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Inhibition Control by Continuous Extractive Fermentation Enhances De Novo 2-Phenylethanol Production by Yeast

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ABSTRACT

Current microbial cell factory methods for producing chemicals from renewable resources primarily rely on (fed-)batch production systems, leading to the accumulation of the desired product. Industrially relevant chemicals like 2-phenylethanol (2PE), a flavor and fragrance compound, can exhibit toxicity at low concentrations, inhibit the host activity, and negatively impact titer, rate, and yield. Batch liquid-liquid (L-L) In Situ Product Removal (ISPR) was employed to mitigate inhibition effects, but was not found sufficient for industrial-scale application. Here, we demonstrated that continuous selective L-L ISPR provides the solution for maintaining the productivity of de novo produced 2PE at an industrial pilot scale. A unique bioreactor concept called "Fermentation Accelerated by Separation Technology" (FAST) utilizes hydrostatic pressure differences to separate aqueous- and extractant streams within one unit operation, where both production and product extraction take place - allowing for the control of the concentration of the inhibiting compound. Controlled aqueous 2PE levels ($0.43 \pm 0.02\,g\,kg^{-1}$) and extended production times (>100 h) were obtained and co-inhibiting by-product formation was reduced, resulting in a twofold increase of the final product output of batch L-L ISPR approaches. This study establishes that continuous selective L-L ISPR, enabled by FAST, can be applied for more economically viable production of inhibiting products.

1 | Introduction

The production of chemicals from renewable biomass through fermentation has emerged as a crucial alternative to address pressing environmental concerns arising from our heavy reliance on fossil resources. However, the transition to sustainable chemical production through fermentation has been slow, despite numerous academic examples of microbial cell factories capable of producing various chemical compounds. To a large extent, these proof-of-concept production hosts have not yet achieved industrial scale. The widespread adoption of microbial cell factories is hindered by key performance indicators in fermentation, namely, titer, yield, and rate, often being disappointingly low (Konzock and Nielsen 2024). These indicators serve as benchmarks for evaluating the cost-effectiveness of engineered cell factories, and they frequently fall short compared to traditional chemical processes (Schmidt 2005). Maintaining high fermentation productivity becomes particularly challenging when the product compounds have growth-inhibiting properties, as is the case with the flavor and fragrance ingredient 2-phenylethanol (2PE). Applications of 2PE range from cosmetics to cleaners and detergents, as well as flavor enhancement in foods ranging from dairy, processed fruits and vegetables to chewing gum and confectionary (http://data.europa.eu/eli/reg_impl/2012/872/oj) (Martínez-Avila et al. 2018;

Mitri et al. 2022). To achieve cost-effective biotechnological production of chemicals from renewable resources, both strain and process innovation are imperative. Innovative process technologies that enhance strain performance and provide more concentrated product streams are essential (Virklund et al. 2023).

The market for 2PE is estimated to US$60 million and is expected to grow at over 5.5% compound annual growth rate between 2022 and 2028. The majority of the 10 kT 2PE global market currently relies on cost-effective chemical synthesis (~US$ 5 kg^{-1}) (Hua and Xu 2011; Martínez-Avila et al. 2020). However, the lack of substrate stereo-selectivity in 2PE chemical synthesis leads to the formation of undesired by-products, necessitating environmentally harmful purification steps (Etschmann et al. 2002; Kirm et al. 2005). European and United States regulatory agencies have limited the use of food-grade 2PE to natural sources, including botanical and microbiological sources (European Parliament and of Council 2008). This has gradually shifted production from chemical to biological sources but at a higher cost. The market volume resulting from plant extraction is estimated to 3 T (~US$1000 kg^{-1}) while the market of biosynthetic 2PE issued from microbial fermentation is estimated to 72 T at a cost of ~US$300 kg^{-1} (Wang et al. 2019).

Biotechnological approaches, such as whole-cell bioconversion of L-phenylalanine and de novo biosynthesis from sugars, have been explored as alternatives to natural extraction routes from rose petals for 2PE (Chantasuban et al. 2018; Reverchon, Della Porta, and Gorgoglione 1997). Aerobic fed-batch cultures of *Saccharomyces cerevisiae* resulted in a maximum 2PE concentration of 3.8 $g\,L^{-1}$ through the bioconversion of L-phenylalanine (Stark et al. 2003). 2PE was also produced de novo (from glucose) by engineered *S. cerevisiae* in controlled aerobic batch cultivation, achieving a concentration of 2.1 $g\,L^{-1}$ with a productivity of 0.052 $g\,L^{-1}\,h^{-1}$ (Hassing et al. 2019). While these are some of the highest reported titers and productivities in scientific literature, they still fail to reach the values required for competitive industrial fermentation processes. Typically, in fermentative production systems, productivity needs to be in the range of 0.5 to 1.0 $g\,kg^{-1}\,h^{-1}$ for cost-effective production, preferably reaching 2.0 $g\,kg^{-1}\,h^{-1}$ for commodity chemicals (Straathof 2014). For bulk processes, the product yield on substrate must be close to the theoretical maximum, which for 2PE from glucose is 0.34 $g\,g^{-1}$ (Crater and Lievense 2018; Konzock and Nielsen 2024).

2PE is known for its ability to inhibit microbial growth, as evidenced by its historical use as a bactericidal agent (Lingappa et al. 1969; Liu et al. 2014). Concentrations of 2–4 $g\,L^{-1}$ have been reported to completely inhibit oxidative metabolism in yeasts like *S. cerevisiae* (Carlquist et al. 2015; Hassing et al. 2019; Hazelwood et al. 2006; Stark et al. 2003; Tönjes et al. 2023). To bridge the gap between desired overall production rates and current rates, a different process mode of operation, such as in situ product removal (ISPR), can be considered. ISPR is a method for controlling product inhibition by removing the product during production (Freeman, Woodley, and Lilly 1993; Salas-Villalobos and Aguilar 2023; Tönjes et al. 2023; Woodley et al. 2008). ISPR techniques are typically categorized based on the phase through which the inhibiting product is removed: gas-liquid (G-L, e.g., stripping), solid–liquid (S-L, e.g., adsorption), and liquid-liquid (L-L, e.g., extraction). Numerous removal techniques are available and have been applied to 2PE and similar molecules (Červeňanský, Mihaľ, and Markoš 2020; Etschmann et al. 2015; Etschmann, Sell, and Schrader 2005; Fabre, Blanc, and Goma 1998; Janković, Straathof, and Kiss 2024). Although various ISPR methods have specific limitations for scalability and industrial application, L-L ISPR has shown promise at small scales and is proposed as a viable route to industrial application (Arroyo-Avirama et al. 2022; Červeňanský, Mihaľ, and Markoš 2020; Qian et al. 2019; Stark et al. 2002).

