUCTIVITY

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Key words: Metabolomics, CHO cell culture

Chinese hamster ovary cells are the most commonly used expression system in the production of monoclonal antibody therapeutic drugs. The biomanufacturing industry has made significant advances in increasing protein titers of these cell cultures by over 100-fold since the 1980s to gram-per-liter ranges, and much of this progress has been made via increasing cell density and viability. However, even next generation processes are approaching the limits of how high cell densities can be reached with available technologies. On the other hand, the specific productivity (qP) of the cell lines, though much higher now than at the advent of biologics production, has not been improved to the same degree, and advances on this front are needed to attain higher titers in shorter times. In this work, a library of twelve cell lines, having a wide range of qPs but all derived from the same parental cell line and expressing one of two different antibodies, was investigated using an untargeted metabolomics approach. Spent medium samples were collected from each fed-batch culture at two time points. BioCAn (Biologically Consistent Annotation), a recently developed automated annotation tool, was used to determine the most likely identities of features detected in LC-MS data from these cell lines. A correlation analysis was then performed to find annotated features that were significantly associated with either cell growth (37 features), qP (32 features), or both (56 features). Interestingly, all features associated with cell growth showed a negative correlation, while all features associated with qP showed a positive correlation. To investigate whether metabolites positively correlated with qP reflect endogenous metabolic activity beneficial for productivity, several metabolites were added to the culture medium at varying concentrations. We found that supplementing the medium with one or more select metabolites could improve qP without negatively impacting cell growth. We next evaluated whether these metabolites could be used as biomarkers to identify clones with potential for high productivity, as current screening methods can falsely eliminate clones due to sub-optimal culture media or process conditions. Together, these studies demonstrate opportunities for using untargeted metabolomics to achieve higher titer in biologics production processes. Further, the identification of biomarkers has potential to shorten cell line development timelines, which is on the critical path to biologics manufacturing.

AN AUTOMATED DATA-DRIVEN PIPELINE FOR IMPROVING HETEROLOGOUS ENZYME EXPRESSION

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A key challenge in cellular biomanufacturing of fuels, chemicals, and pharmaceuticals is that many pathway enzymes have very low activity, limiting overall titers and productivities. One reason is that enzymes are marginally stable under their native conditions, and expression in a different environment can thermodynamically favor the unfolded state. Additionally, overexpression can result in aggregation because natively expressed proteins are close to their solubility limit.

This challenge suggests an engineering solution: engineer pathways enzymes to be stable in their biomanufacturing chassis. However, this is difficult because: (a.) many enzymes do not have high-throughput activity screens needed for directed evolution; (b.) there are few or no structures available; (c.) there are often multiple limiting enzymes; (d.) most mutations confer small benefits to stability; and (e.) the plurality of stability-enhancing mutations decrease catalytic efficiency.

I will present a culmination of my group's approach to solve the above challenges, in effect automating the design of stable, active enzymes from limited combinatorial datasets. This engineering strategy involves user-defined precise mutagenesis[1,2], deep sequencing to evaluate the functional effect of nearly all possible single point mutants on solubility[3], Bayesian methods to discriminate stable, catalytically neutral from deleterious mutations[3], and computational design to combine up to 50 mutations at once[4]. I will show recently published work on application of this method to improve the pathway productivity of a medicinal alkaloid pathway in *Saccharomyces cerevisiae*[5], and end with the description of a computational pipeline to automate our process for any enzyme of interest.

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PROTEINASE K GOES THERMO-LABILE

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Key Words: Proteinase K, Thermolabile, Next Generation Sequencing, NGS workflow, Heat Labile

Proteinase K, originally from the fungus Tritirachium album, is a highly active serine protease with broad cleavage specificity. This enzyme is widely used to remove proteins/enzymes in nucleic acid samples. However, use of wildtype proteinase K (WTPK) in multi-step enzymatic workflows such as next generation sequencing (NGS) is limited due to its extreme thermostability and ineffective removal by heat treatment. The purpose of this study was to engineer a thermolabile Proteinase K (TLPK) as active as WTPK, which may be fully inactivated at 65°C or below to minimize DNA/RNA damage. Using molecular engineering approaches, we have successfully obtained TLPK. As shown in Figure 1, TLPK is almost as active as WTPK at 37°C using native bovine serum albumin (BSA) as substrate. Importantly, TLPK can be efficiently inactivated within the temperature range of 55°C to 65°C, which is demonstrated by loss of protease activity on bovine serum albumin (BSA) substrate (Figure 2a) and a colorimetric peptide substrate (Figure 2b) after heat treatment. Compared to WTPK, TLPK shows over 20°C more labile to heat inactivation. The melting temperature (Tm) of TLPK is also around 25°C lower than that of WTPK, decreasing from 75.9°C to 50.9°C. TLPK greatly outperforms a broad specificity protease isolated from an arctic marine microbial source, both by specific enzyme activity and thermolability. One of the TLPK applications is it can inactivate heat resistant restriction enzymes such as PvuII and PstI without affecting downstream reactions. The mainstream applications may be its incorporation into multi-step enzymatic workflows such as NGS sample preparation. Unlike WTPK, TLPK can be used to eliminate an enzyme function without contaminating the next enzymatic step in the same reaction vessel. New England Biolabs has tested TLPK and found it to simplify and improve NGS workflows.

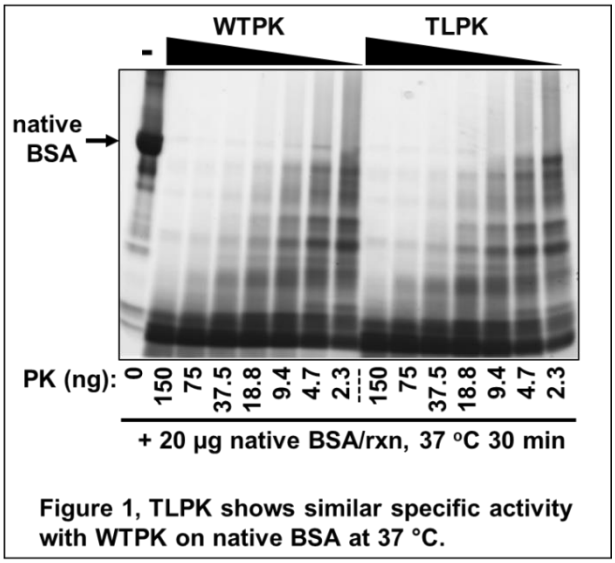

Figure 1, TLPK shows similar specific activity with WTPK on native BSA at 37 °C.

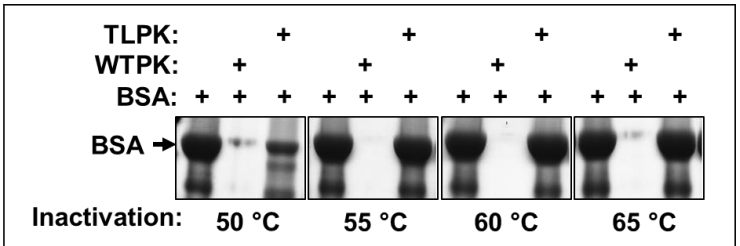

Figure 2a, TLPK is fully inactivated at 55, 60 and 65 °C. BSA was digested by WTPK and TLPK pretreated at 50, 55, 60 and 65 °C for 15 min. SDS-PAGE was used to analyze BSA degradation by proteinase K.

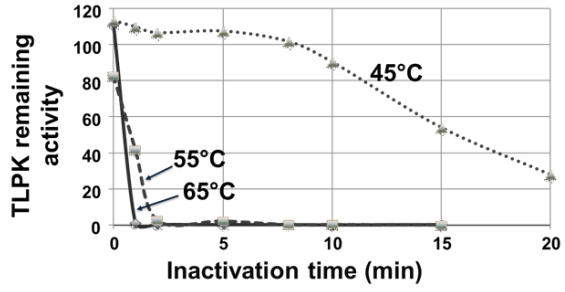

Figure 2b, TLPK is fully inactivated at 55 and 65 °C, assayed using a colorimetric peptide (N-Succinyl-Ala-Ala-Pro-Phe-pNA) as a substrate.

DEVELOPMENT OF SENSITIVE ANTIGEN-DETECTION SYSTEM USING PHOTOACTIVATABLE ANTIBODY FC-BINDING PROTEIN CAPABLE OF INTRODUCING ORIENTED ANTIBODY

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Key Words: Photoactivatable antibody Fc-binding protein, Oriented antibody, Photocrosslinking, Sensitive antigen detection

The photoactivatable antibody Fc-binding protein (PFcBP) allows oriented antibody (Ab) immobilization on various surfaces. Previously, we developed a system for producing the PFcBP containing photo-methionine (pMet) in *E. coli*, which can induce the covalent crosslinking to the Ab-Fc region, by engineering of methionyl-tRNA synthetase and FcBP derived from Protein G (1). In this study, we improved the photo-crosslinking efficiency by multipoint mutagenesis of PFcBP. and optimized the Ab immobilization process. The mutant PFcBP with 7-point substitutions showed the 25-30% enhanced photo-crosslinking efficiency as compared that with 4-point substitutions. The PFcBPs were immobilized onto the solid surfaces using the bifunctional crosslinkers with NHS and maleimide groups, and the Abs were then photo-crosslinked to the PFcBPs upon UV irradiation. The longer spacer arm length of the crosslinker was critical for immobilization of 1xPFcBP with a single Ab-Fc binding domain, but less critical for immobilization of 2xPFcBP with two domains. We also conjugated the PFcBP to the fluorescent beads, and subsequently photo-crosslinked detection Abs upon UV irradiation. Finally, we developed a cassette system capable of introducing capture and detection Abs with orientation onto the PFcBP immobilized chips and fluorescent beads, respectively, and demonstrated the effectiveness of the system in the detection of antigens in sera (Figure 1). We also first prepared the Ab-FcBP conjugates by direct photo-crosslinking of Abs and PFcBPs. After removing free PFcBP by gel filtration, the conjugates were
immobilized onto the maleimide-activated surface. This process allowed more sensitive antigen detection than the sequential Ab-immobilization process.

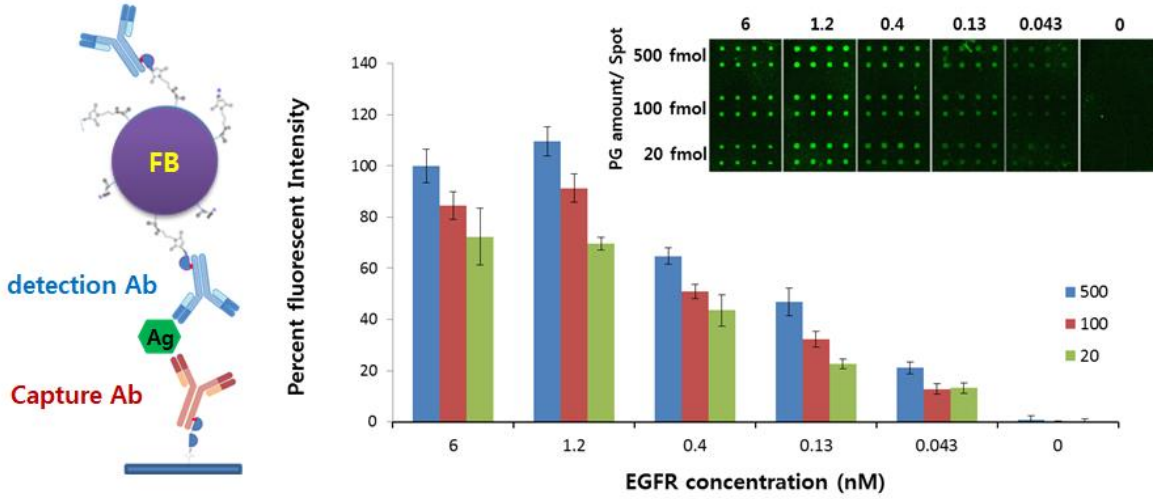

Figure 10 – Antigen detection in sera using the cassette system. The capture antibody and detection antibody were photo-crosslinked to the PFcBP modified array chip and the fluorescent bead (FB), respectively. The antigen (EGFR) diluted in the human sera as indicated was applied onto the capture Ab-chip. The bound EGFR proteins were monitored using the detection-Ab modified FBs.

