Developing a Hybrid Modelling Strategy to Predict Crystal Size Distribution for Reducing CO_2 Footprint

M.R. Boskabadi[a], S. Dommy[a], S.S Mansouri[a]

[a] *Department of Chemical and Biochemical Engineering, Søltofts Plads, Building 228A, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark*

Keywords: Hybrid modeling, Crystal size distribution, CO_2 foot-print reduction, Biomanufacturing

Abstract

Batch crystallization is a common downstream process in biomanufacturing and food industries. These operations are abundant in chemical and biochemical manufacturing, offering significant flexibility to purify an array of products (Wu et al., 2023). Ensuring the distribution of crystal sizes in the crystallization unit operation output can reduce waste production and the need for recycling streams since the product crystals out of specified size are usually separated as production waste or recycled for reproduction. However, due to the challenges in interpreting the crystallization mechanism, accurately factors estimating parameters such as nucleation, crystal growth rates, and complex aggregation phenomena remains a challenge to predict key parameters of Crystal Size Distribution (CSD) and crystal content(Meng et al., 2019).

In the realm of biomanufacturing and biological applications, sugar is a pivotal component utilized for tasks like cell harvesting and as a nutrient source. Nevertheless, traditional sugar production methods have proven unsustainable, with a substantial CO_2 footprint. Approximately 1.2 kg of CO2 emissions are generated per 1 kg of sugar produced (Brobbey et al., 2023), rendering the overall biomanufacturing chain unsustainable. This issue is particularly critical for bioprocesses reliant on sugar, which necessitate a narrow CSD of sugar crystals. The requirement for a narrow CSD is closely tied to sugar crystallization, a notably energy-intensive stage in sugar production. In cases where sugar crystals do not meet the desired size criteria, they undergo separation in a centrifuge. The separated sugar is then recycled and reintroduced into the boiler and crystallizer, further contributing to the process's energy consumption and environmental impact.

This study presents a novel framework that integrates mechanistic modeling and data-driven techniques to predict CSD. This framework provides insights into crystal size control addressing the challenge of monitoring the particle size distribution for biomanufacturing and food industries. First, a laboratory-scale experiment was conducted on batch sugar crystallization to gather a dataset of sugar CSD over time. The experiment utilized an input flow of mother liquor with an average CSD of 10 µm and a supersaturation of 1.15, with a 6-hour crystallization period at a stirring rate of 200 RPM. The CSD data was obtained using the oCelleScope image analysis method. Additionally, the MATLAB software was employed for solving the Population Balance Model (PBM) using the initial CSD distribution, considering both nucleation and growth phenomena (as depicted in Figure 1). Post-PBM solution, the CSD data was utilized for parameter estimation of nucleation and growth kinetic rates. The results indicate the successful prediction of CSD over time by the model. Finally, the study investigated the CO2 footprint reduction effect of this hybrid model to demonstrate the model's potential to enhance biomanufacturing sustainability.

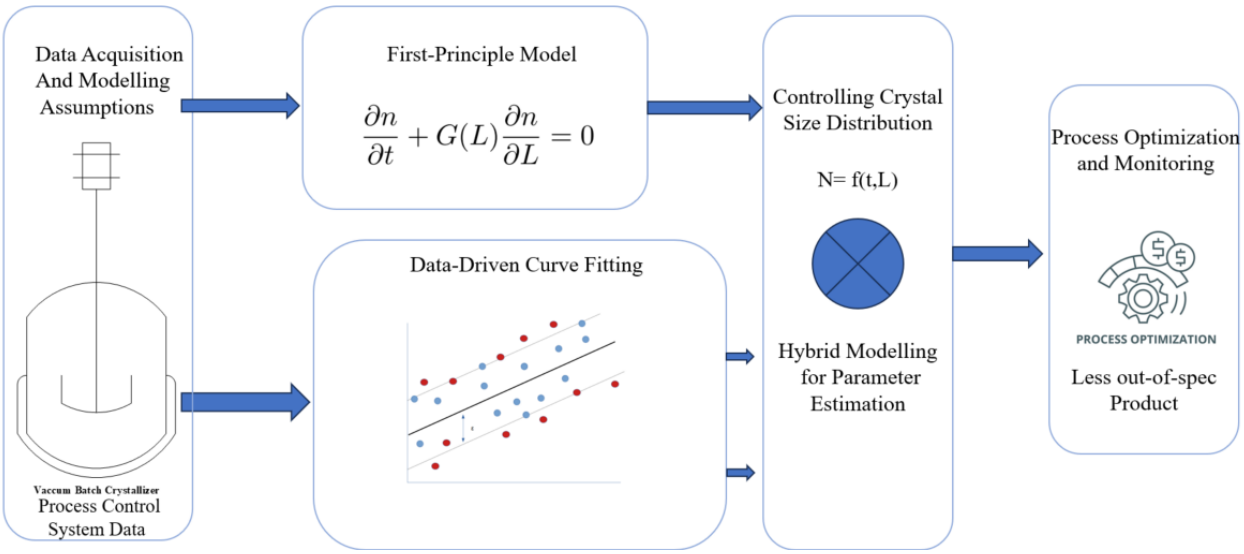

Figure 1: Overall framework of hybrid model to predict crystal size distribution.

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Quantifying Microalgal Growth and Viability: A Machine Learning-based Framework Utilizing HSV-Transformed Microscopic Images

Sh.Shahhoseyni[a], A. Sivaram[a], S. Mohamadnia[b], I. Angelidaki[b], S.Mansouri[a*]

[a] *PROSYS, Department of Chemical and Biochemical Engineering, Technical University of Denmark, Søltofts Plads 227, 2800 Kgs. Lyngby, Denmark.*

[b] *Bioconversion, Department of Chemical and Biochemical Engineering, Technical University of Denmark, Søltofts Plads 228A, 2800 Kgs. Lyngby, Denmark.*

Keywords: Microalgae growth, Microscopic image processing, Machine Learning, Automation

Abstract

Microalgae are versatile unicellular organisms that play pivotal roles in various biotechnology scientific domains including environmental studies, biofuel production, medicine, wastewater treatment, carbon capture, and production of valuable compounds (Borowitzka 2013; Yan et al. 2016). Accurate determination of microalgal biomass is essential for a comprehensive understanding across these disciplines. Swift assessment of a culture's status is crucial for evaluating its growth performance, given the population doubling time of less than one day (Bhattacharya and Shivaprakash 2005). Various methods, including microalgae Dry Cell Weight (DCW), carbon content, optical density (OD) and in vivo chlorophyll-a (chl-a), contribute to understanding microalgae growth dynamics (Schagerl et al. 2022). Alternative parameter such as cell number is utilized to enhance measurement precision, complementing these routine monitoring methods that require caution for interspecies or inter-treatment comparisons (Schagerl et al. 2022; Moheimani et al. 2012). Despite the perceived precision and reliability of cell counting within the scientific community (Stein-Taylor and America 1973), existing methods, particularly manual counting, pose limitations, especially when dealing with species prone to aggregation issues. However, computational tools employing segmentation techniques have emerged to address these challenges, albeit with resource constraints, and issues with aggregation. Advancements in automating processes for microalgae (or any colored microorganisms) sample quantification show promise for next-generation industry applications. By leveraging computational resources, this paper explores the microscopic realm, utilizing *imaging techniques* for a comprehensive approach to quantify microalgae growth and to assess algae content.

In this work, microscopic images of *Chlorella sorokiniana* were obtained using a microscope (TRINOC LED Fluorescence TL534B-SA FL4, Denmark) with the assistance of a 10x lens of the camera (OPTIKA P6FL PRO CAMERA, 6 MP CCD, Italy). The experimental data were collected through Hemocytometer counting (Moheimani et al. 2012). In this method, thick glass chambers are segmented into precisely measured areas and depths. The counted numbers of cells in the hemocytometer chamber are then employed for calculating the total number of cells in a suspension. These images form the primary dataset for computational analysis, utilizing features within the HSV color spaces for precise quantification of microalgal growth and viability. Offering an intuitive representation of colors, Hue signifies color, Saturation represents vibrancy, and Value denotes brightness, with Hue ranging from 0° to 360°, Saturation from 0.0 (grey) to 1.0 (full color), and Value from 0.0 (black) to 1.0 (white). The Hue histogram of an image predominantly containing green elements (here the Microalgae) will typically range between 35 and 85.

Initiating our workflow, the Gaussian blur method is applied to each HSV-transformed image to address defects and prepare them for accurate computational analysis, reducing noise and enhancing overall image quality. Feature extraction focused on mean, median, standard deviation

and skewness of hue histograms are selected for their relevance in capturing the greenness of images, to distill essential information and facilitate mechanistic evaluation of microalgal growth and viability in our study. These features can differ among various species, emphasizing the requirement for individual models tailored to each species that may be distinguished by their unique coloration.

Figure 1 illustrates the workflow utilized for this task alongside the model's performance. Utilizing a ridge regression model on processed data post hyperparameter tuning demonstrates a strong fit to the dataset, indicating the potential for utilizing images to predict microalgae cell count. The limitation with dataset size is addressed by using this simple model form and is also quantified using the learning curve. The learning curve serves to demonstrate the adequacy of the dataset for this modelling task, showing that testing and training mean absolute errors converge as the size of the training set grows, eventually plateauing. This indicates that expanding the training set further does not lead to additional performance enhancements. The R2 value underscores a robust fit to correlate the sample content of microalgae medium.

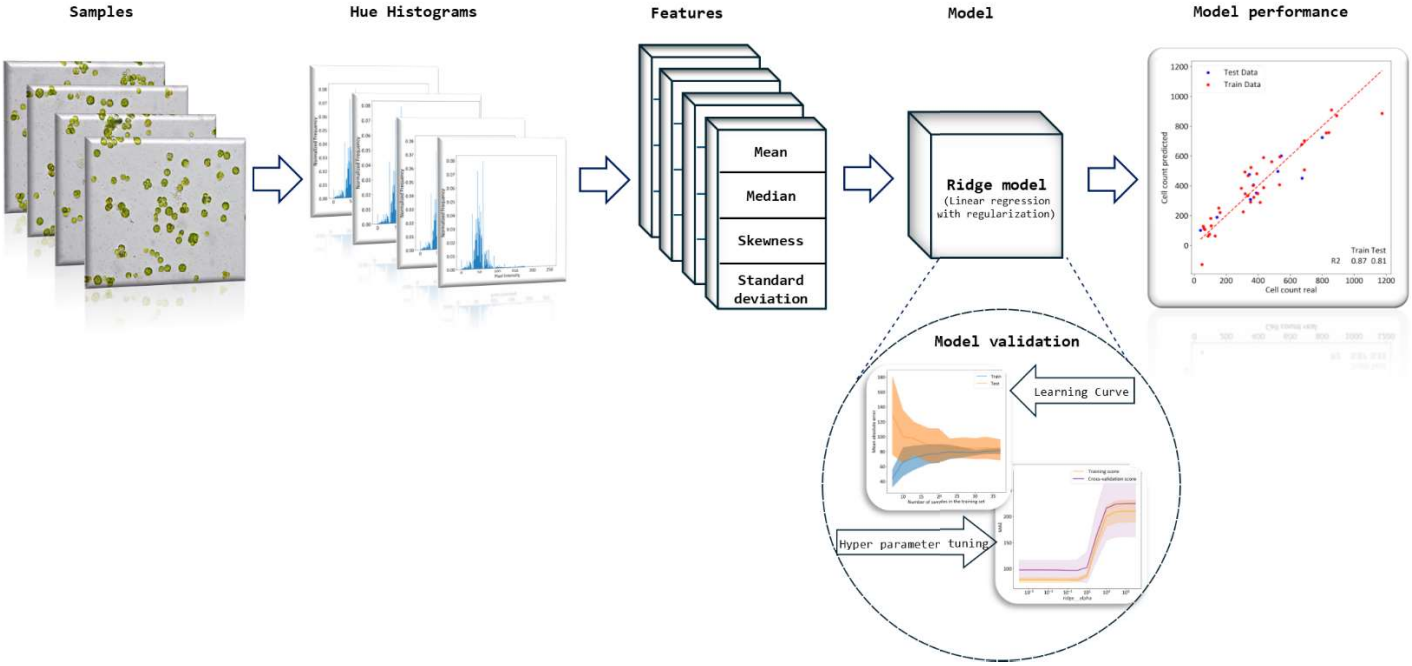

Figure 1) Scheme for the modelling strategy to quantify microalgae growth and to assess algae content.

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Monitoring of Bioprocesses using Explainable Machine Learning

T. Scharl[a], M. Medl[a], A. Dürauer[b]

[a] *Institute of Statistics, University of Natural Resources and Life Sciences, Vienna 1190, Austria*

[b] *Institute of Bioprocess Science and Engineering, Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna 1190, Austria*

Keywords: process control, data-driven models, chemometrics, soft sensors.

Abstract

Initiatives such as Process Analytical Technology, Quality by Design and Continuous Integrated Biomanufacturing have been established to ensure the quality and safety of biopharmaceuticals. Together with further technology development these initiatives will lead to improved process automation, enable real-time release and finally lead to highly controllable and robust biomanufacturing systems. One step towards this vision are statistical models utilizing data streams from multiple physical measurement devices to establish soft sensors for predictive chemometrics and hybrid modeling (Dürauer et al., 2023). To enable model predictive control, real-time information on quantity, purity and potency, - the critical quality attributes – has to be related to critical process parameters. Compared to traditional control strategies, real-time analytics enable control strategies that are more adaptive and precise due to the additional information on the current process state.

One of the goals of the process modelling is a better process understanding through interpretable models. In upstream processing it was shown that random forests are suitable machine learning tools to generate soft sensors that can easily identify the most suitable online variables (Melcher et al. 2015). Real-time monitoring in downstream processing was successfully established in a multiple sensor approach with extensive variable selection for interpretable statistical models (Walch et al., 2019). Recently, we could show that by using deep learning methods like convolutional neural networks in-depth knowledge of the process data, manual variable selection and data preprocessing is not necessarily required to generate highly accurate models. We observed that the models generally exhibited dependencies on correlations that agreed with first principles knowledge, thereby bolstering confidence in model reliability (Medl et al., 2024). Finally, explainability methods such as the proposed permutation/occlusion feature importance for the interpretation of input-output mappings of data-driven models are suitable instruments for building knowledge-based trust in machine learning models.

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Applying an MPC algorithm to the dissolved oxygen level of an intermittent fed-batch process

P. Pably[a], J. K. Huusom[a], J. Kager[a]

[a]*Department of Chemical and Biochemical Engineering, Technical University of Denmark, Building 228A, 2800 Kgs. Lyngby, Denmark*

Keywords: modeling, control, dissolved oxygen, bioprocess

Abstract

Providing cells with ideal physiological conditions for a respective goal is one of the main tasks of a bioreactor. The level of dissolved oxygen (DO) in the broth plays a pivotal role here, as limitations cause shifts in the metabolic activity of the cultivated organisms. These can lead to byproduct accumulation or even cell death. Therefore, controllers are employed to stay above a certain threshold by acting on the agitation, air flow and oxygen partial pressure, where many are based on PID algorithms. In order to counter the nonlinear nature of bioprocesses they were extended by implementing feedback linearization, cascaded controllers or gain scheduling (Babuška et al. 2003). Nevertheless, the downside of their reactive principle can be seen when the system is faced with abrupt changes in nutrient addition. Such conditions can be observed during the transition of phases in a process or because of intermittent feeding profiles in high-throughput small scale multi-reactor systems. The shot-wise addition of the substrate results in sudden drops of the DO signal as described by Kim et al. (2023) and eventually surpass the threshold of the system due to the delayed response of the control loop. The following oxygen limitation can have considerable effects on the health and productivity of the organism, creating a need for better control.

This work explores the potential of a predictive model-based algorithm to prevent oxygen limitations in an intermittent fed-batch process. The oxygen uptake is modeled through the metabolic activity of an aerobic microbial process by employing simple growth kinetics and elemental balances. This demand is met with the oxygen transfer rate, which is connected through a k_LA model established by Van't Riet (1979) to the two actuators of the control loop, the agitation speed and the aeration rate. The parameters of the resulting models are estimated in a laboratory bioreactor setup, using a combination of online and offline signals, as well as the concentrations of the off-gas. The model is then implemented and assessed first as a soft-sensor during a process that simulates an intermittent feeding profile, before incorporating it into an MPC algorithm. The performance of this predictive control strategy is then evaluated and compared to other established technologies, such as PID control. The predictive nature of the MPC is expected to prevent sudden drops in dissolved oxygen and thereby presents a promising control algorithm for small scale reactor systems with intermittent feeding or processes with harsh phase transitions. Since the model is based on simple growth kinetics, it is generically applicable to aerobic cultivations at different scales after parameterizing the reactor specific k_LA model.

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HYBpy: A Python tool for hybrid modeling of bioprocesses

J. Pedreira[a,b], J. Pinto[a], P. Barahona[b], R. Oliveira[a] & R. S. Costa[a]

[a]*LAQV-REQUIMTE, Department of Chemistry, NOVA School of Science and Technology, Universidade NOVA de Lisboa, Campus Caparica, 2829-516, Caparica, Portugal*
[b]*Dept. Informatica Faculdade de Ciencias e Tecnologia, Universidade NOVA de Lisboa, Campus da Caparica, Caparica, 2829-516, Portugal*

Keywords: Python tool, bioprocess digitalization, hybrid mechanistic/ML modeling, Industry 4.0

Abstract

Hybrid models combine state-of-the-art machine learning algorithms (ML) with mechanistic models in the same model structure and is gaining prominence in developing digital twins for Biopharma 4.0 because it offers a flexible balance between prior knowledge and data availability. They have been widely applied in process systems engineering for predictive modeling, process monitoring, model predictive control and design of experiments as well as systems biology problems (Agharafeie et al., 2023). For example, in the last years substantial work in (bio)systems approaches that merge ML/AI and traditional engineering methods were developed, namely the concept of “general bioprocess hybrid model” to describe bioprocess dynamics (Pinto et al., 2022, Teixeira et al., 2007) and for the field of systems biology (von Stosch et al., 2010, Pinto et al., 2023).

Nevertheless, the use of such hybrid modeling techniques has usually been limited. Only a small number of experts (research groups of the in-house tools) have the knowledge and methods to develop such models worldwide. Additionally, a specific open-source and user-friendly software tool to develop a hybrid model is lacking.

To overcome the current limitations, including the dependency on proprietary software, this work aims to provide an open-source, modular and easy-to-use (automated) framework for hybrid models building, simulating and analysis. The outcome is HYBpy, a user-friendly Python tool designed to accelerate the development of decision hybrid models, which offers access to a generalized step-by-step pipeline and intuitive user interfaces. HYBpy provides an added-value for the community interested in hybrid modeling without any programming knowledge or background on hybrid model development, to improve (bio)process understanding and support relevant process interpretation tasks. We demonstrated the utility of HYBPlat through benchmark case studies. The source code is available at the GitHub repository (https://github.com/joko1712/HYBpy-a-Python-tool-for-hybrid-modeling).

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Dynamic prediction of nitrous oxide emissions in a full-scale industrial wastewater treatment plant using a plant-wide model approach

Tianyu Lei[a], Kasper Kjellberg[b], Krist Gernaey[a], Xavier Flores-Alsina[a]
[a]Process and Systems Engineering Centre (PROSYS), Department of Chemical and Biochemical Engineering, Technical University of Denmark. Building, 229, DK-2800, Kgs. Lyngby, Denmark.
[b]Novonesis A/S, Hallas Alle 1, DK-4400, Kalundborg, Denmark.

Keywords: Data reconciliation; Net zero; Virtual assessment

Abstract: The upcoming change of legislation in some European countries like Denmark where wastewater facilities will start to be taxed based on direct greenhouse gas (GHG) emissions will force water utilities / industries to take a closer look at nitrous oxide (N_2O) production. In this paper, a set of mathematical tools are developed and ensembled together to (dynamically) predict N_2O emissions in a full-scale industrial wastewater treatment plant using a plant-wide model. The proposed approach reproduces steady and dynamic state data. It also shows the detailed behavior of ammonium (NH_4^+), nitrates (NO_3^-, NO_2^-), oxygen (O_2) and N_2O changes when alternating aerobic and anoxic conditions. The final version of the paper will further predict and evaluate varied mitigation strategies by scenario analysis with the model.

INTRODUCTION

This case study focuses on the largest industrial wastewater treatment plant (iWTP) in Northern Europe, responsible for handling a pollution load equivalent to 2.5 million population equivalents (PE) within a compact inflow of 12,000 m3/day. Novozymes A/S, the company running the iWTP, has a clear commitment towards a net zero future and has invested substantial amount of time and resources on digital tools. This paper represents one of the earliest scientific outcomes of a highly successful collaboration between industry and academia. A set of mathematical tools predicts mass and volumetric flow, process behavior and greenhouse gas (GHG) emissions (mainly focusing on nitrous oxide).

PLANT CONFIGURATION, MODELS & DATA

A plant-wide model has been developed to comprehensively depict the design and operational conditions of the iWTP under examination. This plant-wide model integrates three key components: 1) biological models, 2) physio-chemical models, and 3) model interfaces (see **Figure 1**).

The biological model (BM) encompasses two critical aspects: an anaerobic digestion model (ADM) and an activated sludge model (ASM). The ADM accounts for influent conditions and processes such as BTL, PRIM, PAT, AGSR, IT, and DEW. Meanwhile, the ASM covers ASR, SEC, and FLOT units. Within the ASM, nitrous oxide (N_2O) biological pathways are explicitly considered, including the hydroxylamine (NH_2OH) oxidation pathway (NN pathway), nitrifier denitrification pathway (ND pathway), and heterotrophic denitrification pathway (DEN pathway). The physio-chemical model (PCM) includes an aqueous phase plus precipitation model and a gas transfer model. Finally, there are ADM/ASM/ADM interfaces integrated before the ASR unit, and ASM/ADM/PCM interfaces incorporated after the SEC and FLOT units (Monje et al., 2022; Solís, et al., 2022 a,b).

Flow diagram and model parameters are adjusted to reproduce the influent, effluent, and process characteristics. A five-week measuring campaign was conducted with grab samples, flow proportional samples and online sensor measurements. Sensor data (DO, NH_X, NO_2^-, NO_3^-) monitors high frequency dynamics within the ASR. Online sensors for dissolved N_2O and gas N_2O were installed and calibrated to collect N_2O data.

The data was dynamically reconciled using a four-steps methodology based on: 1) definition of the identity matrix, 2) curation, processing, cleansing and data analysis, 3) estimation of the missing fluxes, 4) calculation of optimal flows using Lagrange multipliers. The model was calibrated with 24 hours of model prediction and online measurements, while the next 24 hours were validated using RMSE scores regarding model prediction quality.

RESULTS

Steady state simulations

Figure 2 demonstrates that the proposed approach can effectively replicate mainstreams neutralization and mass balancing for activated sludge reactors (ASR) influents (PWW, $AnGSR_{Eff}$, DEW_{Over}) and effluents (ASR_{Eff}). The model can predict biological and chemical N and P removal processes in the aerobic water line (ASR, SEC), accurately projecting volatile fatty acid production and particulate removal processes in the anaerobic water line (PRIM, PAT, AnGSR).

Dynamic simulations

For exemplary purposes, **Figure 3** showcases the results of employing the tools presented in this paper (data reconciliation, influent fractionation, and model predictions) under dynamic conditions for the most important ASR influent ($AnGSR_{Eff}$, DEW_{Over}, bypass from $PREM_{Over}$) and ASR effluent (ASR_{Eff}). Concerning the prediction, it successfully predicts most daily dynamic behavior of total COD (TCOD), total nitrogen (TN), total phosphorus (TP), and total sulfur (TS) concentrations in ASR influent and effluent.

N_2O prediction dynamics

The aerobic/anoxic stages within the bioreactors are controlled by alternating the airflow. **Figure 4** illustrates model simulation results against measurements of the alternating process under high resolution simulations (time step 5 minutes). In the top figure, the first 24 hours data represent model calibration with an average RMSE = 0.92, regarding NH_4^+, NO_2^-, DO, dissolved N_2O and gas N_2O concentrations. The second 24 hours represent the validation period, indicating a high quality of model simulation with an average RMSE = 0.95.

Furthermore, the model successfully describes the detailed bio-chemical nitrogen removal processes in the bioreactors. In the aerobic stage, ammonium (NH_4^+) levels decrease as dissolved oxygen (DO) levels rise, concurrently with an increase in nitrite (NO_2^-) resulting from nitrification processes. During this stage, as shown in **Figure 4** bottom figure, the ND pathway dominates the production of N_2O, particularly during low DO levels (start and end of aeration). N_2O gas is stripped out during aeration, leading to a higher N_2O gas concentration during aeration, which contributes to more than 95% of N_2O emissions. The anoxic stages start when the airflow is regulated to close once NO_2^- reaches a predetermined threshold. NO_2^- is subsequently consumed by denitrification processes during anoxic stages, and the DEN pathway is the dominate N_2O production process in the anoxic stage, contributing to a high dissolved N_2O accumulation. However, the low aeration during anoxic stage leads to a rather small N_2O gas emission with small amount of dissolved N_2O being stripped out.

CONCLUSIONS & FUTURE WORK

The plant-wide model successfully predicts key aspects in various locations of the iWTP under steady-state, dynamic and high-resolution simulations. Under high-resolution simulation, the model is capable for predicting the nitrogen removal processes with a detailed description of N_2O related processes. The model will undergo further validation with longer periods of high-resolution dynamic data regarding N_2O emission rate and emission factors. Furthermore, scenario analysis will be conducted considering various plant operation conditions, followed with plant-wide environmental and economic comparisons and optimizations. The model is not only support verifying hypotheses about the fundamental mechanisms of N_2O production and emissions, but also serve as a valuable tool for optimizing the processes and operations of the iWTP, with a focus on energy efficiency and GHG mitigation.

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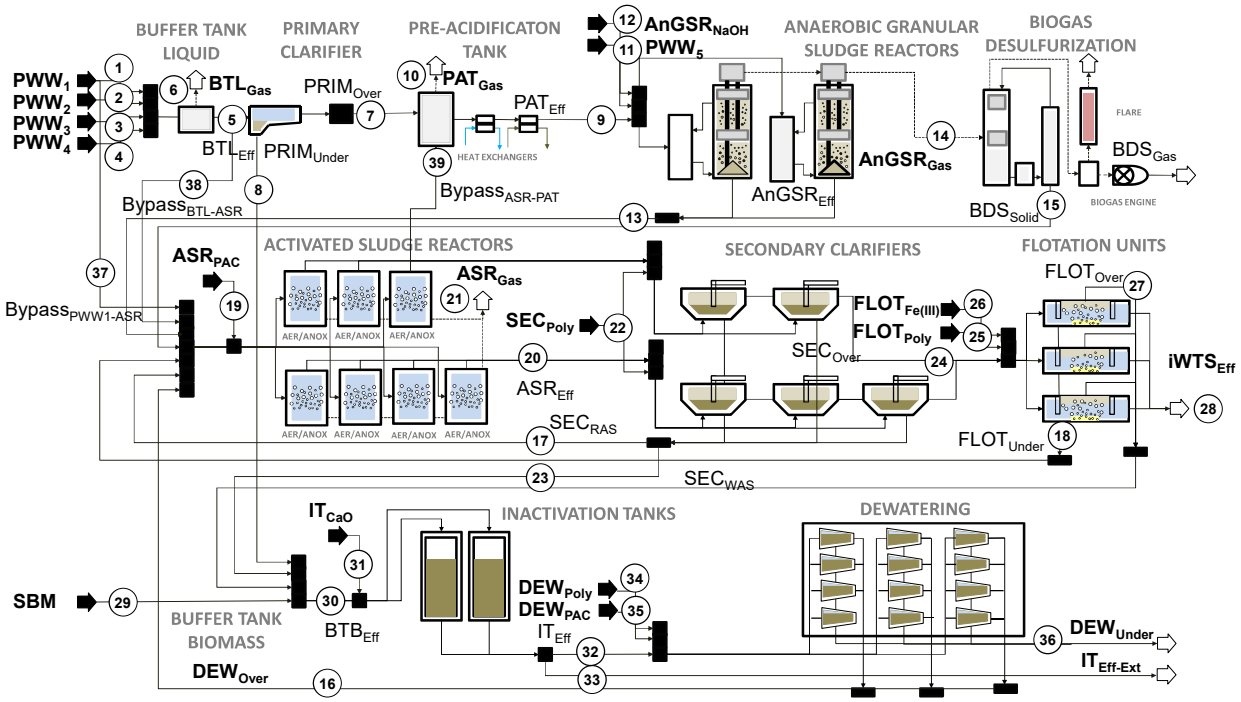

Figure 1. Flow diagram of the iWTS under study: **1–4** Process waste water (PWW_{1-4}), **5,6** - Buffer tank liquid output (BTL_{Eff}, BTL_{Gas}), **7-8 –** Primary clarifier overflow and underflow ($PRIM_{Under}$, $PRIM_{Over}$) **9-10 –** PAT output (PAT_{Eff},PAT_{Gas}), **11** - Process waste water (PWW_5)**, 12 –** Dosing NaOH anaerobic granular sludge reactors ($ASGR_{NaOH}$), **13-14 –** anaerobic granular sludge reactors output ($AGSR_{Liq}$, $AGSR_{Gas}$), **15 –** Elemental sulfur recovered (BDS_{Solid}), **19 –** Dosing PAC activated sludge reactors (ASR_{PAC}), **20-21** - activated sludge reactors output (ASR_{Eff}, ASR_{Gas}), **22** - Polymer dosing secondary clarifiers (SEC_{Poly}), **23,24,17** - secondary clarifiers output (SEC_{WAS}, SEC_{Over}, SEC_{RAS},), **25,26 –** Polymer and Fe(III) dosing flotation ($FLOT_{Poly}$, $FLOT_{Fe(III)}$), **27,18,28** - Flottation units output ($FLOT_{Over}$, $FLOT_{Under}$, $iWTS_{Eff}$), **29 –** Spent biomass stream, **30** - Buffer tank biomass output (BTB_{Eff}), **31** - Quicklime dosing inactivation tanks (IT_{CaO}), **32-33** - Inactivation tanks output (IT_{Eff}, $IT_{Eff-Ext}$), **34,35 –** Polymer and PAC dosing dewatering (DEW_{Poly}, DEW_{PAC}), **36,16** - Dewatering under and overflow (DEW_{Under}, DEW_{Over}), **37-39 –** Bypasses PWW_1 to ASR, BTL to ASR, ASR to PAT. Black/White arrows are inputs/outputs to the whole system.

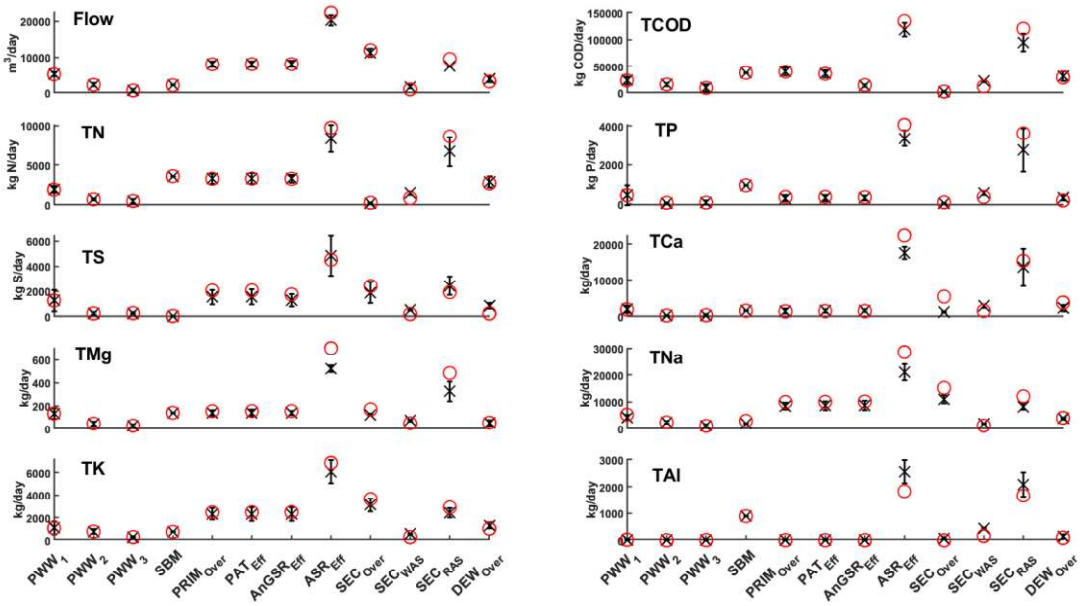

Figure 2. Steady state model predictions (red circles) and reconciled measurements mean and standard deviation (Black crosses and whiskers) for several plant locations (see labels in X-axis).

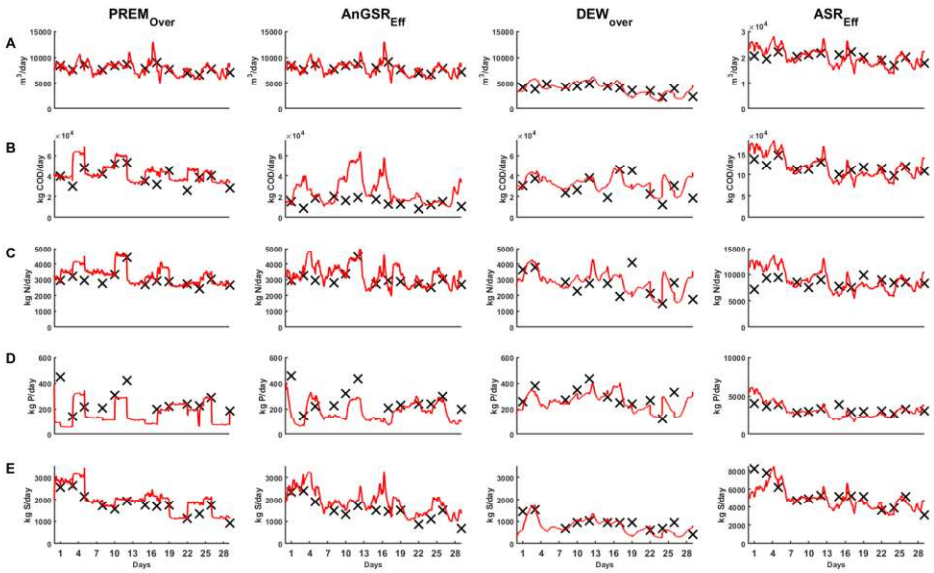

Figure 3. Dynamic model prediction (red solid lines) and reconciled grab-sampled measurement data (black crosses) with **A:** Flows, **B:** TCOD, **C:** TN, **D:** TP and **E:** TS for outflows from PRIM, PAT, AnGSR and DEW.