In an L-L ISPR extractive system, a biocompatible, nonaqueous soluble extractant is added to the fermentation process. A high selective affinity for the product is preferred (Combes et al. 2021; Heintz et al. 2017). Several studies have already demonstrated the advantages of L-L extraction in two-phase aerobic batch processes for 2PE production through L-phenylalanine bioconversion using yeasts such as *S. cerevisiae* (Stark et al. 2002) and *Kluyveromyces marxianus* (Etschmann, Sell, and Schrader 2003; Morrissey et al. 2015). Using oleic acid and polypropylene glycol 1200 as extractants, production rates of 0.13 and 0.33 $g\,L^{-1}\,h^{-1}$ were achieved, showcasing improved process metrics compared to non-ISPR approaches (Etschmann and Schrader 2006). However, batch ISPR processes face limitations in auxiliary phase capacity and maintaining productivity over extended durations (Lee et al. 2019). To overcome these limitations, continuous L-L extractive fermentation could prove to be a breakthrough solution.

Here, these challenges are addressed by introducing continuous liquid-liquid extractive fermentation through employing an integrated bioreactor concept known as Fermentation Accelerated by Separation Technology (FAST) (Oudshoorn et al. 2019). Our study aims to demonstrate the feasibility and advantages of this approach in enhancing the production of 2-phenylethanol at an industrial pilot scale. The *Saccharomyces cerevisiae* strain IMX2179 engineered for de novo 2PE production from glucose (Hassing et al. 2019) was utilized to compare key fermentation performance metrics (titer, rate, and yield) with lab-scale counterparts and traditional fermentation approaches at similar process conditions. Through this investigation, the objective is to further sustainable biochemical production methods for inhibiting compounds by providing insights with regard to key performance parameters (titer, rate, and yield) in a continuous liquid-liquid extractive fermentation process with a novel bioreactor concept.

2 | Materials and Methods

2.1 | Chemicals

All chemicals used in this work were reagent-grade and purchased either from Sigma-Aldrich (St. Louis, MO), Carl Roth GmbH + Co. KG (Karlsruhe, Germany) or Chempri Oleochemicals b.v. (Raamsdonkveer, The Netherlands).

2.2 | Strains and Culture Conditions

The *Saccharomyces cerevisiae* strain IMX2179 (*ura3-52 can1*Δ::-*Spycas9*-natNT2 *aro3*Δ *aro8*Δ $TDH3_p$-$ARO4^{K229L}$-*ENO2*t shrDA $SkTDH3_p$-*PHA2*-$TEF2_t$ shrDB $SePDC1_p$-*ARO2*-$SSA1_t$ shrDC

SeFBA1$_p$-*ARO9*-*ADH1*$_t$ shrDD *SeGPM1*$_p$-*ARO7*^{T226I}-*TEF1*$_t$ shrDE *ENO2*$_p$-*ARO1* *sga1*Δ::*ENO2*$_p$-*TKL1*-*TDH3*$_t$ *tyr1*$_p$Δ::*YEN1*$_p$-*TYR1* X3::*FBA1*$_p$-*ARO3*^{K222L}-*PGK1*$_t$ shrAF *PDC1*$_p$-*EcaroL*-*ADH3*$_t$ *PYK1*^{D146N} pUDE001 (2 μm *URA3* *TDH3*$_p$-*ARO10*-*CYC1*$_t$)) that produces 2PE from glucose as sole carbon source was used in this study (Hassing et al. 2019).

The IMX2179 strain was grown in shake flask in chemically defined medium (SMG_U) adapted from (Verduyn et al. 1992). For lab-scale shake flask cultivations (seed), the media contained (per kg) 20.0 g glucose; 2.3 g urea; 3.1 g potassium hydrogen phthalate; 0.5 g $MgSO_4{\cdot}7H_2O$; 3.0 g KH_2PO_4; 1.0 g concentrated trace metals; 1.0 g concentrated class B vitamins solution (Milne et al. 2015). For demo-scale seed cultivations, the media contained (per kg): 20.0 g glucose; 2.3 g urea; 6.6 g K_2SO_4; 0.5 g $MgSO_4{\cdot}7H_2O$; 3.0 g KH_2PO_4; 1.0 g concentrated trace metals; 1.0 g concentrated class B vitamins solution. The pH was adjusted to 5.5 ± 0.1 with 3 M KOH or 0.5 M H_2SO_4, before sterilization. For all media, the components, except for glucose, vitamins, and trace elements, were combined and autoclaved (L31142, Getinge, Germany) at 121°C for 25 min or sterilized in place. A separate solution of glucose (50% w/w) was also heat sterilized, while vitamins and trace elements solutions were filter sterilized (0.2 μm PES membrane bottle-top filter, Corning, New York, NY).

IMX2179 yeast cultures were stored at −80°C in a ultra-freezer (Fryka-Kaltetechnik B35-95, Esslingen am Neckar, Germany) by adding glycerol to a final concentration of 20% v/v to SMG_U culture broth and dispensed in 1.8 mL aliquots in 2.0 mL cryovials.

Bioreactor cultures were performed in a chemically defined medium (Verduyn et al. 1992) adapted for high cell density fermentations (~120 $g_{CDW}\,kg^{-1}$) (SMG_{HCD}). The adaptation was based on the elemental biomass composition of *S. cerevisiae* (Lange and Heijnen 2001). This medium contained, per kg: 2.5 g citric acid; 10.0 g $(NH_4)_2SO_4$; 8.0 g KH_2PO_4; 15.0 g K_2SO_4; 3.0 g $MgSO_4{\cdot}7H_2O$; 0.2 g Antifoam C; 10.0 g concentrated trace metals; 10.0 g concentrated vitamins stock solution; 20.0 g glucose. The pH was adjusted to 5.0 ± 0.1 with 3 M KOH or 0.5 M H_2SO_4.

