Directional biocatalytic production of high titer hydroxyl acid by the intensified regulation on biocatalysis

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Abstract

Hydroxyl acid has become an important chemical in the field of materials and medicine due to its dual functional modules. Fortunately, Gluconobacter oxydans whole-cell catalysis is on spotlight with promising potential in bio-catalyzing polyhydroxy chemical to produce hydroxyl acids. Therefore, straight-chain primary diols (C2-C6) were investigated as substrates oxidized by G. oxydans. As results, we found a fantastic critical point of methylene-number determining end-products. G. oxydans catalyzes C4 and smaller methylene-number compounds only forming hydroxyl acids, but C5/C6 can be converted to diacids. Furthermore, it was important that we successfully selective and directionality controlled the product of C5/C6 primary diols to hydroxyl acids instead of diacids through the regulation of pH[?]5.5. Finally, we successfully produced nearly 102.3 g/L 5-hydroxyvaleric acid during 48 h with 99.8% yield by sealed-oxygen supply (SOS) biotechnology which is the highest level. These findings have important reference significance for the selective and directionality bioconversion of primary diols into hydroxyl acids and provide a promising path for the industrial development of hydroxyl acids with integrating C2-C6.

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primary diols (C2-C6) were investigated as substrates oxidized by *G. oxydans* . As results, we found a fantastic critical point of methylene-number determining end-products. *G. oxydans* catalyzes C4 and smaller methylene-number compounds only forming hydroxyl acids, but C5/C6 can be converted to diacids. Furthermore, it was important that we successfully selective and directionality controlled the product of C5/C6 primary diols to hydroxyl acids instead of diacids through the regulation of pH[?]5.5. Finally, we successfully produced nearly 102.3 g/L 5-hydroxyvaleric acid during 48 h with 99.8% yield by sealed-oxygen supply (SOS) biotechnology which is the highest level. These findings have important reference significance for the selective and directionality bioconversion of primary diols into hydroxyl acids and provide a promising path for the industrial development of hydroxyl acids with integrating C2-C6.

Keywords:

Hydroxyl acid; Ultra-high titer; Selective and directionality; Whole-cell catalysis;*Gluconobacter oxydans*

1. Introduction

Primary diol is a kind of cheap and convenient substance with a wide range of supply sources from the derivations of epoxy alkanes or olefins (Maihom et al., 2008), aldehydes, alkanes and so on(Mormul et al., 2016; Ichikawa et al., 1995; Liu et al., 2014). Moreover, these diols are also ideal raw materials for making the platform chemicals of hydroxyl acid, which is further converted to various fine chemical intermediates and precursors of biopolymers due to its dual functional modules of hydroxyl and carboxyl groups(Wolinsky et al., 2007; Dunn et al., 2016; Kam and Yoong, 2015). Especially, hydroxyl carboxylic acids are currently in the spotlight of medical polymers industry due to its excellent biocompatibility and biodegradability(Rossi et al., 2004; Jiang et al., 2005; Moore et al., 2005). Moreover, however, the existing hydroxyl acid production technology is difficult to guarantee a high yield and environmental-friendly, either by oxidation or reduction with chemical catalysts and reagents(Zhao et al., 2015; Xia et al., 2018). Moreover, taking 3-hydroxypropionic acid (3-HPA) as an example, it was synthesized from acrolein catalyzed by perchloric acid/sulphur oxide/gaseous hydrochloric acid under high temperature and pressure(Bhattacharyya and Das, 1969). Alternatively, it is prepared by catalyzing 3-hydroxypropanal or 1,3-propanediol in aqueous solution of alkali metal under high temperature and acidic conditions(Pina et al., 2009). In short, these approaches use some toxic catalysts and harsh conditions, which do not meet the development needs of green chemistry. In addition, the preparation of hydroxyl acids from diols mainly involves the oxidation of hydroxyl groups, which makes it difficult to ensure that only one hydroxyl group is catalyzed to carboxyl. For example, 5-hydroxyvaleric acid (5-HVA) which is applied as an intermediate in drug synthesis has been produced by reducing furan compounds with metal catalysts with the yield less than 75%(Asano et al., 2019; Sun et al., 2019). In other words, although the preparation of hydroxyl acid is a feasible industry, the low yield seriously hinders the development of hydroxyl acid industrial production. Fortunately, the whole-cell catalysis of *Gluconobacter oxydans* presents a great possibility for hydroxyl acid production in term of high chemical selectivity, moderate reaction and clean process, compared with chemical methods(Zhao et al., 2015).

