Production of Recombinant Butyrylcholinesterase from Transgenic Rice Cell Suspension Cultures in a pilot scale bioreactor

Kantharakorn Macharoen[1], Min Du[1], Seongwon Jung[1], Karen McDonald[2,2], and Somen Nandi[2]

[1]University of California Davis
[2]University of California, Davis

August 28, 2020

Abstract

Producing recombinant proteins in transgenic plant cell suspension cultures in bioreactors provides controllability, reproducibility, scalability, and low-cost production, although low yields remain the major challenge. The studies on scaling-up to pilot-scale bioreactors, especially in conventional stainless-steel stirred tank bioreactors (STB), to produce recombinant proteins in plant cell suspension cultures are very limited. In this study, we scaled-up the production of rice recombinant butyrylcholinesterase (rrBChE), a complex hydrolase enzyme that can be used to prophylactically and therapeutically treat against organophosphorus nerve agents and pesticide exposure, from metabolically-regulated transgenic rice cell suspension cultures in a 40-L pilot-scale STB. Employing cyclical operation together with a simplified-process operation (controlling gas sparging rate rather than dissolved oxygen and allowing natural sugar depletion) identified in lab-scale (5-L) bioreactor studies, we found consistent maximum total active rrBChE production level of 46-58 μg/g fresh weight in four cycles over 82 days of continuous operation. Additionally, maintaining the overall volumetric oxygen mass transfer coefficient (kLa) in the pilot-scale STB to be equivalent to the lab-scale STB improves the maximum total active rrBChE production level and the maximum volumetric productivity to 85 μg/g fresh weight and 387 μg L-1 day-1, respectively, which are comparable to the lab-scale culture. Here, we demonstrate pilot scale bioreactor performance using a metabolically-regulated transgenic rice cell culture for long-term, reproducible, and sustained production of rrBChE.

Keywords

Butyrylcholinesterase, scale-up, pilot-scale bioreactor, plant cell suspension cultures, semicontinuous culture, rice alpha-amylase 3D

1. Introduction

Plant-based biopharmaceuticals are an alternative for producing human recombinant proteins. Plants provide several advantages over other expression systems, including the ability to grow in simple, low-cost, and chemically defined medium, efficient protein folding and post-translational modification, and the lack of susceptibility to human pathogens (Huang and McDonald, 2009; Santos et al., 2016). Additionally, plant cell suspension cultures grown in a controlled and sterile bioreactor containing animal-free chemically defined medium in an aseptic environment are compatible with regulatory guidelines such as current good manufacturing practices (cGMP). Taliglucerase alfa (TGA) from transgenic carrot cell suspension cultures grown in disposable bioreactors by Protalix Biotherapeutics (Carmiel, Israel) is the first plant-made recombinant therapeutic protein for human use approved by the FDA in 2012; it is used for enzyme replacement therapy of

type 1 Gaucher disease (Fox, 2012; Tekoah et al., 2015). The market price of TGA is 25% lower than its primary competitor, CHO cell-made imiglucerase (Cerezyme®) from Genzyme (Fox, 2012) perhaps indicating the cost-effectiveness of plant cell cultures. Importantly, there is no definitive proof of adverse effects from plant-specific glycans in patients treated with TGA (Shaaltiel and Tekoah, 2016), and the immunogenicity risk associated with anti-plant glycan antibodies in human trials was not apparent (Rup et al., 2017).

Human butyrylcholinesterase (hBChE) circulating in blood plasma is a tetrameric hydrolase enzyme with four ˜85 kDa identical monomers containing 9-*N* -glycosylation sites each (Lockridge, 2015). Human BChE can be used for therapeutic and prophylactic treatment against the exposure to organophosphate (OP) nerve agents and pesticides (Lockridge, 2015). However, the use of purified hBChE from blood plasma for clinical purposes is limited due to its high cost, low yield, and limited availability (Alkanaimsh et al., 2019; Corbin et al., 2016). Among several systems aiming to produce recombinant BChE (rBChE) as a replacement for hBChE, metabolically-regulated transgenic rice cell culture, where the rice α-amylase 3D (RAmy3D) is up-regulated under sugar starvation (Corbin et al., 2016; Huang et al., 1993; Huang et al., 2001; Terashima et al., 1999; Trexler et al., 2002; Trexler et al., 2005) leading to the production of rrBChE, is a promising cost-effective platform most likely due to its low-cost, chemically defined medium. A recent study by our group using technoeconomic analysis showed that the cost of production and purification of rice-made rBChE (rrBChE) was dramatically reduced from the estimated $20,000 for a 400 mg dose from blood plasma to less than $300/dose of pure rrBChE using semicontinuous transgenic rice cell suspensions (Corbin et al., 2020). Here, we demonstrate the use of a 40-L conventional stainless-steel stirred bioreactor (STB) for scaling-up the production of rrBChE in transgenic rice cell suspension cultures under single-stage operations (rice cells naturally deplete sugar in the culture medium without medium exchange) in an uncontrolled dissolved oxygen (DO) condition at a constant aeration rate (Macharoen et al., 2020).

Wild-type plant cell suspension cultures in pilot-scale and industrial scale STBs for the production of plant metabolites have been widely reported (Katō et al., 1972; Noguchi et al., 1977; Schiel and Berlin, 1987; Ulbrich et al., 1985), including *Taxus chinensis* suspended cells grown in a 75,000-L STB at Phyton Biotech®(Ahrensburg, Germany) for the production of paclitaxel, a potent cancer treatment chemical (Imseng et al., 2014). However, the studies of pilot-scale and commercial scale bioreactors for recombinant therapeutic protein productions from transgenic plant cell cultures are scarce. So far, there is only one report using 20-L and 600-L STBs for the scale-up of recombinant green fluorescence protein-hydrophobin fusion (GFP-HFBI) production in transgenic tobacco BY-2 suspension cell cultures (Reuter et al., 2014). In this study, for the first time, we demonstrate the robustness of metabolically regulated transgenic rice cells that can be maintained over long time periods, 80 days of cultivation with four cycles of cyclical semicontinuous operation in a conventional 40-L pilot-scale STB to produce rrBChE. We also show comparable batch culture results in the 40-L pilot-scale bioreactor compared to the 5-L lab-scale bioreactors (Macharoen et al., 2020) using k_La as a scale-up parameter.