2.3 | Lab-Scale Bioreactor Fed-Batch Process

2.3.1 | Seed Culture

The flask and lab-scale bioreactors were inoculated from exponentially growing cells in pre-culture containing 100 mL of SMG_U. Seed medium was inoculated with the contents of one cryovial per flask and subsequently incubated in a S44i Biotechnological shaker (Eppendorf, Hamburg, Germany) at 200 rpm (2.5 cm orbit) and at a temperature of 30°C for 40–48 h, before transfer to the main culture.

2.3.2 | Bioreactor Operation and Sampling

Aerobic fed-batch cultures were performed in 3 L DASGIP DR03F bioreactors controlled with a SciVario twin controller (Eppendorf) with an initial aqueous broth weight of 0.9 kg. A 10% w/w inoculum fraction of aqueous starting weight was applied using the seed culture. For batch overlay processes, 20% v/v extractant (Oleyl Alcohol, 80%–85%, Chempri Oleochemicals) was additionally added before inoculation. A dissolved oxygen (DO) concentration of 30% was maintained with an agitation (1000–1600 RPM) and aeration (compressed air, 0.75 to 1.5 vvm) cascade. pH was controlled at 5.0 ± 0.1 and adjusted with 10% v/v ammonia. Temperature was controlled at 30°C throughout the entire cultivation. Foaming was controlled by adding small amounts of a 10% polypropylene glycol 2000 (P2000; Sigma-Aldrich) solution when required. The fermentation process consisted of three phases: batch-, exponential feed- and constant feed phases. The batch phase was executed until the depletion of ethanol and organic acids, signaled by an increase of the DO signal. The exponential feed program was automated using the expression shown in (Equation 1) and was maintained for 33 h.

$$F_{s,in}(t) = F_{s,in,0} \cdot \exp(k_{ExpFeed} \cdot t). \quad (1)$$

The feed exponent ($k_{ExpFeed}$) was set at 0.12 h^{-1}, and the substrate feed flow start value ($F_{s,in,0}$) at 0.63 $g_{glucose}\,kg^{-1}\,h^{-1}$ to avoid high biomass-specific substrate consumption rate (q_s) and subsequent formation of overflow metabolites like ethanol (Hensing et al. 1995). The maximum- and constant feed rate ($F_{s,in,const}$) was set to 5.0 $g_{glucose}\,kg^{-1}\,h^{-1}$. The feed solution ($C_{s,in}$) consisted of 500 $g_{glucose}\,kg^{-1}$ and was supplemented with 10 g kg^{-1} concentrated class B vitamins solution.

Whole broth samples containing both aqueous- and organic phase fractions were collected periodically and analyzed for extracellular metabolites, dry cell mass concentration, and optical density. Off-gas was analyzed using a DASGIP GA Off-gas Analyzer and interpreted with DASware control software (Eppendorf).

2.4 | Industrial Pilot-Scale Continuous L-L Extraction (FAST) Process

2.4.1 | Seed Train

The seed train comprised two steps. Seed 1 was executed at similar conditions to the seed culturing for bioreactors (New Brunswick Scientific Innova 44, Eppendorf). In the second step (seed 2), aerobic batch cultivation with 20 g kg^{-1} glucose was carried out in a 150 L bioreactor (Getinge, Delft, The Netherlands), with 1% v/v seed 1 culture as inoculum, until the optical density at 600 nm (OD_{600}) reached 5.5. The operational conditions were 30°C, pH 5.0 (maintained by titrating 10% v/v NH_4OH and 2 M H_2SO_4), 0.67 vvm aeration, and agitation speeds ranging from 150 to 550 rpm to maintain a DO of 30% or higher.

2.4.2 | Description of Continuous Overlay With FAST

FAST is a method and apparatus that enables continuous extractive fermentation by integrating fermentation, extraction, and liquid-liquid (L-L) phase separation in a single vessel. The

