

Production of D-Glyceric Acid by a Two-step Culture Strategy Based on Whole-cell Biocatalysis of *Acetobacter tropicalis*



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D-Glyceric acid (D-GA) is a promising chemical for minimizing the toxic effect of acetaldehyde. It has been successfully produced by bioprocesses using glycerol as a substrate. However, high concentrations of glycerol are not beneficial for cell growth. A two-step culture strategy was employed to deal with the ambivalent culture conditions between the growth of *Acetobacter tropicalis* and the biosynthesis of D-GA. The first stage focuses on biomass accumulation with low initial glycerol concentration; the second stage provides D-GA accumulation through whole-cell biocatalysis. Approximately 2-fold of D-GA yield and 4.5-fold of D-GA productivity were gained by the two-step culture strategy compared to the traditional fermentation with 150 g L⁻¹ of glycerol. Our results also showed that *A. tropicalis* was able to catalyze the conversion of glycerol to D-GA in the presence of up to 10 % methanol. These findings have important implications to enhance D-GA yield by strategy optimization and reduce its cost.

Key words:

glyceric acid, *Acetobacter*, two-step culture, biocatalysis, fermentation, cost-effective production

Introduction

Glyceric acid (2,3-dihydroxypropionic acid; GA) is an important intermediate and organic synthetic material, which is widely used in chemical products synthesis¹, cosmetics², and pharmaceuticals³. D-GA has biological functions such as accelerating ethanol and acetaldehyde oxidation in rats⁴ and enhancing the viability of ethanol-dosed gastric cells³. It is also a liver stimulant and cholesterolytic agent in dogs^{5,6}. Therefore, D-GA is a promising chemical for minimizing the toxic effect of acetaldehyde, the metabolite of ethanol.

High purity of D-GA can be gained by microbial oxidation such as fermentation with high concentration of glycerol^{2,7}. However, high glycerol

concentration may have negative effects on cell growth, and the complex compositions of fermentation broth increase the difficulties in D-GA purification. Although desalting electro dialysis was shown to be useful in separating D-GA from broth⁸, the equipment is costly. Enzyme catalysts can avoid the considerable investment in equipment, but the enzyme itself is also costly. Compared with isolated enzymes, whole cell catalysts can be much more readily and inexpensively prepared, and enzymes in the whole cells are more stable than free enzymes as they are protected inside cells. Additionally, the use of pure substrate with whole-cell catalyst should have fewer conflicts between cell growth and production as compared to fermentation. Recently, whole-cell biocatalysis has been successfully applied to produce biodiesels⁹, 6-O-propionyl-D-glucose¹⁰, fructose laurate esters¹¹, and other commercial chemicals. Multistage fermentation was shown

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to be an effective method to facilitate both cell growth and product accumulation^{12,13}. In this study, a two-step culture strategy was utilized to enhance D-GA yield without cell growth inhibition in *A. tropicalis*: low glycerol medium was first applied for cell growth (first stage), and then whole-cell biocatalysis was performed using glycerol as the only substrate to synthesize D-GA (second stage).

Glycerol, the substrate for D-GA, is the main by-product of the biodiesel fuel (BDF) production process. With the rapid worldwide increase in BDF production, glycerol becomes abundant, which leads to a dramatic decrease in price¹⁴. Therefore, biological conversion of glycerol from the BDF process into D-GA is not only an economic use of glycerol, but also a commendable method to cut the cost of D-GA production. However, the raw glycerol derived from BDF production always includes methanol, which is costly to remove by evaporation during BDF manufacturing. Therefore, the use of raw glycerol containing methanol is economically preferable, necessitating the development of technology using glycerol that contains methanol¹⁵. A methanol-resistant mutant of *G. frateurii* was successfully constructed to produce D-GA from 5 % (v/v) methanol-containing glycerol medium¹⁶. However, few studies of methanol resistant ability of *A. tropicalis* have been reported. Therefore, the influence of methanol-containing glycerol on D-GA biosynthesis in *A. tropicalis* in the whole-cell biocatalysis process was also examined in this study.