G. oxydans , a representative obligate aerobic and gram-negative bacterium, had been well-known for its superb applications in oxidations of oxy-compounds such as alcohols, aldehydes and sugars(Gupta et al., 2001; Deppenmeier et al., 2002). With the incomplete oxidation platform of *G. oxydans* , the dehydrogenation of sorbitol(Wang et al., 2013), glucose, glycerol, ethylene glycol(Xia et al., 2018), 2-methyl-1,3-propanediol(Pyo et al., 2012), 1,2-propanediol(Liu et al., 2010) and other alcohols has been realized. This bacterium does not uptakes or metabolite, but catalyzes the above substrates by the periplasm membrane-bound enzymes mainly including alcohol dehydrogenase(Asakura and Hoshino, 1995), aldehyde dehydrogenase(Molinari et al., 1995), glycerol dehydrogenase(Richter et al., 2010) , sorbitol dehydrogenase(Zhou et al., 2019) and releases products into the solutions directly, which greatly improves the biocatalysis efficiency and yield(Zhou and XL, 2017). Moreover, because these membrane-bound enzymes only undergo dehydrogenation without catabolism, the substrates can be oxidized incompletely to corresponding acids or ketones by *G. oxydans* , but not CO_2 and H_2O which can effectively ensures the yield and purity of the products(Hua et al., 2020). In summary,

these special characteristics, coupled with the strict regioselectivity and stereoselectivity of *G. oxydans* , have made it possible to prepare a variety of high value-added products in the industry, and continue to expand the catalytic application field gradually.

As described above, hydroxyl acid are useful chemicals in many important fields, and *G. oxydans* can be employed as a potential hydroxyl acid producing strain. At present, considering that there are more or less technological bottlenecks in the industrial preparation of hydroxyl acids, it is an important opportunity to develop efficient and green hydroxyl acid production pathway by studying the reaction mechanism of*G. oxydans* whole-cell catalysis of primary diol. Hence, in this study, we investigated the straight-chain primary diols (C2-C6) as substrates for hydroxyl acids production by *G. oxydans* . Focus on the chemical selectivity of two hydroxyl groups in order to find a regulatory model to achieve the full preparation of hydroxyl acids (C2-C6), as to provide a common reference for the platform chemical bio-production of hydroxyl acids from polyols.

2. Materials and methods

2.1 Materials

Ethylene glycol (EG), 1,3-propylene glycol (1,3-PG), 1,4-butylene glycol (1,4-BG), 1,5-pentylene glycol (1,5-PG) and 1,6-hexylene glycol (1,6-HG) were obtained from Aladdin Chemical Reagent Corporation. Yeast Extract was purchased from Sigma-Aldrich. Sorbitol, $MgSO_4$, KH_2PO_4, K_2HPO_4, $(NH4)_2SO_4$ and $CaCO_3$ were obtained from Sinopharm Chemical Reagent Co., Ltd. (China). All other chemicals were of analytical grade and were commercially available.

2.2 Microorganism

G. oxydans NL71 was derived from ATCC621. The strain was preserved in sorbitol-agar medium containing 50 g/L sorbitol, 5 g/L yeast extract and 15 g/L agar, at 4 . The inoculum was cultivated in a baffled Erlenmeyer flask at 30 for 24-36 h, with continual agitation at 220 rpm using a shaking table (New Brunswick Scientific). The nutrient medium was composed of 100 g/L sorbitol and 10 g/L yeast extract. The cultured cells were centrifuged at 6000 rpm and 4 , for 5-10 min by freezing centrifuge (Avanti J-26 XP, Beckman Coulter). The centrifuged cells were washed 3 times in sterile saline and sterile water for reserve, respectively(Zhou et al., 2018).

2.3 Whole-cell catalysis

The *G. oxydans* catalyzed bioprocess for primary diols was conducted in a 250-mL Erlenmeyer flask containing 0.5 g/L $MgSO_4$, 1 g/L KH_2PO_4, 2 g/L K_2HPO_4, 5 g/L $(NH4)_2SO_4$ and 5 g/L yeast extract. The previously washed spare cells were measured at a wavelength of 600 nm by ultraviolet spectrophotometry(Yao et al., 2017). Then, the cells with OD_{600} = 2 concentration were centrifuged and inoculated into the catalytic medium containing primary diols. The whole course was performed at a constant temperature of 30 , with continual agitation at 220 rpm using a shaking table. Moreover, the ultra-high titer 5-HPA was conducted in a sealed oxygen supplied bioreactor (SOS-BR) in which pure oxygen (Purity[?]99.9%) was fed into the bioreactor with an oxygen cylinder replacing the air compressor and the pressure of the bioreactor was controlled at 0.03-0.05 MPa by an exhaust valve(Hua et al., 2020).