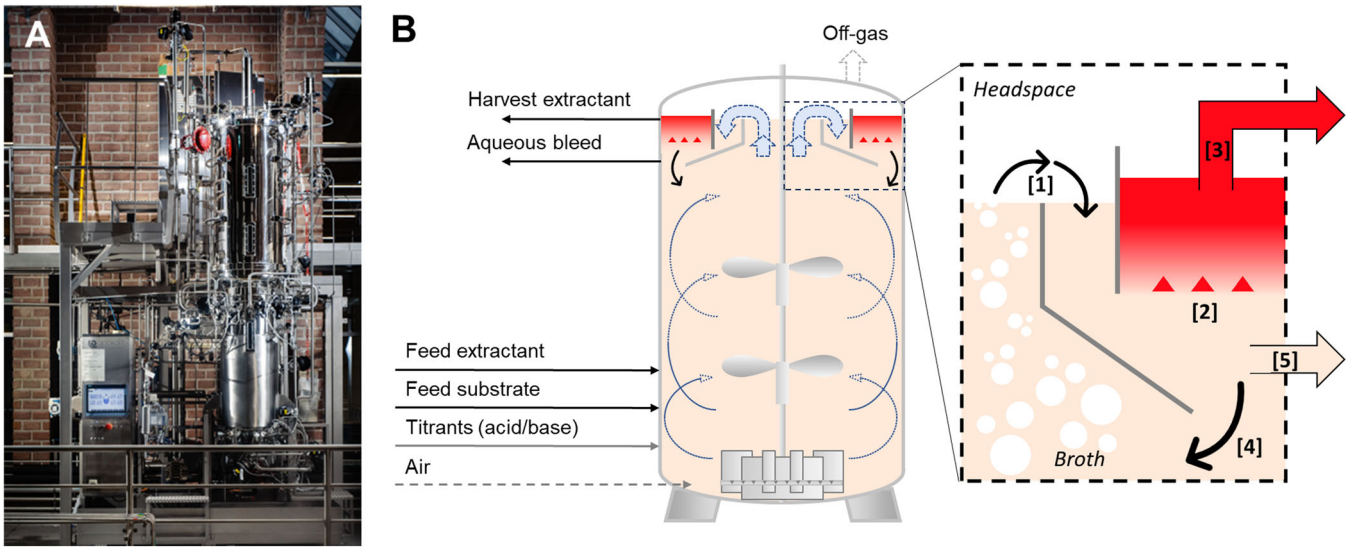

FIGURE 1 | Frontal and schematic views of the FAST bioreactor (500 L). (A) Frontal view of the FAST unit installed in Bio Base Europe Pilot Plant in Ghent, Belgium. (B) Schematic view of the FAST bioreactor with streams entering the system (extractant, substrate, titrants, and air) and exiting the system (harvest enriched with the extractant, bleed enriched in the water phase and off-gas). The transition of the gaseous fermentation liquid to the degassed laminar flow zone in the top, along with the recirculation of the settled aqueous phase from the top to the fermentation zone, are indicated with curved arrows. The detailed schematic of the separation compartment exhibits the following: [1] Aerated- and agitated fermentation broth containing low amounts of extractant enters the riser zone, degasses, and flows into the downcomer zone. [2] In the separation zone, density differences between the extractant and the water phase allow for spontaneous phase separation and extractant enrichment of the top layer. [3] Harvest enriched in the extractant is removed from the top of the separation zone. [4] Biomass is allowed to passively recirculate back to the fermentation compartment of the reactor. [5] If required, an aqueous bleed can be applied from the bottom of the separation zone.

extractive fermentation system is comparable to a standard fermentation reactor and can be either operated as stirred tank reactor (STR) or bubble column (Figure 1A). The extractant (organic phase) is dosed to the system throughout the fermentation process as needed. The extraction process, which controls the aqueous product concentration in the fermentation, occurs predominantly in the fermentation section of the reactor. The major difference with conventional bioreactors is an internal compartment configuration being present at the top segment of the reactor system, as exemplified in the schematic overview of FAST (Figure 1B). This design allows for the internal liquid exchange between the compartments, created by a hydrostatic pressure difference between the degassed liquid and the liquid broth with higher gas hold-up. The pressure difference allows for high continuous internal liquid flow rates. By design, the internal recirculation liquid transitions in a laminar flow regime, allowing gravity-driven L-L phase separation to occur. As the two phases, bulk liquid broth and product rich extractant are now separated, the extractant can be selectively removed in a controlled manner from the system via a harvest line situated in the separation compartment of the bioreactor. Due to the high internal liquid exchange operational window, the system allows a very wide range of applicable liquid-liquid phase separation rates and control of the organic phase hold-up in the fermentation section.

2.4.3 | Bioreactor Operation, Online Monitoring and Sampling

The aerobic continuous extractive fermentation process was performed with a custom-designed proprietary pilot-scale (500 L working volume range) FAST bioreactor installed at Bio Base Europe Pilot Plant (BBEPP, Ghent, Belgium). The FAST bioreactor was inoculated with 9% v/v seed 2 culture. The fermentation process conditions for FAST were identical to that for the lab-scale fed-batch process described earlier, except that the extractant (Oleyl alcohol 80%–85%) was continuously supplied to the system and dosed at a fixed rate of 23.5 g kg^{-1} h^{-1} starting at 11 h after inoculation. A cascade regulation scheme was applied to maintain a DO of 20%. Aeration was regulated with a lower limit of 1.0 vvm, agitation was regulated with a lower limit of 200 rpm. Additionally, vessel pressure was regulated at 2.1 bar de novo.

Samples were collected every 4 h from the fermentation compartment sample line of the FAST system and the separation compartment harvest line (extractant-enriched stream). Samples were stored at −20°C before further processing and analysis. Throughout the fermentation, OD_{600}, cell dry weight, and aqueous phase metabolite concentrations were measured. N_2, O_2, and CO_2 in off-gas were analyzed by mass spectrometry (Prima PRO, Thermo Fisher Scientific, Bleiswijk, The Netherlands).

2.5 | Analytical Methods

2.5.1 | Sample Processing

Whole broth samples containing mixed aqueous and extractant phases were centrifuged at 3.1×10^3 g (Labogene ScanSpeed 1580R, Lillerød, Denmark) until phase separation was obtained. The top layer (clear extractant) and middle layer (clear

supernatant) were subsequently isolated and stored separately at −20°C in 2 mL tubes until further processing was required.

2.5.2 | 2-phenylethanol Quantification

2PE was measured from aqueous- or extractant samples diluted in 99.8% ethanol following the HPLC method as previously described (Hazelwood et al. 2008; Luttik et al. 2008). 2PE concentrations are expressed for the aqueous phase ($C_{2PE,Aq}$; g kg^{-1}), for the extractant phase ($C_{2PE,Extr}$; g kg^{-1}), and for the whole broth (sum of aqueous and extractant phase, $C_{2PE,Act}$; g kg^{-1}). The latter was obtained with the expression shown in (Equation 2):

$$C_{2PE,Act}(t) = \frac{C_{2PE,Aq}(t)\cdot M_{aq}(t) + C_{2PE,Extr}(t)\cdot M_{Extr}(t)}{M_{Extr}(t) + M_{aq}(t)}. \quad (2)$$

2.5.3 | Glucose, Ethanol and Acetic Acid Quantification—Lab-Scale Experiments

Glucose, ethanol, and acetic acid were quantified from fermentation supernatant samples diluted in demineralized water. Samples were analyzed using a Thermo Ultimate 3000 HPLC device (Thermo Fisher Scientific) with an HPX87H column (Bio-Rad, Hercules, CA), operated at 55°C. 0.01 N H_2SO_4 was the mobile phase, at a flow rate of 0.55 mL min^{-1}. Metabolites were detected with a Refractomax refractive index detector and an Ultimate 3400 detector @214 nm (Thermo Fisher Scientific). For data comparison between scales, absolute metabolite and biomass amounts were normalized for the starting weight of the aqueous phase.

2.5.4 | Glucose, Ethanol and Acetic Acid Quantification—Industrial Pilot-Scale Experiments

Glucose, ethanol, and acetic acid were quantified from fermentation supernatant samples diluted in demineralized water. Samples were analyzed using an Agilent 1260 Infinity HPLC device with an Agilent MetaCarb 67H 300 × 6.5 mm column, operated at 35°C. 2.5 mM H_2SO_4 was the mobile phase, at a flow rate of 0.8 mL min^{-1} (Agilent, Santa Clara, CA). Metabolites were detected with an Agilent Refractive Index Detector. For data comparison between scales, absolute metabolite and biomass amounts were normalized for the starting weight of the aqueous phase.