Materials and methods

Strains and culture conditions

A. tropicalis CHM061701 (available in China General Microbiological Culture Collection Center) was first cultivated in 250-mL Erlenmeyer flasks containing 50 mL of glucose medium (pH 6.5) containing 5 g L⁻¹ glucose, 5 g L⁻¹ polypeptone, 5 g L⁻¹ yeast extract, 1 g L⁻¹ MgSO₄·7H₂O at 30 °C, and 200 rpm for 24 h. This was used as seed culture for the traditional culture and two-stage culture.

Traditional culture

Two-and-a-half mL seed culture (inoculum volume: 5 % of glycerol medium) was transferred to 250-mL Erlenmeyer flasks containing 50 mL of glycerol medium (pH 6.5) consisting of 5 g L⁻¹ polypeptone, 5 g L⁻¹ yeast extract, 1 g L⁻¹ MgSO₄·7H₂O, 0.9 g L⁻¹ KH₂PO₄, 0.1 g L⁻¹ K₂HPO₄, and varying concentrations of glycerol (50–200 g L⁻¹), and incubated at 30 °C on a rotary shaker (200 rpm) for 5 d.

Two-stage culture

Culture stage (first stage): This step was similar to the traditional culture. Two-and-a-half mL seed culture was transferred to 250-mL Erlenmeyer flasks containing 50 mL of glycerol medium (pH 6.5) with 50 g L⁻¹ of glycerol, and incubated at 30 °C on a rotary shaker (200 rpm) for 60 h. The cells were then harvested for the second stage.

Whole-cell biocatalysis stage (second stage): The cells from the first stage were harvested by centrifugation at 10,000 rpm for 5 min, and washed twice with 0.9 % NaCl to remove residual culture nutrients. The cells were resuspended in different concentrations of glycerol (normally 150 g L⁻¹), and the OD₆₀₀ of this solution was normalized to 5. Then 50 mL of this glycerol solution was inoculated in a 250-mL Erlenmeyer flask, and the whole-cell biocatalysis process was carried out at 28 °C and 200 rpm for 48 h. Each experiment was carried out in triplicate.

Quantification of glycerol, D-GA and dihydroxyacetone (DHA)

Both of the Glycerol and D-GA contents were analyzed by high-performance liquid chromatography (HPLC) equipped with a Shodex SH1011 Column, and monitor by RID detector¹⁷. The column was maintained at 50 °C. The mobile phase was 5 mM H₂SO₄ solution and the flow rate was 0.8 mL min⁻¹.

DHA was quantified by the visible spectrophotometric method according to Liu¹⁸. Briefly, 1-mL samples were mixed with 9 mL 1 % (w/v) diphenylamine reagent (diphenylamine dissolved in sulfuric acid/acetic acid solution (1:9, v/v)) and incubated in boiling water for 20 min. The samples were then cooled to room temperature and the absorbance at 615 nm was measured immediately.

Results and discussion

Effect of glycerol concentrations on *A. tropicalis* growth and D-GA accumulation

To examine the effect of glycerol concentration on cell growth and D-GA accumulation in *A. tropicalis* CHM061701, glycerol was applied as carbon source to incubate *A. tropicalis* CHM061701. As shown in Fig. 1, *A. tropicalis* CHM061701 grew better in medium with lower concentrations of glycerol (50 g L⁻¹ and 100 g L⁻¹). The cells entered the exponential growth phase after inoculation for 12 h, and entered stationary phase at 72 h (Fig. 1a). The maximum dry cell weight (DCW) gained in these two cultures was 9.65 g L⁻¹ and 7.55 g L⁻¹, respectively. Cells grown in culture with 150 g L⁻¹ and

