# ENLARGING THE SYNTHETIC BIOLOGY TOOLBOX FOR *PICHIA PASTORIS*: GOLDEN GATE CLONING AND CRISPR/CAS9

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State-of-the-art strain engineering techniques for the protein producing yeast host *Pichia pastoris* include overexpression of homologous and heterologous genes, and deletion of host genes. For this purpose overexpression vectors and gene deletion methods such as the split marker technique have been established. For metabolic and cell engineering purposes, the simultaneous overexpression of more than one gene is often needed. Previous approaches employing subsequent steps of overexpression and marker recycling were time- and labor-consuming. Therefore, efficient systems allowing multiple gene overexpression are required, that can be stably integrated into the *P. pastoris* genome. To this end, we developed a synthetic biology toolbox based on Golden Gate cloning to enable efficient construction of complex and versatile over-expression vectors. Up to five different expression cassettes, employing a library of promoters and terminators can be combined into one vector, and successfully integrated into the genomic DNA of *P. pastoris* at targeted loci in one step. Recent trends in synthetic biology, however, go into the direction of building up large and complex reaction networks. To allow for clean and unscarred genetic engineering, a CRISPR/Cas9 based method for gene insertions, deletions and replacements was developed, which paves the way for precise genomic rearrangements in *P. pastoris*. By using this technique precise genomic integrations were performed efficiently without integrative selection markers. The repertoire of genetic techniques developed so far, will provide a wide variety of possibilities to engineer *P. pastoris*. Applications for these synthetic biology tools in cell engineering of recombinant *P. pastoris* will be presented.

# ENGINEERING VACUOLAR SORTING PATHWAYS FOR EFFICIENT SECRETION OF RECOMBINANT PROTEINS

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Recombinant protein production is an expanding branch of biotechnology with increasing economic importance. Currently, 20% of biopharmaceutical proteins and approximately half of the industrial enzymes are produced in yeasts. Many proteins are efficiently secreted by yeast systems, reaching product titers in the g $L^{-1}$ range. The expression of more complex proteins, however, may overwhelm the folding and secretion capacity of the host cells. This triggers the unfolded protein response (UPR), which aims at restoring endoplasmic reticulum (ER) homeostasis. The UPR, in turn, is thought to activate ER-associated protein degradation (ERAD). Alternatively, trafficking of correctly folded proteins can be hampered on their way to the cell exterior leading e.g. to missorting and subsequent degradation in the vacuole.

The methylotrophic yeast *Pichia pastoris* (*Komagataella* spp.) is a popular microbial host for the production of recombinant proteins. Vacuolar protein sorting has not been investigated in detail so far in *P. pastoris*, although there were a few indications that vacuolar mistargeting of recombinant products might occur also in this yeast. Thus we engineered the vacuolar sorting pathways in *P. pastoris* and investigated their impact on extracellular product titers as well as intracellular localization of the recombinant secretory product. Thereby, differences between *vps* (vacuolar protein sorting) mutant strains disrupted in genes involved either in the CORVET or the HOPS tethering complexes became obvious. Moreover, we were able to show that engineering of the vacuolar sorting pathways has a positive impact on heterologous protein secretion, however, in some cases simultaneous inactivation of specific vacuolar proteases was necessary.

Taken together, these studies allowed us to gain deeper insight into the pathways leading to intracellular degradation of recombinant secretory proteins. Based on these findings, approaches how to efficiently adapt the host cell's secretion capacity will be presented, which confirm that impairment of vacuolar protein sorting is an effective means of enhancing secretion of heterologous proteins.

# GENOME-SCALE RECONSTRUCTION OF *SALINISPORA TROPICA* METABOLISM; MICROBIAL ENGINEERING AND ITS APPLICATIONS IN SECONDARY METABOLITE PRODUCTION

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Keywords: genome-scale model, Salinispora sp., secondary metabolites

Bacteria within the order Actinomycetales are a well-known source of natural products such as antibiotics and anticancer agents, and the genus *Salinispora* is no exception. S*alinispora tropica* is a marine actinomycete that produces diverse secondary metabolites, including many that possess pharmaceutical properties such as Salinosporamide A (NPI-0052), a potent anticancer agent, and sporolides, candidates for antiviral compounds. Here, we present the first manually curated genome-scale metabolic model (iCC908) for *Salinispora tropica* strain CNB-440. The reconstruction enables characterization of the metabolic capabilities for understanding and modeling the cellular physiology of this newly sequenced genome. The model was based on physiological and biochemical information of primary and secondary metabolism pathways. The reconstructed stoichiometric matrix consists of 1169 biochemical reactions, 204 transport reactions and 1317 metabolites. A total of 908 structural open reading frames (ORFs) were included in the reconstructed network. The number of gene functions included corresponds to 20% of all characterized ORFs in the *S. tropica* genome. The genome-scale metabolic model was used to study strain-specific capabilities in defined minimal media and to analyze growth capabilities in 41 different minimal growth-supporting environments. These nutrient sources were evaluated experimentally to assess the accuracy of in-silico growth simulations. The model predicted no auxotrophies for essential amino acids, which was corroborated experimentally. The strain is able to use 21 different carbon sources, 8 nitrogen sources and 4 sulfur sources from the nutrient sources tested. Cases where the model was incorrect provided opportunities to gain new insights into the physiology of this specie and generate hypotheses. The incorporation of modifications led to increased accuracy in predicting the outcome of growth/no growth experiments from 76 to 93%. New data, and modifications can be incorporated into the reconstruction to iteratively improve the reconstruction.

Since specialized pathways were included in the reconstruction, growth simulations and in silico gene deletions can be performed by using flux balance analysis (FBA) to dramatically increase secondary metabolites production and yield in *Salinispora* for possible “gene cluster identification” so specific pathways can be cloned in more efficient strains. For example, iCC908 has been used to define a production medium to improve Salinosporamide A production in a recombinant strain with increases over 20% compared to the wild type. This presentation will describe the main features of the metabolic flux analysis and microbial engineering methodology based on reconstruction of the whole metabolism and its applications in the optimization of secondary metabolite production.

# USING SCREENING AND CLASSICAL STRAIN IMPROVEMENT TECHNIQUES TO GET THE BEST PERFORMANCE OF LACTIC ACID BACTERIA

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Key words: texture, *S. thermophilus*, screening, strain improvement

The Gram positive lactic acid bacteria (LAB) produce lactic acid as their main degradation product from sugar fermentation. LAB have been used in bio processed food for thousands of years. Chr Hansen is the largest starter culture producer and the lactic acid bacteria are used in dairy products like cheese, yoghurt, kefir but also in fermented meats like salami sausages, in wine production, and in silage production. The different uses of LABs requires constant search for improved functionalities and scouting for new areas of use. This requires development of better and faster screening technologies to define the next generation of strains and a continued development of classical strain improvement techniques to get even better strains.

Examples will be given on how we use cutting edge technology to find *Streptococcus thermophilus* strains with better texturing capabilities to be used for *e.g.* yoghurt and how we can further improve these traits by natural non GMO techniques.