2. Materials and Methods

2.1 Maintenance of transgenic rice cell cultures

Transgenic rice cell line "9-2", one of the most stable and productive rrBChE lines, generated as previously described (Corbin et al., 2016), has been shown to be stable including expression of rrBChE for more than 4 years (with continuous subculturing on semi-solid media over that time period). The transgenic rice suspension cultures were grown in shake flasks containing NB+S media, which consists of N6 macronutrients (Chu et al., 1975), B5 micronutrients and vitamins (Gamborg et al., 1968), 30 g/L sucrose, and other components as described elsewhere (Macharoen et al., 2020). The shake flasks were maintained in an orbital shaker at 140 rpm and 27@C in the dark and subcultured every 7-8 days.

2.2 Measurement of overall oxygen mass transfer coefficient (k_La)

The measurement of probe response time for a polarographic dissolved oxygen (DO) probe and k_La in a benchtop bioreactor and a pilot-scale bioreactor were performed as previously described (Leth and McDonald, 2017) with slight modifications. The step change from pure N_2 (medical grade) to pure O_2 (medical grade)

was used to measure the response time of a DO probe (Mettler Toledo, Billerica, MA) in deionized (DI) water in two Erlenmeyer flasks saturated with pure N_2 in one flask and pure O_2 in the other flask. To evaluate k_La inside a bioreactor, the DO probe is inserted into the bioreactor containing DI water where temperature was maintained at 27@C. The DI water (4 L in the benchtop bioreactor and 30 L in the pilot-scale bioreactor) was sparged with pure N_2 until the %DO reached 0. Then the gas inlet was switched to industrial air, and the changes of %DO were recorded until a steady state %DO was reached (˜ 100% DO air saturation). Three replicate trials were performed for finding probe response time and k_La values. By using the dynamic method with significant oxygen probe response, the collected data points were fitted in the equations suggested by Blanch and Clark (1997) using the method of least squares in MATLAB® (MathWorks Inc, Natick, MA).

2.3 Inoculum preparation

Combined shake flasks of 7-day-old rice cell suspensions were inoculated at ˜20% v/v (volume of inoculum per total working volume) into a sterile 5-L STB with a height-to-diameter (H/D) ratio of 2 (BioFlo 3000, formerly New Brunswick Scientific, Eppendorf Inc., Hauppauge, NY) containing 4 L of sterile NB+S corresponding to initial inoculation density of 1-2 g DW/L. The bioreactor was operated under conditions similar to those previously described (Macharoen et al., 2020). The agitation of an upward-pumping pitched blade impeller (10.2 cm diameter corresponding to impeller diameter (D_i) to tank diameter (D_t) ratio of 0.60) was kept constant at 75 rpm. The temperature and the aeration rate were maintained at 27@C and 0.2-0.4 vvm (volume of gas sparged per bioreactor working volume per minute), respectively. The cultures were grown until mid-to-late exponential growth phase was reached, typically around day 9-12 of cultivation depending on initial biomass concentration, and ready for the 40-L STB inoculation.

2.4 Pilot-scale bioreactor operation

The pilot-scale cultivation of transgenic rice cell suspension cultures was conducted in a 40-L *in situ* sterilizable STB with i-Control$_{XP}$ control software (Applikon Biotechnology Inc., Foster City, CA) with a H/D of 3. The vessel is equipped with an upward-pumping pitched blade impeller (10.2 cm diameter corresponding to D_i to D_t ratio of 0.38) placed at 23 cm from the base of the impeller shaft that is magnetically driven at the bottom of the vessel. A 24-hole ring sparger (1.5 mm hole diameter) is placed 10 cm above the bottom of the vessel around the impeller shaft. The bioreactor containing DI water was sterilized using the sterilize-in-place (SIP) system at 122@C for 20 min. Then 5x concentrated medium was sterile filtered into the bioreactor through a sterile push valve using a 0.2 µm sterilizing filter capsule (Sartobran® P, Sartorius Stedim North America Inc, Bohemia, NY) connected to a dispensing pressured vessel (Amicon, Millipore-Sigma, Burlington, MA), a liquid holder for filtration, and a compressed air source (10 psig). The amount of concentrated medium filtered into the bioreactor was calculated and prepared to obtain 1x medium after mixing with sterile DI water in the bioreactor. The medium was then agitated at 150 rpm, aerated with sterile compressed air at 0.2 vvm, and the temperature was maintained at 27@C overnight to verify sterility of the medium prior to inoculation.

The plastic tubing connected to the sampling tube in the 5-L STB was aseptically cut and fused to the tubing attached to a push valve inserted into the headplate of the 40-L STB using a sterile tube fuser (Cytiva; formerly GE Healthcare Life Science, Marlborough, MA). Then, the inoculum was transferred from the 5-L STB to the 40-L STB by flowing compressed pure O_2 at 10 psig through a 0.22-µm vent filter connected to the 5-L sparger ring to push the culture into the 40-L STB. The inoculation corresponded to 15-18% v/v (volume of inoculating culture to final working volume of culture). Single-stage operations were used in this study. The first bioreactor run was operated in a cyclical semicontinuous mode where the media exchange to start a new cycle was performed at day 4 since extracellular sugar depletion by cellular uptake. The second bioreactor run was operated in a batch mode. Table 1 summarizes the conditions and bioreactor parameters used in the runs as well as the initial working volume after inoculation and the final working volume in which the culture volume was reduced due to sampling and evaporation. In both runs, the aeration rate of 0.2-0.4 vvm was maintained during the growth phase and 0.2 vvm during the induction phase, while the temperature was maintained at 27@C throughout the operation. The pressure inside the bioreactor was controlled at 0.2 psig. The oxygen uptake rate (OUR) was routinely measured by momentarily halting the

aeration and recording the declining rate of DO. The online culture pH was monitored using Applikon pH sensor but was not controlled.

The media exchange was performed to start a new cycle of the semicontinuous operation in following order. The pressurization, agitation, and aeration were halted to allow rice cell aggregates to settle. A sterile tube fuser (Cytiva; formerly GE Healthcare Life Science, Marlborough, MA) was used to aseptically connect the sterile tubing attached to carboys and the tubing attached to the sampling assembly at the bottom of the bioreactor. Then the spent medium was drained out into sterile carboys through the sampling assembly. Freshly prepared NB+S or NB+0.5xS medium (Table 1) was 0.22-μm filtered into the bioreactor as described earlier. The agitation, aeration, and pressurization were then resumed.