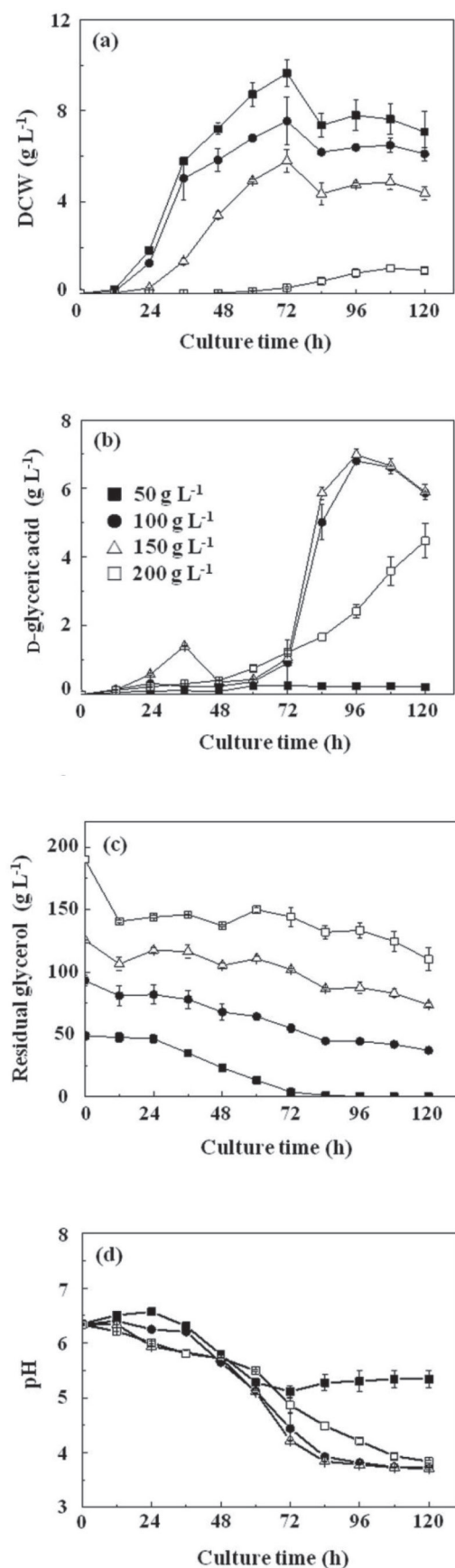


Fig. 1 – Effect of initial glycerol concentrations on cell growth and D-GA yield (a) DCW; (b) D-GA; (c) residual glycerol; (d) pH

200 g L⁻¹ glycerol experienced a rather long lag phase, 24 h and 60 h, respectively. Total biomass decreased with increasing initial glycerol concentration, and only 1.02 g L⁻¹ DCW was harvested in culture with 200 g L⁻¹ glycerol, which was only 10.6 % of that with 50 g L⁻¹ initial glycerol. These results indicated that more than 50 g L⁻¹ glycerol exerted a negative effect on the growth of *A. tropicalis*. Thus, a lower initial glycerol concentration is required to promote cell growth and achieve a relatively high DCW. However, D-GA was undetectable in cultures with 50 g L⁻¹ glycerol which was the best condition for cell growth. D-GA biosynthesis occurred at glycerol concentrations above 100 g L⁻¹ (Fig. 1b). The maximal D-GA yield (6.98 g L⁻¹) was gained in the culture with 150 g L⁻¹ glycerol at 96 h, and the D-GA productivity of *A. tropicalis* was 1.45 g L⁻¹ DCW. Interestingly, the D-GA productivity increased with increasing initial glycerol concentration. In the culture with 200 g L⁻¹ glycerol, the productivity D-GA was up to 4.39 g⁻¹ DCW, however, the cell growth was inhibited severely in this condition (Fig. 1a). These results showed that a high concentration of glycerol was essential for *A. tropicalis* to accumulate D-GA, though it was inhibitory towards cell growth.

All glycerol concentrations in cultures decreased with cell growth (Fig. 1c). In cultures with 50 g L⁻¹ glycerol, glycerol concentration decreased corresponding to cell growth (logarithmic phase), and showed little decrease during stationary phase. However, glycerol concentrations continued to decrease during stationary phase in cultures with glycerol above 100 g L⁻¹. This indicated that glycerol contributed to both cell growth and D-GA biosynthesis in medium with above 100 g L⁻¹ glycerol, but it is only attributed to cell growth in medium containing 50 g L⁻¹ glycerol where little D-GA accumulated. Fig. 1d shows the pH in all cultures decreased with cell growth (24 h–72 h). After 72 h, the pH in the culture with 50 g L⁻¹ glycerol stabilized, presumably due to lack of synthesis of D-GA. The pH in cultures with above 100 g L⁻¹ glycerol continued decreasing until 96 h, and then stabilized around 3.5. These decreases in pH were attributable to D-GA accumulation (Fig. 1b).