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FACILE INTERROGATION OF HIGH-ORDER EPISTASIS BETWEEN DISTAL SITES USING NEXT-GENERATION SEQUENCING

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Key Words: G protein-coupled receptors, protein engineering, deep mutational scanning, next-generation sequencing

Deep mutational scanning (DMS) combines next-generation sequencing and protein engineering to construct sequence-function landscapes and rapidly identify fitness optima. Practical use of these landscapes requires identification of all mutations in each protein variant due to the potential effects of epistasis, the interdependence between residues resulting in non-additive phenotypes. This phenomenon plays an important role in protein evolution and is often a necessary step along the path towards protein fitness optima. However, current methods to assign distal mutations to their corresponding gene are work-intensive, costly, and introduce potential sources of error. To overcome these limitations, we introduce a method compatible with DMS that matches distal mutations to their corresponding gene without additional experimental steps. Using this approach to screen ~2,000,000 unique protein variants, we engineer a human G protein-coupled receptor with a 15-fold improvement in ligand binding affinity and observe prevalent epistasis between distal residues within the ligand binding pocket. Compared to variants containing only proximal substitutions, those harboring missense mutations in distal sites demonstrate significantly greater functional activity in our screen. This method can be applied immediately to all experiments using Illumina next-generation sequencing and provides a facile approach to illuminate complex mechanisms underlying key protein functions.

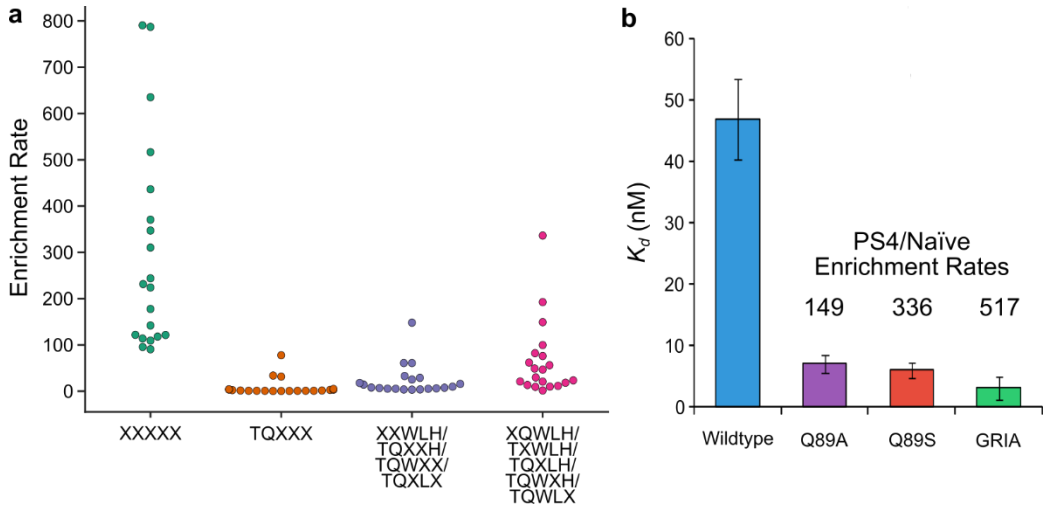

*Figure 11 – (**a**) The top 20 GPCR variants containing at least two distal, mutated residues (XXXXX) exhibit far greater enrichment rates compared to the top 20 variants containing proximal triple (TQXXX), double (e.g. XXWLH), or single (e.g. XQWLH) substitutions. (**b**) Compared to wildtype A_2aR, highly enriched variants, Q89A/S and GRIA, bind [^{3}H]-NECA with ~7 – 15-fold greater affinity (K_d).*

CELLULAR RESPONSES TO CULTURE SUBSTRATES WITH PROGRAMMABLE ANISOTROPY

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Key Words: cell differentiation, cell fate, biomaterial

Physiologically relevant culture substrates are needed to accurately model cell and tissue function *in vitro* to characterize function in both healthy and altered (diseased) states. In addition to their use as model systems, exerting control over cellular function in a biochemical engineering process through cell-substrate interactions may reveal new ways to increase yield or efficiency. While knowledge of cellular responses to elastic substrates has advanced greatly, it was only recently recognized that cellular interactions with viscous components of networks alters mammalian cell spreading, migration, proliferation, and differentiation. Matrix studies have shown varying results in response to stress relaxation timescales however, indicating that multiple factors contribute to the cell's interpretation of its mechanical microenvironment. We hypothesize that there is an additional, critical design parameter that has not been considered: the length scales over which cells sense mechanical properties. This work seeks to investigate these questions using a new type of culture substrate based on cytocompatible liquid crystalline (LC) polymers.

This work focuses on the design, synthesis, and characterization of new biomaterial substrates whose viscoelastic properties can be manipulated by controlling the liquid crystalline (LC) ordering within the material. These materials also have the ability to morph in shape in response to an external stimulus (e.g. light), which may be applied during *in vitro* culture to result in dynamic culture substrates. A unique feature is that order can be programmed from the molecular scale to the macroscale, which permits study of how cells interact with the substrates across different length scales. To enable these studies, liquid crystallinity must be maintained in a hydrated network, which is inherently challenging because swelling of polymers tends to increase the distance between LC molecules to weaken their ordering. This work prepares new LC networks using Click chemistry, which was selected for its efficiency under mild reaction conditions that can be used to incorporate more sensitive biological molecules. This work seeks to combine the dynamic properties of these LC materials with their low cytotoxicity, stability in a hydrated phase, and ability to be processed into scaffolds and gels for use as hydrated and responsive culture substrates. The goals are to first characterize the impact of composition on liquid crystalline ordering and culture substrate properties before quantifying the impacts of substrate anisotropy and mechanics, programmed at different length scales, on mesenchymal stem cell differentiation.
To prepare the materials, alkyne-terminated liquid crystalline monomers (mesogens) and azide-terminated polyether chain extenders (PEO poly(ethylene oxide); PPO poly(propylene oxide)) were synthesized and purified by modifying established reactions. Chain extender molecular weight and composition were varied to afford control over water uptake and LC organization. For one-step LC network synthesis, chemically crosslinked networks were synthesized by polymerizing the mesogens and chain extenders with a tetraazide crosslinker. To enable cell encapsulation, a two-step network synthesis was used, where azide-terminated LC prepolymers were crosslinked in water using multifunctional strained alkyne. Scaffolds were also prepared to enable 3D studies by polymerizing the reactive mixture in the presence of sodium chloride (sieved to 500-600 μm) and extracting the salt once the reaction was complete.
All LC networks were found to organize into the smectic phase. By varying the composition and molecular weight of the chain extender, the material’s elastic modulus and stability of the LC phase was tailored. The networks were found to display reversible shape changing, where the films extended in the LC phase and contracted in the isotropic phase. Composition was found to impact the ability of the network to change shape and the amount of strain generated. Additionally, stress relaxation experiments conducted in the hydrated state showed that networks that were isotropic were found to respond elastically, but LC networks displayed more viscous responses. Mesenchymal stem cells incubated with extractable materials displayed no differences in cellular toxicity compared to tissue culture controls. Cells were found to attach and proliferate on the hydrated LC networks, but attachment was found to be about 50% that of the tissue culture plastic. Adsorption of gelatin with fibronectin onto the networks successfully increased cell attachment. Cell spreading and differentiation (adipogenic vs. osteogenic) studies are ongoing at the time of abstract submission. Ultimately, this work lays the synthetic groundwork for a new synthetic platform for LC biomaterials that can be adapted to include biological molecules as well as investigates LC network utility as a dynamic culture substrate.

UPSTREAM MICROBIAL PROCESS CHARACTERIZATION WITH SINGLE-USE BIOREACTORS FROM 15ML TO 50L

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Key Words: high-throughput, fermentation, single-use, ambr, BIOSTAT STR

Developing biological and industrial molecules derived from microbial fermentation relies upon performant bioreactors to allow a rapid scale up to commercial batches. For this it is relevant to minimize any possible risks while developing a process that fits the industry quality standards. The choice of a well characterized system plays an important role from R&D through to production stages. The aim of this poster is to provide evidence to demonstrate the benefits of a microbial process developed using single-use, high throughput, and scalable upstream solutions. The method chosen to showcase this consistency is based on the DECHEMA Guidelines for Engineering Characterization principles and with the Zurich University of Applied Sciences, ZHAW. DECHEMA guidelines include a set of standard conditions for bioreactor characterization. By using process development and pilot scale bioreactors like the ambr 15f, ambr 250, and BIOSTAT STR 50, it is possible to accelerate development timelines and ensure process success.

EFFICIENT MICROBIAL BIOCONVERSION OF BROWN MACROALGAE OBTAINED THROUGH PROFITABLE HIGH-DENSITY SEA CULTIVATION USING MODIFIED MICROBIAL STRAINS TO PRODUCE COMMODITY AND SPECIALTY CHEMICALS: A DEVELOPING BLUE CHEMICAL INDUSTRY IN CHILE

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Key Words: profitable microbial conversion, brown macroalgae carbohydrates, metabolic engineering, dynamic metabolic models, metabolic potentiality maps.

Plant biomass is considered a promising feedstock for large scale sustainable bio-based green chemistry. However, only the use of agricultural or forestry residues is viable, since they do not compete for land with feed crops and have competitive costs. Moreover, carbohydrate recovery from these sources is always difficult due to their high lignin content. Alternatively, macroalgae are competitive sources of carbohydrate-rich biomass not requiring land or fresh water for its production. *Macrocystis pyrifera* is one of the fastest-growing macroalgal species with high CO_2 fixation efficiency, highly-abundant and accessible carbohydrates. We demonstrated that it can be cultured in temperate seas, yielding 124 ton/Ha/yr, and can be economically profitable at a 10-hectare scale [1,2]. Microbial and enzymatic algal biomass bioprocessing has been also undertaken by our group. We demonstrated the technical feasibility of producing ethanol at a pilot industrial scale by fermenting algal carbohydrates with a genetically modified *Escherichia coli* [3]. However, ethanol production, even with high productivities, was not commercially viable. To make algal biomass bioconversion profitable, we performed a large metabolic engineering and synthetic biology project to discover combinations of metabolic pathways, regulation, carbohydrate sources –algal or not– and alternative bioproducts that maximize microbial efficiency and commercial viability. Using a genome-scale reconstruction of *Saccharomyces cerevisiae*'s metabolism, we demonstrated that redox ratio constraints and the preferential use of NADH or NADPH for alginate metabolism were key for *S. cerevisiae* conversion of alginate:mannitol carbohydrate sources [4]. However, yeast use makes chemical processes technically and economically unfeasible for low value products due to their inability to produce extracellular enzymes for alginate lysis. By means of dynamic metabolic models developed for *E. coli*, we demonstrated that the main metabolic process bottleneck is microbial carbohydrate metabolization and that algal carbohydrate composition is a key determinant of fermentation efficiency. Using a multi-objective optimization strategy focused on microorganism growth, energy levels and redox ratio conservation, we also showed that ethanol production from algal biomass is incompatible with *E. coli*'s metabolism, due to low energetic and redox efficiencies obtained from alginate using host microorganism metabolic pathways. We then used high-performance parallel computing to develop a metabolic potentiality map for *E. coli* in which we explored more than 10.000 possible combinations of metabolic pathways that could be built in our strain to convert brown macroalgae carbohydrates with high efficiency, considering the best combinations of knock-outs and overexpressions to be introduced in *E. coli*'s central metabolic pathways. With this technique, we identified other valuable chemicals, such as succinic, aspartic, gluconic and levulinic acids, and complex aromatic and aliphatic biomolecules can be efficiently produced from *Macrocystis* with specifically modified strains for each product. The bulk of our research fostering algal feedstock production and industrial bioconversion in Chile will be presented in this work.

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TEMPERATURE REGULATION AS A TOOL FOR ENABLING AND PROGRAMMING SYNTHETIC MICROBIAL COMMUNITIES

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Key Words: microbial communities, ecology, temperature, community composition

As target applications grow more elaborate, researchers are developing new approaches to program increasingly complex functionality into synthetic biology platforms. One emerging approach is engineering cooperative, multi-species synthetic microbial communities, which offer significant potential advantages compared to single species systems for numerous applications such as biosynthesis of target compounds through complex pathways enabled by division of labor. However, population dynamics, inter-species interactions, and differing ecological niches of resident microorganisms also introduce complexities that must be addressed to achieve effective and robust synthetic microbial communities. One fundamental challenge is regulation of community composition. At the most basic level, maintaining coexistence of resident community members is required to enable the desired community level functionality. Additionally, community composition often needs to be tuned to optimize overall functionality. For example, when a complex pathway is divided into multiple components hosted by different community members, fluxes through different enzymatic reactions can be coordinated through modulation of each sub-population size to maximize overall efficiency. This type of microbial community manipulation has not been fully utilized in synthetic biology applications, likely due in part to limited available tools. Here we develop temperature regulation as a general tool to enable coexistence and control community composition in synthetic microbial communities. We demonstrate that rationally selected constant temperature regimes can be used to enable coexistence of species from distinct thermal niches. Furthermore, cycling temperature regimes can be used to regulate relative species abundance in microbial communities. We employ mathematical modeling to design cycling temperature regimes for desired community compositions and related features. As microbial communities are increasingly used in a variety of applications, we envision that tools for modulating community composition will continue to expand and we see temperature regulation as a powerful new approach in this area.

