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Scaling up advanced biofuels and biochemical production

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Zusammenfassung

In der biotechnologischen Produktion von Kraftstoffen und Chemikalien ist die effiziente Produktabtrennung nach wie vor in vielen Fällen technisch problematisch und auch mit erheblichen Kosten verbunden. Öl-artige Verbindungen haben die Tendenz zu Emulsionen zu führen, was energieintensive Zentrifugation oder den Einsatz emulsionstrennender Zusätze zur Folge hat. Zusätzliche Unit Operations zur Phasentrennung können beispielsweise durch den Einsatz der sogenannten *in situ* Produktabtrennung (ISPR) vermieden werden. Ziel des vorliegenden Projektes war Scale-up Modelle der neuen kosten-effizienten ISPR-Technologie *Fermentation Acceleration by Separation Technology* (FAST) zu entwickeln und im Pilotmassstab für die fermentative Produktion von Produkten wie Biokraftstoffen oder Chemikalien zu demonstrieren. Das einfache Prinzip der Technologie könnte deutlich niedrigere Investitionskosten als derzeit etablierte Verfahren ermöglichen. Insgesamt könnte dies zu einer Verringerung der Kosten und des Energieverbrauchs für die Bereiche Biokraftstoffe und Chemikalien führen.

Das vorliegende Projekt hat sich auf die Anwendung der FAST 100 Technologie zur mikrobiellen Produktion von Sesquiterpenen und von Biobutanol konzentriert. Sesquiterpene, Verbindungen die insbesondere in der Aromen- und Geruchsstoffindustrie Anwendung finden, werden herkömmlicherweise aus natürlichen Ressourcen gewonnen. Der Projektpartner Firmenich hat in der mikrobiellen Produktion von Sesquiterpenen jahrelange Erfahrung. Über die letzten 30 Jahre, hat Bio-butanol wachsendes Interesse für verschiedene Anwendungen auf sich gezogen. Als Kraftstoff weist Butanol beispielsweise bessere Eigenschaften als Ethanol auf und findet auch in der chemischen Industrie breite Anwendung als Lösungsmittel. Bio-butanol kann fermentativ nachhaltig hergestellt werden. Sowohl in der Produktion von Sesquiterpenen, als auch in der Produktion von Bio-butanol spielt Produkttoxizität/-inhibition eine wichtige Rolle. Die FAST 100 Technologie könnte dies über *in situ* Produktabtrennung verbessern.

Während der Projektlaufzeit wurden die Zielsetzungen und Milestones erfolgreich bearbeitet. Verschieden bakterielle Stämme für die fermentative Produktion von Terpenen wurden konstruiert und in verschiedenen Massstäben getestet, ebenfalls mit der FAST-Technologie. Es musste leider festgestallt werden, dass der hohe Sauerstoffbedarf der terpenproduzierenden Stämme nicht mit der operationellen Breite des FAST 100 Systems kompatibel ist. Die Produktion von Bio-butanol mit dem FAST 100 System konnte jedoch erfolgreich durchgeführt werden. Es wurden ausserdem Energie- und Treibhausgasaspekte für verschiedene Szenarien der anaeroben Butanolproduktion evaluiert. Die experimentellen Ergebnisse und die techno-ökonomische Evaluierung zeigen das Potenzial der getesteten Technologie auf.

Résumé

Dans les procédés biotechnologiques de production de carburants ou de molécules chimiques, l’étape de séparation reste une étape difficile et souvent couteuse. Les produits huileux ont tendance à former des émulsions ce qui nécessite l’addition de produits désémulsifiants. L'utilisation de techniques de séparation in-situ des produits (ISPR) permettrait d'éviter l’utilisation de certaines unités de séparations additionnelles. L'objectif de ce projet était le développement de modèles à grandes échelle et de valider à l'échelle pilote une nouvelle méthode rentable de séparation in-situ, dénommée Fermentation Acceleration by Separation Technology (FAST), des produits de fermentations. La simplicité du concept pourrait permettre des économies d'investissement significatives par rapport aux installations classiques et d’une manière générale une réduction des coûts et de la consommation en énergie dans le secteur de la production de biocarburants et de produits chimiques.

Ce projet s'intéresse à l'implémentation de la technologie FAST 100 à des procédés de production microbienne de sesquiterpènes et bio-butanol. Les sesquiterpènes sont des molécules généralement isolées de ressources naturelles et ont une grande importance dans l'industrie des arômes et parfums. L'entreprise Firmenich, partenaire du projet, possède de nombreuses années d'expérience dans la production microbienne de sesquiterpènes. Au cours des 30 dernières années, le bio-butanol a suscité un intérêt croissant pour diverses applications telles que les carburant, le butanol ayant de meilleures propriétés que l'éthanol, ou comme solvant dans l'industrie chimique. Le bio-butanol est généralement produit de manière durable par fermentation. La toxicité des sesquiterpènes et du butanol sont des facteurs importants affectant leur production par fermentation. La technologie FAST 100 pourrait permettre d'aborder ces problèmes de toxicité en implémentant des solutions de séparation in situ.
Au cours du projet, tous les aspects et objectifs ont été adressés avec succès. Plusieurs lignées de bactéries modifiées pour la production de terpènes ont été testées à différentes échelles en incluant la technologie FAST. Nous avons malheureusement constaté que la forte demande en oxygène des souches productrices de terpènes n'était pas compatible avec la gamme opérationnelle du système FAST. Au contraire, la production de bio-butanol a été réalisée avec succès avec le système FAST. De plus, pour la production de butanol en condition anaérobique, les besoins en énergie et la formation de gaz à effet de serre ont été estimées pour différents scénarios. Les données expérimentales ainsi que les évaluations technico-économiques mettent en évidence les avantages que peut apporter la technologie FAST 100 à l'industrie.

Summary

Product separation in the biotechnological production of fuels and chemicals is still technologically problematic and costly. Oil type products have the tendency to emulsify and require energy-intensive centrifugation or the need to add demulsifying chemicals. Extra separation unit operations could be (at least partially) avoided when using *in-situ* product removal (ISPR) technology. The overall objective of the presented project was to develop scale-up models and to demonstrate a novel cost-effective *in situ* recovery technology, Fermentation Acceleration by Separation Technology (FAST) at pilot scale for fermentation derived products such as advanced biofuels or chemicals. The simplicity of the concept could lead to significantly lower investments for production plants compared to currently used installations. Overall, this would lead to cost and energy savings in the biofuels, chemicals and related sectors.

The presented project focused on the application of FAST 100 technology to microbial sesquiterpene and bio-butanol production processes. Sesquiterpenes are important compounds for the flavour and fragrance industry that are usually isolated from natural resources. Firmenich has more than a decade of expertise on the microbial production of sesquiterpenes. In the last 30 years, bio-butanol has attracted interest among a wide range of industries; it shows better properties for fuels than ethanol while it is also applied as common solvent in the chemical industry. Bio-butanol is usually obtained by fermentation which contributes to more sustainable manufacturing. For both, sesquiterpenes and bio-butanol, the biological production suffers from product toxicity. FAST 100 technology might tackle this issue by overcoming toxicity limitations with *in situ* product removal.

During the course of the project all the goals and milestones were successfully addressed. Different bacterial strains lineages for the production of terpene molecules were generated and tested at different scales and including the FAST technology. It was identified that the oxygen demand for the tested production of terpenes was not compatible with the operational ranges of oxygen transfer of the FAST

100 system. On the opposite, successful production of bio-butanol was achieved in the FAST 100 system. In addition, energy and Total Green House Gases (GHG) aspects were evaluated for different scenarios of anaerobic butanol production. The experimental results obtained, together with the techno-economical evaluation, is a good showcase for industry to support the benefit of the FAST 100 system.

Main findings

Different bacterial strains lineages for the production of terpene molecules were generated and tested. Although initial challenges related to titers, productivity and genetic stability were faced; strains that exceed the targeted titers (>10 g L^{-1}) and productivity (2 $mg_{product}$ g_{cells}^{-1} h^{-1}) were obtained. In addition, the bacterial strains were tested in the *in situ* product recovery system FAST 100. The recovery of the product was higher than 80% and the calculated rate of recovery was above of 5 g L^{-1} h^{-1}.

