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Method Article

A method to simplify bioreactor processing for recombinant protein production in rice cell suspension cultures

Kantharakorn Macharoen[a], Karen A. McDonald[a,b], Somen Nandi[a,b,*]

[a] Department of Chemical Engineering, University of California, Davis, CA 95616, USA
[b] Global HealthShare® Initiative, University of California, Davis, CA 95616, USA

A B S T R A C T

Transgenic plant cell suspension culture is a promising platform for recombinant protein production. Rice cell suspension culture is one of the systems that has been developed due to its unique metabolically-regulated promoter, rice alpha-amylase 3D (*RAmy3D*), that is up-regulated in sugar-deprived medium. Using the *RAmy3D* promoter system in transgenic rice cell suspensions results in two phases of the culture, the growth phase and the induction phase. Conventionally, medium exchange is performed to remove residual sugar and induce recombinant protein. In this work, a simplified production process is demonstrated in a 5-L bioreactor, including reduction of sugar concentration in the initial culture medium, elimination of the media exchange operation, and uncontrolled dissolved oxygen (DO) with constant aeration. The simplified method significantly improves the accumulation level of a recombinant protein, protein purity, and productivity compared to the conventional method. This method also reduces costs associated with material and labor.

- The method of simplified bioreactor processing includes single-stage culture, uncontrolled dissolved oxygen (DO) but controlled inlet air flowrate, and lower (50% reduction) initial sucrose concentration in the culture medium.
- This method improves recombinant protein production level and productivity compared to the conventional method.
- This method reduces material and labor costs.

A R T I C L E I N F O

Method name: Simplified bioreactor processing
Keywords: Plant-made biopharmaceutical, Plant cell suspension cultures, Single-stage batch culture, Bioreactor operation, *RAmy3D* promoter
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* Corresponding author at: Department of Chemical Engineering, University of California, Davis, CA 95616, USA.
E-mail addresses: kamcdonald@ucdavis.edu (K.A. McDonald), snandi@ucdavis.edu (S. Nandi).

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Specifications Table

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Resource availability:	Not applicable

Method details

Background

Metabolically-regulated rice cell suspension culture has been shown to be a promising cost-effective platform to produce biopharmaceuticals [1]. Traditionally, rice cell suspension cultures containing the rice alpha-amylase 3D (*RAmy3D*) expression system (*RAmy3D* promoter, signal peptide and terminator) are grown in sugar-rich medium to generate biomass, and then the spent medium is removed and replaced with sugar-free medium to induce recombinant protein expression since the *RAmy3D* promoter is strongly activated under sugar starvation conditions [2–5]. In other words, a two-stage culture, growth phase followed by an induction phase, is commonly implemented by changing the media between sugar-rich and sugar-free media. The media exchange is usually performed when rice cells reach the mid-late exponential growth phase to ensure high metabolic activity of the rice cells and high cell density prior introducing the sugar-free medium [6,7]. However, removing spent medium containing residual glucose and/or fructose and replacing it with sugar-free medium requires time and effort, increases the risk of contamination, results in the loss of cell biomass, and could be challenging to perform in a large-scale bioreactor. The simplified method of operating a rice cell culture bioreactor presented here includes eliminating the media exchange (i.e. resulting in a single-stage culture), providing oxygen by compressed air sparging at a constant rate, and using 50% of the initial sucrose concentration in the medium to shorten batch cultivation time and improve productivity.

Materials and chemicals

Transgenic rice cells engineered with the *RAmy3D* expression system for biopharmaceutical production were generated as previously described [3,6]. Sugar-rich medium contains 4.1 g/L modified Chu/Gamborg basal [8,9] (NB basal; Phytotech Labs, Shawnee Mission, KS), 300 mg/L of casein hydrolysate, 250 mg/L of L-glutamine, 2 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D), 0.02 mg/L of kinetin, 30 g/L of sucrose (Sigma-Aldrich, St. Louis, MO) in full-strength sucrose (denoted as NB+S medium) or 15 g/L sucrose in half-strength sucrose (denoted as NB+0.5×S), and 250 mg/L of L-proline (Fisher Scientific, Fair Lawn, NJ). Rice cell suspensions grown in shake flasks were subcultured in fresh NB+S medium every 7-9 days.

