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Probiotics derived from circular feedstock via lactic acid bacteria and yeast fermentation

Stanislav Rudnyckyj, Laura Sini Sofia Hulkko, Mette Hedegaard Thomsen.

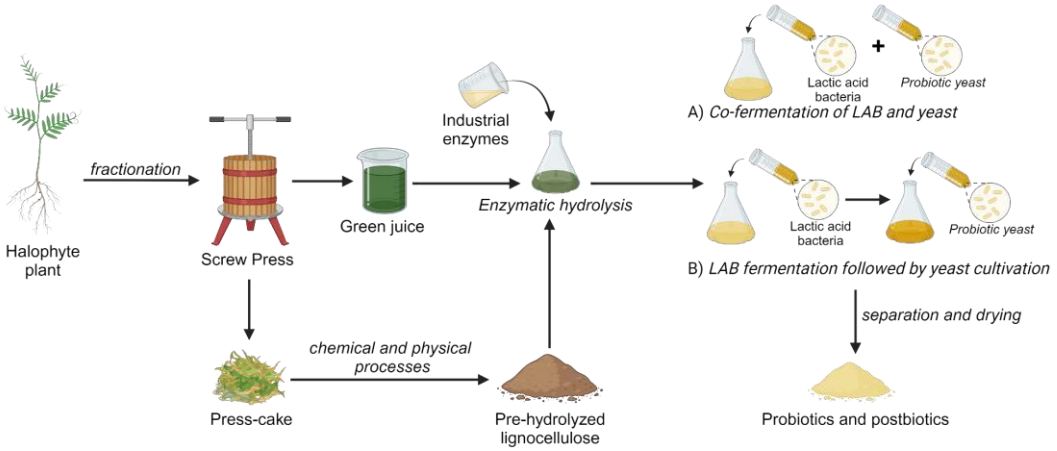

Figure 1: Investigated biorefinery concept of residual halophyte biomass

Halophytes are emerging supercrops due to their large diversity and natural adaptivity. Their main benefit lies in their suitability for cultivation in marginal lands, thus avoiding competition with traditional farm crops [1]. Moreover, due to their ability to tolerate and accumulate salt, they are used for the phytoremediation of salt-affected soils [2]. Besides their use in saline agriculture, they have also proven to be a promising species for CO_2 capture [3]. There is significant interest in halophytes due to their abundance in high-value bioactive compounds such as phenolics, carotenoids, vitamins, and more [4]. In a halophyte-based biorefinery, the focus is on extracting high-value compounds from juiced fibers. The leftover green juice can serve as a medium for enzymatic hydrolysis and fermentation, while the extract-free halophyte lignocellulosic fibers can be enzymatically saccharified and fermented with probiotic bacteria and yeast to produce functional animal feed, a process investigated in this study. To conclude, commercializing halophytes could greatly enhance soil quality, prevent further soil degradation, and take advantage of their natural carbon sequestration properties, while also leading to the production of high-value compounds and functional feed.

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Machine Learning for Advanced Growth Media Optimization with a Fully Automated Microbioreactor

Frédéric M. Lapierre[1,3], Dennis Raith[2,3], Mariela Castillo-Cota[2,3], Jonas Bermeitinger[3], Robert Huber[1]

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Bioprocesses play a pivotal role in the efficient manufacturing of specialized products such as antibiotics and enzymes. Selecting appropriate microbial strains and optimizing culture conditions are vital to ensuring the economic feasibility of these processes. One major hurdle is developing a fermentation medium that meets the specific nutritional demands of the strain, a task that is often labor-intensive and timeconsuming. Currently, Design of Experiments (DoE) is the standard approach used for growth media development. Although DoE helps to reduce the number of experiments, this approach has its limitations, particularly when addressing nonlinear systems. In addition, two-stage DoE processes (screening and optimization) may discard factors too early, deeming them irrelevant before they can prove significant in later optimization stages. In this context, Machine Learning (ML) algorithms for optimization offer a potentially more efficient alternative.

We introduce a fully automated microbioreactor system that autonomously mixes nutrients from various stock solutions and tests their efficacy through iterative cycles, removing the need for manual adjustments. The system integrates a BioLector microbioreactor, which facilitates the parallel cultivation of 48 distinct cultures in a microtiter plate while simultaneously tracking key bioprocess parameters such as biomass, dissolved oxygen (DO), and pH levels in real time. The OT-2 liquid handling system automates tasks such as medium preparation, plate sterilization, and inoculation.

