Research Article

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Potential of sugar beet vinasse as a feedstock for biocatalyst production within an integrated biorefinery context

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Abstract

BACKGROUND: This work explores the feasibility of vinasse as an inexpensive feedstock for industrial biocatalyst production within the context of an integrated sugar beet biorefinery. As an exemplar, production of CV2025 ω-Transaminase (ω-TAm) in *Escherichia coli* BL21 was studied.

RESULTS: Characterisation of vinasse showed that it comprised mainly of glycerol along with several reducing sugars, sugar alcohols, acetate, polyphenols and protein. Preliminary results showed *E. coli* BL21 cell growth and CV2025 ω-TAm production were feasible in cultures using 17% to 25% (v/v) vinasse with higher concentrations demonstrating inhibitory effects. The D-galactose present in vinasse facilitated auto-induction of the pQR801 plasmid enabling CV2025 ω-TAm expression without addition of expensive Isopropyl-β-D-thiogalactopyranoside (IPTG). Assessment of different vinasse pre-processing options confirmed simple dilution of the vinasse was sufficient to reduce the concentration of polyphenols to below inhibitory levels. Optimisation experiments, carried out using a controlled, 24-well microbioreactor platform, showed supplementation of diluted vinasse medium with 10 g L^{-1} yeast extract enabled enhancements of 2.8, 2.5, 5.4 and 3-fold in specific growth rate, maximum biomass concentration, CV2025 ω-TAm volumetric and specific activity, respectively. Investigation into the metabolic preferences of *E. coli* BL21 when grown in vinasse showed a preference for D-mannitol utilisation before simultaneous metabolism of glycerol, D-xylitol, D-dulcitol and acetate. Scale-up of optimised conditions for batch CV2025 ω-TAm production to a 7.5 L stirred tank reactor (STR) was demonstrated based on matched volumetric mass transfer coefficient (k_La). The results showed good comparability with respect to cell growth, substrate consumption and CV2025 ω-TAm production representing over a 700-fold volumetric scale translation. Further enhancements in CV2025 ω-TAm production were possible in the STR when operated at higher k_La values.

CONCLUSION: This work describes the promising application of vinasse for production of microbial enzymes and insights into carbon source utilisation in complex feedstocks. Exploitation of vinasse as a fermentation feedstock could be further extended to other processes involving different microorganisms and target enzymes.

Supporting information may be found in the online version of this article.

Keywords: *E. coli* BL21 metabolism; integrated biorefinery; microbioreactor; sugar beet vinasse; transaminase

INTRODUCTION

Vinasse is the stillage released following distillation of yeast fermentation broth during bioethanol production from either sugarcane or sugar beet.[1] It represents one of the main waste streams discharged by biorefineries in India, South America and Europe, which normally integrate sugar manufacturing plants with ethanol distilleries. In Brazilian and Indian biorefineries for example, the average volumetric production of vinasse is reported to be 10 to 15-fold greater than the ethanol generated.[2,3]

Currently, vinasse is mainly exploited for soil mineralisation and as an additive for fertilisers and animal feeds due to the high content of organic nutrients.[4] However, the market for these applications is not predicted to grow at the same rate as for biofuel production. A large amount of vinasse is still disposed of into water streams, from some manufacturing sites, causing an adverse impact on the aquatic ecosystem due to the presence of toxic compounds.[5] The increasing production of vinasse, as a consequence of the growing demand for bioethanol

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and other biofuels, along with the adverse impact that might result from vinasse disposal, has led to investigation of alternative opportunities for exploitation of vinasse as an industrial feedstock.

The presence of high concentrations of glycerol in the stillage makes vinasse an interesting carbon and energy source for microbial fermentation. A number of studies have addressed the exploitation of vinasse for production of various value-added products such as polyhydroxyalkanoate, PHA,[3,6] and xylitol.[7] Furthermore, vinasse has also been used as one of the medium components for the production of *Spirulina maxima* biomass for nutraceutical applications.[8] The recycling of vinasse to support yeast-based ethanol fermentation has also been addressed.[9] To date, there is no published work reporting the utilisation of vinasse for *Escherichia coli* growth and industrial biocatalyst production. As *E. coli* is widely used for recombinant protein production, it is interesting to investigate the feasibility of developing cost effective fermentation strategies utilising vinasse as a renewable feedstock.

Current work in our laboratory is investigating the utilisation of sugar beet pulp (SBP) as a renewable feedstock within the context of the AB Sugar Wissington biorefinery. Methods for the selective release of pectin from SBP have been established and for separation of the main monosaccharide components: L-arabinose and D-galacturonic acid.[10] These have numerous industrial applications for production of biopolymers[11] and hyperbranched polyesters and plasticisers.[12] We are also interested in the enzymatic conversion of both L-arabinose and D-galacturonic acid into novel chiral aminopolyols that have important applications as pharmaceutical intermediates.[13,14] Within this context, it is interesting to consider the use of vinasse for on-site production of the enzymes used for SBP hydrolysis and in the enzymatic syntheses. This would help integrate the biocatalyst supply chain and improve overall process economics.

One important class of industrial enzyme being examined as part of our integrated biorefinery concept are the ω-transaminases (ω-TAm). ω-TAm catalyzes the transfer of an amine group from an amino-donor, to an acceptor ketone, yielding a secondary amino compound and a ketone by-product.[15,16] The catalytic reaction is mediated by a cofactor, pyridoxal 5′ phosphate (PLP) that binds as a prosthetic group. ω-TAm has been shown to be useful for industrial synthesis of amino acids and chiral amines.[17] The enzyme is important in the pharmaceutical industry as preparative materials for production of neuorological, immunological, anti-hypertensive and anti-infective drugs.[18,19] We have previously cloned and expressed a range of transaminases in *E. coli* for bioconversion applications. One of them is the CV2025 ω-TAm, which is regarded as the best transaminase as it shows high enantioselectivity against the (*S*)-enantiomer for a broad range of amine susbstrates. This enzyme will serve as the model case of the present work.

The aim of this work is to establish a strategy for utilisation of sugar beet vinasse as a fermentation feedstock for production of CV2025 ω-TAm in *E. coli* BL21 (DE3). Furthermore, it will demonstrate the utility of a high throughput microbioreactor platform for rapid screening of fermentation conditions and scale-up of the optimised process. To achieve this, the work will entail characterisation of the sugar beet vinasse, evaluation of vinasse for production of CV2025 ω-TAm and pre-treatment of vinasse for use as a fermentation feedstock. Following establishment of the principle, the work will then focus on optimisation of CV2025 ω-TAm production from vinasse, using a high throughput microbioreactor, and scale-up to a laboratory scale reactor based on an established procedure. The results from this work will provide an early insight into the feasibility of cost effective and sustainable production of industrial biocatalysts within an integrated sugar beet biorefinery.

MATERIALS AND METHODS

Microorganism and enzyme

Escherichia coli BL21 (DE3) containing plasmid pQR801 that incorporates the ω-transaminase gene from *Chromobacterium violaceum* (CV2025 ω-TAm) was used throughout this study. The ω-TAm contains an N-Terminal His_6-tag (GenBank accession no. NP_901695) as established previously.[20] Working stock cultures were stored in a 20% (v/v) glycerol solution at −80 °C.

Media

All chemicals used in this work were obtained from Sigma-Aldrich (Gillingam, UK) unless otherwise stated and were of the highest purity available. Reverse osmosis (RO) water was used in all experimental procedures. Complex medium consisted of (g L^{-1}): glycerol, 30; yeast extract, 5; KH_2PO_4, 13; K_2HPO_4, 10; $MgSO_4{\cdot}7H_2O$, 1; NH_4Cl, 0.2; NaCl, 5 and trace elements, 150 µL L^{-1}. The trace element solution was made up as previously described[21] and prepared in 5 N HCl. The composition is as follows (g L^{-1}): $FeCl_3{\cdot}6H_2O$, 10; $MnSO_4{\cdot}H_2O$, 10; $CaCl_2{\cdot}2H_2O$, 2; $CoCl_2$, 0.2; $ZnSO_4{\cdot}7H_2O$, 2; $Na_2MoO_2{\cdot}2H_2O$, 5; $CuCl_2{\cdot}2H_2O$, 30; H_3BO_3, 30. Before sterilisation, the pH of the medium was adjusted to 7 using 1 mol L^{-1} NaOH or1 mol L^{-1} HCl. The trace element solution was sterilised by filtration through a 0.22 µm pre-sterilised filter (Milipore, Billerica, USA). Phosphate components were autoclaved separately. These and the trace element solutions were added to the other media components aseptically prior to fermentation.

The sugar beet vinasse was supplied from the AB Sugar biorefinery at Wissington, UK. Prior to use, suspended solids were removed by centrifuging the raw vinasse at 4000 rpm for 30 min at 4 °C (Avanti J-E Centrifuge, Beckman Coulter, California, USA). The resulting liquid fraction was then stored at −20 °C. Pre-processing of vinasse was evaluated as illustrated in Fig. 1. In Option 1, clarified vinasse was diluted with RO water and then the pH was adjusted to 7 before it was filtered through a 0.22 µm sterile filter. In Option 2, the vinasse pre-treatment included an additional activated carbon (AC) adsorption step.