ACCELERATING THROUGHPUT OF ANALYTICS WITH ONE-CLICK DATA ANALYSIS

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Key Words: Microscale, Automation, High-Throughput, Process Analytics, Apps

The downstream process development for antibody-based biopharmaceuticals has been applying a platform approach based around Protein A affinity chromatography for many years. Process development can be performed at laboratory scale, but with the adoption of microscale techniques and automation, process development throughput can be enhanced. Being able to do more with less material enables the downstream process development to start with small scale bioreactor-derived feedstreams and can therefore start earlier in the development timeline.

For example, 96-well microplates allow multiple binding and elution conditions to be evaluated for a mixed-mode chromatography step in a less than 2% of the time it would take to screen these same conditions, with a similar saving in feed material requirements. The benefit of being able to perform many chromatography experiments is however bottlenecked by the ability to obtain meaningful analytical data from the large number of samples taken and the analytical method used. The Host Cell Protein ELISA assay has a limited throughput and requires two software packages for analysis. A 93% increase in efficiency was achieved by using a MATLAB™ app to automate this analysis.

The further optimization and process understanding performed as the clinical development progresses can also exploit the benefits of the automated microscale techniques. Moving from 96-well plates to 600µL microscale columns allows the chromatography experiments to be transformed into a format that provides a more representative separation to that achieved at more conventional laboratory scales. However, to obtain useful comparative information that translates to larger scale requires the UV absorbance data to be converted into a chromatogram. Although scientifically straight forward, this process requires the transfer of large amounts of data into a plot-able format. Manual copying of data across formats is not ideal, but apps created in MATLAB™ that can be deployed on any PC are able to rapidly transfer the data from defined analytical formats to generate recognisable chromatograms.

The use of Design of Experiment methodologies should work well with the automated parallel operations of the microscale format, however, converting the experimental design into a suitable automation script to perform the different experiments can be challenging. Using apps for this purpose allows processes to be more consistent and provides a saving in time and reducing the number of repeats due to automation errors.

APPLYING METABOLIC MODELS FOR CONTROL IN ORDER TO ENHANCE ALGAL GROWTH AND LIPID PRODUCTION

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Key Words: *C. vulgaris*, Metabolic models, Control, Fatty acid methyl ester (FAME)

A number of metabolic models have been developed in different algae species in the past five years. In this study, a metabolic model of *C. vulgaris* was applied for controlling algal growth and lipid production. This method optimized nutrient supply by characterizing algal metabolic pathways under different conditions. The approach was validated for autotrophic growth under nitrogen replete condition, in which nitrate requirement was lowered while retaining robust algal growth. Furthermore, this approach was also applied for nitrogen limited environments. Previous studies have found *C. vulgaris* can accumulate high lipid content in biomass, which can be hydrotreated to biodiesel, while decreasing algal growth under nitrogen limitation [1]. Our method optimized the nitrate supply to sustain algal growth while still producing fatty acids efficiently under nitrogen limited conditions. Compared with *C. vulgaris* cultures with complete nitrogen withdrawal, fatty acid methyl esters (FAME) increased substantially. This metabolic model-based approach will have applicability for optimizing nutrient inputs and biomanufacturing across a wide spectrum of organisms ranging from prokaryotes to eukaryotes used to produce a variety of biotechnology products in coming decades.

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GENOME SCALE MODEL RECONSTRUCTION OF THE METHYLOTROPHIC YEAST *OGATAEA POLYMORPHA*

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Key words: *Ogataea (Hansenula) polymorpha,* metabolic model, phenotype microarray experiments, methylotrophic yeast

Ogataea polymorpha is a thermotolerant, methylotrophic yeast with significant industrial applications. It is a promising host to generate platform chemicals from methanol, derived e.g. from carbon capture and utilization streams. Full development of the organism into a production strain requires additional strain design, supported by metabolic modeling on the basis of a genome-scale metabolic model. However, to date, no genome-scale metabolic model is available for *O. polymorpha*. To overcome this limitation, we used a published reconstruction of the closely related yeast *Pichia pastoris* as reference and corrected reactions based on KEGG annotations. Additionally, we conducted phenotype microarray experiments to test *O. polymorpha*'s metabolic capabilities to grown on or respire 192 different carbon sources. Over three-quarter of the substrate usage was correctly reproduced by the model. However, *O. polymorpha* failed to metabolize eight substrates and gained 38 new substrates compared to the *P. pastoris* reference model. To enable the usage of these compounds, metabolic pathways were inferred from literature and database searches and potential enzymes and genes assigned by conducting BLAST searches.

To facilitate strain engineering and identify beneficial mutants, gene-protein-reaction relationships need to be included in the model. Again, we used the *P. pastoris* model as reference to extend the *O. polymorpha* model with this relevant information.

The final metabolic model of *O. polymorpha* supports the engineering of synthetic metabolic capabilities and enabling the optimization of production processes, thereby supporting a sustainable future methanol economy.

ENHANCED SYMBOLIC REGRESSION TO INFER BIOCHEMICAL NETWORK MODELS

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Key words: symbolic regression, genetic programming, biological networks, systems biology

Biological systems can be represented as complex networks illustrating the relationships and connections among biochemical species. Complex networks can uncover vital information regarding specific pathways or network bottlenecks, helping to reveal novel discoveries relevant to a variety of applications. Biological networks, however, are often highly interconnected and non-linear in nature making development of a comprehensive model challenging. Large amounts of data can be acquired to elucidate specific pathways, but deducing the entire network topology requires more rigorous computational techniques. There are *in silico* techniques, including evolutionary algorithms, to predict network topologies using information from experimental data. Biological networks can be decomposed into a system of differential equations under mass action kinetics assumptions describing the rate of change of the various biochemical species in the network. Symbolic regression can be used to generate a system of equations from acquired data.

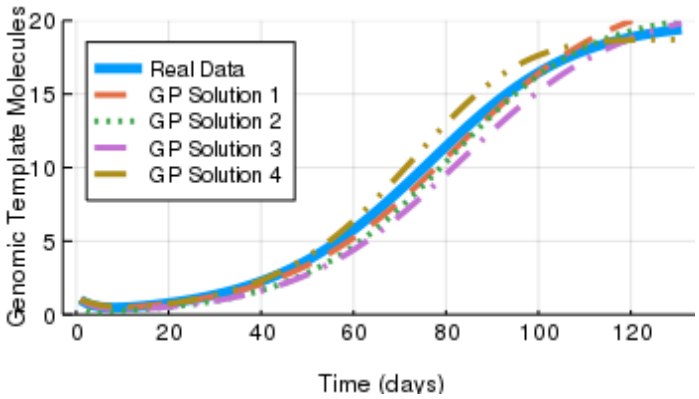

Figure 12: The best solutions generated by the genetic program for viral DNA replication dynamics show good agreement with the data generated by the original model.

In this work, we employed *genetic programming*, a stochastic optimization method, to generate an ensemble of symbolically regressed equations describing intracellular viral kinetics. Due to the highly inter-connected and nonlinear nature of these systems, it can often be computationally infeasible to find a solution for an unconstrained system. To address these hurdles, the complexity of the differential equations and the search space for the kinetic parameters were constrained. First, we assumed that the kinetic equations regressed could only be zero, first, or second order. Higher order equations are rarely observed in nature and therefore excluded to narrow the pool of potential reaction combinations. To limit the search space for rate constants, data acquired from the system were used to make an assisted stochastic guess. From the data, in addition to the actual value for each species at each time point, it was possible to determine an approximate derivative value at each time point. It was then possible to backcalculate a range of potential rate constants and randomly choose one from that range. An initial guess selected from the ranges generated were used to estimate the parameters using simulated annealing for the entire differential equation system.

Instead of only considering the best solution from each simulation, information about the system from an ensemble of models was gathered. Once the genetic program completed, stability analysis was employed to extract only stable and practical solutions from the set of best models. As shown in Figure 1, results of the best models generated via genetic programming bounded the results of the original model. By evaluating the ensemble of equations, it was possible to look for terms that appeared in the majority of these equations. The more frequently terms appeared, the more confident we were that the relationship was part of the real network. Our simulations were run for 100 generations and were completed on a standard desktop computer in less than a day using simulated annealing for parameter estimation. Other parameter estimation algorithms tested took significantly longer, including the Nelder-Mead algorithm which took approximately seven times as long on the same computer. In the future, we plan to extend this algorithm to more complicated systems including multi-omic networks where extensive data can be collected to discover or corroborate different multi-omic mechanisms.

SLOW GROWTH RATE TRIGGERED TRANSITION TO A PSEUDOHYPHAL LIFESTYLE OF THE PROTEIN PRODUCTION HOST PICHIA PASTORIS

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Key Words: *Pichia pastoris*, epigenetics, pseudohyphal growth, specific growth rate

Specific growth rate is an important process control parameter for industrial protein production. In the widely used yeast protein production host *Pichia pastoris*, growth rate is known to significantly impact protein expression and secretion [1]. In that regard, glucose-limited chemostat cultivations carried out over a wide range of specific growth rates have revealed that slow growth rates can trigger a pseudohyphal phenotype in *P. pastoris* [2]. Such phenotypes are undesirable during large-scale protein production processes since they can lead to foam production. In *Saccharomyces cerevisiae* pseudohyphal growth is controlled by *FLO11*, a member of the *FLO* gene family, which is a group of genes encoding cell surface proteins responsible for conferring a diverse array of adhesion-related phenotypes and reported to be controlled by epigenetic mechanisms. *P. pastoris* also carries a number of *FLO* genes but their functions and regulatory patterns are yet unknown. Thus, we set out to investigate this gene family to shed some light on how pseudohyphal growth and other adhesion phenotypes are triggered and regulated in *P. pastoris*.

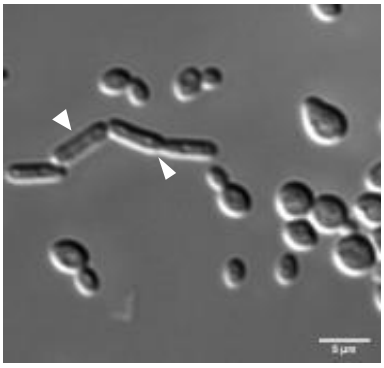

Figure 1- P. pastoris cells displaying pseudohyphal phenotype

We ran glucose-limited chemostat cultivation, initiated first at a specific growth rate (μ) of 0.1 h^{-1}, then switched to a slower growth rate of 0.05 h^{-1} and finally switched back to the faster growth rate of 0.1 h^{-1}. We observed that after two residence times in the slow growth rate condition, some cells in the population took a more elongated form. With increasing residence times in this condition, the phenotype grew stronger, with some cells taking up a branched pseudohyphal appearance, as seen in figure 1. Interestingly, after switching back to the faster growth rate, some pseudohyphal cells persisted, indicating that this phenotype might be under stable epigenetic regulation. Preventing pseudohyphae formation by knocking out the master transcription regulator of many members of the *FLO* family decreased foaming and additionally showed better performance for protein production in fed-batch cultivations.

RT-PCR and RNASeq helped us to identify three *FLO* genes, including *FLO11*, which showed an interesting pattern of regulation at the slow growth rate. We further carried out FAIRE-Seq to analyze open chromatin regions under the different growth rates and two of the *FLO* genes identified before showed stable silencing upon switching to the slow growth rate. Similar chemostat cultivations with knock-outs of these three genes revealed that unlike in *S. cerevisiae*, pseudohyphal growth in *P. pastoris* requires the involvement of not just *FLO11* but also the two other identified *FLO* genes. Investigations using reporter strains expressing green fluorescent protein tagged to these two *FLO* proteins have led us to speculate that these could be acting as signal proteins whose expression might be necessary for expression of *FLO11*, which then triggers the initiation of pseudohyphal growth. Thus our data and observations point to a more complex regulation mechanism of the pseudohyphal phenotype in *P. pastoris* compared to *S. cerevisiae*.