It was identified that the oxygen demand, kLa > 800 h^{-1}, for the tested production of terpenes was higher than standard continuous stirred tank reactor at scale (300 and 500 h^{-1}) and not compatible with the operational ranges of oxygen transfer in the FAST 100 system.

The developed rate-based model of the system allowed scale-up calculations and was able to perform process evaluation. An identified limitation in the model was the individual oil droplet kinetics to obtain the oil split.

A successful fermentation was conducted in the FAST 100 system for the production of bio-butanol. Energy and Total Green House Gases (GHG) aspects were evaluated for different proposed scenarios. The effective productivity increase in the different scenarios corresponded to an overall better use of subsequent DSP operations, significantly lowering the operational utility requirements. Overall GHG equivalents for the main operational utilities can be significantly reduced by a factor of ~ 3 (28 % compared to 100 % in the reference case). This demonstrated that the use of FAST 100 technology can significantly lower the process energy and main utility requirements.

Contents

Abbreviations

DAB	Delft Advanced Biorenewables
TUD	Technical University of Delft
BPF	Bioprocess Pilot Facility
WP	Work Package
FAST	Fermentation Acceleration by Separation Technology
GEOR	Gas Enhanced Oil Recovery
CDW	Cell Dry Weight
DO	Dissolved Oxygen
OD600	Optical Density measured at wavelength 600 nm
DSP	Downstream Processing
LCA	Life cycle analysis
BuOH	Butanol
ABE	Acetone-Butanol-Ethanol
GHG	Greenhouse gas(es)
ISPR	In-situ product removal
F&F	Food and flavour
IPP	Isopentenyl pyrophosphate
DMAPP	Dimethylallyl pyrophosphate
FPP	Farnesyl pyrophosphate
MVA	Mevalonate pathway
CSTR	Continuous stirred tank reactor
CAPEX	Capital expenditure
OPEX	Operating expense

1 Introduction

Currently, product separation in the biotechnological production of fuels and (fine) chemicals is still technologically challenging. It requires, generally, costly and energy-intensive process steps which include addition of demulsifiers, the need of co-solvents for extraction, processing aids and separation processes such as centrifugation and distillation. These unit operations could be (at least partially) avoided with the hereby proposed in situ product recovery technology.

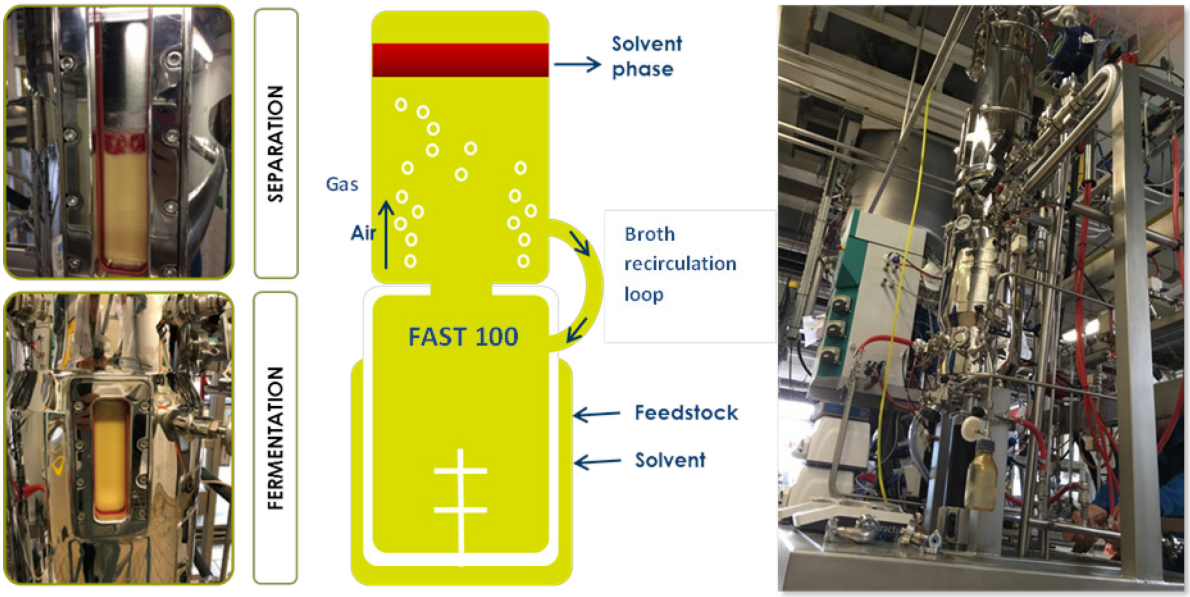

Figure 1. Schematic overview of the FAST 100 reactor. As is visible the FAST 100 reactor consists of two compartments. At the bottom a fermentation compartment is situated with above the separation compartment. Both compartments are connected and broth is recirculated via an external loop. The feedstock and solvent is dosed via the fermentation compartment and the solvent phase is harvested via the separation compartment.

The integrated reactor system, FAST (Fermentation Acceleration by Separation Technology), developed by Delft Advanced Biorenewables (DAB) and the Technical University of Delft (TUD), facilitates multi-phase cultivations and phase separation within a single equipment. The in situ product recovery is enabled by liquid-liquid extraction. The FAST 100 reactor (Figure 1) has a separation zone in the top section of the reactor and its fermentative section at the bottom of the reactor. Both compartments are connected and broth is recirculated via an external loop. The feedstock and solvent is dosed via the fermentation compartment and the solvent phase is harvested via the separation compartment. This technology shows several potential advantages: it can be operated in a continuous mode, it increases the productivity of product inhibited processes (due to toxicity); and it decreases downstream capital costs. Especially for product inhibited fermentations, this approach can allow significant increases in fermentation duration and overall fermentation productivity. Overall, this would lead to cost and energy savings in the biofuels, chemicals and related sectors.

The presented project focused on the application of FAST technology to microbial sesquiterpene and bio-butanol production processes. Sesquiterpenes are important compounds for the flavour and fragrance industry that are usually isolated from natural resources. Firmenich, with more than a decade of expertise on the microbial production of sesquiterpenes, routinely elucidates biosynthetic pathways in plants and microorganisms leading to various terpene hydrocarbons and engineered microorganisms

for the in vivo production of these interesting molecules by heterologous expression of the pathway enzymes **[1]**. Many members of the sesquiterpene family of molecules show certain level of toxicity which can render the production process inefficient.

In the last 30 years, bio-butanol has attracted interest among a wide range of industries; it shows better properties for fuels than ethanol while it is also applied as common solvent in the chemical industry **[2]**. Bio-butanol is usually obtained by fermentation which contributes to more sustainable manufacturing. It is produced together with acetone and ethanol by the microorganism. However, the biological production suffers from low productivity because of butanol toxicity at aqueous concentrations above 10 g L^{-1}. DAB & TUD technology might tackle the productivity issue by overcoming toxicity limitations with in situ product removal.

The overall project objective was to develop scale-up models and to demonstrate a novel cost-effective *in situ* recovery technology at pilot scale for fermentation derived products such as advanced biofuels or chemicals. Our specific objectives were (i) to develop a robust strain with a reproducible protocol for the production of a representative compound (Firmenich, DAB); (ii) to predict the performance of the fermentation and product recovery process of an integrated multiphase separation in an up-scaled fermenter (TUD, DAB); (iii) to demonstrate the in situ recovery technology on pilot scale for fermentation of a representative F&F intermediate that also has fuel-like physical-chemical properties (BPF, DAB, Firmenich); (iv) techno-economical evaluation of the integrated bioreactor concept for biofuel and F&F ingredient manufacturing (DAB, SkyNRG, Firmenich).

This report covers the results of the project that was part of an ERA-NET Bioenergy call with Dutch partners DAB, TUD, BPF and SkyNRG; and the Swiss partner Firmenich.