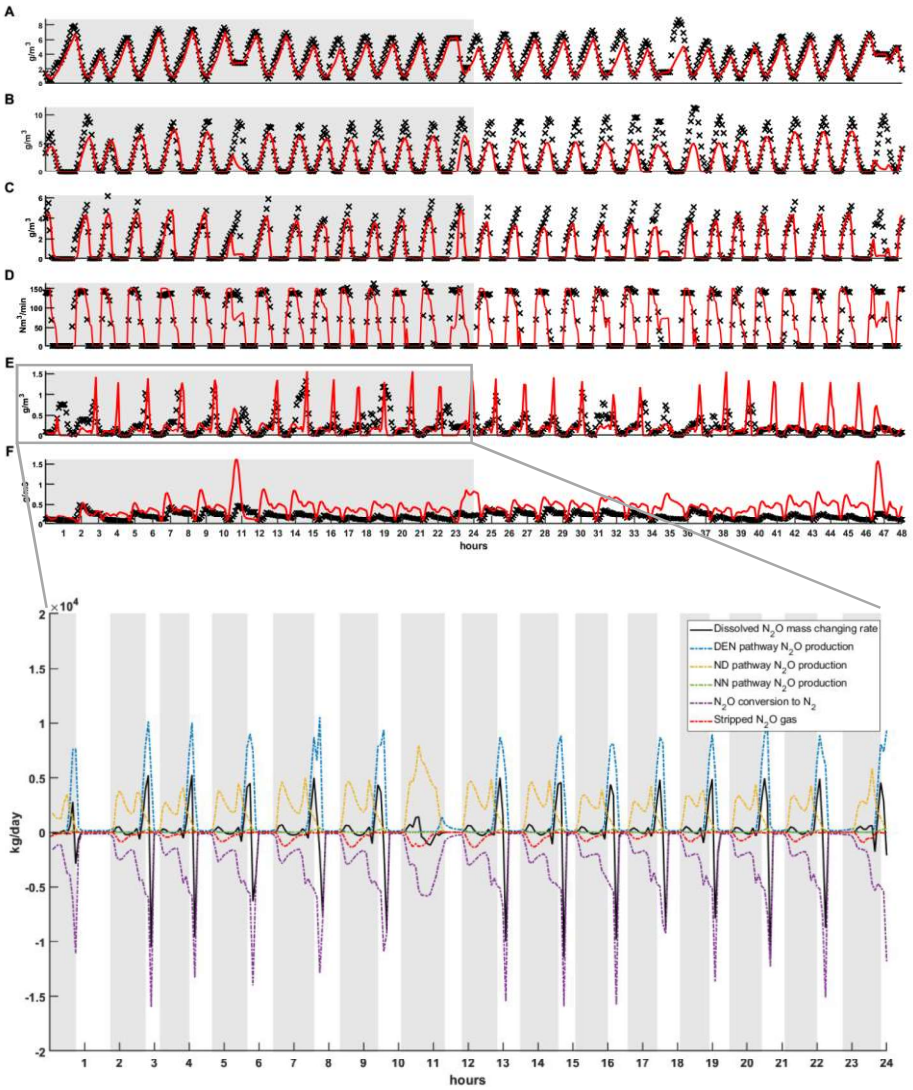

Figure 4. Top: High resolution dynamic model prediction (red solid lines) and online sensor measurements data (black crosses) comparison with **A:** NH_4^+, **B:** NO_2^-, **C:** DO, **D:** Airflow, **E:** Dissolved N_2O **F:** Gas N_2O concentration. The first 24 hours marked in grey represent the calibration period. **Bottom:** N_2O production with ND (yellow), NN (green), and DEN (blue) pathways in ASR during calibration period. Grey parts represent the aerated stage, while white parts are the anoxic stage.

A Continuum Transport Model of Monospecies Biofilm-particulate interactions

Raghu Krishna Moorthy[a], Eoin Casey[a]

[a]*School of Chemical and Bioprocess Engineering, University College Dublin, Belfield, Dublin, D04V1W8, Ireland*

Keywords: Biofilm, continuum model, particulate matter, residence time.

Abstract

The design, scale-up and optimisation of biofilm-based processes, particularly for wastewater treatment operations, is a mature field (Wanner *et al*., 2006; Wei and Yang, 2023). However a neglected area is the interaction between particulate organic matter (in the bulk phase) and the attached phase (biofilm). Mathematical models are useful for the analysis of the complex processes inherent in such systems (Wanner and Gujer, 1986; Duddu *et al*., 2009). Time scales for substrate diffusion and rate of growth and decay of biomass during development of monospecies biofilm are estimated to differ by around two orders of magnitude (Wanner *et al*., 2006).

Microfluidic systems are increasingly used to investigate mass transfer characteristics in the biofilm development (Ford and Chopp, 2020; Wei and Yang, 2023). A high surface-to-volume ratio in these systems provided an increase in rate of mass transfer (Ford and Chopp, 2020). It potentially leads to longer residence time for substrate uptake in the attached phase. On introduction of dominant convective forces, fast substrate diffusion from bulk phase into biofilm may occur due to the reduced diffusion distance. It is hypothesized that the increase in residence time at different flow rates and short diffusion time actively promote the interaction between particulate organic matter and biofilm. The goal of the present study is to develop a fully continuum model to explain the trends of kinetics of particulate organic matter and its interactions with the biofilm, when coupled to these hydrodynamic flows.

A simple set of one-dimensional transport equations that constitutes a two-compartment model for different biomass rate expressions is formulated. The fully continuum model represented the mass and momentum balance for substrate and particulate transport within a microfluidic domain. The residence time distribution at different flow rates are coupled with these expressions, to describe the particulate interactions at the biofilm interface. Substrate flux from bulk flow to the biofilm is predicted to explain the effect of flow regime on the biofilm thickness. Case studies with laminar flow (Reynolds number between 0.01 to 10) are considered to simulate static or dynamic biofilm development at different residence times and theoretical rate expressions, and are implemented using MATLAB®.

From preliminary numerical solutions for assumed Monod kinetics, substrate flux is found to increase with an increase in inlet flow rate. The above mathematical model is compared to the existing, monospecies biofilm models based on the fully continuum description and to be validated numerically within acceptable error limits (root mean square error less than 20%). The results indicate the potential of a continuum description to unfold the mechanisms that govern the particulate matter during biofilm formation in confined environments.

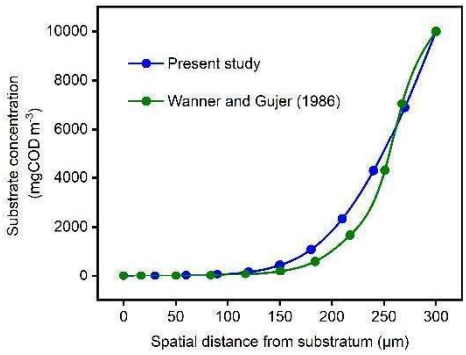

Figure 1: Simple 1D fully continuum model for biofilm growth (present study) in comparison to the existing model in literature

Acknowledgement

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Impact of yeast fermentation on the functional properties and chemical composition of defatted (*Vicia villosa)* flour

Negin Yousefi.[a], *Behdad Shokrollahi Yancheshmeh*[b], *Mohammad Amin Mohammadifar*[b], *Krist V. Gernaey*[a]

[a]*Center for Process Engineering and Technology (PROSYS), Department of Chemical and Biochemical Engineering, Technical University of Denmark*
[b]*Research group of Food Production Engineering, National Food Institute, Technical University of Denmark*

Presenting author email: Negyou@kt.dtu.dk

Abstract

Plant-based protein sources are becoming more and more popular as an alternative to animal protein sources because of their improved environmental sustainability and association with a decreased risk of cardiovascular disease (Olukomaiya et al., 2020). Legumes are important for the human diet because of their functional and health-promoting qualities. They are also thought to be abundant providers of fiber, proteins, carbohydrates, certain minerals, fatty acids, and vitamins. However, their digestion and bioavailability are restricted by the presence of anti-nutritional factors (ANFs), which may be overcome by soaking, boiling, or fermenting processes (Yancheshmeh et al., 2022; Garrido-Galand et al., 2021). Within the Fabaceae (legume) family, the genus *Vicia villosa* has around 150 species. In addition to their effects on human nutrition, *V. villosa* seeds have positive health effects and may be used as a treatment for conditions including liver cirrhosis, hypertension, Parkinson's disease, and renal failure (Berber and Yaşar, 2011). Fermented feeds have gained popularity as nutritious additions to diets and as functional foods that may improve consumer health (Olukomaiya et al., 2019). They may include lactic acid bacteria cells or live or dried yeast cells in addition to proteins, minerals, and vitamins. These components may have probiotic or prebiotic effects on the digestive system's microbiota (Wang et al., 2018). To the best of our knowledge, there is a lack of information regarding the influence of fermentation on the functional and physicochemical properties of *V. villosa* seeds. This research focused on the investigation of proximate composition and functional aspects of *V. villosa* defatted flour (VDF) after a 72-hour fermentation process with bakers yeast (*Saccharomyces cerevisiae)* to improve their functionality in the food industry. Moderate acidity fermentation led to a considerable rise in the density, amounts of fat, protein, moisture, total ash, and total phenolic contents, while a significant reduction in the mass fraction of carbohydrates was found. Color parameters, protein solubility, water holding capacity (WHC), oil binding capacity (OBC), emulsifying capacity (EC), emulsion stability (ES), foaming capacity (FC), and foaming stability (FS) were also evaluated. The fermented VDFs showed decreased ($p < 0.05$) protein solubility and water holding capacity but increased oil binding capacity. In addition, fermented VDFs demonstrated a reduction in color attributes (L* and b*). In contrast, its emulsifying capacity, emulsion stability, foaming capacity, and foaming stability significantly increased. This investigation shows evidence that yeast fermentation modified the functionality of VDFs and can be used as a functional food ingredient.

Keywords: Plant proteins, Defatted (*Vicia villosa*), Yeast fermentation, Chemical composition, Functional properties.

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Characterization and Valorisation of Cork By-Products for Sustainable Applications

L. Campos[a,b,c*], M. Henriques[b], A.C.A. Veloso[b,c], C. Silva[d], S.I. Mussatto[a]

[a] *Department of Biotechnology and Biomedicine, Technical University of Denmark. Søltofts Plads, Building 223, 2800, Kongens Lyngby, Denmark*
[b] *Research Centre for Natural Resources Environment and Society (CERNAS), Polytechnic Institute of Coimbra, Bencanta, 3045-601 Coimbra, Portugal*
[c] *CEB - Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4715-057 Braga, Portugal*
[d] *CITEVE – Technological Center for the Textile and Clothing Industries of Portugal, Rua Fernando Mesquita, 4760-034 Vila Nova de Famalicão, Portugal*

Keywords: cork by-products; cork powder; black condensate; cork boiling wastewaters; chemical characterization.

Abstract

Portugal is the leader in cork oak (*Quercus suber*) production worldwide (~50%, corresponding to 100,000 ton/year) (APCOR, 2020; DGAE, 2019). After cork is extracted from the trees, the cork planks undergo several treatment processes till the final product is obtained. This process generates a substantial amount of by-products (~25%,), namely cork powder (CP), black condensate (BC) and cork-boiling wastewaters (CBW) (Carriço et al., 2018, 2023; Mota et al., 2022), which are still underutilised. Both the industry and researchers are seeking solutions for their valorisation since they are seen as environmental pollutants and not as raw materials for valuable applications. Although cork has been broadly investigated, there is a shortage of knowledge in the characterization and valorisation of cork industrial by-products (Carriço et al., 2018) .

The present work focused on the characterization of the cork by-products with the aim of designing possible sustainable applications in (bio)processes. The by-products were analysed in terms of total solids, ash, pH, electrical conductivity (EC), fat, macro and micronutrients (C, N, P, Ca, Na, K, Mg, Fe, Mn, Cu and Zn), extractives (in dichloromethane, ethanol and water), suberin and lignin content (soluble and insoluble). To be analysed, BC underwent a drying pre-treatment (45 ºC, 24 h) due to its high-water content of 43.2%, while CBW was filtered (G2) to remove the suspended solids (0.09 g/L).

BC was the by-product with the highest total solids and ash contents (93.7% and 36.0%, respectively), followed by CP (87.2% and 3.6%, respectively) and CBW (0.31% and 0.25%, respectively). CBW presented the highest pH (7.7) and EC (5044 µS/cm), while CP had the lowest values (5.1 and 401 µS/cm, respectively). Fat was only determined in the solid by-products with CP having a fat content of 4.3% and BC of 2.8% (values in w/w, dry basis).

Regarding macronutrients, in general, carbon was present in greater concentration in the samples, followed by nitrogen. Magnesium was the macronutrient with the lowest concentration in BC and CBW, while phosphorus was below the quantification limit (1.0 mg/L) in CP. For the micronutrients (Fe, Mn, Cu, Zn), Fe was present in the highest concentration in all by-products. BC was the by-product with the highest concentration of all the micronutrients. It was not possible to quantify zinc in CP and Cu in CBW as the concentrations were below the quantification limit (0.017 mg/L and 0.068 mg/L, respectively).

Only the solid matrices were submitted to extractives and suberin quantification. Overall, total extractives were higher in CP (16.0%) than in BC (8.5%). However, different solvents resulted in different extraction yields. In BC, dichloromethane gave an extraction yield of 5.8%, while ethanol

and water yielded around 1.4% each. In CP, ethanol resulted in the highest extraction yield (7.9%), followed by water (5.1%) and dichloromethane (2.9%). CP proved to be very rich in suberin (33.4%) compared to BC (0.24%). Lignin was quantified into insoluble and soluble lignin. Insoluble lignin was higher in CP (47.3%), while soluble lignin was higher in BC (42%). In CBW, only soluble lignin was quantified, with a result of 28%.

In conclusion, this study highlights the potential of cork by-products for application in (bio)processes, especially due to their high contents of carbon and nutrients. In addition, their physicochemical properties and valuable constituents demonstrate their feasibility for reuse in various fields. This not only contributes to reducing environmental pollution but also aligns with sustainable practices by reusing waste as valuable inputs for other processes.

Acknowledgement

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Towards Carbon Neutrality: Early-Stage Assessment of Zero Emission Biotechnologies

G. J. A. Brouwer[a], J. A. Posada[a]

[a]*Department of Biotechnology, Delft University of Technology (TU Delft), Van der Maasweg 9, Delft, 2629HZ, The Netherlands*

Keywords: CO_2 Conversion, Circularity, Industrial Biotechnology, Biorefinery, Technology Assessment.

Abstract

Drastic action is required to curb greenhouse gas emissions and safeguard our planet. This entails adapting our lifestyles and innovating within current value chains to reduce our carbon footprint. Industrial biotechnology, identified as one of the six key technologies to combat climate change (European Commission, 2017), offers sustainable alternatives to fossil fuel-derived products and a way to achieve net-zero CO_2 emissions by 2050 (Horowitz, 2016). However, true sustainability can only be obtained with circularity (Lieder & Rashid, 2016) and non-competition with food resources. Therefore, new value chains are required with processes that can convert CO_2 into products using renewable energy sources (e.g., green electricity and green H_2). So called, zero emission biotechnologies (ZEBs) like syngas fermentation recently commercialized by LanzaTech (Köpke *et al*., 2020) or microbial electrosynthesis (Jourdin *et al*., 2020) can be instrumental technologies to achieve zero emission in the long-term. Unfortunately, the road from invention to commercial production is long in industrial biotechnology, and most biotechnologies that look promising after laboratory development fail to cross the valley of death and reach industrial scale (Kampers *et al.,* 2021). Therefore, it is important to be able to identify, early-on, promising ZEBs for industrial scale. However, comparison of different technologies at an early-stage is a challenging task, and scientific literature has been limited to heterogeneous catalytic or other types of fermentative processes (Posada *et al*., 2013, Moncada *et al*., 2015 & Moncada *et al*., 2017). Therefore, an early-stage sustainability analysis framework was developed to assess novel ZEB concepts with different biotechnology-product combinations. The ZEB concepts assessed were 1. microbial electrosynthesis (CO_2 to chemical building block: ethanol), 2. enzymatic conversion (CO_2 to chemical building blocks: CO and formic acid), 3. co-culture (from CO_2 to high value products), 4-5. mixed culture (CO_2 or formic acid to high value products) and 6. monoculture to convert ethanol to a high-value product like single-cell protein. The early-stage sustainability analysis was done to first identify the technical, economic and environmental bottlenecks and opportunities, and then select the ZEB concepts with the largest potential to achieve carbon neutrality for large scale production. Results comprise of both a framework for the early-stage sustainability assessment of (zero emissions) biotechnologies and a ranking (from 'most promising' to 'least promising') of the six different biotechnology–product combinations assessed.

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Optimized enzymatic hydrolysis of *Cynara cardunculus* and *Arundo donax* biomass

A. Posilipo[a], M.E. Russo[b], F. Lanzillo[a], F. Raganati[a], P. Salatino[a], A. Marzocchella[a]

[a]*Dipartimento di Ingegneria Chimica dei Materiali e della Produzione Industriale, Università degli Studi di Napoli Federico II, P.le V. Tecchio 80, 80125, Napoli, Italy*
[b]*CNR-Istituto di Scienze Tecnologie per l'Energia e la Mobilità Sostenibili, P.le V. Tecchio 80, 80125, Napoli, Italy*

Keywords: cellulase, enzyme adsorption, biocatalyst consumption.

Abstract

The development of sustainable biorefinery processes goes through the selection of available lignocellulosic feedstock among agro-food wastes and crops fit for the valorization of marginal lands. In addition, efforts are still necessary to reduce the impact of biocatalyst consumption on the overall costs of the process and thus on the competitiveness of bio-based products on the market as alternatives to fossil counterparts. The present study was conceived to optimize the use of two lignocellulosic biomass largely available in the Mediterranean area and adapted to recent climate changes: *Cynara cardunculus* (Barracosa et al., 2019), and *Arundo donax* (Scordia & Cosentino,2019). These two case studies were addressed in an experimental investigation aimed at the minimization of cellulase cocktail dosage. The rational design of process conditions was based on the assessment of enzyme adsorption on the lignocellulosic biomass particles and the overall rate of production of monomeric sugars (mainly glucose and xylose) as schematically represented in Fig. 1. Diluted alkali hydrolysis was applied as a reliable and effective reference pretreatment for delignification of the substrate prior to enzymatic hydrolysis (Russo et al., 2022). As a proof of concept, hydrolysates from cardoon and *A. donax* saccharification were supplemented as carbon sources to *Thermotoga neapolitana* batch cultures, a thermophilic gram-negative bacterium of primary interest for bio-hydrogen production (Xu & d'Ippolito, 2023).

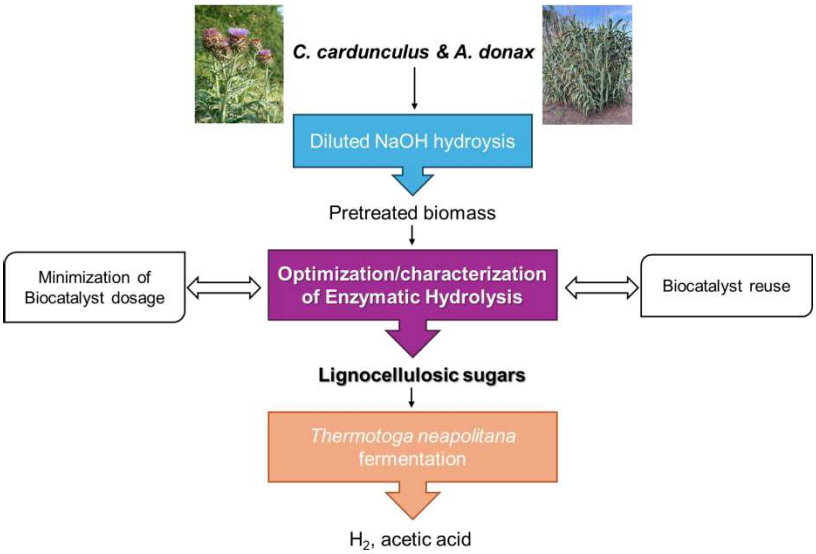

Figure 1: scheme of the experimental study concept

Results provided information on the adsorption of cellulases from a commercial cocktail on the pretreated biomasses. Maximum adsorption capacities were assessed upon pretreatment and compared with those observed for raw biomasses. The diluted alkali pretreatment made the substrate hydrolysable despite a not extended increase in the enzyme adsorption capacity and a shift from linear to Langmuir-type adsorption equilibrium behaviour. The almost complete irreversibility of the adsorption of cellulases on pretreated biomasses suggested partial recovery and reuse of the unbound biocatalyst fraction. The enzyme dosage for each biomass was minimized to 7-4 mg adsorbed enzymes/g dry biomass to approach glucose yield above 61-90%. Recovered unbound cellulases were tested for enzymatic hydrolysis of cardoon biomass and provided not negligible glucose yield (51.5%) at 2 mg/g adsorbed biocatalyst loading.
The cultivation of *T. neapolitana* was successfully carried out for 200 h at 75°C. Hydrolysate were diluted to 6.0 g/L glucose and 2.0 g/L xylose, a synthetic medium with equal sugar content was used as a control. Upon a 60 h lag phase, the cultivation reached 1.17 OD600nm, 1.2 g/L acetic acid and 30% H_2 production in the gas outlet (1.4 atm) at 140 h. The next steps will include the test of cardoon hydrolysate as a carbon source for *T. neapolitana* cultivation and the dosage/composition adjustment of recovered unbound cellulases.

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High-rate production of isobutyric and n-butyric acid from H_2 and CO_2 in a hollow-fibre membrane biofilm reactor: operation and process modelling

Q. Mariën [a,b], X. Flores-Alsina [c], U. Aslam [a,b], K.V. Gernaey [c], A. Regueira [a,b,d], R. Ganigué [a,b]

[a] *Center for Microbial Ecology and Technology (CMET), Ghent University, Coupure Links 653, 9000 Ghent, Belgium.*
[b] *Center for Advanced Process Technology for Urban Resource Recovery (CAPTURE), Frieda Saeysstraat 1, 9052 Ghent, Belgium.*
[c] *Process and System Engineering Center (PROSYS), Department of Chemical and Biochemical Engineering, Technical University of Denmark, Søltofts Plads 228A, 2800 Kgs. Lyngby, Denmark.*
[d] *CRETUS, Department of Chemical Engineering, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain.*

Keywords: Carbon capture and utilization, gas fermentation, acetogen, chain elongation, *Clostridium luticellarii*.

Abstract

Converting CO_2 from industrial off-gases to added value products via (bio)catalytic processes is being pursued as a strategy to minimize carbon emissions. Acetogens are strict anaerobic bacteria that harbor the Wood-Ljungdahl pathway, one of the most energy efficient carbon fixating pathways in nature, making them interesting whole-cell biocatalysts for biological carbon capture and utilization (Claassens et al., 2019). *Clostridium luticellarii* is an acetogen uniquely capable of producing n-butyric and isobutyric acid from H_2 and CO_2 (Mariën et al., 2023). Both n-butyric and isobutyric acid have extensive market applications as platform molecules for the production of food additives, fragrances, antibacterial agents and as precursors for bioplastics (Lang et al., 2014; Moscoviz et al., 2018). Since both chemicals are currently produced from petrochemically derived propylene (Bauer, 2011), establishing a direct production route from CO_2 and H_2 (derived from e.g. renewable electricity via water electrolysis) could greatly improve their sustainability as platform chemicals while simultaneously capturing carbon.

Production rates per volume of reactor determine to a large extent the economic viability of bioprocesses. However, establishing high-rate acetogenic processes from H_2 and CO_2 is currently hampered by two main bottlenecks: limited gas-liquid mass transfer rates and low acetogenic cell densities (Bengelsdorf et al., 2018). Hollow-fiber membrane biofilm reactors (HFMBR) are capable of addressing both bottlenecks simultaneously as they provide a large surface area for mass transfer and retention of cells as biofilms (Elisiário et al., 2021). Here, we investigated the production of n-butyric and isobutyric acid from H_2 and CO_2 by *C. luticellarii* in a HFMBR operated continuously for 80 days. To stimulate the production of n-butyric and isobutyric acid over acetic acid, the H_2:CO_2 ratio was gradually increased from 2.5 to 5.0 during operation. Concentrations of acetic, n-butyric and isobutyric acid of respectively 8.93 ± 0.22 g L^{-1}, 1.56 ± 0.04 g L^{-1} and 1.51 ± 0.06 g L^{-1} were achieved at the highest H_2:CO_2 ratio and a dilution rate of 0.25 d^{-1}. These correspond to production rates of 2.23 ± 0.06 g L^{-1} d^{-1} for acetic acid and 0.77 ± 0.02 g L^{-1} d^{-1} for C_4 products.

Subsequently, a holistic process model was developed to gain deeper insights into the performance of the system and how to optimize its operation. The model enveloped the autotrophic production of the acids, as well as mass transfer of H_2 and CO_2 through the membrane, biofilm diffusion and convection, and biofilm growth and detachment. Calibration against experimental data confirmed the accuracy of the model and simulation framework in reflecting the production dynamics (Figure 1A). Next, the developed model was used to perform scenario analyses. A first analysis revealed the importance of controlling the biofilm at its

optimal thickness since biofilms thicker than ~200 µm will cause large pH gradients throughout the biofilm that can lower cell activity, while biofilms thinner than ~50 µm will result in low volumetric production rates due to a lack of immobilized cells. Finally, the impact of dilution rate and specific membrane surface area was investigated, highlighting that increasing membrane surface area enhances the volumetric production rates while dilution rates can be used as a tool to steer between high-rate production of acetic acid or C_4 products (Figure 1B). Overall, these combined experimental results and model-generated insights can inform future optimization of HFMBRs for the production of added-value products from H_2 and CO_2.

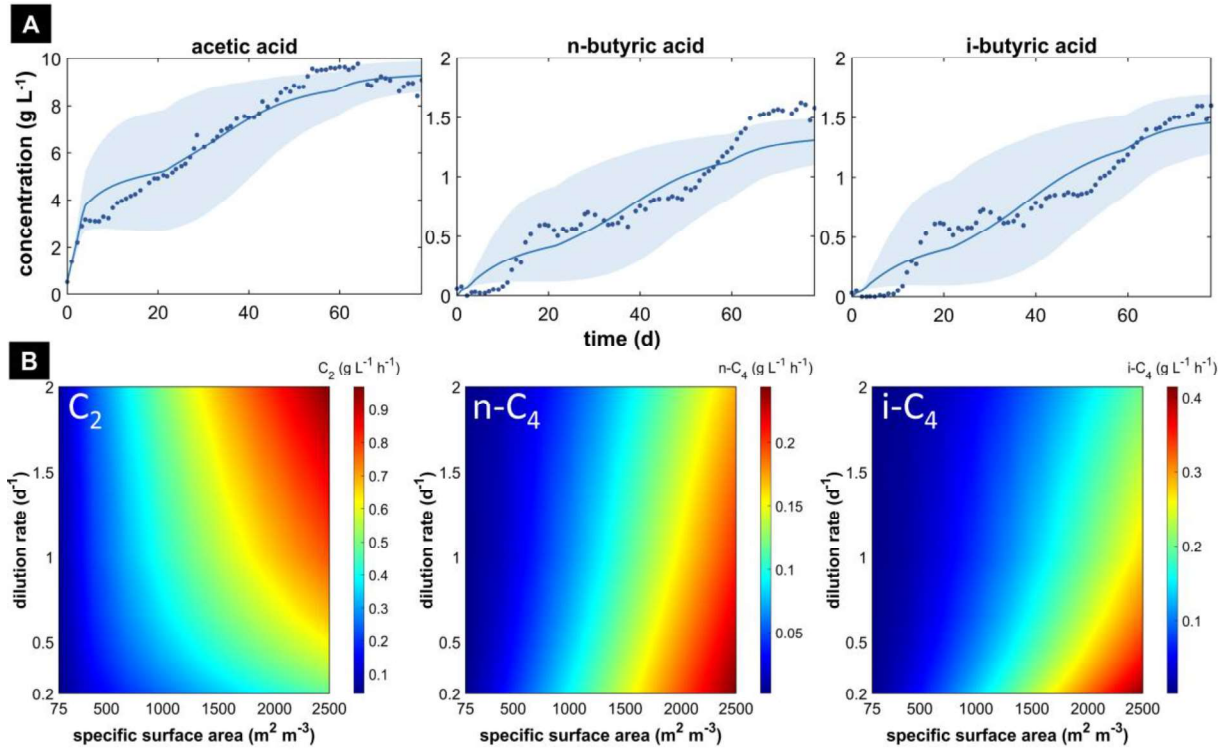

Figure 1 – (A) Fit between the experimental data (filled circles) and the calibrated model (solid line). The shaded area represents the 95% confidence interval. **(B)** Scenario analysis of the impact of dilution rate and specific surface area on steady state volumetric production rates. C_2 = acetic acid, n-C_4 = butyric acid, i-C_4 = isobutyric acid.

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Animal by-product streams as complex carbon and nitrogen sources for polyhydroxyalkanoate production

S. Waldburger[a], B. Gutschmann[a], T. Grimm[b], N. Nafisi[a], K. Harms[a], P. Neubauer[a], S. L. Riedel[a, c]

[a]*Technische Universität Berlin, Institute of Biotechnology, Chair of Bioprocess Engineering, Ackerstraße 76, 13355 Berlin, Germany*
[b]*ANiMOX GmbH, Max-Planck-Str. 3, 12489 Berlin, Germany*
[c]*Berliner Hochschule für Technik, Department VIII – Mechanical Engineering, Event Technology and Process Engineering, Environmental and Bioprocess Engineering Laboratory, Seestraße 64, 13347 Berlin, Germany*

Keywords: polyhydroxyalkanoate, *Ralstonia eutropha*, biopolymer, circular economy, animal by-products.

Abstract

Modern society demands sustainable, bio-based and biodegradable alternatives to plastics derived from fossil fuels, given the persisting demand for plastic materials. Various microbes form polymers called polyhydroxyalkanoates (PHAs) as an energy and carbon reservoir. PHA's structure can influence its qualities, which bear parallels to many other polymers derived from fossil fuels, especially the copolymer poly(hydroxybutyrate-*co*-hydroxyhexanoate) [P(HB-*co*-HHx)] is of rising interest for the commodity polymers industry (Thiele et al., 2024).

Animal by-products can serve as an affordable substitute for raw materials in bioplastic production, thereby reducing the environmental and ecological impact of the process. To obtain pure substrate phases and high substrate yields, pre-treating animal materials is essential. To produce a fat, fat-protein, and protein phase, the animal materials were hydrolyzed, pretreated, and phase separated (Saad et al., 2021).

In *Ralstonia eutropha* cultivations, streams of various animal by-products can be used in place of both carbon and nitrogen sources (Gutschmann et al., 2023). This study assessed the fat and protein fractions of porcine by-product streams hydrolyzed at 130 to 160 °C in *R. eutropha* laboratory-scale bioreactor cultivations under nitrogen limitation. Using various animal fat-phases, up to 59 g L^{-1} cells with 80 wt% p(HB-*co*-18mol%HHx) could be generated, with results comparable to raw substrates like fructose or canola oil. When producing PHA in a laboratory bioreactor, the protein phase was assessed as the only source of nitrogen and evaluated regarding the molecular weight distribution, sterilization, cultivation course, and impact on PHA generation. By replacing fine chemicals, the use of the complex nitrogen source can save cultivation expenses and accelerate *R. eutropha* growth prior to the PHA synthesis phase.

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Performances of an immobilized recombinant thermostable carbonic anhydrase as biocatalyst for CCU processes.

M. Di Giacomo[a], M. E. Russo[b], I. Antonopoulou[c], P. Salatino[a], A. Marzocchella[a]

[a]*Dipartimento di Ingegneria Chimica dei Materiali e della Produzione Industriale, Università degli Studi di Napoli Federico II, P.le V. Tecchio 80, 80125, Napoli, Italy*

[b]*CNR-Istituto di Scienze Tecnologie per l'Energia e la Mobilità Sostenibili, P.le V. Tecchio 80, 80125, Napoli, Italy*

[c]*Biochemical Process Engineering, Division of Chemical Engineering, Department of Civil, Environmental and Natural Resources Engineering, Luleå University of Technology, SE-97187 Luleå, Sweden*

Keywords: carbonic anhydrase, enzyme immobilization, magnetic nanoparticles, CO_2 capture and utilization.

Abstract

The increase of CO_2 atmospheric concentration in the last decades is judged as one of the main responsible of the earth global warming and climate change as well as the driving force behind the development of advanced technologies aimed at decreasing adverse environmental impacts (e.g., greenhouse effect, increasing ocean acidity and sea levels, ice melting and desertification). The achievement of the targeted net decrease of CO_2 emission asks for the deployment of efficient carbon capture and storage technologies, alongside CO_2 utilization processes (CCUS) (Gabrielli et al., 2020). Among the most developed capture processes, absorption-based CO_2 capture has potential applications across several industrial sectors. Recent scientific findings underline the essential role of biocatalysis in the development of environmentally friendly and sustainable CCUS solutions. The use of the enzyme carbonic anhydrase (CA, E.C. 4.2.1.1) is a valuable choice for the development of biomimetic CO_2 capture in aqueous solutions (de Oliveira Maciel et al., 2022; Russo et al., 2022). The use of aqueous solutions instead of amine-based solvents - coupled with the low energy requirements - makes the biomimetic approach appealing as CCUS strategy. The hydration reaction catalyzed by CAs - conversion of CO_2 into bicarbonate ions with simultaneous proton release in aqueous alkaline solutions - improves the overall CO_2 absorption rate and provides an intermediate carbon vector (bicarbonate solutions) ready for storage and conversion/utilization (Marra et al., 2024).

An ultrastable CA from *Desulfovibrio vulgaris* (DvCA8.0) has recently been characterized as a biocatalyst for efficient and rapid carbon capture from flue gas. Through direct evolution, the recombinant form exhibited remarkable stability up to 107°C in the presence of 4.2 M alkaline amine solvent at pH>10 (Alvizo et al., 2014).

The present contribution reports results regarding DvCA8.0 covalently immobilized for the first time on magnetic nanoparticles (MNPs), synthesized through the co-precipitation of Fe^{2+} and Fe^{3+} ions and activated with carbodiimide. MNPs exhibit notable characteristics - including a large specific surface area and quick response to external magnetic fields – that facilitate the separation and recovery from dispersed solid slurries (Peirce et al., 2018). The preparation of MNPs_DvCA8.0 was characterized by high immobilization yield (~90% mass basis) and enzyme loading close to 50 mg/g. Performances of free DvCA8.0 and MNPs-DvCA8.0 as biocatalysts for CO_2 absorption were investigated in a lab-scale experimental setup equipped with a closed mechanically stirred cell (Fig.1).