**COMBINED ENGINEERING OF DISACCHARIDE TRANSPORT AND PHOSPHOROLYSIS FOR ENHANCED ATP YIELD FROM SUCROSE FERMENTATION IN *SACCHAROMYCES CEREVISIAE***

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Key Words: Free-energy conservation, ATP, Facilitated diffusion, Chemostat, Yeast physiology

Anaerobic industrial fermentation processes do not require aeration and intensive mixing and the accompanying cost savings are beneficial for production of chemicals and fuels. However, the free-energy conservation of fermentative pathways is often insufficient for the production and export of the desired compounds and/or for cellular growth and maintenance. To increase free-energy conservation during fermentation of the industrially relevant disaccharide sucrose by *Saccharomyces cerevisiae*, we first replaced the native yeast α-glucosidases by an intracellular sucrose phosphorylase from *Leuconostoc mesenteroides* (*Lm*SPase) (Figure 1). Subsequently, we replaced the native proton-coupled sucrose uptake system by a putative sucrose facilitator from *Phaseolus vulgaris* (PvSUF1). The resulting strains grew anaerobically on sucrose at specific growth rates of 0.09 ± 0.02 $h^{-1}$ (*LmSPase*) and 0.06 ± 0.01 $h^{-1}$ (*PvSUF1*, *LmSPase*). Overexpression of the yeast *PGM2* gene, which encodes phosphoglucomutase, increased anaerobic growth rates on sucrose of these strains to 0.23 ± 0.01 $h^{-1}$ and 0.08 ± 0.00 $h^{-1}$, respectively. Determination of the biomass yield in anaerobic sucrose-limited chemostat cultures was used to assess the free-energy conservation of the engineered strains. Replacement of intracellular hydrolase with a phosphorylase increased the biomass yield on sucrose by 31%. Additional replacement of the native proton-coupled sucrose uptake system by PvSUF1 increased the anaerobic biomass yield by a further 8%, resulting in an overall increase of 41%. By experimentally demonstrating an energetic benefit of the combined engineering of disaccharide uptake and cleavage, this study represents a first step towards anaerobic production of compounds whose metabolic pathways currently do not conserve sufficient free-energy.

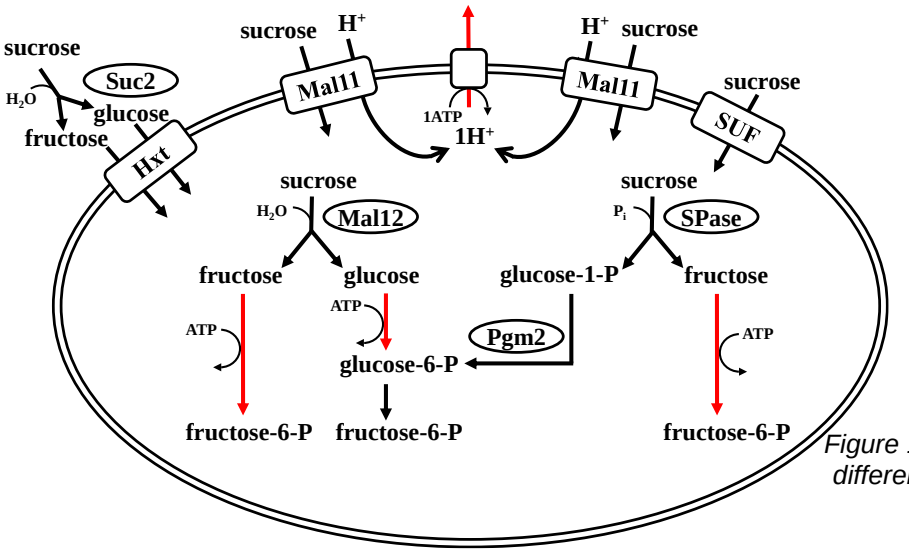


*Figure 1 – Schematic representation of different strategies for the uptake and cleavage of sucrose.*

# ENGINEERING OF *ESCHERICHIA COLI* PROTEIN EXPRESSION PROCESS DEVELOPMENT

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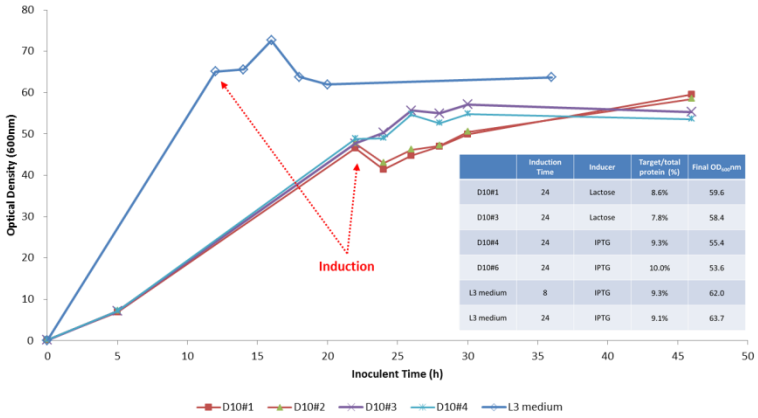
Key Words: *Escherichia coli*, DoE, protein drug, expression, fermentation

It almost 30% protein drugs are expression by *Escherichia coli*, because of rapid growth and high production yield. We have developed E.coli base system for recombinant protein expression, scFv, Fab and vaccine. In this study we introduce example about process development for nutrient components selection. Shaker flasks were used for different nitrogen and carbon components screening by DoE. Seven media formulations for *E. coli* fermentation were used in this study. By changing nitrogen and carbon source ratio, product titer of target protein could be optimized, at least 1.4 folds increased. The best result from shaker flask was used in 250 mL parallel fermenter and pH, dissolved oxygen, feeding/induction strategy were evaluated. The processes from seed culture to harvest only require 64 hours. The optimized time was reduced to 32 hours. The result showed that both target protein expression and cell density value were comparable, but the total process time was significantly reduced by half.




| | Induction Time | Inducer | Target/total protein [%] | Final $OD_{600nm}$ |
|---|---|---|---|---|
| D10#1 | 24 | Lactose | 8.6% | 59.6 |
| D10#3 | 24 | Lactose | 7.8% | 58.4 |
| D10#4 | 24 | IPTG | 9.3% | 55.4 |
| D10#6 | 24 | IPTG | 10.0% | 53.6 |
| L3 medium | 8 | IPTG | 9.3% | 62.0 |
| L3 medium | 24 | IPTG | 9.1% | 63.7 |

*Figure 1. The result of Escherichia coli Growth condition and target protein titer by different process.*

**GRAM LEVEL scFv EXPRESSION PLATFORM OF *PICHIA PASTORIS***

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Key Words: Pichia pastrois, secretion, methanol, scFv, antibody.

The methylotrophic yeast *Pichia pastoris* secretion expression system has been developed for the antibody fragments (scFv) production platform. The platform includes three technology platforms, the first one is strain generation, the second is fermentation process development in 250 ml fermentor and the last is process scale up to 5 L. A recombinant scFv went through clone generation, include signal peptide tool box, normally yield 2.5 mg/L titer in deep well. Through the fermentation process development of induction medium composition and feeding strategy by Eppendorf Dasgip parallel 250 ml mini fermentor. During induction step, feeding 100% methanol as induction medium can only produce less than 50 mg/L scFv while feeding methanol-sorbitol mixture can significant increase the production yield to 306 mg/L in five days, about 6-folds increase in productivity. With the supply of additional nitrogen source during glycerol feeding step or at induction step, higher scFv production with 510 mg/L can be achieved. Thus, following the medium composition optimization, the production titer was improved 10 folds in 250 ml mini-fermentor stage. Moreover, when we switched the induction medium feeding strategy from DO-stat to the stepwise feeding, the titer increased form 510 mg/L to ~1000 mg/L and yielded another 2- folds improvement. During medium composition and feeding strategy optimization at 250 ml mini fermentor scale, the production titer could increase 20 folds. Overall, the production titer increased 400 folds from cell line generation to 250 ml fermentation parameter optimization. Furthermore, the process parameter can be scale-up to 5 L fernentor achieving > 1 g/L. Recent progress to include BIP in the expression vector gave at least 2 fold improvement in scFv titer in shake flask, the new clone will be optimized in our established 250 ml and 5 L fermentation platform.