2 Work packages and Milestones

The reported project is part of an ERA-NET Bioenergy project funded by 3 national administration offices. The ERA-NET project runs parallel with another long term funded project by the Dutch government. An overview of the Work Packages (WP) structures is depicted in Figure 2.

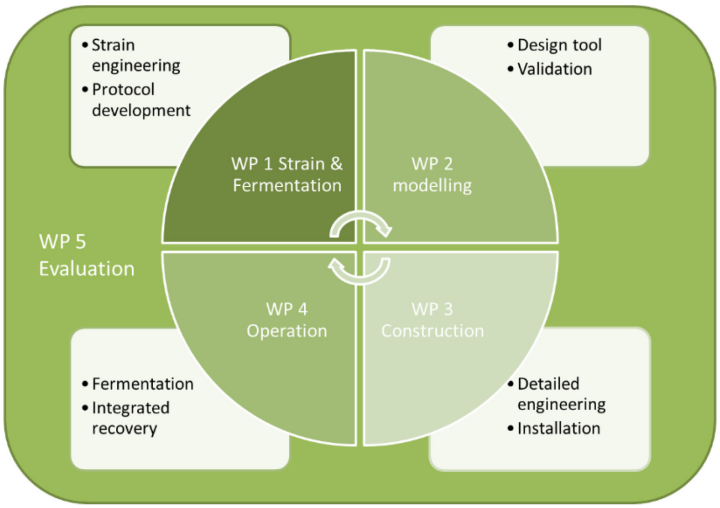

Figure 2. Defined work packages of the integrated projects to develop, demonstrate, model and upscale the intensified bioreactor for cost effective biofuel and chemical production.

The structure included:

- WP 1 Strain engineering & Fermentation development: To develop a robust strain with a reproducible fermentation protocol.
- WP 2 Equipment modelling: To develop a design approach for the equipment to be linked to overall process modelling.
- WP 3 Design and construction: Detail design, construction, installation and testing of proposed system in the existing fermentation pilot facility.
- WP 4 Up-scaled process demonstration: Demonstration of integrated technology on pilot scale.
- WP 5 Technology evaluation and exploitation: To develop a model approach to evaluate the technology.

In order to achieve the above mentioned goals, the following milestones were set:

1) First engineered strain (>10 g L^{-1} final titer and genetically stable).
2) The basic of design delivered by TUD to BPF.
3) Detailed engineering design for construction pilot reactor; Go/No-Go decision point.
4) Optimized protocol delivered to the BPF for fermentation runs (productivity of ≥ 2.0 $mg_{product}$ g_{cells} h^{-1} in lab-scale fermentations).
5) Pilot reactor ready for test run in WP4.
6) Start operating reactor & protocol adjustments; crude product analysed by Firmenich (Validation of model; product quality OK).
7) DAB validates model platform.
8) Validation of the integrated bioreactor on pilot scale (recovery ≥ 80%; recovery rate 5 g L^{-1} h^{-1}). Completion of environmental impact analysis.
9) Dissemination of the pilot work (2 conferences; 3 publications).
10) Yearly technology evaluation (report to BFE).

3 Strain development for terpene production

Firmenich R&D team has expertise in transferring newly discovered biosynthetic pathways from natural environments into tamed microbes aiming for the production of interesting molecules in a stable and sustainable manner **[1]**. Two molecules, members of the sesquiterpene family of natural compounds, were of interest at the moment of this project. These molecules are termed Terpene1 and Terpene2 throughout this report. Both sesquiterpenes have similar physico-chemical properties and it was therefore expected to behave similarly in the recovery process development.

For microbial production of sesquiterpenes, two main obstacles were identified. On the one hand, the enzymes (sesquiterpenes synthases) should have enough activity to efficiently convert the precursor (Farnesyl pyrophosphate, FPP) into the desired molecule, and, on the other hand, enough supply of FPP should be ensured for the sesquiterpene synthase to work properly. FPP supply was ensured by the heterologous expression of the mevalonate (MVA) pathway, present naturally in several organisms.

Two strategies for heterologous expression of MVA were put in place; these strategies were evaluated sequentially during the course of this project. In the first strategy, a MVA pathway was assembled and

expressed in bacteria using a plasmid system. A second strategy, involved the genomic integration of a MVA pathway in a bacterial chassis.

The production of the two selected terpenes in the engineered bacterial strains was guided by the expression of specific terpene synthases. Each synthase was expressed in plasmid systems where gene expression was controlled by a set of different regulatory sequences (promoters and terminators). Firmenich's expertise allowed for the evaluation and optimization of the plasmid system for synthase expression.

Four strain lineages were constructed and tested in cultivation for the production of the interesting sesquiterpenes. Cultivations were carried out using standard conditions and media at different scales (ranging from 2 mL to 2 L). The characteristics of each strain lineage are described in Table 1.

Table 1. Main characteristics of four strain lineages constructed in the framework of this project.

Lineage name	MVA expression system	Sesquiterpene synthase	Synthase expression system
BipTE1	Plasmid	Terpene1 synthase	Plasmid
IntTE1	Integration	Terpene1 synthase	Plasmid
BipTE2	Plasmid	Terpene2 synthase	Plasmid
IntTE2	Integration	Terpene2 synthase	Plasmid

Lineage BipTE1 delivered peak titers of 418 ± 88 mg L^{-1} (n=2) of Terpene1 after 48 h of cultivation in 2 mL of media. In addition, fed-batch cultivations in 2 L reactors were performed. In defined media with oxygen-based controlled glycerol feeding, the titer reached a maximum closed to the targeted titer of 10 g L^{-1} in approximately 4 days. Maximum cell densities of >273 OD600 (equivalent to ~100 g L^{-1} dry cell weight) were achieved (Figure 3).

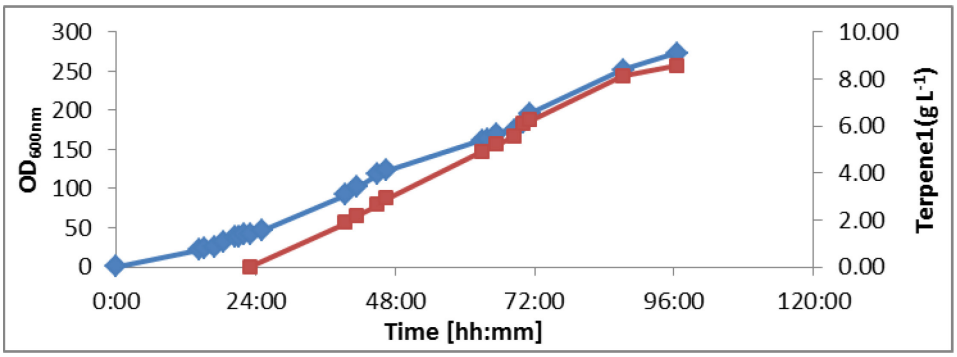

Figure 3. High cell density fermentation of engineered *E. coli* strains from BipTE1 lineage, co-expressing a Terpen1 synthase. Time courses of Terpene1 accumulation (red) and cell growth (blue) are shown.

Two main drawbacks were observed with the BipTE1 lineage. First, a set target titer of 10 g L^{-1} was not achieved and second, the genetically stability of the bacterial lineage was not optimal, plasmid loss was systematically observed. With the aim to improve genetic and culture stability, the lineage IntTE1 was constructed. Functional characterization of strains similar to IntTE1, using a set of selected terpene synthases, showed greater genetic stability resulting in longer cultivation time, higher cell density and higher terpene peak titer than the corresponding bi-plasmid based strains (~10 g L^{-1}) (Data not shown).

Surprisingly, similarly to BipTE1 lineage, the obtained Terpene1 titer was in the order of ~400 mg L^{-1}, indicating that the limiting step for the production is the synthase rather than the bacterial strain. Since

the optimization of the synthase would likely require protein engineering, a rather risky and time consuming task, the production of this terpene was discontinued.

Two extra lineages, BipTE2 and IntTE2, were developed and tested. Under optimized conditions, the IntTE2 lineage reached higher titers than previously observed in a cultivation volume of 2 mL, a 3-fold improvement over the BipTE2 lineage (Figure 4). Based on these results, it was expected that this strain could reach fermentation titers higher than the milestone of 10 g L^{-1}.