Free DvCA8.0 was characterized by remarkable enhancement of the CO_2 capture absorption rate. The MNPs_DvCA8.0 biocatalyst was characterized by performances close to those of the free enzyme and no relevant leaching of the enzyme.

The biocatalyst was also tested for CO_2 utilization purposes through accelerated weathering of limestone (AWL) tests (Caserini et al., 2021). $CaCO_3$ powder from a paper and pulp mill (14 μm average diameter) was used as diluted slurry in distilled water (0.4%w) according to previous study (De Oliveira Maciel et al., 2024). Preliminary results proved an increase in the limestone dissolution rate during CO_2 absorption tests in the presence of DvCA8.0. The assessment of the performances of MNP-DvCA8.0 as solid biocatalyst for AWL and its potential magnetic field assisted separation from the limestone slurry for reuse/recycling was also carried out.

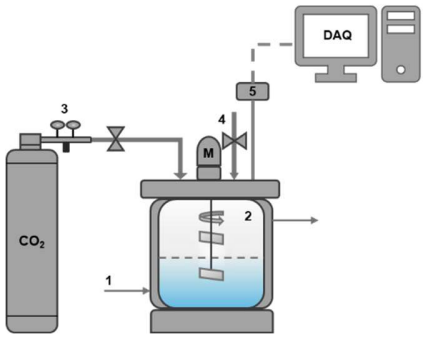

Figure 1. Experimental apparatus. (M) Variable rate stirrer motor; (DAQ) unit for data acquisition and analysis; (1) Water stream from the thermostatic bath to the water jacket; (2) Stirred cell; (3) CO_2 line; (4) Liquid inlet port; (5) Differential Pressure Transducer

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Finding Potential Valorisation Routes for the Production of Bioproduct from Potato Peels

L. Van der Hauwaert[a], A. Regueira[a,b], E. Zondervan[c] , M. Mauricio Iglesias[a]

[a]*CRETUS, Department of Chemical Engineering. Universidade de Santiago de Compostela, Spain. Rúa Lope Gómez de Marzoa, s/n. 15782, Spain*
[b]*Center for Microbial Ecology and Technology (CMET), Ghent University, Coupure links 653, B-9000 Ghent, Belgium*
[c]*Sustainable Process Technology (SPT), University of Twente, Enschede 7500, Netherlands.*

Keywords: Biorefinery design, Superstructure optimization, Agricultural waste management, OUTDOOR

Abstract

Handling agricultural waste is essential to minimizing its environmental footprint, particularly in terms of methane emissions, and advancing towards a circular economy (Gontard et al., 2018). Potato peels are an example of a major agricultural waste stream produced by various food-industries, (e.g., those producing starch), reaching up to 140 000 tons of potato peels annually (Sampaio et al., 2020). As a result, this research focuses on finding processing pathways to transform potato peels into high-value products, thereby optimizing the use of this waste stream.
Promising compounds with significant market value include: Polyhydroxyalkanoates (PHA) as potential bioplastics; phenolic compounds as antioxidants in both the food and cosmetic industries; and proteins for food and animal feed applications (Torres and Domínguez, 2020).
As an increasing number of potential products and processes are being developed, finding the optimal biorefinery design for this feedstock requires a systematic method of evaluation. Additionally, these new potential processing pathways have yet to be compared to classic processing routes (e.g., involving anaerobic digestion) and synergies between existing and emerging technologies have yet to be investigated. As a result, this study employs superstructure optimization techniques to explore early-stage biorefinery designs utilizing emerging and existing technologies. Superstructures are mathematical representations that include all possible design alternatives and configurations for a particular system or process. By optimizing this superstructure, the most efficient, cost-effective, or otherwise optimal system configuration for a given set of constraints and objectives can be identified (Mencarelli et al., 2020).
To generate this superstructure, a panel of experts with academic and industrial backgrounds was assembled to identify potential valorisation pathways (Table 1). Table 1 outlines five main processes, with each participant of the panel assigning a score to indicate the importance of incorporating a specific technology into the superstructure. Additionally, upstream processes, downstream processes and products were identified for these principal technologies. According to the questionnaire conducted, the most interesting pathway to include, according to the panel, was acidogenic fermentation, especially for the production of PHA. Other processes were also discussed, including extrusion for food/feed applications, composting, and distillation for alcohol production. A more detailed overview of the preliminary superstructure can be found at the following link: https://bit.ly/3x0QhTa. However, the design of these biorefineries is not without challenges and comes with significant uncertainty due to for example, feed stock variability. For this reason, exploring processing routes with superstructure optimization under uncertainty is crucial.
With this approach we aim to create early stage biorefinery designs that are robust against uncertainty (e.g., due to feedstock composition or market prices) and which is guided by expert opinions from industry and academia. With these processing routes promising avenues for

sustainable biorefinery operations can be explored, addressing critical issues in waste management, and the circular economy.

Table 1: Potential upstream and downstream processes of 5 main processes discussed with a panel of experts. A score for the principal technologies was also established to identify the importance of a technology being implemented in the superstructure

Process	Score	Upstream processing	Downstream processing
Anaerobic digestion	3.7	Alkaline or acid treatment, milling , freeze drying, bio-pulping , Organosolv pretreatment	1.Biogas combustion for combined heat and power production 2. Methane oxidizing bacteria for microbial protein production 3. CO_2 for hydrogenotrophic microorganisms to produce biomethane
Acidogenic fermentation	4.3	Removal of inhibitors, alkaline or acid treatment, milling , freeze drying	1.Fermentation broth is fed to a mixed culture fermentation to produce PHA 2. Fermentation to produce medium chain carboxylates
Fermentation to Lactic acid	3.6	Removal of inhibitors, alkaline or acid treatment, milling , freeze drying	1.Purification/polymerization of lactic acid to produce PLA 2. Medium chain carboxylates for PHA production
Fermentation to ethanol	3.1	Removal of inhibitors, alkaline or acid treatment, milling , freeze drying	1.Purification and extraction of Ethanol from the fermentation broth
Starch extraction	3	Milling, hydrothermal processing, supercritical CO_2	1.Extraction of proteins and drying of starch for food applications

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Online Monitoring of the Polyol Lipid Liamocin accelerates Development of Novel Biosurfactant Production Process

M. Dicke[a,c], P. Kohl[b,c], T. Tiso[b,c], M. Mann[a,c], J. Magnus[a,c]

[a]*Chair of Biochemical Engineering (AVT.BioVT), RWTH Aachen University, Forckenbeckstr. 51, 52074 Aachen, Germany*
[b]*Institute for Applied Microbiology (iAMB), RWTH Aachen University, Worringer Weg 1, 52074 Aachen, Germany*
[c] *Bioeconomy Science Center (BioSC), Forschungszentrum Jülich, 53435 Jülich, Germany*

Keywords: Biosurfactants, Liamocin, *Aureobasidium pullulans*, Polyol lipid

Abstract

Surfactants are surface-active compounds, commonly found in many products such as detergents, emulsifiers, and foaming agents. Today, most surfactants are produced based on fossil crude oil and therefore contribute to global pollution. Sustainable alternatives to conventional surfactants are microbially produced biosurfactants such as liamocins. Liamocins are a novel group of polyol lipid biosurfactants consisting of a polyol headgroup and an ester of three to four 3,5-dihydroxy decanoic acids (Price *et al.*, 2013). Interestingly, due to their water insolubility, their higher density than water and their secretion into the medium, liamocins form a second oil-like phase during fermentations, simplifying product recovery. Furthermore, Manitchotpisit *et al.* (2011) reported fluorescent behavior of liamocin oils produced by various *A. pullulans* wildtype strains. Based on this, a tool for online monitoring of liamocin formation in small scale cultivations could further accelerate strain engineering and process development.

In this study, small scale characterization of the process is done by employing fluorescence and gas transfer rate measurements in microtiter plate and shake flask cultivations. Using the Kuhner TOM System, characteristic oxygen and carbon dioxide transfer rate profiles were recorded for liamocin production in shake flasks. These profiles allow to distinguish between growth and liamocin production. Fluorescence of the produced liamocin oil was characterized by recording 2D-fluorescence spectra in an in-house build BioLector (Berg *et al.*, 2022). These fluorescence spectra revealed characteristic fluorescence peaks and specific wavelength combinations were chosen for potential online monitoring of liamocin formation. For this, an in-house build prototype capable of monitoring fluorescence and oxygen transfer rates in microtiter plates (Ladner *et al.*, 2016) is used. At certain wavelengths, differences between liamocin-producing and non-producing strains of *A. pullulans* could be observed. Based on these findings, a potent tool to online monitor liamocin production is to be developed. This novel fluorescence-based screening system could accelerate and reduce development efforts for a sustainable industrial production process.

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Harnessing Ocean Water for Sustainable Cellulose Biomanufacturing

Divya Dharshini Uma Shankar[a], Suresh Sudarsan[a], Sumesh Sukumara[a]

[a]*The Novo Nordisk Foundation Centre for Biosustainability, Technical University of Denmark, Kongens Lyngby 2800, Denmark.*

Keywords: Cellulose, Ocean Water, Sustainability assessments, Biomanufacturing

Abstract

Cellulose is a versatile and abundant material traditionally sourced from cotton plants [1][2]. The methods for optimal extraction led to significant freshwater consumption and the use of synthetic chemicals [3]. To address these issues, alternative sources of cellulose production are being explored [4]. Our research aims to develop a sustainable alternative to produce cellulose by harnessing the power of common microbes and using abundant ocean water sources. The study delves into the potential of ocean water for bacterial cellulose production, specifically addressing its influence on production using the native cellulose producer microorganism, *Komagataeibacter xylinus* DSM 2325. We have successfully achieved this major milestone by growing it by 50% solution of ocean water with promising results of producing 1.1 g/l of bacterial cellulose under optimized laboratory conditions. The environmental impact assessment highlights that by using ocean water there is an ~60% and ~70% reduction in consumption of land and water respectively when compared to the traditional methods. The techno-economic analysis indicates that cellulose produced from ocean water offers significant implications for the industry, necessitating a reassessment of current practices and embracing ocean water as a critical driver of sustainability and economic viability in cellulose biomanufacturing.

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High throughput optimization of cyanobacterial processes in novel cultivation devices

L. Poduschnick[a,c], A. Nakielski[b,c], M. Mann[a,c], I. Axmann[b,c], J. Magnus[a,c]

[a]*Chair of Biochemical Engineering, RWTH Aachen University, Worringerweg 1, 52062 Aachen, Germany*
[b]*Institute for Synthetic Microbiology, Heinrich Heine University Düsseldorf, Universitätsstraße 1, 40225 Düsseldorf, Germany*
[c]*Bioeconomy Science Center (BioSC), Forschungszentrum Jülich, 53435 Jülich, Germany*

Keywords: Bioprocess optimization, Cyanobacteria, Small-scale cultivation, Squalene, Illumination intensity.

Abstract

Cyanobacteria pose as an interesting host for biotechnological production processes with regard to closing industrial carbon cycles and thus minimizing industry greenhouse gas emissions. This is the case since cyanobacteria possess the ability to fix CO_2 directly through photosynthesis. Compared with land plants, cyanobacteria exhibit higher photosynthetic efficiency (Branco Dos Santos *et al.*, 2014), are more accessible to genetic engineering techniques and usually their cultivation sites do not compete with arable land (Dietsch *et al.*, 2021).
Nonetheless, production processes involving cyanobacteria or microalgae are still scarce in the biotechnological industry. This is most often associated with high production costs that stem from growth media (Ashokkumar *et al.*, 2019) or the application of artificial lighting (Blanken *et al.*, 2013). Especially, the optimization of lighting conditions can substantially lower the cost of a cyanobacterial cultivation by increasing the cell density through optimization of the incident illumination intensity or optical path length of the photobioreactor (Pfaffinger *et al.*, 2019). Moreover, the composition of the applied light source with regard to different wavelength regimes can influence growth and product synthesis (Nur *et al.*, 2023). Additionally, limitations of cyanobacterial growth by media components are still not fully understood (van Alphen *et al.*, 2018). Thus, it is of great importance to understand and optimize these factors in a cyanobacterial production process starting from early process development for these processes to become viable alternatives.
In our work, we employ two novel photobioreactor systems in microtiter and shake flask scale to cultivate a genetically engineered *Synechocystis sp.* PCC6803 strain, with the ability to produce squalene, an interesting terpene with applications in the vaccine and cosmetics industry (German *et al.*, 2023). By employing small-scale cultivation systems, we enable high throughput experiments towards optimizing the illumination conditions and the media composition. The set-up in microtiter scale comes equipped with a custom-made LED module that can illuminate the wells of a microtiter plate with 48 individually dimmable white light LEDs and thus facilitates the execution of up to 48 parallel illumination intensity experiments (Loogen *et al.*, 2021). The system in shake flask scale on the other hand allows the combination of LEDs with different emission spectrums at different individual illumination intensities (Beuel *et al.*, 2021). Both systems are combined with the **R**espiratory **A**ctivity **MO**nitoring **S**ystem (RAMOS) (Anderlei & Büchs, 2001) or µRAMOS (Flitsch *et al.*, 2016) respectively. While RAMOS devices enable the continuous measurement of oxygen and carbon dioxide transfer rates throughout cultivations, µRAMOS devices solely measure the oxygen transfer rate continuously.
By measuring the oxygen transfer rate, limitations of the cultivation caused by nutrients or light can be identified (Anderlei *et al.*, 2004). Moreover, the overall net oxygen transfer is a measure of photosynthetic activity which in turn is directly correlated with carbon dioxide fixation in cyanobacteria. Thus, by comparing the integral of net oxygen transfer with the integral of incident

illumination, it is possible to determine and subsequently maximize the effective quantum yield and carbon dioxide fixation during cyanobacterial cultivations. In our experiments, we could uncover limitations induced by media components as well as lighting by online monitoring the oxygen transfer rate. Furthermore, our measurements revealed that the oxygen transfer rate reaches a constant maximum value regardless of media concentration and incident illumination intensity in the photo saturated regime. We believe that this value constitutes an intrinsic characteristic of the photobioreactor system, being the maximum biomass fraction that is photosynthetically active. Thus, this parameter will prove crucial in the optimization and scale-up of cyanobacterial production processes as well as in understanding limitations induced by lighting. Overall, these results showcase the unexploited potential of utilizing cyanobacteria for future bioeconomic processes.

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Continuous extraction of VFAs produced during dark fermentation of food wastes using submerged anaerobic membrane bioreactor

J.S. Guez[a], O. Kanine[a], A.R. Fleitas Garcia[a], M. Reynaud[b], M. Goncalves[b,c], C. Gonzalez[c,d,e], P. Fontanille[a,b] and C. Vial[a]

[a]Université Clermont Auvergne, Clermont Auvergne INP, CNRS, Institut Pascal, 63000 Clermont-Ferrand, France. [b] S.A.S Bio-Valo, 63200 Riom, France. [C] Biotechnological Processes Unit, IMDEA Energy, Madrid, Spain [d] Department of Chemical Engineering and Environmental Technology, University of Valladolid, 47011, Spain [e] Institute of Sustainable Processes, Valladolid, 47011, Spain

Keywords: Dark fermentation, Volatile fatty acids, Food waste, Hydraulic retention time, Population analysis, Submerged anaerobic membrane reactor

In the current environmental context, reducing the share of fossil fuels to produce platform molecules has become a key issue. Among the different technologies, dark fermentation (DF) is a process allowing the production of green Volatile Fatty Acids (VFAs) using complex organic substrates as food wastes, which must be recovered. The so-called VFAs are considered as potential marketable chemicals but can also be used as carbon source and converted into a vast number of valuable bioproducts (Singhania et al., 2013). To help these organic products break into the market and become more widely available, the OLEOFERM project (sustainable OLEOchemicals bioproduction from carboxylates via oleaginous FERMentation) aims to maximize carbon recovery from the waste feedstock in order to produce VFAs suitable for subsequent downstream production of microbial oils by oleaginous yeasts. For this, an innovative submerged membrane bioreactor was used to operate dark fermentation and extract, in the continuous mode, sterile liquid fraction containing VFAs directly usable as a substrate.

Two depackaging food waste (DFWs) obtained from a biogas plant (Biovalo, Riom, France) were tested for the production of VFAs. Both substrates were characterized by HPLC, NIR spectroscopy using the IR-SCAN® method to estimate the COD/VS conversion factor. DFW2 contained more than 30 g/L of mono-di saccharides, while DFW1 was around 7 g/L. These differences suggested that DFW1 may had advanced fermentation due to industrial transport, resulting in a lower sugar concentration and higher concentrations of ethanol and lactate, compared to DFW2. Population analysis also showed differences, as DFW1 contained mainly *Weissella*, *Leuconostoc*, *Streptococcus*, and *Lactobacillus*, while DFW2 contained mainly *Lactobacillus* and *Leuconostoc*.

Process parameters were previously optimized within several batch experiments carried out in a 3 L stirred tank reactor. It was chosen not to add inoculum nor heat treated- substrate since it did not exhibit any influence on the performance in terms of VFAs produced (data not shown). In continuous cultures, two different hydraulic retention times (HRTs) were tested, *i.e.,* 4 and 8 days. The pH was set at 6.0 and the temperature maintained at 37°C. Then continuous fermentation was realized using submerged membrane bioreactor. The setup previously published by authors (Trad et al., 2015) was used to define adapted transmembrane pressure (TMP) across a hollow fiber membrane.

The comparison between HRT 4 and 8 days for DFW1 at 37°C, showed a significant difference in the conversion of organic matter into VFAs. Specifically, the HRT of 4 days showed a conversion yield of 30% COD_{VFAs}/COD_{in}, while the 8-day HRT demonstrated a more efficient conversion of 37%. The result was confirmed by the total concentration of VFAs, approximately 23 g/L at 4-day HRT, which increased to 27 g/L at 8-day HRT. VFAs profiles remained remarkably consistent across both experiments, with acetate, butyrate, and propionate being the predominant species. For DFW2, the trend was nearly the same. As for DFW1, the 8-day HRT exhibited a better conversion yield of 38% compared to 36% with a 4-day HRT. Interestingly, DFW2 showed a higher conversion yield than DFW1 for both HRT values. Additionally, the total VFAs concentration was slightly higher at 8-days HRT, reaching 26 g/L against 23 g/L at 4-days HRT. Both, the 4-day and 8-day HRTs experiments, exhibited a consistent VFA profile composed of acetate, butyrate, and caproate.

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Table 1: Total VFAs (g/L), percentage of VFAs (%w/w), conversion yield COD_{VFAs}/COD_{in} (%) and caproate selectivity (%) in continuous cultures with different DFWs and hydraulic retention time (days)

SUBSTRATE	DFW1	DFW1	DFW2	DFW2
HRT (Days)	4	8	4	8
OLR (gCOD/L.D)	30	15	30	15
COD_{VFAs} (g COD/L)	34.44 ± 2.29	42.51 ± 1.08	40.32 ± 1.05	43.22 ± 1.06
Total VFAs (g/L)	23.3 ± 0.82	27.3 ± 1.00	23.32 ± 0.40	25.69 ± 0.59
HAc (% w/w)	40.4 ± 0.81	32.1 ± 0.60	21.17 ± 0.30	22.77 ± 0.17
HPro (%w/w)	16.8 ± 0.05	15.22 ± 0.60	8.49 ± 0.13	12.61 ± 0.14
isoHBu (%w/w)	2.54 ± 0.05	3.15 ± 0.10	2.26 ± 0.04	2.32 ± 0.01
HBu (%w/w)	33.99 ± 0.10	33.61 ± 0.50	37.04 ± 0.13	37.55 ± 0.32
isoHVal (%w/w)	3.99 ± 0.04	5.52 ± 0.1	5.43 ± 0.03	5.00 ± 0.13
HVal (%w/w)	1.25 ± 0.09	3.77 ± 0.30	9.4 ± 0.15	8.57 ± 0.06
HCa (% w/w)	1.04 ± 0.09	6.63 ± 0.30	16.2 ± 0.42	11.17 ± 0.25
COD_{vFAs}/COD_{in} (%)	30.09 ± 0.01	37.15 ± 0.004	35.83 ± 0.01	38.40 ± 0.01
Selectivity C_6 * (%)	1.33	8.78	21.13	15.13

$$(*): Selectivity\ indicator\ (\%) = \frac{\%\ \text{caproic acid}}{(\%\ \text{acetic acid} + \%\ \text{butyric acid} + \%\ \text{isobutyric acid} + \%\ \text{caproic acid})}$$

It was shown that the genus *Anaerococcus* was prevailing in the different experiments (except for DFW1 at 4 day-HRT). The hypothesis was raised that the properties of the substrate may have an effect on the profiles of VFAs produced, as DFW2 (different composition to DFW1 and less pre-fermented than DFW1) favored the production of longer chain fatty acids with a caproate selectivity reaching 21% for DFW2 at an HRT of 4 days.

To optimize the production of VFAs, and separate HRT and solid residence time, a submerged anaerobic membrane bioreactor was developed for the continuous extraction of sterile product. Substrate addition was carried out according to the HRT of 8 days, with a daily addition of 250 ml of substrate, divided into two equal portions. Coupling the reactor with the membrane allowed daily withdrawal of permeate equal to the supplied feed volume. The permeate owing to the membrane and its 0.2 µm porosity, effectively maintained a VFAs composition identical to that of the medium, while excluding the organic matter present within the reactor. Despite of the use of heterogeneous DFWs, the reversible fouling mechanism of the hollow fiber was under control during more than 30 days, using a gas scouring and backwashing strategy. In these conditions, the submerged membrane bioreactor allowed the continuous extraction of a sterile liquid phase containing 32 g/L of VFAs directly usable as substrate, reaching a caproate selectivity of 11% and a high conversion yield of 53%.

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Microfluidic Filtration Device for High Throughput Vaccine Process Development

A. Nusrat Jahan[a], Nicolas Szita[a], Sheng-Ching Wang[b], Marco PC Marques[a]

[a]*Department of Biochemical Engineering, University College London, UK*
[b]*Research & Development, Merck, Rahway, NJ USA*

Keywords: Tangential Flow Filtration, Microfluidics, Downstream Processing

Abstract

Sustainable vaccine manufacturing requires cost-effective, scalable, robust, and efficient processes. While process development efforts have largely focused on improving bioreactor yields, downstream yield is equally important since it can account for a significant contribution to the overall manufacturing costs. Key challenges in downstream processes are the optimisation of filtration and chromatography purification steps. For chromatography development, there are well-established microscale experimentation tools such as resin slurry plates and microscale columns to enable efficient screening studies. However, for tangential flow filtration (TFF), there is a lack of well-understood and cost-effective small-scale screening devices that can identify optimal process parameters for achieving efficient filtration yields for industrial production.
Microfluidic tangential flow filtration (µTFF) devices can be engineered to optimise filtration unit operations and reduce the cost of process development for large molecules downstream processing due to some of their key characteristics, such as low feed volume, laminar flow across the system and the opportunity to integrate sensor technology. These characteristics will enable the study a variety of filtration products with different biochemical and physical properties, using µTFF devices as screening tools. However, there has not been enough efforts to bring these µTFF devices forward.

We have developed and characterised a novel parallel µTFF device for high throughput filtration process development. This device can be used to screen optimal conditions for the purification of viral vectors and vaccines. The prototype has been tested in concentration mode (5-10x with BSA feed concentration up to 10 $g.L^{-1}$) at fluxes ranging between 29.6 LMH to 101.1 LMH (70 to 240 $\mu l.min^{-1}$) at constant 15 psi transmembrane pressure (TMP). Results have shown no aggregation of protein during filtration with minimum retentate yields of 40%. Furthermore, robust operation was achieved (e.g. no internal leaks observed) which suggest that higher yields are achievable at low flow rates with constant TMP.
Integration with sensing technology allows the implementation of automated control strategies, removing operator-induced variability, therefore improving reproducibility and data quality. Our approach has the potential to deliver scale-relevant data, significantly reducing development times and process costs.

Advancing Bioprocessing with Continuous Microfluidic Platforms"

M.A. Wahab [a, b] C. Domingues [b], A. M. Azevedo [a], V.Chu [b], J.P. Conde [b], M.R. Aires-Barros [a]

[a]*iBB—Institute for Bioengineering and Biosciences, Associate Laboratory i4HB—Institute for Health and Bioeconomy and Department of Bioengineering, Instituto Superior Técnico, Universidade de Lisboa, Lisboa, Portugal*

[b]*Instituto de Engenharia de Sistemas e Computadores Microsistemas e Nanotecnologias (INESC MN), 1000-029 Lisboa, Portugal*

Keywords: Continuous biomanufacturing, integrated processing, microfluidics, microbioreactor, cell lysis, aqueous two-phase extraction.

Abstract

Microfluidic devices have emerged as indispensable tools for advancing the development of novel biomanufacturing methodologies, utilizing their capability to efficiently explore a vast array of variables while minimizing reagent consumption. However, despite their capacity to streamline bioprocess design, the modular potential of these microfluidic devices remains largely untapped, with predominant applications concentrating on individual unit operations. Integrated continuous bioprocessing stands ready to revolutionize bioproduction by simultaneously reducing costs, optimizing yields, and minimizing environmental footprints compared to traditional batch operations.

In this study, we present the development of an integrated microfluidic device tailored for continuous processing, demonstrating its capability to seamlessly connect various unit operations. The system integrates a micro-chemostat module for continuous production of a target protein, a chemical cell lysis module for efficient protein release, and a purification and concentration module utilizing aqueous two-phase systems (ATPS). This setup enables the simultaneous screening of multiple conditions for each operation, elucidating their collective impact on the final product. (Figure 1) (Wahab, Domingues, Azevedo, Chu, Conde and Aires-Barros, 2024).

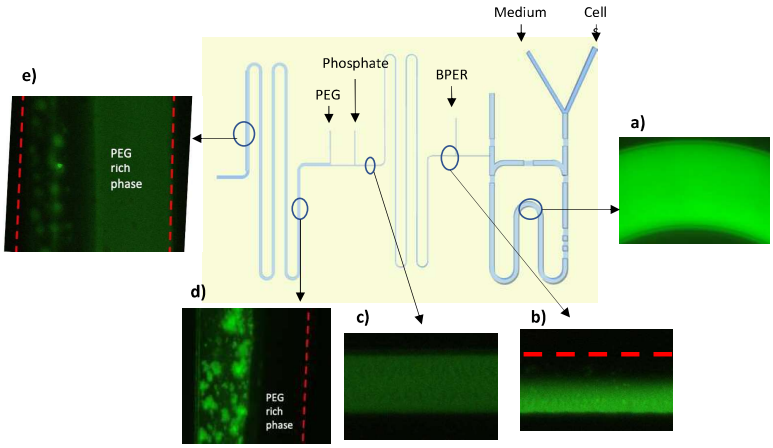

Figure 1: Integrated microfluidic device operation showing: (a) GFP production in a microbioreactor; (b,c) lysis; and (d,e) ATPE

As a model system, we employed a recombinant Escherichia coli (E. coli) strain engineered to produce green fluorescent protein (GFP). This choice facilitated the evaluation of GFP production, lysis efficiency, and partition coefficient via fluorescence microscopy. Over the course of a week-long experiment, continuous medium supply sustained GFP production, while a chemical lysis solution (B-PER®) ensured continuous protein extraction. Subsequently, the extracted GFP was clarified and concentrated in the PEG-rich phase of a PEG/phosphate ATPS system, exhibiting a partition coefficient of 2.

Operating in continuous mode, this integrated device holds the potential for seamless integration of upstream with downstream processing modules, offering a promising pathway for scalable and efficient bioprocessing.

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Helix Lab Research and Education Center, Kalundborg

Anette Birck[a], Thomas Neergaard[a]
[a]*Helix Lab, Campus Kalundborg 3, 4400 Kalundborg*

Keywords: MSc thesis projects, industry collaboration, bio-production, industrial sustainability, industry 4.0.

Abstract

Helix Lab is a first-rate knowledge center in Kalundborg strengthening collaboration between industry and academia. It supports and accelerates the translation of new academic research into specific solutions that will drive industry 4.0 and the green transformation in the industry.

Danish and international MSc students enrolled at Danish universities have the opportunity to do their master thesis project work on bio-production, circular production and industry 4.0 in collaboration with the Kalundborg industry at Helix Lab.

After the first five successful semesters, Helix Lab has proven to be an attractive learning community that supports and develops talents locally, and at the same time strengthens the collaboration between the universities and Kalundborg bio industry regarding research and innovation.

Helix Lab, Kalundborg

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Isolation and quantification of alginate in choline chloride-based deep eutectic solvents

Wimar Reynaga-Navarro[a]

[a]*Bioprocess Engineering, Wageningen University PO Box 16, Wageningen 6700 AA, the Netherlands*

Keywords: Alginate, Deep Eutectic Solvents, Precipitation

Abstract

Brown seaweed is an abundant marine resource mainly exploded for its content of an industrial-relevant hydrocolloid: Alginate. This phycocolloid represents a relevant biomaterial with a broad set of current and potential applications. It is used as a thickener and as a gelling agent in the food industry; or within the medical sector, as a coating material for improving drug delivery and for the production of dental imprints. The alginate extraction comprises several steps with different basic or acid chemical additions. Additionally, other chemicals such as paraformaldehyde could be added to remove pigments and for seaweed preservation to avoid the costly drying process (Saji et al., 2022). Together with the harsh chemicals, the usage of large amounts of water for better handling of the viscous alginate solution, makes the process unsustainable.

One important green technology recently explored for the valorisation of bio-compounds more sustainably, is the utilization of Deep Eutectic Solvents (DES). These types of solvents are defined as a mixture of two or more components: Brønsted-Lewis bases and acids. They possess high melting points individually but when mixed the melting point of the mixture decreases dramatically. Normally composed of a Hydrogen-bond acceptor (HBA) and a Hydrogen-bond donor (HBD). Hydrogen bonds between the compounds determine their good solvent properties (Naik et al., 2022). Deep eutectic solvents have drawn attention since they can be prepared from natural and biodegradable compounds, the so-called Natural Deep Eutectic Solvents (NaDES). Eutectic mixtures could be obtained from mixing ammonium quaternary salts such as choline chloride with amides, polyols, sugars, organic acids, amino acids, etc. This makes their manufacture potentially more sustainable and inexpensive. Additionally, as many combinations could be achieved and with different proportions of each compound, a DES can be tuned to provide the optimal conditions for the target (s) compound extraction. Several investigations have been conducted to extract compounds such as lipids, pigments, and polyphenolic compounds with potential bioactive applications with relative success (Kaoui et al., 2023). However, the extraction of macromolecules represents a bigger challenge due to the complex interactions that could come up with the size and the chemical nature of the molecule.

Before the extraction, a reliable method for alginate quantification needs to be developed. The polymer content is normally determined, based on the analysis of its uronic acids which are released after an acid hydrolysis process. Colorimetric methods exist, however, unexpected reactions occur with some DES compounds. Besides, care must be taken to maintain the structure integrity of the target molecule after the extraction for further applications, and the analytical procedure should reflect this. Therefore, the separation of alginate from DES for analytical purposes should be accomplished. Alginate in water solutions could be chemically separated via precipitation with acid, ethanol or calcium-rich solutions and the pellet used for further analysis. This research work aimed to evaluate different chemical methods to separate the alginate from DES solutions for quantification purposes and identify potential interactions between the solvents and the biopolymer.

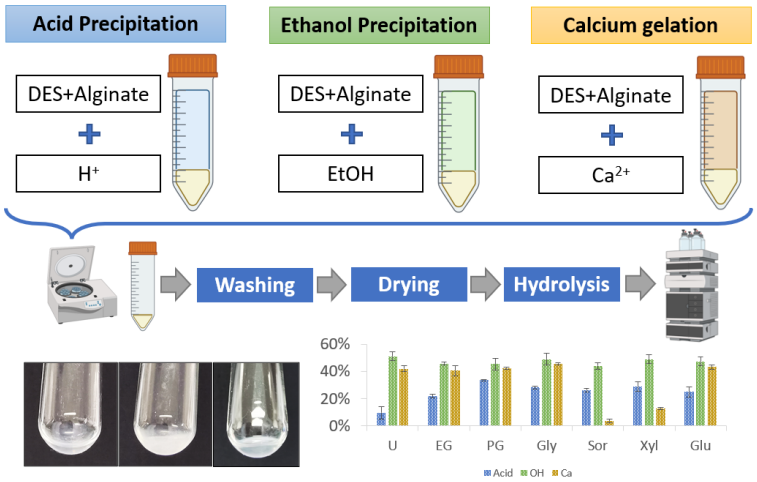

Figure 1. Approach considered for the isolation and quantification of alginate in DES.

DES containing sodium alginate were subjected to precipitation with sulfuric acid 0.2 M (pH 1.6), ethanol-water mixture (80 % v/v) and calcium chloride (1 % w/v CaCl2·2H2O) (Figure 1). Alginate in precipitates was quantified and used to evaluate the performance of each separation technique. The highest recovery yields (51.2 ± 1.3 %) were obtained using the ethanol-water mixture followed by calcium chloride (45.7 ± 1.2 %), except for polyols (e.g. sorbitol). The lowest recovery yields were obtained with acid, with a particularly low recovery yield when urea was used as HBD (9.6 ± 1.3 %) (Reynaga-Navarro, 2024). This research work provides insights into the feasibility of chemical techniques for alginate separation from DES and it will contribute to developing a sustainable seaweed biorefinery platform using this emerging technology.

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Optimizing productivity of Capture SMB processes using an iterative process design approach

L. J. Appoldt[a], Matthias Wiendahl[a], L. Aumann[b], T. Müller-Späth[b]

[a]*Novo Nordisk A/S, Hagedornsvej 1, 2820 Gentofte, Danmark*
[b]*ChromaCon AG, Technoparkstrasse 1, 8005 Zürich, Switzerland*

Keywords: Continuous chromatography, Continuous Capture, Process Design, Protein A, Modelling

Abstract

Continuous capture chromatography using twin columns, also called Capture Simulated Moving Bed (CaptureSMB, or "cSMB") is a chromatographic technique promising higher resin utilization, higher productivity and lower buffer consumption than traditional single column capture processes. The design of cSMB processes is more challenging than the design of batch processes because each "switch" of a given column is influenced by the load from the previous switch and it is in turn influencing the subsequent switch. An iterative approach is necessary to identify optimal loading conditions.

Currently, process design of cSMB processes is based on a single column breakthrough curve and feeding the breakthrough data into a model. However, this concept leads to a sub-optimal process as it is based on a single loading flow rate, while effectively, the cSMB process uses two different loading flow rates in the interconnected and the parallel load phases and the chosen flow rate is not necessarily optimal.

We are presenting an approach where different flow rates and different breakthrough curves are used as model inputs together with pressure-flow dependencies for the resin of interest. Using the improved design procedure of cSMB, significantly higher productivities were achieved than with the regular procedure. The effect was confirmed for a given in-house model process (mAb purification using Protein A affinity chromatography) where productivities could be increased by approximately 50% which has been confirmed at laboratory scale. The presented approach was also evaluated using mechanistic modelling. Finally, Implications of the new cSMB design approach for large-scale manufacturing are discussed.