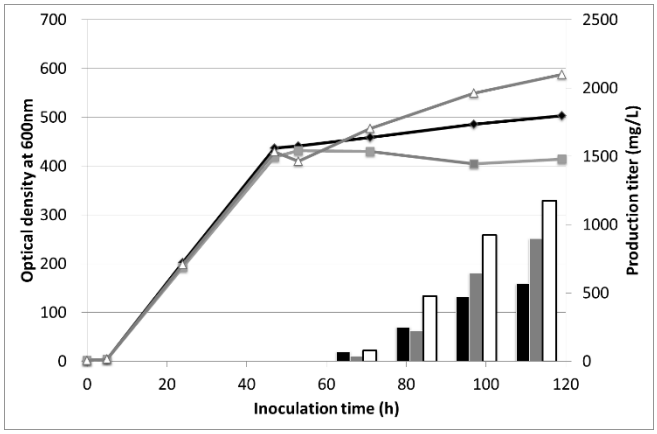


*Figure 1. Biomass optical density profile and production titer using different feeding strategy.*
*White column and triangle, methanol and sorbitol mixture feed by constant feeding rate.*
*Gray column and squared, methanol and sorbitol i feed separately by using constant feeding rate and DO-stat feeding strategy, respectively;*
*Black column and diamond, methanol and sorbitol mixture feed by using DO-stat feeding strategy;*

# REDOX POTENTIAL CONTROL IN ANAEROBIC CLOSTRIDIUM BEIJERINCKII FERMENTATION USING SINGLE-USE VESSELS

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Key Words: Clostridium, redox potential, anaerobic fermentation, single-use fermentor

Redox potential is an important physiochemical factor which measures the tendency of the medium to acquire electrons. In *Clostridium beijerinckii* fermentation, redox potential indicates the status of the $NAD(P)^+$ pool regeneration which directs the electron flow leading to solvent production including butanol. In this study, anaerobic *C. beijerinckii* fermentation was conducted in Eppendorf BioBLU® 3f Single-Use Vessels controlled by the BioFlo® 120 bioprocess control station. The parameters being monitored throughout the fermentation were redox potential and pH using ISM® redox/pH sensors. The objectives of this study were (1) to investigate the effects of redox control on the growth and butanol production of *C. beijerinckii*; and (2) to validate the suitability of the BioFlo 120 and BioBLU 3f Single-Use Vessel for anaerobic fermentation applications. When *C. beijerinckii* was grown without redox control, a continuous change of redox potential was observed in the broth. When fermentation ended at 124 h, the optical density at 600 nm ($OD_{600}$) was 0.8, glucose consumption was 33 % and butanol production was limited. When the redox potential was controlled at -500 mV by redox sensor guided addition of $Na_2S{\cdot}9H_2O$ solution, the $OD_{600}$ was 1.6, glucose consumption was 51 %, and butanol production showed a 2-fold increase. In summary, with the combination of ISM redox sensor and BioBLU Single-Use Vessel, the high variability of redox potential during *C. beijerinckii* fermentation can be actively controlled to drastically increase biomass growth and solvent production.

# PRODUCTION, IMMOBILIZATION AND SYNTHESIS OF PHARMACOLOGICAL DERIVATIVES OF LIPASE B FROM *CANDIDA ANTARCTICA* IN *PICHIA PASTORIS*

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Key Words: Pichia pastoris, production strategy, core-shell supports, CALB, myo-inositol.

Lipase B from Candida antarctica (CALB) is widely used because of its excellent enantioselectivity. Producing this recombinant lipase in Pichia pastoris has advantages since it can be cultured in simple media and can reach high cell densities. This capability is especially important when using a constitutive promoter for lipase production, as here. The $P_{PGK}$ promoter is similar to the well-known $P_{GAP}$ promoter and also circumvents the need for inducing production with methanol, which is a hazard when used on a large scale and would increase the downstream production costs, which could be prohibitive for pharmaceutical products.

This study tested two main fermentation strategies: continuous and fed-batch. In both cultures, different specific growth rates occurred (0.05, 0.10, 0.15 and 0.18$h^{-1}$), and process parameters (qP, qS, YX/S, YP/X, YP/S) were evaluated in order to properly compare them. The highest specific production rate achieved with a continuous culture was 57.71 U/gX.h with $\mu$=0.15 $h^{-1}$ and 16 U/gX.h with $\mu$=0.14 $h^{-1}$ for a fed-batch culture. Productivity decreased dramatically near the $\mu$max (0.18 $h^{-1}$) for P. pastoris (57.6% lower). The best strategy for production was calculated over a three-month period. In both cases, the enzyme is secreted to the supernatant and purification is needed to ensure that only LIPB participates in further reactions. The immobilization process is ideal because purification and concentration is achieved in only one step, reusability is made possible, and in certain cases, stability and efficiency are boosted. Hydrophobic core-shell polymeric supports synthesized by a combined suspension and emulsion polymerization process have shown good potential for lipase immobilization procedures and were used in this study, compared to traditional supports such as Accurel, in order to determine their efficiency.

After the enzyme was immobilized, the reactions included the resolution of (±)-1,3,5-O-benzyl-myo-inositol (DL-1) via acylation using vinyl acetate in hexane, and resolution of (±)-1,2-O- isopropylidene-3,6-di-O-benzyl-myo-inositol (DL-2) via acylation using vinyl acetate (solvent-free system). The support used directly affected the reaction, but trends were observed. In general, the recombinant lipase produced (LIPB) had higher resolutions than the commercial lipase (CALB, Novozym 435). In the resolution of DL-1 and DL-2 via transesterification (using different media), LIPB immobilized in Accurel or PS-co-DVB/PS-co-DVB showed more activity per enzyme molecule than CALB immobilized in similar supports, while when immobilized in PMMA-co-DVB/ PMMA-co-DVB the activities of the two enzymes were similar. The recombinant LIPB immobilized on PS-co-DVB proved to be the most efficient in the enantioselective resolution of both racemic derivatives, DL-1 and DL-2. The productivity for DL-2 resolution was 50% higher than the commercial Novozym 435, and the new derivative was operationally more stable than Novozym 435. The products obtained had a high level of purity (ee of 99% for both derivatives). Both products of the enantio-selective reaction, L-2 and L-5, obtained from the racemic derivatives (DL-1 and DL-2, respectively), are intermediates from different pharmacological pathways involved in the synthesis of building blocks for drugs that inhibit the etiological agent of Chagas disease, Trypanosoma cruzi.

**IMPROVEMENT OF RETINOIDS PRODUCTION IN RECOMBINANT *E. COLI* USING GLYOXYLIC ACID**

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Key Words: Retinoids, Organic acid, Pyruvate, Glyoxylic acid, *E. coli*

Isoprenoids are the most chemically diverse compounds found in nature. They are present in all organisms and have essential roles in membrane structure, redox chemistry, reproductive cycles, growth regulation, signal transduction and defense mechanisms. In spite of their diversity of functions and structures, all isoprenoids are derived from the common building blocks of isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Optimization of IPP synthesis pathway is of benefit to mass production of various isoprenoids. There are two pathways of 2-C-Methyl-D-erythritol-4-phosphate (MEP) and mevalonate (MVA) for IPP synthesis. Prokaryotes including *E. coli* generally use MEP pathway whereas MVA pathway is used in eukaryotes.

To improve isoprenoid production, it was performed the deletion of genes in *E. coli,* which are involved in both formation of fermentation by-products such as organic acids and alcohols, and consumption of precursors of MEP and MVA pathways, pyruvate and acetyl-CoA. As a result, we were able to develop a strain with improved fermentation productivity and carbon source utilization efficiency, the mutant strain was called AceCo. Higher lycopene production was achieved in the AceCo strain compared to the wild type MG1655 strain due to no formation of the inhibitory by-products. However, retinoids production of AceCo strain decreased to a half of that of MG1655 strain.

The decrease of retinoids production was presumed to be related to glyoxylate cycle. Glyoxylic acid was thus added in various concentrations into the culture media. It increased the retinoids production in the AceCo strain by up to 80% after 72 hours when 10g/L glyoxylic acid was added. In MG1655 strain, the supplementation of 1g/L glyoxylic acid improved the retinoids production by 3-folds at 24 hours. This work was supported by the grant (NRF-2016R1A2B2010678 and NRF-2016M1A2A2924237) from the National Research Foundation, Korea.