2.5 Sampling and biomass measurements

Samples were routinely taken by opening the sampling assembly that was connected to a pre-sterile collecting bottle. After that, a clean magnetic stir bar was put in the collecting bottle to homogenously stir the culture, and then four ˜10 mL cultures were separately aliquoted into four centrifugal tubes. The actual culture volume and settled cell volume were recorded prior to centrifugation at 3,200 x g (GS 6KR, Beckman Coulter, Inc., Brea, CA) for 20 mins. The packed cell volume of each tube was recorded, and the supernatant was collected and stored at 4@C for further analyses. One tube was saved for protein quantifications, while the other three tubes were used for determining fresh weight (FW) and dry weight (DW) as described (Corbin et al., 2016) using pre-weighed 1.6 μm Binder-Free Glass Microfiber filters (Whatman GF/A 4.7 cm, GE Healthcare Life Sciences, Pittsburgh, PA). To quantify DW, the biomass was dried in an oven at 65@C for 24 h and weighed and reweighed until a stable value was obtained.

2.6 Sugar measurements

The sample supernatant was pipetted into a 96-well plate at 250 μL and placed in the YSI 2900 Biochemistry Analyzer (Xylem, Inc., Rye Brook, NY) to measure sucrose and glucose concentrations in the culture medium. To ensure the sugar concentrations were within the calibration ranges, samples were sometimes diluted with distilled water prior the measurement.

2.7 Quantification of active rrBChE

Fresh biomass was washed and extracted with cold (4@C) 100 mM sodium phosphate + 100 mM NaCl buffer pH 7.4 at a ratio of 1 mL extraction buffer per 1 gram FW biomass as previously described (Corbin et al., 2016). The extracted mixture was then centrifuged, and the supernatant was transferred to a new tube and stored at 4@C until analysis. A modified Ellman activity assay (Ellman et al., 1961) was used to quantify active rrBChE in the cell extract (cell-associated rrBChE) and the medium (culture medium rrBChE). The assay was performed by adding a 150 μL of Ellman's substrate to a 50 μL of sample, diluted with the extraction buffer if necessary, in a 96-well plate and measuring the absorbance at 405 nm for 3 min at 25@C with a spectrophotometer (SpectraMax 340PC, Molecular Devices, Sunnyvale, CA) where the initial rate was in the linear range of 200-500 mOD/min. Three replicates of each sample were measured. The specific activity of 260 U/mg crude rrBChE was assumed throughout this study (Alkanaimsh et al., 2016).

2.8 Total soluble protein (TSP) measurement

TSP in the sample was determined by the Bradford assay (Bradford, 1976) using Bio-Rad protein assay dye reagent (Bio-Rad, Hercules, CA). The standard curve of 0.05 to 0.5 mg/mL bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) was generated along with the samples. The absorbance at 595 nm of the samples and standards were analyzed in triplicate with a SpectraMax 340PC spectrophotometer (Molecular Devices, Sunnyvale, CA).

2.9 Metabolic activity assay

Triphenyl tetrazolium chloride (TTC) solution was used to determine rice cell metabolic activity as described elsewhere (Macharoen et al., 2020). In brief, the TTC solution was added to fresh rice cells and incubated

at room temperature for 24 h in the dark. The red formazan stain in active rice cells was extracted, the absorbance was measured at 485 nm, and then the absorbance was normalized by g FW.

2.10 Western blot analysis

SDS-PAGE using a 4-20% gradient gel (Mini-PROTEAN precast gels, Bio-Rad, Hercules, CA) under reducing conditions with 5% β-mercaptoethanol was performed at 200 V for 37 mins. Then the gel was transferred to a 0.45 μm nitrocellulose membrane for 90 mins at 100 V and blocked with 5% non-fat dry milk (NFDM) in PBST overnight. The blotted nitrocellulose membrane was washed with PBST buffer three times at 5-min intervals and incubated with the mouse BChE (D5) antibody HRP (Santa Cruz Biotechnology, Dallas, TX) diluted in 10 mL 5% NFDM at a 1:1,000 dilution for 1 h at room temperature on a rotary shaker at 60 rpm. The blot was then washed with PBST buffer three times for 5 min each. Clarity enhanced chemiluminescence (ECL) substrate (1 mL) was dropped over the blot to detect BChE bands using chemiluminescent mode in the ChemiDoc system (Bio-Rad, Hercules, CA). Human BChE derived from blood plasma, kindly provided by Dr. Douglas M. Cerasoli (US Army Medical Research Institute of Chemical Defense, USAMRICD), was used as a control.

3. Results and Discussion

3.1 Cyclical semicontinuous pilot-scale bioreactor operation

3.1.1 Growth kinetics and sugar consumption

Transgenic rice cell growth profiles and sugar levels of the cyclical semicontinuous bioreactor run is shown in Figure 1a. As expected, the biomass concentration in each cycle increased during the growth phase and somewhat dropped during the induction phase due to the lack of exogenous carbon source as also seen in our previous semicontinuous rice cell cultures (Corbin et al., 2016; Trexler et al., 2005). The growth periods in cycles 1-4 were 26, 21, 10 and 9 days, respectively (Table S1). Whereas the lag phase was not known in cycle 1 and not observed in cycles 3 and 4, the rice cell culture in cycle 2 likely experienced ˜4-day lag phase (semi-log plot; data not shown) because rice cells probably took some time to acclimatize in the presence of a large amount of fresh NB+S medium (the working volume was increased from 13 L, the final working volume of cycle 1, to 32.5-L, the initial working volume of cycle 2) (Table 1). In addition, the agitation rate in cycle 2 was increased to 150 rpm compared to 100 rpm in cycle 1; as a result, rice cells likely took time to adjust themselves to the new environment. Note that the culture sample at day 0 of cultivation was not taken to avoid any risks of contamination and to test the sterility of inoculation process.

The maximum specific growth rates (μ_{max}) of cycles 1-4 were comparable at 0.10 ± 0.02, 0.07 ± 0.01, 0.10 ± 0.04 and 0.08 ± 0.03 day^{-1}, respectively, corresponding to the doubling times (τ_D) of 6.7 ± 1.1, 9.8 ± 1.8, 6.8 ± 2.4 and 9.3 ± 3.8 day, respectively (Table S1) . The μ_{max} values in the semicontinuous run were somewhat lower than our previous report for lab-scale semicontinuous bioreactor operation (Corbin et al., 2016) where μ_{max} of 0.15 ± 0.01 and 0.22 ± 0.01 day^{-1} and τ_D of 4.7 ± 0.34 and 3.2 ± 0.12 day were found in the first and second cycle, respectively, using the same transgenic rice cell line and NB+S medium. The discrepancy between the two studies is likely due to different hydrodynamic stress since baffles were equipped in this study but not in the lab-scale study. Also it could be due to different oxygen transfer, especially near the bioreactor wall, or from different D_i/D_t ratios since k_La was not maintained between the two scales.

