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RAPID COMMUNICATION

Improving techno-economics of bioproduct glycolic acid by successive recycled-cell catalysis of ethylene glycol with *Gluconobacter oxydans*

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Abstract

Bioconversion of ethylene glycol (EG) to glycolic acid (GA) by the whole-cell of *Gluconobacter oxydans* in an aired stirred tank reactor (ASTR) with continuous substrate feeding yielded over 220 g/L of GA. However, the bioreactor productivity declined to an unfavorable level of 0.63 g/L/h due to negative feed-back by GA which inhibited the reaction. To overcome this problem, based on results obtained from techno-economic comparative analysis, we set up a successive recycled-cell catalytic bioprocessing ASTR, and carried out five consecutive cycles stably during 240 h. At the end of this process, total 490.7 g GA was accumulated with over 90% yield, and an average bioreactor productivity of 2.04 g/L/h. The twin strategies of end-product titer control and cell-recycling successfully demonstrated the large scale applicability of EG bioconversion to GA.

Keywords Glycolic acid · Ethylene glycol · Successive recycled-cell catalysis · *Gluconobacter oxydans* · Techno-economics

Introduction

As the simplest α-hydroxyl acid containing both hydroxyl and carboxyl groups, glycolic acid (GA) has significant industrial potential [1]. It is widely used in the production of chemicals such as adhesives, in metal cleaning, as a dye-stuff additive, etc. [2, 3]. The most important commercial use of GA is in the synthesis of biodegradable polyglycolic acid (PGA), an ideal packaging material, [4] and poly (Lactic-co-Glycolic Acid) (PLGA) for medical applications [5]. The market for GA in 2011 was USD 93.3 million (40 million kg) and expected to reach USD 203 million in 2018 [6].

Industrial production of GA mainly relies on chemical synthesis using chloroacetic acid or hydroxy acetonitrile hydrolysis [7]. Both approaches have several shortcomings such as high costs, environmental pollution and a complicated purification process. On the contrary, bio-catalysis using living microorganisms has the advantage of mild reaction conditions, good selectivity and high purity. *Gluconobacter oxydans*, a representative gram-negative, aerobic and acidophilic bacteria, is known for its versatile bio-oxidation capabilities on hydroxyl-aldehydes, hydroxyl-ketones and sugars, and is, therefore, utilized for the industrial production of aldonic acid, 1,3-dihydroxyacetone [8], xylonic acid [9], furonic acid [10] and other oxidation products. We used *G. oxydans* for the bioconversion of cheaply available ethylene glycol (EG) substrate into the more expensive GA [11].

Many studies have reported GA bio-production using natural *G. oxydans* strains [12, 13]. The highest quantity of GA obtained using Fed-batch production in an aired stirred tank reactor (ASTR) was 74.5 g/L GA with a productivity of 1.49 g/L/h. Even after introducing an in-situ resin adsorption column, only 93.2 g/L of GA was obtained at the productivity of 1.86 g/L/h. Recombinant *G. oxydans* overexpressing the membrane-bound alcohol dehydrogenase could accumulate 113.8 g/L GA at the productivity of 2.53 g/L/h [14]. Although the biotechnological optimization of cell catalysis has come a long way, more effective and competitive bioprocesses need to be developed with special focus on the economical aspect. Therefore, the primary objective of this

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study was to simplify the bioconversion of EG to GA and improve the latter's productivity using a natural *G. oxydans* strain.

Materials and methods

Microorganism and culture conditions

Gluconabcter oxydans NL71, derived from the strain of ATCC 621, was maintained at 4 °C on sorbitol-agar medium containing sorbitol (50 g/L), yeast extract (5 g/L), and agar (15 g/L).

Gluconabcter oxydans NL71 inocula were grown in 50 mL medium containing sorbitol (100 g/L) and yeast extract (10 g/L) for 24–36 h in a continuously shaken 250-mL Erlenmeyer flask at 220 rpm and 30 °C. The cell pellet was harvested by centrifugation at 6000 rpm for 5 min [15].

Bioconversion of EG was carried out in a 3 L ASTR in 1 L fermentation broth containing 0.5 g/L $MgSO_4$, 1 g/L KH_2PO_4, 2 g/L K_2HPO_4, 5 g/L $(NH4)_2SO_4$, 25 g/L yeast extract and1 g/L sorbitol for every 20 g of EG [9]. Sorbitol acted as a cofactor for cell growth and metabolism. The aeration rate in the fermenter was consistent and the pH was adjusted to 5.5 by adding NaOH.