2.4 Analytical methods

The enzyme activity under the different acetate concentrations was determined in a 200 μL mixture composed of 170 μL DCPIP, 20 μL 100 g/L substrate sorbitol, and 10 μL *G. oxydans* with OD_{600}=2. The 96-well plate was placed in a Microplate Reader (INIFINITE 200 PRO, Tecan Austria GmbH) set at 220 rpm and 30, and the absorbance measured at 600 nm after every 30 seconds. The absorbance was then linearly fitted to

obtain a slope. The enzyme activity was finally calculated using formula (1)(Peters et al., 2013):

$$U = \frac{V_t \frac{A}{t}}{V_s \lambda \varepsilon} \qquad (1)\backslash n$$

The titer of EG, 1,3-PG, 1,4-BG, 1,5-PG, 1,6-HG, hydroxyl acid and diacid catalyzed by *G. oxydans* were detected by high performance liquid chromatography (HPLC) (Agilent 1260 series) equipped with differential Detector. The separation column used was Aminex Bio-Rad HPX-87H column and 5 mM H_2SO_4 was used as mobile phase at a flow rate of 0.6 mL/min.

Due to the difference of experiment data, three parallel assays were performed for each experiment to ensure reliability of results.

3. Results and discussion

3.1 Whole-cell catalysis process of primary diols oxidized by G. oxydans

The dual functional modules of hydroxyl acids give them the application prospects in many high-end fields. However, the traditional technology for hydroxyl acid preparation not only releases a large amount of waste, but also produces serious by-product. Hence, employing *G. oxydans*as the core catalyst to explore green and environmentally friendly preparation process of hydroxyl acids is an effective approach to solve the current industry bottlenecks. In order to explore the reaction mechanism of *G. oxydans* whole-cell catalyzing linear primary diols, five diols were selected as substrates for kinetic study with OD_{600}=2.

As shown in Figure 1, the bioprocesses for catalysis of EG, 1,3-PG and 1,4-BG (100 mmol/L) by*G. oxydans* were described respectively. As shown in the figure, the final products of EG, 1,3-PG and 1,4-BG could only be catalyzed to glycolic acid (GA), 3-HPA and 4-hydroxybutyric acid (4-HBA), respectively, but could not be further catalyzed to diacids. Moreover, the average consumption rates of EG, 1,3-PG and 1,4-BG were 10.89, 20.4, 24.8 mmol/L/h, respectively. Compared with chemical method, the catalytic rate and purity of the products are satisfactory. Therefore, the results confirm that *G. oxydans* has a promising future in the production of hydroxyl acids (C2-C4) from primary diols. In addition, Figure 1 also shows the bioprocess for the catalysis of 1,5-PG and 1,6-HG (100 mmol/L) by *G. oxydans* respectively. Surprisingly, when the methylene number was [?] 5, the primary diol reaction processes showed qualitative differences. As shown in the figure, 1,5-PG and 1,6-HG catalysis followed a step-by-step process, with further catalysis to glutaric acid (GTA) and adipic acid (AA) when primary diols were oxidized to hydroxyl acids. Moreover, in terms of catalytic rate, the catalytic processes of 1,5-PG and 1,6-HG were very similar. The substrate consumption time for 100 mmol/L were less than 3 h and 4 h, while the average substrate consumption rates reached 31.22 and 45.50 mmol/L/h, respectively. Apparently, although the reaction efficiency of C5/C6 was excellent, the product quality was not satisfactory due to the byproducts. However, at present, hydroxyl acid is receiving much research attention in materials science, due to its excellent properties of two functional modules. Hence, we need to further explore the regulation of cheap substrates primary diols oxidized by *G. oxydans* to lay a theoretical foundation for finding the technology of selective regulation to prepare hydroxyl acids form primary diols.