During the initial experiment, 48 different media compositions are determined using a DoE-based approach. After the first cultivation cycle, the system automatically cleans and sterilizes the microtiter plate, preparing it for the next round of medium development. From the second cycle onward, the medium compositions are generated by an ML algorithm using Bayesian Optimization. Fresh pre-cultures are automatically introduced into each of the 48 growth media, and this process is repeated until an optimal medium formulation is identified.

The preliminary data sets are promising. The chosen model organism was MICPrelevant bacterium *Sporosarcina pasteurii*. The ML-optimized medium showed a 34% improvement in the maximum backscatter value compared to the medium optimized through DoE. The ML algorithm is set to be further diversified and applied to additional strains to validate its broader applicability.

Assessing the physiological, regulatory and gene expression changes affecting global microbial metabolism upon fermentation process upscaling: a multiomics study

Laura García Plaza, Lei Yang, Rasmus John Normand Frandsen, José Luis Martinez Ruiz

The successful scaling of fermentation processes from laboratory to industrial scales is crucial for optimizing microbial production of valuable chemicals. However, scaleup introduces biological, chemical, and physical challenges that can lead to reduced microbial performance under industrial conditions. A deeper understanding of microbial physiology and metabolism during scale-up is essential for addressing these issues.

This study employs a multi-omics approach, integrating transcriptomics, proteomics, phosphoproteomics, and metabolomics, to investigate Escherichia coli, a model industrial organism, in conditions that mimic large-scale reactors. By combining genome stability and transcriptional data with high-resolution mass spectrometry, we aim to capture the complex signalling networks and metabolic changes that occur during fermentation upscaling.

In particular, we focus on a previously genetically engineered *E. coli* strain designed to produce high titers of tyrosine, a precursor for sustainable chemicals. Initial work on this strain, has shown high production efficiency in lab-scale fermentations, but the fermentation process has not been optimized carefully yet.

Through this project, we aim to use systems biology approaches to better understand the physiological adaptations and robustness of microbial host that occur during scaleup. Providing these insights will enable the rational design of more efficient, scalable fermentation processes for sustainable chemical production.

Extraction of intracellular green fluorescent protein from *Escherichia coli* with hydrophobic deep eutectic solvents

Tjalling Gijsbert Tjalsma, Philipp Pably, Yannick Patrice Didion, Ziran Su, Magdalena Malankowska, Julian Kager, Manuel Pinelo

As the fermentation industry is expanding, it is vital to develop sustainable downstream processing methods of intracellular compounds. In comparison to other solvents employed in solid-liquid extraction, deep eutectic solvents (DESs) possess distinct characteristics that enhance their sustainability. Hydrophobic deep eutectic solvents can act as cell wall permeabilization agents and hence initiate intracellular compound release. Hydrophobic DESs may be more effective than hydrophilic DESs in downstream processing because they can form a bilayer system with water, enhancing separation and extraction efficiency. However, the technique has been studied poorly because only limited sets of DESs and cellular compounds have been used. Therefore, the primary objective of the presented study was to assess the potential of hydrophobic DESs for intracellular protein extraction of *Escherichia coli* containing intracellular green fluorescent protein (GFP). Particular attention was given to the characteristics of the DESs related to their extraction performance, which was measured by the total derived protein content and the GFP yield. These findings can inform future applications of sustainable downstream processing of fermentation broths or other biomasses.

The saltier, the better: a deep look into salt stress tolerance in yeast through adaptive laboratory evolution

Pablo Torres-Montero, Gloria Muñoz-Fernández, José L. Martínez

The non-conventional yeast *Debaryomyces hansenii* is a species commonly found in various environments such as dairy products, fermented foods, or marine habitats. Its importance and interest lie on its remarkable stress tolerance capabilities. Its halotolerant/halophilic behaviour has put it on the spotlight as eukaryotic model organism for osmotic and salt tolerance. In addition to salty environments, this yeast can thrive in diverse and challenging environments. It can utilize numerous different carbon sources and grown in a wide range of pH and temperatures. However, despite being reported to grow in the presence of up to 4 M sodium chloride concentrations (as a reference, sea water is roughly 0.5 M), the mechanisms behind this noteworthy ability, their internal relationships, and how to link them to pH and temperature are pieces of a bigger puzzle which is still to be assembled. For these reasons, we propose an adaptive laboratory evolution approach to decipher sodium adaptation mechanisms while also harnessing the potential of *D. hansenii* for practical applications in biotechnology.