Whole-cell catalysis

Three catalysis techniques were tested for the efficient bioconversion of EG to GA—Fed-batch catalysis (FBC), Continuous feeding catalysis (CFC), and Successive recycled-cell catalysis (SRC). FBC and CFC were adapted in ASTR by, respectively, adding the substrate intermittently and continuously. Just because of this operation, parallel experiments were hard to be implemented. For intermittent substrate addition, we analyzed the composition of the fermentation br [13] oth at regular intervals, and added 20 g/L of the substrate very time its concentration dropped below a set threshold. This step was repeated several times during the catalysis process. CFC on the other hand was carried out by means of a peristaltic pump that continuously fed a constant quantity of the substrate (20 g/L) into the broth.

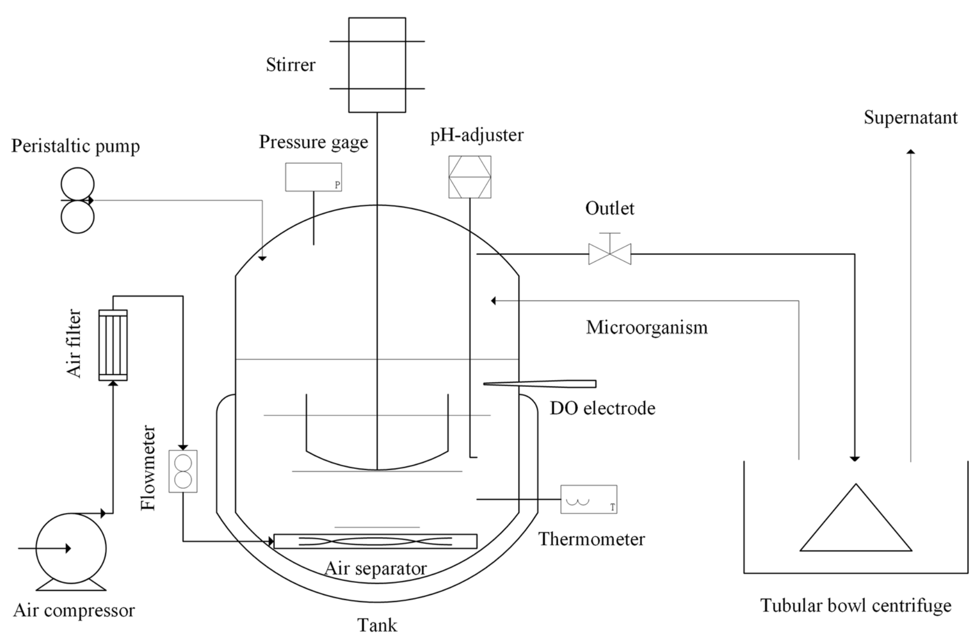
For SRC, the *G. oxydans* inoculum was recycled after every 48 h of catalysis, which involved continuous substrate feeding in the ASTR (BioFlo 115, New Brunswick 69 Scientific Co., Inc.), and the vessel was equipped with a peristaltic pump. The design of the bioreactor suitable for FBC, CFC and SRC is showed in Fig. 1. After each 48 h catalysis cycle, the fermentation broth and *G. oxydans* cells were separated by centrifugation at 6000 rpm for 10 min by tubular bowl centrifuge. The pelleted cells were reloaded with 1 L of fresh medium containing 20 g/L EG and then the centrifugal supernatant was detected by HPLC. Cell-recycling was repeated for a total of 5 times, which resulted in along continuous reaction.

Analytical methods

GA and EG were purchased from Macklin, and yeast extract from Sigma. All other chemicals were of analytical grade and were commercially available.

The concentrations of GA and EG were measured using high performance liquid chromatography (HPLC) (Agilent 1260) equipped with Aminex Bio-Rad HPX-87H column

Fig. 1 Diagram of the ASTR for successive production



and 5 mM/L H_2SO_4 was used as the mobile phase at 0.6 mL/min. GA yield (%) was calculated by dividing the total concentration of GA obtained in the end by the total EG concentration, and then multiplying by the constant 0.805. Bioreactor (volume) productivity (g/L/h) of GA was calculated by dividing the GA titer by the reaction time and volume of the fermentation broth. Production rate of GA was calculated by differential value of GA production vs reaction time.