Activated carbon adsorption

The pre-treatment of vinasse using AC was performed using a modified literature method.[3] Pre-treatment occurred in a 100 mL shake flask with a working volume of 10 mL, an initial pH of 2 and shaking frequency of 160 rpm at 25 °C. Different concentrations of AC (5–20% (w/v)) and incubation times (1–24 h) were tested. The pre-treated vinasse was then centrifuged at 12 000 rpm for 40 min at 4 °C. Quantification of the remaining polyphenols is as described later. For use as fermentation media, the pre-treated vinasse was then diluted six-fold with RO water and the pH was adjusted to 7 prior to filtration through a 0.22 µm filter.

Microbioreactor (MBR) system

A Micro-24 reactor system (Pall Corporation, Port Washington, USA) was used as a high throughput platform for parallel

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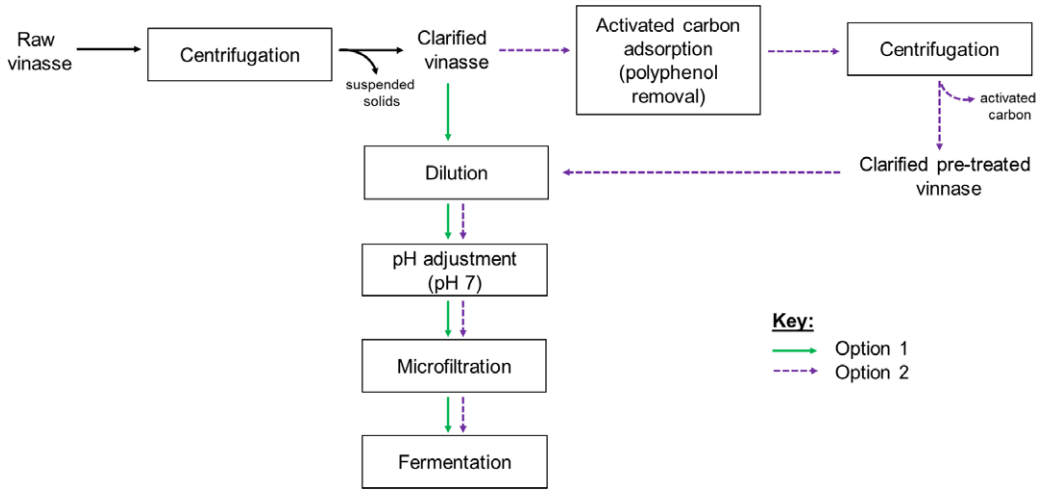

Figure 1. Pre-treatment options evaluated for use of sugar beet vinasse as a fermentation feedstock for industrial enzyme production in *E. coli* BL21.

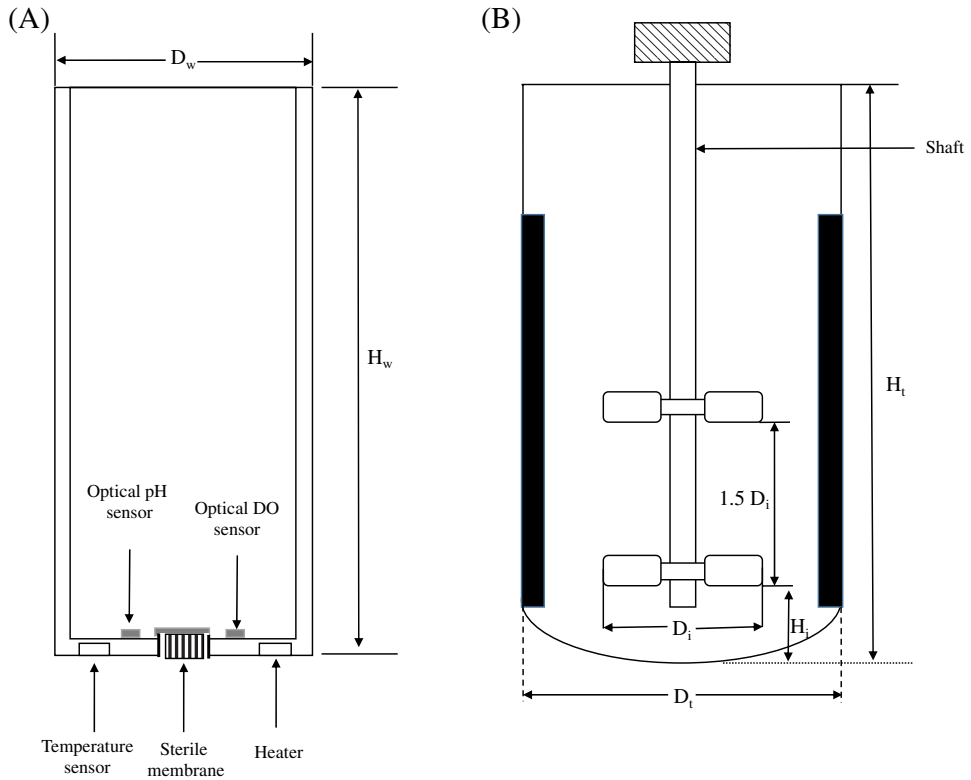

Figure 2. Schematic diagram of (A) an individual well from the REG cassette showing location of sparger (sterile membrane) and optical sensors, and (B) the geometry of the 7.5 L STR (BioFlo 310, New Brunswick, Hertfordshire, UK) used in this work. D_i, impeller diameter; D_t, tank diameter; D_w, well diameter; H_i, impeller height; H_t, tank height; H_w, well height.

evaluation of microbial cell growth and biocatalyst production. The reactor allows 24 simultaneous experiments in a single-use cassette with independent monitoring of process parameters (pH, dissolved oxygen (DO) and temperature) in each well via fluorescent pH and DO sensing patches (PreSens-Precision Sensing, GmbH, Regensburg, Germany). Fig. 2(a) shows a schematic diagram of an individual well from a directly sparged, regular (REG) cassette indicating the location of the sparger (sterile membrane) and optical sensors. The pH was controlled one-sided either by using 15% (v/v) NH_3 for cultures using a complex medium or 100% (v/v) CO_2 in the case of vinasse medium (flow rate of 10 standard cubic centimetres per minute (sccm)). The DO was controlled by a blending of pure oxygen and nitrogen (flow rate of 6.5 sccm for each gas). These gases are introduced to each vessel through a 0.2 µm membrane at the bottom of the well. Thermistors and heaters on the base plate allow the monitoring and control of temperature in each well. The cassette was clamped onto the Micro-24 by an applied vacuum. Control was facilitated via MicroReactor Control software (Pall Corporation, USA). During inoculation, media loading and sampling, the cassette was withdrawn from the Micro-24 and manipulations performed in a biological safety cabinet.

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7.5 L stirred tank reactor (STR)

Batch fermentations were performed in a 7.5 L stirred glass reactor (BioFlo 310, New Brunswick, Hertfordshire, UK) as illustrated in Fig. 2(b). The aspect ratio of the reactor is 1.79:1 and it consists of two, six-bladed Rushton impellers ($D_i = 59$ mm, $D_i/D_t = 0.25$) and four equally spaced baffles. The temperature was monitored by a thermocouple and controlled by the circulation of water in the external jacket of the reactor. The pH was monitored using an Ingold gel filled pH probe (Ingold Messtechnik, Urdorf, Switzerland) while the DO was controlled using a polarographic oxygen electrode (Ingold Messtechnik). Prior to sterilisation, the pH probe was calibrated using standard buffers at pH 7 and 4. The calibration for the DO probe was performed using pure nitrogen and air for the deoxygenation and oxygenation, respectively. The air sparged into the reactor was sterilised using a 0.2 μm filter.

Inoculum preparation

A glycerol stock vial (600 μL) of *E. coli* BL21 (DE3) was aseptically inoculated into 100 mL of sterile medium in a 1 L-baffled shake flask. Except for preliminary studies, the medium formulation used for inoculum preparation was the same as used in the subsequent fermentation. Kanamycin was added to a final concentration of 0.15 g L^{-1}. The culture was incubated on an orbital shaker (Adolf Kuhner AG, Birsfelden, Switzerland) at 250 rpm (shaking diameter 25 mm) for 12 h at 37 °C. The inoculum concentration for subsequent fermentations was standardised at 0.1 g_{dcw} L^{-1} (after addition).

Fermentation conditions

For shake flask cultures, fermentations were carried out in 250 mL baffled flasks with a working volume of 20 mL. Prior to inoculation, sterile kanamycin was added to the medium to a final concentration of 0.15 g L^{-1}. All cultures were shaken on an orbital shaker at 250 rpm at 37 °C. Whenever stated, sterile isopropyl-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mmol L^{-1} at mid-exponential phase. All fermentations were performed in triplicate. Cell growth was followed by removing about 3 mL samples approximately every 2 h followed by optical density (OD) measurement.

MBR fermentations were carried out using REG cassettes, fitted with Type D caps with a working volume of 6.5 mL in each well. The wells were filled aseptically with a sterile medium followed by the addition of kanamycin to a final concentration of 0.15 g L^{-1} prior to the inoculation. Antifoam, sterile 50% (v/v) propylene glycol (PPG), was added to each well at a working concentration of 1 mL L^{-1} as required. Cultures were shaken at 800 rpm throughout the cultivation. The set-points in each well were at a temperature of 37 °C and a pH and DO of 7% and 30%, respectively. In the case of a synthetic medium, the culture was induced with 0.1 mmol L^{-1} IPTG after 6 h of inoculation. Cell growth was monitored by removing 300 to 650 μL samples at specified times and measuring OD.