A high concentration of glycerol was a prerequisite to D-GA biosynthesis in *A. tropicalis*, though it had a negative effect on cell growth. Previously, a fed-batch culture with glycerol feeding was applied to avert this problem¹⁷. Here we performed a two-step culture to deal with the ambivalent culture conditions between cell growth and D-GA biosynthesis. In the first stage (culture stage), the medium with low initial glycerol (50 g L⁻¹) was selected for cell growth. Cells were then harvested at stationary phase, and transferred to a highly concentrated glycerol solution to induce D-GA biosynthesis, the second stage (whole-cell biocatalysis stage). Here,

glycerol was the single substrate, and *A. tropicalis* was used to catalyze glycerol to D-GA. Optimization of conditions for the whole-cell biocatalysis stage are described in the following text.

Optimization of conditions for the whole-cell biocatalysis process

During the whole-cell biocatalysis stage, the DCW tended to be stable and increased only slightly in the first 12 h (Fig. 2). D-GA biosynthesis was triggered immediately, and the maximum D-GA yield (ca. 3.99 g L⁻¹) was found after catalyzing for 36 h. Glycerol was the only substrate in the whole-cell biocatalysis stage, so cell growth was inhibited because of starvation after depletion of the nutrients that had accumulated inside cells during the previous stage. Since there is competition between cell growth and D-GA synthesis, the alleviation of the competition may be an efficient strategy for the enhancement of D-GA yield by allowing the cells to focus on catalyzing the conversion of glycerol to D-GA.

Effect of initial glycerol concentration on D-GA yield in the whole-cell biocatalysis process

A series of glycerol concentrations (50, 75, 100, 125 and 150 g L⁻¹) were applied to study the effect of glycerol concentration on D-GA yield on the whole-cell biocatalysis system. Both D-GA yield and the substrate conversion rate increased with increasing glycerol concentrations (Fig. 3). D-GA yield reached 8.5 g L⁻¹ when cultures contained 150 g L⁻¹ glycerol. The maximum conversion rate of glycerol to D-GA (60.5 %) was observed at 125 g L⁻¹ glycerol, with a D-GA yield of 7.65 g L⁻¹, and a D-GA productivity of 3.4 g g⁻¹ DCW. Since little cell growth was observed in the whole-cell biocatalysis process, the substrate was considered to be converted to product and consumed for maintenance of live cells. There is competition between cell growth and D-GA biosynthesis, a greater glycerol conversion rate for D-GA gained as expected was probably due to the lack of cell growth.

Effect of pH

The pH decreased dramatically during fermentation due to cell growth and D-GA accumulation (Fig. 1d). This change in pH might in turn affect D-GA accumulation. To demonstrate the effect of pH on D-GA accumulation, 0.1 M phosphate buffered saline (PBS) was applied to alleviate the pH reduction during the whole-cell biocatalysis process. The pH of the glycerol solution with or without PBS was set to be 7.0, which was the best initial pH observed in a previous study (data not shown). As shown in Fig. 4, the pH values in solutions with