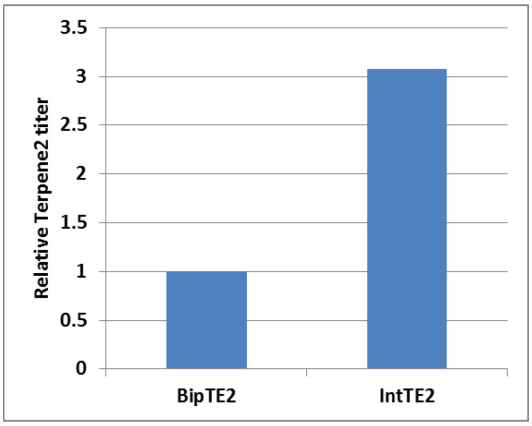

Figure 4. A Terpene2 synthase was expressed in the plasmid-based and integrated strain backgrounds, lineages BipTE2 and IntTE2 respectively. Cultures were performed in 2 mL volume. Shown is the relative Terpene2 titer after 48 h growth.

Additionally, the construction of a yeast strain capable to produce large quantities of sesquiterpenes was evaluated as alternative host in the frame of this project (data not shown).

4 Fermentation protocol transfer and implementation

Firmenich's microbial cultivation protocol was adapted and up-scaled to the 20 L reactor system and the 100 L integrated pilot reactor, FAST 100, by DAB. Fermentations with lineages BipTE2 and IntTE2 showed properties that might cause limitations in the further testing of the FAST process. Interestingly, high-density fermentations using an organic solvent generated creamy emulsions that are not suitable for the operation window of the gas enhanced oil recovery (GEOR) method.

In order to overcome this challenge, the more stable and high-producing lineage IntTE2 was used for further development in cultivations aiming to achieve low cell densities (<25 gCDW kg^{-1}). These fermentation runs were termed F050 (-A and -D) and F052. In addition, several protocol changes were implemented mainly to decrease the emulsion stability, namely, the pH set point (decreased), timing of addition of second phase (dodecane) and temperature after induction. With this strategy, the offline recovery experiments still did not result in the recovery of clear oil.

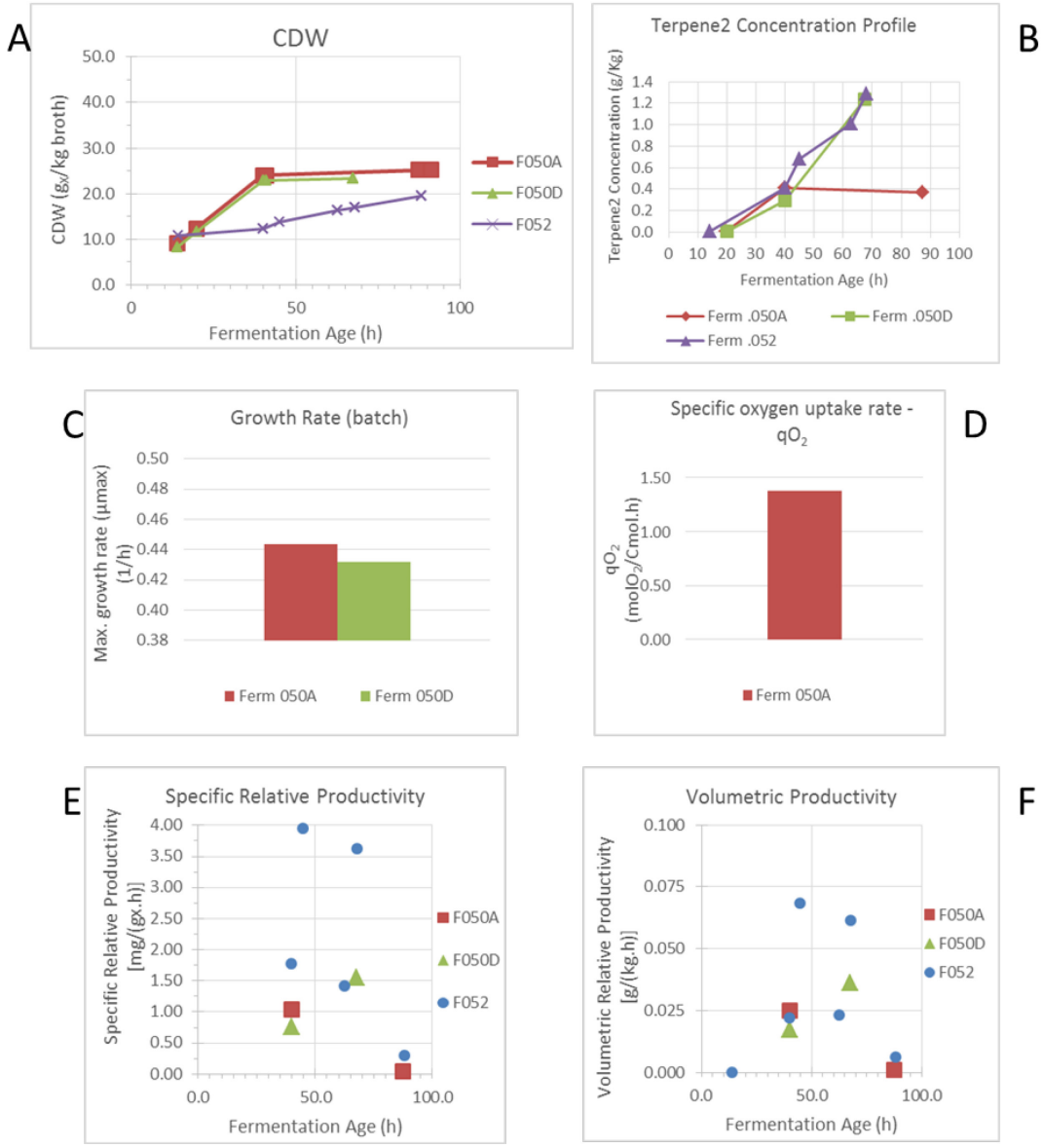

Figure 4. Biomass (A) and product (B) concentration profiles, overview of maximum kinetic parameters (C and D) and Productivity (E and F) obtained in fermentations F050 and F052.

As intended, the biomass concentrations obtained varied from 5 to 25 gCDW kg^{-1}. Kinetics parameters such as maximum biomass growth rate (µmax) and maximum oxygen specific uptake rate (qO2) were derived from online and offline measurements performed during the batch phase. The amount of product obtained per kilogram of broth variated between fermentations, according to strains, fermentation settings, control strategy followed, and bioreactor systems used. The highest volumetric productivity registered in the 20 L system was obtained in fermentation F052, at fermentation age of 45 h (ca. 16 h after induction) – 0.07 g kg^{-1} h^{-1}, matching a specific relative productivity of 4.0 mg g_X^{-1} h^{-1}. A summary of the results can be seen in Figure 4. Given the observed oxygen limitations in batch phase with the producing strains, there are evidences that these strains have a high oxygen demand.

5 Feasibility evaluation of the production of terpenes with FAST 100

In cooperation with Firmenich, DAB developed a Terpene2 production process in the FAST 100. Additionally, an economic evaluation was performed. It considers only the integrated reactor itself but not CAPEX or DSP costs. Different assumptions were made for the computational model; first, it was assumed that all cells have the ability to produce Terpene2 at the same rate; second, the model input values were obtained experimentally, except for the physical properties such as densities, interfacial density and vapor pressure which were acquired from literature. A summary of the cultivation runs is given in Table 2.