Hybrid Technology as a Solution for Biomanufacturing Challenges

Getachew S. Molla[a], Ted Johanson[a], Manos Papadakis[a], Niels Banke[a]

[a]*DSM-Firmenich; HMO Innovation & Business Development, Kogle Alle 4, 2970 Hørsholm, Denmark*

Keywords: Hybrid, HMOs, reaction equilibrium, transmembrane transporters

Major inherent limitations of enzyme catalyzed (*in vitro*) and whole-cell catalyzed (*in vivo*) biotransformation processes are the inability to change the reaction equilibrium position and ineffective product transportation out of cells, respectively. While *in-situ* product removal techniques can be used to shift a reaction equilibrium of an *in vitro* process it is predicated on substantial differences in chemo-physical properties between the substrate(s) and product(s), which is often not the case. Product export out of cells in an *in vivo* process can be achieved using transmembrane transporters. However, it is not trivial to find suitable transporters capable of exporting big and complex products. Moreover, they need to be very specific and not also export precursor molecules, which typically contain the same molecular structures recognized by the transporter binding sites. A novel technology that solves these inherent limitations was developed by integrating *in vitro* and *in vivo* approaches into a hybrid process. The hybrid technology was proven to be efficient for the biomanufacturing of several different complex human milk oligosaccharides (HMOs) such as lacto-N-difucohexaose I (LNDFH-I), lacto-N-fucopentaose III (LNFP-III), lacto-N-fucopentaose II (LNFP-II), 3'-sialyl-3-fucosyllactose (FSL), sialyllacto-N-neotetraose (LST-c) and sialyllacto-N-tetraose (LST-a). Results from the hybrid production of a neutral (LNDFH-I) and a charged (LST-a) HMO are presented in this abstract. Figure 1 shows a schematic illustration of the hybrid process for the production of LNDFH-I.

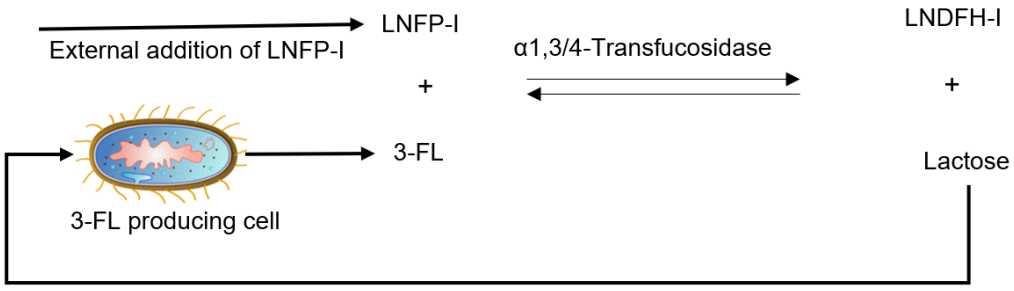

Figure 1: A schematic overview of hybrid process technology illustrating the production of LNDFH-I in a one-pot reaction by combining in vivo formation of 3-fucosyllactose (3-FL) and in vitro transfucosylation of lacto-N-fucopentaose I (LNFP-I) with in-situ recycling of the side-product lactose.

An *in vitro* synthesis of LNDFH-I by α1,3/4-transfucosidase catalyzed transfucosylation of lacto-N-fucopentaose I (LNFP-I) utilizing 3-fucosyllactose (3-FL) as a fucosyl donor led to 43% conversion of the supplied LNFP-I and producing a mixture at equilibrium consisting of 15% 3-FL,17% lactose, 35% LNFP-I and 33% LNDFH-I (wt./wt.). In comparison a hybrid process, which combined the *in vivo* 3-FL formation from lactose by fermentation and *ex vivo* transfucosylation of externally added LNFP-I into LNDFH-I by the action of the same α-1,3/4-transfucosidase, achieved 99% conversion of the supplied

LNFP-I and produced a final mixture consisting of 37% 3-FL, 0.7% lactose, 0.8% LNFP-I and 61% LNDFH-I (wt./wt.). The high conversion in the hybrid process could be achieved as the enzymatic reaction equilibrium was circumvented by *in-situ* recycling of the side-product lactose (released in the enzymatic step) into the fucosyl donor 3-FL.

An *in vitro* LST-a process catalyzed by a α-2,3-transsialidase using lacto-N-tetraose (LNT) as acceptor and 3'-sialyllactose (3'-SL) as sialyl donor achieved 57% conversion of the supplied 3'-SL and produced a mixture at equilibrium consisting of 18% lactose, 21% 3'-SL, 18% LNT and 43% LST-a (wt./wt.). A corresponding hybrid process using the same α-2,3-transsialidase which combined the *in vivo* production of LNT from lactose with the enzymatic transsialylation of the formed LNT using externally supplied 3'SL as sialyl donor, achieved full conversion of the 3'-SL and produced a final mixture of 35% LNT and 65% LST-a (wt./wt.), with no residual lactose or 3'-SL.

Abstract for stickiness assay:

Authors: Alexander Findeisen, Ole Simonsen

Title: *Understanding stickiness of protein formulations, from Lab to Process*

Abstract: The stickiness of enzyme formulations is an important factor in industrial production, especially for drying processes like spray drying, freeze drying or fluid bed drying. In this study, we investigate the influence of water content and temperature on the stickiness of different formulations.

A usual approach for describing stickiness of material is the use of its glass transition temperature. The temperature where a material transits from a rubbery into a crystalline state. There are several methods established measuring the glass transition temperature, most frequently used are Differential Scanning Calorimetry (DSC), Dynamic Mechanical Analysis (DMA) or Thermomechanical Analysis (TMA).

Because of practical reasons and the complexity of our formulations we investigated the use of alternative methods like a texture analyzer and a rotating drum with both controlled humidity and temperature. The results from these experimental setups can then be used for generating regime map to be able to control our processes more efficient and to get a deeper understanding of our process interactions.

A future vision of this project is the implementation of stickiness into models to create better digital twins or digital shadows of our internal production processes.

Effect of dynamic rheology on the gas bubble size distribution at 180 L pilot scale

E. O. Willer, J. M. Woodley

Department of Chemical and Biochemical Engineering, Technical University of Denmark, Søltofts Plads 228A, 2800 Kgs. Lyngby, Denmark

Keywords: Viscosity, Gas bubble size distribution, Pilot scale, & Oxygen mass transfer.

Abstract

Gas-liquid mass transfer phenomena are essential for many biochemical processes, such as aerobic fermentation. Some aerobic fermentations have been shown to result in increased viscosity through the fermentation due to increased biomass or product formation (Sadino-Riquelme et al., 2022). Importantly, viscosity has been shown to influence increasing gas bubble size, affecting gas-liquid mass transfer (Ali & Solsvik, 2021). For other oxygen-dependent enzyme-based reactions, viscosity can also be important, such as with oxidase and oxygenase catalysed reactions. Therefore, investigating the effect of liquid properties such as viscosity on gas-liquid mass transfer is essential. Previous studies have investigated the general influence on the volumetric mass transfer coefficient (K_La) of various operating parameters, and liquid properties. However, understanding the separate effects of liquid properties on the liquid film coefficient (K_L) and interfacial area (a) is needed to fully understand oxygen mass transfer. In this work, results will be presented from experiments aimed at measuring the gas-liquid interfacial area and how gas bubbles change with increasing viscosity under dynamic rheological conditions (such as with power law fluids). The aim is to predict the viscosity effect on gas bubble size distribution, focusing on oxygen mass transfer.

Different experimental methods are used to reach this goal. Experiments are conducted in a 180 L working volume transparent reactor. The dynamic gassing out method is used to determine the volumetric mass transfer coefficient (K_La), and to determine the oxygen concentration, four optical oxygen probes are used (Pyroscience Oxrobsc O_2). They are distributed in the reactor to check for oxygen concentration gradients. Moreover, a shadowgraphic endoscope (Dantec Dynamics) is used to determine the gas bubble size. The endoscope is placed at different heights to determine the impact of viscosity and geometry on the gas bubble size distribution at different heights in the reactor. A power law liquid mimics an increasing viscosity similar to industrial fermentations, and the liquid viscosity is determined by a rotational shear rheometer (Discovery HR-2 Hybrid, T.A. instruments).

The experimental results suggest that dynamic rheology affects the gas bubble size distribution and, thereby, the overall gas-liquid mass transfer coefficient. The reactor configuration seems to have an essential influence

on gas bubble size distribution, and altering the parameters can enhance the interfacial area and, thereby, the oxygen transfer capacity of the reactor.

Acknowledgement

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Pickering emulsions for efficient lipase catalysed synthesis of peracids as a precursor for epoxidation of alkenes

L. Adomi[a], S. Bhutta[b], M. Ansorge-Schumacher[b], A. Drews[a]

[a]*HTW Berlin, Wilhelminenhofstrasse 75A, Berlin 12459, Germany*
[b]*TU Dresden, Zellescher Weg 20b, Dresden 01217, Germany*

Keywords: *Candida antarctica* lipase B, Multiphasic biocatalysis, Pickering emulsions

Abstract

Due to the growing emphasis on environmental conservation and sustainability, there is an increasing demand to substitute the conventional harsh oxidative functionalization procedure, crucial for synthesizing various organic compounds, with biocatalysis. *Candida antarctica* lipase B (CalB), a catalyst widely utilized in industrial applications, has been investigated as an efficient biocatalyst for the oxyfunctionalization of alkenes (Meyer-Waßewitz et al. 2017a). Catalytic oxyfunctionalization of alkenes by lipase usually consists of a two-step reaction: first, the enzymatic oxidation of carboxylic acids using green oxidants to form peracids (PA), and then the subsequent spontaneous oxidation of alkenes by the peracids (Meyer-Waßewitz et al. 2017a). Studies have explored lipase-catalyzed oxyfunctionalization of alkenes in various reaction systems, which are however still challenged with enzyme instability and/or low specific reaction rate (Su et al. 2021).

Pickering emulsions (PEs) have garnered attention as effective reaction medium for multiphasic biocatalytic reactions and have been increasingly studied for more than a decade (Ansorge-Schumacher and Plikat 2022, Wu et al. 2011). However, investigations into enzymatic reactions in PEs have primarily focused on hydrolysis (Wei et al. 2016), transesterification (Heyse et al. 2021), hydrogenation (Zhang et al. 2018), and dehydration (Bago Rodriguez et al. 2021) reactions. To the best of our knowledge, there is no literature that investigated oxyfunctionalization in PEs.

Given the still existing challenges of lipase catalyzed oxyfuntionalization and the efficiency of PEs for multiphasic reactions, this work aims to investigate the first, enzymatic step of lipase catalyzed continuous oxyfunctionalisation of alkenes (peracids synthesis) in PEs, using a membrane reactor that retains the dispersed phase (dp) containing the enzymes. Specifically, the work will evaluate the PE composition and operating parameters that influences the enzymatic synthesis of peracids which serves as a precursor/intermediate for the epoxidation of alkenes.

Initial investigation shows that the synthesis of peracids in PE is indeed influenced by the composition of PEs. The type of nanoparticles used for stabilizing the PE and enzyme concentration significantly influenced the peracids formation as shown in Fig. 1. However, the concentration of the nanoparticles and liquid/liquid phase fraction only had a marginal effect. Additionally, at a hydrogen peroxide concentration of 225 mM in the influent solution and a yield of 62% (Fig. 1), a specific reaction rate of 12.2 $mmol h^{-1} g^{-1}$ was achieved (Adomi and Drews, 2024), which is over 4 times the specific reaction rate reported by Meyer-Waßewitz et al. (2017b) (2.9 $mmol h^{-1} g^{-1}$) at hydrogen peroxide concentration of 192 mM in the influent solution for a single-organic phase reaction medium and immobilized CalB.

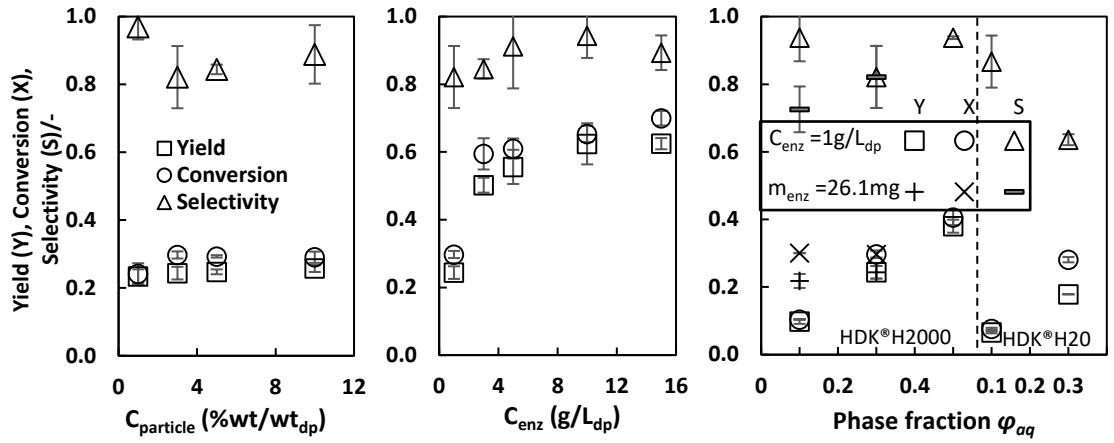
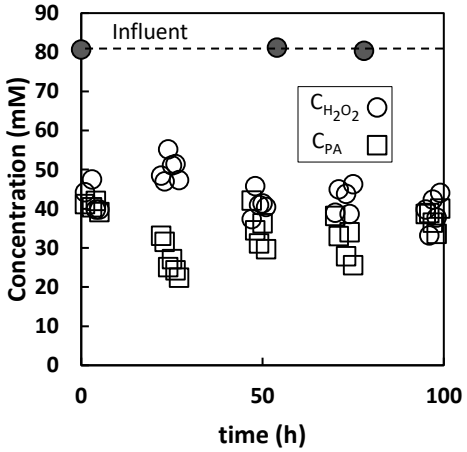


Figure 1: Influence of PE composition on lipase catalyzed peroxyacetic acid synthesis. $C_{H_2O_2,in} = 225\ mM$. *(Adomi and Drews, 2024)*



Further investigation shows that CalB still retained 92% of the initial activity (Fig. 2) after 100 h of continuous operation, which is 24% more than the value reported by Meyer-Waßewitz et al. (2017b) (74% of CalB initial activity retained) for a single-organic phase reaction medium after the same hours of long term operation. This remarkable enzyme stability against deactivation is probably due to the protective environment provided by the nanoparticles against the detrimental effect of the oxidant. This talk will cover the influence of PE composition and operating conditions which will provide essential information for kinetics modelling and process optimization.

Figure 2: Concentration of hydrogen peroxide and peroxyacetic acid (PA) over 100 h of operation. C_{enz} *= 5 g/*L_{dp}*,* $C_{particles}$ *= 3%wt/*wt_{dp}*,* τ*= 2.54 h,* φ_{aq} *= 0.3. (Adomi and Drews, 2024)*

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Mathematical modelling of the oxygen transfer rate (OTR) as a first step towards the development of a digital twin

M. Lemperle [a], M. Albino [a], P. Ramin [a], J. Kager [a], B. Cassells [b], S. Stocks [b], K. V. Gernaey [a]

[a]*Process and Systems Engineering Center (PROSYS), department of Chemical and Biochemical Engineering, Technical University of Denmark, Building 228A, 28000 Kgs. Lyngby*
[b]*Novonesis, Fermentation Pilot Plant, Krogshoejvej 36, 2880 Bagsværd, Denmark*

Keywords: Oxygen transfer rate, Modelling, Digital twin,

The oxygen transfer rate (OTR) is often a limiting factor when targeting maximum yield in a fermentation process. Understanding the OTR is therefore critical for improved bioreactor performance, as dissolved oxygen often becomes the limiting factor in aerobic fermentations due to its inherent low solubility in liquids such as in fermentation broths[1]. With the long-term aim of establishing a digital twin framework, the initial phase of development involves mathematical modeling of the OTR in a pilot-scale bioreactor, hosting the filamentous fungus *Aspergillus oryzae* using an elaborate experimental design.

The experimental design is specifically tailored to the interplay of the factors influencing the OTR e.g., airflow, back-pressure and agitation speed. Through a set of 4 fermentation, a full-factorial experimental design with three factors (aeration, agitation and pressure) and two levels (high and low) is designed. Concluding the 2^3 factorial design, 8 different unique patterns of factors and two centre points were investigated in four different fermentation processes. Two fermentations were conducted with the highest and lowest values of the factors, respectively. The other two fermentations were carried out with different patterns from the full factorial design, each initiated in a subsequent step change manner for a prolonged period, starting after the outgrowth phase of the fermentation. In this methodology, the entire design space is covered in 4 fermentation processes.

Viscosity, which describes the flow behavior of a liquid, is sometimes neglected as a factor affecting the oxygen transfer because its behavior in the bioreactor is not always well understood. The OTR in fermentation processes, particularly in low cell density fermentations, typically disregards the influence of viscosity on OTR. However, this approach becomes unreasonable when using fungal strains to produce proteins due to their inherent higher observed viscosity. Since viscosity plays a crucial role in determining the mass transfer properties in the chosen fungal process, understanding its effects is essential for modeling the OTR[2].

Therefore, cell dry weight and off-line viscosity measurements were taken from each of the above-mentioned industrial based fermentation processes throughout the fermentation. The subsequent analysis aims to decipher the relationships between the OTR and the agitation, aeration, head pressure and viscosity, thus providing the basis for an accurate and reliable mathematical model of OTR. Following Ostwald-de Waele's power law correlation between shear stress and apparent viscosity, various empirical models, including Metzner and Otto and Perez et al. were investigated to assess which correlates the shear stress most accurately.

This physical OTR model presents the first step towards developing a digital twin, aiding with operational decisions for fermentation processes.

Acknowledgement

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Solving Chromatography models in CADET-Julia

Jesper Frandsen[a], Jan Michael Breuer[b], Eric von Lieres[b], Johannes Schmölder[b], Jakob Kjøbsted Huusom[a], Krist V. Gernaey[a], Jens Abildskov[a]

[a]*Dept. of Chemical and Biochemical Engineering, Technical University of Denmark, Søltofts Plads, Building 228A, 2800 Kgs. Lyngby, Denmark*
[b]*Forschungszentrum Jülich, IBG-1: Biotechnology, Jülich 52428, Germany*
Corresponding author: jespfra@kt.dtu.dk

Keywords: Chromatography, Modelling, Numerical methods, Discontinuous Galerkin, Julia

Abstract

Chromatography plays a pivotal role in the purification of biopharmaceutical products and proteins. Continuous chromatography stands out as a promising approach to boost productivity while minimizing solvent usage compared to traditional batch chromatography. However, the implementation of continuous chromatography is notably intricate. It necessitates the utilization of multiple columns arranged in series and/or parallel configurations within open or closed loops (Schmidt-Traub et al., 2020).
While modeling can guide the design and operation of continuous chromatography, it is essential to acknowledge the computational challenge posed by solving the underlying partial differential equations (PDEs). The simulation time required for these models can be substantial. Notably, for the simulated moving bed (SMB), the simulation time can become significant as all columns must be solved simultaneously until cyclic steady state (He et al., 2020).

The prolonged simulation times underscore the need for fast solvers. To address this challenge, Meyer introduced an arbitrary order Discontinuous Galerkin spectral element method (DGSEM) for solving the general rate model in batch chromatography (Meyer et al., 2020). Recently, Breuer derived a slightly different DGSEM variant and implemented it using C++. The resulting code is open-source and publicly accessible through the CADET-Core software (github.com/modsim/CADET) (accessed 28/02/2024) (Breuer et al., 2023; Leweke & von Lieres, 2018).
While C++ is a programming language renowned for its high computational performance, it is a low-level compiled language and thus demands more programming expertise compared to languages such as Python. In contrast, Python is a dynamic high-level programming language that is easy to use but at the expense of lower computational performance. Meanwhile, the programming language Julia leverages the advantages of C++ and python as it is a dynamic, high-level programming language that simultaneously generates fast, low-level machine code (Bezanson et al., 2017). Furthermore, Julia has a broad range of Ordinary Differential Equations (ODE) solvers for stiff problems (Rackauckas & Nie, 2017), which are often encountered in chromatography modelling (Kumar & Lenhoff, 2020).
The CADET-Core is a fundamental system solver with various unit operations including chromatography columns, crystallization, filtration, reactions etc. It supports arbitrary sequence and networks of unit operations, tubes, valves, tanks etc. Given the extensive functionality and complex codebase, there is a need for more agile and accessible code. For example, the two most used isotherm models, the steric mass action isotherm for ion-exchange chromatography and the Langmuir isotherm, cannot always explain resulting chromatograms (Kumar & Lenhoff, 2020). Especially for complex chromatography modes with various adsorption phenomena present, such as mixed-mode chromatography, the knowledge base and selection of isotherm models available

is limited (Kumar & Lenhoff, 2020). Thus, to mathematically describe the adsorption behavior accurately, new isotherm models are needed. To implement isotherm models easily while maintaining high performance computation of chromatography models, the CADET-Julia package was developed. The Julia package has implemented the DGSEM for chromatography models in a simplified codebase with ease of implementation of new models for rapid prototyping and customized isotherms. The ease of implementing new isotherms is demonstrated by the fact that specifying the analytical Jacobian is not necessary to achieve very high solver performance. Hence, the isotherm models can be specified arbitrary and take many factors into account. The CADET-Julia code can be found on Github (github.com/jespfra/CADET-Julia) (accessed 28/02/2024).

In this study, extensive benchmarks of CADET-Julia to the C++ implementation in CADET-Core are made in terms of convergence of error and simulation time for different complex continuous setups. The various Julia solvers will be tested for various chromatography models and setups. The performance differences between the CADET-Julia and CADET-Core will be discussed.

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Hybrid semi-parametric Modeling vs Physics-informed Neural Network: A Comparative Study Applied for Bubble Column Aeration

P. Jul-Rasmussen[a], M. Kumar[b], J. Pinto[b], R. Oliveira[b], X. Liang[c], J. K. Huusom[a]

[a]*Process and Systems Engineering Center (PROSYS), Department of Chemical and Biochemical Engineering, Technical University of Denmark, Kongens Lyngby, DK-2800, Denmark*
[b]*REQUIMTE/DQ, Faculty of Science and Technology, University Nova de Lisboa, Campus de Caparica, 2829-516 Caparica, Portugal*
[c]*Center for Energy and Resources Engineering (CERE), Department of Chemical and Biochemical Engineering, Technical University of Denmark, Kongens Lyngby, DK-2800, Denmark*

Keywords: Hybrid semi-parametric model, Physics-informed neural network, Bubble column aeration

Abstract

As data is becoming more easily accessible in the (bio)chemical industry, there is a renewed interest in leveraging data-based methods for the improvement of process operations (Udugama et al., 2020). Even though vast amounts of data are available for said processes, it is often serially correlated and with low variation due to the operation of the processes at specified set points. As a result "purely" data-driven modeling approaches are often inadequate in predicting process outcomes, and there is a high risk of the models extrapolating when used for process optimization. As a way of mitigating these problems, *a priori* process knowledge can be incorporated into the development of data-based models.

Process knowledge and data-driven methods can be combined in "hybrid semi-parametric modeling" (hereinafter referred to as hybrid modeling) (Thompson and Kramer, 1994). Over the last 30 years, hybrid modeling has been gaining popularity and a large number of applications of hybrid modeling have been published (Sansana et al., 2021). Hybrid modeling offers the possibility to model well-known phenomena using a parametric model, such as first principles equations, while unknown or uncertain phenomena are modeled using a nonparametric model, e.g. neural networks. In typical applications of hybrid modeling, feedforward neural networks are used for predicting mass transfer coefficients in material balances for bubble column aeration (Jul-Rasmussen et al., 2024) or for predicting kinetic parameters for population balances in flocculation processes (Nazemzadeh et al., 2021), but also more advanced nonparametric models such as long short-term memory (LSTM) networks have been used for predicting specific reaction rates for material balances in a HEK293 process (Ramos et al., 2024).

A new and promising way of including process knowledge in data-driven modeling is using "Physics-informed Neural Networks" (Raissi et al., 2019). In physics-informed neural networks, the physical knowledge about the system is implemented in the cost function used for training the neural networks, rather than enforcing the mathematical structure as done in hybrid models. Physics-informed neural networks can be used for approximating both ordinary differential equations and partial differential equations, using automatic differentiation for differentiating the neural networks. Through the cost function, both boundary/initial conditions and the mathematical structure of the ordinary/partial differential equation are imposed for the neural network to learn these system characteristics. Physics-informed neural networks have been used for solving lumped kinetic models for chromatography processes (Tang et al., 2023) and for model predictive control of CSTR systems (Zheng and Wu, 2023).

In this work, a comparative study of hybrid modeling and physics-informed neural networks is performed based on the same pilot-scale bubble column aeration case as used by Jul-Rasmussen et al. (2024). Both modeling approaches are applied to the same data obtained from experiments performed at the pilot plant at the Technical University of Denmark. The bubble column is used for investigating oxygen mass transfer using the hydrogen

peroxide-catalase method to determine the oxygen transfer rate and volumetric mass transfer coefficient ($K_L a$). The physical knowledge included in both modeling approaches is based on species conservation balances, while $K_L a$ is considered as the unknown phenomenon. Furthermore, a sensor drift is observed for the dissolved oxygen sensor in the bubble column, meaning that the models must be able to account for a sensor bias, which is different depending on the time the experiment was performed. The study seeks to investigate strengths and weaknesses in the two modeling approaches, comparing the prediction accuracy, extrapolative capabilities, and ease of interpretation.

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DNS of an extreme case of a plate with holes as inlet for a bubbly gas-liquid photobioreactor.

B. Martínez López[a], G. Guizard[b], R. Escudié[b], H. Gómez[c], G. Capson-Tojo[,b]

[a]*UMR 1208 Ingénierie des Agropolymères & Technologies Emérgentes, Université de Montpellier, INRAE, Institut Agro, 34060 Montpellier, France.*

[b]*INRAE, LBE, 102 Avenue des étangs, F-11100 Narbonne, France*

[c]*School of Mechanical Engineering, Purdue University, 585 Purdue Mall, West Lafayette, 47907 IN, USA*

Keywords: mathematical modeling, gas-liquid flow, photobioreactor, openFoam, VOF, DNS.

Abstract

The goal of the research project twins4GasPBR is to develop an open-source digital twin of a gas-fed photobioreactor, and to validate it experimentally. Since we are aiming for a 3D CFD-based model, one of the most important aspects with respect to realism, and that will allow better control over upscaling, is an appropriate reproduction of the geometry of the reactor prototype. As well, we intend to reproduce a somehow realistic a bubbly flow, hence the choice of a volume of fluid (VOF) model. This is a step forward with respect to the 0-d CSTR models that are often used to represent such systems (Tsapekos et al., 2022), and more specifically, to those that use purple phototropic bacteria (Puyol et al., 2017).

Our current experimental setup (figure 1) is a cylinder with a liquid height of 0.64 m and a radius of 0.05 m. In order to facilitate the solubilization of the gas in the liquid, a micro-diffuser is used as gas inlet. This diffuser is an extreme version of a plate with holes: the pore diameter is only of a few μm, which means that a circular surface with r = 0.05 m is covered by an uncountable number of them. This poses two exceptional challenges that question the state of the art of modeling and simulation: i) generation of such a complex mesh and ii) the simulation of a realistic bubbly flow using an amount of computing power that does not make it prohibitive for practical applications. The present work intends to show how to overcome these difficulties.

With respect to i) as of today, and specially on places that work with applications of mathematical modelling, rather than on the development of modeling tools, it is common practice to use the built-in meshers that are shipped with the software. This means that the geometry must be drawn either point-by-point (COMSOL, 2019), or with an external CAD tool, and then imported into the simulation environment. Although current CAD packages ("FreeCAD," 2022) allow for parametric drawing, both solutions are too manual, and hence not adapted to deal with hundreds or thousands of inlet patches.

With respect ot ii), in the case all the inlets could be drawn to some extent, simulation of such a system would require a prohibitive amount of computing power. The VOF model relays on a diffuse interface approach, in which the interface (in this case between the liquid and the gas) spans several volume elements. Consequently, the quality of the numerical results will be extremely dependent not only on the general coarseness of the mesh, but on the ratio of faces

per inlet patch. Additionally, in order to avoid artificial deformation of the bubbles, the cells should as close to a cubic shape as possible.

The mesh generation problem has been dealt with by developing the tool *blockMeshScript*, that assists on the generation of a mesh that represents the reactor and the diffuser only requiring as inputs the pore size, their spacing, the radius and the height of the liquid. The output of this tool is a specification file that is compatible with the mesh generation tool *blockMesh*, that ships with openFoam (Greenshields, 2020).

This tool was then used for a mesh consistency test that allowed to establish the amount of faces per inlet patch that is necessary in order to reproduce the bubbly flow numerically without the need of turbulence models. Finally, a number of meshes that increase the amount of pores and decrease their radius were generated, and used to establish the liquid height at which bubble coalescence occurs. These two numerical experiments will allow to find a compromise between physical realism and practicality. Finally, the numerical bubble distributions were compared with experimental data.

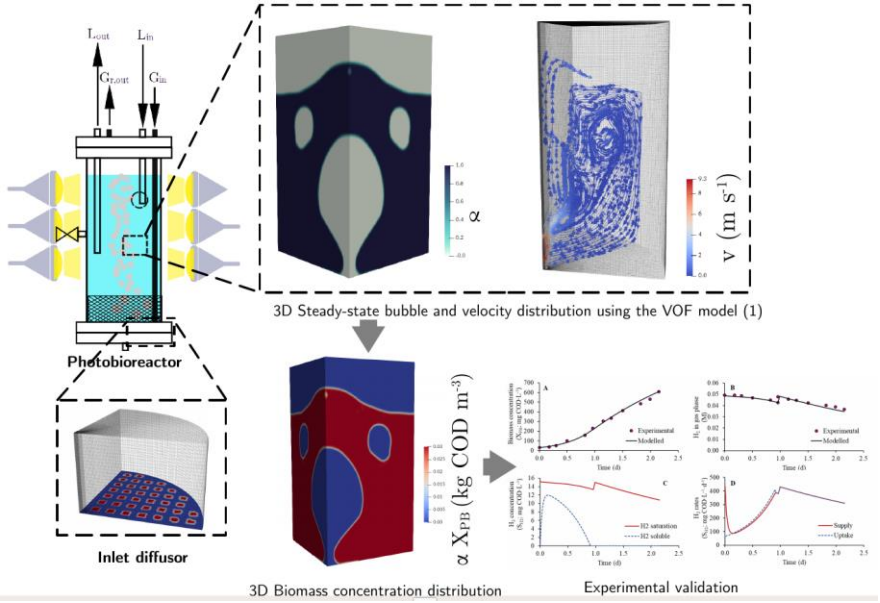

Figure 1: diagram of the photobioreactor, highlighting the inlet diffusor and the bubbles

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Understanding Variation on an Industrial *Aspergillus oryzae* Fermentation Process – A Study Across Scales

Mariana Albino[a], Carina L. Gargalo[a], Ulrich Krühne[a], Mads O. Albæk[b], Gisela Nadal-Rey[b], Krist V. Gernaey[a]
[a]*Process and Systems Engineering Center (PROSYS), Department of Chemical and Biochemical Engineering, Technical University of Denmark, Building 228A, 2800 Kgs. Lyngby, Denmark*
[b]*Novonesis A/S, Fermentation Pilot Plant, Krogshoejvej 36, 2880 Bagsvaerd, Denmark*

Keywords: scale-down, fermentation, *Aspergillus oryzae*, rheology, industrial scale

Abstract

Industrial fermentation processes have long been used, however, there is little literature available reporting data from such big scales, most often due to confidentiality issues. For this reason, it is hard to improve said processes based on scientific publications since most studies concern small-scale reactors. Studying large-scale processes in detail to understand the sources of batch-to-batch variation is essential since it is one of the biggest challenges in industrial biomanufacturing.

This contribution concerns an *Aspergillus oryzae* fermentation process with considerable batch-to-batch variation at both production and pilot scales. The aim is to understand how process variables and parameters affect the fermentation process performance. A key element of filamentous fungi fermentation processes is understanding the relationship between the biomass concentration and the broth's rheology. This is because an increase in broth viscosity, as biomass concentration increases, can reduce mass transfer, potentially impacting process performance (McIntyre et al. 2002). This study first analyzed on-line process data, as well as off-line samples for biomass concentration and viscosity, to identify possible sources of batch-to-batch variation in production.

A meaningful way to gain process understanding for optimization is through scale-down studies since experiments on production scale are not feasible. With an adequate scale-down model, it is possible to study the large-scale process and find leads for improved process performance (Noorman 2011). Thus, the process was downscaled to pilot scale for a detailed examination of crucial process parameters, feeding settings and agitation power, and their influence on the process' KPIs (product titer and yield coefficients). The findings were compared with those at large scale, revealing that the yield of product on substrate was generally superior at the large scale. Lastly, a multi-omics (metabolomics, proteomics and transcriptomics) analysis was carried out for fermentation processes under varying agitation and aeration conditions. This was done to assess and gain a deeper understanding of their effect on the cells' metabolic behavior. In conclusion, the collected and analyzed data allow a deeper understanding of the process differences across scales, which can ultimately be used for process optimization.

Acknowledgment

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Development of computational tools for modelling and scale-up of industrial bioreactors

D.D. McClure[a], J.M. Kavanagh[a], D.F. Fletcher[b], W. Gu[b], G. Nadal-Rey[c] and K.V. Gernaey[c]

[a] *Department of Chemical Engineering, College of Engineering, Design and Physical Sciences, Brunel University, Uxbridge, London UB8 3PH, UK*
[b] *School of Chemical and Biomolecular Engineering, J01 The University of Sydney, Darlington, 2006, Australia*
[c] *Process and Systems Engineering Center (PROSYS), Department of Chemical and Biochemical Engineering, Technical University of Denmark, Building 228A, 2800 Kgs. Lyngby, Denmark*

Keywords: Scale-up, Bioreactor modelling, Computational Fluid Dynamics

Abstract

Biotechnology offers the opportunity for the sustainable production of a wide range of compounds, however a major challenge is successfully scaling-up processes from laboratory to industrial scale. Performing large-scale experimental work is generally costly and time-consuming, so there is an increasing trend to use computational tools for the modelling and scale-up of industrial bioreactors (Straathof et al., 2019). Development of such computational tools firstly aims to provide understanding of the conditions found inside industrial-scale equipment, and from this understanding identify ways in which the reactor design and operating conditions can be improved in order to deliver increased process performance.