The growth phases in cycles 3 and 4 were shorter than cycles 1 and 2 (Figure 1a) due to the use of half-strength sucrose (Table 1) and their higher initial biomass concentrations (Table S1). Sucrose gradually decreased due to its conversion to glucose and fructose by rice cell wall invertases and reached 0 g/L at day 24 of cycle 1, day 20 of cycle 2, day 10 of cycle 3, and day 8 of cycle 4 (Table S1) following by glucose depletion a few days later (Figure 1a). The shorter period of the growth phase is desirable not only to improve productivity but also to minimize energy and materials supply to the bioreactor. It is pertinent to mention here that the initial sucrose concentration in cycle 2 was ˜20 g/L rather than 30 g/L since the newly added medium was diluted by the remaining culture, while NB+0.5xS used in cycles 3-4 was prepared by taking this dilution into account.

3.1.2 Oxygen uptake rate (OUR) and specific oxygen uptake rate (SOUR)

The measurement of OUR is a simple non-invasive method to monitor metabolic activity of the culture, while the SOUR measurement is the OUR normalized by biomass dry weight. During the exponential growth phase, the OUR increased in all cycles, and the SOUR was fairly constant in cycles 2-4, while the OUR and SOUR trends in cycle 1 are somewhat different from the rest as seen in Figure 1b. The difference in the OUR and SOUR patterns in cycle 1 may be the result of differences in agitation rates, which influence overall oxygen transfer rates, between cycle 1 and other cycles. The maximum OUR and SOUR in cycles 1-4 were 0.96, 1.38, 1.49 and 1.91 mmol $O_2L^{-1}h^{-1}$, respectively, and 0.44, 0.35, 0.25 and 0.24 mmol O_2 g $DW^{-1}h^{-1}$, respectively (Table S1), which are generally higher than our previous report of 0.52-1.25 mmol $O_2L^{-1}h^{-1}$ for the maximum OUR and 0.18-0.29 mmol O_2 g $DW^{-1}h^{-1}$ for the maximum SOUR (Corbin et al., 2016) likely due to different bioreactor conditions. The decreasing OUR and SOUR during the induction phases correlated to the reduction of metabolic activity (Figure S1a) probably due to cell quiescence from sugar starvation and/or cell lysis. However, the OUR, SOUR, and metabolic activity increased again after the medium exchange to fresh sugar rich media (Figure 1b). The pattern of increasing OUR and SOUR during the growth phase but decreasing during the induction phase was also found in previous studies using a two-stage transgenic rice cell semicontinuous suspensions under the RAmy3D promoter (Corbin et al., 2016; Trexler et al., 2005).

3.1.3 Production, purity, and productivity of rrBChE

With the RAmy3D signal peptide in the gene construct, we previously reported a significant amount rrBChE in both culture medium and rice cell aggregates in the screening process (Corbin et al., 2016) and a 5-L STB using single-stage batch culture and simplified bioreactor processing (Macharoen et al., 2020). Figure 1c shows the accumulation of culture medium and cell-associated rrBChE during the induction phases in cycles 1-4. Total active rrBChE reached its maximum level at 3-day post induction (dpi) in cycle 1 but 4 dpi or later in cycles 2-4. We stopped the induction phases in cycles 2-4 at 4 dpi to be consistent with cycle 1 and to not excessively exhaust rice cell metabolic activity (Figure S1a) since allowing rice cells one day longer in the induction phase could impact the cell growth in the subsequent cycle. The maximum total active rrBChE in cycles 1-4 were comparable at 58.2 ± 13.4, 46.1± 6.7, 49.8 ± 5.5, and 48.2 ± 6.4 μg /g FW, respectively (Table 2), suggesting that the transgenic rice cells are stable and robust over 82 days of cultivation in a cyclical semicontinuous operation. While Gagnon et al. recently reported a constant steady-state volumetric productivity of IgG from CHO-K1 cells in a 100-L continuous stirred-tank bioreactor (CSTR) linked with a perfusion bioreactor operation for 83 days (Gagnon et al., 2019), here, we demonstrate the reproducible production of a plant-made therapeutic recombinant protein in a 40-L STB using simplified-bioreactor processing over 82 days of semicontinuous operation. To best of our knowledge, this is the longest plant cell culture bioreactor run.

The purity of rrBChE is determined by the ratio of the concentration of rrBChE (mg/L) to the total soluble protein (TSP; mg/L) multiplied by 100 to convert to a percent of total protein. The maximum purities of cell-associated rrBChE in cycles 1-4 were 0.91 ± 0.07%, 0.69 ± 0.03%, 0.80 ± 0.06%, and 0.79 ± 0.06%, respectively (Table 2), while our previous study reported 0.27-0.55% g rrBChE/g TSP (Corbin et al., 2016). Improvement in the purity of rrBChE in the crude extract may facilitate the downstream processing. Maximum purities of culture medium rrBChE were also quite high at 0.80 ± 0.06%, 0.72 ± 0.08%, and 0.61 ± 0.01% in cycles 2, 3, and 4, respectively (not available in cycle 1; Table 2). It could be implied that the greater proportion of culture medium rrBChE than cell-associated rrBChE in cycle 2 at 4 dpi (Figure 1c) resulted in higher maximum purity of rrBChE in the culture medium than in the cell extract, and vice versa in cycle 4, whereas the maximum purities of culture medium and cell-associated rrBChE in cycle 3 were comparable due to similar maximum rrBChE levels in both sources.

The volumetric productivities in cycles 1 and 2 using full strength sucrose NB+S were comparable at 76 ± 25 and 92 ± 15 μg $L^{-1}day^{-1}$, respectively (Table 2). Using half-strength sucrose (NB+0.5xS) in cycles 3 and 4 also gave comparable volumetric productivities at 226 ± 26 and 271 ± 48 μg $L^{-1}day^{-1}$, respectively (Table 2), which are at least 2.4-fold higher than cycles 1 and 2 and at least 1.2-fold higher than our previous

report, 36 ± 8 μg $L^{-1}day^{-1}$ in cycle 1 and 184 ± 17 μg L^{-1} day^{-1} in cycle 2 (Corbin et al., 2016). The specific productivities in cycles 1-4 were 20.5 ± 6.7, 24.0 ± 3.8, 42.4 ± 5.1 and 41.1 ± 7.6 μg g $DW^{-1}day^{-1}$, respectively, indicating that using NB+0.5xS improved specific productivity at least 1.7-fold. In other words, the use of half-strength sucrose in the cyclical semicontinuous single-stage culture operation with the simplified bioreactor processes improves volumetric productivity and specific productivity primarily due to shortening of growth phase. Altogether, we demonstrate that our transgenic rice cell suspensions cultivated in a pilot-scale STB are robust and stable over long term (82 days) cyclical semicontinuous operation in which cleaning, sterilizing and inoculating are minimized resulting in saving the cost of rrBChE production in which our recent technoeconomic analysis showed that the overall cost of rrBChE production and purification has significantly reduced in semicontinuous operation compared to batch operation (Corbin et al., 2020).