STR fermentations were performed at a working volume of 5 L. The vessel was initially filled up with 4 L of water with 10 g L^{-1} yeast extract and was then sterilised at 121 °C for 20 min. Upon cooling to room temperature, concentrated vinasse was then added to the vessel. Filtered kanamycin with a final concentration of 0.15 g L^{-1} was aseptically added to the reactor prior to inoculation. The appropriate volume of inoculum that corresponded to a standardised initial cell concentration at 0.1 g_{dcw} L^{-1} was aseptically added to the reactor. Cultures were performed at 37 °C and pH was maintained at pH 7 by the addition of 8.5% H_3PO_4 (v/v) or 28% NH_4OH (v/v). The DO was controlled at 30% via cascade control of air and pure oxygen at 5 L min^{-1} (1 vvm). Sterilised antifoam, PPG, was added periodically as required. Cell growth was followed by removing 5 mL samples and measuring OD.

Cell recovery and lysis

Harvested culture broth was centrifuged at 13 000 rpm for 40 min at 4 °C (Hettich Universal 320 Benchtop Centrifuge, GMI Inc, Tuttlingen, Germany). The supernatant was used for glycerol, acetate, sugar and sugar alcohol determination as described below. The pellets were resuspended in 2 mmol L^{-1} pyridoxal 5′-phosphate (PLP) in 50 mmol L^{-1} 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7.4. The cells were then disrupted using a Soniprep 150 sonicator (MSE Sanyo, Japan) with 10 cycles of 10 s on and 10 s off at 10 μm amplitude. The disrupted cells suspension was again centrifuged and the clarified lysate recovered for protein quantification and enzyme activity analysis.

Measurement of volumetric mass transfer coefficient (k_La)

Determination of k_La in the MBR was based on the dynamic gassing out method.[22] In the deoxygenation stage, the pH control loop was used to sparge pure nitrogen until the DO readout reached 0%. Following that, during the oxygenation stage, both the DO and pH control systems were turned off. The shaking frequency was set at a predetermined value, between 500 and 800 rpm, while the aeration rate was varied from 1.0 to 10 sccm. Air was then sparged until the DO reached saturation. The change in the measured DO profile was recorded by the MicroReactor software. All experiments were conducted at 37°C. The wells were filled with 6.5 mL of fermentation medium containing 1 mL L^{-1} PPG.

The k_La values were determined from Eqn (1) by plotting $\ln(1 - C_L/C_L^*)$ against time and k_La represents the slope of the graph.

$$\ln\left(1 - \frac{C_L}{C_L^*}\right) = -k_L a\,(t) \tag{1}$$

where C_L is the dissolved oxygen concentration in the liquid phase (kg m^{-3}) and C_L^* is the saturated oxygen concentration in the liquid phase (kg m^{-3}). Whenever the value of $1/k_La$ is less than the probe response time, then it is necessary to correct the k_La value by using Eqn (2).

$$C_p = \frac{1}{t_m - \tau_p}\left[t_m \exp\left(\frac{-t}{t_m}\right) - \tau_p \exp\left(\frac{-t}{\tau_p}\right)\right] \tag{2}$$

where C_p denotes the normalised DO at time t, $t_m = 1/k_La$ (s) and τ_p represents the probe response time (s).

Measurement of k_La values in the 7.5 L STR was also based on the dynamic gassing out method. All experiments were performed at 37 °C with an agitation speed of 200 to 1000 rpm and airflow rate of 5 to 10 L min^{-1}. Changes in DO were monitored and recorded by DASGIP Control 4.5 software (Eppendorf, Juelich, Germany). The probe response time in the 7.5 L STR was determined by first immersing the probe in a beaker containing water that had been deoxygenated by nitrogen gas. Once the DO readout was stabilised at 0%, the probe was transferred swiftly to the reactor that contained water at 100% saturation.

For the 7.5 L STR the measured k_La values were correlated to reactor operating conditions based on a correlation of the form shown in Eqn (3).[18]

$$k_L a = a\left(N^3 D_i^2\right)^b \left(v_s\right)^c \tag{3}$$

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where N is agitation speed (rpm), D_i is impeller diameter (m), v_s is superficial velocity (m s^{-1}) and a, b and c represent fitted coefficients. Experimental k_La values were used as initial 'guess' values to solve for the coefficients (a, b and c) using the multiple linear regression analysis module in MATLAB (Matlab R14, MathWorks, USA).

Analytical techniques

OD measurements of culture broth were determined at 600 nm using an Ultrospec 500 Pro spectrophotometer (Amersham Bioprocess, Amersham, UK). Whenever necessary, the sample was diluted with RO water such that the measured OD value was in the range 0.2–0.8. The OD was then translated to a dry cell weight (DCW) based on a previously established standard curve for each medium.

Total protein concentration in cell lysates was determined based on the Bradford assay.[23] Bradford reagent was used along with bovine serum albumin (BSA) as the standard protein. 50 µL of diluted clarified lysate was mixed with 1 mL of Bradford reagent and the mixture was incubated at room temperature for 5 min. The absorbance of the reaction mixture was then measured at 595 nm and translated into protein concentration based on a previously established BSA calibration curve.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed on a Mini −155 Protean II system (Bio-Rad Laboratories Inc., Hemel Hempstead, UK). Precast gels (10 cm × 12 wells) of SDS 10% (w/v) were run in Tris-glycine buffer system. Clarified lysate was mixed with Lamelli 4× concentrated protein sample buffer and heated to 99 °C in a polymerase chain reaction (PCR) machine (Techne Ltd, Cambridge, UK) for 16 min. Following that, 15–20 µg of the total protein of the clarified lysate suspension was loaded in each lane. The first lane was loaded with 3 µL of EZ-Run Prestained Rec Protein Ladder (Thermo Fisher Scientific Inc, UK) as the protein molecular weight marker. The gel was run at a power of 175 V for about 40 min. The gel was stained with an appropriate amount of Coomassie Blue solution, that consists of 0.1% (w/v) Coomassie Blue R-250, 40% (v/v) methanol and 10% (v/v) acetic acid, for 1–2 h on a rocking table (Genetic Research Instrumentation Ltd., Essex, UK). Next, the gel was de-stained overnight with de-staining buffer (10% (v/v) acetic acid, 30% (v/v) methanol and 60% (v/v) RO water). The gel was finally visualized and analysed on a Gel–Doc–it bioimaging system using Labworks 4.5 software (Bioimaging Systems, Cambridge, UK).

The activity of CV2025 ω-TAm was determined based on the reaction between methylbenzylamine (MBA) and pyruvate, yielding acetophenone (AP) and L-alanine.[24] 20 µL of lysate sample was mixed with 180 µL of substrate buffer (50 mmol L^{-1} phosphate buffer pH 7.4 containing 11 mmol L^{-1} MBA, 11 mmol L^{-1} pyruvate, 1.25% (v/v) dimethyl sulfoxide (DMSO) and 0.1 mmol L^{-1} PLP) in a 96-well, flat-bottomed microtiter plate (Radleys Discovery Technologies, Essex, UK). Throughout the reaction, the increasing absorbance was measured at 280 nm and at 30 °C, every 20 s for 2 min. The extinction coefficient, ε of AP obtained in this work was 0.8477 mmol L^{-1} cm^{-1}. The activity was expressed in units (U) where 1 U represents the amount of enzyme that catalyses production of 1 µmole of AP per minute.

Polyphenols was determined according to the Folin-Ciocalteu (FC) method.[25] Gallic acid (GA) was used as a standard. 100 µL of the test sample was mixed with 100 µL of FC reagent and equilibrated for 2 min prior to addition of 800 µL of 5% (v/v) sodium carbonate solution. The reaction mixture was placed in a water bath at 40 °C for 20 min. Following that, the absorbance of the mixture was read at 740 nm and was then translated into GA concentration based on a standard curve.

Table 1. Characterisation of sugar beet vinasse composition from two separate batches provided by AB sugar (Wissington biorefinery, UK). Errors represent one standard deviation about the mean (n = 3)

Parameters	Batch 1	Batch 2
pH	4.9	5.4
Glycerol (g L^{-1})	187.6 ± 2.8	183.6 ± 2.3
D-Mannitol (g L^{-1})	8.0 ± 0.0	5.3 ± 0.1
D-Galactose (g L^{-1})	5.5 ± 0.0	10.2 ± 0.1
D-Xylitol (g L^{-1})	5.4 ± 0.1	6.5 ± 0.1
D-Fructose (g L^{-1})	2.0 ± 0.0	2.1 ± 0.1
D-Dulcitol (g L^{-1})	2.0 ± 0.0	1.5 ± 0.0
Acetate (g L^{-1})	1.7 ± 0.0	7.2 ± 0.1
Total protein (g L^{-1})	5.1 ± 0.1	11.5 ± 0.2
Polyphenols (g_{eq} L^{-1})	6.0 ± 0.0	7.1 ± 0.2

Glycerol and acetate were quantified using a Dionex HPLC system consisting of an ASI-100 automated sample injector, P680 HPLC pump and STH 585 column oven. The system was fitted with an Aminex HPX-87H ion exclusion column (300 mm × 7.8 mm, Bio-Rad Labs, Richmond, CA, USA), injection volume of 10 µL, column oven temperature of 60 °C with 5 mmol L^{-1} H_2SO_4 as mobile phase at a flow rate of 0.6 mL min^{-1} for 30 min, monitored with a refractive index detector (RefractoMax 520 ERC) controlled by Chromeleon client 7.20 software. Retention times for glycerol and acetate were 13.3 and 14.8 min respectively.