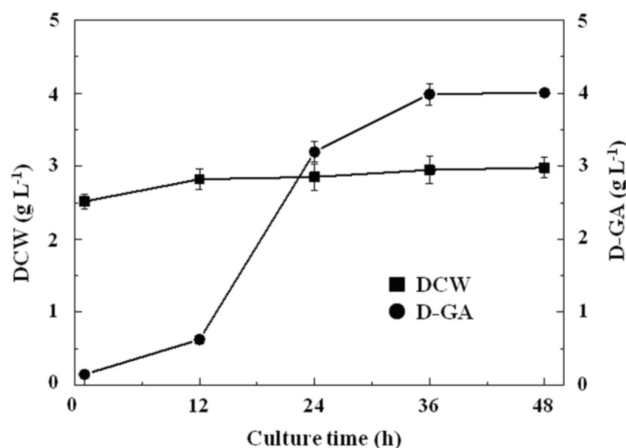


Fig. 2 – Effect of culture time on cell growth and D-GA yield

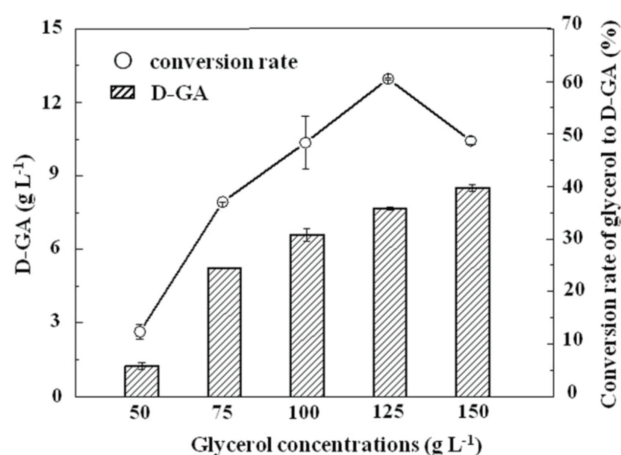


Fig. 3 – Effect of initial glycerol concentrations on D-GA yield in the whole-cell biocatalysis stage. Conversion rate of glycerol to D-GA: $D-GA \text{ concentration (mol L}^{-1}) / (\text{initial concentration of glycerol (mol L}^{-1}) - \text{final concentration of glycerol (mol L}^{-1}) \cdot 100 \%$.

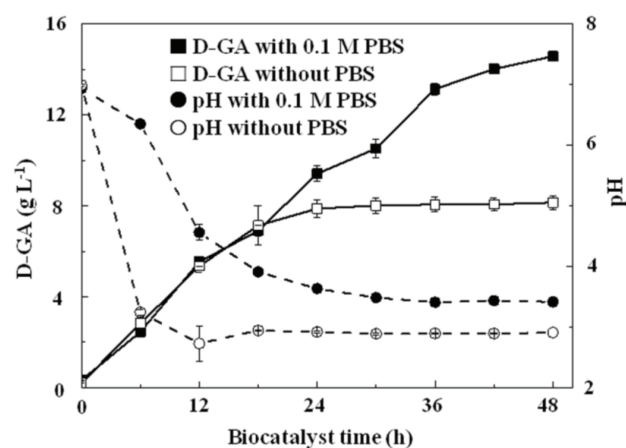


Fig. 4 – Effect of pH buffer on D-GA biosynthesis and pH in culture

or without PBS dropped acutely, though those with PBS decreased slower. The pH in solution with 0.1 M PBS reduced to 3.4 after 24 h, and then stabilized around 3.5 until 48 h. However, the pH in solution

without PBS decreased to 2.9 at 18 h, and then stabilized around 3.0. During the first 18 h, the D-GA accumulation trends in solution with PBS were the same as the control (without PBS). Although D-GA biosynthesis stopped in the control at 24 h, it kept synthesizing in solution with PBS until 48 h. The D-GA yield in solution with 0.1 M PBS was up to 14.57 g L^{-1} (D-GA productivity: $6.48 \text{ g g}^{-1} \text{ DCW}$), which was 79 % higher than that without PBS. This result suggested that D-GA formation was inhibited by a pH lower than 3.0. By addition of 0.1 M PBS, the pH decreased slower during the whole-cell biocatalysis process, and finally stabilized around 3.5, which served to prolong the D-GA biosynthesis. Therefore, pH controlling strategy is a potential method to further increase D-GA yield in the whole-cell biocatalysis stage, especially the biocatalyst process scale-up to pilot scale in jar fermentor. In addition, the D-GA yield and D-GA productivity gained by this two-stage culture with PBS was approximately 2-fold and 4.5-fold compared to the traditional fermentation with 150 g L^{-1} of glycerol, respectively.