Table 2. Summary of the experiments in the pilot reactor FAST 100

	DBF 18.001	DBF 18.003	DBF 18.004	DED 18.001
Product harvest [kg·h^{-1}]	0.002	0.003	0.00006	0.0005
Product harvest [kg·a^{-1}]	16.16	20.53	0.45	3.79
Solvent harvest [kg·a^{-1}]	190.35	1,607.05	101.22	3,065.49
Solvent feed [kg·a^{-1}]	1,796.32	1,680.00	1,707.04	3,744.00
Org. phase recovery [%]	26.8	95.2	5.9	81.9
Clear oil recovered	Yes	No	No	No
Solvent used	Dodecane	Castor oil	Castor oil	Castor oil

During the course of the project, two different organic solvents were used, dodecane and castor oil. While the recovery using dodecane was only 26%, the recovery was 80-95% when castor oil was used. This difference is linked to the droplet diameter. The results are in line with known physical behavior of dispersed droplets that bigger droplet diameters have higher buoyancy driving force and will therefor allow faster and more phase separation and recovery.

In the tested system, the product should be harvested as a component of a “clear oil” composed of organic solvent and Terpene2 floating on top of the aqueous phase in the separation compartment. Although the recovery of the organic phase was larger when castor oil was used, the experiments with this organic solvent lead to a creamy phase that contained small amounts of aqueous phase and biomass. The extra components in the recovered phase resulted in the addition of extra steps for DSP.

Unexpectedly, Terpene2 titers lower than the set 10 g L^{-1} were measured in the fermentation compartment. Unfortunately, the product yield YP/S was about 0.06 kg kg^{-1}. Despite the different attempts to increase titers and yield by process development, they did not resulted in a dramatic increase.

The FAST 100 has a fixed cross sectional separation area [m^2] and has been shown to be able to recover organic phase/product phase [L] in a given time [h] at the rates listed in Table 3. In accordance to the targeted percentage of product’s phase recovery and rate, the listed rates can be expressed/interpolated. On total organic fraction [g] per reactor volume [L] per time [h] it would be 41.25 g organic phase per total reactor volume [L] per time [h]. Highest obtained (DBF18001) Terpene2 concentration in organic phase has been 150 g.kg^{-1} (a fraction of 0.150 calculated from the maximum theoretical separation capacity). Therefore, the listed recovery can either be seen as 41 (total organic phase) g.L.h^{-1} or 6.1 (Terpene2) g.L.h^{-1}.

(Auxiliary) organic phase and/or product separation efficiency is achievable under the correct combination of fermentation protocol, organic phase and operational conditions of the FAST-100 reactor. The optimal phase separation achieved has been high and close to the model predicted theoretical maximum values.

Table 3. Key performance indicators as set in the project proposal (FAST 100 reactor).

KPI's	FAST 100 target	Achieved max.
Recovery efficiency of oil phase	80%	96.5%
Rate oil phase recovery	8.8 $L \cdot m^{-2} \cdot h^{-1}$	165 $L \cdot m^{-2} \cdot h^{-1}$

In addition, it was noticed that production of Terpene2 was a high oxygen demand process. An oxygen limitation was encountered in the recirculation compartment, preventing the bacteria to utilize oxygen until they reach the fermentation compartment again. Although this challenge could be addressed by increasing the air supply or concentration of oxygen provided, other problems such as foaming and security concerns prevented their implementation. Unfortunately, the oxygen demand by the bacteria could not be optimally achieved within the constraints of the proposed system.

The experiments were also used to calculate potential economics for Terpene2 production and identify important cost factors. The economic evaluation of different scenarios considers only the integrated reactor itself but not CAPEX or DSP costs. The low yield resulted in high raw material cost contribution. Other elements such as utilities and waste disposal play a minor role in the economics.

The production of Terpene2 was demonstrated and the continuous in situ product removal method is able to extract the product and recover the majority of the organic phase in the separation compartment of the reactor. Since the operational window of the in situ product removal and optimal conditions for productivity of the microorganism do not overlap due to the high oxygen demand of the terpene producing strain, it was decided to turn to a different microbial production system with lower oxygen demand.

6 Model set-up description

In order to allow scale-up and flowsheet calculations with aqueous product concentration as main input variable, an integrated rate based reactor model was developed. Its main goal is to be able to describe the experimentally obtained separation capacity/oil phase recovery of the integrated reactor. A mathematical model tool comprised of several models, briefly explained below, was developed, with the integrated reactor model being the key feature.

Inoculation train model.

The inoculation model determines the amount of seed tanks required, the final biomass concentration and the consumption of substrate. The calculations are based on yield coefficients (product/substrate) of and the maximum growth rate of Firmenich's terpene producing strains. It is assumed that the cells grow without inhibition and lag phase operated in a batch.

Integrated reactor model.

The integrated reactor model focuses on the continuous (pseudo) steady state production phase of the cultivation. The model builds on scale dependent physical mass transfers and phase dependent properties as well as kinetic fermentation information. The model gives a process solution under steady-state for given input variables that might be feasible or not. In order to make it flexible, different choices can be set by the user: 1) Aerobic or anaerobic, 2) Bubble column or stirred tank reactor, 3) Blending or extraction, and 4) Experimental microbial q-rates or stoichiometric reaction data.

In order to use the model, the user needs to know the dimensions of the reactor, microorganism specific consumption/production rates, the partitioning of solvent/product in the aqueous and organic phase and an estimate for droplet diameters. The remaining input variables can be obtained by estimations or literature data. The outputs of the model are a harvest rate, product and solvent concentration in the harvest, limitations in extraction or aeration, recirculation flow and limitations in aeration or product inhibition.

Purification.

The model focusses on the following points of the process: 1) Compression of the aeration gas, 2) Condensation of the off-gas, 3) Centrifugation of the harvest stream of the integrated reactor, and 4) Distillation of product and solvent. The outputs of the model are utility consumption such as heating and cooling, and sizing of certain equipment.

Model results and validation with experimental data.

The model was validated with experimental results (Table 4) with focus on the oil phase separation and recovery and gas-phase hold-up in the riser section of the reactor. The model is able to describe gas hold-up and oil separation adequately.

The comparison of the experimental oil split and liquid split is difficult because it is not measured during the experiment. The detail on individual oil droplet behaviour and specific kinetics is limited, but can be improved further. The oil split can be estimated indirectly from the oil concentrations in each compartment. The gas holdup of the riser can be determined with the measured gas outlet flow and the density of air.

The development of the integrated reactor model took roughly 6 months including performing experiments and literature research. After the model validation and the parameter fitting, the model can be applied for scaling up systems and new potential applications. Currently, the process assessment can be reduced to 2 to 3 weeks. This assumes that the new unknown process fulfils the assumptions of the models, e.g. continuous steady state process, product extractable in a lighter second phase and that microbial q-rates are given.

Table 4. Experimental and model result comparison

	DBF 18.001	Model 18.001	DBF 18.003	Model 18.003	DBF 18.004	Model 18.004	DED 18.001	Model DED 18.001
Oil split into sep.	0.641	0.982	0.965	0.987	0.934	0.994	0.873	0.997
Harvest rate [$kg \cdot h^{-1}$]	0.023	0.023	0.198	0.197	0.0125	0.0125	0.379	0.380
$x_{in,oil,sep}$	0.132	0.132	0.290	0.290	0.151	0.151	0.217	0.216
Gas holdup riser	0.100	0.099	0.065	0.067	0.084	0.088	0.057	0.059
K_f	-	55.6	-	41.15	-	30.8	-	49.4

Discussion organic phase separation and overall performance.

The campaign of experiments (DBF 18.001 to DED.18.005) explored a wide range of fermentative systems for sesquiterpene production. DAB's novel reactor (FAST 100) system was operated as fermentation vessel with integrated separation. The FAST 100 reactor system is able to (dependant on fermentation broth and organic oil phase interaction) separate substantial amounts of organic liquid phase for all the presented scenarios. The FAST 100 has therefor showed the applicability of extractive fermentation systems for sesquiterpene production.

In the FAST 100 system, the maximum (auxiliary) organic phase separation achieved was 165 L.day-1 (on a ~70 L total liquid). The highest product titre of sesquiterpene (DBF.18.001) was 150 g.kg-1 in dodecane. This was the case when using a minimum solvent dosing strategy as product formation rate was the limiting factor for our production system. The organic phase handling and separation capacity of the reactor exceeded its extractive organic phase capacity for the used strain.