Sugars and sugar alcohols were quantified using a Dionex ion chromatography system ICS-5000+ (Thermo Fisher Scientific Inc, Sunnyvale, USA) with AminoPac SA10 column (4 × 250 mm). The separation was performed at 30 °C for 30 min using gradient elution with 0.5–15 mmol L^{-1} KOH as the mobile phase at a flow rate of 0.25 mL min^{-1} and controlled by Chromeleon client 7.20 software. Retention times for D-xylitol, D-dulcitol, D-mannitol, D-maltitol, D-galactose and D-fructose were 2.3, 2.6, 2.8, 6.2, 12.1 and 18.7 min, respectively.

RESULTS AND DISCUSSION

Characterisation of sugar beet vinasse

Vinasse is a heterogenous mixture consisting of suspended solids and a 'blackish' liquid fraction. Unlike previous reports that focused on environmental aspects,[3,5] vinasse characterisation in this study focused on the composition of fermentable sugars and related compounds. Table 1 outlines the composition of vinasse obtained from two different batches throughout this work. Vinasse exhibited an acidic pH in the range 4.9–5.4, which is in agreement with literature values.[3,4,8,26,27] The acidic pH of vinasse can be associated with organic acids formed during yeast fermentation.

The concentration of glycerol in vinasse was found to range from 184 to 188 g L^{-1}, making it a rich source of fermentable carbon and energy. The vinasse also contains several sugars such as D-fructose and D-galactose. These sugars are believed to originate from D-raffinose, a trisaccharide composed of D-galactose, D-glucose and D-fructose, which is found in the sugar beet.[28] No D-glucose or D-raffinose were detected in vinasse and are assumed to be utilised during the preceeding yeast fermentation. Additionally, sugar alcohols including D-mannitol, D-xylitol and D-dulcitol were also

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identified with concentrations of less than 10 g L^{-1}. The presence of sugar alcohols as by-products of ethanol-producing yeasts has been discussed in the literature.[29] The type of sugar alcohols and their amounts produced vary with different yeast strains.[30]

Apart from potential fermentable carbon sources, vinasse also possesses cell growth inhibitors such as acetate and polyphenols. The acetate concentration ranged from 1.7 to 7.2 g L^{-1}, which is in agreement with some previous work[31] but lower than reported in others.[27] The vinasse polyphenol concentration ranged between 6.6 and 7.1 g L^{-1} (expressed as GA equivalent). Previous works reported the concentration of polyphenols in vinasse in the range between 1.9 and 2.5 g L^{-1},[7] or in a much lower amount (<1 g L^{-1}).[6]

Literature also indicates that the variation of the physico-chemical characteristics of vinasse can be attributed to the type of crop that it originated from[5] as well as variation in the ethanol fermentation and distillation processes. In general, the characterisation results obtained here suggest that vinasse has potential for use as a fermentation feedstock as explored in the following section.

Preliminary studies on vinasse as a fermentation feedstock

Preliminary batch fermentation studies were carried out in shake flasks. The initial aim was to study the influence of vinasse concentration and IPTG induction on cell growth and CV2025 ω-TAm activity. Different concentrations of vinasse (17–100% (v/v)) were prepared using RO water as a diluent. The pre-processing procedure is described as Option 1 in Fig. 1, which involved simple dilution and pH adjustment of the vinasse.

Figure 3 depicts *E. coli* BL21 (DE3) growth kinetics using vinasse and a complex fermentation medium as a control. It was observed that in both IPTG-induced and non-induced cases, the vinasse concentration of 50% and 100% (v/v) exerted an inhibitory effect on cell growth. The inhibitory effect is most likely caused by the polyphenols present in vinasse that remained at concentrations between 3 and 6 g_{eq} L^{-1}. The influence of acetate on cell growth is not thought to be significant since its maximum concentration of 1.7 g L^{-1} (from Batch 1, Table 1, before dilution) is much lower than the inhibitory level reported for *E. coli* of about 5 g L^{-1}.[32]

In fermentations using 17% and 25% (v/v) vinasse, comparable cell growth profiles were observed in both non-induced and induced cultures. The maximum biomass concentration obtained in non-induced cultures using 17% and 25% (v/v) vinasse was about 70–80% of that using the complex medium while for induced cultures it was 60–63%. The good cell growth suggests that the *E. coli* cells are tolerant to the lower concentration of polyphenols present. Previous literature reports have suggested the feasibility of sugar cane/sugar beet vinasse as growth media for several microorganisms such as *Chlorella vulgaris*,[33] *Haloarcula marismortui*,[3] *Spirulina maxima*[8] and *Debaryomyces hansenii*[7] but there has been no study reported for *E. coli* thus far.

The corresponding CV2025 ω-TAm titres from the fermentations using 17% and 25% (v/v) vinasse were also determined. Figure 4 shows the comparison of CV2025 ω-TAm specific activity obtained from the non-induced and IPTG-induced fermentations using both vinasse and a standard complex medium. In induced cultures, the results showed that the CV2025 ω-TAm specific activities achieved at 12 and 24 h using both vinasse media were comparable and consistent with the levels of cell growth (Fig. 3). The maximum specific activity attained in vinasse medium was about 70–72% of that in the complex medium. Interestingly, CV2025 ω-TAm expression was observed in the non-induced vinasse fermentations. The maximum CV2025 ω-TAm specific activities achieved in 17% and 25%

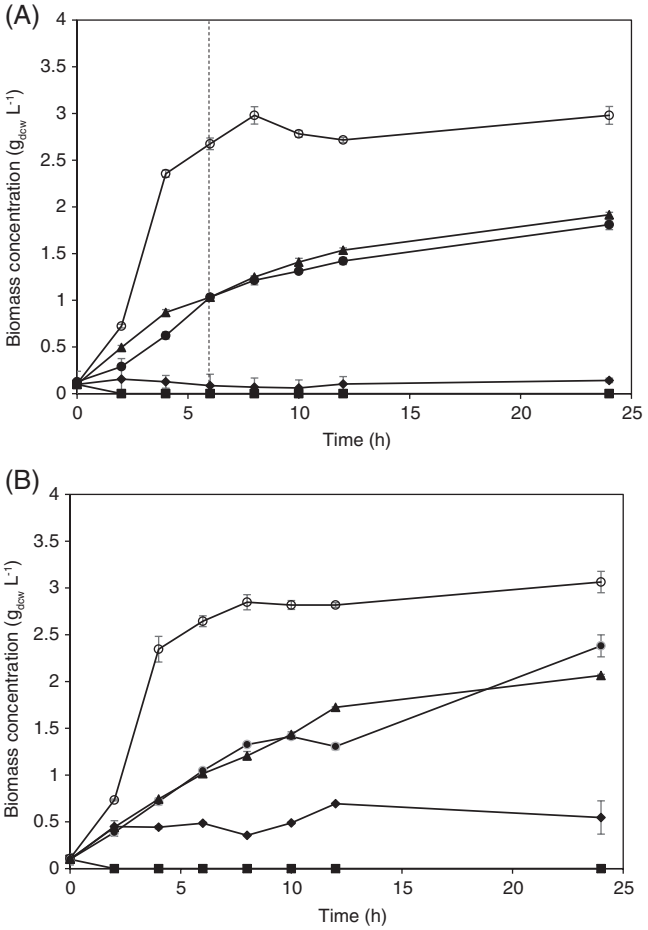

Figure 3. Comparison of batch fermentation kinetics of *E. coli* BL21 cultured in shake flasks with (A) IPTG induction, and (B) no IPTG induction using: (○) complex medium; (▲) 17% (v/v) vinasse; (●) 25% (v/v) vinasse; (◆) 50% (v/v) vinasse; (■) 100% (v/v) vinasse. Vertical dotted line indicates the point of IPTG induction. Error bars denote one standard deviation about the mean (n = 3).

(v/v) vinasse medium were 90 and 89 U g_{dcw} L^{-1}, respectively; this is 2.6-fold higher than that obtained in the non-induced complex medium fermentations and around 80% of the maximum value attained in the complex medium cultures with IPTG induction.