D-GA produced by whole-cell biocatalysis using methanol-containing glycerol

Methanol is a major ingredient of raw glycerol derived from the BDF industry, and it is costly to remove. Therefore, it is necessary to investigate the effect of methanol on D-GA yield in the whole-cell biocatalysis process. Glycerol (125 g L^{-1}) containing different concentrations of methanol (0, 2 %, 4 %, 6 %, 8 %, and 10 %, v/v) was used as substrate in the whole-cell biocatalysis process. Since DHA was the major by-product of D-GA yield from fermentation, it was also monitored in this experiment. As compared to glycerol without methanol, D-GA yield gained in glycerol with 2 % methanol decreased by 48.8 %, and the ratio of D-GA to DHA reduced from 5 to 2. (Fig. 5). However, when methanol increased from 2 % to 10 %, D-GA yield did not decrease substantially, and the ratio of D-GA to DHA was stable at 2–3. A 29 % reduction in D-GA yield was found when methanol concentrations increased from 2 % to 10 %. These results suggested that methanol inhibited the biosynthesis of D-GA in *A. tropicalis*, and caused a change in product selectivity between D-GA and DHA during the whole-cell biocatalysis process. However, these effects of methanol were not concentration-dependent and probably attributable to the fact that inhibition of methanol on mADH glycerol dehydrogenation is noncompetitive¹⁵. mADH is a key enzyme in D-GA yield which catalyzes the oxidation of glycerol to glyceraldehyde^{5,19}. The reduction in D-GA yield in the methanol-supplemented catalysis was probably due to the methanol-induced inhibition of

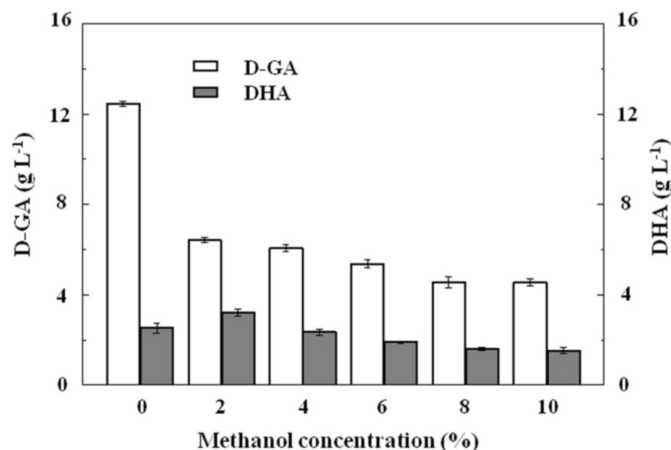


Fig. 5 – Effect of methanol on the yield of D-GA and DHA

mADH-catalyzed oxidation at a high concentration of glycerol¹⁵. Although the D-GA yield was reduced by one half relative to the methanol-free conditions, *A. tropicalis* was first found to be able to catalyze the conversion of glycerol to D-GA in the presence of up to 10 % methanol. This tolerance to methanol is comparable to *G. frateurii* in fed-batch culture¹⁵.

Using 10 % methanol-containing glycerol as substrate, D-GA yield remained 4.5 g L^{-1} with whole-cell biocatalysis. As both cell growth and D-GA biosynthesis were inhibited by methanol¹⁵, glycerol without methanol was applied in the first stage to avoid the inhibition of methanol on cell growth; then raw glycerol containing methanol up to 10 % could be used in the second stage to reduce the cost of materials. Taken together, these results suggested that the two-stage strategy with a whole-cell biocatalyst for D-GA yield by *A. tropicalis* was an efficient method for producing D-GA from methanol-containing glycerol. The potential of using raw glycerol as substrate would decrease the cost of raw material, and the simple composition of D-GA broth from the whole-cell biocatalysis make the following separation easier and cheaper. In addition, the equipment required for this two-step culture strategy is all normal equipment for fermentation industry, which make industrial yield easy. Therefore, this is a promising technology for producing D-GA at low cost. Further studies on optimization of conditions for the whole-cell biocatalysis using raw glycerol as a substrate, especially pH control, will be necessary to enhance D-GA yield by the two-stage culture strategy, which is part of our on-going work.

Conclusions

A novel two-stage culture including whole-cell biocatalysis was developed to deal with the conflict between cell growth and D-GA yield at high con-

centrations of glycerol. Approximately 2-fold D-GA yield and 4.5-fold D-GA productivity were gained by this two-stage strategy compared to the traditional fermentation with 150 g L⁻¹ glycerol. This strategy also worked well when using methanol-containing glycerol as a substrate. Though the D-GA yield had reduced by one half relative to the methanol-free conditions, *A. tropicalis* was first found to be able to convert glycerol into D-GA in the presence of 10 % methanol. These findings have important implications for the production of D-GA by biocatalyst process, including culture conditions optimization, scale-up strategy, and cost reduction.

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