2.5.5 | Biomass Quantification—Lab-Scale Experiments

To determine cell dry weights from lab-scale tests, broth was collected in 15-mL test tubes that had been previous dried and weighed (m_1) and centrifuged for 10 min at 3.1×10^3 g (Labogene ScanSpeed 1580R). The cell pellet was washed with demineralized water and centrifuged at the aforementioned conditions. Tubes containing the cell pellet were dried at 105°C for 24 h (Firlabo AC 240, Froilabo, France), before being weighed (m_2). The cell dry weight was calculated as the difference between m_1 and m_2 divided by the total sample weight, expressed in g_{CDW} kg^{-1}. Optical density was determined at 600 nm (OD600) with samples diluted to be in the 0.1–0.3 A.U. range with a spectrophotometer (Evolution 60, Thermo Scientific). Demineralized water was used as a blank sample.

2.5.6 | Biomass Quantification—Industrial Pilot-Scale Experiments

Five milliliters broth samples were spun down at 3.1×10^3 g (Labogene ScanSpeed 1580R) an subsequent pellets were washed in reverse osmosis (RO) water. The washed pellet was spun down and resuspended in RO water, from which cell dry weight was determined using a Sartorius MA37 Moisture Analyzer operated at 130°C. Optical density was determined at 600 nm (OD_{600}) with samples diluted in demineralized water until a reading was obtained between 0.25 and 0.75 A.U. (Cary 60 Spectrophotometer, Agilent Technologies).

3 | Results

3.1 | Characterization of IMX2179 Performance in Carbon-Limited Fed-Batch Conditions

To validate the adverse effects of 2PE accumulation on *Saccharomyces cerevisiae*, the strain IMX2179 (Hassing et al. 2019) was cultivated in fed-batch mode without introducing a second organic phase (Figure 2A). The strain IMX2179 incorporates a series of modifications including the improvement of the supply of phosphoenolpyruvate by expression of a pyruvate kinase variant, the elimination of *p*-hydroxy-phenylethanol formation without causing a tyrosine auxotrophy, and the concurrent engineering of both the aromatic amino acid biosynthesis pathway and the Ehrlich pathway (Hazelwood et al. 2008; Luttik et al. 2008). These mutations facilitate a more efficient connection between glycolysis and the pentose phosphate pathway, thereby optimizing the carbon flux toward 2PE production. In these conditions, up to 2.6 g kg^{-1} of 2PE accumulated after 48 h; with no further increase in concentration thereafter (Figure 2A). Notably, at 48 h when the maximum titer was achieved, a sharp decline in the oxygen uptake rate (OUR) was measured, suggesting a significant reduction in metabolic activity that could be attributed to toxic levels of 2PE.

To alleviate the toxicity of 2PE, an organic phase overlay was introduced to the fed-batch process. Oleyl alcohol was selected due to its known biocompatibility and favorable distribution coefficient (K_d, hereinafter referred to as partitioning (P)) for 2PE (De Brabander et al. 2021; Welton 2015). A flask-scale growth study confirmed that oleyl alcohol is indeed biocompatible with *Saccharomyces cerevisiae* IMX2179, showing comparable growth to reference growth without overlay added (Supporting Information S1: Figure S1). Moreover, P was estimated to be between 15 and 25 kg_{aq} kg_{org}^{-1} (Supporting Information S1: Figure S1). By adding 20% v/v oleyl alcohol to the batch medium, the 2PE production period was extended by 30 h based on OUR, and the total 2PE concentration more than doubled, reaching 6.1 g kg^{-1} at 76 h (Figure 2B). However, even

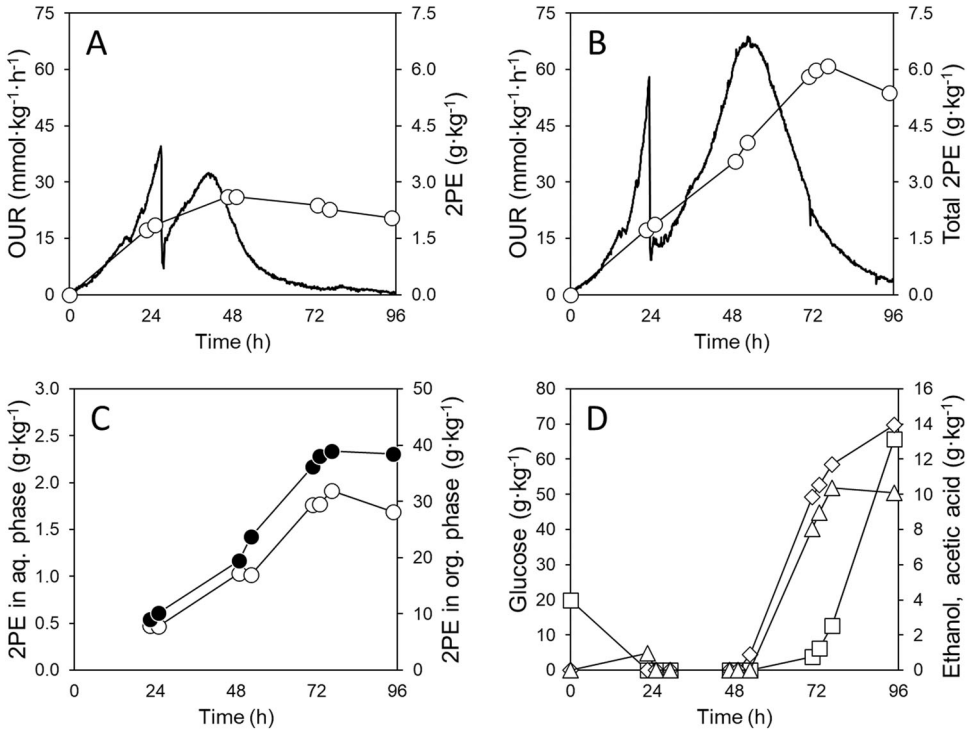

FIGURE 2 | Oxygen uptake, product- and byproduct formation, glucose accumulation, and phase concentration profiles of 2PE-producing IMX2179 in carbon-limited fed-batch non-overlay and batch overlay modes of operation. All fermentations were glucose-limited fed-batches at pH 5.0°C and 30°C, with oleyl alcohol as extractant where applicable. Fermentations were performed with sequential done feeding mode, an exponential feed was applied to the batch phase which was changed to a constant feed of 5.0 $g_{gluc}\,kg^{-1}\,h^{-1}$ for the production phase. (A) OUR (black line) and total 2PE (open circles) of a fed-batch production process without overlay. (B) OUR and total 2PE (open circles) data of a fed-batch with 20% v/v oleyl alcohol overlay. (C) The 2PE in aqueous phase (open circles) and organic phase (closed circles) concentrations during a fed-batch with 20% v/v oleyl alcohol batch overlay. (D) residual glucose (open square), ethanol (open triangle), and acetic acid (open diamond) concentration profiles during a fed-batch with 20% v/v oleyl alcohol batch overlay. The data shown are derived from representative duplicate experiments for which data is provided in Supporting Information S1: Figure S2.