SDS-PAGE analysis of the soluble intracellular protein obtained from each fermentation, is shown in Supporting Information, Figure S1. This confirms the expression of CV2025 ω-TAm (molecular weight 51 kDa) in both induced and non-induced cultures employing vinasse medium. CV2025 ω-TAm expression in the non-induced cultures is most likely due to the presence of significant concentrations of D-galactose in vinasse (Table 1). This can act as an inducer of enzyme expression from plasmids such as pQR801 that contain the *lac* promoter due to the similarity in structure to IPTG and lactose.[34]

To confirm this auto-induction by D-galactose, we carried out a separate experiment (data not shown) by incorporating D-galactose at similar concentration to that in 17% (v/v) vinasse, in the complex medium from the beginning of the cultivation. The results showed significant expression of CV2025 ω-TAm confirming the role of D-galactose as an inducer to the *lac operon* in *E. coli* BL21 (DE3) pQR801. As IPTG is expensive and toxic, elimination of its use in the fermentation using vinasse medium gives an additional benefit to vinasse as a fermentation feedstock. This is

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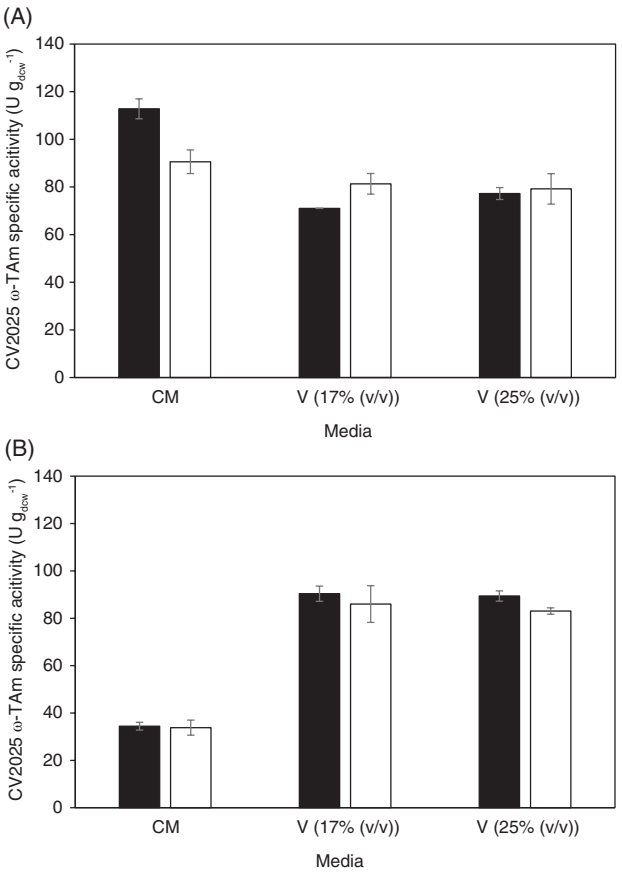

Figure 4. Specific activity of CV2025 ω-TAm from fermentations with (A) IPTG induction, and (B) no IPTG induction at: (■) 12 and (□) 24 h. Data shown for fermentations from Fig. 2 using complex medium (CM), 17% (v/v) and 25% (v/v) vinasse (V). Error bars denote one standard deviation about the mean (n = 3).

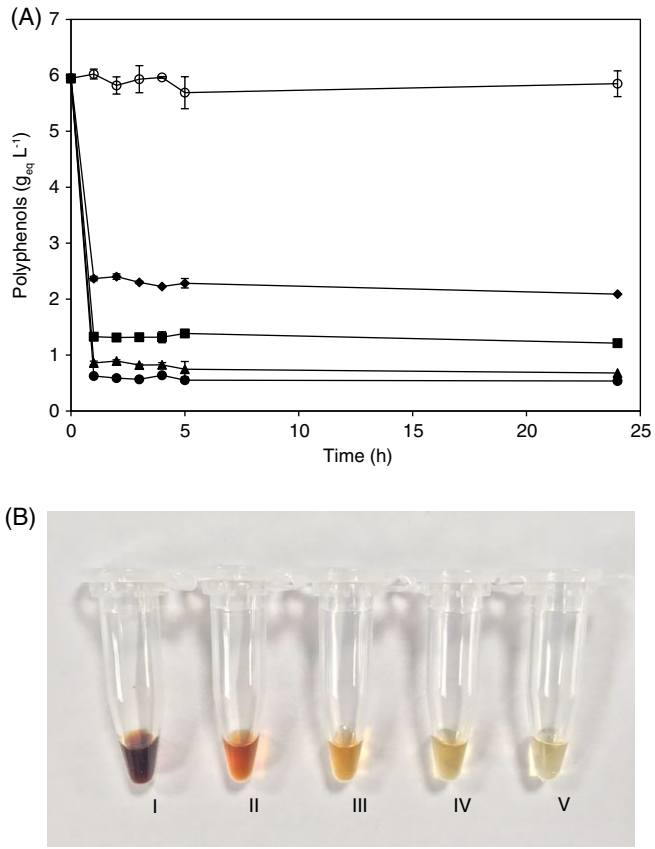

Figure 5. (A) Kinetics of polyphenol removal in untreated (control) and activated carbon (AC) pre-treated vinasse using different concentrations of AC: (◆) 5% (w/v); (■) 10% (w/v); (▲) 15% (w/v); (●) 20% (w/v) and (○) control. Error bars denote one standard deviation about the mean (n = 3). (B) Appearance of the untreated and AC pre-treated vinasse from the experiments shown in Fig. 4(a): I, untreated vinasse; II, pre-treated vinasse 5% AC (w/v); III, pre-treated vinasse 10% AC (w/v); IV, pre-treated vinasse 15% AC (w/v); V, pre-treated vinasse 20% AC (w/v). Samples taken and photographed after 24 h of incubation.

an important finding since the cost of IPTG for enzyme induction would represent a significant contribution to the overall Cost of Goods at large scale.

While auto-induction in vinasse-based fermentation media has not previously been reported in the literature, auto-induction by D-galactose in synthetic media has been noted in several published works.[34,35] Auto-induction of benzaldehyde lyase production by *E. coli* BL21 when sugar beet molasses was used as the fermentation medium has been reported.[36] It could thus be expected that other types of sugar-based wastes that contain galactose might potentially exhibit the auto-induction phenomenon when utilised as production media.

Pre-treatment of vinasse by activated carbon adsorption

Two options to prepare the vinasse for use in fermentation were evaluated (Fig. 1). While Option 1 involved simple dilution, Option 2 involved AC adsorption for polyphenol removal. Literature shows that adsorption processes using AC or other adsorbent materials are among the best options for removal of polyphenolic compounds from natural feedstocks.[26]

The influence of AC concentration and incubation time on polyphenol removal was first investigated. Fig. 5(a) shows the change in polyphenol concentration in vinasse using different pre-treatment conditions. The profiles are compared with a control case that had no AC added. The extent of polyphenol reduction was found to be proportional to the concentration of AC used. Pre-treatment of vinasse with 15% and 20% (w/v) AC led to a maximum polyphenol removal of 85–89%, comparable with the findings of Pramanik and co-workers.[3] Overall, a rapid decrease in polyphenol concentration was observed whereby the adsorption process reached an equilibrium concentration after 1 h.

Figure 5(b) compares the physical appearance of the untreated and pre-treated vinasse using different AC concentrations. As clearly shown, decolourisation was achieved in the pre-treated vinasse. The intensity of the brown colour was proportional to the concentration of polyphenol remaining in the pre-treated vinasse confirming that the colour is associated with the presence of the polyphenolic compounds.

The impact of untreated and AC pre-treated vinasse on *E. coli* BL21 (DE3) fermentations and CV2025 ω-TAm production was next investigated. All of the AC pre-treated vinasse media were first diluted with RO water to 17% (v/v) based on the previous results. Figure 6(a) shows the time course of cell growth using the dilute untreated and pre-treated vinasse media at different AC concentrations. Generally, all profiles followed a similar trend with regard to cell growth whereby a stationary phase was reached after about 12 h of incubation. The specific growth rate observed in all fermentations using the pre-treated vinasse media ranged between

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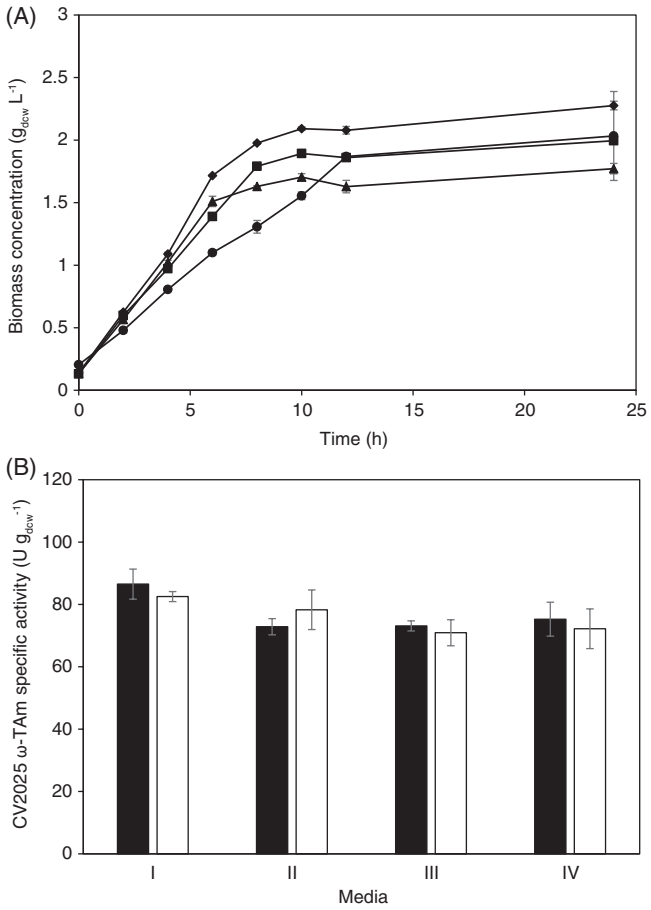

Figure 6. (A) Comparison of batch fermentation kinetics of *E. coli* BL21 cultured in shake flasks using diluted vinasse, untreated and pre-treated with different AC concentrations: (◆) 5% (w/v); (■)10% (w/v), (▲)15% (w/v) and (●) no AC. (B) Specific activity of CV2025 ω-TAm from *E. coli* BL21 fermentations shown in Fig. 5(a) using dilute untreated vinasse (I) and dilute pre-treated vinasse with 5% (w/v) AC (II), 10% (w/v) AC (III) and 15% (w/v) AC (IV) at: (■) 12 and (□) 24 h. Error bars denote one standard deviation about the mean (n = 3).