3.2 Selective regulation for hydroxyl acid production from C5 diols by pH control

Previous studies show that the carbon chain length of the substrate directly determines the product type of whole-cell catalysis. When C[?]5, the primary diols are oxidized to form intermediate hydroxyl acids, which are further converted to diacids. Nowadays, in the current organic acid industry, the preparation of diacids has been approaching maturity and there are cheaper and more convenient production methods. Nevertheless, there are still many bottlenecks in the industrial preparation of hydroxyl acids, such as low yield, impure products, toxic raw materials, high cost and other fatal defects. In 2019, Keiichi et al employ over supported platinum catalysts only obtain 62% yield of target products including 5-HVA, δ-valerolactone, and methyl 5-hydroxyvalerate(Asano et al., 2019). The result not only has a low yield, but also contains abundant

derivatives, which seriously affects the purity of the products. Moreover, in 2021, Hee et al fermentative production of 5-HVA by metabolically engineered *Corynebacterium glutamicum* . Finally, about 55 g/L 5-HVA and 10 g/L GTA were produced during 28 h fermentation [18]. Therefore, targeted regulate of the process for selective catalysis of high-grade primary diols (C[?]5) is a promising way to develop efficient and green hydroxyl acid industrial preparation.

As shown in Figure 2, we conducted the whole-cell catalysis of 1,5-PG under different pH gradients, including pH=2.5, 3.5, 4.5, 5.5, 6,5. Surprisingly, the results showed that when pH[?]5.5, GAT would not be produced even if the substrate 1,5-PG completely bio-oxidized to 5-HVA. However, when pH is less than 5.5, the whole-cell catalysis would show two-stage reactions, that is, 1,5-PG would generate 5-HVA in the first step, and then 5-HVA would be catalyzed to GTA. It is noteworthy that the result under pH=2.5 was contrary to the law we obtained, because it is difficult for cells to maintain normal physiological activity under extremely acidic conditions, and *G. oxydans* has lose catalytic ability after 2 h.

In order to verify the accuracy of pH regulation, we further used the entire cells as the crude enzyme, and measure the enzyme activity of 1,5-PG and 5-HVA as substrates by employing the microplate reader. The enzyme activity was calculated by Formula 1, and the results are shown in Table 1. From the results, we can clearly see that when 1,5-PG was employed as the substrate, the catalytic ability was stronger with the decrease of pH, which was similar with the previous whole-cell catalysis. As for 5-HVA as the substrate, when pH[?]5.5, *G. oxydans* has no catalytic activity at all, which means that *G. oxydans* has no ability to transform 5-HVA to GTA under this condition. In summary, the results of 1,5-PG as the substrate indicated that the lower pH was, the higher catalytic efficiency would be. At the same time, when 5-HVA was used as substrate, the results indicated that the pH must exceed 5.5 to accurately control the product to 5-HVA. Therefore, the proposed scheme of pH-regulated whole-cell catalysis provided a green and high-quality process for the industrial production of 5-HVA. Finally, in order to directional obtain ultra-high titer of 5-HVA, we chose pH=5.5 to conduct bioreactor experiment.

3.3 The whole-cell catalysis for ultra-high titer of 5-HVA production in SOS-BR

The background of the previous section mentioned that the maximum yield of 5-HVA prepared by biological method is 55 g/L during 28 h, and with 10 g/L byproduct (GTA) produced. However, we found that pH regulation can selectively and directionally prepare 5-HVA by *G. oxydans* . Therefore, based on the fact, in-depth development of SOS technology for ultra-high titer production of 5-HVA is the key procedure to establishing the feasibility of industrial by biological method.

Consequently, SOS-BR which was designed for whole-cell catalysis by aerobic microorganisms with incomplete oxidation was employed as intensified technology to conduct 5-HVA bio-production, with the results shown in Figure 3. From the perspective of production, 102.3 g/L of 5-HVA accumulated during 48 h whole-cell catalysis with an average productivity of 2.1 g/L/h. Simultaneously, the production of the first 10 h was 58.9 g/L with the productivity of 5.9 g/L/h which has exceeded the supreme level of 5-HVA (55 g/L during 28 h). It was importantly that the broth contained only 0.2 g/L substrate and without any diacid production at 48 h, that is, the yield of 5-HVA was as high as 99.8%. Moreover, concerning the dissolved oxygen (DO) level, SOS-BR could always maintain a high DO level during whole-cell catalysis process, which met the demand of *G. oxydans* for oxygen. At the same time, due to the strict sealing environment of the entire system, the use cost of oxygen was greatly saved, and the economy of the entire bioprocess was improved. In conclusion, combined with pH control and SOS-BR technology, we successfully achieved the preparation of 5-HVA with superior quality and ultra-high titer, which provides a promising avenue for the industrialization of 5-HVA and even hydroxyl acids.