The performance of a bioreactor depends on the conditions experienced by the microorganisms, which in turn depends on mixing and mass transfer within the reactor (Nadal-Rey et al., 2021). Various approaches exist to model such systems, each having advantages and disadvantages. For example, Computational Fluid Dynamics (CFD) based models provide a high degree of information about the hydrodynamics within bioreactors. Integration of such models with Lagrangian particle tracking can provide further information about the conditions experienced by microorganisms. However, such models have the disadvantage of a high computational demand. Contrastingly, simpler models based on systems of ordinary differential equations have a much lower computational demand, while sacrificing the resolution offered by CFD.

In this presentation available computational tools for modelling bioreactors will be examined. These include Computational Fluid Dynamics (CFD) models (including models using Lagrangian particle tracking), compartment models and hybrid modelling approaches where several models are synthesized. Use of these models will be discussed in the context of industrial fermentation, looking at examples from pilot-scale (~50 L) to industrial-scale (~90,000 L), and considering different reactor designs, microorganisms and operating conditions. Advantages and disadvantages of different modelling approaches will be discussed, and suggestions for further work in the area will be highlighted.

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Unraveling the single cell behavior of *Escherichia coli* producing L-phenylalanine in a scale-down bioreactor by automated real-time flow cytometry analysis of multiple fluorescences

P. Arulrajah[a,b], D. Weuster-Botz[b] and A-L. Heins[a]

[a] *Hamburg University of Technology, Insitute of Bioprocess and Biosystems Engineering, Denickestraße 15, 21073 Hamburg, Germany*
[b] *Technical University of Munich, Chair of Bioprocess Engineering, Boltzmannstraße 15, 85748 Garching, Germany*

Keywords: population heterogeneity, automated-real time flow cytometry, fluorescent reporter strains, bioprocess monitoring

Abstract

Nowadays, biotechnological production has emerged as a sustainable alternative to chemical processes (Soetaert and Vandamme, 2006). However, scaling up from laboratory- to industrial-scale often results in productivity loss (Schmidt, 2005), partly attributed to arise of phenotypic population heterogeneity. Despite this phenomenon being well-established, its mechanistic understanding remains limited (Fernandes et al., 2011), sparking an ongoing debate regarding its impact on bioprocess outcomes. Therefore, it is crucial to quantitatively investigate phenotypic population heterogeneity with temporal resolution. Building upon this, a bioprocess-phase-specific assessment, enables the development of strategies to positively influence the level of population heterogeneity through cell physiology-based bioprocess control strategies. A popular method to study population heterogeneity in bioprocesses are fluorescent reporter strains, which express one or several fluorescent proteins together with cellular genes of interest. Hence, each fluorescence, detectable through flow cytometry analysis, correlates to a specific cellular characteristic. By applying automated real-time flow cytometry (ART-FC), monitoring of cellular characteristics on a minute scale without supervision over several days is enabled. For studying the effect of mixing insufficiencies on cellular physiology, scale-down bioreactors (SDB), for instance, consisting of a well-mixed stirred-tank (STR) bioreactor and a coiled flow inverter (CFI) in a bypass (Hoang et al. 2023), simulating a gradient zone, are applied.

We chose to study the production of the industrially relevant amino acid L-phenylalanine from glycerol with *Escherichia coli* FUS4 (pF81$_{Kan}$) (Weiner et al. 2017) in a fed-batch process. This process consists of three phases: an initial batch phase, followed by a two-step biomass production phase, and culminating in the product formation phase after induction with IPTG. The *E. coli* FUS4 (pF81$_{Kan}$) strain was transformed into a quadruple reporter strain by integrating three fluorescent proteins in the chromosome (Hoang et al., 2023) enabling to monitor oxygen availability (CyOFP1), general stress response (mTagBFP2) and growth (mEmerald) on single cell level. The fourth fluorescent protein, mCardinal2, is expressed from the IPTG-inducible plasmid to follow single-cell product formation. For uninterrupted monitoring, *at-line* bioprocess analytics for the evaluation of key performance parameters were supplemented by integrating an automated sampling and sample processing unit (OnCyt Microbiology AG) into the bioprocess setup allowing ART-FC analysis every 20 minutes. Initially, this bioprocess was conducted in a homogeneous STR as a reference. Afterward, it was transferred to the SDB, whereby the mean residence time inside the CFI was set to 102.63 s and 403.30 s, respectively. On population level, comparing STR and SDB cultivations revealed decreased biomass and L-phenylalanine concentrations by up to 15% with increasing residence time in the CFI.

On single cell level, predominantely monomodal distribution were found during the biomass production phase in the STR reference process, suggesting uniform conditions inside the bioreactor. However, the introduction of a highly concentrated feed led to arise of a less active subpopulation comprising approximately 5 % of the total cell population. In contrast, the cultivation in the SDB with a residence time of 102.63 s in the CFI revealed a bigger subpopulation of around 14 % during the biomass production phase. This subpopulation diminished upon the application of a second, higher concentrated feed, resulting in a broader, single population by the end of the biomass production phase. Additionally, heterogeneous distributions were observed for oxygen availability, with three distinct populations evident at the transition to higher concentrated feed, which later merged into one broader distribution. Surprisingly, cultivation in the SDB with a mean residence time of 403.30 s in the CFI exhibited more homogeneous fluorescence distributions of oxygen availability and growth compared to the SDB cultivation with lower residence time. After induction with IPTG, STR cultivations exhibited solely monomodal distributions, while SDB cultivations displayed heterogeneous responses. Notably, when the residence time was set to 102.63 s, approximately 17% of the population exhibited reduced single cell growth. Furthermore, although no distinct subpopulations were observed, both the oxygen availability and product formation marker displayed tailing toward lower fluorescence intensities. When the residence time in the CFI was set to 403.30 s, heterogeneous growth distributions with tailing towards lower fluorescence intensities were found. Moreover, distributions for oxygen availability and product formation were less heterogeneous in these cultivations. Our results suggest cellular adaptation to enhance robustness against fluctuating conditions during experience of extended gradients whereas weaker gradients did not seem to significantly induce these processes.

Furthermore, the population heterogeneity level increased towards bioprocess end, characterized by wide distributions, possibly due to the onset of by-product accumulation. This can aid in predicting process termination based on cell physiology or developing intervention strategies to enhance bioprocess performance. It was further noted that subpopulations mainly emerged during late biomass production and L-phenylalanine production phase, prompting consideration of heterogenous cell induction. Another parameter conducive to the development of physiology-based process control is the overall feeding strategy, especially during the biomass production phase, given the pronounced heterogeneities observed therein. In conclusion, our investigations underscore the potential of beneficially influencing the level of phenotypic population heterogeneity to enhance the robustness of bioprocesses.

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Development of a pressurized bioprocess for psilocybin production in *Aspergillus nidulans*

S. Weiser[a,b], J. E. Kufs[c], B. Bardl[a], D. Hoffmeister[a,b], V. Valiante[a], L. Regestein[a]

[a]*Leibniz Institute for Natural Product Research and Infection Biology – Hans Knöll Institute (Leibniz-HKI), Beutenbergstraße 11a, 07745 Jena, Germany*
[b]*Faculty of Biological Sciences, Friedrich Schiller University Jena, Bachstraße 18k, 07743 Jena, Germany*
[c]*Faculty of Technology, Bielefeld University, Universitätsstraße 25, 33615 Bielefeld, Germany*

Keywords: filamentous fungi, pressurized bioprocess, overpressure

Abstract

Aerobic microbial bioprocesses are indispensable for producing many biotechnological products. Due to the low solubility of oxygen, this essential substrate needs to be constantly supplied during bioprocesses. Filamentous fungi can form diverse morphologies from dispersed mycelia to dense pellets in liquid cultures (Cox et al., 1998). Especially, freely dispersed growth often increases the culture broth viscosity resulting in mixing problems accompanied by extremely reduced air bubble dispersion and inhomogeneous nutrient supply. Moreover, the shear sensitivity of filamentous structures can limit stirrer speed adaptations to counteract mixing issues. Besides aeration rate, bubble dispersion, and aeration with elevated oxygen concentrations, overpressure is a measure to generally increase gas solubilities, and thus, gas transfer (Knoll et al., 2007). Therefore, the application of overpressure presents a promising strategy to overcome oxygen limitation without the need for increased stirrer speeds for filamentous fungi.

At Leibniz-HKI, the filamentous fungus *Aspergillus nidulans* was genetically modified for heterologous production of psilocybin, a promising prodrug candidate in psychotherapy (Hoefgen et al., 2018). To maximize psilocybin formation, the influence of hydromechanical stress and the application of overpressure on growth and product formation were investigated in 7 l stirred tank reactors and 7.5 l pressure reactors. Within the tested conditions, increasing stirrer speeds correlated with a process time reduction. The pressurization further shortened process time and enabled oxygen-unlimited cultivation at reduced hydromechanical stress.

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Bioprocess Microfluidics 2.0: Towards Standardised Microfluidic Platforms for Applications in Bioprocessing

M.P.C Marques and N. Szita

University College London (UCL), Biochemical Engineering, Gower Street, London, WC1E 6BT, United Kingdom

Keywords: bioprocessing, microfluidics, microreactors, standardization, platform technologies

Abstract

Standardisation facilitates the commercial uptake of new technologies. It is a de-risking approach to reduce failure rates which in turn makes the development of products more economical. Standardisation for example enables the manufacturers to produce off-the-shelf components. These lead to enhanced reliability in the fabrication process, resulting in yield improvement and ultimately in the mentioned cost reductions. Standards facilitate the communication between supplier and end-user, they standardize and thus streamline any testing protocols, and they strengthen manufacturing practices and commerce between different customers and companies around the world (Reyes, 2019). Standards are particularly valuable for low to medium high production volumes, such as those known for microfluidics manufacturers. With the ISO norm 22916:2022, a set of minimum specifications have already been defined for the interoperability of microfluidic components.

Platform technologies relate to the principle of standardization, and quite a few microfluidic platforms have been successfully developed (Haeberle, 2007). With these platforms, many possibilities and opportunities exist to manipulate liquids, for example to mix them for a specific reaction, or to separate out compounds, and to integrate detection methods, and to create automated workflows. We have identified two challenges particular to Bioprocess Microfluidics (Marques & Szita, 2017), which are not typical to the analytical systems where microfluidic applications originally stem from. Unless a specific high-throughput screening application or an analytical tool is sought, microfluidic devices for bioprocessing must accommodate comparatively large volumes. For cell therapy and regenerative medicine, it might be chambers where organoids or 3D cell clusters, such as embryoid bodies, can fit. In biopharmaceutical processing and microbial fermentations, large cell densities of cells in suspension facilitate the comparability with larger scales. And for small molecule drugs, a significant number of immobilized biocatalysts is required to avoid variability in biocatalyst load. The second challenge ties in with the first one. It is often cumbersome to reliably and reproducibly insert the bio-material into microfluidic devices. Unlike with larger reactors, it is non-trivial to create access ports into the small structures, and flowing the bio-material from an up- or downstream port can negatively affect either the bio-material itself or induce variability of the amount of bio-material loaded.

We have developed a unique microfluidic design (Reichen, 2012) which contains a resealable lid with which the afore-mentioned challenges are addressed. With this device, we have demonstrated a gamut of bioprocessing applications: immobilized enzymes for the synthesis of active pharmaceutical ingredients, mammalian cell culture for the production of monoclonal antibodies, adherent cell transfection, expansion, and differentiation for regenerative medicine, and CAR T cell expansion for the immunotherapies. At the conference we intend to give an overview of the results we obtained with this design for the different applications mentioned.

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Novel Fusion Proteins for Identifying Preferred CAR-T Cells

Justin Fisher[a], Scott Dessain[b] and William J. Kelly[a]

[a]Villanova University, 800 Lancaster Ave, Villanova PA 19085 USA

[b]OCMS Bio, LLC 3700 Horizon Drive, King of Prussia PA 19406 USA

Keywords: Fusion Protein, CAR-T cells, selection, Interferon

Abstract

The commercialization of the first Chimeric Antigen Receptor (CAR) T cell therapies in 2017 proved to be a major advancement in personalized cancer treatment [1-3]. Despite this success there has been a large variation in responses and unpredictable toxicity in patients- which is partially attributed to inter-patient heterogeneity of CAR-T product infusion [4]. Future development of CAR-T therapies will likely seek to overcome variations in responses and unpredictable toxicity in patients by addressing the interpatient heterogeneity of CAR-T products. An interesting approach for improving clinical success of CAR-T cell therapies is the selection of specific CAR-T subpopulations. The CAR-T procedure fundamentally alters the cytokine secretion phenotypes of the T cells, with both $CD4^+$ and $CD8^+$ CAR-T cells. Specifically, IFN-γ which is released in large amounts in activated $CD8^+$ T cells, is a key moderator of cell-mediated immunity [5]. Thus, a 'cytokine-optimized' CAR-T product would balance the levels of cytokine production for optimal activation and efficacy yet avoid the pitfalls of overstimulation. In this research, a novel fusion protein, containing an IFN-γ scFv fused to the CD19 extracellular domain, has been designed and characterized to capture expressed IFN-γ exclusively on the surface of the expressing CAR-T cells.

Size identification of the fusion protein using SDS-PAGE gel electrophoresis confirmed the fusion protein produced was of the estimated molecular mass and correct glycosylation. ELISA assays confirmed the binding of the novel fusion protein to various concentrations of IFN-γ, including in the presence of competitor mAb's. ELISA assays also confirmed the binding of the CD19 ECD to the common anti-CD19 scFv, FMC63, through use of an FMC63-Fc protein. Binding of the fusion protein to anti-CD19 CAR expressing T cells was confirmed using flow cytometry and the sensitivity range was evaluated. This fusion protein can successfully identify individual CAR-T expressing cells and select CAR expressing cells based on their CAR expression levels, and work continues to evaluate this approach in the identification of cell-specific IFN-γ secretion.

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Multi-Objective Optimization and Time-scale Analysis for the Production of Valuable Products in Gas Fermentations

D. Pfaff[a], J. A. Horvath[b,c], P. Ramin[a], S. Pflügl[b,c], J. Kager[a]

[a]*Department of Chemical and Biochemical Engineering, Technical University of Denmark, Søltofts Plads, Building 228, 2800, Kgs. Lyngby, Denmark*
[b]*Institute for Chemical, Environmental and Bioscience Engineering, Research Area Biochemical Engineering, Technische Universität Wien, Gumpendorfer Straße 1a, 1060 Vienna, Austria*
[c]*Christian Doppler Laboratory for Optimized Expression of Carbohydrate-active Enzymes, Institute of Chemical, Environmental and Bioscience Engineering, Technische Universität Wien, Gumpendorfer Straße 1a, 1060 Vienna, Austria*

Keywords: gas fermentation, thermophilic acetogen, *Thermoanaerobacter kivui*, mechanistic model, multi-objective optimization, time scale analysis

Abstract

In a time of global climate change, there is an increasing interest in conversion and capture of gaseous carbon (e.g. CO_2, CO) from air, industrial blast furnace gas or biomass gasification due to its high potential as a carbon source for microbial production of biofuels and biochemicals. Acetogenic bacteria can reduce carbon to a variety of chemicals such as butyrate, formate, acetate and ethanol via the Wood-Ljungdahl pathway. In this regard the thermophilic acetogen *Thermoanaerobacter kivui* is a promising biocatalyst for the conversion of CO_2 and CO to acetate as it has a high growth rate, is adaptable to variations in the gas feed, and does not require expensive media (Schwarz and Müller, 2020; Regis *et al.*, 2024).

The goal of this study was to understand the response behavior and operational sensitivities of continuous gas fermentations, using *T. kivui* as a case study. First a mechanistic model based on the current understanding of the system was developed. Numerical simulations were performed to explore the process design space, identify optimal operating points in terms of balancing high acetate yields and CO_2 emissions, and determine system response times and stability in steady states.

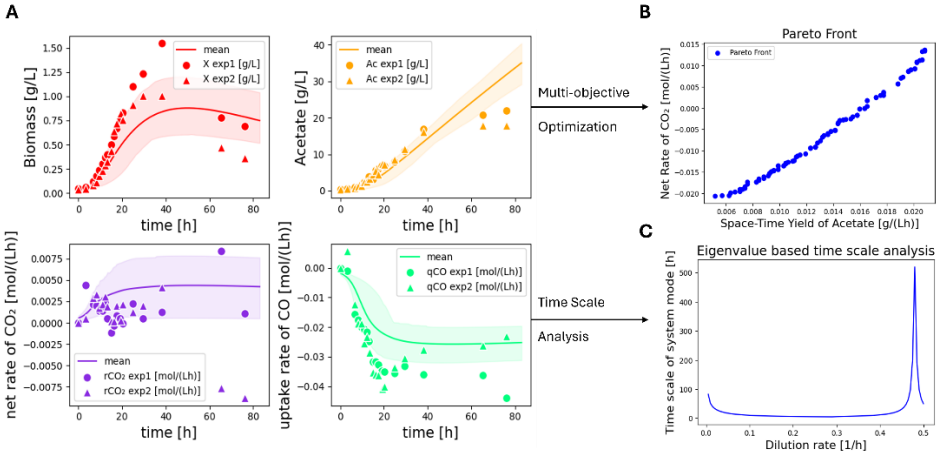

Figure 1: (A) Model fit of the general mechanistic model to two experimental data sets. The shaded areas correspond to 90% confidence interval resulted from 200 Monte-Carlo simulations with a parameter uncertainty of 30% on all parameters, (B) Pareto front of competing objectives, maximizing the space-time yield of acetate while minimizing the net rate of CO_2, (C) Time scale of the system derived from Jacobian matrix in dependence of the dilution rate.

Experiments with *T. kivui* were performed in a stirred tank reactor (V=0.20 L). The mechanistic model was then developed based on process knowledge and a kinetic model developed by (Ruggiero et al., 2022) for another acetogen. Before performing a local parameter estimation to fit the model to experimental data, the identifiability of the kinetic parameters was determined based on the local sensitivity matrix (Brun *et al.*, 2002). Subsequently, the Markov Chain Monte Carlo method was applied to estimate a global set of parameters and their uncertainties for a general model fitted to several experiments based on the method described by Hernández Rodríguez et al. (2019). The general model was then used to determine optimal operating points for the gas feed composition of H_2, CO, and CO_2 and the dilution rate. The optimal operating range lies on a Pareto front due to the conflicting objectives of maximizing the space-time yield of acetate while minimizing the net rate of CO_2 production (Acosta-Pavas *et al.*, 2023). Time-scale analysis was used to calculate the system response times in a steady state with respect to deflections in the state variables based on the Jacobian matrix (Klinke and Finley, 2012).

The model outcomes showed a good performance (normalized RMSE<0.2), as indicated in Fig. 1A, revealing correct description of the main underlying processes (e.g. microbial growth, acetate production as well as uptake rates of H_2, CO, and CO_2 and inhibitions) and identification of the kinetic parameters. Sensitivity analysis revealed the significance of the kinetic parameters related to the uptake of CO such as the yield coefficient $Y_{co,x}$, the maximum uptake rate of CO as well as the coefficients reflecting the inhibitory effect of CO on the uptake of H_2 and the growth. Using multi-objective optimization, it was possible to pinpoint the optimal operating range, shown in Fig. 1B, with the optimum of a gas composition as 0.76:0.16:0.08 for H_2:CO:CO_2, respectively, and a dilution rate of 0.026 h^{-1}. Time-scale analysis provided insights on the system response time for different dilution rates. As indicated in Fig. 1C, the characteristic time of the system is rapidly increasing in the area of washout (0.4-0.5 h^{-1}). This is associated with reduced responsiveness and stability of the system in that region.

Overall, the study highlights the advantage of model-based analysis for gas fermentation. By using a kinetic model, the research deepened the understanding of fermentation mechanisms and identified key parameters under varying conditions. This was demonstrated for less studied organism *T. kivui* as a case study.

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Hydrodynamics in FAST™ Bioreactors

R.M. da Costa Basto[a,c] , A. Oudshoorn[b], K. Steinbusch[b] , R.F. Mudde[a] and L.A.M. van der Wielen[a,d].

a. Departments of Biotechnology and Chem. Engineering, Delft University, Delft, The Netherlands
b. Delft Advanced Biorenewables BV, Alexander Fleminglaan 1, 2613 AX Delft, The Netherlands.
c. Jansen Biologics, Leiden, The Netherlands
d. Bernal Institute and Department of Chemical Sciences & Engineering and Bernal Institute, University of Limerick, Republic of Ireland

Keywords: integrated, extractive, multiphasic fermentations of phase splitting compounds

Abstract

Manufacturing of novel categories of hydrophobic, phase-separating bioproducts, ranging from commodity building blocks for fuels and materials to high-value flavors and fragrances as well as bioproducts that inhibit their production rates such as terpenoid and aromatic compounds (Hassing et al, 2019), can be drastically improved by in-situ liquid-liquid separation in advanced systems such as DAB.bio's integrated FAST™ bioreactors (Cuellar et al, 2013; Heeres et al, 2014; Pappas and Oudshoorn, 2024). FAST™ (Fermentation Accelerated by Separation Technology) bioreactors have been shown to be robust and scalable integrated bioreactor technology that enhances yields and significantly minimizes capital and operational expenses.

Delft Advanced Biorenewables BV (DAB.bio) as originally a TU Delft spin-out and the team at TU Delft have been engaged in a long-term joint development of FAST™ technology, with DAB.bio focusing on scale-up, piloting and demonstration plants for commercially relevant products such as butanol and phenylethanol (Pappas and Oudshoorn, 2024), and TU Delft on a focused series of underpinning, fundamental research projects (Heeres et al, 2015, 2016; Pedraza et al, 2018; Da Costa Basto et al, 2019, 2020, 2024).

Sofar, the fundamental flow phenomena and published scale-up studies in multiphase bioreactors have been described mostly by engineering correlations based on limited comprehensive data across scales. This study explores computational fluid dynamics (CFD) and further experimental validation at small (2 L) and pilot (100 L) scales of integrated FAST™ bioreactors as a robust tool for modeling, analysis, and design of these complex bioreactors.

The central component of the FAST™ bioreactor is a central downcomer through which the emulsion of broth and hydrophobic, phase splitted 'oily' product droplets enters a concentric separation section from a central riser. Within this section, oil droplets ascend for recovery while oil-free broth flows horizontally back to the fermentation compartment. Two different model systems utilizing commercially relevant solvents (Dodecane and Oleyl Alcohol) were employed successfully to document and analyze FAST™ bioreactor performance.

A CFD model was developed to investigate the impact of emulsion inlet velocity and specific geometries on oil recovery in non-coalescing systems. The model predicted enhanced oil recovery at lower velocities and generally describes 'oil' recoveries reasonably accurately.. Additionally, it describes the (hydraulic load conditions at increased scale for) development of mixing patterns

such as roll cells in the critical recirculation zone. separation channel which affect proper operational performance. and guides further optimization of reactor internals and operation.

This study demonstrates the usefulness of model-based predicting of trends in experimental FAST bioreactor operation. Further refinement is useful to enhance model accuracy. Further key challenges relate to implementing realistic coalescence models in the CFD framework and obtaining in-line measurements of droplet size distribution and flow phenomena within the FAST's internals

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The value of a Designer in Bioprocess Engineering

J. Carvalho Pereira, P. Carrion-Gordon, M. Ottens

Department of Biotechnology, Delft University of Technology, van der Maasweg 9, Delft, 2629 HZ, The Netherlands

Keywords: education; biotechnology; bioprocess design; modeling and simulation; scale-up

Abstract

Delft University of Technology offers Engineering Doctorate (EngD) programmes in Bioprocess Engineering, aiming at the translation of academic developments into real-life applications. These two-year salaried design traineeships offer an application-focused alternative to traditional PhD positions, and provide trainees with a solid basis for an accelerated start in an industrial career.

A very strong engineering background for the Designer in Bioprocess EngD programme is requested: mathematics, mass and heat transfer, and experience with modeling and simulation software tools. In the first year of the programme, the EngD designers follow a dedicated curriculum involving relevant courses, interactive workshops, and group design assignments in close cooperation with industrial partners. In the second year, they perform their Individual Design Project, typically seconded within a company, working on real business cases covering from small start-ups to large multinational companies in the biobased, biopharmaceutical, food, cosmetics, bioplastics, and wastewater sectors, among others. In addition to technological topics, the participants acquire professional skills in areas such as stakeholder management, personal and project management, and communication.

Our industrial partners, on the other hand, benefit from fully supported collaborations delivering a fit-for-purpose (tailor-made) design project at a competitive cost, executed by one or more of our selected trainees under the supervision of TU Delft's principal investigators and experts, plus an experienced design coach. Examples of previous design assignments are:

- Downstream process development for a novel single cell protein production technology
- Raman-based chemometric model development for mammalian cell culture production
- Techno-economic feasibility of a chromatographic process to obtain a concentrated stream of antibodies from dairy products
- Evaluation of continuous manufacturing for enzyme production
- Continuous processing of food bio-chemicals: development of a CFD model for protein concentration via ultrafiltration
- Bio-based platform chemicals: sequential screening & selection of technical options

Besides promoting collaboration and fostering knowledge and technology transfer between academia and industry, our industrial partners additionally become part of the academic network of TU Delft and other TU Delft-wide initiatives, such as the Delft Process & Product Technology Institute and Delft Bioengineering Institute, furthering academic-industry initiatives for a sustainable future.

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www.tudelft.nl/engd

Cleaning in Place of whey protein fouling: a mechanistic model of mass removal rate in dairy systems

Yimin Zhang[a], Frans W.J. van den Berg[a], Mogens L. Andersen[a], Behnaz Razi Parjikolaei[b], Serafim Bakalis[a]

[a]*Department of Food Science, Faculty of Science, University of Copenhagen, Rolighedsvej 26, 1958 Frederiksberg C, Denmark*
[b]*Arla Foods Ingredients P/S, Soenderupvej 26, 6920 Videbaek, Denmark*

Keywords: Cleaning in place, process modeling, whey protein fouling

Abstract

Clean-in-Place is a standard operation in the dairy industry that requires significant resources in terms of time, water and energy. Amid a growing sustainability awareness, optimizing CIP to minimize water and energy usage is gaining urgency. Existing research typically concentrates on the chemical interactions between protein and alkaline (Xin, 2004) or the protein layer mechanical properties (Helbig, 2022), however, real-world cleaning involves multiple mechanisms. Our work focuses on cleaning the protein fouling inside plate heat exchangers used for pasteurization. We have developed a transparent, rectangular channel with a metal base to mimic a heat exchanger channel. We choose the rig geometry, liquid flow rate, and heating profile enable the creation of a fouling layer representative to those found in industrial pasteurization. By circulating hot sodium hydroxide through this channel, we replicate the CIP process. Cameras monitor the fouling layer thickness at distinct positions along the 30cm channel, while UV-Vis spectroscopy measures the effluent protein concentration over time, quantifying the mass removal rate of the whole channel. The observed thickness reduction across the channel demonstrates a uniform two-stages pattern, with the rate of the first stage being consistent across location and the second stage onset varying by location. Figure 1 illustrates the distinct phenomena associated with the two stages of cleaning, highlighting the diverse mechanisms observed. We explain the position-dependent rate of thickness removal using a ad-hoc model that incorporates the fouling layer density. This model accounts for the observed trends in the mass removal rate measured in the effluent stream (i.e. global cleaning in the entire channel).

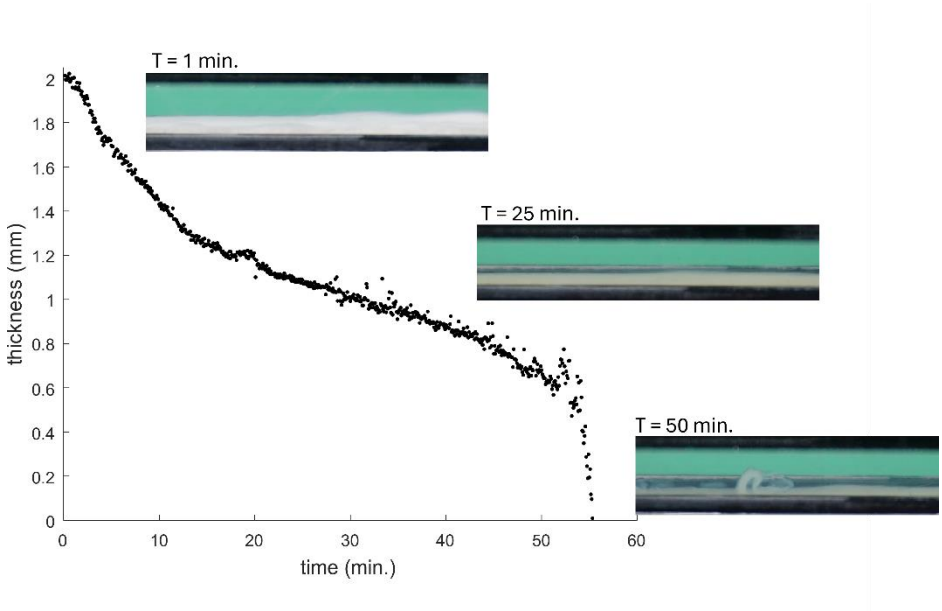

Figure 1: A typical thickness curve of fouling layer and its corresponding phenomena. Fouling layer changes from white to yellow and from flat to curly.

Acknowledgement

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CFD modelling of a mixing tank of 200L for fully formulated biologics solutions.

J. W. Hartvig[a], S. Erkul[b], M. Nielsen[b], and S. Lemoult[a]

[a]*Danmarks Tekniske Universitet, Lyngby, 2800, Denmark*
[b]*Fujifilm Diosynth Biotechnologies Denmark, Biotek allee 1, Hilleroed 3400 , DenmarK*

Keywords: Fill and Finish, LevMixer ®System, Biologics, Mixing tank.

Abstract

During the production of monoclonal antibodies drug product, freezing is a common method used to preserve the formulated product. However, when thawed, significant concentration gradients can develop, necessitating the use of a mixing tank to ensure uniformity before filling into syringes or vials.
To minimize product wastage, a 1L tank was explored as a scale-down model for predicting the behavior of a larger 200L cubical tank equipped with single-use bag technology and a magnetic impeller drive system. The 1L tank maintained key ratios of impeller diameter to tank width and tank width to tank height, enhancing the similarity in mixing characteristics between the two tanks.
Colorimetric and conductivity-based experiments on the 1L scale-down model revealed a strong exponential correlation between mixing time and impeller rotation speed. Computational fluid dynamics (CFD) models accurately predicted mixing behavior for both the 1L and 200L tanks, with similar results observed in simulations and experiments.
Moreover, shear strain rate, used as an indicator for product damage and surfactant foaming, scaled between the two tanks with impeller diameter and rotation rate. Scaling formulas were developed to equate mixing conditions between the 1L and 200L tanks, yielding results within a 5.3% margin of error.
Additionally, colorimetry experiments and two-phase flow CFD simulations illustrated the distribution of insoluble particles and immiscible liquids in both tanks, showing aggregation in the corners.
CFD was additionally utilized to predict the distribution of insoluble particles in the two tanks.

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Evaluation of Lipid-Nanoparticle Stability after Pump-induced Processing

N. Beckert[a], J. Hubbuch[a]

[a]*Institute of Process Engineering in Life Sciences – Section IV: Biomolecular Separation Engineering, Karlsruhe Institute of Technology (KIT), 76131 Karlsruhe, Germany*

Keywords: lipid-nanoparticle (LNP), micropump, stability, processing.

Abstract

The use of lipid-nanoparticles (LNPs) for delivering nucleic acids has become widely popular in clinical studies, including vaccine development and other innovative therapies (Jung et al. 2022). LNPs typically consist of four lipid types, each playing a critical role in stability, delivery, and cellular uptake: cationic or ionizable amino lipids, helper phospholipids, cholesterol, and PEGylated lipids. The production of LNPs involves rapid mixing of a lipid-containing ethanol phase with an aqueous nucleic acid-containing phase. The increasing polarity of the surrounding media during the mixing process induces the electrostatic interaction of the phosphate backbone of the nucleic acid and the amino lipid. Further increasing polarity yields lipid particles with an electron-dense core containing the nucleic acids. To eliminate residual ethanol and neutralize the pH, cross-flow filtration (CFF) is employed. All processing steps, particularly CFF applications, necessitate transportation through pumping. While the effects of pumping and shear stress on proteins have excessively been studied (Fanthom et al. 2023), LNP stability has only been evaluated for storage and filtration so far (Mehta et al. 2023).

This study presents a systematic approach to evaluate the influence of pump-induced stress on LNP quality attributes in a lab-scale setup. The combination of online Z-average determination using dynamic light scattering (DLS) during pump-induced transportation and offline analytics to evaluate LNP quality attributes yields a comprehensive picture of LNP quality and stability during processing. To reduce the required volumes and minimize the scale, a piezoelectric titanium-based microfluidic pump with passive spring valves (Bußmann et al. 2021) is used. For a full set of analytics, the in-process sampling took place and was monitored in terms of stability. Besides size monitoring via DLS, the further LNP quality attributes surface charge, lipid composition, and encapsulation efficiency of the nucleic acids are assessed by electrophoretic light scattering, reversed-phase high-performance liquid chromatography coupled with a charged aerosol detector, and fluorescence-based assays.

In the second part of the study, LNPs loaded with nucleic acid molecules of different sizes were tested using the same pumping process. The LNP formulation parameters, such as nitrogen-to-phosphate ratio, lipid concentration, and lipid ratio, as well as process-related parameters, such as flow rate ratio and total flow rate, were kept constant. This allowed us to evaluate the impact of nucleic acid molecular weight and length on the stability of LNP in isolation.

In conclusion, a combination of online and offline monitoring of LNP size, along with offline analysis of LNP quality attributes can help to accurately identify pumping-induced quality issues. Moreover, this analytical panel and lab-scale setup can be utilized in the future to assess different pump types or tubing types helping to increase process knowledge and understanding for LNP large-scale processes.

Acknowledgement

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Pichia expression platform design for biopharmaceutical protein production

E. Antonov[a], D. Degreif[a]

[a]*Sanofi-Aventis Deutschland GmbH, Industriepark Hoechst, 65926 Frankfurt am Main, Germany*

Keywords: *Pichia pastoris*, Nanobodies, methanol-free production system

Abstract

Pichia pastoris (syn. *Komagataella phaffii*) is one of the cornerstones of Sanofi's microbial expression platform. The use of the methanol-inducible expression system for production was the golden standard for years, delivering high titers and unsurpassed quality. However, for large scale manufacturing, the use of methanol as toxic substance increases the complexity of HSE & safety requirements. Hence, Sanofi's microbial expression platform is looking for an alternative system that is capable of matching the performance of methanol-based processes but without the drawbacks associated with the use of methanol. Having a safe- and eco-friendly process design in mind, Sanofi is working on establishing a methanol-free expression platform for Nanobody® molecule production. Recent developments to optimize the methanol-free process have emphasized the potential of this new platform to achieve commercially competitive productivity and product quality. Further progress in the field of strain-engineering and process design will aid to advance the *Pichia*-based production platform further.