0.21 and 0.25 h^{-1}, which were higher than in the culture using untreated vinasse (0.17 h^{-1}). The maximum biomass concentrations obtained from cultures using both untreated and pre-treated vinasse were comparable and in the range 1.8–2.3 g_{dcw} L^{-1}, indicating that AC pre-treatment has limited further impact on cell growth after vinasse dilution.

Figure 6(b) depicts the CV2025 ω-TAm specific activity attained in these cultures after 24 h incubation. The CV2025 ω-TAm specific activities attained in cultures employing AC pre-treated vinasse were found to be comparable with that achieved using the dilute, untreated vinasse. Comparing this performance with the results obtained in the preliminary studies, it is clear that the polyphenol concentration in 17% and 25% (v/v) vinasse media, which were approximately 1.0 and 1.5 g_{eq} L^{-1}, respectively, did not significantly inhibit cell growth and biocatalyst production. These results suggest that removal of the polyphenols in vinasse before fermentation is not required provided that dilution reduced the level of polyphenols below the minimum inhibitory concentration. Simple dilution of the vinasse is less laborious than AC adsorption and would be easy to implement within a biorefinery context using recycled water streams. Subsequent work will thus be based solely on dilution (Option 1, Fig. 1)

Table 2. Kinetic parameters for *E. coli* BL21 growth on complex and various vinasse-based media during medium optimisation experiments in the controlled microbioreactor system. Biomass growth profiles shown in Supporting Information, Figure S2. Errors represent one standard deviation about the mean (n = 3). DV, dilute vinasse; TE, trace elements; YE, yeast extract.

Medium	Specific growth rate, μ (h^{-1})	Maximum biomass concentration, X_{max} (g_{dcw} L^{-1})
Complex medium	0.36 ± 0.01	11.5 ± 0.6
DV only	0.13 ± 0.00	4.5 ± 0.2
DV + 150 μL L^{-1} TE	0.12 ± 0.01	4.7 ± 0.3
DV + 1 g L^{-1} NH_4Cl	0.27 ± 0.01	6.8 ± 0.1
DV + 1 g L^{-1} $(NH_4)_2SO_4$	0.26 ± 0.02	7.7 ± 0.1
DV + 1 g L^{-1} YE	0.34 ± 0.02	6.1 ± 0.3
DV + 5 g L^{-1} NH_4Cl	0.22 ± 0.01	7.9 ± 0.1
DV + 5 g L^{-1} $(NH_4)_2SO_4$	0.23 ± 0.01	8.4 ± 0.2
DV + 5 g L^{-1} YE	0.35 ± 0.03	7.7 ± 0.5
DV + 10 g L^{-1} YE	0.36 ± 0.02	11.4 ± 0.2
DV + 15 g L^{-1} YE	0.38 ± 0.01	13.1 ± 0.3

Optimisation of CV2025 ω-TAm expression using vinasse medium

Following the establishment of the pre-processing steps for vinasse utilisation, subsequent work aimed to further enhance biocatalyst production using the high throughput, controlled MBR to explore a range of further medium additions, i.e. trace elements and nitrogen-containing substrates such as yeast extract, NH_4Cl and $(NH_4)_2SO_4$, on cell growth and biocatalyst production. The rationale behind these supplementation experiments was to replace any nutrients in the vinasse that have either been fully utilised during yeast fermentation or are degraded during distillation. Table 2 compares the fermentation kinetic parameters for *E. coli* BL21 (DE3) grown on synthetic and various vinasse-based media. The corresponding maximum CV2025 ω-TAm volumetric and specific activities are shown in Fig. 7.

Assessment of the effect of trace elements on biocatalyst production showed that there was no significant effect on cell growth rate or maximum biomass concentration. Even if these had been largely utilised in the initial bioethanol fermentation any remaining nutrients would have been concentrated during the distillation step. While an increase in CV2025 ω-TAm specific activity by 21% was observed in the supplemented cultures, the volumetric activity was comparable with that in the base vinasse medium.

Evaluation of nitrogen source addition in the range of 1–5 g L^{-1} had a more notable impact. The maximum biomass concentration and specific growth rate were found to increase 1.4–1.9-fold and 1.7–2.7-fold, respectively (Table 2). In particular, it was observed that the addition of yeast extract increased CV2025 ω-TAm production in comparison with the other nitrogenous substrates evaluated. For example, addition of 1 g L^{-1} of $(NH_4)_2SO_4$ to the dilute vinasse promoted a 1.4-fold increase in CV2025 ω-TAm volumetric activity whereas in the fermentation supplemented with 1 g L^{-1} of yeast extract, the titre increased 3.4-fold. Supplementation of vinasse with yeast extract at 5 g L^{-1} showed the greatest improvement in maximum biomass concentration and CV2025 ω-TAm specific activity, increasing 1.7 and 3.7-fold, respectively compared with the non-supplemented culture. The presence of vitamins, minerals and transcription enhancers, such as cyclic adenosine

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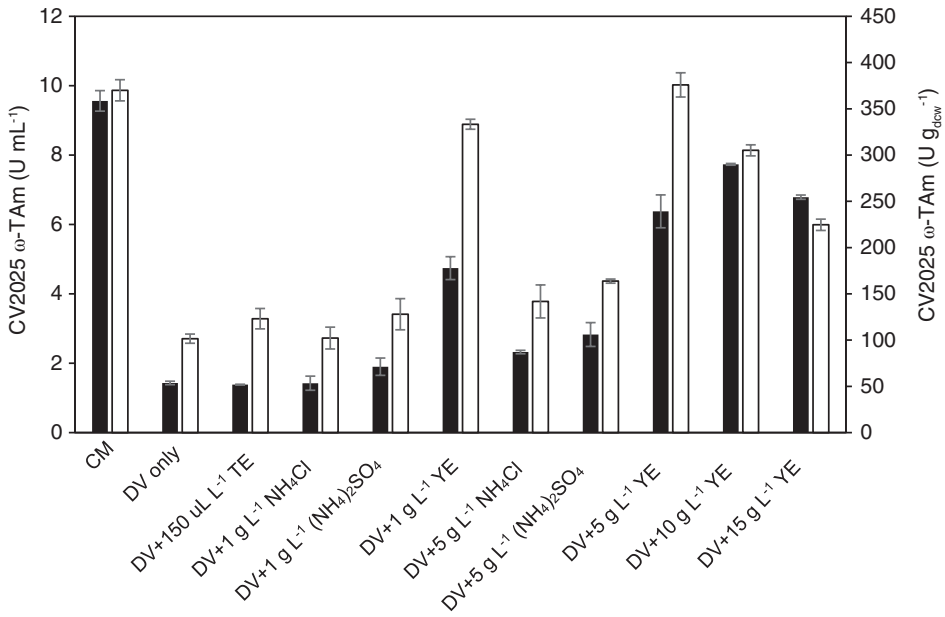

Figure 7. Optimisation of CV2025 ω-TAm expression using a controlled microbioreactor system: maximum (■) volumetric and (□) specific activity of CV2025 ω-TAm from *E. coli* BL21 fermentations using complex medium and various vinasse-based media. Error bars denote one standard deviation about the mean (n = 3). CM, complex medium; DV, dilute vinasse; TE, trace elements; YE, yeast extract.

monophosphate (cAMP), in the yeast extract[37] may also facilitate recombinant protein expression as previously reported.[38,39]

Yeast extract supplementation was subsequently extended by investigating the effect of higher concentrations from 10 to 15 g L^{-1}. It was found that the maximum biomass concentration increased proportionately with the increase in yeast extract concentration. In terms of the cell growth rate, the cultures supplemented with 10 to 15 g L^{-1} yeast extract yielded an enhancement of about 2.8 to 2.9 times higher than the non-supplemented culture.

Increasing the yeast extract concentration to 10 g L^{-1} had a positive effect on CV2025 ω-TAm production. With regard to enzyme titre (U mL^{-1}), the culture supplemented with 10 g L^{-1} yeast extract achieved the highest activity approximately 5.4-fold greater than the non-supplemented culture. Moreover, the specific activity obtained in this optimised vinasse medium represented 81% of that attained using the complex medium.