Adaptive laboratory evolution (ALE) is a powerful technique that allows us to investigate how organisms adapt to new environments over time. It involves subjecting populations of organisms to controlled conditions for multiple generations, allowing the observation of evolutionary changes along the way. We postulate *D. hansenii* as candidate for ALE, talking the advantage of its innate tolerance towards salt, and trying to push it by using sodium chloride concentration as the main selective pressure. Different experimental configurations will be tested, from traditional shake flask serial passage to instrumented bioreactors, and compared. Growth characterization will be used to initially assess the phenotypic characteristics of the resulting strains. These data are to be combined later with omics data, and an integrative analysis will be executed to identify newly acquired characteristics and trade-offs resulting from the evolution process.

Optimal feed rate to induce Crabtree effect

Kasper Weinreich Rask, Tom Schmidt Hansen, Ana-Helena Caetano, Ioannis Skiadas, **Julian Kager**

DTU, Chemical and Biochemical Engineering, Pilot Plant

S. cerevisiae is a widely used organism in biotechnological production and as a model organism for education. The yeast can growth anaerobically where sugar is reduced to ethanol but also aerobically with full reduction of sugar through the TCA cycle.

In addition to that the yeast shows also the so called “Crabtree” effect where under aerobic condition and high sugar availabilities ethanol is produced as byproduct [1]. In aerobic cultivations the Crabtree effect needs to be avoided to ensure full conversion of sugar into biomass and product without the formation of ethanol. One way to avoid this is a strict limitation of sugar by employing a low nutrient feed rate or a low dilution rate in continuous cultivation. On the other hand, this strict limitation yields in low turnover rates.

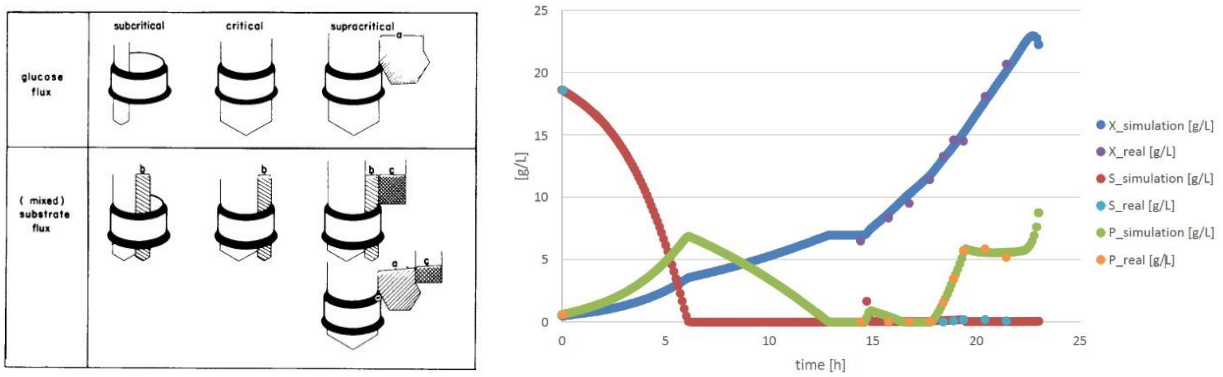

Figure 1 Different metabolic pathways of S. cerevisiae and an exemplary dynamic experiment and model fit including all metabolic states

Within this thesis best nutrient addition strategies are explored to find the best trade-off between high growth and turnover rates and low ethanol production through the “Crabtree” effect. The Study uses wildtype *S. cerevisiae* in batch and fed-batch operation with different feed addition rates. The growth, respiratory quotient, substrate uptake and ethanol production rate are determined for the different feed phases.

Based on the collected dataset a commonly used kinetic yeast model [1] is fitted to find out physiological properties of the strain and the model is used to propose optimal (interesting) feed regimes, which are potentially verified in Lab-scale. This model assisted process development strategy ensures to find out best dynamic feed supply with a lower amount of expensive experimentation.