with this delay, OUR sharply declined after 55 h, reaching nearly zero by 72 h. The aqueous phase 2PE increased from 0.5 g kg^{-1} at 24 h to nearly 2.0 g kg^{-1} at 76 h (Figure 2C). This suggests that, in both non-overlay and batch overlay conditions, the loss of metabolic activity and the levelling off of 2PE production occurred when the aqueous concentration approached 2.0 to 2.5 g kg^{-1}, consistent with findings reported in previous studies (Etschmann and Schrader 2006; Stark et al. 2003). Concurrently, the 2PE content in the organic phase increased from 10 to 39 g kg^{-1} within the same timeframe, indicating the achievement of a partitioning of approximately 20 $kg_{aq}\,kg_{org}^{-1}$.

Furthermore, a sudden production of acetate and ethanol was observed 48 h into the 20% overlay fed-batch, occurring immediately after a decrease in the OUR and preceding an increase in residual glucose in the aqueous phase (Supporting Information S1: Figure S2A,B). Within this timeframe, the levels of acetate and ethanol reached 10 and 8.0 g kg^{-1}, respectively. The presence of these metabolites alongside 2PE production could potentially lead to synergistic inhibitory effects and an increased loss of viability, as evidenced by the changes in OUR and glucose accumulation (Figure 2D), as well as a decline in OD_{600} (Supporting Information S1: Figure S2). This data exemplified the restricted effectiveness of an additional auxiliary phase in mitigating product inhibition, underscoring that inhibition remains unavoidable when operating in batch overlay mode. Different metabolic states are indeed achieved by not controlling the concentration of the inhibiting product. The objective of continuous overlay is to regulate the level of the inhibiting product to prolong the duration of productive fermentation.

3.2 | Continuous Extraction Performance in the Fast Bioreactor

The aerobic continuous L-L extractive fermentation was conducted using a custom-designed proprietary FAST industrial pilot-scale system with a working volume of 500 liters. Meanwhile, the fermentation was operated as a conventional fed-batch bioprocess. The primary liquid inlet streams included the feed, extractant, and base, while the main liquid outlet streams consisted of extractant-enriched harvest and bleed (Figure 1). Throughout the entire process, the harvest rate closely matched the extractant feed rate, and the same control was applied to the substrate feed and bleed rates (Figure 3A). Additionally, the densities of the harvest and bleed lines remained constant, ensuring consistent compositions of aqueous and extractant fractions over an extended fermentation period (Figure 3B). The densities of the extractant and harvest remained close to that of oleyl alcohol (845 kg m^{-3}), while the feed and bleed densities matched those of the feed solution and broth (1200 and 1020 kg m^{-3}, respectively).

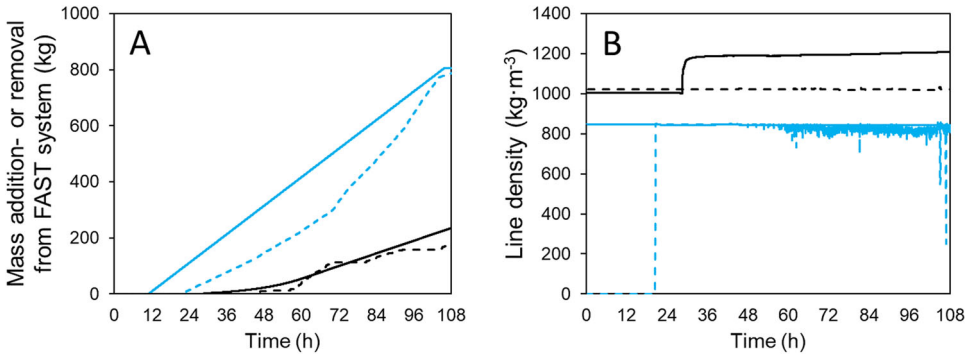

FIGURE 3 | Mass- and density monitoring of the extractant (blue line), harvest (dashed blue line), substrate (black line), and bleed (dashed black line) lines during fermentation with FAST. Measurements were performed with in-line devices during glucose-limited fed-batch 2PE production with continuous overlay in the FAST bioreactor (oleyl alcohol). Substrate feeding was activated at 28 h, and continuous overlay mode (fixed rate extractant dosing and harvesting) was started at 10 h. Bleed was activated according to aqueous level when required. (A) Mass totalizers for liquid in- and outgoing streams and (B) measured stream densities for liquid in- and outgoing streams.

The FAST bioreactor design decoupled the amounts of broth and extractant phases that could be present in the system at any given time, thereby expanding the scope of process design for fermentation optimization. Consequently, it became possible to control the concentration of the aqueous product throughout the fermentation process, which could be predetermined in advance. This contrasts batch overlay operations where such control is unattainable. In total approximately 800 kg of extractant was dosed. In relation to the working volume of the reactor, this is more than 100% overlay—thereby exceeding traditional batch overlay system capacities. The volume of the aqueous phase exiting the reactor, relative to the product output per unit reactor volume, is substantially lower. This reduction is directly linked to improvements in volumetric productivity. As a result, the overall footprint of the production process is minimized, and the energy costs do not scale proportionally with the amount of product produced.

