pH regulatory divergent point for the selective bio-oxidation of primary diols during resting cell catalysis

Xia Hua
Nanjing Forestry University
ChenHui Zhang
Nanjing Forestry University
Jian Han
Nanjing Forestry University
Yong Xu (xuyong@njfu.edu.cn)
Nanjing Forestry University

Research Article

Keywords: Hydroxyl acid, Whole-cell catalysis, Oxidation, Sealed-oxygen supply, pH regulation

Posted Date: March 17th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1453062/v1

Abstract

Background

Hydroxyl acid is an important platform chemical that covers many industrial applications due to dual functional modules. At present, the traditional technology for hydroxyl acid production mainly adopts the petroleum route with benzene, cyclohexane and butadiene and other non-renewable resources as raw materials which violates the law of green chemistry development. Conversely, it is well-known that biotechnology and bioengineering techniques possess several advantages over chemical methods, such as moderate reaction conditions, high chemoselectivity, and are environmental-friendly. However, there still exist some major obstacles for the industrial application of biotechnology as compared with chemical engineering. The critical issue in the competitiveness between bioengineering and chemical engineering is products titer and volume productivity. Therefore, based on the importance of hydroxyl acids in many fields, exploring a clean, environmentally friendly, efficient and practical production technology for the hydroxyl acids preparation is the core purpose of this study.

Results

To obtain high-purity hydroxyl acid, a microbiological regulation employing *Gluconobacter oxydans* for its bioproduction was constructed. We achieved a critical point of chain length, determining the end-products. *G. oxydans* catalyzed diols with chain length ≤ 4, forming hydroxyl acids, and converting 1,5-pentanediol and 1,6-hexanediol to diacids. Based on this principle, we successfully synthesized 75.3 g/L glycolic acid, 83.2 g/L 3-hydroxypropionic acid, and 94.3 g/L 4-hydroxybutyric acid within 48 h. Furthermore, we directionally controlled the products of C5/C6 diols by adjusting pH, resulting in 102.3 g/L 5-hydroxyvaleric acid and 48.8 g/L 6-hydroxycaproic acid instead of diacids. Combined with pH regulation and cell-recycling, we prepared 271.4 g 5-hydroxyvaleric acid and 129.4 g 6-hydroxycaproic acid in 6 rounds.

Conclusion

In this study, a green scheme of employing *G. oxydans* as biocatalyst for superior-quality hydroxyl acids (C2-C6) production is raised up. The proposed strategy commendably demonstrated a novel technology with simple pH regulation for high-value production of hydroxyl acids via green bioprocess developments.

Introduction

Traditional petrochemical resources consumption result in environmental pollution, causing toxicity, carcinogenicity, and biological aggregation of harmful chemical substances. Moreover, excessive use of coal, oil and natural gas by humans makes the non-renewable resources on earth increasingly scarce [1, 2]. Hence, to overcome the dilemma of insufficient resources and inefficient utilization, green synthesis

methods are developed, and these environmentally friendly economics has become an important technology impetus in recent years [3, 4]. Consequently, green methods for amino acids, vitamins, polymers, and other chemicals production have been recently reported [5, 6].

Hydroxyl acids ($HOCH_2$-$(CH_2)_n$-COOH) are important bioresource intermediates containing both hydroxyl and carboxyl groups at terminal positions [7, 8]. Because of the unique properties of dual-functional module, hydroxyl acids can be converted to various fine chemical intermediates and biopolymer precursors [9–11]. Especially, they are in the spotlight of the medical polymers industry due to their excellent biocompatibility and biodegradability [12–14]. However, the existing hydroxyl acid production technology, oxidation or reduction via chemical catalysts or biocatalysts, has low yield and high environmental toxicity [15–17]. In chemical catalysis, serious by-products are generated due to the redox relationship between hydroxyl and carboxyl groups [18]. For example, the most common method for glycolic acid (GA) synthesis in the industry is chloroacetic acid or hydroxy acetonitrile hydrolysis [19]. However, the raw materials applied in two processes have high toxicity and strong corrosivity, causing environmental pollution and safety issues. Moreover, the preparation of hydroxy acids by fermentation is also a hot research area, although no fermentation technology has been employed in industry. At present, many hydroxyl acids have been successfully synthesized by employing recombinant microorganisms such as *Escherichia coli* [20, 21], *Klebsiella pneumoniae* [22, 23], and *Corynebacterium glutamicum* [24, 25]. However, fermentation technology has some inherent disadvantages of high cost and long reaction cycle, limiting its use in large-scale industrial production [26]. Therefore, the development of a green and environmentally friendly technology is required for preparing high-quality hydroxyl acids.