Within the context of an integrated sugar beet biorefinery it is noted that commercial yeast extract is typically manufactured from *Saccharomyces cerevisae,* which is the same strain used for bioethanol production at the AB Sugar Wissington facility. There is thus the potential to integrate lysis of the yeast recovered post-fermentation to generate the required yeast extract on-site. This has been reported for lactic acid production using autolysed yeast[40] or by enzymatic hydrolysis[41] as alternatives to conventional thermochemical processing. In either case, the on-site production of yeast extract may eliminate the dependence on external sources and may help minimise the overall cost of production.

Understanding *E. coli* BL21 (DE3) metabolism in vinasse medium

Having shown the utility of vinasse as a fermentation medium, it is of fundamental interest to understand the differential rates of fermentable carbon source metabolism by *E. coli* BL21 (DE3) when grown on this complex feedstock. Figure 8(a) shows the time course of cell growth and substrate consumption throughout the fermentation using diluted vinasse supplemented with 10 g L^{-1} yeast extract. The initial phase of cell growth up to 12 h was accompanied by a decrease in D-mannitol concentration while the concentration of all the other sugars and sugar alcohols remained largely unchanged. A decrease of glycerol concentration was then observed, which only began once the D-mannitol had been fully depleted. This occurred during the mid-exponential phase of cell growth between 12 and 24 h. Additionally, it was seen that along with the reduction of glycerol there was a simultaneous drop in D-xylitol and D-dulcitol concentrations as well as acetate. Acetate was seen to be generated particularly during the period of D-mannitol metabolism where a concentration of up to 6 g L^{-1} was produced during exponential growth before it was re-metabolised. Throughout the fermentation the concentrations of D-fructose and D-maltitol remained constant indicating that they were not utilised during *E. coli* BL21 (DE3) growth in the vinasse medium.

The above results suggest that D-mannitol was the favoured carbon and energy source during the initial stage of *E. coli* BL21 (DE3) growth in the vinasse medium followed by glycerol. This may be attributed to carbon catabolite repression (CCR), whereby the secretion of enzymes responsible for the metabolism of the secondary substrate is supressed when the preferred carbon and energy source is present.[42] Examples of CCR phenomena in *E. coli* include repression of lactose metabolism in the presence of glucose[43] and consumption of arabinose over xylose.[44] To date, the occurrence of CCR between D-mannitol and glycerol has not been reported in the literature.

As discussed previously in the characterisation studies, there was no D-glucose detected in the vinasse. Glucose is known to be the preferred substrate for many microorganisms including *E. coli*.[42] The phenomenon of diauxic growth in *E. coli* BL21 (DE3) between glucose, as the primary substrate and other sugars such as sorbitol, rhamnose, xylose, arabinose and galactose[43] has previously been reported. To further elucidate the principles of *E. coli* BL21 (DE3)

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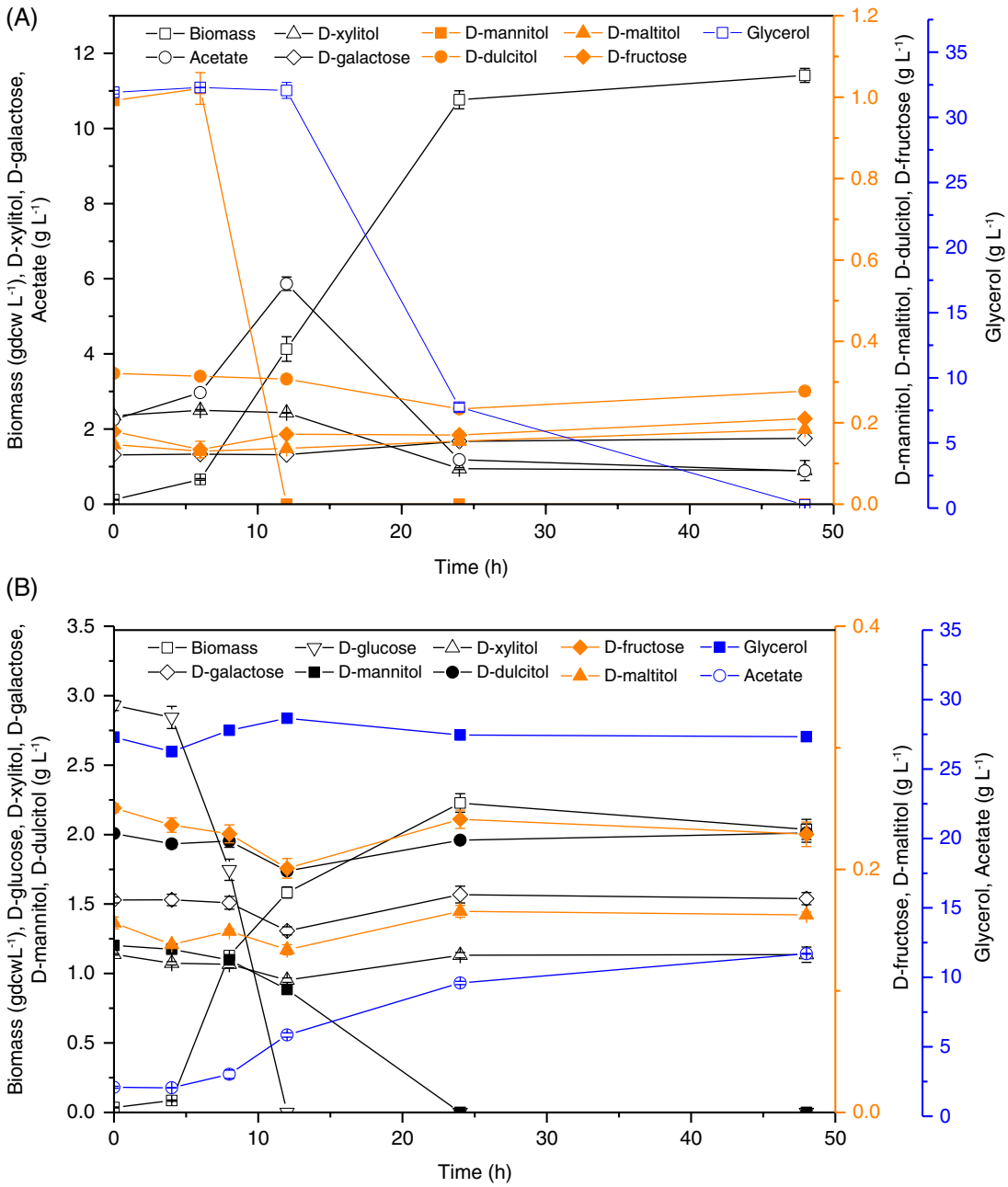

Figure 8. Carbon source utilisation, cell growth and acetate formation during batch fermentation of *E. coli* BL21 cultured on (A) dilute vinasse medium supplemented with 10 g L^{-1} yeast extract, and (B) dilute vinasse medium supplemented with 10 g L^{-1} yeast extract and 3 g L^{-1} D-glucose in the controlled microbioreactor system. Error bars denote one standard deviation about the mean (n = 3).

metabolism when grown on vinasse medium, cultures were carried out in the presence of added D-glucose. A low concentration of D-glucose was chosen here to facilitate its rapid consumption during the fermentation.

Figure 8(b) illustrates the time course of cell growth and carbon source consumption in the fermentation using the vinasse medium supplemented with 10 g L^{-1} yeast extract and 3 g L^{-1} D-glucose. In this case D-glucose was metabolised first with a rapid decrease in concentration seen between 4 and 12 h; this corresponded to a reciprocal increase in biomass concentration from 0.1 to 1.6 g L^{-1}. The consumption of D-mannitol up to 12 h was small with only about 26% being used. Significant utilisation of D-mannitol was then observed between 12 and 24 h when the D-glucose concentration became limiting. This suggests that although D-glucose and D-mannitol were found to be utilised simultaneously, *E. coli* BL21 (DE3) metabolism favours D-glucose over D-mannitol. In an early report on D-mannitol metabolism in *E. coli*,[45] it was revealed that when *E. coli* cells were grown in the presence of D-glucose and D-mannitol, the metabolic pathway of the latter is not hampered by the presence of the former.