Finally, the entire process is illustrated in Figure 4. The study focused on the whole-cell catalysis for the selective and directionality preparation if hydroxyl acids. At present, the treatment of primary diols has been mature which making diols a cheap chemical(He et al., 2018; Kim et al., 2020). Furthermore, in the downstream treatment of hydroxyl acid, electrodialysis system can be applied to simultaneously acidify and concentrate the fermentation broth, and the obtained high titer of hydroxyl acids can be easily crystallized(Du

et al., 2021). In summary, the entire process can realize highly selective oxidation and directional regulation of hydroxyl acids, which greatly promotes the industrial production of hydroxyl acids with C2-C6.

4. Conclusions

The whole-cell catalysis of primary diols by *G. oxydans* with different methylene-numbers were significantly different in products. Primary diols with C[?]4 will only be oxidized to the hydroxyl acids, while primary diols with C[?]5 will be further oxidized to diacids. Importantly, the catalysis of C5 diols with the pH control successfully selective and directionality produce hydroxyl acids without any diacids, and the output is the highest. In conclusion, the whole-cell catalysis of *G. oxydans* for hydroxyl acids bio-production was established for industrialization. Moreover, these findings provided valuable insights for promoting environmentally friendly industrial production of hydroxyl acids (C2-C6).

Abbreviation

EG: Ethylene glycol; 1,3-PG: 1,3-propylene glycol; 1,4-BG: 1,4-butylene glycol; 1,5-PG: 1,5-pentylene glycol; 1,6-HG: 1,6-hexylene glycol; GA: glycolic acid; 3-HPA: 3-hydroxypropionic acid; 4-HBA: 4-hydroxybutyric acid; 5-HVA: 5-hydroxyvaleric acid; GTA: glutaric acid; AA: adipic acid;*Gluconobacter oxydans: G. oxydans* ; DO: dissolved oxygen; SOS: sealed-oxygen supply: SOS;

Declarations

Author's contributions

XH designed the project, performed experiments, analyzed data, and prepared the manuscript. JH and CHZ helped to analyze the data and revised the manuscript. YX supervised the project and revised the manuscript. All authors read and approve the final manuscript.

Conflicts of interest

The authors declare no competing financial interest

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Figure caption

Table 1. The enzyme activity parameters of crude enzyme (entire cells) with 1,5-PG and 5-HVA as substrate respectively

Figure 1. The whole-cell catalysis of EG, 1,3-PG, 1,4-BG, 1,5-PG, 1,6-HG by *G. oxydans*

Figure 2. The whole-cell catalysis of 1,5-PG by *G. oxydans* with different pH regulation

Figure 3. The whole-cell catalysis of 1,5-PG by *G. oxydans* with the regulation of pH control at 5.5 and SOS-BR technology

Figure 4. The entire process for hydroxyl acids production, including upstream treatment to produce primary diols (first step), the whole-cell catalysis to prepare hydroxyl acids (second step), and downstream treatment to crystallize products (third step)

Table 1.

Group	Enzyme activity (U) (1,5-PG)	Enzyme activity (U) (5-HVA)
2.5	0.78±0.07	1.37±0.14
3.5	0.55±0.04	0.48±0.08
4.5	0.21±0.04	0.11±0.4
5.5	0.17±0.03	0
6.5	0.09±0.02	0
7.5	0.06±0.01	0

Figure 1.