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Use of cpGFP to monitor the real-time signal response of bacterial stress during fermentation processes

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This study addresses challenges in bacterial fermentation, including monitoring the accumulation of cAMP, indicative of glucose availability. Through real-time measurements, we anticipate gaining insights into fermentation dynamics and facilitating the timely adjustment of process parameters.

Glucose is often used in fermentation as a carbon source being readily metabolized by bacteria. Limitation of glucose in *E. coli* triggers the synthesis of cyclic AMP (cAMP) which serves as an activator for the transcription of other-sugar genes in the carbon catabolite regulation. Alterations in glucose availability and consequent shifts in gene regulation during the fermentation processes might decrease the productivity and product yield.

In this work, we make use of a biosensor based on a circularly permuted fluorescence protein (cpFP)[1] previously developed for real-time monitoring of cAMP in living eukaryotic organisms[2]. The biosensor comprises a rearranged fluorescent protein (cpGFP) fused to a cAMP-binding domain, the cyclic nucleotide-binding domain (CNBD) derived from a gram-negative bacterium, *Mesorhizobium loti*. Upon binding to cAMP, CNBD changes conformation and enhances fluorescence emission, enabling real-time detection of cAMP concentration in the cell.

Circularly permuted fluorescent proteins represent a class of rearranged proteins that maintain their native fold and function while offering versatility in sensor design. Although extensively studied in eukaryotic cells, particularly mammalian systems, our work introduces the application of cpFP-based biosensors in bacteria. The cpFP-based biosensor is therefore expressed in a low copy plasmid vector suitable for *E. coli, B. subtilis* and lactic acid bacteria, and the detectable shifts in fluorescence intensity were observed from a constitutively high transcribed promoter.

Quantification of fluorescence intensity (or cAMP synthesis) was followed during exponential growth in micro bioreactors (Biolector II) in a population of cells exposed to different growth conditions, with or without glucose. Our preliminary results focus on the functionality of cpFP-based biosensors in *E. coli*. These biosensors were expressed through highly expressed-inducible promoters. Further enhancements to the biosensors involve identifying bacterial promoters that alleviate cellular burden and adjusting the metabolite binding site to match the range of intracellular metabolite levels. Once optimized, these biosensors hold the potential to monitor and provide insights into cellular stress at a single-cell level, which will be tested in in picoscale in a dynamic microfluidics device in collaboration with the University of Bielefeld and in a bubble microreactor in collaboration with TU Braunschweig.

Keywords: Biosensor, circularly permuted fluorescence protein, carbon catabolite regulation.

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Laerke.eu: An Online Pressure Sensor System for Monitoring Gas Formation and Consumption in Vials

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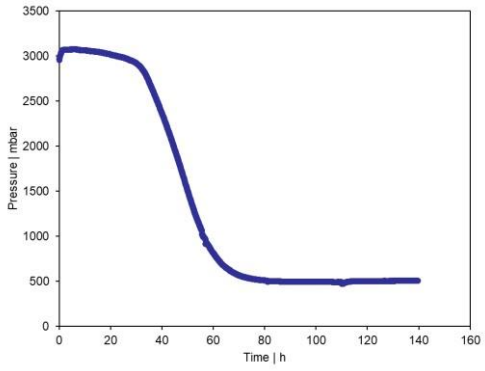
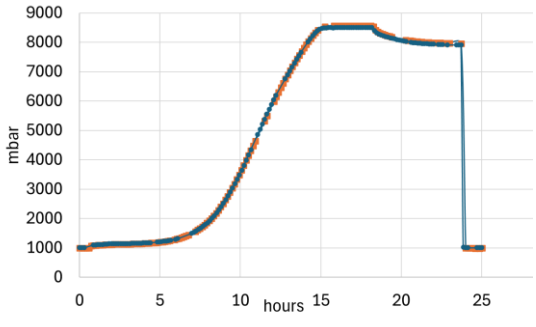
Lærke is a pressure sensor system developed by DTU Biosustain in collaboration with FabLab RUC. It enables real-time, non-invasive pressure measurements in vial experiments, offering a continuous online data acquisition method.