3.3 | Characterization of IMX2179 Performance in Carbon-Limited Fed-Batch Conditions With Continuous Overlay Operation in FAST Bioreactor

The 2PE-producing *S. cerevisiae* strain IMX2179 was cultivated in the FAST bioreactor with a continuous overlay mode. The glucose feed rate was maintained at a level that ensured the OUR remained close to 80 mmol kg^{-1} h^{-1} (Figure 4A). In contrast to the two previously described operation modes, the OUR did not decline and continued to increase even after 96 h. This sustained microbial activity resulted in ongoing 2PE accumulation per unit of dosed glucose (Figure 4B). The end concentration of 2PE per working volume reached 16.6 g kg^{-1}, which was nearly twice as high as that achieved in the 20% v/v overlay cultures (Table 1). Notably, the 2PE yield on glucose remained constant at 0.04 g g^{-1} throughout the constant feed phase. An average 2PE production rate of approximately 0.20 g kg^{-1} h^{-1} was attained during the final 50 h of the continuous extractive fed-batch process. Furthermore, the improved production profile was accompanied by continuous biomass accumulation, reaching OD_{600} = 160 A.U. at 84–96 h (as shown in Figure 4C), representing an approximate biomass concentration of 35 g_{CDW} kg^{-1}. By changing the overlay mode from batch- to continuous overlay, the cumulative 2PE yield on glucose increased from 0.032 to 0.050 g g^{-1} (+56%), the productivity increased from 0.095 to 0.16 g kg^{-1} h^{-1} (+68%), and the total accumulated product increased from 9.05 to 16.6 g kg^{-1} (+ 83%) (Table 1). In contrast to the non-overlay and batch overlay modes, during the carbon-limited feed phase in the continuous overlay mode the levels of acetic acid and ethanol remained below 0.66 and 0.85 g kg^{-1}, respectively, and residual glucose was zero (Supporting Information S1: Figure S3). This, combined with an aqueous 2PE concentration below 0.5 g kg^{-1}, effectively prevented potential co-inhibition.

4 | Discussion

To date, in situ product removal approaches were limited in terms of both rate and yield due to product inhibition. Typically, the process becomes constrained by product inhibition once a certain concentration point is reached, and increasing the extractant volume indefinitely can negatively impact biomass growth, metabolism, and overall process economics (Crater and Lievense 2018). Two technological innovations, namely de novo 2PE production and continuous extractive fermentation, were combined to enable a feasible 2PE production process with regard to volumetric productivity and product yield.

First, 2PE was produced through de novo biosynthesis from sugar by an engineered strain of *S. cerevisiae*—an approach that did not involve the costly use of L-phenylalanine as a direct precursor. Second, a new bioreactor concept based on continuous and controlled in situ addition and removal of an extractant phase was introduced. The primary advantage of the continuous mode of the extractive phase rests in its ability to maintain a consistent 2PE concentration in the aqueous phase throughout the fermentation. Tight control of 2PE levels was demonstrated in both the aqueous and organic phases (Figure 4D): (i) continuous dosing and removal of the extractant after 24 h promptly reduced the 2PE concentration in the aqueous phase, (ii) the 2PE aqueous concentration was held below 0.50 g kg^{-1} during the exponential feed phase, well below the inhibitory concentration of 2.5 g kg^{-1}, allowing for sustained production for up to 100 h, and (iii) aqueous 2PE levels

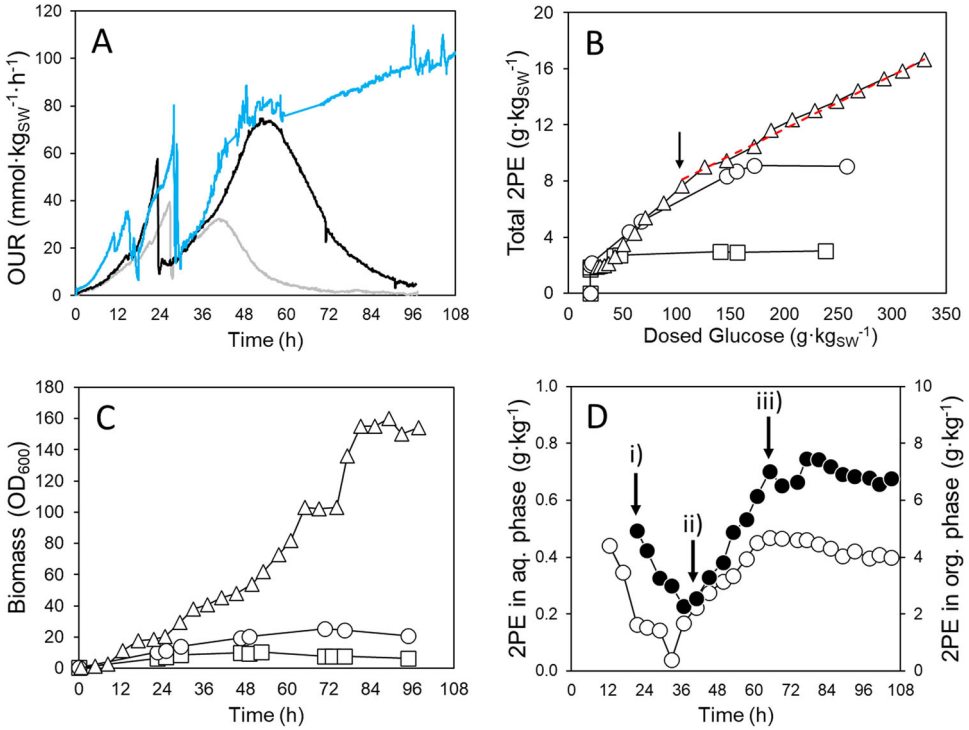

FIGURE 4 | OUR, yield, biomass, and phase concentration plots for the continuous overlay carbon-limited fed-batch 2PE production fermentation process with IMX2179 compared to 20% v/v oleyl alcohol batch overlay and non-overlay. All fermentations were glucose-limited fed-batches at pH 5.0°C and 30°C, with oleyl alcohol as an extractant. Fermentations were performed with sequential feeding modes: an exponential feed was applied after the batch phase, which was changed to a constant feed of 5.0 $g_{gluc}\,kg^{-1}\,h^{-1}$ for the production phase. (A) The OUR (unit) (normalized for aqueous starting weight, SW) profiles of non-overlay (gray line), batch overlay (black line), and continuous overlay (blue line). (B) The 2PE concentration per dosed glucose for non-overlay (open square), batch overlay (open circle) and continuous overlay (open triangle). The data for the aqueous phase were normalized by the starting broth weight (SW). (C) The biomass data expressed in OD unit (600 nm) for non-overlay (open square), batch overlay (open circle), and continuous overlay (open triangle). Panel D shows PE concentration profiles in aqueous phase (open circle) and organic phase (closed circle) of a fed-batch with continuous oleyl alcohol overlay. Point (i) denotes the start of the continuous dosing phase- and removal of oleyl alcohol. Dosing rate was maintained throughout the remainder of the fermentation process. Point (ii) indicates the start of exponential glucose feed phase, and point (iii) the start of the constant glucose feed phase. The data shown are derived from representative duplicate experiments for which the data is provided in the Supporting Information.