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ENGINEERING A BLUE LIGHT INDUCIBLE SPYCATCHER SYSTEM (BLISS) AS A TOOL FOR PROTEIN PHOTOPATTERNING AND OPTOGENETICS

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Key Words: bioconjugation, optogenetics

The SpyTag-SpyCatcher protein conjugation system has recently exploded in popularity due to its fast kinetics and high yield under biologically favorable conditions in both *in vitro* and intracellular settings. We imagine we can further expand the utility of this system by introducing the ability to spatially and temporally control the conjugation event. Taking inspiration from photoreceptor proteins in nature, we designed a method to integrate light dependency into the protein conjugation reaction. The light-oxygen-voltage 2 domain of *Avena sativa* (AsLOV2) undergoes a dramatic conformational change in response to blue light. We have thus genetically fused the SpyTag into the AsLOV2 domain to create a Blue Light Inducible SpyCatcher System (BLISS). In this design (Figure 1), the SpyTag is blocked from reacting with the SpyCatcher in the dark, but upon irradiation with blue light, the Jα-helix of the AsLOV2 undocks to expose the SpyTag. We screened several likely insertion points in the Jα-helix, and found a variant with desirable light switching behavior where after one hour of irradiation, the reaction is 80% complete, while only 10% of the AsLOV2-SpyTag protein reacted in the dark. This reaction can be quenched within minutes by returning the reaction to the dark. We demonstrated the spatial aspect of this light control mechanism through photopatterning proteins onto Ni-NTA coated slides. As our system is made solely from protein components, which can be genetically encoded, we can extend the same spatiotemporal control of proteins inside cells. We anticipate BLISS will be a strong tool for fabricating protein microassays, crafting biomaterial composition, as well as optically controlling enzyme activity and protein localization in cells.

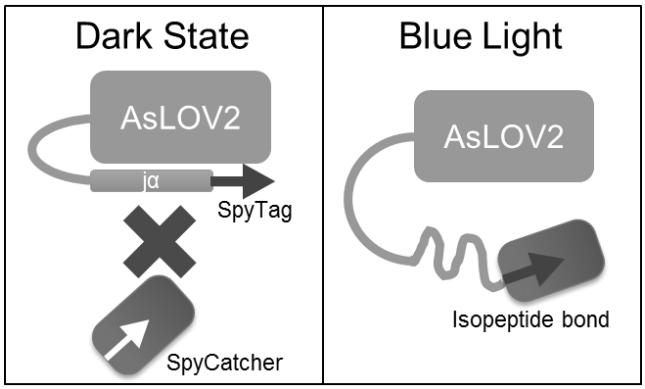

Figure 13 – BLISS design for photoactive protein conjugation

EPIGENETIC MODIFICATION OF NEURAL GENES BY THE NEURON RESTRICTIVE SILENCER FACTOR

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Key Words: NRSF, methylation, acetylation, master transcriptional regulator.

The Neuron Restrictive Silencer Factor (NRSF) is the master transcriptional repressor of the neural phenotype and has been shown to regulate hundreds of neural genes. It is becoming very clear that NRSF maintains repression of gene expression by recruiting chromatin modifiers to target gene regions. Previous work of ours showed that the small molecules, forskolin and isobutylmethylxanthine (IBMX), could induce neural-like differentiation in mesenchymal stem cells by causing downregulation of NRSF and de-repression of neural gene expression[1]. We next set out to determine if there were epigenetic changes in the promoter regions of NRSF target genes. In our work, we look at changes in the methylation of DNA in the promoter regions through bisulfite conversion and sequencing, as well as the acetylation status of nearby histones through ChIP. NRSF is also dysregulated in several neurological diseases. In particular, repression of certain ion channels involved in the electrophysiological properties of neurons may underlie conditions such as neuropathic pain and epilepsy. Work has shown that in the disease state the genes for these ion channels show repressive epigenetic marks[2]. Using dCas9, we are able to bring chromatin modifiers to specific regions of the genome. Here, we use a dCas9-Tet1 fusion to demethylate NRSF regulated genes. As a proof of principle, we show that by reversing repressive epigenetic marks on genes that contribute to neurological disease, that the epigenetic activity of NRSF itself could be a therapeutic dimension.

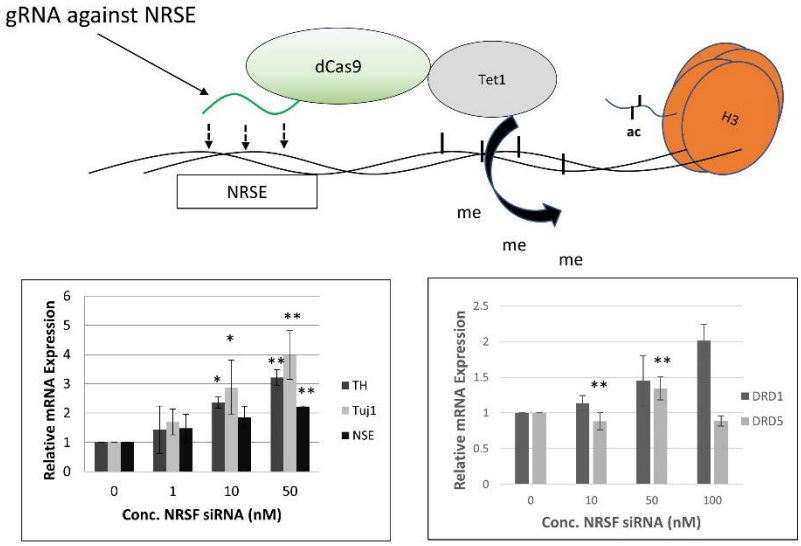

Figure 14 – Downregulation of NRSF causes de-repression of several neural genes. dCas9 fused to chromatin modifiers could reverse repressive epigenetic marks and guided to NRSF controlled genes.

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OPTICAL CONTROL OF EXOPOLYSACCHARIDE PRODUCTION IN *SINORHIZOBIUM MELILOTI* FOR STUDYING BIOFILM FORMATION AND WATER RETENTION

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Key Words: rhizosphere, soil, exopolysaccharide, optogenetics, synthetic biology

The rhizosphere contains many types of microbes interacting with plant roots, creating a complex symbiotic system. Microbial processes occurring in the rhizosphere are essential to the productivity of terrestrial ecosystems. In particular, exopolysaccharide produced by soil microbes allows dynamic regulation of soil moisture by modulating water transport. We have demonstrated that purified microbial exopolysaccharide (EPS) impacts soil water retention through enhancing the variability of water distributions in the soil microstructure. However, the impact of EPS on water transport in soil is not understood due to complex interaction of microbial EPS with soil microstructure and particle surface properties. To decipher the causal role of EPS in soil microstructures, we set out to develop engineered soil bacteria with spatially regulated EPS biosynthesis capabilities. Here we report genetic engineering of soil bacterium *Sinorhizobium meliloti* to enable *in situ* spatial control of EPS production. We show that the photo-sensitive transcription factor EL222, derived from *Erythrobacter litoralis*, allows robust control of gene expression in *S. meliloti*. Essential genes in the type II EPS (a major component of EPS from *S. meliloti* in the soil) production pathway were identified, and deletion strains were generated. Complementation of the essential gene using a synthetic promoter controlled by EL222 led to robust light-activated production of EPS. Optimization of the engineered genetic construct was performed by varying promoters, ribosome binding sites, and using alternative start codons. Using the engineered EPS production strain, we observed rapid settlement of EPS producing *S. meliloti* in liquid culture, and selective biofilm formation quantified by a crystal violet staining assay. This approach enables spatially regulated EPS production and biofilm formation. We will demonstrate control of gene expression in a synthetic soil microsystem that emulates aggregated sandy loam soil. We will also report our current progress on using these new strains of soil bacteria to study the impact of EPS production on water drying rate in the synthetic soil microsystem. We anticipate that the engineered genetic constructs will be broadly applicable for dissecting gene function in a defined population of microbes in the rhizosphere.

RAPID ASSAYS AND CONTINUOUS IN-SITU BIOSENSORS FOR BIOPROCESS MONITORING

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Key Words: Biosensor, Bioprocess Monitoring, Glucose

Fast, accurate, and precise measurements are critical in the biofuels, biochemical, biopharma, and biotechnology industries. Bioprocessing operations require rapid measurement techniques during cultivations to ensure product quality, ensure batch-to-batch consistency, increase product yield, improve process efficiency, and prevent expensive shutdowns. Although continuous sensors exist for dissolved oxygen, pressure, temperature, and pH, organic chemical concentrations must be measured using expensive equipment that can take hours or days to yield results. OptiEnz Sensors provides a biosensor platform for rapid assay or continuous monitoring of organic chemicals. The platform consists of an instrument with an attached sensor probe and replaceable sensor caps with multiple sensor spots. The sensor tips are unique to each chemical to be measured. The platform includes PC-based software for product configuration, display of results in both digital and graphical formats, and data export to process control systems. This technology is available in two platforms: A rapid assay system capable of quickly measuring the analyte concentration in samples and an in-situ probe for continuous, aseptic measurements in a bioreactor. The sensors are accurate, specific, and quantitative. OptiEnz has identified over 50 organic chemicals that can be measured using this technology and has constructed and tested biosensors for 22 of them. Of particular importance to biotech process monitoring are sensors for glucose, ethanol, lactate, glutamate, glutamine, xylose, lactose, glycerol, butanol, and methanol. The rapid assay system is on the market now for glucose and ethanol concentration measurement and a continuous sensor for in-situ glucose concentration monitoring is in customer trials.

DESIGNER BIOSENSORS FOR ENGINEERED METABOLIC PATHWAY OPTIMIZATION

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Key Words: Biosensors, Transcription Factors, Semi-Rational Engineering, Adipic Acid

Synthetic biology techniques aimed at constructing artificial metabolic pathways in genetically modified microorganisms are emerging as important, sustainable methods to produce biofuels, pharmaceuticals and value-added chemicals. To reach industrially relevant scales, challenges related to pathway bottlenecks and system optimization must be addressed. Since these pathways are usually built of multiple enzymes, improving pathway efficiency by processes such as enzyme directed evolution offers a solution to these limitations. However, screening methods for the majority of products of these enzymatic pathways are laborious and inefficient. The purpose of this work is to utilize transcriptional repressor-based biosensors, predominantly from the TetR family, to develop fast and high-throughput detection methods of artificial metabolic products.

Transcriptional repressors bind specific effectors or effector families, which limits their usability as biosensors in many engineered pathways. This project aims at expanding the toolbox of repressors available by engineering their effector-binding domains to respond to alternative effector molecules. As a proof of principle, using a semi-rational approach, we will engineer repressors to respond to intermediates of an engineered metabolic pathway to adipic acid that has been derived from the shikimate pathway. Adipic acid is a precursor of nylon and plastics and is currently produced unsustainably from petrochemicals, with worldwide annual demands of over 2 million tonnes. Our “designer” biosensors will be utilized to improve yields of an adipic acid-producing yeast strain. Particularly, they will be used as genetic circuits within engineered strains for pathway dynamic control, which is a method for *in vivo* real-time control of gene expression. As well, these biosensors will be used as screening tools for the directed evolution of pathway enzymes.

IMPROVING 1,3-BUTANEDIOL PRODUCTION IN *E. COLI* USING A PROTEIN ENGINEERING APPROACH

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Key Words: deoxyribose-phosphate aldolase, 1,3-butanediol, crystal structure, protein engineering, biotechnology.

Traditional chemical production processes have high yields but require harsh reaction conditions and use non-renewable feedstocks derived from petroleum [1, 2]. These processes have a negative impact on the environment, which motivates the development of more sustainable processes as replacements [2]. Advances in systems metabolic engineering over the past thirty years have given rise to bioprocesses where engineered microbes make chemicals from natural feedstocks under mild reaction conditions [1]. The promise of the field has also resulted in financial resources being made available to the development and commercialization of bioprocess. According to a recent report by Ontario Genomics [3], global investment in the field is projected to be at $38.7B in 2020, a 12-fold increase from what it was at in 2013.