Fig. 1 shows a conventional 5-L stirred-tank bioreactor (STB; BioFlo 3000, New Brunswick Scientific, Edison, NJ is now Eppendorf, Inc., Hauppauge, NY) with a height-to-diameter ratio of 2 equipped with a pitched-blade impeller of 10.2 cm diameter used in the simplified bioreactor processing method. A ring sparger, thermowell, and sampling/harvest tube were assembled with the bioreactor headplate by following the manufacturer's manual (baffles were not used in these studies). The pH sensor and the polarographic DO sensor from Mettler Toledo (Billerica, CA) were used in this method in which pH and DO were not controlled but monitored using FoxyLogic Fermentation Program version 4.4.4.

Bioreactor set-up (modified from the manufacturer's manual and [6,7])

1. Check and ensure all o-rings are intact.
2. Link the opening of acid and base addition ports with a small and autoclavable plastic tubing since the ports are not used (i.e. culture pH is not controlled).

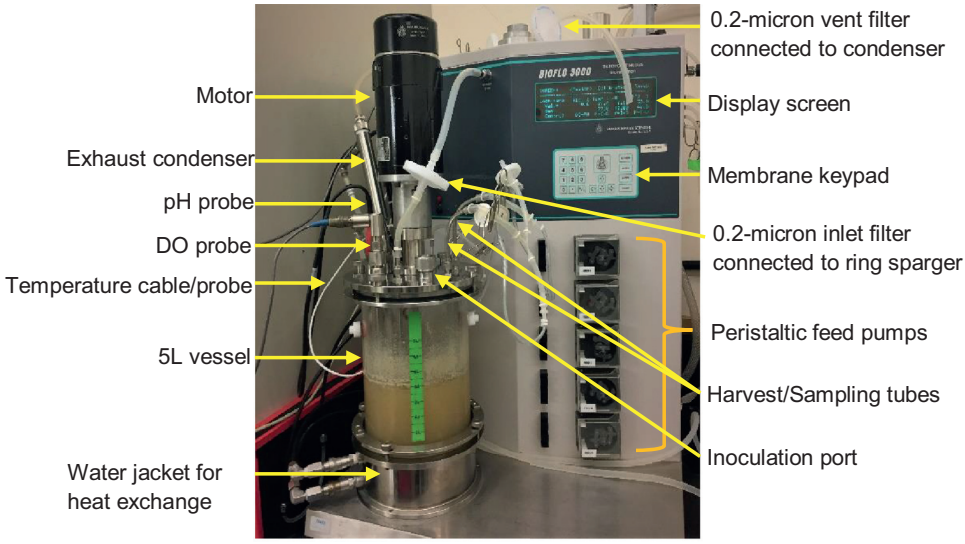

Fig. 1. Bioreactor assembly for metabolically regulated transgenic rice cell suspension cultures. Aeration was set to "manual" with 100% air with the flow rate of 0.2–0.4 volume of gas per culture volume per minute (vvm).

3. Clamp plastic tubing lines connected to the opening of the harvest/sampling ports and the ring sparger with 0.2 µm vent filters at their ends with metal pinch clamps.
4. Calibrate the pH sensor with standard buffers at pH 4 and pH 7.
5. Prepare three liters of NB+0.5×S and fill it in the bioreactor vessel.
6. Place the bioreactor headplate on the top of the bioreactor vessel and secure it with the headplate clamp knobs
7. Insert the pH sensor and the DO sensor through their ports.
8. Insert the exhaust condenser in its port, with the condenser outlet connected to a "Y" fitting and plastic tubing lines terminating in 0.2-µm vent filters.
9. Tighten the headplate fittings on each port, except the inoculation port that is loosened to avoid pressure build up during autoclaving.
10. Autoclave the bioreactor at 121 °C for 40 min.
11. After autoclaving, allow the bioreactor to cool down overnight. The inoculation port is tightened once it is cool enough to handle.
12. Place the bioreactor on the console, set agitation at 200 rpm and temperature at 27 °C, and then sparge compressed air into the bioreactor at 2 vvm (volume of gas sparged per working volume per minute) for several minutes to allow the medium to be saturated with air.
13. Calibrate the DO sensor by setting the span at 100% when the medium is fully saturated and at equilibrium with air. Detach the instrument cable from the DO sensor to set the zero to 0% DO.
14. Adjust the agitation to 75 rpm and the aeration to 0.2 vvm.

Operation of the bioreactor

1. Detach the bioreactor from the console, spray 70% ethanol over it, and then place it inside a laminar flow hood or biosafety cabinet (BSC).
2. Combine shake flask cultures inside the hood, and transfer the combined culture into the bioreactor through the inoculation port to obtain 20 ± 5% v/v (volume of inoculating suspension to final working volume of culture), depending on inoculum biomass density to achieve a target initial biomass density greater than 1.5 g dry weight/L, corresponding to working volume of 3.5–4.0 L.