TABLE 1 | Comparison of accumulated product, production rates, and yields at end of fermentation and productive fermentation time for glucose-limited fed-batch 2PE production processes with *S. cerevisiae* IMX2179 for non- (94.7 h), batch- (94.7 h) and continuous (105 h) overlay modes of operation.

	Non overlay	20% v/v batch overlay	Continuous overlay
Total accumulated 2PE ($g_{2PE}\,kg_{SW}^{-1}$)	3.00	9.05	16.6
Productive fermentation time (h)	46.5	76.5	105 (entire fermentation)
Overall average volumetric production rate ($g_{2PE}\,kg_{SW}^{-1}\,h^{-1}$)	0.032	0.095	0.16
Cumulative product yield on dosed glucose ($g_{2PE}{\cdot}g_{Gluc}^{-1}$)	0.011	0.032	0.050

Note: Accumulation- and rate numbers are normalized for aqueous starting weight in the reactor for comparison between scales.

remained stable at $0.43 \pm 0.02\,g\,kg^{-1}$ throughout the constant feed phase. Together with the mass flow rate data (Figure 3A), it was illustrated that control of the 2PE concentration in the aqueous phase was achieved through the internal hydrodynamic regimes characteristic of the FAST system. One could argue that the cost–benefit of de novo 2PE production in FAST might be offset by the additional volume of extractant required for continuous product removal. However, the increased 2PE productivity and the ability to recycle the organic phase after distillation mitigate the need to use more extractant in FAST fermentation than in more traditional overlay processes.

The cumulative amount of 2PE that was produced with the FAST bioreactor ($16.6\,g\,kg^{-1}$) is, to our knowledge, the highest to date—even when compared to L-phenylalanine bioconversion approaches. The highest titers previously reported

were in L-phenylalanine co-fed batch L-L ISPR systems: 9.8 g kg^{-1} (Chreptowicz and Mierzejewska 2018) and 12.6 g kg^{-1} in batch ISPR systems (Stark et al. 2002). As exemplified by the OUR and residual metabolite levels, the FAST 2PE process was not near a deterioration point and could have been extended to achieve an even higher product output per bioreactor volume, provided a maintained production rate of 0.20 g kg^{-1} h^{-1}. Though comparison of product output was possible with previously reported values, due to the de novo production approach it was not deemed useful to compare yield and productivity with L-phenylalanine bioconversion approaches. The results presented here underscore the critical advantage of continuous L/L in situ product removal for 2PE production from glucose in *S. cerevisiae*. Biomass-specific rates in a system that produces inhibiting compounds may be modeled by extending the Pirt equation with terms for inhibiting product concentrations (Straathof 2023). With this expression, it is evident that stabilizing the concentration of inhibiting product through continuous product removal enables more predictable metabolic behavior in terms of, among others, specific substrate consumption rate (important for avoiding Crabtree ethanol formation) and maintenance requirements. Any bioprocess that is in agreement with the extended Pirt kinetic relation for inhibition would be benefited with continuous product removal. Determining the optimum between aqueous inhibiting product concentration with regard to growth rate and substrate consumption and product removal economics (i.e., extractant usage) then becomes a previously unavailable tool through which a continuous extractive bioprocess can be designed.

Although the impact of 2PE concentration in the aqueous phase was not systematically investigated, a comparison of metabolite accumulation (e.g., ethanol and acetate) between static and continuous overlay conditions provided initial insights into the importance of maintaining the product/inhibitor concentration in the aqueous phase (González-Ramos et al. 2016). High residual 2PE in the aqueous phase in the 20% static overlay-fed batch (2.0 g kg^{-1}) led to the accumulation of ethanol and acetate. The significance of the presence toxic overflow products (i.e., acetic acid and ethanol) is further emphasized by considering that inhibition terms for multiple compounds per kinetic parameter can add up or even multiply, which may lead to spiraling processes once accumulation of these co-inhibiting compounds has started (Seward et al. 1996; Stark et al. 2003; Straathof 2023; Wang et al. 2011). The ability to prevent the occurrence of such synergistic inhibitory effects by preventing the co-accumulation of 2PE and overflow by-products (ethanol and acetate) can only be attained in a continuous extractive system.

Next to intensification of the fermentation and extraction operational units, resulting organic concentrated product streams will also facilitate efficient downstream processing (DSP) and product purification through existing techniques, infrastructure, and principles that are already established in the chemical industry. Potential by-products could also be extracted and contaminate the organic phase, preventing their formation is therefore essential for a cleaner product stream and reducing downstream purification efforts. Consequently, a continuous extractive fermentation process provides a more constant environment conducive to achieving higher product yields, both quantitatively and qualitatively.

5 | Conclusions

To date, the toxicity of 2PE has hindered the industrial-scale production of this flavor compound from sugar sources. Here, we successfully demonstrated that by using the FAST system—which integrates fermentation, separation, and extraction—industrial-scale applicability in terms of performance can be attained for toxic biomolecules. The extension of the fermentation process beyond the limitations of noncontinuous ISPR methods was enabled and scaled up, as evidenced by the 500 L working volume of the industrial pilot-scale bioreactor used in this study. Due to the uncoupled liquid phases in the FAST system, the aqueous concentration of the inhibiting product was controlled, with decreased production of (co-inhibiting) overflow metabolites as a result. The higher and sustained productivity achieved through this mode of operation can serve as a more economically viable foundation for the fermentative production of other growth-inhibiting bio-compounds.

Author Contributions

Alessandro Brewster: conceptualization, investigation, writing–original draft, visualization. **Arjan Oudshoorn:** methodology, writing–original draft, supervision. **Marion van Lotringen:** methodology, validation, investigation, writing–review and editing. **Pieter Nelisse:** conceptualization, methodology. **Emily van den Berg:** validation, investigation. **Marijke Luttik:** validation, investigation. **Jean-Marc Daran:** conceptualization, writing–review and editing, supervision.

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Conflicts of Interest

Alessandro Brewster, Arjan Oudshoorn, Marion van Lotringen, Pieter Nelisse, and Emily van den Berg are employees at DAB.bio.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.