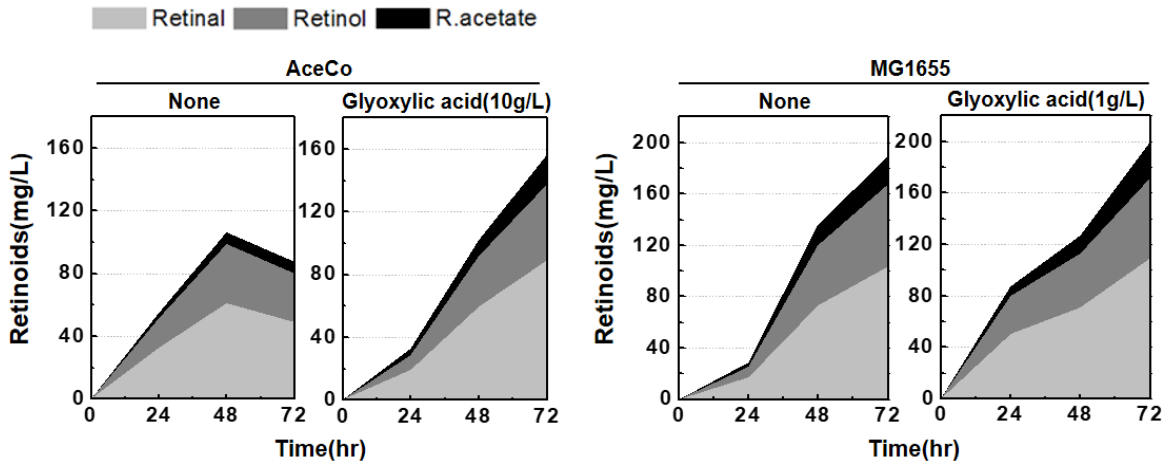


*Figure 2 – Production of Retinoids from AceCo and MG1655 strain harboring pT-DHBSR and pSNA with or without supplementation of glyoxylic acid. Culture was carried out at 30℃ for 72 hours in 2YT medium containing, 2% (v/v) glycerol, and 0.2% (w/v) arabinose with overlay of 5mL dodecane.*

# SEQUENTIAL WHOLE CELL CONVERSION PROCESS FOR PRODUCTION OF D-PSICOSE AND D-MANNITOL FROM D-FRUCTOSE

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Key Words: D-Psicose, D-Fructose, D-Mannitol, Whole-cell conversion, *Corynebacterium glutamicum*

Rare sugars, which exist only limited quantities naturally, have received considerable attention because of its various specific nutritional and biological functions. Likewise, D-psicose (D-ribo-2-hexulose or D-allulose), a C-3 epimer of D-fructose, has many uses which include reducing intra-abdominal fat accumulation, protecting pancreas beta-islets and improving insulin sensitivity. Especially, D-psicose has only 0.3% calories compared to sucrose, while it has 70% relative sweetness. Additionally, in 2012, D-psicose was approved as a food additive and designated as Generally Recognized As Safe (GRAS) by Food and Drug Administration (FDA). Despite such abundant advantages, there is no economical way of mass production of D-psicose. Recently, biological production of D-psicose from D-fructose using D-psicose 3-epimerase (DPE) has been developed. However, the conversion yield is below 30%, which causes an undesirable increase of purification cost because of the similar solubility of D-psicose and D-fructose. Thus, we addressed the problem by converting the residual fructose, after the reaction of D-psicose production, to D-mannitol, which has a low solubility. The sequential whole cell conversion reactions for D-psicose and D-mannitol allow a convenient and economic purification of both products. This work was supported by a grant from the Next-Generation BioGreen 21 Program (SSAC, grant#: PJ01106201), RDA, Korea.

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**OPTIMIZATION OF ISOPRENE PRODUCTION USING A METABOLICALLY ENGINEERED ESCHERICHIA COLI**

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Key Words: Isoprene, C5 terpenoid, Metabolic engineering, Synthetic rubber, Platform chemical

The volatile C5 hydrocarbon, isoprene is an important platform chemical, which has been used in the manufacture of synthetic rubber for tires and also has the potential for various other applications such as elastomers and adhesives. Moreover, isoprene is convertible to biofuel blend stocks such as C10 gasoline, C15 diesel, and jet fuels because of its higher energy content than other biofuels. Although isoprene is currently derived from petroleum, its sustainable supply has been suffered from price fluctuation of crude oil, high refining cost and energy consumption, and low recovery yield of pure isoprene. As an alternative, the biologically produced isoprene (bio-isoprene) has been developed rapidly for the last decade. Bio-isoprene is synthesized from dimethylallyl diphosphate (DMAPP), which is derived from mevalonate (MVA) pathway or the methylerythritol phosphate (MEP) pathway, by isoprene synthase.

In this study, metabolic engineering for enhanced production of bio-isoprene was performed by deletion of relevant genes and optimization of culture condition. In comparison of isoprene production between *E.coli* DH5α and MG1655, lower isoprene production was observed in MG1655. The lower isoprene production in *E. coli* MG1655 was ascribed to the presence of *recA* gene which is absent in the DH5α strain. The deletion of *recA* gene in *E.coli* MG1655 allows higher isoprene production than *E. coli* DH5α. Moreover, the optimized expression of isoprene synthesis pathway with 0.03mM IPTG induction enhanced the isoprene production up to 2,850 mg/L. Overall, isoprene production through the optimization was improved by 28.5-fold compared to the initial production of MG1655 strain.

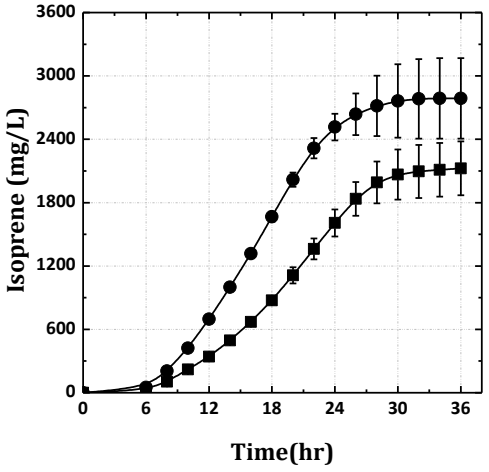


*Figure 3 – Comparison of isoprene production from E. coli MG1655ΔrecA transformed with the pTSNK-sPtispS-MVA and pSNA plasmids (square), and the pTSNK-sPtispS-MVA plasmid alone (circle). Culture was carried out for 36hr in TB medium containing 2% glycerol. The recombinant strain harboring pTSNK-sPtispS-MVA was induced with 0.03mM IPTG.*

This work was supported by C1 Gas Refinery Program through the NRF funded by the MSIP (NRF-2016M3D3A1A0193246).

**PRODUCTION OF α-BISABOLOL FROM METABOLICALLY ENGINEERED ESCHERICHIA COLI**

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Key Words: Bisabolol, RBS modulation, *Escherichia coli*, Metabolic engineering

α-Bisabolol is a natural-occurring sesquiterpenoid with applications in cosmetics as whitening and soothing agent. It is synthesized from the universal precursors, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are generated either through the mevalonate (MVA) pathway or the 2C-methyl-D-erythritol-4-phosphate (MEP) pathway. Farnesyl pyrophosphate (FPP) synthase (IspA) then catalyzes the condensation of IPP and DMAPP to the linear FPP, which is rearranged and cyclized to α-bisabolol by bisabolol synthases.

Here, we compared the capacity of 5 α-bisabolol synthases from *Lippia dulcis*, *Streptomyces citricolor*, *Santalum spicatum*, *Matricaria recutita*, and *Artemisia annua* for α-bisabolol production. MVA pathway and FPP synthase were also overexpressed to supply sufficient FPP for bisabolol synthesis in the recombinant *E. coli*. Bisabolol synthase from *M. recutita* (MrBBS) shows the highest activity of bisabolol synthesis, and 75 mg/L/$OD_{600}$ of bisabolol was produced in a test-tube culture. We further optimized the expression level of IspA and MrBBS by modulation their RBS strength. The 24 bisabolol synthesis operons with different RBSs were assessed for their performance on bisabolol synthesis. By this approach, the best strain is able to produce bisabolol with a capacity of 220mg/L/$OD_{600}$ in a test tube culture. The consequence of host strain optimization led to an increase in bisabolol production to 300 mg/L/$OD_{600}$, which presents a 4-fold increase over the initial engineered strain. This work was supported by a grant (NRF-2016R1A2B2010678) from the National Research Foundation, MSIP, Korea.