Results and discussion

Fed-batch catalysis

Compared to the Erlenmeyer flasks subjected to continuous shaking, ASTR significantly enhanced the kinetics of cell bio-catalysis due to its agitator blade that trapped the air into small bubbles of the fermentation broth, and greatly improved oxygen distribution in the broth [16]. Therefore, ASTR was used in our study to increase the output of GA and improve the catalytic performance of the bacterial strain. In addition, a pure oxygen-aerated stirred tank reactor (O-ASTR) has also been devised but that did not significantly increase GA output compared to the regular ASTR, suggesting that the aeration in the latter can provide enough oxygen to support *G. oxydans* growth and bioconversion of EG to GA. With the additional aspect of lower costs, later experiments were designed in ASTR. Previous studies showed that GA production by whole-cell bio-catalysis was inhibited by both the substrate and product through negative feed-back mechanisms. Since EG concentration above 20 g/L significantly inhibited the reaction, FBC with intermittent substrate feeding was designed to overcome substrate inhibition.

As shown in Fig. 2, the quantity of GA accumulated within 240 h was 191.4 g/L with a 94.0% yield. Both the product yield and the action time were significantly higher than reported in previous studies, and was most likely a result of the yeast extract which provided sufficient restrictive factors and nutrition. As seen in a large number of early optimization experiments, regardless of whether the catalysis was carried out in Erlenmeyer shaken flasks or ASTR, GA production and the reaction time improved enormously with increasing concentrations of yeast extract (data not shown). We hypothesized that it was not the nitrogen or protein in the yeast extract that aided GA production, but vitamin B. Simultaneously, a study examining any possible role of vitamin B is currently underway. During the first 48 h of fermentation, the productivity of GA increased to 1.70 g/L/h, at which point the GA output was 81.39 g/L, and production rate remained constant at 0.55 g/L/h thereafter till 240 h. This indirectly proved that yeast extract increased the catalytic activity of *G. oxydans* as well as reduced the

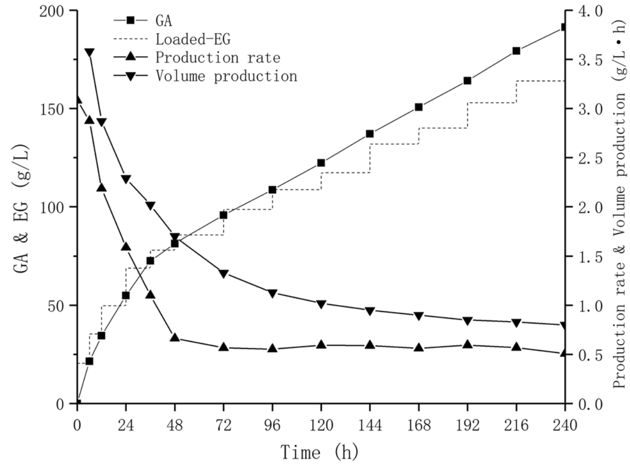


Fig. 2 Reaction process of the whole-cell catalysis in ASTR was operated by FBC. Recorded concentrations of EG and GA, bioreactor productivity and production rate of catalysis

toxic effects of both EG and GA on *G. oxydans*. After 240 h of catalysis, the total volume productivity sunk to 0.8 g/L/h, although it did not affect the overall catalytic process and the end product still had enough space to accumulate. Taken together, the metrics of GA production by the above method are encouraging and offer a potentially new efficient biological method for industrial GA production.

Continuous feeding-cell catalysis

The combination of the intermittent substrate feeding FBC and yeast extract has significantly improved bio-production of GA by *G. oxydans*. Nevertheless, the main objective of this study was to simplify the bioprocess and improve bioreactor productivity. Therefore, we made a further improvement of replacing the manual intermittent substrate feeding by automatic continuous feeding via a peristaltic pump. This approach has two advantages: (a) it simplifies the whole process, and saves considerable time and labor, and (b) it avoids the problem of insufficient reaction kinetics due to substrate depletion.

As shown in Fig. 3, GA production followed overall similar trends with both the FBC and CFC approaches in the ASTR. When compared horizontally at the 48 h timepoint, CFC showed a certain improvement in both GA production and bioreactor productivity that reached 92.95 and 1.94 g/L/h, respectively. Similarly, after 48 h of CFC, the production rate was slightly higher and remained constant at around 0.69 g/L/h. The final quantity of GA obtained after 240 h of catalysis was 224.71 g/L at the yield of 98.3%, and the final bioreactor productivity was 0.94 g/L/h. Compared to FBC, GA production and bioreactor productivity increased by 17.4 and 17.5%, respectively, in CFC, with ample space for further product accumulation. In conclusion,