Recently, a novel aldolase-based pathway for producing 1,3-butanediol (BDO) in *E. coli* was reported by Nemr et. al [4, 5]. 1,3-BDO is a commercially viable product as it is used in formulations in cosmetics products, and as a precursor for pharmaceuticals [2]. This pathway involves the conversion of pyruvate to acetaldehyde via the EutE enzyme from *E. coli*, followed by the conversion of acetaldehyde to 3- hydroxybutanal via the enzyme BH1352 – a Deoxyribose-phosphate aldolase (DERA) – from *Bacillus halodurans* and subsequently by the conversion of 3-hydroxybutanal to 1,3-BDO via the enzyme PA1127 (an aldo-keto reductase) from *Pseudomonas aeruginosa* [5].

We examined the crystal structure of BH1352, which revealed key residues involved in catalytic activity in the substrate binding pocket. We show that two DERA mutants F160Y and F160Y/M173I improve the production of 1,3-BDO 5-fold and 6-fold respectively in bench-scale bioreactors [6].

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ACCESS TO *N*-ALKYLATED AMINO ACIDS BY MICROBIAL FERMENTATION

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Key Words: *Corynebacterium glutamicum*, *N*-methylated amino acids, Imine reductase, Alternative feedstocks, Site-directed mutagenesis

N-methylated amino acids are found in many pharmaceutically active compounds and have been shown to improve pharmacokinetic properties as constituents of peptide drugs since *N*-methylation of amino acids may result in conformational changes, improved proteolytic stability and higher lipophilicity of the peptide drug.[1] *N*-methylated amino acids are mainly produced chemically or by biocatalysis, however with low yields or high costs for co-factor regeneration.

First, we established a fermentative route for production of *N*-mehtyl-L-glutamate by *Pseudomonas putida* from glucose and glycerol. Interception of the C1 assimilation pathway of *Methylobacterium extorquence* yielded *N*-methyl-L-glutamate titers of 17.9 g L^{-1} in fed-batch cultivation.[2] Due to high substrate specificity of this C1 assimilation pathway genes, we continued with an independent pathway for extension of the product range. Therefore, we focus on pathway-design for *N*-methylated amino acids by the industrially relevant production host *Corynebacterium glutamicum*. Metabolic engineering of *C. glutamicum* led to an expanded product range of proteinogenic amino acids like L-valine[2] but also ω-amino acids like γ-aminobutyrate and diamines like putrescine[3]. The rare imine reductase DpkA from *P. putida* KT2440 catalyzes the reductive methylamination of pyruvate as side activity. Implementation of DpkA into the central carbon metabolism of the pyruvate overproducing *C. glutamicum* strain ELB-P[4] yielded *N*-methyl-L-alanine production. Optimization of carbon- and nitrogen ratios of the minimal medium allowed production of up to 10.5 g L^{-1} when cultivated in shake flasks. *N*-methyl-L-alanine titers of 31.7 g L^{-1} with a yield of 0.71 g per g glucose were achieved in fed-batch cultivation[5]. Due to the somewhat relaxed substrate scope of DpkA, the product portfolio of *N*-methylated amino acids produced by fermentation could be successfully extended. Changing the base strain to a glyoxylate producing *C. glutamicum* strain[6] achieved production of 2.6 g L^{-1} sarcosine, the *N*-methylated glycine derivative, from glucose. Sarcosine production based on the second generation feedstocks xylose and arabinose led to higher product titers than glucose-based production and optimization of substrate composition led to a titer of 8.7 g L^{-1} sarcosine. This is the first example in which a *C. glutamicum* process using lignocellulosic pentoses is superior to glucose-based production.

By mutation of the active site of DpkA, a mutant with higher specific activity towards glyoxylate (30.3 ± 2.7 U mg^{-1}; wild type enzyme 25.7 ± 1.8 U mg^{-1}) was identified. Therefore, the mutant DpkA^{F117L} was incorporated into the production strain and enabled faster sarcosine production. Additionally, this mutation led to an increased activity towards reductive ethylamination of glyoxylate (31.2 ± 1.1 U mg^{-1}; wild type enzyme 25.3 ± 3.2 U mg^{-1}). As a result, the fermentative production of *N*-ethylglycine showed enhanced volumetric productivity compared to the strain harboring the wild type enzyme.

Fermentative access to *N*-methylated amino acids was achieved by two independent pathway designs. First, we enabled *N*-methyl-L-glutamate production by pathway interception in *P. putida.* Additionally, introduction of the imine reductase gene *dpkA* from *P. putida* into various 2-oxoacid producing *C. glutamicum* strains extended the product range. Optimization of medium composition, preferred substrate specificity of the strain or the enzyme itself resulted in excellent production yields.

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ENGINEERED METABOLISM FOR CHEMICAL PRODUCTION FROM ONE-CARBON SUBSTRATES

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Key Words: C1 feedstocks, native & synthetic methylotrophy, *Methylomicrobium buryatense*, *Escherichia coli*

One-carbon (C1) compounds, including carbon dioxide, carbon monoxide, formate, methanol, and methane, are attractive feedstocks for fuel and chemical production due to their availability and sustainability. However, the efficient and economical utilization of these feedstocks can be challenging for traditional chemical processes due, in part, to their diffuse nature. As a result, biological processes are gaining increased attention as alternatives due to safer, milder processing conditions and potential for scale-down, which may allow for decentralized, distributed chemical manufacturing that can make better use of these resources (*Science 355, 38, 2017: doi: 10.1126/science.aag0804*). In order for this potential to be realized, however, significant advances in the performance of C1-metabolizing enzymes, pathways and microorganisms must be achieved.

In this talk I will discuss our recent efforts to engineer and implement biological C1 utilization for chemical production. In one approach, we have leveraged the existing C1-utilization pathways of native methanotroph *Methylomicrobium buryatense* 5GB1 for the production of industrially relevant products such as lactate (*JIMB 45:379, 2018*). In an alternative approach, we engineered a synthetic metabolic pathway for C1 conversion to multi-carbon products that is distinctive from and orthogonal to any known metabolic network (*Nat. Chem. Biol., 2019, MS in Revision*). This C1 elongation pathway is enabled by our discovery that an enzyme involved in mammalian α-oxidation (2-hydroxyacyl-CoA lyase, HACL) can catalyze the condensation of formyl-CoA, an activated C1 molecule, with aldehydes of varying chain lengths. We have prototyped the pathway using a cell-free system with different C1 substrates and showed operation by synthesis of glycolaldehyde, glycolate, ethylene glycol, acetaldehyde, and lactate. We also demonstrated *in vivo* feasibility through the synthesis of glycolic acid and ethylene glycol by *E. coli* using formaldehyde as the sole carbon source. Our work establishes the potential for biotechnological applications of HACL, which includes both bioconversion of C1 feedstocks as well as synthetic methylotrophy and autotrophy.

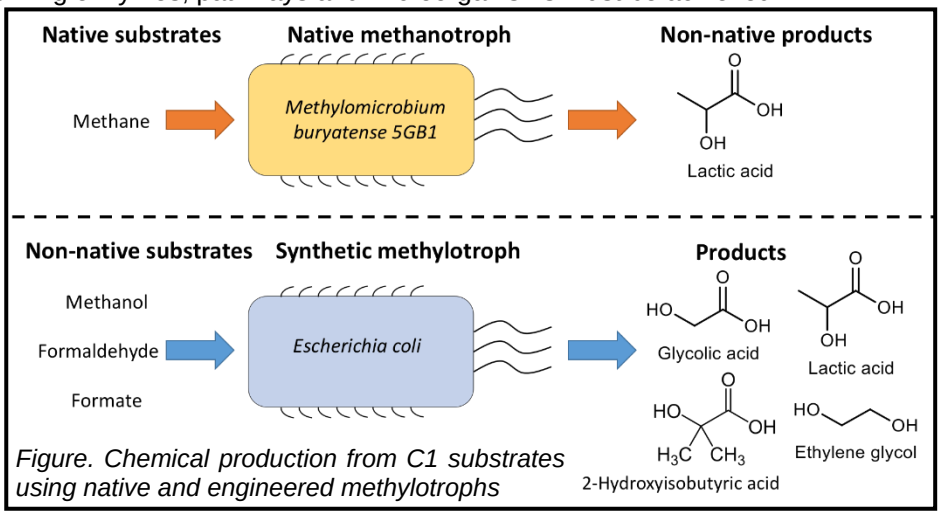

Figure. Chemical production from C1 substrates using native and engineered methylotrophs

METABOLIC ENGINEERING OF YEAST FOR INCREASED PRODUCTION OF CYCLOPROPANE FATTY ACIDS

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Key Words: phospholipid, triacylglycerol, lipid, cyclopropane fatty acid synthetase, *Saccharomyces cerevisiae*

Biological production of chemicals and fuels using whole cells is an important and growing segment of manufacturing and among the various forms, microorganisms are the most successfully utilized. In particular, yeasts such as *Saccharomyces cerevisiae* are both widely used production organisms and metabolic models for oleaginous yeasts. Fatty acid-containing lipids are one example of moderate value, highly versatile chemicals produced by yeasts that are used in a broad range of industries for lubrication, cosmetics, fuels and polymers.

Production levels of standard fatty acids by yeasts has increased enormously over the past 10 years through the application of metabolic pathway engineering, flux analysis, computational approaches and to a lesser extent, bioprocessing improvements. Combined, these advances have brought yeast-based fatty acid production close to commercial reality. Functionalized fatty acids such as those containing hydroxyl or cyclopropyl groups are more valuable as chemical feedstocks and are an attractive target for yeast production as commercial supply is limited. Cyclopropane fatty acids, possessing a strained 3-membered ring and having a saturated chain, are especially attractive as they have application in cosmetics and specialty lubrication. However, cyclopropyl fatty acids present greater challenges for metabolic engineering as they are not produced naturally by yeast.

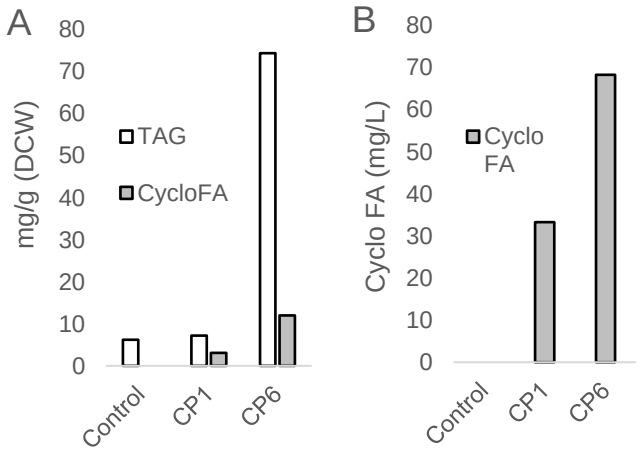

Figure 1. Production of triacylglycerol (TAG) or cyclopropyl fatty acids (cycloFA) in TAG on A) a dry cell weight basis (mg/g DCW) or B) in productivity terms (mg/L) in S. cerevisiae expressing EcCFAS only (CP1) or together with genes to increase standard fatty acids (CP6).

When we expressed the *Escherichia coli* cyclopropane fatty acid synthetase (EcCFAS) in *S. cerevisiae,* both *cis*-9,10-methylene-hexadecanoic and -octadecanoic acids were identified in the phospholipid and triacylglycerol fractions of the cell. Furthermore, EcCFAS expressed in cells engineered for increased lipid production through increases in fatty acid synthesis, accumulation and sequestration in lipid droplets, increased cyclopropyl fatty acid content 4-fold in triglyceride and yield increased to 68.3 mg/L (Fig 1 A& B; Peng et al. in press). This result is very promising for yeast production and there is great potential to improve content further as the triacylglycerol fraction had just 16% present as cyclopropyl fatty acid whereas phospholipid remained enriched at 40%. To further improve yield and purity of this fatty acid in yeast through metabolic engineering, we have undertaken a systematic study of location(s) of cyclopropane fatty acid synthesis, assessed the ability of native yeast enzymes to process the exotic fatty acid, examined potential substrate limitations and the heterologous expression of acyl handling genes. The outcomes of these approaches will be described and show the path towards improving the production of cyclopropyl and other similarly synthesized high value exotic fatty acids in yeasts.