Different solvent were applied to the system such as dodecane, castor oil or the full food grade organic phase like soya oil. All can be used in extractive based sesquiterpene fermentations. The organic phases showed different interactions with the extracellular components present in the broth and had various degrees of phase separation, leading to difference in clear oil and organic phase cream-like fractions in the recovered organic phase.

The final experiment (DED.18.005) showed stable continuous production of sesquiterpene coupled with continuous auxiliary phase removal. The production titre was limited by oxygen transfer limitations, which are a direct result of the scale-up of laboratory systems to large scale fermentation vessels. The current strain's oxygen demand was not within the operational range of the system. Nevertheless the potential of ongoing fermentation coupled to continuous auxiliary phase removal has been shown.

7 Microbial production of Bio-butanol in FAST 100

Since the oxygen demand of terpene production was not in the operational range of the reactor system tested here, DAB scouted other production processes that were suitable for the FAST system. The selection was made for anaerobic ABE fermentation to produce bio-butanol. A batch followed by continuous regime ABE fermentation in the FAST 100 system was performed with *C. beijerinckii*). Bio-butanol is currently considered as a potential alternative for bio-fuel. Bio-butanol has higher energy content than ethanol and existing infrastructure can be used for the distribution. Butanol can be used directly as blend-in fuel or upgraded to jet fuel **[3]**.

After initial piloting tests to check emulsion stability and microbial behaviour (data not shown), the solvent selected with the highest potential for an in-situ product removal reactor FAST 100 ABE-fermentation was oleyl alcohol. The parameters of the FAST 100 ABE-fermentation run (DED.18.004) are summarized in Table 4.

Table 4. DED.18.004 operational conditions.

Parameters		FAST 100
Inoculation train		2 transfers of 24 h
Process time	Days	5
Temperature	°C	37
Vessel pressure	Bar	0.2
pH set-point after 18h		Cascade on from pH 5.3
pH control		Only base

Parameters		FAST 100
Solvent		Oleyl alcohol
DO set-point	%	0
Stirring	RPM	Off<100
Nitrogen flow ferm. compartment	VVM	0.5
Liquid volume batch	L	70
Time batch phase	h^{-1}	24
Substrate conc. batch medium	$g_{gluc} \cdot L^{-1}$	90
Substrate conc. feed semi-continuous mode	$g \cdot L^{-1}$	>20
Dilution rate	h^{-1}	0.06
Oleyl alcohol feed rate	$kg \cdot h^{-1}$	3.5
Antifoam 204 Sigma after 18h	Cascade	Cycle time: 3s Dead time: 15s

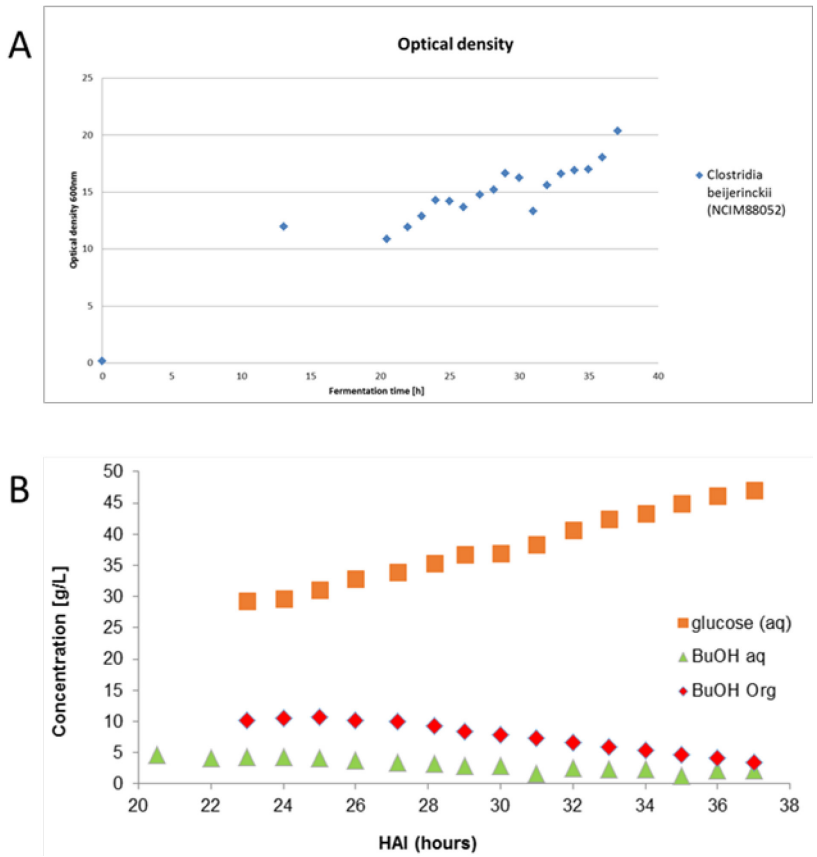

Figure 5. (A) Optical density, blue; (B) glucose, orange; butanol titer in aqueous phase, green; and butanol titer in organic phase, red; during the fermentation run DED.18.004.

During the fermentation test, a maximum bio-butanol titer in the aqueous phase of 4.7 g L^{-1} was achieved (Figure 5) during the batch step. The switch to continuous regime was successful and the organic phase removal during continuous production was validated however a matching productivity and product removal was not maintained. Productivity and bio-butanol titers decreased over time. Different recommendations for the improvement of the process are: use of different solvent with a partition

coefficient higher than the one for oleyl alcohol, metabolic engineering to increase butanol tolerance and or increase strain productivity.

In addition, the reactor model used for Terpene production was modified into an anaerobic reactor model. The new business case considers a production capacity of minimum 30 kt a^{-1} butanol. A techno-economic evaluation was performed for anaerobic production of butanol in industrial scale of min. 30 kt per year. Different scenarios were proposed:

1) Scenario 1: 30 kt·a^{-1} butanol, productivity 2.1 g·L^{-1}·h^{-1} (integrated butanol production, main scenario for comparison to reference case)

2) Scenario 2: 40 kt·a^{-1} butanol, productivity 2.1 g·L^{-1}·h^{-1}, same amount of reactors as reference case (increased revenues)

3) Scenario 3: 12 kt·a^{-1} butanol, productivity 0.8 g·L^{-1}·h^{-1}, same amount of reactors as reference case (oleyl alcohol with m=4) [DED.18.004 run]

4) Scenario 4: 30 kt·a^{-1} butanol, productivity 2.6 g·L^{-1}·h^{-1} (smaller integrated reactor volume with higher productivity)

5) Reference case: 30 kt·a^{-1} butanol, productivity 1.25 g·L^{-1}·h^{-1}

Table 5. Detailed parameters and assumptions of Scenarios 1 and reference.

	FAST 2.1 g·L^{-1}·h^{-1} (scenario 1)	1.25 g·L^{-1}·h^{-1} (scenario Reference)
Production capacity [kt·a^{-1}]	30.3	30.6
Productivity [g·L^{-1}·h^{-1}]	2.1	1.25
Reactor volume [m^3]	200	200
Reactors required	16	21
Product yield [g_P·g_S^{-1}]	0.35	0.35
q_P [g·g^{-1}·h^{-1}]	0.41	0.41
$c_{inhibition}$ [g·L^{-1}]	20	20
$c_{biomass}$ [g·L^{-1}]	25	25
Production time [d]	19	19
Gas flow [vvm]	0.5	2
Solvent partitioning	15	-
OPEX [M€·a^{-1}]	45.5	56.7
CAPEX [M€]	87.3	117.9

Scenarios 1 and 5 (Reference) are detailed in Table 5. The reference scenario comprises gas stripping as ISPR with a productivity of 1.25 g·L^{-1}·h^{-1}. Gas stripping represents (one of) the current industrial production systems for bio-butanol. Although the reference scenario is preferred in terms of process simplicity, gas stripping is limited in its selectivity and separation capacity. Higher gas flows lead to unacceptable equipment requirements and raw material costs. In contrast to the reference scenario, the FAST technology can be scaled up to higher productivities leading to better and smarter usage of equipment. Higher productivities result in less CAPEX. Compared to the reference scenario, OPEX are smaller because of reduced raw material und utilities consumption (data not shown). Especially the N_2 costs, the electricity and cooling water costs are reduced compared to the reference scenario.