3.2 Determination of the volumetric oxygen mass transfer coefficient (k_La)

Even though we succeeded in producing consistent rrBChE in the cyclical semicontinuous single-stage culture operation in the 40-L STB described in previous section, the rrBChE production levels and productivities were still lower than what was obtained in a single-stage benchtop bioreactor culture under simplified bioreactor processing (Macharoen et al., 2020). Our hypothesis was that maintaining k_La, as a scale up parameter, and bioreactor configuration such as eliminating baffles between the lab-scale and pilot-scale bioreactors would provide comparable growth kinetics and rrBChE production kinetic parameters between the two scales likely due to similar hydrodynamic stress and oxygen transfer capabilities.

The dynamic gassing out method as described in Blanch and Clark (1997) was used to determine the k_La values in a lab-scale bioreactor and a pilot-scale bioreactor using different polarographic DO probe and DI water. The DO probe response times used in the lab-scale and pilot-scale bioreactors were 25.3 ± 1.5 s and 30.9 ± 1.9 s, respectively. Figure 2 shows the k_La values obtained in a 5-L STB with constant agitation rate at 75 rpm (Figure 2a) and a 40-L STB with a constant aeration rate 0.4 vvm (Figure 2b) where baffles were not equipped in the two scales. Under normal aeration rates used in the 5-L STB, 0.2-0.4 vvm during the growth phase and 0.2 vvm during the induction phase, the k_La values were found in the range of 8.7 ± 0.3 h^{-1} to 13.2 ± 0.1 h^{-1}(Figure 2a), while the k_La values in the range of 8.1 ± 0.2 h^{-1} to 9.7 ± 0.1 h^{-1} in the 40-L STB were found using different agitation rates between 75-250 rpm at a constant aeration rate 0.4 vvm (Figure 2b). Generally k_La values around 10 h^{-1} are adequate during the growth phase of various plant cell cultures (Takayama and Akita, 2006). Increasing agitation rate in the 40-L STB does not appear to have a strong effect on k_La in this case probably due to a slightly undersized impeller compared to bioreactor diameter (Di/Dt of 0.38). Within the testing agitation range, the single undersized impeller may not have a chance to break up rising-up bubbles outside of the impeller due to comparable sparger ring diameter to impeller diameter. The agitation rate of 150 rpm and the aeration rate of 0.4 vvm were used to investigate the growth kinetics and rrBChE production kinetics in a single stage batch operation in the 40-L STB without baffles to compare to the results in the 5-L STB.

3.3 Batch pilot-scale bioreactor operation using k_La as a scale-up parameter

3.3.1 Growth profile, sugar consumption, OUR and SOUR

Figure 3a shows the growth profile and sugar consumptions of the transgenic rice cell culture grown in NB+0.5xS medium in the pilot-scale bioreactor using single-stage batch operation with simplified bioreactor processes. The rice cells continued growing until day 8 of cultivation; no lag phase was not observed in this run (data not shown). The initial and final biomass concentrations in the growth phase were 2.05 ± 0.05 and 5.85 ± 0.13 g DW/L, respectively (Table S1). The μ_{max} and τ_D were 0.16 ± 0.03 day^{-1} and 4.4 ± 0.9 day, respectively (Table S1), which are comparable to the μ_{max} of 0.20 ± 0.04 day^{-1} and τ_D of 3.5 ± 0.8 day obtained in the lab-sale bioreactor run under similar k_La and simplified bioreactor operation (Macharoen et al., 2020) but substantially different from what was obtained in the cyclical semicontinuous operation as described earlier. Tanaka et al., reported that *Catharanthus roseus* cell suspension cultures in the shake flask with two baffles had lower growth rate and maximum biomass concentration than the shake flask without baffles (Tanaka et al., 1988). Thus, the presence of baffles in the semicontinuous run likely generates different

hydrodynamic stress and oxygen transfer capability compared to the batch run, resulting in different $\mu_{max}$and τ_D.

Sucrose concentration dramatically declined during the first few days of cultivation and reached undetectable levels by day 5 of cultivation, while glucose concentration became undetectable at day 8 of cultivation, indicating the start of the induction phase, or 0 dpi (Figure 3a). This sugar consumption pattern is also observed in the lab-scale culture using the same bioreactor process and k_La (Macharoen et al., 2020). In other words, maintaining k_La as a scale-up parameter likely provides equivalent growth kinetic parameters, i.e. μ_{max}, τ_D and rate of sugar consumption between lab-scale and pilot-scale transgenic rice cell suspension cultures.

The OUR pattern in this run (Figure 3b) is similar to cycles 3-4 in the semicontinuous run presented in previous Section 3.1.2 (Figure 1b) in which the maximum OUR of 1.76 mmol O_2L^{-1} h^{-1} (Table S1) was reached simultaneously with the start of sucrose depletion (2-4 days prior to the induction phase). In addition, the OUR and the metabolic activity (Figure S1b) profiles are alike, indicating that rice cells are likely most metabolically active at the beginning of sucrose depletion. The SOUR reached its maximum at 0.46 mmol O_2 g DW^{-1} h^{-1} (Table S1), which is equivalent to what obtained in the lab-scale culture under the same conditions (Macharoen et al., 2020), at the early exponential growth phase and then gradually decreased toward the end of growth phase (Figure 3b). A possible explanation for this is that the portion of active cell aggregates to total cell aggregates at the beginning of this run was greater than for cycles 3-4 in which the proportion of accumulated nonviable cells may increase over the long cultivation period. During the induction phase, OUR, SOUR and metabolic activity dramatically decreased as expected. The DO and pH profiles in this run (Figure S2) are also similar to the profiles found in the lab-scale study (Macharoen et al., 2020) suggesting that maintaining k_La in this study is adequate to reproduce growth kinetics (e.g. μ_{max}, τ_D, OUR and SOUR) and culture conditions (e.g. DO and pH patterns) between a lab-scale (5-L) production to a pilot-scale (40-L) production.