Peng, L He, VS Haritos. Enhanced Production of High-Value Cyclopropane Fatty Acid in Yeast Engineered for Increased Lipid Synthesis and Accumulation, Biotechnology Journal, https://doi.org/10.1002/biot.201800487

DEVELOPMENT OF ENGINEERED CHROMATIC ACCLIMATION SENSOR WITH STRICT AND REVERSE RESPONSE TO LIGHT SIGNAL, AND APPLICATION TO OPTOGENETIC CONTROL IN CYANOBACTERIA

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Key Words: Chromatic acclimation sensor, Cyanobacteria, Optogenetic control, Strict gene regulation, Two-component system,

Genetic regulation and metabolic engineering enabled cyanobacteria to produce renewable chemical compounds from carbon dioxide via photosynthesis. Optogenetic control enables to precisely regulate the timing and level of gene expression without chemical inducer which is environment-hazardous. We recently developed a green-light regulated gene expression system in a model cyanobacterial strain *Synechocystis* sp. PCC6803 (hereafter PCC6803) [1] and a fast-growing marine cyanobacterial strain *Synechococcus* sp. NKBG15041c (hereafter NKBG15041c) [2] using a PCC6803-derived chromatic acclimation sensor, CcaS/CcaR two-component system [3]. However, the regulation of gene expression by CcaS is not strictly controllable and the background expression level under non-inductive condition is not negligible. Furthermore, altering the direction of gene expression, that is induction under red-light and repression under green-light, may expand its flexibility as one of the genetic tools.

To obtain stricter and versatile system, we fabricated engineered CcaSs focusing on its domain structure using *Escherichia coli* expression system. One of the engineered CcaSs, CcaS#11, showed reverse response to light signal, i.e. inducible under red-light and strictly repressible under green-light [4]. To investigate the potential application and versatility of CcaS#11 as the red-light regulated gene expression system in cyanobacteria, we next introduced CcaS#11/CcaR two-component system and GFP_{uv} as a probe of gene expression into PCC6803 after knocking out genomic CcaS/CcaR two-component system to exclude the interference. In this strain, the gene expression was induced under red-light and strictly repressed under green-light as we expected. Then, we applied this system to NKBG15041c. Similarly, red-light inducible gene expression with 2-fold higher ON/OFF ratio compared with the original system was successfully observed in NKBG15041c. Remarkably, there was no leaky expression under green-light, indicating that this system enables strict regulation of gene expression by light signal.

In conclusion, we successfully constructed the engineered CcaS, CcaS#11, with strict and reverse response to light signal. Then we also confirmed its versatility and applicability as the red-light regulated gene expression system with strict regulation in cyanobacteria. Further development of the light regulated bioprocess will be expected using cyanobacterial hosts with this system, as a cell factory for the renewable chemical compounds production.

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PHENOTYPIC DESIGN CHOICES FOR ENHANCED TWO-STAGE MICROBIAL PRODUCTION PROCESSES

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Key Words: Two-stage fermentation, Metabolic Modeling, Dynamic Control, Dynamic FBA, Optimal Production Processes

Microbial metabolism can be harnessed to produce a broad range of industrially important chemicals. Many microorganisms naturally produce some important compounds but do so with low efficiency. To target a more diverse range of chemicals, pathways for non-natural products can be designed and implemented. However, in order to improve these microbes toward the target of industrial production, their metabolism must be engineered by controlling metabolic flux through key pathways. The merits of microbial production processes are often measured using three key variables: titer, rate and yield (TRY). Each of these variables has an impact on the economic viability of any microbial production process. Previous research into improving these TRY metrics have examined the efficacy of decoupling microbial growth from chemical production to achieve enhanced production rates. However, there has been limited research into the choice of microbial phenotype for the growth and production stages of two-stage production processes. Moreover, the substrate uptake rates of microbes drop significantly upon reducing the growth rate, adding to the need for intelligent phenotype selection while designing strains for two-stage processes. In this work, we present a two-stage optimization framework that scans the phenotypic space of microbial metabolism to identify the correct choice of phenotypes during growth and production stages, along with the optimal time to switch between these stages to achieve required TRY values. Through this framework and using *Escherichia coli* as a model organism, we compare the performance of two-stage fermentation processes where dynamic pathway regulation is involved with one-stage fermentation processes that have static intervention strategies implemented for a range of naturally produced chemicals. Our results indicate that while one stage processes are better at achieving optimal yields, two-stage processes outperform them in achieving optimal production rates even after incorporating the effects of reduced substrate uptake rates during the production stage. We anticipate that this optimization framework would be invaluable in designing microbial strains and fermentation processes for industrial chemical production.

MODEL-GUIDED METABOLIC ENGINEERING OF *PSEUDOMONAS TAIWANENSIS* VLB120 FOR THE PRODUCTION OF METHYL KETONES

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Keywords: methyl ketone, *Pseudomonas*, novel microbial cell factory, model-guided metabolic engineering

Aliphatic methyl ketones are discussed as promising novel diesel blendstocks because of their favorable cetane numbers. To achieve sustainable production of these compounds, bio-based production in engineered microbes is followed and successful synthesis in *Escherichia coli*[1,2,3] and *Pseudomonas putida*[4] has recently been shown. In this presentation, we report on the metabolic engineering of *Pseudomonas taiwanensis* VLB120[5] for the production of saturated and monounsaturated medium chain methyl ketones (C11, C13, C15, C17). Major arguments for the use of this microbe are its metabolic versatility, high tolerance of organic solvents[5] and ease of cultivation. *P. taiwanensis* VLB120 can grow on various carbon sources besides glucose such as glycerol, an important by-product of biodiesel production, as well as on major components of biomass hydrolysate such as xylose, organic acids and aromatic compounds, e.g., 4-hydroxybenzoate[4]. Further, its superior redox cofactor regeneration capability[6] might benefit the synthesis of the reduced, aliphatic target compounds. The transformation of *P. taiwanensis* VLB120 into a microbial cell factory for methyl ketone production was achieved by: (i) overproduction of the fatty acyl-CoA synthetase FadB to increase acyl-CoA availability, (ii) oxidation of acyl-CoA to a trans-2-enoyl-CoA by a heterologously expressed acyl-CoA oxidase from *Micrococcus luteus*, (iii) conversion of this intermediate to β-hydroxyacyl-CoA and further oxidation to a β -ketoacyl-CoA by overexpression of the native *fadB* gene, (iv) increased thioesterase activity by overexpression of *fadM* to form free β -keto acids, which spontaneously decarboxylate to methyl ketones. The 1st generation production strain yielded 550 mg L^{-1}_{aq} methyl ketones in a batch fermentation with *in situ* product extraction into a second organic layer of *n*-decane. Further strain optimization was guided by metabolic modeling, which suggested an additional deletion of the acyl-CoA thioesterase II (*tesB*). TesB hydrolyzes acyl-CoA to free fatty acids, hence, reverses the desired FadD reaction. In a simple batch fermentation, the proposed gene deletion resulted in a 2.5-fold increased product titer of 1.4 g L^{-1}_{aq} while 9.4 g L^{-1}_{aq} were reached in fed-batch cultivations. Additional, successful strategies tested in parallel were the deletion of the *pha* operon, responsible for polyhydroxyalkanoate synthesis and deletion of a *fadA* homologue in the 1st generation production strain, with the later resulting in an even 4-fold improvement of the product titer. While the production of 9.4 g L^{-1}_{aq} is already the highest reported titer of recombinantly produced methyl ketones so far, consolidation of all successfully tested engineering strategies holds great promise to significantly boost methyl ketone production in *P. taiwanensis* VLB120 to even higher titers. Overall, the results of this study underline the high potential of *P. taiwanensis* VLB120 for the production of methyl ketones and highlight model-guided metabolic engineering as a means to rationalize and accelerate strain optimization efforts.

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CORYNEBACTERIUM GLUTAMICUM AS A PLATFORM STRAIN FOR THE PRODUCTION OF A BROAD VARIETY OF TERPENOIDS

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Key Words: *Corynebacterium glutamicum*, volatile terpenoids, carotenoids, torulene.

Corynebacterium glutamicum is a natural carotenoid producing bacterium used in the million-ton-scale amino acid biotechnology that has been engineered for isoprenoid production[1]. The native membrane-bound carotenoid decaprenoxanthin is a rare C50 carotenoid. Volatile terpenoids such as valencene[2] and patchoulol[3] could be produced upon deletion of the first step of the specific carotenoid pathway and heterologous expression of the FPP synthase gene *ispA* from *E. coli* and terpene synthases from plant origin. However, these strains produced a yet unidentified carotenoid and only when all carotenoid biosynthetic genes were deleted, a colorless strain resulted. Expressing a codon optimized ADS from *Artemisia annua* in the white strain, amorphadiene, the volatile precursor for artemisinin was produced. For production of volatile terpenoids a dodecane overlay was used, a condition in which *C. glutamicum* benefits from its robust myco-membrane. Recently, we showed production of membrane-bound carotenoids with different length and/or cyclization status: bicyclic C50 sarcinaxanthin[4], bicyclic C40 astaxanthin[5], the linear lycopene[6] and the linear C50 bisanhydrobacterioruberin[7]. This indicated that the *C. glutamicum* myco-membrane accepts these linear and bicyclic carotenoids.
Here, we tested if the mono-cyclic C40 torulene can be produced by *C. glutamicum*. For this, a lycopene-overproducing strain was used as a platform strain to heterologously express a codon optimized lycopene cyclase/phytoene synthase gene *crtYB* from the torulene producing yeast *Sporidiobolus pararoseus*. This strain was analyzed in regard to its ability to transform lycopene into torulene under different membrane triggering conditions in order to enhance productivity of membrane-bound compounds.

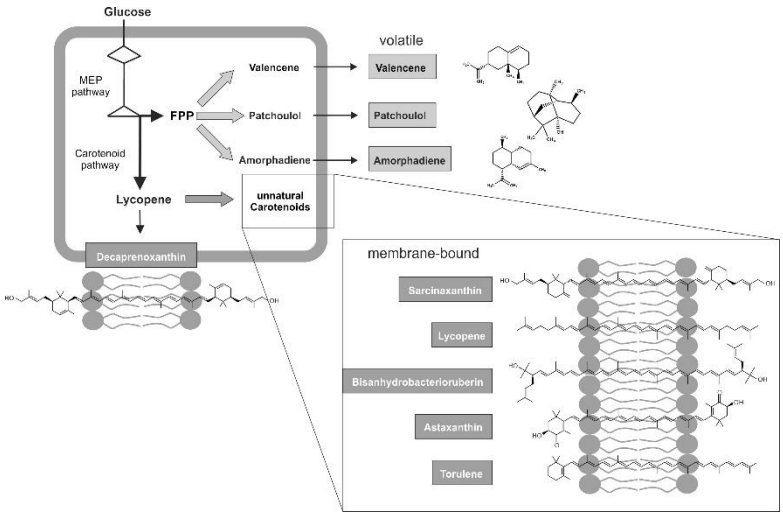

Figure 15 – Production of volatile and membrane-bound terpenoids with C. glutamicum.

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A GENETIC SWITCH FOR STABLE, LONG-TERM FERMENTATIVE PRODUCTION OF ANABOLIC PRODUCTS IN YEAST

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Key Words: farnesene, synthetic biology, fermentation, genetic switch, manufacturing

Amyris is the integrated renewable products company that is enabling the world's leading brands to achieve sustainable growth. Amyris applies its innovative bioscience solutions to convert plant sugars into hydrocarbon molecules and produce specialty ingredients and consumer products. Production and marketing of the molecule farnesene (Biofene®) has already been commercialized with production scale in some markets. Farnesene has many applications as a renewable feedstock for polymers, nutraceuticals and cosmetics. To reduce the production cost of farnesene, at Amyris we engineer strains using a state-of-the art industrial synthetic biology platform to have high titer, yield, and productivity, and we perform fermentations in 200 m3 vessels over the course of many days or weeks. The challenge is that high-producer cells grow more slowly than spontaneous mutant low- or non-producer cells, especially in the nutrient-unlimited conditions of the seed train expansion, and yet must comprise the vast majority of the population. We have successfully addressed this challenge by developing an industrially-scalable genetic switch to successfully maintain high performance throughout lengthy fermentations. This genetic switch uses maltose (a cheap, non-toxic and metabolizable molecule) to control transcription such that when maltose is added in the seed train, product formation is shut off. This increased the growth of high-producer cells, resulting in higher inoculum purity and improved performance in bioreactors.