Despite the improvement in OPEX and CAPEX when applying the FAST technology, the techno-economic analysis has shown that bio-butanol production is only competitive to conventional butanol

when a high productivity is achieved. High sugar and N_2 costs limit the profitability of bio-butanol production. The minimum productivity required for economic profitability is 7.8 $g \cdot L^{-1} \cdot h^{-1}$ using the FAST technology at given boundary conditions (Figure 6). The assumed market (manufacturing) price is 1.3 $€ \cdot kg^{-1}$.

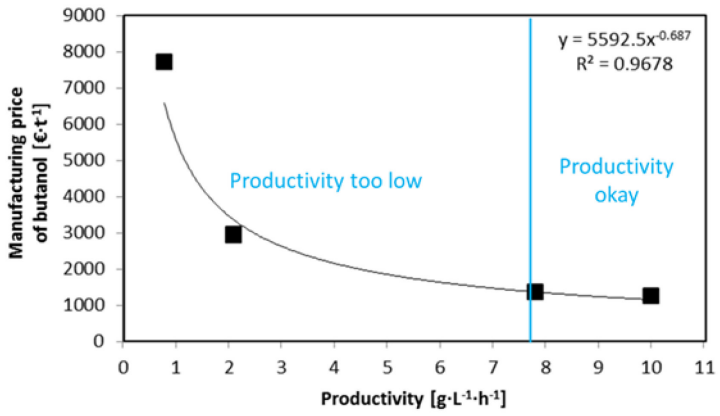

Figure 6. Minimum productivity required for competitiveness with conventional butanol.

In order to intensify the process further, strain engineering and solvent selection is necessary. The separation capacity of the FAST reactor can be improved by increasing the cross-sectional area of the separation compartment. This can be realized by reactor design optimizations.

Table 6. Additional OPEX for butanol conversion to jet fuel in the first year and CAPEX

Year 1	FAST 2.1 $g \cdot L^{-1} \cdot h^{-1}$ (scenario 1)
Total variable operational costs [k€]	2,985.1
• Dehydration	657.8
• Oligomerization	852.8
• Isomerization	1,474.5
Total fixed operational costs [k€]	2,127.9
• Dehydration	614.6
• Oligomerization	794.5
• Isomerization	718.8
CAPEX [k€]	19,140.3
• Dehydration	4,386.3
• Oligomerization	8,174.5
• Isomerization	6,579.5

For the production of jet fuel, conversion of butanol is necessary. The cost related to this conversion was also taken into account. Only scenario 1 with 2.1 $g \cdot L^{-1} \cdot h^{-1}$ were compared to current market options. The conversion of n-butanol into jet fuel consists of a dehydration step to n-butene, an oligomerization step to C12-chains and an isomerization step to jet fuel. Compared to the n-butanol production the additional OPEX of the butanol upgrade are small (Table 6). Adding additional reactors for the butanol upgrade leads to higher CAPEX. Since jet fuel is a bulk chemical, the CAPEX are important but not significant compared to the variable cost. The production capacity was a consequence of obtaining natural non-negative integers for the amount of reactors and losses in DSP.

The price gap between bio-jet fuel from n-butanol and fossil jet-fuel is enormous with a difference of 2,635 €·t-1. The highest cost contributor is feedstock costs with up to 75% (Figure 7). The increase in

reactor productivity has a significant impact on CAPEX. Since less reactors are required with a high productivity, the CAPEX reduction is about 200 €·Mt^{-1} jet fuel at this scale.

We showed that the bio-butanol production is technically feasible applying DAB's FAST reactor. However, the economic feasibility is doubtful with the current technology due to high raw material costs. The FAST technology has the potential to achieve high reactor productivities which would lead to economic competitiveness.

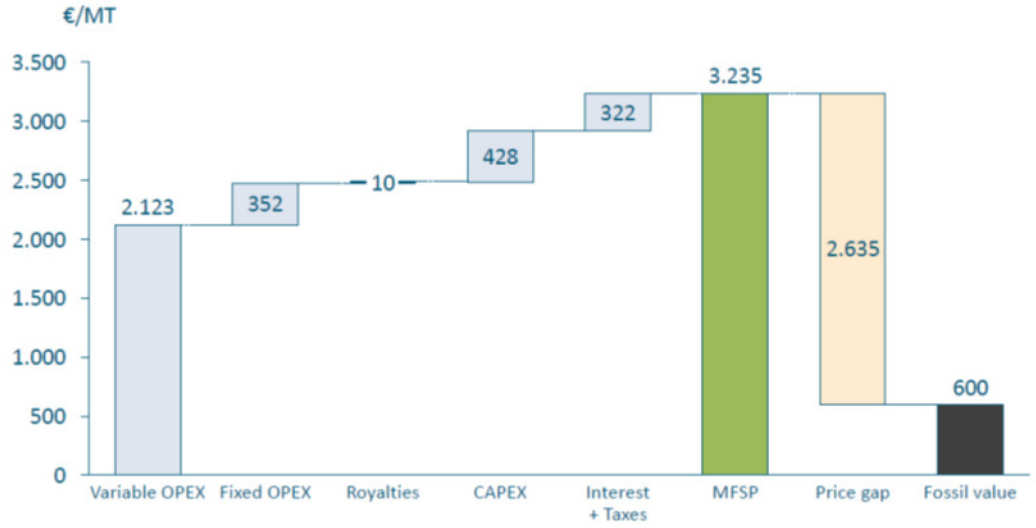

Figure 7. Cost build-up of minimal fuel selling price of FAST scenario 1.

One advantage of the FAST technology is to reach higher productivities and thus, use less equipment. This translates in the need of fewer utilities. The FAST technology enables savings in electricity, cooling water and wastewater treatment. Total Green House Gases (GHG) equivalents (per kT BuOH product) are listed in Table 6. Based on this GHG equivalents, the electricity consumption can be calculated (Table 7) using the conversion factors in Table 8.

Table 6. Total Green House Gases (GHG) equivalents for Butanol production (Kg CO2 per kT butanol).

GHG equivalents (kg CO2 per kT BuOH)	Scenario 1	Scenario 2	Scenario 3	Scenario 4	Reference
Electricity	3.99E+05	3.86E+05	1.38E+05	8.17E+04	2.03E+06
Cooling water losses	4.06E+03	4.02E+03	8.35E+04	3.94E+03	1.19E+04
cooling duty	1.50E+02	1.19E+02	8.79E+01	1.52E+02	4.52E+02
heating duty	3.20E+05	2.41E+05	9.84E+05	3.70E+05	5.65E+05
waste water treatment	2.76E+03	3.53E+03	1.43E+03	2.76E+03	3.97E+03
Total (GHG kg CO2 per kTa BuOH)	7.26E+05	6.34E+05	1.21E+06	4.59E+05	2.61E+06
Percentage (%) relative to reference	28	24	46	18	100

Table 7. Electricity consumption data.

Electricity [kW.h·a-1] per [kTa BuOH]	Scenario 1	Scenario 2	Scenario 3	Scenario 4	Reference
Compressor	6.33E+05	6.25E+05	2.08E+06	5.00E+05	4.00E+06

Electricity [kW.h·a-1] per [kTa BuOH]	Scenario 1	Scenario 2	Scenario 3	Scenario 4	Reference
Condenser	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
Integrated reactor	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
Centrifuge	1.63E+04	1.63E+04	5.08E+04	1.80E+04	0.00E+00
Distillation column	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
Heat exchanger (E-101, E-102, E-103)	0.00E+00	0.00E+00	2.08E+06	5.00E+05	0.00E+00
Decanter	1.67E+05	1.48E+05	2.83E+05	1.67E+05	1.53E+05
Total	8.16E+05	7.89E+05	2.83E+05	1.67E+05	4.15E+06

Table 8. GHG conversion factors for utilities.