Strategies for Preservative Loss in Pharmaceutical Manufacturing: Study of Phenol Diffusion in Silicone Tubing

Jinyuhan Wang, Pau Cabaneros, Pedram Ramin, Andreas J. Kunov-Kruse, Krist V. Gernaey

Novo Nordisk A/S, Novo Alle, Bagsværd 2880, Denmark
Technical University of Denmark, Anker Engelunds Vej, Kongens Lyngby 2800, Denmark

Keywords: PAT, Raman, silicone tubing

Abstract

Silicone tubing, characterized by its flexible mechanical properties, is extensively used in the filling process of pharmaceutical manufacturing. However, a significant loss in the concentration of preservatives diffuses when the production line is interrupted during the filling process (Saller et al., 2017). This loss is primarily attributed to diffusion phenomena.

The present study focuses on the diffusion process of phenol as one of the most used preservatives. Diffusion of phenol through silicone tubes is studied using Raman spectroscopy. As a robust technique in Process Analytical Technology (PAT), Raman spectroscopy offers non-invasive, rapid direct measurements. Mathematical modelling is developed combining Raman spectral data to facilitate effective understanding of diffusion mechanisms.

Two phases of diffusion are identified: the initial phase involves the permeation of phenol into the silicone tube layer, followed by a secondary phase where, upon reaching saturation, the phenol diffuses into the surrounding atmosphere. The latter phase appears to represent a steady state, while the former is characterized by an exponential decrease in phenol concentration.

This investigation on diffusion dynamics of phenol consequently leads to a data-driven understanding which is crucial for ensuring the integrity of the filling process. Moreover, the potential use of Raman spectroscopy as a real time monitoring tool in filling process is highlighted and worth further study.

Acknowledgement

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Design, simulation, and economics of a manufacturing process for mesenchymal stromal cell (MSC) and their extracellular vesicles (MSC-EV)

Ricardo M. Silva(1), Sara Sousa Rosa(1), José A. L. Santos(1), Ana M. Azevedo(1) and Ana Fernandes-Platzgummer(1,2)

(1) Institute for Bioengineering and Biosciences, Department of Bioengineering, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001 Lisbon, Portugal

(2) Associate Laboratory i4HB-Institute for Health and Bioeconomy, Instituto Superior Técnico, Universidade de Lisboa, Lisbon, Portugal

Keywords: Mesenchymal stromal cells; Extracellular Vesicles; Manufacturing platform; SuperPro Designer

Abstract

Extracellular vesicles (EV) are a heterogenous population of membrane vesicles secreted by cells, behaving as valuable information carriers, fundamental to cell-cell communication through the exchange of their cargoes. For this reason, EV have emerged as a new cell-free strategy with clinical applications for disease diagnosis, prognosis and therapy, working as non-invasive biomarkers and drug delivery vehicles, with high efficiency and sensibility. However, taking EV to the clinic requires an investment in the development of cost-effective manufacturing processes capable of delivering great quantities of high-quality product. From an upstream perspective, these include culturing cells in large 3-dimensional (3D) bioreactors, in order to maximize cells number and secreted EV, while from a downstream point of view we require scalable techniques capable of handling large product quantities. So far, different downstream strategies have been implemented based on the differential ultracentrifugation or PEG precipitation. These techniques, although efficient at lab-scale, do not perform well at large scale. Additionally, can also form protein aggregates which decreases the purity of final the product, while the high centrifugal forces can damage exosome integrity.

In this work we explore the integration into an industrial-scale process, simulated in SuperPro Designer, and the advances achieved by our group in the field of mesenchymal stromal cell-derived EV (MSC-EV) (Fernandes-Platzgummer *et al.*, 2016; Silva *et al.*, 2023). The process starts with MSC culture in two-dimensional (2D) system, and gradually expanding up to a 10*L* bioreactor. To accurately describe our process a macrochemical equation was included detailing nutrient and oxygen consumption, and metabolites and EV secretion. After cell culture, a stream rich in 5.4×10^9 MSC and 6.9×10^{13} EV is collected. Due to their different sizes, both products are easily separated, following different downstream steps. MSC are detached from microcarriers and dosages formulated. MSC-EV, on the other hand, are filtered to remove debris, collecting 4.3×10^{13} EV, which are further processed using the strategy proposed by our group in earlier work, consisting of nuclease digestion followed by anion exchange chromatography (Silva *et al.*, 2023). With this strategy, 2.39×10^{13} EV can be recovered, yielding 55% of EV.

The designed plant has a capital investment of nearly 65 million € and an annual operating cost of 20 million €, and can run 38 batches annually, corresponding to 1406 clinical-grade doses of MSCs ($>1 \times 10^7$ cells/dose) and 9082 clinical-grade doses MSC-EV (1×10^{11} particles/dose). In this constructed scenario, a minimum selling price of 1566€ and 16000€ can be calculated for a dose of 10 and 100 million cells, respectively. For MSC-EVs, the selling prices are substantially lower compared to MSC – 308€ for smaller doses containing $\times 10^{10}$ EV, and 3082€ for larger doses with 1×10^{11} EV. Yet, by increasing the number of staggered units, the annual number of batches can be increased, allowing to reduce the selling

prices. Indeed, when compared with established MSC-approved therapies and calculated reimbursement values (Pereira Chilima *et al.*, 2018), our prices are competitive for different dosages.

At the end, our results highlight the cost-effectiveness of the MSC-EVs approach when compared to MSC doses, partly due to the scalability and robustness of our proposed EV purification platform. Moreover, economic results reveal the potential of the co-production of both MSC and MSC-EV clinic products, with capacity to increase the number of products manufactured per batch, creating more attractive selling prices.

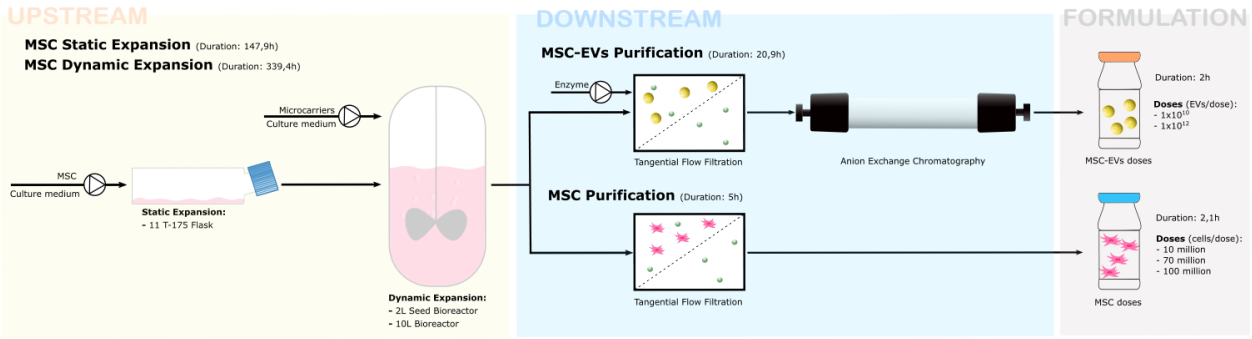

Figure 1: Schematic representation of MSC and MSC-EV co-manufacturing process.

Acknowledgement

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Understanding the reconstitution of pharmaceutical protein powders

T. Weywadt[a,b], A. Gulotta[a], M. Oskolkova[a], T. Eifler[b], U. Krühne[b]
[a]Injectable Finished Products, Novo Nordisk A/S, Novo Allé, 2880 Bagsværd, Denmark
[b]Department of Chemical and Biochemical Engineering, Technical University of Denmark, Søltofts Plads 227, 2800 Kgs. Lyngby, Denmark

Keywords: Powders, Reconstitution, Formulation, Active Pharmaceutical Ingredients

Abstract

In this project, we investigate the underlying mechanisms governing the dissolution of active pharmaceutical ingredients (API) from a powder state. The API is dissolved either in water or an excipient solution. Here, a range of relevant APIs are characterized in terms of physicochemical characteristics aiming to identify limiting factors in the reconstitution of the protein in solution.

Previous research performed by Lillford and Fryer (1998), Börjesson et al. (2016), and by Andersson (2020) has gone into the production and reconstitution of powders in the food industry, specifically the dairy industry. Here, reconstitution is shown to be limited by factors such as fish-eye formation and poor imbibition of the powder into the liquid. These are both indicators of poor penetration of the liquid through the powder.

We look at dissolution of a range of pharmaceutically relevant API which are characterized in terms of e.g. powder particle size distributions, porosity, contact angle etc. to be able to identify limiting factors to the reconstitution. The aim of this is to find linkage between the characteristics of the API and the dissolution, yielding quicker process with reduced variability, while also minimizing the potential stressing of the pharmaceutical molecules.

Figure 1: Principal drawing of a formulation process in the production of injectable drug products.

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Improving Monoclonal Antibody Manufacturing: Experimental Insights into Membrane Selection for Single-Pass Tangential Flow Filtration (SPTFF)

J. Kaiser[a,b], J. Krarup[a], M. Pinelo[b], U. Krühne[b]

[a]*Novo Nordisk A/S, Novo Allé, 2880 Bagsvaerd, Denmark*
[b]*Technical University of Denmark (DTU), Department of Chemical and Biochemical Engineering, Søltofts Plads 228A, 2800 Kgs. Lyngby, Denmark*

Keywords: Ultrafiltration (UF), monoclonal antibodies (mAbs), downstream processing, pharmaceutical manufacturing

Abstract

Purifying biopharmaceuticals such as monoclonal antibodies (mAbs) is a complex process that involves multiple steps including chromatography and ultrafiltration (UF), where the latter is utilized to concentrate the product or to exchange buffers. Single-pass tangential flow filtration (SPTFF), a special UF technology, has become an effective tool in the biopharmaceutical industry for increasing product concentrations, reducing large volumes, and as a result, enhancing process performance (Madsen et al., 2022). High concentrations are achieved in a single pass through a specific “multi-stage” design, as opposed to conventional “single-stage” tangential flow filtration (TFF), which requires multiple recirculation cycles to reach the target concentration. However, the multi-stage design of SPTFF presents challenges in understanding filter design aspects like membrane cassette configuration and membrane selection and their impact on process performance, especially when compared to conventional TFF.

This study conducted an experimental evaluation of the influence of membrane material, molecular weight cut-off (MWCO), and product on permeate flux and membrane cleaning. The experiments were carried out using three distinct membrane cassettes and two types of mAbs. Through pressure-flux excursions, we observed that permeate fluxes are significantly influenced by the membrane material, MWCO, and mAb. The findings highlight the superiority of regenerated cellulose (RC) membranes over polyethersulfone (PES) membranes in terms of fluxes and the restoration of water permeability after cleaning. Moreover, product-specific factors and operating conditions were found to significantly affect the permeate flux.

In conclusion, the selection of an appropriate membrane is pivotal for reducing fouling and achieving consistent permeate fluxes after cleaning. The insights from this study can guide the choice of suitable membranes for mAb concentration via SPTFF, thereby improving the efficiency and reducing costs of mAb manufacturing.

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Development of an integrative dynamic model associating morpho-rheological patterns of bioprocesses with filamentous microorganisms

L. Schumann[a,b], A. Dinius[a,b], Z. J. Kozanecka[a,b], J. Büchs[c], L. Fillaudeau[d], R. Krull[a,b]

[a]*Institute of Biochemical Engineering, TU Braunschweig, Rebenring 56, Braunschweig 38106, Germany*
[b]*Center of Pharmaceutical Engineering, TU Braunschweig, Franz-Liszt-Straße 35a, Braunschweig 38106, Germany*
[c]*AVT – Biochemical Engineering, RWTH Aachen, Forckenbeckstraße 51, 52074 Aachen, Germany*
[d]*Toulouse Biotechnology Institute, Bio & Chemical Engineering, Université de Toulouse, CNRS 5504,INRAE 792, INSA, 135 Avenue de Rangueil,31077 Toulouse, France*

Keywords: filamentous microorganisms, cellular morphology, rheology, kinetic modeling, machine learning.

Abstract

The cultivation of filamentous microorganisms for the production of organic acids or antibiotics has vast application potential in food and pharmaceutical industries, generating annual sales totaling several billion dollars (Meyer et al., 2016). The bioprocess performance of such operations directly depends on mass transfer limitations arising from the change in cellular morphology and the rheological behavior of the broth during the course of the cultivation. Equally, the cellular morphology often persists as the bottleneck of productivity for various industrial processes, as different morphologies cause mixing issues or limit mass transfer to the cells.

In submerged cultivations, filamentous microorganisms typically show a very complex morphological life cycle, starting as spores, via germ tubes and the micromorphological hyphal network to the macromorphological appearance. Depending on the process conditions, the same microorganism's macromorphology can vary between loose hyphal networks called dispersed mycelium, dense and nearly spherical pellets, as well as several intermediate forms. It is not yet possible to predict which of the different morphologies is optimal for the ultimately pivotal product formation, as it is eminently strain-specific. It is reported that cultivation in the pelleted form is often advantageous for the production of secondary metabolites (e.g., antibiotics), while primary metabolites (e.g., enzymes) are usually synthesized in dispersed mycelium. Generally, a macromorphology of small fluffy pellets appears to be preferred in literature as oxygen and substrate limitations in the pellet's center are diminished by reduced pellet size and higher porosity. Furthermore, mixing problems are avoided due to a decreased viscosity compared to dispersed mycelium. The morphology can be engineered through various environmental parameters, which have been extensively studied in literature. Amongst these are the implementation of micro- and macroparticles in the cultivation broth, the variation of inoculum concentration, as well as the supplementation of inorganic salts (Dinius et al., 2023). Further, the substantial impact of fluid dynamic stress on pellet size, particularly the power input through aeration and agitation has been repeatedly reported and recently emphasized by Waldherr et al. (2024). The influences of physico-chemical parameters can also be diametrically opposed, depending on which specific microorganism is considered. In practice, a population of filamentous microorganisms behaves very heterogeneously despite identical cultivation conditions, so that growth and metabolic activity are subject to very strong fluctuations.

The presented research project aims to overcome the two following substantial challenges for the effective cultivation of filamentous microorganisms: Firstly, a consensus on the optimal cellular morphology which can be transferred beyond the microorganism genus is yet to be agreed on and is intended to be established in this project. Secondly, this work attempts to close the remaining

knowledge gap in adequate description of strain-independent correlations between the morpho-rheological changes and the subsequent performance parameters of macro morphological structures, rheology, substrate utilization, and productivity.

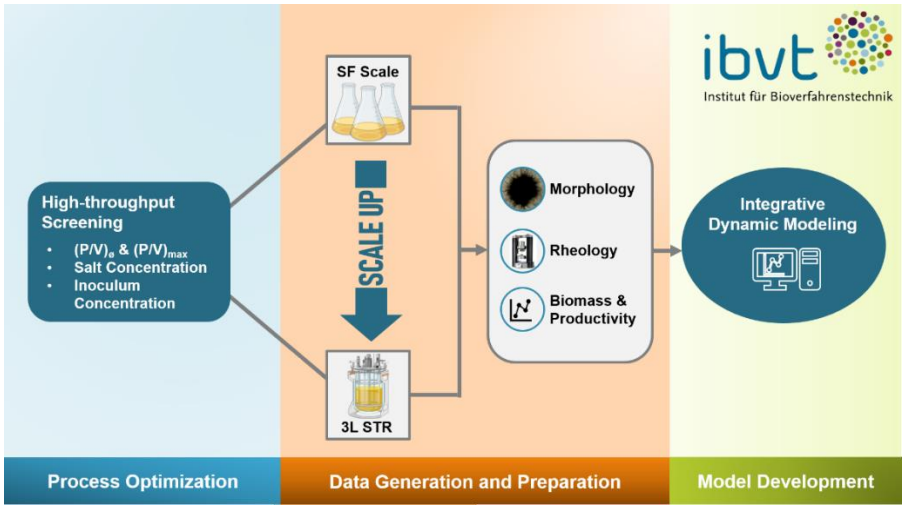

Figure 1: *Workflow for establishing a dynamic integrative model of rheological and morphological developments during cultivation of filamentous microorganisms to predict growth and productivity.*

The projects subsequent workflow is shown in **Fig. 1** and can be structured in three different phases. As a first step, optimal process conditions will be obtained. Therefore, a high-throughput screening approach serves to further understand the correlation between different cultivation parameters. Various process conditions will be investigated on shake flask scale, including the concentration of inoculum concentration and inorganic salts, as well as the volumetric power input and hydromechanical stress (maximum local volumetric power input). The corresponding viscosity and cellular morphology will be determined by in-situ morpho-granulometry. Based on the results, optimized process conditions will be developed for different scales up to a 3 L stirred tank bioreactor. To attain knowledge beyond strain-specific data, the experiments are conducted for organisms with both growth-associated and non-growth-associated product formation kinetics. The thereby identified experimental correlations between biological and physical cultivation parameters will subsequently serve as the foundation for evolving a comprehensive integrative dynamic model, enabling the prediction of biomass growth and productivity based on current morphological and rheological patterns. In particular, morphological complexity will be modeled by pellet and hyphal network descriptors based on the analysis of distribution functions. The broth viscosity will be characterized in a unified rheological model integrating shear rate, biomass concentration, as well as cellular morphology. Both models will further be combined to establish unified and predictive morpho-rheological models with integrated growth and product formation kinetics. Different machine-learning approaches will be employed and compared to determine the most robust method for all considered strains and scales.

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Innovative and circular growth of *Chlorella vulgaris* mutants feeding with grass fiber residues toward food applications

S. Mohamadnia[a], S.S. Mansouri[a], M.F.L. Olsen[b], P.E. Jensen[b], I. Angelidaki[a,*]

[a]*Department of Chemical and Biochemical Engineering, Technical University of Denmark, Søltofts Plads 228A, 2800 Kgs. Lyngby, Denmark.*
[b]*Department of Food Science, University of Copenhagen, Rolighedsvej 26, 1958, Frederiksberg C, Denmark.*

Keywords: Heterotrophic cultivation, *Chlorella vulgaris*, Random mutagenesis, Microalgae, Protein.

Abstract

As the world moves to more environmental preservation and focuses on a healthy diet, people are beginning to substitute animal protein in their diets with alternative protein sources in recent years. Specifically, microalgae have been regarded as an alternative source of protein since they offer a higher protein content (between 50% and 71% DW) and comparable or better quality of protein than most common protein sources (meat, egg, soybean, milk, etc.). In addition, their biomass is well-known for polysaccharides, polyunsaturated fatty acids (PUFAs), amino acids, and vitamins (Alishah Aratboni et al., 2019). Microalgae grow faster than other photoautotrophs in diverse growth conditions and can survive in a wide range of environments without needing arable land or potable water (Taelman et al., 2015). Considering these characteristics, microalgae have the potential to emerge as a significant substitute food source for a growing population in the future. However, one major barrier is that the consumer acceptance of foods enriched with microalgae depends on organoleptic characteristics like color, taste, and smell. Particularly for green microalgae like *Chlorella*, chlorophyll gives the biomass a distinct green color and grassy taste. Thereby, to improve its acceptability as a novel protein source, it is required to modify the unfavorable organoleptic properties by reducing or removing chlorophylls from their biomass. A classical breeding technique known as random mutagenesis has been used to lower the amount of chlorophyll in various microalgal strains. Note, the mutants with higher protein contents and/or lower amounts of chlorophyll strains are generated by exposing the target cells to chemical mutagenic agents, such as ethyl methane sulfonate, or physical mutagenic agents, such as UV or gamma lights (Liu et al., 2023). On the other hand, using waste for microalgal cultivation can upgrade low-value raw materials into high-value products, helping to decrease the cost of microalgal protein production. In this study, we explored the feasibility of using mutant heterotrophic fermentation to convert grass residues into protein. Hence, *Chlorella vulgaris* chlorophyll-deficient mutants were obtained from UV mutagenesis (Fig. 1) and were cultivated in two different grass residues, hydrolysates and brown juice, as alternative substrates for growth.

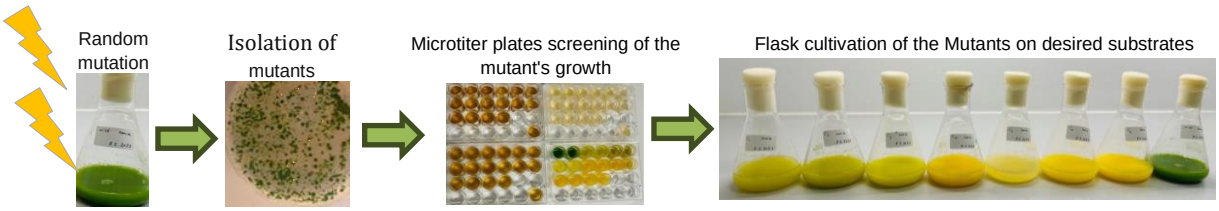

Figure 1: Random mutagenesis and screening of the growth of the mutants and wild type in grass fiber residues (hydrolysates and brown juice) under heterotrophic conditions.

Hydrolysates, with two different dry matter contents (2 and 15%), were obtained from enzymatic hydrolysis of ensiled grass fiber by cellulase. Brown juice as the side stream of the grass protein production process was provided after heating the green juice and separating the protein

concentrate. Next, the growths in hydrolysates, brown juice, and synthetic media (control) were screened using 24-well cell culture plates. Two mutants (C4 and C6) were selected from 8 initial mutants for further optimization and growth analysis in 250-mL flasks. Fig. 2 demonstrates during 5 days of heterotrophic cultivation, all strains reached higher Dry Cell Weight (DCW) in brown juice compared to the synthetic media and hydrolysates. This indicated that mutants could take up glucose from the media under sufficient nitrogen conditions. In the current study, faster biomass growth of mutants, 2.92 g/l dry cell weight of C4, was achieved under the optimum C/N ratio (C/N=1.5) in brown juice. In other words, hydrolysates and synthetic media have higher C/N ratios, 8 and 3.15, respectively, which exceed the optimal condition (C/N=1.5) causing nitrogen sources to become the limiting factor for biomass accumulation.

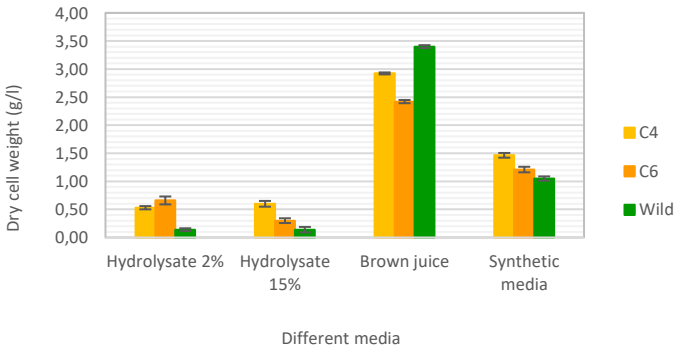

Figure 2: Dry cell weight (DCW, g l^{-1}) of Chlorella vulgaris wild type and mutants grown in 250-mL Erlenmeyer flasks under heterotrophic conditions, in different media.

This is also evident from the specific growth rates of C4 and C6 mutants cultivated in the brown juice (Table 1) which are 0.43 and 0.52 d^{-1}, respectively, and 2 times greater than those cultivated in the synthetic media.

Table 1: Maximum biomass productivities and growth rates of Chlorella vulgaris WT and mutants C4 and C6 in 250-mL Erlenmeyer flasks in Brown juice and synthetic media.

Strain	Biomass productivity (g $l^{-1}d^{-1}$)		Specific growth rate (d^{-1})	
	Brown juice	synthetic media	Brown juice	synthetic media
Wild type	0.61±0.03	0.26±0.04	0.33±0.04	0.16±0.02
C4	0.59±0.02	0.24±0.04	0.43±0.02	0.27±0.03
C6	0.53±0.03	0.27±0.05	0.52±0.03	0.26±0.04

Due to the better growth performance of the two chlorophyll-deficient mutants, C4 and C6, in brown juice compared to the synthetic media, they will scale up, at FermHub Zealand, to evaluate the growth performance in the pilot-scale fermenter and determine their feasibility and potential for applications in the food and nutraceutical industries for novel products based on microalgal biomass.

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Iron(III) cross-linked hydrogels based on *Alteromonas macleodii* Mo 169 exopolysaccharide

P. Concórdio-Reis[a,b], M. Martins[a,b], D. Araújo[a,b], V. D. Alves[c], X. Moppert[d], J. Guézennec[e], M. A. M. Reis[a,b], F. Freitas[a,b]

[a]*Associate Laboratory i4HB – Institute for Health and Bioeconomy, School of Science and Technology, NOVA University Lisbon, 2829-516 Caparica, Portugal.*
[b]*UCIBIO – Applied Molecular Biosciences Unit, Department of Chemistry, School of Science and Technology, NOVA University Lisbon, 2829-516 Caparica, Portugal*
[c]*LEAF—Linking Landscape, Environment, Agriculture and Food Research Center, Associated Laboratory TERRA, Instituto Superior de Agronomia, Universidade de Lisboa, 1349-017 Lisboa, Portugal*
[d]*Pacific Biotech BP 140 289, 98 701 Arue Tahiti, French Polynesia*
[e]*AiMB (Advices in Marine Biotechnology), 17 Rue d'Ouessant, 29280 Plouzané, France*

Keywords: Hydrogel, Iron crosslinking, Extracellular polysaccharide (EPS), *Alteromonas macleodii* Mo169, Blue biotechnology

Abstract

Hydrogels are three-dimensional structures of hydrophilic nature capable of holding large quantities of water without dissolving (Darge et al., 2019; Yang et al., 2022). Although hydrogels can be fabricated from synthetic polymers, hydrogels based on polymers of natural origin such as polysaccharides have several advantageous properties, including enhanced biological activity, excellent biocompatibility, low immunogenicity, predictable biodegradability, and physical similarities with tissues (Darge et al., 2019; Jiang et al., 2020; Wang et al., 2020; Yegappan et al., 2018). Moreover, the abundance of carboxyl, hydroxyl, or amine groups in polysaccharide structures provides a great platform for post-modification and functionalization (Li and Lin, 2021). Thus, these soft biomaterials hold great potential in a wide range of applications, such as scaffolds in tissue engineering, vehicles for drug delivery, biomaterials for wound management, contact lenses, and immunomodulation (Darge et al., 2019; Hu and Xu, 2020; Jiang et al., 2020; Kang et al., 2019; Yang et al., 2022; Yegappan et al., 2018).

Different physical and chemical crosslinking methodologies can be employed in the preparation of polysaccharide hydrogels (Dave and Gor, 2018). One of those strategies, ionotropic gelation, is based on the formation of coordination bonds between the polymer's negatively charged functional groups and metal cations (e.g., K^+, Ca^{2+}, Zn^{2+}, Mg^{2+}, Cu^{2+}, or Fe^{3+}) [5–8,10]. Recently, polysaccharide-based hydrogels crosslinked with the trivalent iron cation have attracted interest due to their remarkable properties that include high mechanical stability, stimuli-responsiveness, and enhanced absorptivity.

In this study, a Fe^{3+} crosslinked hydrogel was prepared using the biocompatible extracellular polysaccharide (EPS) secreted by the marine bacterium *Alteromonas macleodii* Mo169. The hydrogel preparation conditions were investigated to obtain homogenous and structurally stable hydrogels. The impact of Fe^{3+} and EPS concentrations on the hydrogels' strength was evaluated through response surface methodology (RSM) and the obtained hydrogels were characterized in terms of their composition, morphology, mechanical, and swelling properties. Hydrogels with mechanical strengths (G') ranging from 0.3 kPa to 44.5 kPa were obtained as a result of the combination of different Fe^{3+} (0.05–9.95 g L^{-1}) and EPS (0.3–1.7%) concentrations. All the hydrogels had a water content above 98%. Three different hydrogels, named HA, HB, and HC, were chosen for further characterization. With strength values (G') of 3.2, 28.9, and 44.5 kPa, respectively, these hydrogels can meet the strength requirements for several specific applications. Their mechanical resistance increased as higher Fe^{3+} and polymer concentrations were used in

their preparation (the compressive hardness increased from 8.7 to 192.1 kPa for hydrogel HA and HC, respectively). In addition, a tighter mesh was noticed for HC, which was correlated to its lower swelling ratio value compared to HA and HB. Overall, this study highlighted the potential of these hydrogels for tissue engineering, drug delivery, or wound healing applications.

Acknowledgement

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Investigating the growth kinetics of acidophiles isolated from arsenic-bearing waste

H. Maniak[a], A. Pawlowska[b]

[a]*Department of Micro, Nano and Bioprocess Engineering, Wroclaw University of Science and Technology, Wyb. Wyspianskiego 27, 50-370 Wroclaw, Poland*
[b]*Department of Process Engineering and Technology of Polymer and Carbon Materials, Wroclaw University of Science and Technology, Wyb. Wyspianskiego 27, 50-370 Wroclaw, Poland*

Keywords: iron Fe^{2+} oxidation, Monod constants, microbial consortium, biomass determination, *Acidithiobacillus ferrooxidans*

Abstract

Bioleaching is a process of biological extraction of metals from minerals conducted by microorganisms that utilize various metabolic mechanisms to convert insoluble into soluble forms of metals. While bioleaching presents an advantageous method for extracting valuable metals from natural deposits, it also entails the inadvertent release of these metals into the environment by naturally existing microorganisms within heaps and ores. Such a phenomenon contributes to the mobility of toxic metals, which is crucial for the environment. Currently, significant attention is directed toward contemporary industrial zones using circular economy for metal recovery, while investigating and monitoring historical and exploited mining areas remains relatively limited. This issue is particularly severe in post-mining areas. Mining and processing of ores generate a significant amount of waste, which is typically stored in open-air conditions. As a result of the natural bio-oxidation of sulfide minerals, acidic effluents are formed, which can pose an environmental threat due to the presence of sulfates(VI) and toxic elements such as arsenic, cadmium, chromium, copper, lead, mercury, nickel or zinc (Newsome and Falagán, 2021).

The biodiversity of microorganisms capable of oxidizing metal compounds includes archaea, bacteria, fungi, and yeast (Sajjad et al., 2019); these are resistant to high levels of metal concentration and high acidity. Naturally occurring and the most extensively characterized is the bacterium *Acidithiobacillus ferrooxidans* (Bonnefoy and Holmes 2012). Several studies of bioleaching have been carried out using *A. ferrooxidans* monoculture (Zhang et al. 2018; Santaolalla et al. 2021; Kang and Wang 2022) and also mixed cultures (Wang et al. 2014; Heydarian et al. 2018). Many scientific studies investigated the growth kinetics of pure cultures of *A. ferrooxidans* using 9K medium and $FeSO_4$ as substrate (Silverman and Lundgren 1959). However, the kinetic constants varied considerably and required consideration of additional parameters such as product inhibition, initial Fe^{3+} ion concentration, and inoculum size. A model for the growth kinetics of *A. ferrooxidans*, including all assumed parameters, was presented for the monoculture (Molchanov et al. 2007).

So far, no growth kinetics model has been presented for the consortium isolated from arsenic-containing waste. Our research showed that such a consortium consists predominantly of *A. ferrooxidans*, and *Acidiphillum cryptum*. We determined a standard curve for the relation of cell biomass [mg] against absorbance λ = 500 nm and Monod equation constants. We also investigated surface characteristics, such as surface charge, which are important when bacteria are in contact with the mineral surface (e.g. in bioleaching). It was shown that in the case of *A. ferrooxidans*, zeta potential depends on their growth history (Blake et al. 1994). To test whether changing culture conditions with

different iron(II) content affects the surface charge of bacterial cells, the zeta potential was measured using Zetasizer 2000 (Zetasizer, Malvern, United Kingdom) at a constant ionic strength of 10^{-3} M KCl, pH 2.0. The oxidation rate of iron was measured using the titrimetric method. Our research aims to better understand the acidophilic bacteria consortium's kinetic growth isolated from arsenic-bearing waste. The expected results will contribute significantly to the fields of metal recovery and environmental remediation.

Acknowledgement

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Investigating pH fluctuations as factor deteriorating large-scale performance of industrial relevant prokaryotic strain

G. Avolio[a], R. Takors[a]

[a]*Institute for Biochemical Engineering, University of Stuttgart, Allmandring 31, 70569 Stuttgart, Germany*

Keywords: Large-scale, fermentation, pH fluctuations, *C. glutamicum*, heterogeneities

Abstract

The industrial biotechnology market size has largely increased over the last few years with revenues growing at an average annual rate of > 10% considering the US market data (Carlson, 2016). In this scenario, the fermentation segment has always become more important from the economic point of view and for the environment to reduce greenhouse gas emissions and reach the carbon neutrality goal.

However, despite the growing bioprocess engineering industry, the transition from laboratory to large-scale cultivations remains a major risk. This is because laboratory scale conditions often differ from industrial-scale reactors, leading to unpredictable performance losses during bioprocess scaled-up. For instance, pH control through the addition of highly acidic or alkaline solutions combined with longer mixing times observed in large-scale tanks can lead to pH gradients within the reactor (Lara et al., 2006). If these large-scale limitations can be identified, we can mimic their effect with “scale-down” reactors at a laboratory scale (Oosterhuis, 1984).

In this study, we make use of a Stirred Tank Reactor – Plug Flow Reactor scale-down system (described by (Löffler et al., 2016)) to expose an engineered *Corynebacterium glutamicum* aminobenzoate producer strain to pH fluctuations, mimicking what cells experience in large-scale reactors. We evaluate the effect of oscillating pH on the transcriptome, the adenylate energy charge, and process parameters such as titers, rates, and yields. Due to the stress of external pH shifts, we expect a change in the internal ATP concentration along the PFR due to the energy required to maintain the intracellular pH. Moreover, we characterize the short-term (minutes) and long-term (hours) adaptations of the transcriptome during stressed cultivation. With the combined information we can potentially elucidate native regulatory mechanisms and suggest engineering strategies to build more robust strains for future industrial applications.