3.3.2 Production of rrBChE

The total active rrBChE during the induction phase is shown in Figure 3c. The culture medium rrBChE was significant compared with the cell-associated rrBChE starting at 2 dpi. The active rrBChE levels in the medium and cell extract at 5 dpi were 23.0 ± 3.8 µg/ g FW and 61.6 ± 0.6 µg/ g FW, respectively, corresponding to total active rrBChE of 84.6 ± 13.8 µg/ g FW (Table 2), which is at least 1.5-fold higher than the semicontinuous cycles previously described in Section 3.1.3 but comparable to the results acquired from the lab-scale study at 5 dpi (Macharoen et al., 2020). The accumulation of total active rrBChE may or may not increase after 5 dpi; however, keeping rice cells longer in the induction phase may lead to a decrease in viable cell density (Figure S1b), longer recovery times, and may not increase rrBChE productivities.

Figure 3d shows the increase of cell-associated rrBChE purity in the induction phase due to the increase of cell-associated rrBChE production level (Figure 1c) and the decrease of TSP (data not shown). This rrBChE purity pattern is similar to the lab-scale study (Macharoen et al., 2020) . The maximum purity of cell-associated rrBChE of 1.87 ± 0.26% g rrBChE/g TSP (Table 2) was at least 2-fold greater than cycles 1-4 in the semicontinuous run but 1.25-fold lower than previous study (Macharoen et al., 2020), whereas the maximum purity of culture medium rrBChE of 0.55 ± 0.02% g rrBChE/g TSP was at most 1.5-times lower than cycles 2-4 in the semicontinuous run. Overall, the maximum total active rrBChE concentration and maximum cell-associated rrBChE purity was significantly improved in this run compared with the semicontinuous run.

Figure 4 shows a Western blot analysis under reducing conditions of rrBChE samples during the induction phase. Crude cell extracts from 0-5 dpi were loaded into lanes 1-6, respectively, with an equal volume of 20 µL. As a result, the rrBChE band intensity represents the concentration of combined active and inactive cell-associated rrBChE when the extraction ratio was maintained at 1 g FW per 1mL extraction buffer. The rrBChE bands at 0 and 1 dpi (lanes 1 and 2, respectively) were not visible due to the low concentrations of rrBChE (˜4 and ˜12 ng active rrBChE/µL, respectively), while the intensity of rrBChE bands increased,

as expected, from 2 dpi to 5 dpi (lanes 3-6, respectively) indicating the increase of rrBChE concentrations in the crude extract. Lane 7, under reducing condition, represents hBChE derived from blood plasma as a positive control showing the majority of monomeric hBChE (around 85 kDa) and a small fraction of dimeric hBChE. Lane 8 was loaded with 30 μL of 45X concentrated rrBChE from the culture medium showing slightly different molecular weight compared to the control and rrBChE from crude extract. Comparing with monomeric hBChE, the monomeric rrBChE bands from both crude extract and concentrated medium appear slightly below 85 kDa may be due to fewer occupied N -glycosylation sites in rrBChE resulting in smaller$N$ -glycan structures (Corbin et al., 2018; Kolarich et al., 2008). The rrBChE bands at 5 dpi from the crude extract (lane 6) and the culture medium (lane 8) show different molecular weights probably due to different N -glycoforms suggesting that rrBChE in the culture medium is most likely secreted to the rice cell wall and then the medium via the secretory pathway.

The maximum volumetric and specific productivities of total active rrBChE in this 13-day batch cultivation were 387± 66 μg L^{-1} day^{-1} and 76.1 ± 13.2 μg g DW^{-1} day^{-1} (Table 2), respectively, resulting in 1.4-fold and 1.8-fold, respectively, greater productivities than what obtained in cycles 3-4 in the semicontinuous run. The different rrBChE production levels and productivities between the semicontinuous run and the batch run was probably caused by different hydrodynamic stress (with and without baffles in the semicontinuous run and the batch run, respectively) and overall mass transfer. It could be interpreted that maintaining k_La as a scaling-up parameter in a bioreactor and eliminating baffles improves rrBChE productivities and allows comparable results between the lab-scale bioreactor and pilot scale bioreactor using the similar bioreactor conditions (Macharoen et al., 2020). By replicating the growth kinetics and rrBChE production kinetics in the pilot-scale bioreactor as previously found in the lab-scale bioreactor, we are successful in scaling up the production of rrBChE using k_La as a scaling-up parameter.

4. Conclusion and future prospects

The cyclical semicontinuous operation in a pilot-scale bioreactor using simplified bioreactor processing yielded consistent maximum total active rrBChE production levels in the four cycles over 82-days of operation showing the potential robustness and stability of the transgenic rice cell cultures for rrBChE production. Here, in best of our knowledge for the first time, we report the longest plant cell culture bioreactor with reproducible production of a plant-made therapeutic recombinant protein in a pilot-scale bioreactor. Using k_La as a scale-up parameter, the pilot-scale batch culture without baffles provides comparable growth kinetics, maximum total rrBChE production level, maximum volumetric productivity and maximum specific productivity as obtained from the lab-scale bioreactor. Based on Western blot analysis, the culture medium rrBChE is likely the extracellular rrBChE that is secreted to the medium via the secretory pathway guided by the RAmy3D signal peptide. Altogether, we demonstrate a simple, reproducible, and scalable platform for rrBChE production.

Future work will investigate medium optimization to improve rrBChE production level and productivities. A cyclical semicontinuous pilot-scale bioreactor operation without baffles and simplified bioreactor processes using k_La as a scale-up parameter is worth studying in a long-term operation (>6 months). Exploring strategies and methods to mitigate bioreactor wall growth of cell aggregates and cell lysis is also needed in a long-term semicontinuous bioreactor operation reported as a better cost-effective operation than batch operation for plant cell-made biopharmaceuticals (Corbin et al., 2020).

Acknowledgements

This work was supported by the Defense Threat Reduction Agency (DTRA, #HDTRA1-15-0054). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the DTRA. Kantharakorn Macharoen would like to acknowledge the Royal Thai Government Scholarship.

Conflicts of interest

The authors declare no conflict of interest.