In the culture shown in Fig. 8(b), the switch to a third carbon source after D-mannitol consumption was not observed (Fig. 8(a)). This is more likely due to retardation of cell growth associated with the accumulation of acetate that reached nearly 10 g L^{-1} by 24 h. As a consequence, the maximum biomass concentration only reached 2.2 g L^{-1} compared with 11.4 g L^{-1} when cultured on the

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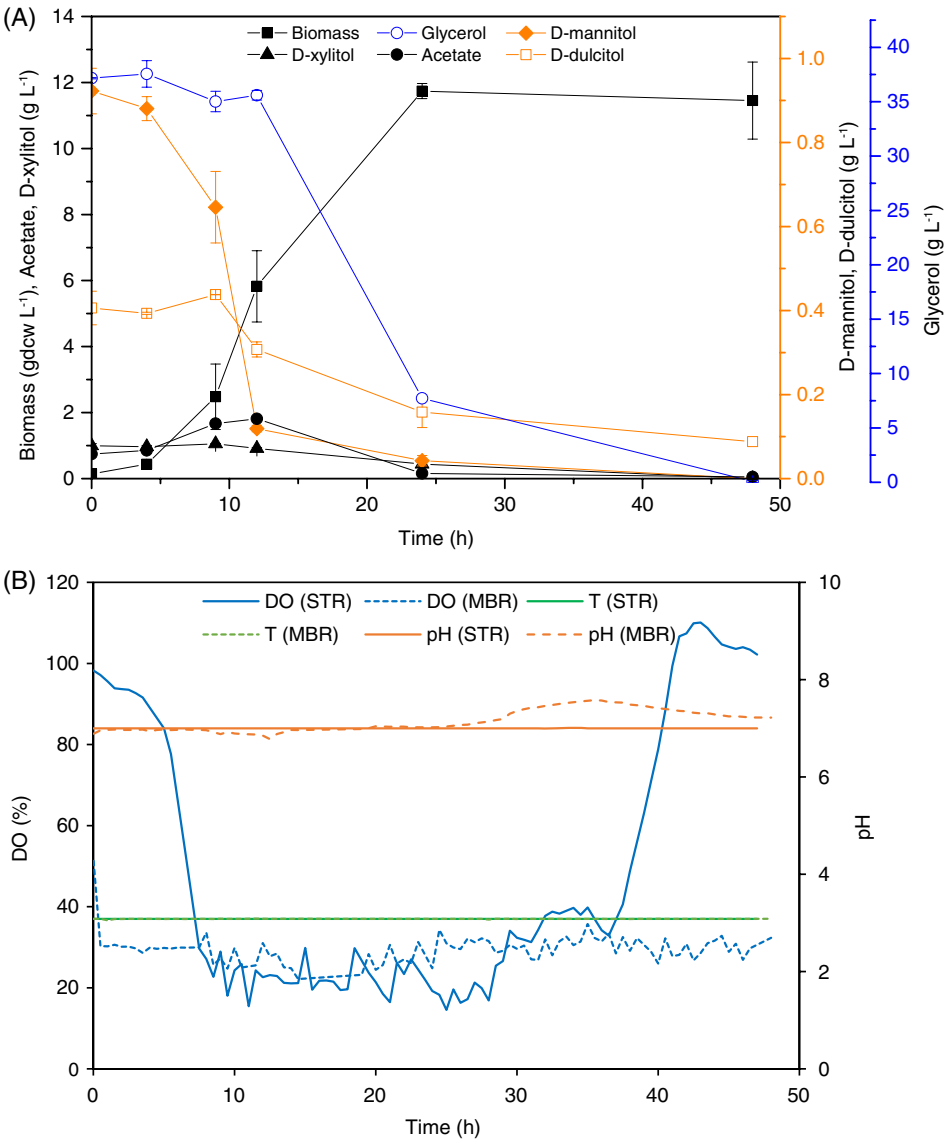

Figure 9. (A) *E. coli* BL21 growth, carbon source utilisation and acetate production in a 7.5 L STR, and (B) comparison of online DO, temperature and pH profiles in the controlled MBR and 7.5 L STR operated at a matched $k_L a$ value (66 h^{-1}). Error bars denote one standard deviation about the mean (n = 3).

same medium without glucose addition. This significant increase in acetate concentration, not seen in Fig. 8(a), is believed to be due to D-glucose metabolism, which generated approximately 4.6 mol of acetate per mole of D-glucose consumed. Moreover, the acetate was not re-metabolised at this high concentration.

The key finding of this metabolic study is that, between D-glucose and D-mannitol, the former remains the favoured carbon source for *E. coli* BL21 (DE3). Furthermore, the absence of D-glucose in vinasse facilitates glycerol utilisation, which actually constitutes the main carbon source of the feedstock making it the favoured carbon source from an economic perspective.

Overall, the hierarchy of carbon source utilisation by *E. coli* BL21 (DE3) when cultured in the yeast extract supplemented vinasse medium is proposed to be:

D-mannitol > glycerol > D-xylitol, D-dulcitol

An understanding of this hierarchy is important since industrial production of CV2025 ω-TAm within an integrated biorefinery would most likely occur by fed-batch fermentation. In developing a fed-batch process this preferential utilisation must be taken into account. In this case, glycerol might serve as the best feeding option since it appears to be the limiting substrate for cell growth (after D-mannitol) and is, itself a relatively cheap by-product of the biofuel industry.[46]

Fermentation scale-up from MBR to STR at matched $k_L a$

Having established optimum conditions for CV2025 ω-TAm production in the controlled MBR, it was desired to scale-up biocatalyst production to a conventional 7.5 L STR. In the small-scale studies the diluted vinasse was sterilised by filtration prior to fermentation (Fig. 1) but this would not be economically viable at larger scale. In the context of an integrated sugar beet biorefinery the pasteurised vinasse, after ethanol distillation, could be used directly for fermentation once it had cooled to 37 °C. A series of small-scale experiments showed comparable cell growth and CV2025 ω-TAm production using either filtered or pasteurised

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vinasse (data not shown) hence the latter method was used in these scale-up studies.

In related work, the oxygen mass transfer coefficients of both the MBR and the STR over a range of operating conditions with the vinasse medium were determined.[47] The maximum k_La value achieved in the MBR was 67 h^{-1} while that for the STR was 322 h^{-1}. In this work scale-up of CV2025 ω-TAm production from the controlled MBR to the 7.5 L STR was performed at a matched k_La value of 66 h^{-1}. The shaking frequency in the MBR was 800 rpm at a specific aeration rate of 1 vvm. For the STR, Eqn 3 was used to determine the corresponding agitation speed at this k_La and aeration rate, which was calculated to be 298 rpm. The same seed culture was used to inoculate both vessels that contained the same pasteurised vinasse medium supplemented with 10 g L^{-1} yeast extract.

Figure 9(a) shows the fermentation profile in the 7.5 L STR at 66 h^{-1}. Using the optimised vinasse medium there is good comparability between cell growth and carbon source utilisation at this scale and that seen previously in the MBR (Fig. 8(a)). The same trends in substrate consumption are clearly seen in both reactors suggesting that *E. coli* BL21 (DE3) metabolism is the same at the two different scales and in the two different culture formats (shaken and stirred). The highest CV2025 ω-TAm specific activities in the controlled MBR and 7.5 L STR were achieved at 48 h, which were 246.5 and 224.7 U g_{dcw}^{-1}, respectively. Generally, the CV2025 ω-TAm specific activity obtained from fermentations in both reactors was found to be reproducible with less than 14% difference recorded at several time points (12, 24 and 48 h).

k_La was used as a scale-up basis as we have previously shown it is a suitable basis for scale-up of aerobic *E. coli* cultures in microwells where the rate of oxygen mass transfer can be limiting.[48] In the MBR used here it was also possible to control DO levels by gas blending during the most rapid periods of cell growth. In these experiments DO was controlled at 30% in both reactors and the measured DO profiles in both the MBR and the 7.5 L STR are shown in Fig. 9(b). Along with excellent control of the culture pH, this figure shows that this minimum DO level is maintained at both scales. This further confirms that the cells at the two scales are in similar metabolic states during the key stage of the cultures.

Given that higher k_La values can be achieved in the STR the possibility of further enhancing CV2025 ω-TAm production was explored. A further STR fermentation was thus performed at 818 rpm corresponding to a 4-fold increase in k_La to 264 h^{-1}. The results showed an increase in maximum biomass concentration and volumetric CV2025 ω-TAm activity by about 1.4 and 1.9-fold, respectively to 16.7 g_{dcw} L^{-1} and 10.6 U mL^{-1}. These results indicate that there is further potential to improve the fermentation performance by increasing k_La once initial scale-up of the optimised culture conditions has been verified. At the current level of biomass production 1 L of vinasse is able to produce approximately 4 g of CV2025 ω-TAm with an activity of 13 U mL^{-1}. Even allowing for enzyme losses during purification (and immobilisation) it appears easily feasible to produce the required quantities of enzymes for SBP hydrolysis and bioconversions on site from vinasse.

CONCLUSIONS

This work has demonstrated the utility of sugar beet vinasse as a fermentation feedstock suitable for production of industrial biocatalysts within an integrated biorefinery context. The research was facilitated by the use of a high throughput controlled MBR platform that enabled rapid and parallel data acquisition. Production of the CV2025 ω-TAm by *E. coli* BL21 (DE3) fermentation, highlighted several potential benefits including simple pre-processing requirements (dilution), auto-induction of enzyme expression and direct use of the pasteurised vinasse post-distillation. Supplementation of vinasse with 10 g L^{-1} yeast extract resulted in enhancements of 2.8, 2.5, 5.4 and 3-fold in terms of specific growth rate, maximum biomass concentration and CV2025 ω-TAm volumetric and specific activity, respectively. The metabolic preference of *E. coli* BL21 (DE3) in the presence of the various carbon sources in vinasse was shown to be for D-mannitol after which simultaneous metabolism of glycerol, D-xylitol, D-dulcitol and acetate occurred. A 769-fold scale-up of ω-TAm production was demonstrated from a 10 mL miniature bioreactor to a 7.5 L STR on the basis of matched k_La. The scale-up results showed good comparability in terms of cell growth, substrate consumption and biocatalyst production kinetics. Future studies should consider further intensification of biocatalyst production through development of fed-batch operations as well as integration of fermentation and bioconversion processes using whole cell biocatalysts.

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Supporting Information

Supporting information may be found in the online version of this article.

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