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A broad-host-range expression platform to facilitate chassis screening

Smith[a], J. Milton[b], B. Franklin[a,b]

Ácil De Almeida Will[a]*, Viviënne Mol[a]*, Kristoffer Bach Falkenberg[a], Ivan Pogrebnyakov[a], Charlotte Beck[a], Anna Lyhne Skøttrup[a], Alex Toftgaard Nielsen[a], Sheila Ingemann Jensen[a]

[a]*Novo Nordisk Foundation – Center for Biosustainability (DTU Biosustain), Kemitorvet 220, 2800 Kongens Lyngby*

Keywords: broad-host-range tool, inducible promoter, chassis screening

Abstract

Microbes have proven extremely valuable for the bioproduction of proteins and compounds, with applications in medical, environmental, and industrial contexts. Even though a vast number of bacterial species has been identified, the process of choosing the most suitable one as a host for a desired application still needs optimization. A barrier arises from the limited availability of genetic engineering approaches suitable for a broad range of organisms. This hinders comparison between model organisms and restricts the consideration of non-model organisms, often overlooked due to the lack of functional engineering tools. In this study, it is provided a modular, single vector-based expression platform, centered around the well-known promoter system tetR-pTet, inducible by anhydrotetracycline. This system has been presented in several studies with different modifications to improve its functionality in specific organisms. However, here, we prove that one single version can be compatible with a wide range of bacteria. In all the studied microbes, the promoter system was shown to be tight and titratable, within an 84-fold dynamic range. It enables easy screening of recombinant proteins and pathways in both mesophilic and thermophilic, and Gram-negative and Gram-positive hosts. Overall, this platform enables simple screening of heterologous expression and production in a broad variety of hosts.

Phage-free and scalable mass production of artificial single-stranded DNA with *Escherichia coli*

N. Hafner[a,b], N. Nemazadeh Somehsaraei[a], M. N. Honemann[b], H. Dietz[b], D. Weuster-Botz[a]

[a]*Technical University of Munich, Biochemical Engineering, 85748 Garching, Germany*
[b]*Technical University of Munich, Biomolecular Nano-Technology, 85748 Garching, Germany*

Keywords: Phagemid particle, ssDNA, *E. coli*, helper plasmid, high cell density fermentation, scale-up

Abstract

Large quantities of artificial single-stranded DNA (ssDNA) with user-defined sequences and lengths are increasingly required to exploit the potential of new technologies such as DNA origami. These DNA nanoparticles are currently being explored as drug delivery systems or as therapeutic agents for virus neutralization. In principle, biotechnological mass production of ssDNA can be achieved using *Escherichia coli* in a fed-batch process via the secretion of phagemid particles derived from the filamentous M13 phages.

Preliminary work recently succeeded in producing non-infectious phagemid particles with user-defined ssDNA, allowing for the first time the cross-contamination free and thus safe biotechnological production of ssDNA with *E. coli* at any contract manufacturer. For this, an artificial phagemid is used with one gene of the M13 phage genome together with the custom target sequence, and *E. coli* with an optimized helper plasmid encoding the other genes of the M13 phage. In absence of the helper plasmid, the phagemid particles are not capable of self-replication. However, this cross-contamination free production of ssDNA was so far solely possible in high cell density fermentations on a lab-scale. Scale-up of this lab-scale process to an industrial scale will need large amounts of phagemid particles for the infection.

In this work, we aimed to establish a new infection strategy of *E. coli* with helper plasmid at an early stage of the high cell density cultivation process. Different infection densities between a multiplicity of infection (MOI) of 10^{-6} and 10^{-2} transforming unit per colony-forming unit (tfu cfu^{-1}) were evaluated already at the beginning of the batch phase in a 2.5 L stirred tank bioreactor (Figure 1). A MOI of 10^{-3} tfu cfu^{-1} was identified as compromise between process runtime and ssDNA yield. Compared to the previous infection 5 hours after initiating the exponential feeding phase, the amount of phagemid particles necessary for infection was thus reduced by a factor of 250,000.

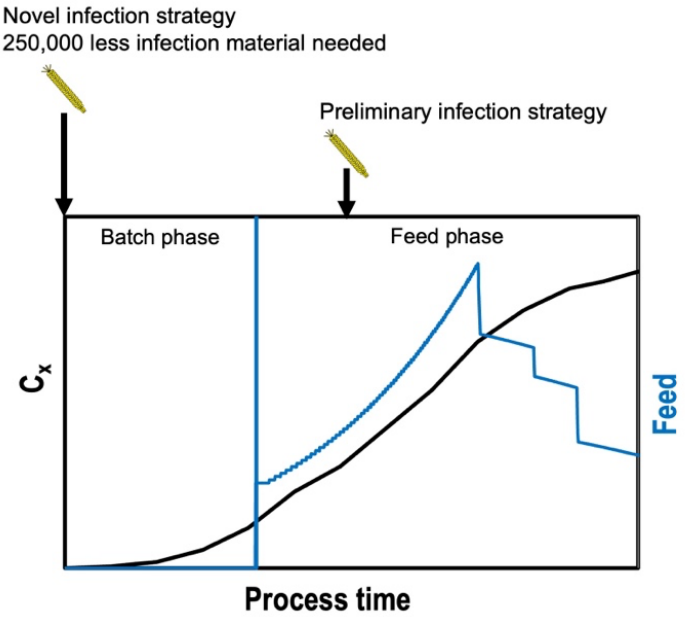

Figure 1: Novel infection strategy to reduce the amount of infection material in high cell density fermentations with engineered *E. coli* for the production of artificial single-stranded DNA

The early infection strategy was successfully transferred to a 25 L- and a 450 L-scale, achieving ssDNA concentrations of > 100 mg L^{-1} within a process time of one day on both scales. In addition, the early infection strategy was successfully evaluated with an increased user-defined ssDNA sequence, demonstrating general usability. With the increased ssDNA sequence a 42-helix bundle was fold and analyzed with negative stain transmission electron microscopy (TEM).

The ssDNA was purified according to the protocol of Kick et al. (2017) on both scales by first separating the *E. coli* cells via centrifugation. The phagemid particles in the culture supernatant were precipitated with polyethylene glycol 8000 and NaCl and resuspended. To isolate the ssDNA, the phagemid particles were alkaline lysed and ssDNA precipitated in the presence of ethanol.

Acknowledgement

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Production of a HA-like polysaccharide by *Vibrio* sp. Mo 245 using glycerol as sole carbon source

B. Almeida[a,b], C. A. V. Torres[a,b], X. Moppert[c], F. Freitas[a,b], M. A. M. Reis[a,b]

[a]*UCIBIO, Chemistry Department, NOVA School of Science and Technology, NOVA University Lisboa,Campus da Caparica, 2829-516 Caparica, Portugal*
[b]*Associate Laboratory i4HB – Institute for Health and Bioeconomy, School of Science and Technology, NOVA University, Campus da Caparica, 2829-516 Caparica, Portugal*
[c] *Pacific Biotech, BP 140 289 Arue Tahiti, French Polynesia*

Keywords: polysaccharide, glycerol, marine bacteria, bioproduct

Abstract

In recent years, interest in hyaluronic acid (HA) has grown in the biomedical and cosmetic industries due to its significant water retention capacity, viscoelastic properties, and biocompatibility (Baier et al., 2003). However, challenges associated with its production using *Streptococcus zooepidemicus* strains, namely limited cell growth, byproduct inhibition and safety concerns due to pathogenicity arose the need to find more sustainable alternatives (Liu et al., 2011). Several marine bacteria have been investigated as producers of glycosaminoglycans (GAGs) that possess physicochemical and biological properties similar to HA. In particular, the strain *Vibrio* sp. Mo245 has been reported to produce a novel HA-like polysaccharide, which is a promising alternative to the HA produced by the current methods due to its composition and viscoelastic and gelling properties (Martin-Pastor et al., 2019). In this study, *Vibrio* sp. Mo245 was cultivated, for the first time, using glycerol as the sole carbon source to produce the HA-like polysaccharide (HA-like EPS) in a 24 h fed-batch bioreactor cultivation run (2 L working volume), operated at 30 °C pH controlled at 7.20. The dissolved oxygen concentration was controlled at 40%, with a constant aeration of 1 SLPM. The strain grew at a maximum specific growth rate of 0.40 h^{-1}, reaching a biomass concentration of 20.6±0.70 g/L within 24 h of cultivation. EPS synthesis began at the end of the exponential phase (at 7 h of cultivation) and continued throughout the stationary phase. The culture produced the HA-like EPS, attaining a final concentration of 2.71±0.03 g/L and a productivity of 0.11 g/L.h, higher than the productivity previously reported for glucose (Raguénès et al., 1997). The high molecular weight HA-like EPS was recovered from the cell-free supernatant by dialysis and characterized by HPLC in terms of neutral sugars, uronic acids and hexosamines. The produced polysaccharide was mainly composed of glucosamine, glucuronic acid and rhamnose. When using glucose as a carbon source, similar composition in terms of glucuronic acid and glucosamine was obtained (Martin-Pastor et al., 2019), but galacturonic acid and galactosamine were also detected, unlike what was observed in this work. This study demonstrated that glycerol is a suitable carbon source for *Vibrio* sp. Mo245 cultivation and HA-like EPS production. The presence of rhamnose in the produced HA-like EPS may confer it additional interest, as polysaccharides enriched in this sugar monomer are reported to possess biological activity (Roca et al., 2015). Therefore, the use of glycerol not only supported good cell growth and EPS production, but it also resulted in a HA-like EPS with distinct characteristics.

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Exploring Lignolytic Bacteria Potential for Enhanced Lignin Valorization

B. Ruengsrichaiya [a], A. Y. Romero [b], S. I. Mussatto [a]

[a] *Department of Biotechnology and Biomedicine, Technical University of Denmark. Søltofts Plads, Building 223, 2800, Kongens Lyngby, Denmark*
[b] *Department Engineering and science, Monterrey Institute of Technology and Higher Education, Av. Eugenio Garza Sada 2501 Sur, Tecnológico, 64849 Monterrey, N.L., Mexico*

Keywords: Lignolytic Bacteria, lignin degradation, lignin valorization, inhibitors.

Abstract

Lignin, a crucial component of lignocellulosic biomass, represents a rich source of naturally occurring aromatic compounds. Traditionally, it is obtained as a by-product in pulp and paper industries and biorefineries. However, its enormous potential remains underexploited as most large-scale industrial processes that use plant polysaccharides from lignocellulose usually burn lignin for energy generation. In the last years, more attention has been given to transforming lignin into value added-products including fuels, chemicals and materials. Nevertheless, utilizing lignin as a fermentation medium can be challenging, as it can inhibit the growth of bacteria. Moreover, there is a lack of information regarding the lignin degradation pathway as well as studies on the tolerance of lignin derivative compounds by various bacteria. Based on that, the present study aimed to assess and comprehend the degradation pathway as well as the inhibitory effect of lignin derivative compounds on three distinct lignolytic bacterial strains: *Oceanimonas doudoroffii*, *Pseudomonas alloputida* (commonly known as *Pseudomonas putida* KT2440), and *Cupriavidus necator* DSM545. The results revealed that all the tested strains were able to utilize lignin derivative compounds as a sole carbon source at different concentrations. This opens up new opportunities for using lignin in higher value applications. By enhancing our understanding of lignin degradation pathways and the inhibitory effects of lignin derivative compounds on bacteria, this study contributes to the advancement of sustainable methods for converting lignin into high-value products, thereby fostering the development of a circular and sustainable economy.

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Dynamic modelling of pH over time in known chemical systems using scientific computing methods

P.E. Carstensen[a,b], J. Bendsen[a], J.B. Jørgensen[a],

[a]*DTU Compute, Scientific Computing, Building 303B, 2800 Lyngby, Denmark*
[b]*The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark*

Keywords: Mathematical Modelling, Chemical Modelling, Optimization, Ordinary Differential Equations

Maintaining optimal pH is crucial in chemical production, as it directly influences formation, quality, and quantity of the product. While pH can be readily measured in physical systems, digital twin systems or in-silico experimentation require an estimation of pH (Gustafsson et al 1995).

The model introduces a pH predictor specifically designed for known chemical systems, utilizing equality constrained problems, estimated Gibbs free energy (Dick 2019) and the ideal gas law for accurate predictions of pH over time. The model utilizes stoichiometric matrices, making it adaptable for both small and large chemical systems.

The problems are defined in a well-stirred tank with a constant volume, where in and outflow can be defined. This allows the system to be described by ordinary differential equations, allowing for tracking of the various concentrations of chemical substances as well as the pH over time.

The model includes examples of methanol synthesis in water and phosphoric acid in water with ammonia, carbonic acid, nitric acid, and sodium hydroxide as the control. These examples demonstrate the model's ability to accurately estimate the pKa-values of PO_4^{3-} to H_3PO_4, CO_3^{-2} to H_2CO_3 and NH_3 to NH_4^+, in a single system with a total of 18 species.

The examples are written as optimization problems in Julia with JuMP (Lubin et al. 2023) as well as our own implementation of optimization algorithms. The model is written in Julia to prioritize speed and open-source accessibility, enabling efficient problem-solving.

The model offers a fast and reliable solution for optimizing pH levels in chemical production. Its adaptability and computational efficiency make it an asset for improving production efficiency and product quality in closed loop well-stirred chemical tanks.

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Water Purification and Selective Recovery of Metals from Industrial Effluents and Natural Waters Contaminated with Non-Degradable Heavy Metallic Ions

A. Mitic[a], C. Gargallo[a], O. Falholt[a]
[a]*BioScavenge ApS, Algade 2D, 4281 Gørlev, Denmark*

Keywords: water purification, recovery of heavy metals, mining effluents, electronic waste, contaminated ground waters

Abstract

Pollutants like industrial gasses, dust, waters, sludges, coal combustion residues, etc. are often contaminated with non-degradable heavy metals. Some of the most polluting players are the military, agriculture, and various industries. Particular examples are fuel and power industries that discharge 2.4 million tons/year of arsenic, cadmium, chromium, copper, mercury, lead, selenium, and zinc. The metals industry annually contributes with 0.39 million tons of the same metals while wetland disposals add 0.72 million tons each year. Such discharged metals are considered as large threats to public health with strong environmental impacts and huge economic effects.

Instead of being considered as contaminants, metals from polluted waters could be converted into valuable raw materials and used for various purposes. Water itself is also considered as a valuable resource. For instance, it is estimated that water consumption will rise by 400% before 2050 particularly in the westernized world. Combined with climate changes and increased water scarcity in some countries, clean water is anticipated to be a very valuable commodity.

The concept developed here is depicted in Figure 1. It is based on a cascading approach involving 2 stages. Stage 1 deals with high hydraulic loads and pre-selective up-concentration of metallic ions. Stage 2 creates brines with individual metallic ions enabling their further valorizations in various forms.

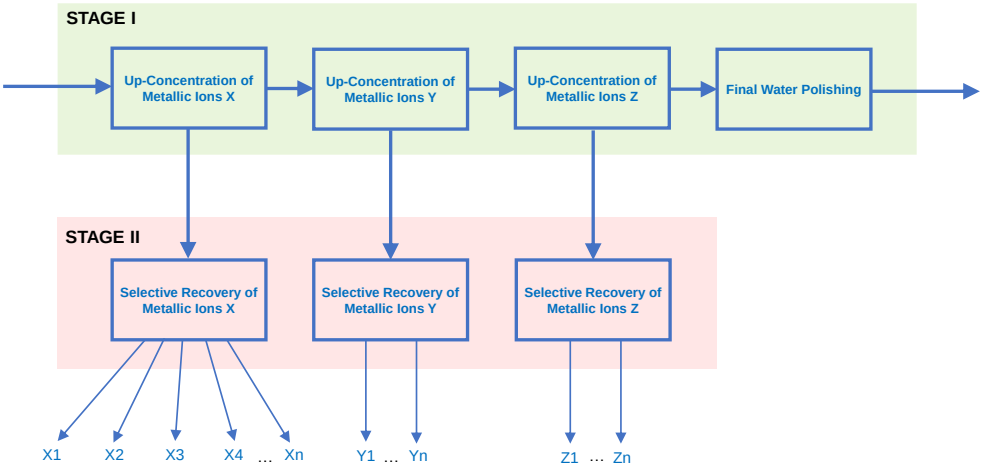

Figure 1: Concept for the Recovery of Metals and Water Purification

The concept was tested on effluents containing more than 70 impurities dissolved in water. Despite a high diversity of metallic ions and their various concentrations, this modular design managed to deliver clean brines containing most valuable metallic ions. Good examples are copper, scandium, nickel, cobalt, aluminum, and magnesium. Estimated carbon-footprint of the applied concept was up to 10 kg per metric ton of treated water while the levelized cost of purified water was represented with a negative number (appx. -2 Euros per metric ton of treated water). This essentially implies on a high value of metallic ions that are trapped in this type of effluents. The achieved final purity of water was rather high allowing its reuse or harmless disposals to the environment.

This modular design is also adjustable to various types of contaminated waters. Good examples are effluents from the electronic industry polluted with rare earth elements, as well as water streams from the industry of batteries that are polluted with cadmium, nickel, and cobalt. Industries as pharma, mining, and even tanning with their chromium problems, can all benefit from this type of recovery processes. Furthermore, water effluents from wetlands, polluted seawater, acid mine drainages, etc., could also be considered as suitable raw materials.

Brewer's spent grain as a single source feedstock for sustainable lactic acid production

B. Akkoyunlu[a], N. Arul Manikandan[a], K. Amulya[a], H. Vohra[a], B. Freeland[a,*]

[a]*School of Biotechnology, Dublin City University, Glasnevin, Dublin 9, Ireland*

Keywords: Brewer's spent grain, enzymatic hydrolysis, fermentation, L-lactic acid, circular economy

Abstract

Brewer's spent grain (BSG) constitutes approximately 85% of the total mass of solid by-products in the brewing industry and it is primarily used in animal feed and being disposed of in landfills. (Zeko-Pivač et al., 2022). Due to the high nutritional composition of BSG (including cellulose, non-cellulosic carbohydrates, proteins and polyphenols), it has the potential to be used as sole feedstock for biotechnological applications such as lactic acid fermentation. Lactic acid (LA) is an essential platform chemical that has numerous applications in various industries. Currently, the majority of LA production is via microbiological fermentation, primarily because it yields optically pure products compared to chemical synthesis, mainly using corn starch, sugarcane and sugar beet as feedstock (Mailaram et al., 2023). Using these feedstocks are expensive since they can account for up to 70% of the total production cost, and they compete with food products. Therefore, using by-products from various industries as feedstock for lactic acid fermentation is becoming an important opportunity for decreasing the costs and contributing to the circular economy. This study suggests BSG as a self-sufficient and cost-effective feedstock for LA fermentation and aims to investigate batch, fed-batch and continuous modes of operation. Enzymatic hydrolysis was used as the pre-treatment step to prepare the fermentation broth for lactic acid producing bacteria. Process parameters such as initial glucose concentration, carbon to nitrogen source ratio and inoculum concentration were investigated. Preliminary results showed that a maximum lactic acid concentration of 114.4 g/L can be achieved with batch mode when 120 g/L initial glucose concentration was used. Further research will include fed-batch and continuous operation modes where the effect of different feeding strategies and dilution rates on lactic acid production will be investigated. This research also aims to create a framework for future bioprocess applications where BSG is used as a self-sustaining feedstock to produce a range of bioproducts.

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Selecting and cultivating photosynthetic microorganisms in anaerobic digestion effluent from agricultural waste for bioplastic production

E. Sventzouri[a], K. Pispas[a], G. Manthos[a], M. Kornaros[a,]*

[a]*Laboratory of Biochemical Engineering & Environmental Technology (LBEET), Department of Chemical Engineering, University of Patras, 1 Karatheodori Str., University Campus, 26504 Patras, Greece*

Keywords: microalgae, cyanobacteria, bioplastics, anaerobic digestate, bioremediation

Abstract

Over the last years, the production and consumption of plastic materials are growing exponentially. Plastic-derived pollution has several impacts on both the environment and human health. To this end, alternative solutions to replace petroleum-based plastics are necessary. Polyhydroxyalkanoates (PHAs) are biodegradable polymers of biological origin with similar thermal and mechanical properties to conventional plastics that can be used in a wide range of applications. Microalgae and cyanobacteria cultivation for bioplastic production seems to be a promising solution (Mastropetros *et al.*, 2022). However, the main bottleneck in its widespread application is the high operational cost, with the cultivation medium accounting for up to 50% of the total cost (Medeiros Garcia Alcântara *et al.*, 2020). Cultivation in wastewater can significantly enhance the economic feasibility of the process, while this approach also contributes to a sustainable waste management system.

In this study, ten microalgal and three cyanobacterial species were evaluated, during a two-stage cultivation strategy, in terms of their ability to grow in the liquid fraction of anaerobic digestate from agricultural wastes as well as in terms of the simultaneous accumulation of PHB. More specifically, *Parachlorella kessleri*, *Chlorella vulgaris*, *Chlorella minutissima, Tetraselmis tetrathele*, *Chromochloris zofigiensis, Acutodesmus obliquus*, *Euglena gracilis*, *Chlorococcum oleofaciens, Botryococcus braunii*, *Coelastrella vacuolata*, *Arthrospira platensis, Nostoc muscorum* and *Synechocystis* sp. were cultivated in 10% v/v digestate, at 26 ± 2 °C with photon flux density of 500 μmol m^{-2} s^{-1}. The same strains were also tested in a nitrogen depleted synthetic medium, under the same cultivation conditions, to enhance PHB accumulation. Among these species, *T. tetrathele*, *C. zofigiensis*, and *A. platensis* did not manage to adapt to the specific substrate. *C. minutissima* exhibited the highest biomass production, 2.6 g L^{-1}, while the removal of chemical oxygen demand, total nitrogen, and total phosphorus were 37%, 98%, and 100%, respectively. However, *C. minutissima* did not accumulate PHB during the second stage of the cultivation. *Synechocystis* sp. is a promising candidate for bioplastic production. After 32 days of nitrogen starvation, PHB content reached 15% of cell dry weight.

This study is a primary stage of selecting the most suitable species not only for the production of high value-added products but also for the bioremediation of the used substrate, promoting the circular economy context.

Acknowledgement

This research work was financially supported by the research program “ALGAPLAS: Microalgae-based Digestate Valorization towards Sustainable Bioplastics Production” (Project Number: 4799), funded by the Hellenic Foundation for Research and Innovation (H.F.R.I.) under the “2nd Call for H.F.R.I. Research Projects to support Faculty Members & Researchers”.

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Effect of Freeze Drying Process on Surface-driven Properties of Bacterial Cells

A. Panetsou[a], A. C. Mendes[a], I. S. Chronakis[a], H. NasriLari[b]

[a]*DTU-Food, Technical University of Denmark, Søltofts plads B227, 2800 Kgs, Lyngby, Denmark*
bProcess Design, Research and Development, Novonesis A/S, 2970 Hørsholm, Denmark

Keywords: Freeze-drying, bacterial cell, Streptococcus thermophilus, FTIR, hydrophobicity.

Abstract

Streptococcus thermophilus (ST) is a key acidifying bacteria used by dairy industry as starter culture. Freeze drying has been widely used in biotechnology industry for producing frozen bacterial cells including STs. It is well-known that the freeze-drying profile and process parameters can have significant impact on the functionality of the final product. However, the underlying principle behind it has not been yet thoroughly investigated. In this work, we have applied different freeze-drying profiles on a formulated ST-4458, and stored the freeze-dried products for one month at high temperature of 37°C. The acidification performance of the stored samples was evaluated by CINAC milk acidification activity test. We primarily showed how different drying profiles result in variation in acidification performance during the storage of the products. Further, at a more profound level, we examined the freeze dried granulates during their storage using hydrophobicity measurement and FTIR analysis. The results demonstrated that there is a strong correlation between surface-driven properties such as hydrophobicity of bacterial cells and chemical composition of cell membrane with the acidification performance of those samples.

Acknowledgement

We thank the members of Process Design Department, Fermentation Lab, and Biochemical Assays Department of Novonsis A/S for their technical support.

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Effect of the Freeze-Thaw Method on the Extraction and Purity of Phycocyanin from Cyanobacterial Biomass

K. Pispas[a], E. Sventzouri[a], G. Manthos[a], M. Kornaros[a,*]

[a]*Laboratory of Biochemical Engineering & Environmental Technology (LBEET), Department of Chemical Engineering, University of Patras, 1 Karatheodori Str., University Campus, 26504 Patras, Greece*

Keywords: pigments, cyanobacteria, extraction, phycocyanin

Abstract

Phycocyanin (PC) is a water-soluble protein that has gained significant popularity in recent years as a natural blue colorant. It is derived from Cyanobacteria and Rhodophyta and has diverse applications in food, cosmetic, and pharmaceutical industries (Mastropetros *et al.*, 2023). Beyond its coloring properties, phycocyanin also exhibits antioxidant and anti-inflammatory properties. Currently, commercial PC products are primarily derived from the well-known cyanobacterium *Arthrospira platensis*. However, there are numerous photosynthetic microorganisms that can produce phycocyanin at similar concentrations and purity. Notably, current limitations to phycocyanin applications are related to the extraction methods (low yield and purity) and the lack of stability of the substance during storage (Pez Jaeschke *et al.*, 2021).

This study investigates the effect of the number of freeze-thaw (Moraes *et al.*, 2011) cycles on the extraction of phycocyanin from the wet biomass (Figure 1) of four different cyanobacteria (*Arthrospira platensis*, *Chlorogloeopsis fritschii*, *Phormidium* sp., and *Synechocystis* sp.). The study also examines the effect of five different extraction solvents (Tris-HCl buffer, phosphate buffer, calcium chloride solution, 3D water, Tap water) at different pH values.

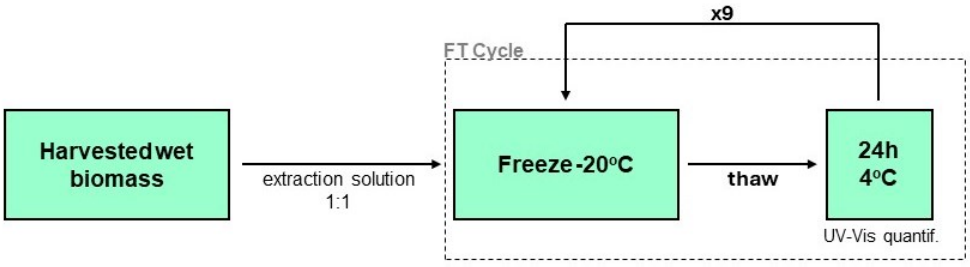

Figure 1: Flowchart of the extraction process followed

The results showed that *Synechocystis* sp. exhibited the highest phycocyanin content among the examined cyanobacteria. For *A. platensis*, using Tris-HCl buffer as the extraction solvent yielded the maximum PC concentration from the first cycle, while the use of sodium phosphate buffer resulted in satisfactory results from the second cycle of extraction. Tris-HCl yielded similar results for *C. fritschii* (achieving 66% of the maximum yield from the first cycle), whereas using phosphate buffer or 3D water resulted in a maximum PC content of 12% w/w by the sixth cycle. For *Phormidium* sp., the maximum concentration of the pigment was achieved from the first cycle of

extraction using both 3D and tap water. Interestingly, the use of calcium chloride solution led to an exceptional purity ratio in this species. *Synechocystis* sp. and *C. fritschii* exhibited a purity ratio greater than 1, for the scenario of Tris-HCl buffer and 3D water, respectively. Although there is a different optimal extraction solvent for each species, Tris-HCl buffer is considered capable of extracting sufficient amount of the pigment for all species from the first cycle.

Acknowledgement

This research work was financially supported by the research program “ALGAPLAS: Microalgae-based Digestate Valorization towards Sustainable Bioplastics Production” (Project Number: 4799), funded by the Hellenic Foundation for Research and Innovation (H.F.R.I.) under the “2nd Call for H.F.R.I. Research Projects to support Faculty Members & Researchers”.

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Bio-refining of Bacterial Biomass for Astaxanthin Recovery: Semi-Switchable Solvent Extraction and Recovery Protocol

E. Mrotek[a], V. Sapone[a], M. Bravi[a]

[a]*Dept. Chemical Engineering Materials Environment, University of Rome La Sapienza, via Eudossiana 18, Rome, 00184, Italy*

Keywords: Astaxanthin, Paracoccus carotinifaciens, switch, recovery, fatty acids

Abstract

Astaxanthin, a naturally occurring carotenoid pigment that belongs to the xanthophyll family is renowned for its exceptional antioxidant properties, which are significantly stronger than those of other carotenoids such as beta-carotene and lutein. Astaxanthin is highly valued for its various health benefits and wide applications in pharmaceuticals, nutraceuticals, and cosmetics industries. The bacterium *Paracoccus carotinifaciens* is a rich natural source of astaxanthin and offers several important advantages over synthetic alternatives, such as increased bioavailability and bioactivity, complexity of natural astaxanthin and minimization of the environmental impact of the antioxidant production process.

This study explores the potential of refining astaxanthin-rich biomass from microbial source to obtain a purified mixture of carotenoids rich in astaxanthin devoid of unwanted components such as proteins and carbohydrates. The astaxanthin recovery process consists of two main steps. Initially, carotenoids are extracted from the biomass using short-chain fatty acids with carbon chain lengths ranging from 2 to 8, resulting in the separation of an extract and a residual fraction. Subsequently, the addition of water and a strong base (sodium or ammonium hydroxide) triggers a transition of the solvent from hydrophobic to hydrophilic, causing the precipitation of the carotenoids as a dark red solid (Figure 1). This solid can be easily separated from the liquid phase, representing the final product. Results indicate that the solvent effectively dissolves approximately 90% of the astaxanthin and other carotenoids present in the biomass during the first step, leaving behind a whitish residue. Importantly, the carotenoids can be recovered in solid form with high efficiency during the second stage of the process.

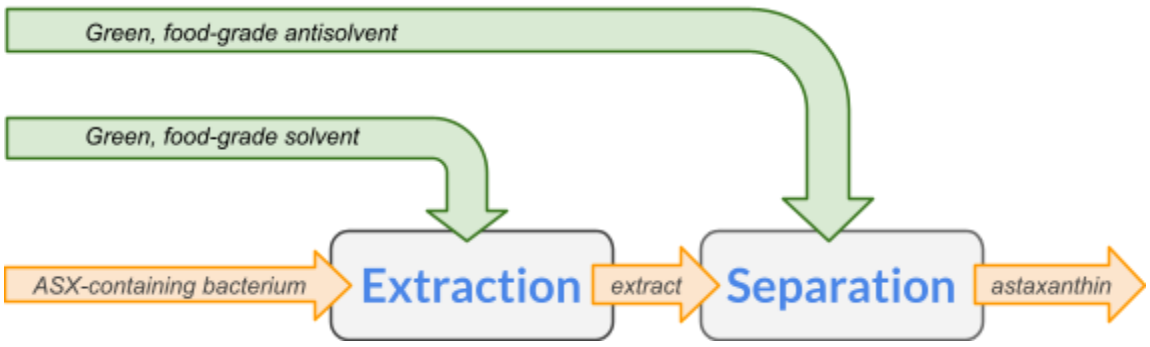

Figure 1: Process scheme for Astaxanthin extraction and recovery from bacterial biomass

Acknowledgement

The continuous support of professor Philip G. Jessop and his research group during the development of this study is gratefully acknowledged.

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Production, characterization, and modification of biopolymers for application in zinc-based batteries

D. Lammers[a,b], R. Krull[a,b], K. Dohnt[a,b]

[a]*Institute of Biochemical Engineering, Technische Universität Braunschweig, Rebenring 56, 38106 Braunschweig, Germany*
[b]*Braunschweig Integrated Centre of Systems Biology (BRICS), Technische Universität Braunschweig, Rebenring 56, 38106 Braunschweig, Germany*

Keywords: Xanthan, alginate, cellulose, gel polymer electrolytes

Abstract

Biopolymers, as a response to the ever-increasing demand for more environmentally friendly and sustainable alternatives to fossil fuel-based products and processes, gained the attention of modern scientific research. Driven by their promising properties in terms of biodegradability, functional diversity, and accessibility, the importance of biopolymers as alternative materials has rapidly increased. Biopolymers not only find applications in biomedical, food, or packaging industries but also demonstrate potential in battery technology, meeting the demands for safer, more sustainable, and cost-effective battery materials. As alternatives to today´s predominant Lithium-ion batteries (*LIBs*), zinc-based batteries are considered suitable candidates for one of the "beyond lithium-ion" technologies due to their safer aqueous alkaline electrolyte as well as lower cost and specific power and energy close to *LIBs* (Parker et al., 2018). Natural biopolymers such as xanthan or alginate and cellulose of bacterial origin are thus herein investigated as replacements for current battery components. Utilizing these biopolymers to form gel polymer electrolytes (*GPEs*) provides a promising opportunity to avoid common challenges of zinc-based batteries, such as zinc anode derived dendrite growth or shape-change during battery cycling.

The biotechnological production of the natural biopolymers xanthan from *Xanthomonas campestris*, alginate from *Azotobacter vinelandii* and cellulose from *Komagataeibacter xylinus* was investigated and characterized at different scales from shake flasks to 20 L bioreactors. After isolation and purification, the biopolymers demonstrated their suitability as a *GPE* in a nickel-zinc battery (*NiZnB*). Compared to the reference *NiZnB* with a glass fiber separator and a liquid alkaline electrolyte, the biopolymer-based *GPEs* revealed their potential in terms of higher state of health and cycle life values in the long term. Both parameters are directly related to the battery performance. However, the biopolymer-based *GPEs* need improvement concerning long formation processes and poor capacity retention. To this end, we produced biopolymers with varying molecular weights by selected cultivation conditions. Gel permeation chromatography was used to estimate the differences in molecular weight of the biopolymers and revealed a direct correlation between molecular weight and battery performance. These results indicate the versatile applicability of biopolymers and are an important step towards a more sustainable battery development.

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Hybrid modelling for prediction of the biopolymer based mixed matrix membrane performance in gas separation.

C.Casado-Coterillo[a], O.A. Prado-Rubio[b], J.K. Huusom[c]

[a]*Universidad de Cantabria, Av. Los Castros, 39005 Santander, Spain.*
[b]*Universidad Nacional de Colombia, 170003 Manizales, Colombia*
[c]*Technical University of Denmark (DTU), DK-2800 Lyngby, Denmark*

Keywords: Chitosan biopolymer mixed matrix membranes; MOF fillers; CO_2/N_2 separation; Maxwell phenomenological model; Hybrid modelling, Machine learning.

Abstract

Membrane technology has long been a promised next generation gas separation technology that has drawn research interests during past decades. To advance the progress of membrane materials and process development which potentially satisfy the urgent needs climate emergency, accurate prediction of membrane performance has to be efficiently optimized, which is even more difficult if not only performance, but also sustainable fabrication is considered (Xie et al. 2021). Therefore, there is a need to develop high-fidelity models capable to represent underdeveloped green technologies. Chitosan-ionic liquid composite membranes have been modified by different fillers to improve the CO_2 permeation, separation, and stability for different applications in the decarbonization of industry. Previous modelling efforts have used Maxwell modified equations to correlate CO_2 and N_2 permeation with the composition of mixed matrix membranes (Casado-Coterillo et al. 2015). Despite sufficiently accurate predictions, the model parameters are locally dependent and lack interpretability, thus still show a gap to contribute to the fabrication of better scalable membranes with sustainable criteria. Recently, machine learning approaches have attracted researchers' attention at their potential for introducing experimental and simulation data as input to train models, thus predict membrane performance from their properties. However, the interpretation and applicability in gas separation applications is not straightforward, especially if we consider novel materials (Wang et al. 2023). Therefore, hybrid modelling approaches involving mechanistic aspects combined with data-driven tools may help closing the gap between present and future membrane behavior understanding to help the technology development forward to meet the promises towards a sustainable society and industry (Galinha & Crespo, 2021). Hybrid neural network models (HNN) have been applied in bioprocess applications, mainly the pharma sector, following the semi-parametric approach where physical laws are directly incorporated int the model structure (Agharafeie et al. 2023). Hybrid models provide mechanistic understanding to identify the gaps caused by the variables still not fully covered by the deterministic models' hypotheses (Prado-Rubio & von Stosch, 2017).