References

Alkanaimsh S, Corbin JM, Kailemia MJ, Karuppanan K, Rodriguez RL, Lebrilla CB, McDonald KA, Nandi S. 2019. Purification and site-specific N-glycosylation analysis of human recombinant butyrylcholinesterase from Nicotiana benthamiana. *Biochem. Eng. J.* **142** :58–67. https://doi.org/10.1016/j.bej.2018.11.004.

Alkanaimsh S, Karuppanan K, Guerrero A, Tu AM, Hashimoto B, Hwang MS, Phu ML, Arzola L, Lebrilla CB, Dandekar AM, Falk BW, Nandi S, Rodriguez RL, McDonald KA. 2016. Transient Expression of Tetrameric Recombinant Human Butyrylcholinesterase in Nicotiana benthamiana. *Front. Plant Sci.* **7** :1–13. https://doi.org/10.3389/fpls.2016.00743.

Blanch HW, Clark DS. 1997. Biochemical Engineering. Boca Raton, FL: CRC Press, Taylor & Francis Group. Chemical Industries.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72** :248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Chu CC, Wang CC, Sun C, Hsu C, Yin KC, Chu CY, Bi FY. 1975. Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. *Sci. Sin.***18** :659–668. hhttps://doi.org/10.1360/ya1975-18-5-659.

Corbin JM, Hashimoto BI, Karuppanan K, Kyser ZR, Wu L, Roberts BA, Noe AR, Rodriguez RL, McDonald KA, Nandi S. 2016. Semicontinuous Bioreactor Production of Recombinant Butyrylcholinesterase in Transgenic Rice Cell Suspension Cultures. *Front. Plant Sci.* **7** :412. https://doi.org/10.3389/fpls.2016.00412.

Corbin JM, Kailemia MJ, Cadieux CL, Alkanaimsh S, Karuppanan K, Rodriguez RL, Lebrilla CB, Cerasoli DM, McDonald KA, Nandi S. 2018. Purification, characterization, and N-glycosylation of recombinant butyrylcholinesterase from transgenic rice cell suspension cultures.*Biotechnol. Bioeng.* **115** :1301–1310. https://doi.org/10.1002/bit.26557.

Corbin JM, McNulty MJ, Macharoen K, McDonald KA, Nandi S. 2020. Techno-economic analysis of semi-continuous bioreactor production of biopharmaceuticals in transgenic rice cell suspension cultures.*Biotechnol. Bioeng.* **n/a** :bit.27475. https://doi.org/10.1002/bit.27475.

Ellman GL, Courtney KD, Andres V, Featherstone RM. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity.*Biochem. Pharmacol.* **7** :88–95. https://doi.org/10.1016/0006-2952(61)90145-9.

Fox JL. 2012. First plant-made biologic approved. *Nat. Biotechnol.* **30** :472–472. https://doi.org/10.1038/nbt0612-472.

Gagnon M, Nagre S, Wang W, Coffman J, Hiller GW. 2019. Novel, linked bioreactor system for continuous production of biologics.*Biotechnol. Bioeng.* **116** :1946–1958. https://doi.org/10.1002/bit.26985.

Gamborg OL, Miller RA, Ojima K. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.***50** :151–158. https://doi.org/10.1016/0014-4827(68)90403-5.

Huang J, Sutliff TD, Wu L, Nandi S, Benge K, Terashima M, Ralston AH, Drohan W, Huang N, Rodriguez RL. 2001. Expression and Purification of Functional Human α-1-Antitrypsin from Cultured Plant Cells.*Biotechnol. Prog.* **17** :126–133. https://doi.org/10.1021/bp0001516.

Huang N, Chandler J, Thomas BR, Koizumi N, Rodriguez RL. 1993. Metabolic regulation of α-amylase gene expression in transgenic cell cultures of rice (Oryza sativa L.). *Plant Mol. Biol.* **23** :737–747. https://doi.org/10.1007/BF00021529.

Huang T-K, McDonald KA. 2009. Bioreactor engineering for recombinant protein production in plant cell suspension cultures. *Biochem. Eng. J.* **45** :168–184. https://doi.org/10.1016/j.bej.2009.02.008.

Imseng N, Schillberg S, Schürch C, Schmid D, Schütte K, Gorr G, Eibl D, Eibl R. 2014. Suspension Culture of Plant Cells Under Heterotrophic Conditions. In: . *Ind. Scale Suspens. Cult. Living Cells* . Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA, pp. 224–258. https://doi.org/10.1002/9783527683321.ch07.

Katō K, Shiozawa Y, Yamada A, Nishida K, Noguchi M. 1972. A Jar Fermentor Culture of Nicotiana tabacum L. Cell Suspensions. *Agric. Biol. Chem.* **36** :899–902. https://doi.org/10.1007/s00253-017-8241-5.

Kolarich D, Weber A, Pabst M, Stadlmann J, Teschner W, Ehrlich H, Schwarz H-P, Altmann F. 2008. Glycoproteomic characterization of butyrylcholinesterase from human plasma. *Proteomics***8** :254–263. https://doi.org/10.1002/pmic.200700720.

Leth IK, McDonald KA. 2017. Growth kinetics and scale-up of Agrobacterium tumefaciens. *Appl. Microbiol. Biotechnol.***101** :4895–4903. https://doi.org/10.1007/s00253-017-8241-5.

Lockridge O. 2015. Review of human butyrylcholinesterase structure, function, genetic variants, history of use in the clinic, and potential therapeutic uses. *Pharmacol. Ther.* **148** :34–46. https://doi.org/10.1016/j.pharmthera.2014.11.011.

Macharoen K, McDonald KA, Nandi S. 2020. Simplified bioreactor processes for recombinant butyrylcholinesterase production in transgenic rice cell suspension cultures. *Biochem. Eng. J.* **In press** :107751. https://doi.org/10.1016/j.bej.2020.107751.

Noguchi M, Matsumoto T, Hirata Y, Yamamoto K, Katsuyama A, Kato A, Azechi S. 1977. Plant Tissue Culture and Its Bio-technological Application. Ed. W Barz, E Reinhard, M H Zenk. *Plant Tissue Cult. Its Bio-technological Appl.* Berlin, Heidelberg: Springer Berlin Heidelberg 85–94 p. Proceedings in Life Sciences. https://doi.org/10.1007/978-3-642-66646-9.

Reuter LJ, Bailey MJ, Joensuu JJ, Ritala A. 2014. Scale-up of hydrophobin-assisted recombinant protein production in tobacco BY-2 suspension cells. *Plant Biotechnol. J.* **12** :402–410. https://doi.org/10.1111/pbi.12147.