The system ensures high-frequency data collection without the risk of contamination or interference that can arise during manual sampling, particularly in microbial experiments. This capability facilitates the detailed analysis of gas formation and consumption in both biological and chemical experiments. Lærke is particularly suited for studies investigating microbial H_2/CO_2 consumption, methane production, or CO_2 formation during fermentation processes involving yeast or other microorganisms. When integrated with online optical density (OD) measurements, it offers comprehensive insights into the interplay between biomass growth and gas-uptake dynamics in small-scale vial experiments. Each system is built using a Wi-Fi-enabled microprocessor connected to three high-accuracy pressure sensors mounted on a custom-designed printed circuit board (PCB). Sterile, replaceable needles are attached to each sensor. The microprocessor samples pressure data, which is stored locally and can be accessed via Wi-Fi or transmitted to a server for real-time plotting and visualization. The system operates within a pressure range of 0-30 bar, with a resolution of 0.2 mbar. All data is stored in CSV format, facilitating manual analysis.

Lærke provides a high-resolution, cost-effective solution for monitoring vial-based experiments, offering an accessible alternative to larger bioreactors equipped with online mass spectrometry for gas analysis.



Mixed microbial community cultivated in minimal medium with synthetic syngas at 60°C. Initial pressure: 3000 mbar; final pressure: 500 mbar.



Yeast culture grown in a medium containing 80 g/L glucose and 80 g/L yeast extract. Pressure increases from 1000 mbar to 8500 mbar.

This work is part of The Fermentation Based Biomanufacturing initiative (FBM) funded by the Novo Nordisk Foundation. Grant number: NNF17SA0031362.

Evaluating the Capability of MidInfrared Spectroscopy for RealTime Prediction of Substrate and Metabolite Concentrations in Fermentation Processes

Maria Eleni Mazaraki* and Meryem Irem Sipahi*,Christos Panos" Carlos Casado Vazquez", Krist V. Gernaey*, Julian Kager*

**DTU, Chemical and Biochemical Engineering*

"Novo Nordisk A/S

This study focuses on the evaluation the capability of MidInfrared (MIR) spectroscopy for realtime prediction of substrate and metabolite concentrations in fermentation processes. The objective was to develop and validate calibration models that accurately predict concentrations of key metabolites such as acetate, ethanol, glycerol, and glucose in a fedbatch Saccharomyces cerevisiae fermentation process. Four Partial Least Squares (PLS) models were tested to evaluate their potential for realtime concentration predictions and monitoring. The models were developed using datasets comprising pure synthetic samples, mixed synthetic samples, and inline fermentation data. The most accurate predictions were achieved using inline spectral data from actual fermentations, highlighting the importance of representative training data. The study underscores the significance of using highquality, representative training data that captures the true variability of industrial fermentation processes, along with robust modeling techniques.

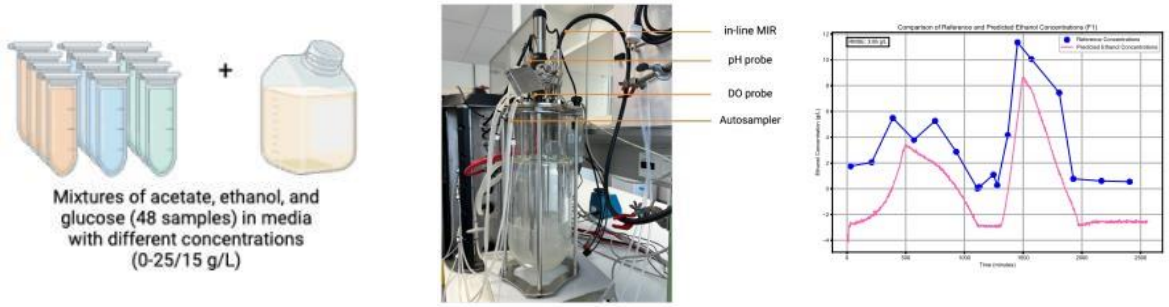


Figure 1 establishment of PLS models with synthetic mixtures and application on real bioreactor data

The findings are particularly relevant for the application of chemometrics in Process Analytical Technology (PAT), illustrating the potential of PLS models to enhance process efficiency and product consistency through realtime monitoring and control. Future research directions will include integrating advanced machine learning algorithms with PLS models to handle complex, nonlinear relationships, expanding training datasets to include a broader range of fermentation conditions, and ensuring transferability of the successful prediction models across various MIR spectroscopic instruments. The integration of sophisticated realtime data acquisition and processing technologies, will be crucial in advancing bioprocess monitoring and optimization.

On-farm testing on a Stick.

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Abstract

We describe an easy to use 'cow side' –test, which the dairy farmer will be able to use in his daily routine in order to identify which animals to treat with antibiotics and which not to treat.