**ENGINEERING OF CORYNEBACTERIUM GLUTAMICUM FOR THE SECRETORY PRODUCTION OF RECOMBINANT PROTEINS VIA TAT-DEPENDENT PATHWAY**

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Key Words: *Corynebacterium glutamicum*, Tat-dependent secretion, synthetic promoter, plasmid copy number, fed-batch cultivation

*Corynebacterium glutamicum*, which has been an industrial producer of various L-amino acids, nucleic acids, and vitamins, is now also regarded as a potential host for the secretory production of recombinant proteins since it exhibits numerous ideal features for protein secretion: (i) it has a single cellular membrane as a gram-positive bacterium, which allows proteins to be easily secreted into the extracellular medium. (ii) *C. glutamicum* secretes only a few endogenous proteins into the culture medium, which allows the simpler purification of target proteins in downstream process. (iii), secreted proteins from *C. glutamicum* can be kept stable because extracellular protease activity is rarely detectable. To harness its potential as an industrial platform for recombinant protein production, the development of an efficient secretion system is necessary. To achieve this goal first, we engineered several genetic parts in *C. glutamicum*: (i) synthetic promoters, (ii) plasmid copy number, (iii) signal peptides, (iv) co-expression of secretion machinery proteins. Using the engineered host-vector systems, gram-scale production of recombinant proteins could be achieved in fed-batch cultivation.

# EXPRESSION AND DOWNSTREAM PURIFICATION OF INSULIN MOLECULES IN *PICHIA PASTORIS*

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Key Words: Insulin, Pichia pastoris, polyelectrolyte

In the next decade and beyond global demand for insulin is expected to rise significantly requiring additional manufacturing capacity. The next generation of insulin manufacturing plants will likely be based on new and robust expression and bioprocess platforms that are flexible, safe, simple to implement in a manufacturing setting and capable of step improvements in productivity and cost compared to current manufacturing techniques. Towards this end, *Pichia pastoris* expression systems has been evaluated for insulin due to its capacity to secrete a variety of heterologous proteins and its ability to grow to high cell densities. Under the well-characterized, tightly regulated AOX1 promoter, yields of 1.5 to nearly 3 g/L of purified insulin have been reported.[1,2,3] However, methanol is a very volatile substance requiring specialized facilities, which can hamper large-scale production. Downstream processing of insulin precursors also requires use of organic solvents which can also burden manufacturing. We report development of an insulin process using a constitutive promoter expression system in place of the inducible AOX1 promoter, and a simplified downstream purification process using precipitation. Fermentations were carried out in 2 L scale bioreactors and culture supernatant collected after 65 hours. A design of experiment (DoE) was performed to identify optimal conditions for polyelectrolyte precipitation of the recombinant protein using polyvinyl sulfonic acid (PVS).[4] The resulting pellets were then analyzed via SDS-PAGE and HPLC.

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# REPLACING ANIMAL-BASED HYDROLYSATES IN BIOPHARMACEUTICAL PROCESSES WITH ANIMALFREE AND CHEMICALLY DEFINED ALTERNATIVES TO REDUCE REGULATORY CONCERNS

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Many biopharmaceutical products are routinely manufactured with biological systems (i.e. mammalian and/or microbial cell lines) and consequently, concerns regarding the product's quality, efficacy and safety are raised. Due to the complexity of these "biological systems," many regulatory agencies intensely scrutinize these products prior to approving them for human usage. When working with biological systems, it is presumed that the cultivating medium plays a significant role in product manufacturing; specifically, in terms of product efficacy, quality and titer. To achieve cost efficiency and titer requirements in commercial scale processes, protein hydrolysates are classically used complex sources of nitrogen to support cellular growth and productivity. Over the past century, the main types of protein hydrolysates are typically made from animal-based materials such as milk (e.g. casein), muscle tissue, and organ meats (i.e. heart, brain, spleen). These animal-based hydrolysates have been shown to play a crucial role in vaccine production and food fermentations where high-density, productive cultures are required to achieve the final output. However, with incidences of bovine spongiform encephalopathy occurring over the past decade, regulatory bodies have become more concerned with the use of animal-based materials in fear of transmitting animal diseases to humans. To address these concerns, many hydrolysate suppliers are starting to produce vegetable-based hydrolysates (i.e. soy, wheat, corn, pea, etc.) and yeast extracts. In addition to these vegetable sourced hydrolysates, a handful of companies are developing chemically-defined media and/or supplements to completely remove hydrolysates from media formulations. As a leading, global manufacturer of complex nitrogen sources, Kerry offers a multitude of various protein hydrolysates, yeast extracts and chemically defined media to meet the needs and desires of numerous markets.

# SYNTHETIC BIOCATALYTIC MODULES FOR ENHANCED TRANSFORMATION OF BIOLOGICAL WASTE PRODUCTS

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Key words: solid-binding peptides; silica; enzyme modules; pathway assembly; biomass

Many insoluble materials can be used as carriers for the immobilisation of enzymes. Solid-binding peptides (SBPs) are short amino acid sequences that can act as molecular linkers to direct the orientated immobilisation of proteins onto solid materials without impeding their biological activity [1]. Silica-based materials like silica and zeolite have been found to be suitable matrices for enzyme immobilisation in industrial processes. They are inexpensive, offer high mechanical strength and stability, are chemically inert and can be deployed over a wide range of operating conditions. We have constructed biocatalytic modules that are based on the incorporation of a silica-binding SBP ('linker') sequence into several genes for thermostable enzymes to facilitate the immobilisation of the proteins onto silica-based matrices, enabling the hydrolysis of both simple and complex polysaccharides. We have shown also that the procedure is suitable for the construction of complex enzymological pathways.

In proof of concept experiments, the linker (L) sequence was attached to the N- or C-terminus of three thermostable hemicellulases isolated from thermophilic bacteria using genetic engineering techniques [2]. The resulting L-enzymes remained active after fusion and displayed the same pH and temperature optima but differing thermostabilities in comparison to their corresponding enzymes without linker. The linker facilitated the rapid and simple immobilisation of each L-enzyme onto zeolite, resulting in the construction of 'single enzyme biocatalytic modules'. All three L-enzymes co-immobilised onto the same zeolite matrix resulted in the formation of 'multiple enzyme biocatalytic modules', which were shown to degrade various hemicellulosic substrates effectively in a 'one-pot' reaction.

Cell-free synthetic biology circumvents many of the limitations encountered by *in vivo* synthetic biology by operating without the constraints of a cell. It offers higher substrate and enzyme loading and the facile optimisation of enzyme ratios. Some of the challenges of this approach include costly enzyme preparation, biocatalyst stability, and the need for constant supplementation with co-factors. To overcome these challenges, we have developed a molecular toolbox that facilitates the construction of biocatalytic modules with predefined functions and catalytic properties. It consists of three interchangeable building blocks: (a) low-cost inorganic matrices (e.g., silica, zeolite), (b) matrix-specific SBPs and (c) thermostable enzymes. The rational combination of these building blocks allows for flexibility and a 'pick, mix' and re-use' approach with multiple biocatalytic modules available for the assembly of natural and non-natural pathways. Individual immobilised enzymes can be combined rationally to assemble recyclable and product-specific reactions.

We present preliminary results relating to the construction of two synthetic pathways for the conversion of organic wastes such as coffee and plant biomass. The pathway assembly process allows for rapid evaluation for proof of concept and for assessing the parameters for a synthetic pathway, which are very labour- and time-intensive by the *in vivo* approach.