Electricity (kWh)	0.489	kg CO2/kWh
cooling water	0.039	kg CO2/ton H2O
waste water (ton)	0.32	kg CO2/ton WWT
heating duty (MJ)	0.074	kg CO2/MJ

The relative change in utilities is shown in Figure 8. The impact of working at low productivity in the FAST 100 system (scenario 3) is clearly reflected in the energetic requirements. Especially the consumption of cooling water that is required for the condensation of off-gases and distillation unit differs for the scenarios. Due to gas flow of 2 vvm in the gas stripping scenario, a large quantity of water is removed with the off-gas. The consumption of less resources results in less production of waste and more efficient use of resources.

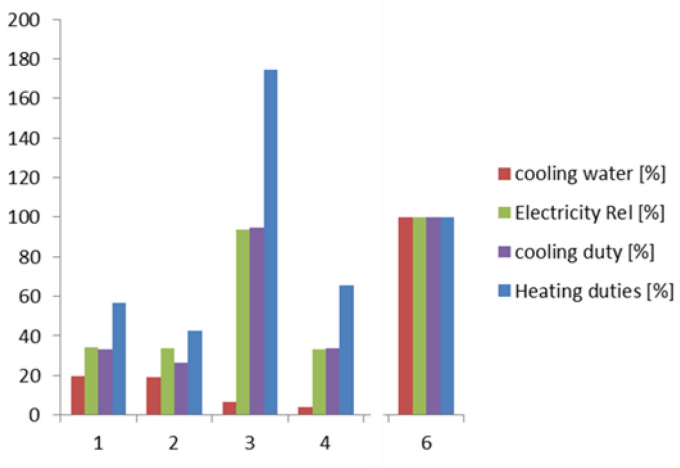

Figure 8. Relative change in utilities for the different modelled scenarios (1-4) compared to the reference case (6). All changes are expressed per kT butanol.

Pereira *et al*. (2015) and Montano (2009) showed with their studies that the bio-butanol production scores positively in the impact categories abiotic depletion and global warming **[4, 5]**. This is attributed to the electricity savings and/or electricity production from by-products of the bio-butanol production. The FAST technology also cuts electricity consumption assuming beneficial effects on the two former impact categories. Other impact categories such as ozone layer depletion, marine aquatic eco-toxicity and photochemical oxidation benefit from bio-butanol. Overall, Pereira and Montano assess bio-butanol environmentally friendlier than butanol obtained from the petrochemical route. Despite the more positive

effect of bio-butanol production on the environment compared to the petrochemical route, two impact categories worsen significantly: acidification and eutrophication. One of the reasons is represented by the raw material used for the fermentation. The feedstock source and fertilizer usage on the agricultural stage have great impact on eutrophication and acidification. So far, glucose was used as substrate in the business case presented. To alleviate the mentioned categories, a second generation feedstock should preferably be used.

8 Conclusions

During the course of the project all the goals and milestones were successfully addressed. Different bacterial strains lineages for the production of terpene molecules were generated and tested. Although initial challenges related to titers, productivity and genetic stability were faced; strains that exceed the target milestone (>10 g L^{-1}) were obtained. In addition, Firmenich's fermentations protocols were successfully transferred and implemented by DAB. Within this transfer and implementation, the targeted productivity of 2 $mg_{product}$ g_{cells}^{-1} h^{-1} was exceeded. Moreover, the bacterial strains were tested in the in situ product recovery system FAST 100. The recovery of the product was higher than 80% and the calculated rate of recovery was above of 5 g L^{-1} h^{-1}.

The developed rate based model of the reactor system was able to adequately predict overall oil recovery and gas hold-up for the different fermentation scenarios. The model allowed scale-up calculations and, combined with flow-sheeting calculations, was able to perform process evaluation. An identified limitation in the model was the individual oil droplet kinetics to obtain the oil split. Further improvements can be made here.

It was identified that the oxygen demand for the tested production of terpenes was not compatible with the operational ranges of oxygen transfer in the FAST 100 system, at least not under conditions that will lead to an economically feasible process. Oxygen demand (kLa) was > 800 h^{-1} at lab scale fermentations. Oxygen transfer during normal continuous stirred tank reactor (CSTR) at scale is normally between 300 and 500 h^{-1}. The oxygen demand of the strain at high cell density is therefor too high for conventional CSTR reactors at scale. This technology was nevertheless identified to be interesting for application in aerobic fermentation, as the FAST 100 is comparable in oxygen transfer to normal at scale systems.

A successful fermentation in batch/continuous regime was conducted in the FAST 100 system for the production of bio-butanol. In addition, energy and Total Green House Gases (GHG) aspects were evaluated for 5 proposed scenarios of anaerobic butanol production where one scenario (scenario 3) corresponded to the current performance of the technology using the FAST 100 system.

The effective productivity increase in the different scenarios corresponds to an overall better use of subsequent DSP operations, significantly lowering the operational utility requirements. Overall GHG equivalents for the main operational utilities can be significantly reduced by a factor of ~ 3 (28 % for scenario 1 compared to 100 % in the reference case). This demonstrates the use of FAST technology can significantly lower the process energy and main utility requirements.

For these reasons, successful pilot runs that show the above mentioned improvement together with the techno-economical evaluation is a good showcase for industry to support the benefit of the FAST system.

9 Outlook and next steps

Based on the experimental work presented and the results of the model, the scaling-up of the existing FAST 100 system is feasible. Efficient choice of organic/liquid phase allows its separation to such an extent that it can be applied to larger systems. Depending on the fraction of organic phase to be recovered during a day of operation, the current vessel can be scaled-up to 200 m^3.

Scale-up calculations performed on the reactor systems show high potential in relation to organic phase removal capacity as long as the product can be extracted to the organic phase. Product partitioning is preferably with a distribution constant (product fraction organic over water) >10. For sesquiterpene this is easily achieved for most solvents.

An envisaged path for commercialization of the technology is the upgrading/retrofitting of existing fermenters, in particular at CMOs or dedicated production lines of products that suffer from limitations, such as product inhibition, that can be addressed with the FAST 100 system.

Additionally, DAB is currently performing anaerobic fermentations using the FAST 100 system as these processes can run without the bottleneck of oxygen transfer and are in line with the outlook of showing a significant production rate increase for extractive fermentation on fermentation inhibited by product. Due to the continuous product removal, the effective volumetric production rate [$g.L.^{-1}h^{-1}$] can be pushed up significantly. It is therefore expected that the business case compared to other production pathways can be improved significantly. For this reason, successful pilot runs that show this productivity improvement together with the techno-economical evaluation will be a good showcase for industry to support the benefit of the FAST system. The next steps in technology development will be (i) demonstrating the benefit from FAST on several other, preferably commercial, processes and (ii) to scale them to demonstration level.

10 Communication

During the course of this project, different scientific publications and presentations were generated.

Scientific publications

- Pedraza-de la Cuesta, S., Keijzers, L., van der Wielen, L.A.M., Cuellar, M.C. Integration of Gas Enhanced Oil Recovery in Multiphase Fermentations for the Microbial Production of Fuels and Chemicals (2018) 13 (4), art. no. 1700478.
- Cuellar, M.C., Straathof, A.J.J. CHAPTER 4: Improving Fermentation by Product Removal (2018) 2018-January (55), pp. 86-108.
- Heeres, A.S., Heijnen, J.J., van der Wielen, L.A.M., Cuellar, M.C. Gas bubble induced oil recovery from emulsions stabilised by yeast components (2016) 145, pp. 31-44.

Congresses with oral presentations on the FAST in 2018

- ECO-BIO by Kirsten Steinbusch, Dublin 3-6 March 2018.
- Symposium on Biotechnology for Fuels and Chemicals by Kirsten Steinbusch, Florida USA April 29 - May 2, 2018.
- Nederlandse Biotechnologen Congres NBC-18 by Marina Elisiário in Ede, Netherlands, May 22, 2018.
- World BIO industrial biotechnology, by Kirsten Steinbusch, Philadelphia, USA by July 2018

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