GENE SOURCE SCREENING AS A METABOLIC ENGINEERING TOOL FOR FLAVONOID PRODUCTION

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Key Words: flavonoids, *Saccharomyces cerevisiae*, gene source selection, metabolic engineering

Flavonoids are plant secondary metabolites with advantageous health benefits and great potential in the food industry. Current production techniques are facing limitations due to climate changes, small plantation sizes and long growth cycles. Metabolic engineering food-grade microorganisms can provide an alternative solution. However, the current production yield is limited by low enzymatic activities. Proper gene selection can be a solution to this problem. Gene source analysis for 4-coumarate:coenzyme A ligase (4CL) and chalcone synthase (CHS) was performed based on evolution and protein structures. The analysis identified several potential enzyme gene sources, which were expressed in Saccharomyces cerevisiae. The flavonoid production was quantified by HPLC with the aim to find gene sources producing the highest amount of flavonoids. Two new enzyme gene sources, 4CL from *Medicago truncatula* and CHS from *Vitis vinifera* had been found to provide the highest naringenin production yield. The amount of naringenin produced was 28-fold higher as compared to the reference strain expressing Arabidopsis thaliana. The results demonstrated that gene screening and the combination of enzymes from the correct gene source could greatly improve flavonoid production. For the future, this could help commercialize flavonoid production, which would result in natural food preservatives and additives.

DESIGNER RHAMNOLIPID PRODUCTION

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Key Words: designer rhamnolipids, metabolic engineering, Pseudomonas, biosurfactants

Rhamnolipids are biosurfactants featuring surface-active properties that render them suitable for a broad range of applications, e.g., in detergents, food, bioremediation, medicine/pharmacology, and crop science. These properties include their emulsification and foaming capacities and their ability to lower the surface tension. Further, aspects like biocompatibility and environmental friendliness, both features of rhamnolipids [1] are becoming increasingly important. Rhamnolipids thus constitute suitable substitutes for synthetic surfactants produced from fossil resources. Native producers of rhamnolipids are mainly pathogenic bacteria like *Pseudomonas aeruginosa*. We previously designed and constructed a recombinant *Pseudomonas putida* KT2440, which synthesizes rhamnolipids by decoupling production from host-intrinsic regulations and cell growth [2]. As most biosurfactants, rhamnolipids are synthesized in mixtures. We here show our approach to alter the native mixture of surfactant molecules to produce specific new-to-nature combinations. The molecular structure (Figure 1) can on the one hand be altered in the hydrophilic moiety by changing the number of rhamnose molecules. We achieved this by using only distinct genes from the native rhamnolipid synthesis pathway. On the other hand, we were also able to change the length of the fatty acids in the hydrophobic part. This chain length is determined by the acyl-transferase (RhlA). Using *rhlA* genes from different organisms enables our microbial cell factory to synthesize molecules with different chain lengths [3]. The different molecular structures have further been shown to feature diverse physico-chemical properties [4]. Exploiting the natural structural diversity will thus allow for the synthesis of designer rhamnolipids tailor-made for specific applications. We thus present a novel approach to use biochemical engineering to create tailor-made products for a more sustainable future.

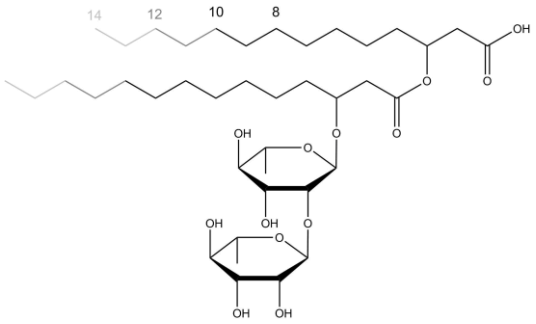

Figure 16 – Molecular structure of rhamnolipids

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SYSTEMS AND SYNTHETIC BIOLOGY ADVANCEMENTS TO IMPROVE *SYNECHOCYSTIS* SP. PCC 6803 STRAIN ENGINEERING IN THE INDUSTRIALLY-RELEVANT CONDITION OF DIURNAL LIGHT-DARK CYCLES

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Key Words: cyanobacteria, metabolomics, synthetic biology, metabolic engineering

Cyanobacteria are an interesting chassis for industrial chemical production due to their ability to utilize sunlight and carbon dioxide as substrates. However, much of the strain engineering has been done under low- and continuous- light laboratory conditions as opposed to the realistic day/night cycle of outdoor sunlight availability. Our lab previously demonstrated that engineered free fatty acid production is decreased in daily light-dark cycles as opposed to continuous light relative to wild-type. This observation motivated system and synthetic biology developments to improve strain engineering efforts specifically in realistic day/night cycles. Toward this goal, we have improved systems biology understanding and developed synthetic biology tools for use in day/night cycles. Specifically, we discovered and characterized four native *Synechocystis* sp. PCC 6803 promoters which enable light-activated gene expression in daily light-dark cycles. We engineered a photobioreactor system which enables diurnal sinusoidal light cycles with peak-light intensities reaching over 1,500 µmol photons m^{-2} s^{-1} and report interesting *Synechocystis* sp. PCC 6803 growth in these conditions. We also developed and implemented a multi-platform 'omics study investigating the dynamic behavior of *Synechocystis* sp. PCC 6803 in sinusoidal day/night cycles. We have engineered and optimized bisabolene production in *Synechocystis* sp. PCC 6803. Together, these advances contribute to the advancement of *Synechocystis* sp. PCC 6803 as an industrially-relevant chassis for chemical production.

COUPLING ENGINEERING OF *SACCHAROMYCES CEREVISIAE* WITH MEDIUM OPTIMIZATION FOR THE PRODUCTION OF ERGOTHIONEINE

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Key Words: Ergothioneine, metabolic engineering, medium optimization, *Sachharomyces cerevisiae*, nutraceutical

Ergothioneine (ERG) is a naturally occurring, exogenous antioxidant that is nonetheless abundant in the human body. It has been shown both to reduce oxidative damage and to be involved in several diseases *in vivo*[1,2]. Therefore, ergothioneine is poised to take a place in the dietary supplement industry. Here we describe the engineering of the yeast *Saccharomyces cerevisiae* and subsequent medium optimization to produce ergothioneine by fermentation. After integrating combinations of biosynthetic pathways from different organisms, we screened yeast strains for their production of ERG. Next, the highest producing strain was engineered with ergothioneine transporters, and its amino acid metabolism was altered by knock-out of Tor1 or Yih1. The bottleneck for ergothioneine production was determined by integration of a second copy of the pathway enzymes. We also optimized the media composition for production of ergothioneine using yeast *S. cerevisiae*. Following these manipulations, we obtained a titer of 630 mg/l in fed-batch cultivation in bioreactors. This work shows that with further engineering of the strain, current chemical synthesis of ergothioneine could be replaced with a sustainable alternative.

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METABOLIC ENGINEERING OF *PSEUDOMONAS PUTIDA* KT2440 FOR ENHANCED RHAMNOLIPID PRODUCTION

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Key Words: *Pseudomonas putida*, rhamnolipid, metabolic engineering, streamlining

The production of chemicals and fuels is mainly based on fossil resources. The reduced availability of these resources and thus the increasing prices for crude oil as well as the resulting pollution of the environment require alternative strategies to be developed. One approach is the employment of microorganisms for the production of platform molecules using renewable resources as substrate. Biosurfactants, such as rhamnolipids, are an example for such products as they can be naturally produced by microorganisms and are biodegradable in contrast to chemical surfactants. The bio-based production of chemicals has to be efficient and sustainable to become competitive on the market. Several strategies can be applied to increase the efficiency of a microbial cell factory, e.g., streamlining the chassis. Here, we show the heterologous production of rhamnolipids with the non-pathogenic *Pseudomonas putida* KT2440 with the aim of increasing the yield. *P. putida* KT2440 is a well-characterized microorganism and its genome is sequenced and well annotated. Thus, the targeted removal of genes is possible and can lead to a reduction of the metabolic burden and by-product formation, which can result in a higher yield. Furthermore, the efficient supply of precursors is an important factor for optimized production processes. Rhamnolipids are amphiphilic molecules containing rhamnose and ß-hydroxy fatty acids. These precursors are synthesized by two pathways, the fatty acid *de novo* synthesis and the rhamnose pathway. We performed gene deletions to avoid the synthesis of by-products, like pyoverdine, exopolysaccharides, and large surface proteins and energy consuming devices as the flagellum. Most of the genome-reduced mutants reached a higher yield compared to the strain with wildtype background. With the best chassis, the yield could be increased by 35%. Furthermore, we conducted the overexpression of genes for precursor supply, either plasmid-based or genomically integrated. In this regard, the genes for the phosphoglucomutase, the complete rhamnose-synthesis pathway operon, and different enzymes in the pathway for acetyl-CoA synthesis were targeted. Various combinations were tested, and the highest yield reached was 51% higher compared to the initial rhamnolipid producer. Finally, a genome-reduced mutant was equipped with the overexpression modules and the rhamnolipid titer was increased from approximately 590 mg/L for the wildtype background to 960 mg/L, which represents a 63% increase. In conclusion, we were able to enhance the yield of rhamnolipids per glucose using metabolic engineering.

ENGINEERING SULFATE DONOR ACCUMULATION IN *ESCHERICHIA COLI* FOR SYNTHESIS OF SULFATED GLYCOSAMINOGLYCANS

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Key Words: Metabolic Engineering, Escherichia coli, 3'-phosphoadenosine-5'-phosphosulfate, Sulfated Glycosaminoglycans

The model bacterium *Escherichia coli* has been extensively engineered for a variety of applications. However, sulfated biomolecules remain a relatively under-explored domain of biologics that can be synthesized using *E. coli.* An important class in this domain are sulfated glycosaminoglycans (GAGs) which are of great pharmaceutical/nutraceutical interest. On-going studies aim at developing efficient and scalable chemical and chemoenzymatic methods to produce these compounds. However, we propose that engineered *E. coli* capable of entirely *in vivo* synthesis of sulfated GAGs will serve as a great alternative to the current state-of-art.

The biosynthesis of sulfated biomolecules relies on the universal sulfate donor, 3'-phosphoadenosine-5'-phosphosulfate (PAPS). PAPS plays the role of a co-enzyme in enzymatic sulfotransferase reactions and donates the sulfate to the substrate. In the first part of this study, we engineered the metabolism of *E. coli* to improve PAPS accumulation around 10000-fold. In the second part, we applied this engineered strain to *in vitro* biosynthesis of chondroitin sulfate (a sulfated GAG).

Certain strains of *E. coli* possess the ability to biosynthesize unsulfated GAG backbones. These have been well-studied and optimized for GAG production. *In vitro* sulfation requires the addition of purified sulfotransferase and an excess of commercially obtained PAPS to the unsulfated GAG. By utilizing PAPS from our engineered strain in the existing setup, we improve the biotransformation method to one in which all components are synthesized from *E. coli*. We also use this system and its comparison to other *in vitro* systems to identify the bottlenecks for total *in vivo* synthesis of sulfated GAGs in *E. coli*. We show for the first time that with this system, we have achieved the synthesis of chondroitin sulfate A without the dependence on the commercially procured PAPS cofactor.

3-HYDROXYPROPIONIC ACID PRODUCTION FROM CRUDE GLYCEROL WITH *LACTOBACILLUS DIOLIVORANS*

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Biodiesel consists of fatty acid methyl esters and is produced via transesterification of long chain fatty acids, derived from vegetable oil, with methanol. The principle by-product of this process, which makes up to 10% of the biodiesel production, is crude glycerol. Since its purification is rather unsustainable, the microbial upgrading to value-added products opens new opportunities. *Lactobacillus diolivorans* metabolizes glycerol to balance its electron household by reducing the intermediate 3-hydroxypropione aldehyde to 1,3-propanediol or oxidizing it to 3-hydroxypropionic acid. This species of *Lactobacillus* is a very effective natural producer of 1,3-propanediol, with titers up to 90 g/L, but as well shows potential for the production of 3-hydroxypropionic acid, which is considered as one of the top twelve value-added platform compounds from biomass according to the US Department of Energy.

In this study, it was shown, that process engineering to manipulate the redox household of *L. diolivorans* is a valuable tool to shift the product pattern. By switching to an aerobic process, the production of 3-hydroxypropionic acid could be improved to titers up to 40 g/L compared to 27 g/L in the anaerobic process. Another target is the feeding strategy, since *L. diolivorans* is not able to use glycerol as sole energy source. The metabolization of sugars like glucose generates excess electrons which favor the reductive pathway for glycerol utilization. Different molar ratios of glucose to glycerol as well as other carbon sources were tested to study their impact on the product pattern. These process engineering approaches together with the relatively high robustness of *L. diolivorans* towards this toxic product are promising steps towards the optimization of 3-hydroxypropionic acid production and render *L. diolivorans* a future host for metabolic modeling targets.