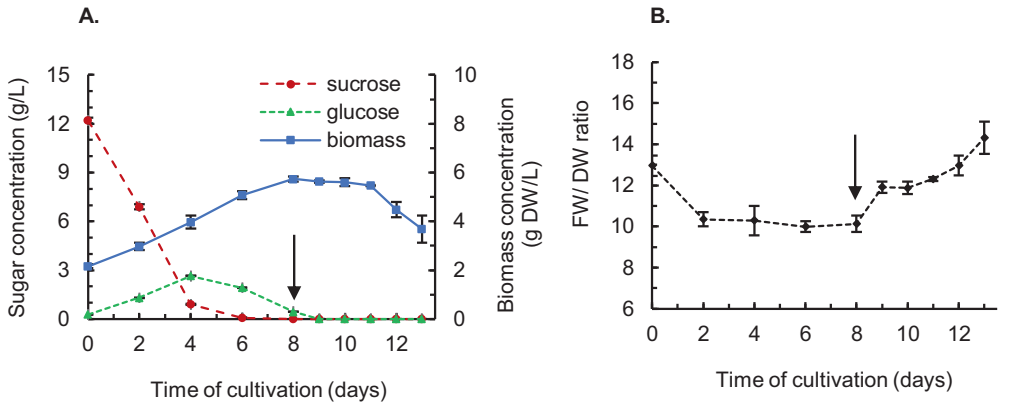

Fig. 2. Metabolically regulated rice cell culture bioreactor for method validation. (**A)** Growth profiles and sugar consumption under the simplified bioreactor processing (uncontrolled DO and no media exchange) and half-strength sucrose of the culture medium. (**B)** Rice cell fresh weight (FW) to dry weight (DW) ratio. Error bars indicate one SD from three biological replicates. Arrows indicate the start of induction (sugar depleted) phase.

3. Insert a pre-sterilized 10-mL dipper to scoop a well-mixed sample while the impeller shaft is manually being rotated to ensure good mixing. Four samples are normally taken for fresh weight (FW) and dry weight (DW) analyses, sugar concentration measurement, and quantification of the recombinant protein level.
4. After sampling, move the bioreactor back to the console and connect electrical sensors (pH, DO and temperature probes), gas sparger, motor, cooling/heating jacket, and chilled-water condenser jacket.
5. Prime the water to the pump inside the console for a minute, and then set the temperature to 27 °C.
6. Set the agitation rate and aeration rate to 75 rpm and 0.2 vvm, respectively, in which 100% of air is selected.
7. Measure oxygen uptake rate (OUR) by halting the aeration temporarily and recording the rate of decrease in DO.
8. Once DO is below 20% at day 2–3 of cultivation, increase the aeration rate to 0.4 vvm.
9. Monitor the pH and DO over the time of cultivation. Once the sugar in the NB+0.5×S culture medium is depleted, the pH and DO will rise, indicating the start of the induction phase.
10. During the induction phase, decrease the aeration rate to 0.2 vvm and collect samples every day for five days.
11. Quantify recombinant protein concentrations and total soluble protein (TSP) concentrations in the culture medium and rice cell extract (typically using a 1:1 ratio of g FW per mL extraction buffer).

Method validation

Transgenic rice cell line for the expression of recombinant human butyrylcholinesterase (BChE), a bioscavenger enzyme that stoichiometrically binds to organophosphorus nerve agents and pesticides [10], was used to validate the method in which the development of the rice cell line was previously described [6,11]. Starting at the initial biomass concentration of 2.2 ± 0.1 g dry weight (DW)/L, rice cell biomass gradually increased and reach its maximum of 5.7 ± 0.1 g DW/L at day 8 of cultivation where sucrose and glucose were 0 g/L and <0.5 g/L, respectively (Fig. 2(A)). The concentration of sucrose in the medium rapidly decreased due to sucrose conversion to glucose and fructose by rice cell wall invertases [12,13] leading to increased glucose concentration in the medium at the beginning of cultivation. In the meantime, glucose and fructose were taken up by the rice cells. At day 4 of