In this work we have developed a 5 steps framework to develop a hybrid model architecture to optimize the prediction of the permeability of chitosan-based mixed matrix membranes for CO_2/N_2 separation (Figure 1, upper section). The baseline uses local optimization and modified Maxwell equation based on experimental permeability and operation temperature (case 1). The modelling approach is upgraded sequentially up to the use of global optimization algorithms including 2 parameters of physico-chemical meaning: void thickness, i.e. the interaction between the components of the membrane matrix and the rigidification or immobilization factor of the dispersed phase in the continuous matrix for all the biobased materials involved at a time (case 2, 3), and to the use of centralized temperature dependence of the permeability (Andersen et al. 2017) (case 4). As result, this approach was able to reduce the confidence intervals of the residual errors with

2 parameters. Finally, a hybrid model is proposed using the residuals of the best phenomenological approach combined in series with machine learning methods (Figure 1, bottom) and more experimental input variables than the mechanistic approach allowed. The MLPNN configuration is known as a quick training to learn how a set of input variables can be linked to an output parameter, thus we estimate the weights and bias parameters through iterative training to reduce the mean square error (MSE) between the model prediction and the experimental results down to 1.3e-3. The correlation between the outliers in all the features that phenomenological models cannot easily include, like water uptake, membrane thickness and density, may provide significance of different input properties of the membrane and process performance that, influencing gas permeability and selectivity, can be used to understand the structure-functional relationship of novel membranes.

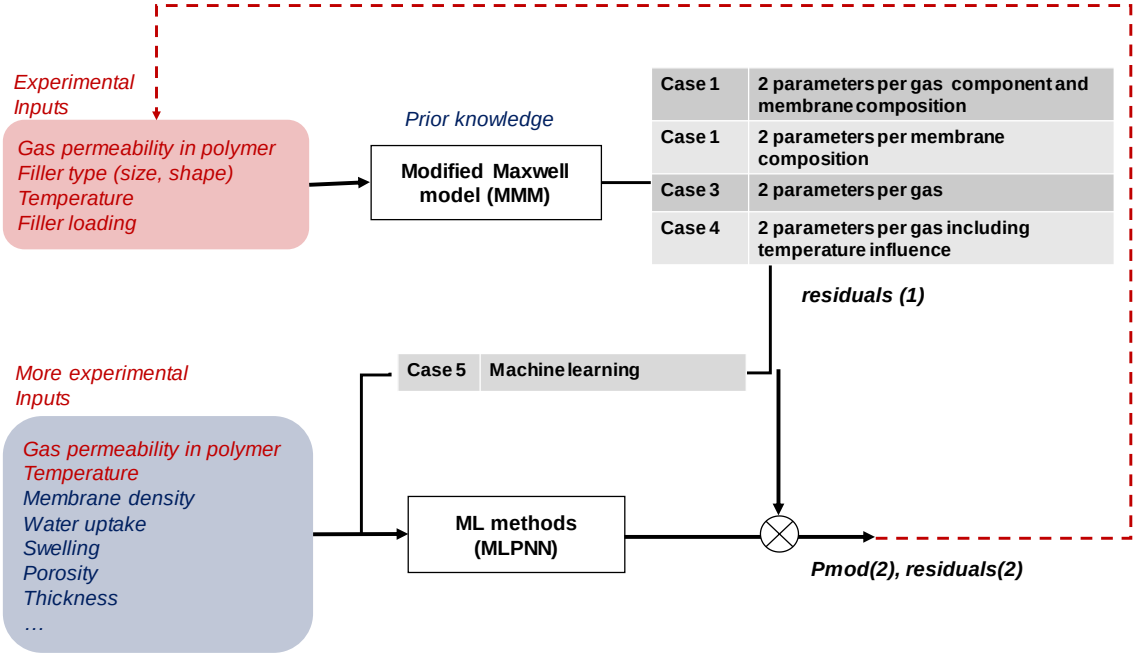

Figure 1: General algorithm for data processing according to proposed hybrid model approach.

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Sustainable PHBV extraction from MMC biomass using Natural Deep Eutectic Solvents (NADESs)

Yannick Patrice Didion[a], Tjalling Gijsbert Tjalsma[a], Ziran Su[a], Magdalena Malankowska[a], Mariana Matos[b], Carla Brazinha[b], Joao Crespo[b], Manuel Pinelo[a]

[a]*Technical Univeristy of Denmark (DTU), Department of Chemical and Biochemical Engineering, Søltofts Plads 228, 2800 Kgs. Lyngby*
[b]*Universidade Nova de Lisboa (NOVA), Faculdade de Ciências e Tecnologia (FCT), Caparica, Setubal, Portugal*

Keywords: Polyhydroxyalkanoates (PHAs), Natural deep eutectic solvents (NADES), extraction

Abstract

As the world shifts away from oil-based products, certain essentials like plastics and detergents remain integral to our modern way of life. One promising approach involves harnessing cell factories, such as specific bacterial and yeast strains, which naturally produce biopolymers akin to conventional plastics. In this context, mixed microbial cultures (MMC) show great promise as they do not require sterile conditions as opposed to pure bacterial cultures (Mondal et al., 2023). The biopolymers produced by MMC have garnered industrial interest due to their dual attributes of biodegradability and biocompatibility, offering potential replacements for some oil-derived plastics, particularly in consumer goods and medical applications.

In this study a downstream process to extract and purify the polyhydroxy(butyrate-co-valerate) (PHBV) produced by MMC is presented. Traditional extraction and purification of PHBV relies on energy intensive cell disruption techniques and toxic solvents such as chloroform (Patrice Didion et al., 2023). Here an optimized and effective alternative process using hydrophobic natural deep eutectic solvents (NADESs) is presented and evaluated, with an emphasis on recycling the solvents that were applied. At the optimized extraction conditions, PHBV yields of 84.7 ± 2.6 wt% at purities ≈ 95 wt% were achieved, while all the applied solvents were recovered and recycled. This result is approaching yields achieved with organic solvents and the hope is that this work will contribute to the more wide-scale introduction of PHBV as a cost effective, environmentally friendly and biodegradable alternative to oil-derived plastics (Didion et al., 2024).

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Accelerating AI-Model Development for Bioprocess Analysis Through Raman Spectroscopy and Innovative Design of Experiments

Joerg Weber[1]*, Andreas Latza[1], Oleg Ryabchykov[1,2], Darina Storozhuk[2], Oliver Valet[1]

1 Biophotonics Diagnostics GmbH, Jena/Germany
2 Leibniz Institute of Photonic Technology Jena, Albert-Einstein-Str. 9, 07745 Jena, Germany
**joerg.weber@bpd.gmbh*

Keywords: Raman Spectroscopy, Artificial Intelligence (AI), Bioprocess Analysis, Inline Measurements, Process Analytical Technology (PAT)

Abstract

Precision in the analysis of bioprocesses, e.g. in pharmaceutical and food production, is crucial. These complex processes, based on the dynamics of living organisms, require efficient and fast analytical tools for direct control and immediate reaction to deviations. Our study highlights the benefits of combining RAMANMETRIX™, an AI-powered software, with Raman spectroscopy for real-time monitoring of complex multi-component bioprocesses. One focus of our research is the development of an innovative approach to experimental design that enables the rapid creation and adaptation of robust models to efficiently monitor and control a variety of bioprocesses.

A concrete example of the application of this technology is the cultivation of a genetically engineered E. coli strain in a bioreactor, where direct inline measurements with a Raman probe inserted into the process enabled the continuous acquisition of spectral data. This method, supported by conventional HPLC analysis, serves as the basis for our reference model and illustrates the effectiveness of inline Raman spectroscopy in combination with advanced AI for immediate monitoring of components such as products, by-products or the carbon source. Our approach, which is not limited to E. coli processes but also applicable to other biological systems, significantly accelerates the model development process and increases the robustness of the developed models.

This innovative approach to experimental design opens up new possibilities for process monitoring and control in bioprocesses and emphasizes the importance of efficient, non-invasive analytics.

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We would like to acknowledge the Leibniz Institute for Natural Product Research and Infection Biology - Hans Knöll Institute (HKI) in Jena, Wasatch Photonics Inc. and Schott AG, Biotech Department. Their support and cooperation was crucial for the successful progress of the research.

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Simplifying mRNA vaccine manufacturing by using immobilised enzymes during *in vitro* transcription reactions

Georgia J.M. Taylor, Sara Sousa Rosa, Paul A Dalby, Marco P.C. Marques

Department of Biochemical Engineering, University College London, Bernard Katz Building, Gordon Street, London WC1H 0AH, United Kingdom

Keywords: mRNA, IVT, immobilisation, magnetic beads, T7 RNA Polymerase

Abstract

Vaccinations play a vital role in stopping the spread of infectious diseases. An upcoming technology ismRNA vaccines, which have proved very effective during the COVID-19 pandemic. These vaccines are now being developed and tested for a panoply of diseases, including infectious diseases and cancers, due to their manufacturing flexibility, safety and precision. Until now, mRNA is produced in cell-free system reactions from a linear DNA template, using RNA polymerase as a catalyst and nucleoside triphosphates (NTPs) as co-substrates, together with other reaction components. Typical production titres are between 2 to 5 g· L^{-1} but recent studies have shown that titres can be increased to 12 g· L^{-1} in both batch and fed-batch mode. Despite the well-defined enzymatic manufacturing process and the tight reaction control, the cost of these vaccines is still prohibitive for LMICs, mainly driven by the cost of goods.

In this work, we explore the use of magnetic beads to immobilize enzymes (e.g. T7 RNA Polymerase, T7RNAP) or substrates present in the IVT reaction. Magnetic beads offer the flexibility to operate IVT reactors in different modes of operation and/or reactor configurations, besides reducing significantly purification steps and reaction components. Enzymes were immobilized on the magnetic beads exploring the biotin–streptavidin chemistry to simplify immobilization workflows. Reaction space was explored with optimum temperature and pH ranging from 41-44°C and pH ranging from 6.3-6.8, respectively. This is in contrast with the free enzyme system optimum conditions where high yields (12 g·L^{-1}) are obtained at 44 °C and pH 6.8. Furthermore, T7 RNA polymerase activity could be maintained for 3 days at 4 °C without significant loss. The magnetic beads allowed for stable operation over 5 cycles. Despite the impact of immobilisation on the kinetic parameters, e.g. $k_{m(app)}$ and k_{cat}, when compared to solution phase enzymes, the immobilisation proved highly robust. This work will contribute to achieving lower production costs, and ultimately making the processes sustainable and affordable to all.

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Proteomic analysis between industrial-scale and laboratory-scale fermentations of a 2'-Fucosyllactose producing *Escherichia coli* strain

R. Labunskaite[a,b], M. Pedersen[a], T. Johanson[a], M. Kilstrup[b]
[a] *dsm-firmenich, Kogle Alle 4, 2970 Hørsholm, Denamark*
[b] *DTU Bioengineering, Søltofts Plads Building 221, 2800 Kgs. Lyngby, Denmark*

Keywords: 2'-FL, proteomics, *Escherichia coli*, fermentation.

Abstract

Human Milk Oligosaccharides (HMOs) are highly valued constituents of human milk, ranking third in abundance after lactose and lipids. HMOs offer numerous health benefits for infants, including protection against infection and inflammation, modulation of intestinal microbiota and development of the immune system (Ray et al., 2019). Among the diverse array of over 200 HMOs found in human milk, 2'-Fucosyllactose (2'FL) stands out as the most prevalent, with current commercial-scale production achieved through *Escherichia coli* fermentation (Bych et al., 2019).

Industrial fermentation processes impose challenging conditions to microorganisms (Wehrs et al., 2019), notably the presence of sugar gradients that significantly impact microbial performance, often resulting in a commonly observed yield gap between industrial-scale and laboratory-scale fermentations. Consequently, it is hypothesized that these sugar gradients play a pivotal role, at least partially, in the observed yield gap.

In this study, pulse-feeding fermentation techniques were employed, with feed additions every 65s or 130s, as scale-down models in high-cell-density fed-batch fermentations. This approach mimicked the sugar gradients encountered in industrial processes, allowing for an assessment of the yield gap between laboratory and industrial scales. Fermentations were meticulously monitored for both 2'FL production and biomass measurements. Additionally, a comprehensive proteome expression analysis was conducted to explore potential insights into the observed yield gap.

Remarkably, the pulse-feeding regime, particularly with a 65s feeding cycle, effectively replicated the 2'FL yield observed in large-scale fermentation settings. However, the protein expression profiles did not exhibit significant convergence with those of industrial-scale fermentations, as compared to the reference and pulse-feeding with a 130s feeding cycle processes. These findings underscore the complex interplay between fermentation dynamics and protein expression, shedding light on avenues for optimizing industrial-scale HMO production.

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A GNN-Transformer Based Reduced-Order Model for Transient Solid-Liquid Mixing in Stirred Tanks

Yu Jiang[a], Jarka Glassey[a,b], Edmond Byrne[a], Xizhong Chen[c]

[a]*Department of Process and Chemical Engineering, School of Engineering and Architecture, University College Cork, Cork, T12 K8AF, Ireland*
[b]*Merz Court, School of Engineering, Newcastle University, Newcastle upon Tyne, NE1 7RU, United Kingdom*
[c]*Department of Chemical Engineering, School of Chemistry and Chemical Engineering, Shanghai Jiao Tong University, Shanghai 200240, PR China*

Keywords: Graph Neural Networks, Predictive Model, Solid-Liquid Mixing, Reduced-Order Model, Transformer.

Abstract

Stirred tanks are widely used across various process industries for the purpose of facilitating reactions and for mixing, including for solid-liquid mixing. In order to accurately model and design solid-liquid processes in stirred tanks, it is important to utilize high-fidelity methods such as computational fluid dynamics (CFD) (Wadnerkar *et al.*, 2016). However, for industrial processes involving large computational domains, CFD simulation is computationally expensive to run. To address the challenge of high computational costs, reduced-order models (ROMs) have been used in fluid dynamics (Wang, Hesthaven and Ray, 2019; Hasegawa *et al.*, 2020b). This is especially the case for transient processes, where there have been applications on reduced order modeling of fluid flows based on the long short-term memory (LSTM) and transformer (Hasegawa *et al.*, 2020a; Hemmasian and Barati Farimani, 2023). In this research, a method that combines Graph Neural Networks (GNNs) with Transformer is used (Han *et al.*, 2022), in the development of a predictive reduced-order models (ROMs) for the transient processes of solid-liquid mixing in stirred tanks. This innovative GNN-Transformer ROM is able to effectively capture the temporal evolution and complex fluid dynamics inherent to multiphase mixing processes. Our model achieves this by leveraging the spatial representation capabilities of GNNs and the long-term dependency modeling strengths of Transformers, resulting in a more accurate and computationally efficient tool for simulating and optimizing stirred tank operations. Preliminary results demonstrate advancements in prediction accuracy and computational speed, offering promising implications for real-time process control and optimization in industrial applications.

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A Novel Digital Tool for Scaling, Optimizing, and Controlling Bioreactors – Rapid Transfer from Lab to Production Floor

Ch. Witz[a,b], P.Eibl[a], J. Khinast[b,c]

[a] *SimVantage GmbH, Inffeldgasse 13, 8010 Graz, Austria*
[b] *Research Center Pharmaceutical Engineering RCPE, Inffeldgasse 13, 8010 Graz, Austria*
[c] *Graz University of Technology, Rechbauerstraße 12, 8010 Graz, Austria*

Keywords: Scale-up, process transfer, bioreactor simulation, lattice-Boltzmann method

Abstract

To ensure optimal design and operation of industrial biofermenters, the underlying critical process parameters such as volumetric mass transfer coefficient, gas-holdup, dissolved oxygen, or sufficient supply of nutrients must be ensured to cater to the specific needs of the microorganisms present in the fermentation broth. These process parameters are highly dependent on the fermenter operating conditions, geometry and scale and can generally not be easily predicted by empirical correlations alone, which is apparent by the vast number of existing correlations and variations of up to orders of magnitude (Yawalkar et al., 2002).

In this work, we present an in-silico approach to predict these critical parameters based on first principles, which utilizes the Lattice Boltzmann Method (LBM) for transient simulations of the liquid phase (Witz et al., 2016). It is based on an in-silico scale-independent simulation method to predict the behavior inside the bioreactor based on first principles up to the largest production scales.

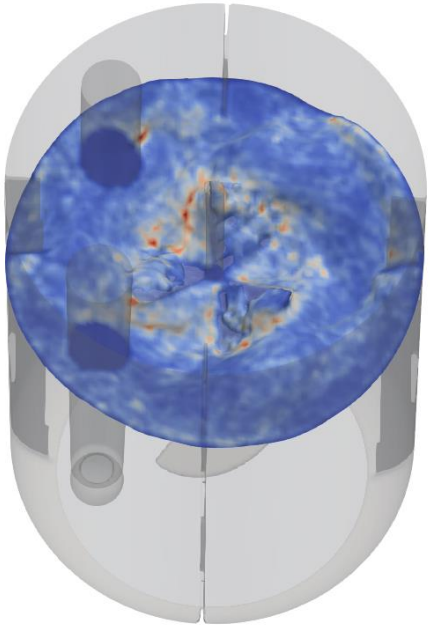

Figure 1: Shear rate on the surface of a commonly used lab reactor

The implementation is optimized for state-of-the-art Graphics Processing Units (GPUs). It includes the motion, breakup, and coalescence of the dispersed gas phase via the Euler-Lagrange method, mass transfer between phases and throughout the fermentation broth, and the movement and distribution of microorganisms. Including the microorganism's movement allows for a detailed analysis of the inhomogeneities within the fermentation broth by tracking the individual organism's lifelines and the environmental conditions of the whole population over time (Haringa et al., 2016).

We will showcase the application of this simulation framework to the optimization of large scale production reactors with 160 m^3 (Bernauer et al., 2022). Additionally, the creation of a scale-down model with single-use lab-scale reactors and a scale-up to a single-use pilot scale reactor is shown. The process is starting with the characterization of the source reactor and then shows the matching of the microclimate of the cells to the target reactors for the case of shear rate. This method's ability to maintain accuracy across scales without re-tuning modeling parameters is a significant advancement in the field. This session aims to provide insights into enhancing the efficiency and effectiveness of industrial biofermentation processes through advanced simulation techniques.

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Scaling up the production of natural compounds in *Streptomyces* using systems biology approaches

S. Brockmann[a], T. Weber[a], C. Licona[b], L. Yang[a]

[a] *Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark*
[b] *FEMSA Biotechnology Center, Tecnologico de Monterrey, 64849 Monterrey, Mexico*

Keywords: Precision Fermentation, Natural Products, Streptomyces, Process Optimization, Process Upscaling

Abstract

Streptomyces contain a rich source of natural products for various applications, including food and agriculture (Jauri et al., 2016). The enzymes needed for synthesis of these products are usually encoded in biosynthetic gene clusters (BGC) that range between 20-250 kb. Industrial production of these compounds is mostly done in native producers using traditional strain-optimization protocols e.g. random mutagenesis and screening. Given the physiological complexity, natural hosts are usually less characterized and difficult to engineer. Thus, it is of great advantage if the target BGC can be expressed in a heterologous Streptomyces host that is well-characterized and easier to manipulate. We are especially interested in producing different classes of natural products (NPs), such as polyketides (PKs) and non-ribosomal peptides relevant for agriculture and sustainability, e.g. Spinosad, a commonly used insecticide.
To bring a target natural compound from lab to biomanufactory, it is critical that a fermentation process can be scaled up. NP producers, such as Streptomyces, shift from primary metabolism to secondary metabolism over the course of fermentation (Jauri et al., 2016). Most valuable NPs including PKs are secondary metabolites produced upon a metabolic switch when cells enter stationary phase. It is still not very clear how the carbon flux is redirected from growth to NP biosynthetic pathways (Wang et al., 2020). Additionally, the choice of the carbon-source can regulate the intracellular production of secondary products, which adds another layer of complexity to optimizing the fermentation process (Sánchez et al., 2010). The aim of this project is to better understand these bottlenecks using systems biology approaches. The production of natural products will be improved using engineering strategies such as dynamic control to develop a robust *Streptomyces* fermentation process that can be scaled up. This will circumvent one of the major bottle necks to heterologous NP production, paving the way for its large-scale synthesis and marketability. This can have a significant impact on the pharmaceutical and agricultural markets as new compounds can be brought to market which are simultaneously more sustainable than existing alternatives.

Various methods will be used in this project. iModulons are groups of co-regulated genes identified using independent component analysis on bacterial transcriptomic data and can provide insights into the complex regulation of gene expression (Rychel et al., 2021). This method will be used to better understand the regulation of the genes responsible for producing natural compounds in *Streptomyces*. Media optimization will be done using a mix of systems biology tools and a design of experiment approach to analyze the best viable conditions for the strain from a production and stress-response perspective. Strain performance tends to increase when fermentation is scaled up due to effects of gradients inside the reactor, product toxicity etc. (Rugbjerg & Sommer, 2019). Therefore, a data-drive scale-up of the fermentation will be used to avoid possible negative effects and to produce a maximum amount of the compound of interest.

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Deciphering the key CAZymes secreted by *Pleurotus floridanus FBCC 469*, cultivated in a liquid-state surface fermentation utilizing an agro-industrial side stream.

Prajeesh Kooloth Valappil[1], Ephraim Akpari[3], Jutta Varis[1], Pekka Oinas[3], Pauliina Lankinen[2], Kristiina S. Hilden[2,4,] Kirsi S. Mikkonen[1,4*]

1 Department of Food and Nutrition, Faculty of Agriculture and Forestry, University of Helsinki, Finland.

2 Department of Microbiology, Faculty of Agriculture and Forestry, University of Helsinki, Finland.

3 Department of Chemical and Metallurgical Engineering, Aalto University, Finland

4 Helsinki Institute of Sustainability Science (HELSUS), University of Helsinki, Finland.

* Corresponding author email address: kirsi.s.mikkonen@helsinki.fi

Edible fungi with the ability to secrete potent enzymes have significant commercial value. As a sustainable food source with rich protein, dietary fibre, and low-fat content as well as their ability to flourish on low-cost agro-industrial waste, Pleurotus attract the attention of both academic and industrial researchers on a global basis. The Carbohydrate-Active enZymes (CAZymes) secreted by this fungus play a pivotal role in carbon recycling. Industries like textiles, food, pulp and paper, biofuels, and pharmaceuticals are always searching for ideal enzymes for their process improvements. The present study aims to identify key CAZymes secreted by *Pleurotus floridanus FBCC 469* in a liquid-state surface fermentation. The LC-MS-based quantitative proteomics analysis identified a total of 1816 differentially expressed proteins. The Carbohydrate-Active enZymes annotation of these proteins was determined using the dbCAN3 meta server by the combined results of HMMER, DIAMOND, and Hotpep tools, as per the CAZy database; which predicted a total of 87 CAZymes, including 45 glycoside hydrolases (GHs), 1 carbohydrate esterase (CE), 9 glycosyl transferases (GTs), 1 polysaccharide lyase (PL), and 28 proteins with auxiliary activities (AAs) in the *P. floridanus FBCC 469* proteome. Significantly, laccase was identified as one of the most highly expressed CAZymes in the proteome. Therefore, a preliminary laccase activity assay was performed, and a high level of activity was measured in the culture liquid. The enzyme produced could be utilized either in its crude form or after purification for diverse commercial applications. The Information gained from the current research study would be useful for understanding the strain from a better perspective.

Key words: CAZymes, Laccase, Pleurotus floridanus FBCC469, LC-MS, proteomics

Enhancing Bioprocess Development Through an Integrated Robotic Minibioreactor Platform: Bridging the Gap Between High-Throughput Screening and Industrial Scale-Up

R. Hassfurther[a], A. Kemmer[a], M.N. Cruz Bournazou[a], P. Neubauer[a]

[a]*Institute of Biotechnology, Chair of Bioprocess Engineering, Technische Universität Berlin, 13355 Berlin, Germany; r.hassfurther@tu-berlin.de*

Keywords: High-throughput experimentation, Automation, Digitalization in Bioprocessing, FAIR data principle

Abstract

The screening process for optimal producers involves numerous combinations of strains and cultivation conditions, making miniaturization essential for conducting these extensive experiments in a cost-effective and practical manner (Long *et al.*, 2014). High-throughput cultivation systems have advanced from simple parallel microtiter plates to sophisticated facilities offering extensive options for monitoring and manipulating cultivation conditions (Hemmerich *et al.*, 2018; Teworte *et al.*, 2022). However, additional challenges must be addressed to better replicate production conditions and reduce the risks associated with scaling up. Industrial bioprocesses are inherently dynamic, and stress factors like induction can cause significant metabolic changes (Lin and Neubauer, 2000). Therefore, careful monitoring and control are crucial to maintaining operations within the desired constraints and ensuring consistent product quality (Dochain, 2003). In miniaturized systems, achieving this is particularly challenging due to limitations in real-time monitoring and the complexity of managing numerous parallel cultivations at very small volumes.

Over the last years, we have developed a sophisticated Integrated Robotic Minibioreactor Platform designed for automated, parallel microbial cultivation that enhances both throughput and data relevance in bioprocess development. This platform enables milliliter-scale parallel cultivations with precise online monitoring and control, closely mimicking industrial bioprocess conditions. By integrating two liquid-handling stations, the system supports automated bioreactor operation alongside at-line analysis, ensuring well-controlled environments and advanced feeding strategies. A central database and application of FAIR data principles facilitate seamless data exchange and fully integrated process control, while a model-based operation algorithm enables the execution of complex cultivation processes, such as scale-down studies and strain characterization, with high accuracy. This approach significantly improves the reliability and transferability of data, bridging the gap between high-throughput experimentation and the demands of industrial-scale processes, ultimately reducing the risk of scale-up failures. Automation plays a crucial role in addressing the reproducibility crisis in science by minimizing human error, standardizing experimental procedures, and ensuring consistent data generation across repeated experiments.

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Mathematical modelling of granulation processes for the manufacturing of biochemical products

Nikolai A. Jessen[a], Ulrich Krühne[a], Krist B. Gernaey[a], Alexander Findeisen[b]

[a]*Department of Chemical and Biochemical Engineering, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark.*

[b] *Novonesis A/S, Smørmosevej 11, 2880 Bagsvaerd, Denmark*

Keywords: Granulation, particle modelling, multiphase modelling, enzyme activity

Granulation processes involve transforming fine powders into larger particles known as granules through agglomeration. These granules serve several purposes including enhancing the uniformity of the final product, increasing the blend's density, allowing it to occupy less volume per unit weight for improved storage and shipment, facilitating volumetric dispensing and reducing dust generation of potentially toxic or hazardous products. Consequently, the ideal characteristics of granules include a spherical shape to enhance flow, a narrow particle size distribution for content uniformity and accurate volumetric dispensing, sufficient fines to fill void spaces between granules, improving compaction and compression properties and adequate moisture and hardness to prevent breakage and dust formation during processing [1], [2]. Despite the transformation of particle technology from an under-funded and widely scattered research endeavor to a thriving globally recognized engineering discipline over the past years, the design and analysis of industrial particulate processes still rely heavily on empirical methods. Granulation processes, in particular, remain one of the least understood and therefore inefficient operations within the process industries [3]. When producing enzymatic products, it is important to maintain adequate control of various process parameters to prevent denaturation of the enzymes in order to maintain a high enzyme activity. The factors influencing enzyme denaturation include temperature, pH, and water content among others [4]. Granulation processes of enzymatic products may therefore risk denaturizing a substantial amount of the enzymes if the process is not properly modelled. New and accurate state-of-the-art models for granulation processes therefore have the potential to greatly contribute to the production of solid enzymatic granules. One way of producing the granules is through the process of spray drying, which has been subject to substantial amount of research in order to optimize and model the mechanisms related to the process. A common modelling approach to the spray drying process is through application of computational fluid dynamics (CFD). Here, the process is modelled as a two-phase flow with the hot air being the continuous phase and the droplets/particles being the discrete phase. Such models have been reported by Salah [5] which used an empirical approach for modelling the drying kinetics of the droplets, and by Hussain et. Al. [6] which also included modelling of the agglomeration and coalescence of the particles within the spray dryer. A second stage in the granulation process may be applied if the characteristics of the particles from the spray dryer are not optimal. Such a process could be a fluidized bed granulator in which the particle size, size distribution and other characteristics may be improved. Modelling of such processes has also been reported utilizing a multiphase

approach within CFD by Börner et. Al. [7]. Another modelling approach involves population balance equations which was reported by Askarishahi et. Al. [8].

This project is a collaboration between The technical University of Denmark (DTU) and Novonesis A/S. The aim of the project is to develop mathematical models that are implemented in DigitalTwin tools to assist in the optimization of a multistage granulation process consisting of spray drying and subsequent fluidized bed granulation which produces enzymatic granules. First principle mechanistic models will be developed which includes all the relevant mechanisms within the process like droplet evaporation, particle collision and particle agglomeration. A hybrid modelling approach may be needed in which the mechanistic models are used in combination with data-based models, in which the data is gathered from various experiments. The main expected results are a state-of-the-art model which can assist in optimizing the multistage granulation process to achieve a high enzyme activity of the granules at low operating costs. Furthermore, a digital platform will be developed, which contains data generated from the model based on multiple different input values. The platform will work as a simplified modelling tool for Novonesis instead of acquiring a license for the more expensive and computer-demanding software that may be needed to run the original model directly.

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Dynamic modelling of microalgae photosynthesis for growth rate predictions under flashing light conditions

A. Pazo Vila[ab], N. H. Norsker[b], K. V. Gernaey[a], J. Kjøbsted Huusom[a]

[a]*Algiecel, Helgavej 26, 5230 Odense, Denmark*

[b] *Department of Chemical and Biochemical Engineering, Technical University of Denmark, Søltofts Plads, 2800 Kgs. Lyngby, Denmark*

Keywords: Microalgae, Modelling, Calvin cycle, Photosystem II, Light – Dark cycles

Abstract

In recent times, microalgae technology has been receiving significant attention due to their promising perspectives in several fields. Daneshvar et al. (2022) compared their potential for carbon capture with other conventional methods, highlighting their efficiency and the valorization of the captured carbon into biomolecules, while Patil et al. (2007) compared different species in terms of their fatty acid composition and total output. However, one of the problems facing microalgae technology is the limited availability of light as cultivations increase in cell density, which in turn hinders biomass concentration and productivity. Nevertheless, results from Nedbal et al. (1996), Xue et al. (2011) and Vejrazka et al. (2011) showed that, when high frequency flashes of light (between 10 Hz to 100 Hz) were used instead of the equivalent continuous illumination, the high intensity pulses caused little to no photoinhibition, and growth rate could be maintained at close to the same value. Several qualitative explanations for this phenomenon, known as light integration, have been proposed by Iluz et al. (2012), based on different physiological mechanisms. It is against this background that we aimed to develop, in this study, a mechanistic model which could describe the most significant physiological mechanisms involved, and also explain and reproduce the empirical observations showing more efficient light utilization and increased photoinhibition tolerance.

For that purpose, and based on the descriptions made by Han et al. (2000) and Yoshimoto et al. (2004) of the photon capture units and of the Calvin cycle respectively, we propose here an integrated dynamic model that describes the interaction between both systems (Figure 1). Our model considers the growth rate of the algae to be proportional to the organic carbon output of the Calvin cycle, whose kinetics are described in terms of the fraction of RuBP molecules active in the cycle, and of the available ATP. The production of ATP is proportional to the quantum yield of the photosynthesis, which depends on the turnover rate of the electron transfer chain. The capacity of the photon capture units to initiate the transfer chain is limited by the state of the D1 macromolecule, in the reaction center of the photosystem II complexes. This is here modeled as either functional or inactive, where the transfer between both states is dependent on the restoration and photodamage kinetics.

The model, which can be applied both to cultivations with continuous illuminations and under brief light flashes, has successfully predicted the expected behaviors, such as I) growth rate decline at high light intensities due to photoinhibition, II) more efficient light utilization by the algae under light and dark cycles, and III) growth enhancement as the frequency of the light and dark cycles increases from 0.1 Hz to 10 Hz.

Our model now opens the door to the utilization of CFD-generated cell streamlines to evaluate how the light and dark patterns induced by the flow impact the overall performance of the reactor. As far as known, the integration of this type of data into complex dynamic growth models has not been reported in the literature. However, it is actually in these scenarios (Richmond, 2004) where the main benefits of light integration would be observed. If an efficient circulation of the liquid in the reactor was achieved, so that all cells experience light flashes regularly as they move closer and then away from the light source, the whole volume of the reactor would become productive. This has the potential to greatly improve the throughput of photobioreactors, particularly of those that operate high cell density systems.

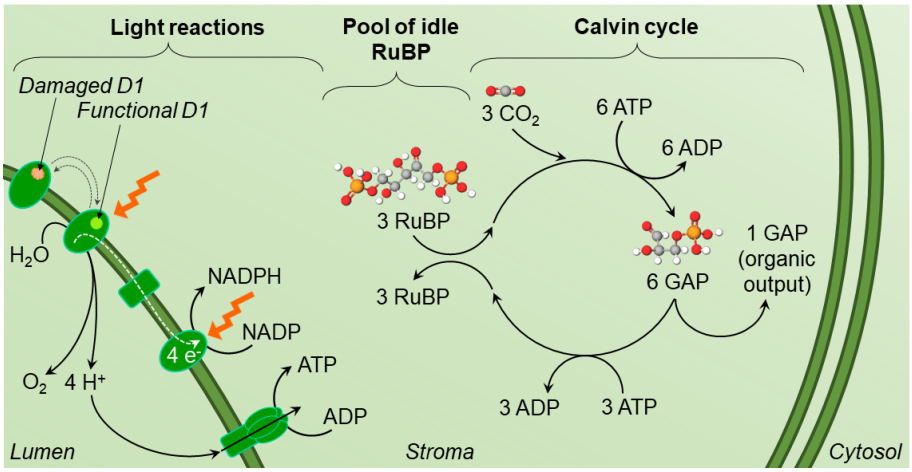

***Figure 1**: Graphical description of the two systems described by the model, the light reactions and the Calvin cycle.*

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