Keywords: On-farm analysis, mastitis test, Gram-negative, Gram-positive, decision tool, cow side test, lab-on-a-chip, point-of-need, point-of-cow, BACT.

1. Background

The need for diagnostic tools in connection with the day-to-day -decision making in the modern milk production facility, has been eminent for years, however - easily accessible tools have been absent.

On-farm analysis using agar-plates has been ported from the veterinary's laboratory and into the barn. The limited success of said otherwise rugged and proven technology, stems from the fact that associated processes, routines and standards that may be considered "first page in the book" for vets and trained laboratory technicians, may pose a hindering barrier for the average farmer and his staff. Today's dairy farmer is experiencing stress from all aspects of their business, so the efforts associated with adopting new tools and procedures should be minimal, otherwise they are not adopted into the daily routine. We have devised a "stick test", that – in terms of ease of use – are akin to a pregnancy test from a super market. In line with the before mentioned level of stress, it is our belief, that the test should provide a simple answer, and that the availability of multiple tests for *different* situations and – not least - for different farmers, is the way to achieve widespread usage of on-farm –analysis. This first test is thus a Gram-positive/negative –test that will provide two simple answers:

Are a given set of **mastitis symptoms** caused by bacteria?

And – if so – are they caused by **Gram-negative** or a **Gram-positive** bacteria?

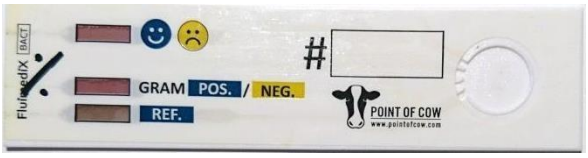
Provided with the answer to these questions, the farmer will be able to make a decision on whether or not to treat a given quarter, as a (mild) Gram-negative bacterial infection is often cleared by the animal *without* antibiotic treatment and because penicillins have little or no effect on Gram-negative bacteria.

In most cases, this is all the answer you want, and you want it without being dependent of making arrangements with and sending milk samples of to - external laboratories.

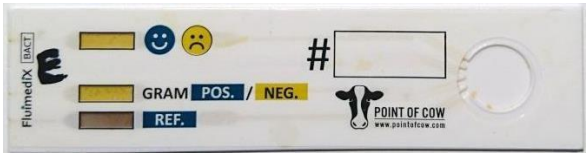
2. FluimediX' Cow side test - BACT

Our test is based on promoted – respectively – suppressed growth of mastitis related bacteria; additionally it has been an object to provide a test with a prolonged storage and with all necessary components included in a stabilized matrix in the test stick itself. The stabilized and lyophilized component matrix is deposited in an injection molded microfluidic structure; the shelf life is **+12 months**. The microfluidic structure additionally comprises a filter structure, which will prevent clumped milk from obstructing the filling of the structure.

BACT – test stick (Gram +/-)



BACT ‘negative’ – no growth



BACT ‘positive’ - ‘E.Coli’

The test stick is filled with 150µl milk via a single use Pasteur pipette, where after it is **incubated 10 to 16 hours**, either in a standard incubator or in our dedicated USB-powered “pocket incubator”

The test result is – as depicted above – clearly identifiable by the naked eye as either ‘**blue’** or ‘**yellow’**.

Developing a kinetic model for the expression of rProt monomer

Ruo-Xian Zhou, Dalimil Ott, Xiyan Li, Krist. V. Gernaey PROSYS, DTU

Antonio Gaetano Cardillo, GSK

Inno4Vac is a collaborative initiative between the public and private sectors aimed at overcoming scientific challenges in vaccine development. The project seeks to create predictive biological and mathematical models to enhance vaccine performance and bio-manufacturing processes. The rProt monomer, is an antigen that plays a crucial role in the bacterium's ability to evade the human immune system by binding to human factor H. Together with GSK and EVI (European vaccine initiative), DTU aims at developing a kinetic model for the fProt expression in an E. coli process based on small-scale bioreactor data. In this poster, we will present the optimal density (OD) and the growth rate model against the experimental data. Apart from that, we will also include different metabolic pathways in the future work.

FermentDB: A Database for High-cell Density Fermentations

Txell Amigó, Novo Nordisk Foundation Center for Biosustainability

Industrial fermentation leverages microorganisms for the synthesis of valuable products. These processes serve as a pivotal technological asset for reducing our dependence on chemicals and products derived from fossil fuels.