RESOLVING GENETIC ENGINEERING SIGNATURES IN YEAST ON-SITE WITH THE MinION AND iSeq

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Key Words: metabolic engineering, synthetic biology, next-generation sequencing.

Generating an assembly that captures all of the genome and plasmid modifications resulting from metabolic engineering is essential for quality control, connecting genotype to phenotype, establishing and protecting intellectual property, and generating “ground truth” for monitoring potential release events. Furthermore, high quality *de novo* assemblies can be used to accurately determine the presence and function of metabolic engineering signatures in an unknown sample. Here, we use two new inexpensive sequencers, the Oxford Nanopore MinION and the Illumina iSeq, to enable fast acquisition of sequence on-site. We also use new processing algorithms to enable fast transformation of sequence information into a high-quality genome assembly. The resulting pipeline can generate *de novo* microbial genome assemblies that capture complete chromosomal pathways and episomal plasmids. We further extend the pipeline to resequence six nonconventional yeasts of interest for metabolic engineering.

To establish the most accurate workflow, we evaluated four nanopore *de novo* assemblers and three polishing algorithms at varying genome coverage depths for the lab strain *S. cerevisiae* CEN.PK113-7D. Our results show that (1) nanopore genome coverage depth must be at least 40X, (2) Flye and Canu are currently the best assemblers due to their combination of structure, completeness, and accuracy, and (3) Illumina data is essential for polishing. Our final pipeline (Figure 1A) generated a better *S. cerevisiae* CEN.PK113-7D assembly than the publicly available reference genome.

We then applied this pipeline to 12 engineered *S. cerevisiae* strains of varying genetic background – including strains from S288C, BY4741, BY4742, CEN.PK, W303a, and brewery lineages. Interestingly, the nanopore assembler Flye was the only software able to resolve both chromosomally-integrated pathways and complete plasmids – and was able to do so even when presented with mixtures of plasmids (Figure 1B). The more widely used nanopore assembler Canu was unable to resolve complete plasmids. We then extended the pipeline to resequence the nonconventional yeasts *Pichia pastoris*, *Hansenula polymorpha*, *Yarrowia lipolytica*, *Kluyveromyces marxianus*, *Debaryomyces hansenii*, and *Xanthophyllomyces dendrorhous*. The resulting *de novo* genomes show significant improvement over the respective references – achieving chromosomal resolution, closing large gaps, and revealing previously omitted genes. Thus, the engineered and “ground truth” assemblies we have created represent an advance in the ability to detect signatures of metabolic engineering and support further metabolic engineering of nonconventional yeasts.

Sequencing is becoming ever more prevalent in research workflows across disciplines, including metabolic engineering. We provide here a pipeline that can accurately determine genotype and resolve complete engineering signatures in unknown samples. This technology can be inexpensively implemented on-site in the many distributed locations where organisms are engineered to obtain high-quality *de novo* genome assemblies. Thus, this pipeline can be widely applied in academic, government, and industry settings to study and monitor engineered organisms without high capital costs and deep coverage depths characteristic of alternative sequencing platforms and algorithms.

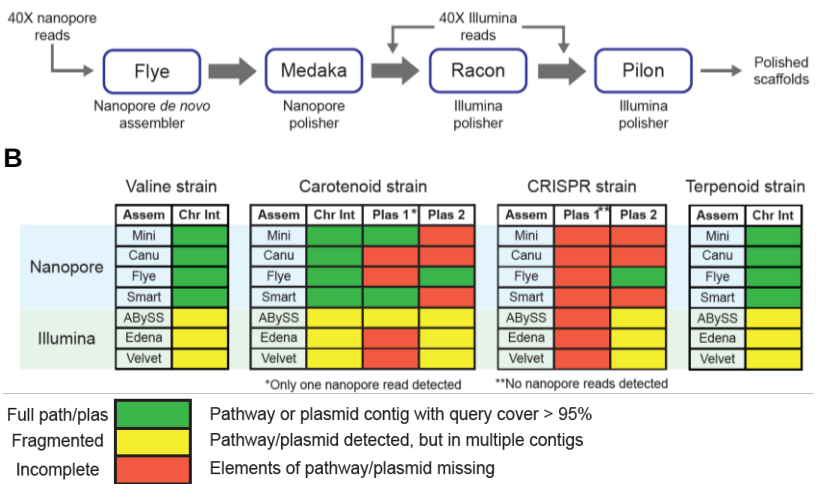

Figure 17 – Resolving genetic engineering signatures. **A.** Bioinformatic workflow to assemble genomes and plasmids. **B.** Scoring the ability of the software to resolve chromosomal pathways and episomal plasmids. Only nanopore data processed with Flye allows complete assembly of chromosomal edits and full plasmids.

STRINGENCY OF ANTISENSE REGULATION VARIES BASED ON VOLATILITY OF mRNA TARGET REGION

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Keywords: antisense, regulation, bacteria, mRNA secondary structure

Bacteria can regulate gene expression by transcribing antisense RNA to interfere with protein translation. Antisense has been shown to control a wide variety of prokaryotic proteins, including membrane proteins, protein toxins, and proteins involved in transport and metabolism. This type of regulation can be used in the production of biologics to optimize the health of the culture and maximize production of the desired product. We examined naturally occurring antisense to enhance design principles for product optimization. We found that the level of secondary structure fluctuation of the antisense binding site varied depending on the function of the target. We hypothesized that stringency of regulation by naturally evolved antisense was driven by the impact of the target molecule on cellular survival. Specifically, high stringency was important for toxin-antitoxin systems where survival depended on high levels of control. Toxin-antitoxin systems rely on effective antisense to prevent the translation of self-damaging proteins. Antisense-based systems regulating transport and metabolism potentially benefited from less stringent antisense control. Basal levels of antisense-regulated proteins involved in metabolic processes could allow for quick adaptation to changing nutrient conditions. More than fifty naturally occurring sense/antisense pairs were analyzed to demonstrate that antisense binding sites correlate to the level of stringency needed in regulating the target protein.

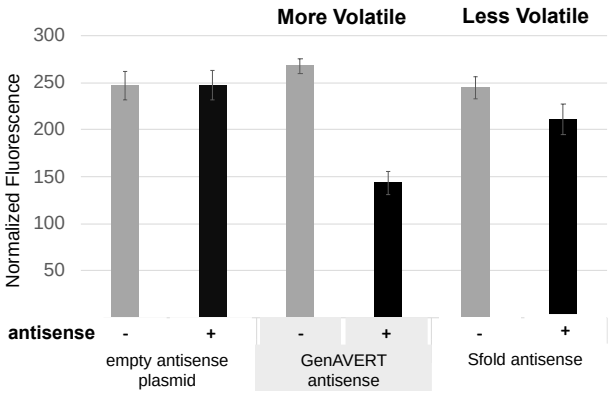

Figure 18 – GFP was expressed in all conditions. Fluorescence normalized by OD_{595} was down regulated more when antisense targeting a more volatile region was induced.

We postulated mRNA secondary structure to be an ensemble of conformations sampling different possible low Gibbs energy states around the global minima. Certain regions break and form hydrogen bonds more frequently, making them more volatile and available for antisense molecules to bind. Less volatile regions resulted in more stable hydrogen bonded secondary structures making accessibility by antisense less likely. By applying an algorithm developed by our lab, GenAVERT, to predict volatile regions of mRNA(1), we were able to examine antisense volatility. Antisense binding regions for targets that encoded toxins were more likely to align with high volatility predictions than other targets. Targeting high volatility regions of toxin mRNAs likely maximized antisense efficacy where stringent control was critical for survival. Less stringent control of metabolic targets could also provide an evolutionary benefit. Analogous to leaky promoter systems, such as the *lac* operon, a basal level of metabolic proteins available when nutrient conditions change would also serve as an evolutionary benefit. A random forest classification was performed to orthogonally verify the results. With 94% accuracy, the random forest was able to correctly predict whether or not an antisense binding region would result in stringent or astringent regulation.

Antisense was also tested in *Escherichia coli* to assess the efficacy of artificial antisense. Antisense sequences designed using the GenAVERT and Sfold algorithms were expressed targeting green fluorescent protein (GFP). GFP fluorescence was downregulated 46% when the more volatile region was targeted (GenAVERT) compared to a 14% decrease when a less volatile region was targeted (Sfold). However, neither sequence resulted in stringent down regulation of GFP fluorescence. The random forest correctly classified both antisense molecules as astringent.

These efforts provided new insight into how bacteria have evolved elaborate regulatory mechanisms. Antisense can regulate its target in a very specific manner based on the volatility of the target region. Our work in understanding antisense has the potential to provide a regulation tool that can be controlled based on expression level needs.

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USING SYNTHETIC BIOLOGY TO ENGINEER FUNCTIONAL PROTEIN-BASED MATERIALS

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Key Words: Self-assembling proteins, biopolymers, protein-based materials, hydrogels, synthetic biology.

While conventional organic polymers and plastics have served various needs of our society for decades, they present important limitations, such as limited biodegradability, biocompatibility and chemical tunability. Protein-based materials represent sustainable, non-toxic and versatile alternatives to these organic polymers. In fact, synthetic biology now allows us to easily customize the sequence of self-assembling proteins to modify their chemical and physical properties. In particular, amyloid proteins can assemble into nanofibers with high surface area, which can be used as structural or genetic scaffold for a variety of applications. Curli nanofibers are a class of amyloids naturally produced by *Escherichia coli* bacteria to promote surface adhesion and biofilm formation. After extracellular secretion of the curli subunits (CsgA), they self-assemble to form highly resistant functional nanofibers. The curli subunits exhibit exceptional structural and mechanical stability, and can be engineering to form a variety of fusion proteins or to display functional point mutations. Through a scalable vacuum filtration-driven process (Figure 1), our group isolates aggregated engineered proteins to form a range of macroscopic materials, including protein hydrogels and free-standing films.[1] The simplicity and scalability of this process allows us to move towards the fabrication of protein-based biopolymers, coatings and composite materials for environmental, energy and biomedical applications.

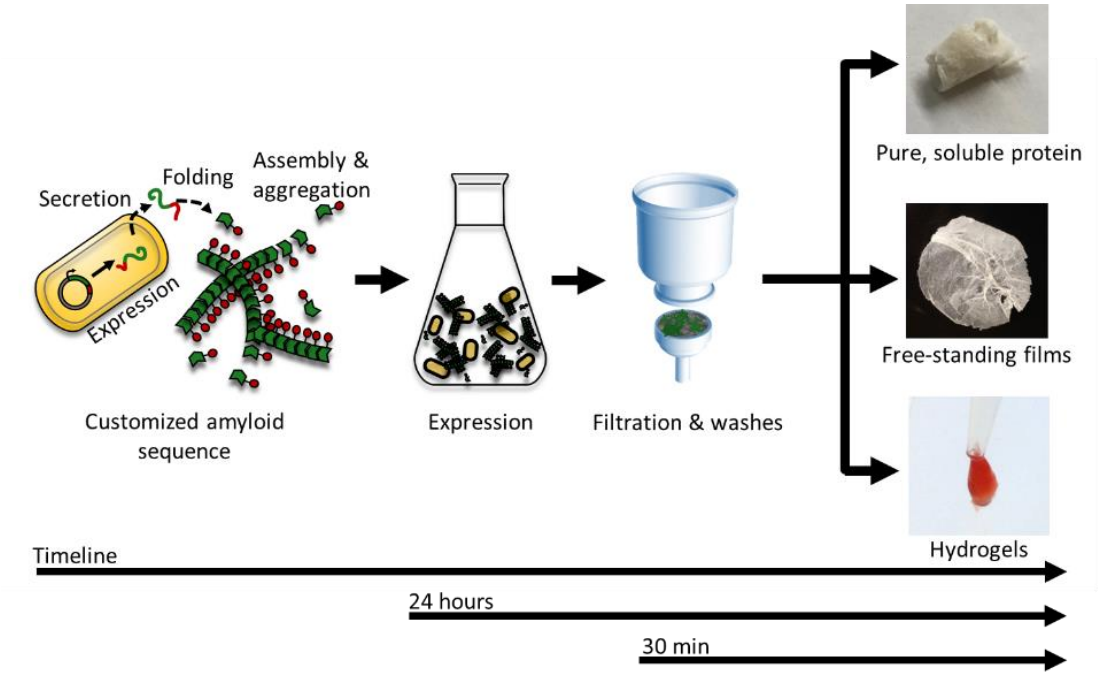

Figure 19 – A scalable process for producing genetically engineered gels, films and soluble protein.[1]

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