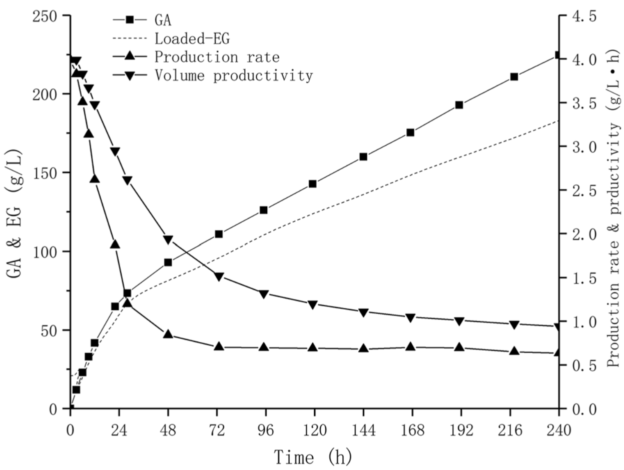


Fig. 3 Reaction process of the whole-cell catalysis in ASTR was operated by CFC. Recorded concentrations of EG and GA, bioreactor productivity and production rate of catalysis

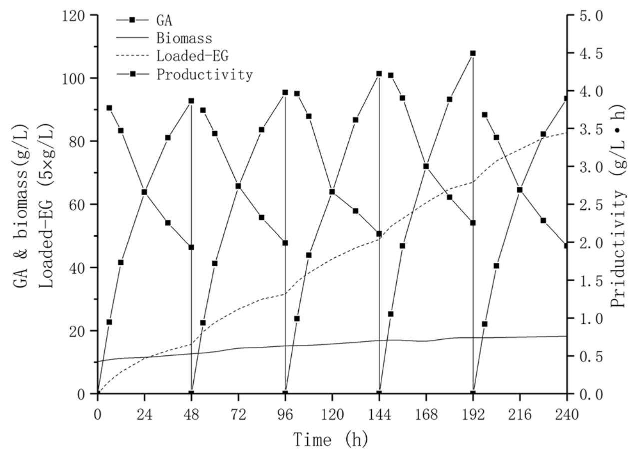


Fig. 4 The profile of EG to GA in ASTR carried out by SRC operation and cell-recycle technique (5 times)

cell bio-catalytic activity can be significantly enhanced by continuous substrate feeding. However, despite improvements in overall production and yields by regulating concentration of EG and increasing that of yeast extract, significant product inhibition still existed after 48 h. Therefore, the bioreactor could not be fully utilized and we could not obtain favorable techno-economic statistics. To overcome this limitation, we adopted the cell-recycling technique where were cycled the bacterial inoculum after every 48 h catalysis cycle.

Successive recycled-cell catalysis

Previous studies have been successful to a certain extent in improving tolerance of *G. oxydans* to substrate and product toxicity. Nevertheless, it is more economical to simultaneously enhance *G. oxydans* catalytic activity and lower product inhibition. As is clear from Fig. 2, the catalysis process could be divided into two stages in 48 h. Since it is difficult to incite *G. oxydans* proliferations [17], recycling the cell load along with a continuous fed-batch technique, i.e. SRC, can help override this physiological limit. The process involves separating and harvesting the *G. oxydans* cells from the fermentation broth and reloading those cells along with fresh medium to make most use of the bioreactor. As shown in Fig. 4, the SRC process went through five rounds of cell recycling. In each catalysis cycle, EG was rapidly converted to more than 90 g/L GA and this bio-catalytic ability of *G. oxydans* could be almost completely recovered when reloaded into the new medium. This once again indicated that the catalytic activity of *G. oxydans* was restricted by product inhibition. In addition, the bacterial biomass increased during the catalysis process due to sorbitol which acted as a cofactor for cell growth and metabolism. Consistent with this increase in biomass, there was a slight improvement in the GA output in each successive catalysis cycle, with the total production increasing from 92.7 to 107.81 g/L till the fourth cycle. However, GA production dropped sharply in the fifth cycle, inevitably due to the accumulating dead and damaged cells over the entire catalysis period. The total GA production after 5 catalysis cycles was 490.7 g at the yield over 90% and an average productivity of 2.04 g/L/h. Compared to CFC, the total GA mass-production and bioreactor productivity increased by 118.4 and 117.0%, respectively, during the same time period. Taken together, our processes have obtained the highest level of bioconverting EG to GA so far with a natural *G. oxydans* strain.

Conclusions

Based on the techno-economic analyses, *G.oxydans* catalyzed production of glycolic acid was markedly improved by employing the SRC fed-batch strategy. We were able to utilize the cells to the greatest extent possible that substantially reduced the costs of cell culture and catalysis. Furthermore, more importantly, our strategies greatly simplified bioprocess and effectively improved bioreactor productivity. Our study provides a practical approach for the industrial bio-production of glycolic acid from ethylene glycol with respect to glycolic acid titer, yield and bioreactor productivity.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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