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# IDENTIFYING THE BEST *PICHIA PASTORIS* BASE STRAIN USING FUNCTIONAL GENOMICS

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Key Words: Functional genomics, *Pichia pastoris*, variant calling, transcriptomics, single nucleotide polymorphism (SNP)

Market sizes for novel breakthrough therapies and growing demand for existing treatments in emerging markets promise to challenge the current capacity for production of biologics. These trends dictate the need for a concomitant paradigm shift in biomanufacturing toward greater productivity for lower cost. Strain engineering is a promising means to realize the greatest returns by increasing the product titer going into downstream processes. Current cellular hosts are approaching saturation of optimal productivity due to lack of deep biological understanding or limitations of the host's intrinsic secretion capacity. We demonstrate an approach informed by functional genomics to understand key performance differences between interchangeably-used variants of the host, *Pichia pastoris*. Genomic variant calling on all USDA-banked and commercially-available strains revealed varying numbers of SNPs relative to the WT strain, Y-11430. Combining transcriptomics and traditional phenotypic assays, the functional impact of these SNPs can inform which host strain is best suited for a given application. Taken together, we have identified key, beneficial SNPs that can be introduced into a WT background to create an IP-free host primed for optimal protein production.

# CASE STUDY: RAMAN IMPLEMENTATION FOR PROCESS LIFECYCLE MANAGEMENT IN FERMENTATION BASED PROCESSES

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Key Words: PAT, RAMAN, spectroscopy.

Existing metabolite analyzers for several inline fermentation based vaccine processes are becoming obsolete in upcoming years. Analyzers are used to support classified parameters and attributes as well as characterization of the fermentation processes. A PAT solution is preferred as a replacement as it would allow for enhanced process understanding and control. Raman spectroscopy has been aligned as a core technology for implementation with varying challenges based on media, organism, processing parameters and attributes being measured. Implementation requires a highly collaborative approach across functions and sites to ensure effective implementation with no interruption to supply. Using a standardized approach to Raman model development and validation, robust models have been developed for 2 product lines with implementation scheduled over the next three years.

# *E.COLI* STRAIN ENGINEERING TO MINIMIZE HOST CELL PROTEIN CONTAMINATION OF RECOMBINANT TARGET PROTEIN

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Microbial hosts are preferentially employed for recombinant protein production, as clone generation is easier relative to eukaryotic host systems. Also, methods for manipulation of microbial genomes are generally more straightforward. Using *E.coli* as a model, we demonstrate a rapid genome engineering method named FAST-GE and compare this method to several other common techniques. Examples of protein expression host genome modification are highlighted where the final objective is isolation of highly pure target protein.

Host cell protein (HCP) analysis is a highly sensitive quality assurance test and is a standard during the purification of therapeutic proteins. In some cases, host genome modification may be necessary to eliminate specific contaminating proteins since contaminant removal by conventional chromatography methods reduces target protein yield too severely. The NiCo21(DE3) strain of *E.coli* is a BL21(DE3) derivative engineered to aid in the isolation of poly-histidine tagged recombination protein. The advantages of employing NiCo21(DE3) for recombinant protein expression will be described.

# SUSTAINABLE PRODUCTION OF β-XANTHOPHYLLS IN *SACCHAROMYCES CEREVISIAE*

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Key Words: Metabolic Engineering, Carotenoids, Xanthophylls, Zeaxanthin, Saccharomyces cerevisiae.

Xanthophylls are a group of $C_{40}$ pigments that belong to the carotenoids family. β-Xanthophylls, such as zeaxanthin, violaxanthin and neoxanthin are derived from β-carotene metabolism, and play a central role in the protection of photo-oxidative damage in plants and algae. These molecules have interesting applications as precursors of commercially relevant natural aromas, like safranal and damascenone. Furthermore, zeaxanthin is also widely used as a nutraceutical to improve ocular health. In this study, we engineered the yeast *Saccharomyces cerevisiae* to biosynthesize zeaxanthin and violaxanthin from glucose. We used integrative vectors to construct a genetic stable β-xanthophylls pathway in a β-carotenogenic yeast strain. To find an effective zeaxanthin biosynthetic enzyme, we compared the titers achieved by bacterial, plant and algal β-carotene hydroxylases. Additionally, we evaluated the effect of the chloroplast transit peptide of plant and algal enzymes on zeaxanthin biosynthesis. The strain that expressed truncated version of *Solanum lycopersicum* β-carotene hydroxylase showed the best performance, reaching up to 4.7 mg/g DCW of zeaxanthin after 72 h cultivation in shake-flasks. Zeaxanthin producing strains were transformed with zeaxanthin epoxidase genes to further extend the pathway to violaxanthin, which was measured by UPLC-MS. To the best of our knowledge, this work presents the highest titer of zeaxanthin in *S. cerevisiae* reported to date, the first zeaxanthin cell factory using β-carotene hydroxylase from plants, and the first heterologous biosynthesis of violaxanthin.

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# COENZYME Q PRODUCTION BY METABOLIC ENGINEERED *ESCHERICHIA COLI* STRAINS

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Key Words: coenzyme Q, ubiquinone, *Escherichia coli*, metabolic engineering.

Coenzyme Q (CoQ) plays an important role as an electron transporter in the respiratory chain. It is formed from a benzoquinone ring and an isoprenoid chain of a specific length depending on the organism. CoQ10 has been used in the treatment of different diseases including Parkinsons, Alzheimer and cardiovascular diseases. In addition, it is used as a dietary supplement and in cosmetic applications due to its important antioxidant property. *Escherichia coli* produces CoQ8 naturally but it is able to produce CoQ10 when an heterologous decaprenyl synthase is expressed. *E. coli* is easy to culture and relatively easy to modify genetically which makes it suitable for the development of an industrial-scale process. In a previous work, we constructed strains unable to produce demethylmenaquinone (DMK) and menaquinone (MK), compounds that compete for both chorismate, precursor of the benzoquinone ring, and the isoprenoid chain. In addition, mutant strains unable to produce enterobactin, high affinity siderophore, synthesized from chorismate, were also constructed. These strains where designed as platforms for the generation of novel CoQ-producing strains. In the present work, the production of CoQ was assessed in the mentioned strains at several culture conditions including the use of different carbon sources (glucose, glycerol and succinate) and different culture strategies (batch and continuous) in a Lab-Scale Bioreactor.

# AUTOMATION AND MINIATURIZATION OF A MICROBIAL FERMENTATION PLATFORM FOR THE PRODUCTION OF ANTIBODY FRAGMENTS

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Key Words: Automation, miniaturization, fermentation.

Automation and miniaturization is currently a hot topic in many industries, and the Bioprocessing industry is no exception. The potential benefits of automating and miniaturizing our Microbial fermentation process development work include reducing our process development timelines by improving our throughput and making better use of available resources.

With these aims in mind, two systems have been implemented in UCB's fermentation process development laboratories, namely the Ambr250 Modular system from Sartorius and the Freedom Evo 200 from Tecan. It has been demonstrated that the Ambr250 modular system is comparable to 5L scale glass STR's over a range of parameters including growth, titre and cell viability. The disposable nature of the system has resulted in a significant saving of man-days per experiment. In combination with this, the Tecan robot has been used to both automate plate based assays and to enable rapid purification of protein samples ready for further product quality analysis to support a QbD approach.

In this work we have considered the challenges that will need to be addressed to reach the ultimate aim of a fully automated miniaturized Upstream Process development platform for E.coli.