Employing high-density fermentation strategies proves to be a cost-effective approach for achieving optimal yields in biomass, extracellular metabolites, intracellular components, or modified substrates. The rate in which fermented products are generated is contingent upon various factors such as concentration of microorganisms, cell density, cellular components, enzymes, temperature, and pH. These systems are difficult to design, operate, and scale up and down, which has a direct impact in crucial parameters such as product titer, rate, and yield. The absence of high-quality fermentation data hinders optimization and prediction strategies that could otherwise mitigate these challenges.

Our goal is to establish a standardized and aggregated database, consolidating highdensity fermentation data for accessibility within the scientific community.

The database will harmonize a previously generated comprehensive dataset from more than 600 fermentations using different strains under varied conditions to produce melatonin and tryptophan. Furthermore, this resource will allow the analysis and visualization of these experiments as well as streamlining the integration of new fermentation datasets. By compiling comprehensive data, we aim to expedite the development of "fermenterphiles" and enhance bioproduction, marking a significant stride toward sustainable industrial practices.

Holistic process understanding and intensification of the Ltyrosine production in E. coli

Mykhaylo Semenov[1] Petrov, Deborah Pfaff[2]

[1]Novo Nordisk Foundation Center for Biosustainability

[2]DTU Chemical Engineering

These interdisciplinary PhD projects are part of the FBM initiative (Novo Nordisk Foundation) will focus on the holistic process understanding and intensification of Ltyrosine production in *E. coli*. A microbial cell factory for L-tyrosine is highly valuable as it has a role as a precursor for several commercially important compounds including pharmaceuticals like L-DOPA, cosmetics and skin care products that are rich in melanin or nutritional supplements like flavenoids and stillbenoids.

To determine the microbial performance the project aims to analyze the process from upstream to downstream at different scales. System biology approaches are deployed to better understand the physiology change and robustness of microbial hosts mimicking industrial fermentation conditions where substrate and oxygen gradients, among others, are present.

As the design and performance of the downstream process is heavily influenced by upstream conditions, such as media composition and product location, the interlinking between both phases will be investigated. Furthermore, modelling of single unit operations is used to increase the process understanding as well as simulating and planning future experiments.

Our combined goal is to develop a pipeline where you can scale up a process by scaling it down first and establish key decision points where the impact on other process stages needs to be considered, potentially saving both time and costs in the development of a bioprocess.

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Deletion of BGCs in *streptomyces* for the construction of a chassis strain

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Abstract:

The genus *Streptomyces* is well known for producing specialized metabolites that exhibit wide spectrum of bioactivities, many of which are encoded by biosynthetic gene clusters (BGCs) (Lee et al., 2020). However, a significant number of BGCs remain cryptic in native strains, being necessary to develop methods to express these yet undiscovered compounds (Rutledge et al., 2015). A reliable option is the construction of a chassis for synthetic biology, for the reconstruction of synthetic cellular behaviour and efficient implementation of biomanufacturing (Yan et al., 2024). This project aims to develop a *Streptomyces* chassis strain by systematically deleting endogenous BGCs using three different methods of CRISPR/Cas: Cas9, Cas3, and cBEST; as well as evaluating each method for efficiency, precision, and potential off-target effects (Whitford et al., 2023).

To fulfil this objective, it is proposed to begin with the bioinformatic identification of BGCs within a new native *Streptomyces* strain, using tools such as AntiSMASH for detailed annotation. The three CRISPR methods offer unique advantages, such as double-strand breaks that facilitate homologous recombination, deletion of large BGC regions, or a base-editing system that permits specific base modifications without double-strand breaks (Tong et al., 2019). Each method is to be assessed for its deletion efficiency, ease of use, and ability to maintain genomic stability. Following each deletion, it is necessary to confirm the absence of target clusters. Finally, it is necessary to characterize the metabolic and transcriptomic profiles to make sure that essential functions remain intact.

This work aims to perform NRPS-encoding BGC deletion in a novel native *Streptomyces* strain to establish a versatile and stable chassis strain for secondary metabolite production of geographically co-isolated strains.

Acknowledgement:

This project is supported by the National Council of Science and Technology (Mexico) and the DTU-alliance PHD between DTU and Tecnologico de Monterrey (DTU, Denmark).

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