Rup B, Alon S, Amit-Cohen B-C, Brill Almon E, Chertkoff R, Tekoah Y, Rudd PM. 2017. Immunogenicity of glycans on biotherapeutic drugs produced in plant expression systems—The taliglucerase alfa story. Ed. Raphael Schiffmann. *PLoS One* **12** :e0186211. https://doi.org/10.1371/journal.pone.0186211.

Santos RB, Abranches R, Fischer R, Sack M, Holland T. 2016. Putting the Spotlight Back on Plant Suspension Cultures. *Front. Plant Sci.***7** :297. https://doi.org/10.3389/fpls.2016.00297.

Schiel O, Berlin J. 1987. Large scale fermentation and alkaloid production of cell suspension cultures of Catharanthus roseus.*Plant Cell. Tissue Organ Cult.* **8** :153–161. https://doi.org/10.1007/BF00043152.

Shaaltiel Y, Tekoah Y. 2016. Plant specific N-glycans do not have proven adverse effects in humans. *Nat. Biotechnol.* **34** :706–708. https://doi.org/10.1038/nbt.3556.

Takayama S, Akita M. 2006. Bioengineering Aspects Of Bioreactor Application In Plant Propagation. In: Gupta, SD, Ibaraki, Y, editors.*Plant Tissue Cult. Eng.* Dordrecht: Springer Netherlands, pp. 83–100. https://doi.org/10.1007/978-1-4020-3694-1_5.

Tanaka H, Semba H, Jitsufuchi T, Harada H. 1988. The effect of physical stress on plant cells in suspension cultures. *Biotechnol. Lett.***10** :485–490. https://doi.org/10.1007/BF01027061.

Tekoah Y, Shulman A, Kizhner T, Ruderfer I, Fux L, Nataf Y, Bartfeld D, Ariel T, Gingis-Velitski S, Hanania U, Shaaltiel Y. 2015. Large-scale production of pharmaceutical proteins in plant cell culture-the protalix experience. *Plant Biotechnol. J.* **13** :1199–1208. https://doi.org/10.1111/pbi.12428.

Terashima M, Murai Y, Kawamura M, Nakanishi S, Stoltz T, Chen L, Drohan W, Rodriguez RL, Katoh S. 1999. Production of functional human α1-antitrypsin by plant cell culture. *Appl. Microbiol. Biotechnol.* **52** :516–523. https://doi.org/10.1007/s002530051554.

Trexler MM, McDonald KA, Jackman AP. 2002. Bioreactor Production of Human α1-Antitrypsin Using Metabolically Regulated Plant Cell Cultures.*Biotechnol. Prog.* **18** :501–508. https://doi.org/10.1021/bp020299k.

Trexler MM, McDonald KA, Jackman AP. 2005. A Cyclical Semicontinuous Process for Production of Human α1-Antitrypsin Using Metabolically Induced Plant Cell Suspension Cultures. *Biotechnol. Prog.***21** :321–328. https://doi.org/10.1021/bp0498692.

Ulbrich B, Wiesner W, Arens H. 1985. Large-Scale Production of Rosmarinic Acid from Plant Cell Cultures of Coleus blumei Benth. In: Neumann, K-H, Barz, W, Reinhard, E, editors. *Prim. Second. Metab. Plant Cell Cult.* Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 293–303. http://link.springer.com/10.1007/978-3-642-70717-9_28.

FIGURE 1

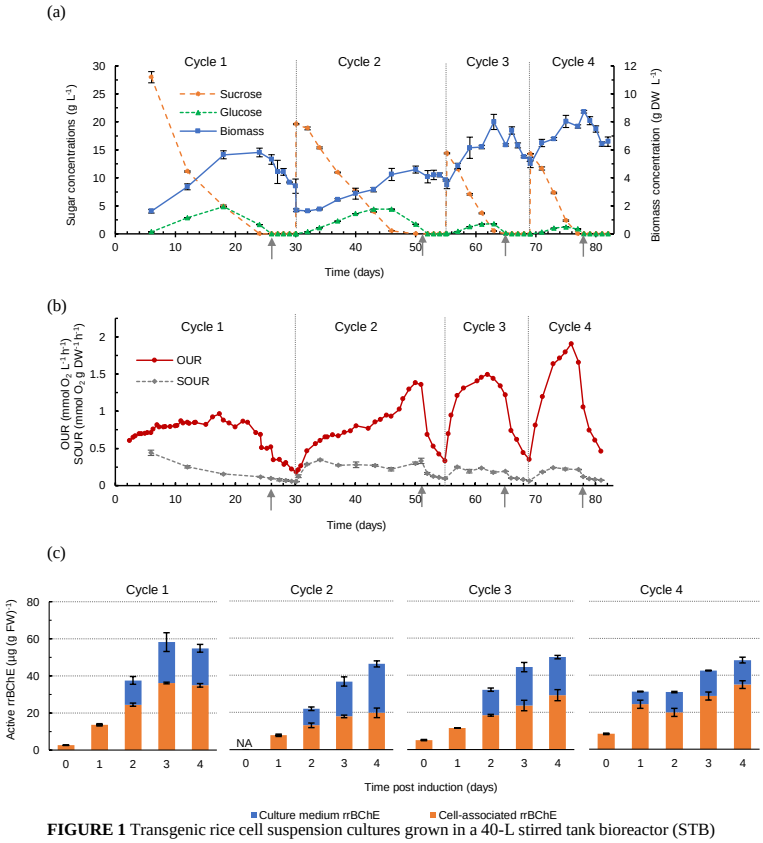

FIGURE 1 Transgenic rice cell suspension cultures grown in a 40-L stirred tank bioreactor (STB) using single-stage, cyclical semicontinuous culture operation: (a) growth profiles and sugar consumption, (b) oxygen uptake rate (OUR) and specific oxygen uptake rate (SOUR), and (c) active rrBChE production levels. Gray arrows indicate the time of induction, and vertical black dotted lines indicate the time of media exchange to start a new cycle. Error bars represent one SD from three replicate measurements. NA, not available.

Hosted file

`Macharoen et al pilot-scale bioreactor Figures.pdf` available at https://authorea.com/users/314815/articles/477416-production-of-recombinant-butyrylcholinesterase-from-transgenic-rice-cell-suspension-cultures-in-a-pilot-scale-bioreactor

Hosted file

Macharoen et al pilot-scale bioreactor Tables.docx available at https://authorea.com/users/314815/articles/477416-production-of-recombinant-butyrylcholinesterase-from-transgenic-rice-cell-suspension-cultures-in-a-pilot-scale-bioreactor