# GENETICALLY ENGINEERED PROBIOTIC *E. COLI* NISSLE TO CONSUME AMINO ACIDS ASSOCIATED WITH ORPHAN METABOLIC DISEASES

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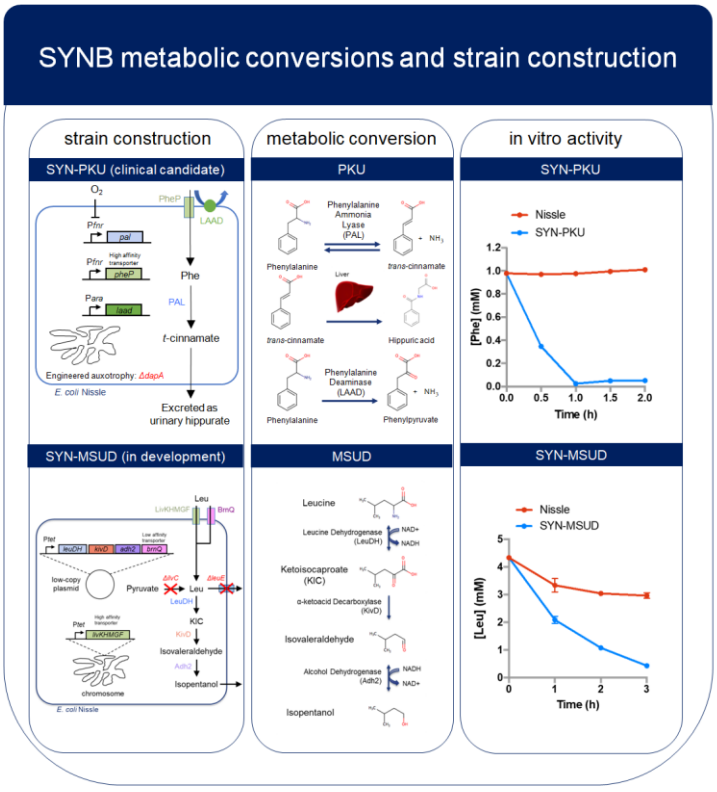
Key Words: Metabolic Disease, Nissle, Synthetic Biology, MSUD, PKU

Orphan metabolic diseases are rare genetic defects that interfere with metabolism due to ineffective or missing enzymes. Two of them, Phenylketonuria (PKU) and Maple Syrup Urine Disease (MSUD) are defined by accumulation of amino acids to toxic levels due to defective metabolism of protein break down products. PKU is caused by a defect in the gene encoding phenylalanine hydroxylase (PAH). MSUD is caused by a defect in a multi-enzyme complex found in mitochondria called branched chain α-ketoacid dehydrogenase “BCKDH”. Without the activity of these enzymes, the amino acid phenylalanine (Phe) in the case of PKU or the branched-chain amino acids leucine (Leu), isoleucine and valine for MSUD build up to neurotoxic levels in the blood and brain, leading to neurological deficits. Current treatment options focus on dietary protein restriction, are insufficient and, unfortunately, can lead to a failure to thrive. Lifelong compliance with a prescription diet is also a concern. We have genetically engineered Nissle, a probiotic strain of E. coli, to reduce serum phenylalanine and leucine levels in patients with PKU or MSUD; preclinical data supporting the activity of these strains are described.




Poster Number 24

# TOWARDS EXTRACELLULAR SECRETION OF RECOMBINANT PROTEINS USING ANTISENSE TECHNOLOGY

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Key Words: E.coli Fermentation, Process Development, Optimization & Characterization.

Biopharmaceutical industries have been exploiting microbial organisms such as Escherichia coli to manufacture recombinant proteins that mostly are intended for therapeutic applications. For extraction of accumulated proteins in the periplasm, a method of cell lysis must take place which has a number of significant drawbacks such as numerous recovery and purification steps which leads to further reduction of the overall yield. Also, as well as the product, contaminants such as DNA and HCP are released which may be difficult to remove. Antisense technology offers a platform that once optimized can reduce the named drawbacks. Antisense RNA can target and inhibit the synthesis of proteins made by the cell, particularly outer membrane proteins in order to facilitate secretion of the recombinant product out of the cell during fermentation. This project aims to investigate the impact of inhibition of synthesis of selected outer membrane proteins on the secretion levels, thus having an effective release system. Various products such as alpha-amylase, Fab fragments have been investigated. In case of high secretion yields, the methodology can be adapted by the industry to eliminate cell lysis steps and the overall number of recovery and purification steps in the manufacturing of recombinant proteins can be reduced. The success of this project will be significantly attractive for the industry and it can lead to a new bioprocess strategy within the industry. Yields of 60% has been achieved, an increase from 10%. Yield defined as % of total product in the supernatant. Potential significance for the industry include elimination of cell disruption steps in a bioprocess, significant reduction in levels of contaminants such as HCP, DNA, proteolytic activity is greatly reduced in the culture medium, reduction in number of unit operations in a bioprocess and reduction in process running cost and time.

# EFFECT OF THE OXYGEN TRANSFER RATE ON OXYGEN-LIMITED PRODUCTION OF PLASMID DNA BY ESCHERICHIA COLI

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Key Words: Oxygen limitation, microbioreactors, plasmid DNA, *Escherichia coli*, *Vitreoscilla* hemoglobin.

Oxygen limitation can increase the pDNA yield in cultures of *Escherichia coli*. Nevertheless, such effect has not been studied systematically. Namely, only cultures at low DOT have been performed, excluding important factors like the oxygen transfer rate (OTR). Moreover, to the best of our knowledge, there is no information regarding the impact of oxygen availability on the topology of the plasmid. The supercoiling of DNA requires energy and it is hypothesized that oxygen availability will affect the produced isoforms. In the present study, we performed fully aerobic and oxygen-limited cultures of *E. coli* bearing a high copy number plasmid. Cultures at $OTR_{max}$ values of 10, 14, 30, 45 (for oxygen-limited cultures) and 110 mmol $L^{-1}$ $h^{-1}$ (for aerobic cultures) were performed in microtiter plates with DOT, pH, biomass (measured as scattered light) and NADH fluorescence online monitoring. To further investigate the impact of oxygen limitation on pDNA topology, an *E. coli* strain constitutively expressing the *Vitreoscilla* hemoglobin (VHb) was used. VHb is known to improve aerobic respiration and consequently ATP generation at low oxygen availability. Our results show that the pDNA yields on biomass ($Y_{pDNA/X}$) were inversely proportional to the $OTR_{max}$ for both strains, and increased more than two-fold in cultures at the lowest $OTR_{max}$, compared to aerobic cultures. Expression of VHb resulted in lower $Y_{pDNA/X}$, compared to cultures of the parent strain. The strain expressing the VHb displayed higher specific growth rates at $OTR_{max}$ of 10, 14 and 30 mmol $L^{-1}$ $h^{-1}$, compared to the parent strain. However, at $OTR_{max}$ of 45 and 110 mmol $L^{-1}$ $h^{-1}$, the growth rate of the parent strain was higher. In general, the specific NADH fluorescence was lower in cultures of the engineered strain, which can be associated to a more oxidized intracellular state, in agreement with the proposed effect of VHb on the cellular metabolism. The pDNA supercoiled fraction (SCF) was maximum in cultures at $OTR_{max}$ of 30 mmol $L^{-1}$ $h^{-1}$, reaching 92.9 % for the wild type strain and 98.7 % for the strain expressing VHb, while no linearized pDNA was detected. This condition was replicated in a 1 L stirred tank bioreactor (STB) for W3110 *recA*$^{-}$, due to the higher productivity of this strain. The performance of cultures in the STB was very similar to that of cultures in the MTP concerning accumulated fermentative by-products, cell growth and pDNA production and SCF.

Altogether, these results show the existence of an optimal $OTR_{max}$ for oxygen-limited production of plasmid DNA. Furthermore, we demonstrate that studies in microtiter plates are excellent to predict culture performance of STB and to scale-up plasmid DNA production cultures.

# METHODOLOGY TO RAPIDLY ASSESS ENZYME CASCADES IN AID OF METABOLIC ENGINEERING OF HOST CELLS

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Key Words: Recycling cascade, sequential pathway, statistical experimental design, chiral amino alcohol.