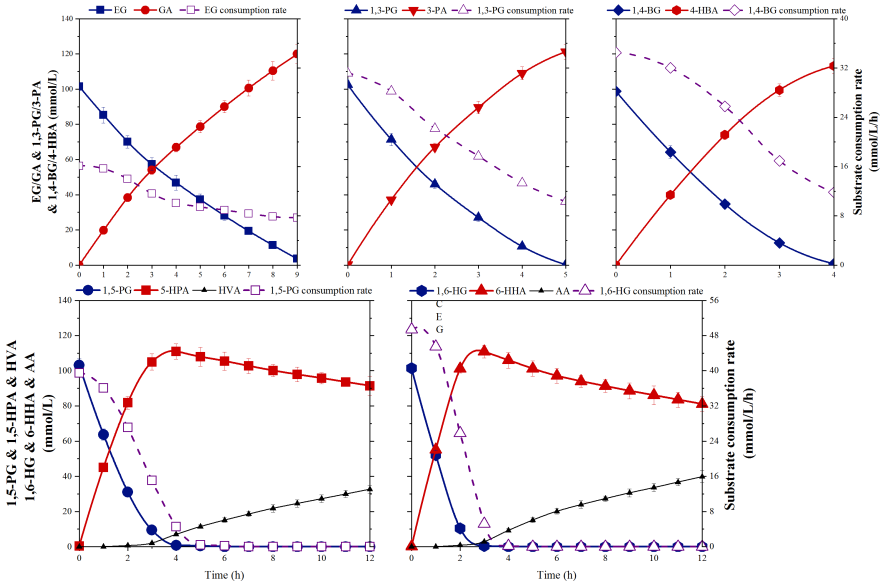

Figure 2.

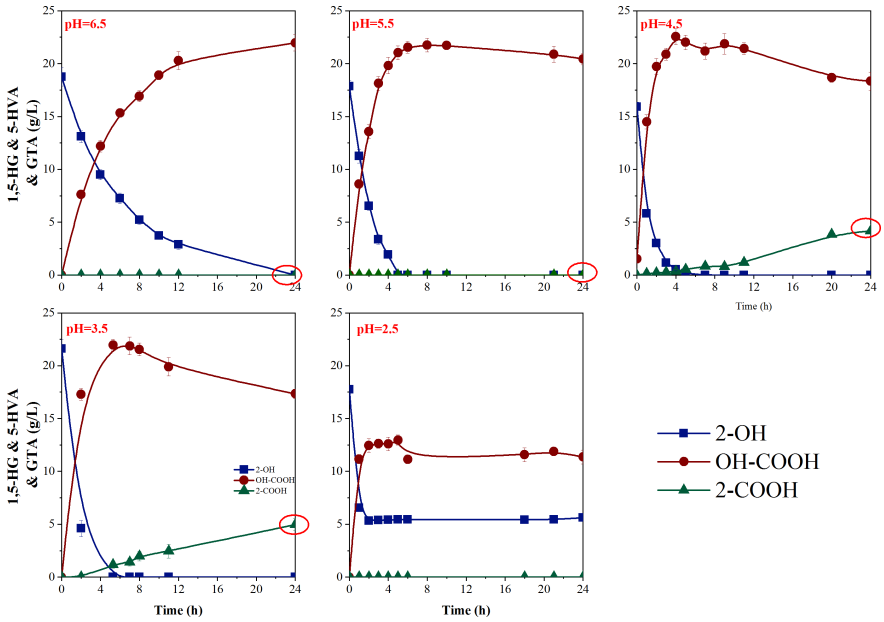

Figure 3.

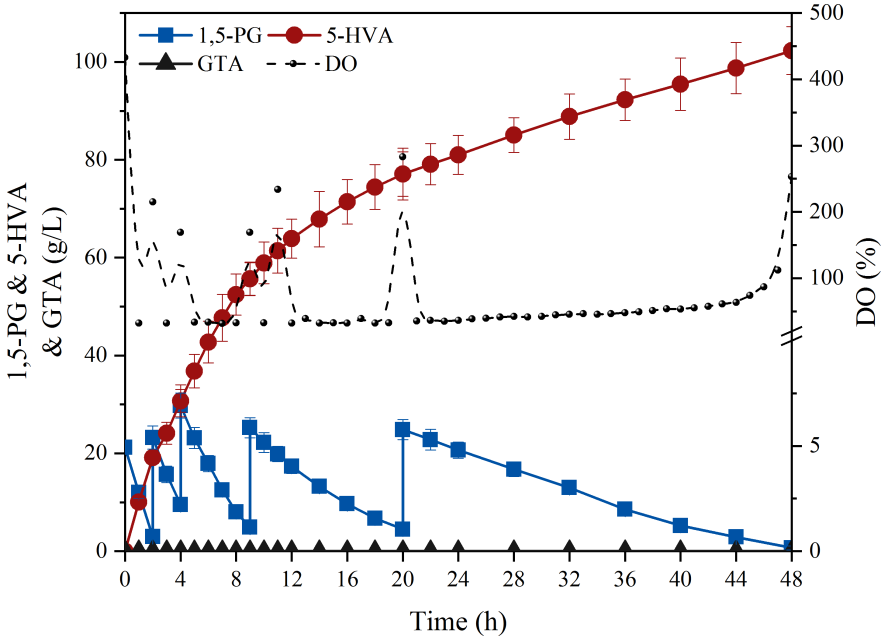

Figure 4.