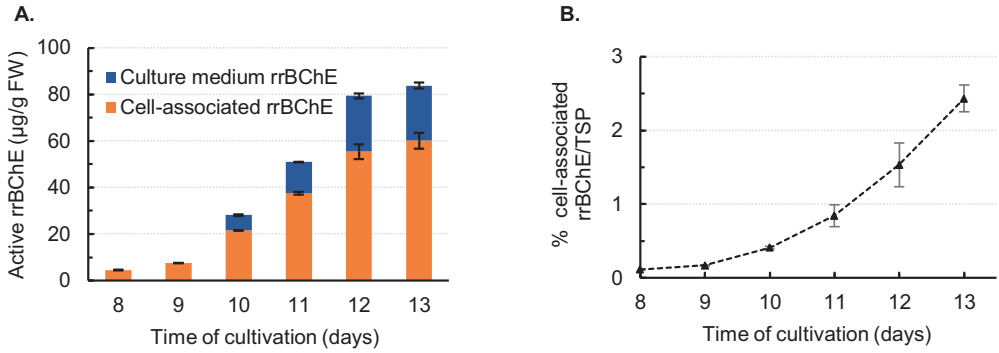

Fig. 3. Production parameters of the validation bioreactor run during the induction phase (day 8–13 of cultivation). (**A**) Active rice recombinant butyrylcholinesterase (rrBChE) with a combination of rrBChE from the culture medium and rice cell extract (cell-associated rrBChE). (**B**) % cell-associated rrBChE per total soluble protein (TSP) as recombinant protein purity. Error bars indicate one SD from three technical replicates.

cultivation the rate of sucrose hydrolyzation was lower than the rate of glucose uptake, resulting in the decrease of glucose in the medium as seen in Fig. 2(A). During the induction phase, after sugar depletion, the biomass concentration decreased due to sugar deprivation in the culture medium, which is normally found in metabolically regulated rice cell suspension cultures under the *RAmy3D* promoter.

The ratio of FW to DW shown in Fig. 2(B) was around 10 g FW/g DW during the exponential growth phase but increased during the induction phase up to 14 g FW/ g DW at day 13 of cultivation (5-day post induction). The increase of FW/DW ratio correlates with the increase of rice recombinant human BChE levels (rrBChE) as seen in Fig. 3(A). The accumulation level of rrBChE increased starting at day 8 of cultivation when the sugar was almost depleted due to the activation of the RAmy3D promoter. While rrBChE in the culture medium in a previous report using two-stage culture and controlling DO at 40% was negligible [6], a significant amount of rrBChE in the culture medium was detected in this run (Fig. 3(A)). The total active rrBChE reached 84 ± 7 µg/ g FW at day 13 of cultivation in which cell-associated rrBChE contributed 60.1 ± 6.5 µg/ g FW, resulting in a significant improvement of rrBChE production compared to 21–25 µg/g FW [6] or 48–53 µg/g FW [11] in conventional two-stage 5-L bioreactor cultures. The purity of rrBChE (% rrBChE/TSP) in crude cell extracts is shown in Fig. 3(B). The purity increased over time during induction as cell-associated rrBChE accumulation increased, while TSP in the rice cell extract decreased, resulting in a maximum purity of 2.43 ± 0.18%. Finally, the maximum volumetric productivity and maximum specific productivity of total active rrBChE improved considerably to 383 ± 31 µg/(L day) and 100 ± 17 µg/(g DW day), respectively, compared to 36–184 µg/(L day) [6] or 174–275 µg/(L day) and 48–55 µg/(g DW day) [11] in conventional two-stage 5-L bioreactor cultures.

Employing a single-stage batch culture can save time and material consumption (US$2.0/L of sugar-free medium; in-house preparation at lab scale pricing) from the medium exchange in traditional two-stage batch culture. Moreover, using NB+0.5×S and single-stage batch culture not only improves rrBChE production level and productivity but also decreases the medium cost by 22% from US$2.3/L of NB+S to US$1.8/L of NB+0.5×S at lab scale pricing. Our recent techno-economic simulation study for commercial scale operation in a new facility also showed that a single-stage batch culture reduces the cost of goods sold for rrBChE by ~5% compared with a two-stage batch culture [1].

Conclusions

The method of simplified plant cell culture bioreactor processing (uncontrolled DO and no media exchange) with half-strength sucrose of the culture medium was validated in a 5-L bioreactor batch culture. The method showed no negative impacts on rice cell growth profiles and sugar

utilization. In contrast, the method significantly improved the maximum accumulation level, purity, and productivity of the recombinant protein. Altogether, implementing the method presented here in other metabolically regulated rice cell suspensions could potentially improve recombinant protein production and minimize production costs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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