Chiral amino alcohols are compounds of pharmaceutical interest as they are building blocks of sphingolipids, antibiotics, and antiviral glycosidase inhibitors. Due to the challenges of chemical synthesis we recently developed two TK-TAm reaction cascades using natural and low cost feedstocks as substrates: a recycling cascade comprising of 2 enzymes and a sequential 3-step enzyme cascade yielding 30% and 1% conversion, respectively. In order to improve the conversion yield and aid the host strain engineering we used a combination of microscale experiments and statistical experimental design. For this we implemented a full factorial design to optimise pH, temperature and buffer type, followed by the implementation of Response Surface Methodology (RSM) for the optimisation of substrates and enzymes concentrations. We achieved 60% conversion for the recycling cascade and 3-fold improvement on the sequential pathway. Based on the results, limiting steps and individual requirements for host cell metabolic integration were identified expanding the understanding of the cascades without implementing extensive optimisation modelling. Therefore, the approach described here is ideal for exploratory work or when the interest is in defining the enzymatic expression levels required for microbial cell factories development.

**ENHANCING THE PRODUCTIVITY OF SUPERCOILED PLASMID UPSTREAM BIOPROCESSING THROUGH PLASMID ENGINEERING.**

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Key Words: Plasmid Bioprocessing; Supercoiling density; Plasmid yield; Plasmid Engineering; *E. coli*

This study was set out to develop an approach for producing highly supercoiled plasmid DNA. Potentially, the level of supercoiling can have an impact on ease of downstream processing. A 7.2kb plasmid was developed by cloning of Bacteriophage-Mu Strong gyrase-binding sequence (Mu-SGS) into 6.8kb pSVβ-Gal. Four *E. coli* strains were transformed with both the modified pSVβ-Gal398 plasmid and pSVβ-Gal. Small scale fermentations and analysis were carried out in triplicate cultures to screen for best performing strains. Two of the four strains selected amplified the plasmids efficiently. There was over 20% increase in the total plasmid yield with pSVβ-Gal398 in both strains. The supercoiled topoisomer content was increased by 5% in both strains leading to a 27% increase in the overall yield. The two strains were investigated further in shake flasks. Increases in supercoiling and plasmid yield were also observed. The extent of supercoiling was examined by superhelical density quantification, with pSVβ-Gal398 maintaining a supercoil density of -0.022 and pSVβ-Gal -0.019 in both strains. The compactness of the plasmid DNA was also quantified by hydrodynamic diameter measurement using the Nanoparticle Tracking Analysis (NTA) and it was observed that pSVβ-Gal398 was more compact with a $D_h$ of 40-59nm compared to pSVβ-Gal with $D_h$ of 70-90nm for both strains examined. The report of this study has shown that plasmid engineered to contain the Mu-phage SGS sequence has a beneficial effect on improving not only the yield of total plasmid but also the supercoiled topoisomer content of therapeutic plasmid DNA during bioprocessing.

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# DEVELOPING BACTERIAL MICROCOMPARTMENTS FOR THE RECOMBINANT PRODUCTION OF PROTEINS

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Key Words: bacterial microcompartments, recombinant proteins, disulphide bonds, bio-therapeutics

In prokaryotes, supramolecular self-assembling protein structures, known as bacterial microcompartments (BMCs), have evolved to encapsulate proteins associated with a number of different metabolic processes, providing a physical diffusion barrier whilst increasing local enzyme concentrations. The modular nature of these structures makes them promising biological platforms for the engineering of synthetic compartmentation within the bacterial cell that have potential to be used as novel nano-bioreactors [1-3]. We are interested in the development of robust synthetic BMC technologies that can be utilised for industrially relevant applications, specifically the spatial segregation of synthetic enzyme cascades for the enhanced production of valuable chemical compounds. BMCs may also be valuable in the production of recombinant proteins. Many therapeutic proteins and antibody fragments require disulphide-bonds for correct folding and activity. Here, we are exploring the potential of BMCs to serve as synthetic cellular organelles within the bacterial cytoplasm of *E. coli* that promote correct protein folding and disulphide bond formation of recombinant proteins, providing an alternative method to traditional approaches (folding in the eukaryotic endoplasmic reticulum and in the periplasm of prokaryotes). Previously, it has been shown that proteins of interest can be compartmentalised by fusing them to targeting peptides, which direct the proteins to the microcompartment shell [4, 5]. In this work, both, the shell (PduABJKNU) and the targeting peptides (P18/ D18) are derived from the 1,2- propanediol utilisation (Pdu) BMC from *Citrobacter freundii* [4]. We first determined the effect of fusing short targeting peptides onto the *E. coli* alkaline phosphatase PhoA, a protein widely used to examine disulfide bond formation *in vivo*, and the sulfhydryl oxidase Erv1p, a catalytic enzyme for the formation of disulphide bonds. The most active fusion proteins were selected for co-production with the BMC shell. For efficient recruitment of these proteins to the BMC, gene expression levels were controlled using tunable promoters and recombinantly produced BMC variants were analysed *in vivo* and *in vitro* using biochemical and biophysical methods. We demonstrated that both, PhoA and Erv1p, are targeted to recombinant BMCs and determined disulphide bond formation of PhoA in the presence and absence of Erv1p when targeted to the microcompartments. Using this approach, a range of other proteins of industrial interest will be tested and the potential for the production and purification of bio-therapeutic proteins and antibody fragments will be determined.

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# FROM SCREENING TO PROCESS OPTIMIZATION: AMBR TECHNOLOGY TO SPEED UP MICROBIAL FERMENTATION PROCESSES

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Session proposals:
- Therapeutic Proteins
- Vaccines

The development of biopharmaceuticals or biotechnological products derived from microbial fermentation is a financially risky endeavor and time consuming process, requiring technical upstream solutions which reduce timelines, increase efficiency, and raise likelihood of success. We have identified in particular the early steps of strain and process development offering best prospects to speed up the entire process significantly by using a reliable screening system. Based on the well-proven ambr® principle we designed with ambr 15 fermentation system to accelerate early stage development of microbial fermentation products. The multi-fermentation unit mimics larger scale bioreactor processes, and is suitable for screening clones, strains or growth conditions. In case studies with industrial partners using *E. coli* and *P. pastoris*, consistent and efficient control of fermentations across a variety culture conditions (e.g. feed, temperature, duration, pH) could be demonstrated. In the succeeding step of process development ambr 250 has been widely applied to speed up the 2$^{nd}$ critical phase of the microbial upstream process development. The larger working volume and the range of features, which this multi-parallel system offers, are superior to common benchtop fermenters. Optical density supervision, off gas analysis, fed-batch processing and advanced control capabilities allow process development for most commercial-scale upstream fermentation processes. In addition to this impressive range of features ambr250 has proven its ability to reliably increase the efficiency of fermentation process development many times through its rapid setup and cleanup, advanced control software, and automation.

# ENUMERATION METHOD AND MEDIUM DESIGN FOR A MIXED CULTURE OF SACCHAROMYCES CEREVISIAE AND CHLORELLA VULGARIS

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Key Words: flow cytometry, autofluorescence, autotroph, heterotroph, symbiosis, photo-bioreactor

The scientific literature shows a rising interest in studies on symbiotic mixed cultures as an innovative bioprocess to increase biomass and lipid productivity. The main issue with mixed cultures appears to be the dominance of one organism over the other during cultivation. In the current work, a methodology is proposed to develop a co-dominant mixed culture of *Saccharomyces cerevisiae GFP* and *Chlorella vulgaris*, in which their growth would be based on mutual symbiosis through recycling $O_2$ and $CO_2$. The first challenge was to develop a rapid and accurate method to distinguish and enumerate each population. We confirmed a method based on flow cytometry to enumerate simultaneously *S. cerevisiae GFP* and *C. vulgaris* through their autofluorescence (GFP protein and chlorophyll respectively). The second challenge was the design of a medium adapted to both organisms and likely to avoid the dominance in mixed culture. The newly designed medium was formulated by combining components from the microalgae growth medium (Bristol medium) and the yeast growth medium (YPD). Monocultures of *S. cerevisiae GFP* and *C. vulgaris* in the newly-designed medium were grown in a 5-liter photo-bioreactor. The designed medium limited yeast overgrowth and enhance the maximum microalgae population and its specific growth rate. The enumeration method and strategy for medium design proposed here can be adapted to bio-processes involving mixed cultures between an autotrophic and a heterotrophic microorganisms.