Biocatalysis, a promising approach for sustainable development of industry in the future, has applications in various fields, including food, chemical, medicine, environmental protection, and energy [27, 28]. Hence, in-depth development and application of biocatalysis for hydroxyl acids production could provide a promising direction for their industrial production. *Gluconobacter oxydans* (*G. oxydans*) is a type of Gram-negative bacterium, known for its incomplete oxidation capability, attributed to the presence of membrane-bound dehydrogenases [29, 30] such as alcohol [31], aldehyde [32], glycerin [33], and sorbitol dehydrogenases [34], which catalyze alcohols and aldehydes to corresponding acids and ketones. Furthermore, membrane-bound dehydrogenases are directly located on the cell membrane, and substrates are oxidized to products and released into the periplasm without carrier transport, considerably improving the catalytic efficiency [35]. These properties enabled the use of *G. oxydans* as a common industrial biocatalyst for the industrial production of vitamin C [36], xylonic acid [37], and gluconic acid [38]. In addition, glycolic acid, furoic acid, 2-hydroxyacetone, and other platform compounds are also preliminarily synthesized on an industrial scale [39, 40]. These special characteristics, coupled with strict regioselectivity and stereoselectivity of *G. oxydans*, have facilitated the large-scale production of hydroxyl acids.

Considering the bottlenecks in the industrial-scale preparation of hydroxyl acids, it is important to develop an efficient and green method by investigating the reaction mechanism of *G. oxydans* whole-cell catalysis of primary diols, employing cheap chemical raw materials from petroleum and wood fiber.

Herein, *G. oxydans* was employed as a core biocatalyst and primary diols were selected as substrates to investigate the regulatory mechanism underlying directed selective synthesis of hydroxyl acids. As results, combined SOS technology, we successfully achieved high-titer GA, 3-hydroxypropionic acid (3-HPA), and 4-hydroxybutyric acid (4-HBA). Furthermore, a green route for whole-cell catalysis can precisely control the production of 5-hydroxyvaleric acid (5-HVA) and 6-hydroxycaproic acid (6-HCA) by pH regulation.

Materials And Methods

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), 1,6-hexylene glycol (1,6-HG), GA, 3-HPA, 4-HBA, 5-HVA, 6-HCA, glutaric acid (GTA) and adipic acid (AA) were obtained from Aladdin Chemical Reagent Corporation (China). 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.

Microorganism

G. oxydans NL71 was derived from ATCC621. The strain was preserved in sorbitol-agar medium containing 50 $g{\cdot}L^{-1}$ sorbitol, 5 $g{\cdot}L^{-1}$ yeast extract, and 15 $g{\cdot}L^{-1}$ agar, at 4 ℃. The inoculum was cultivated in an Erlenmeyer flask at 30 ℃ for 24-36 h, with continuous agitation at 220 rpm using a mechanical shaker (New Brunswick Scientific). The nutrient medium was composed of 100 $g{\cdot}L^{-1}$ sorbitol and 10 $g{\cdot}L^{-1}$ yeast extract. Cultured cells were centrifuged at 6000 rpm at 4 ℃, for 5-10 min in a freezing centrifuge (Avanti J-26 XP, Beckman Coulter). Centrifuged cells were washed 3 times with sterile saline and sterile water, respectively [41].

Whole-cell catalysis

Bioprocess for whole-cell catalysis of 1,6-HG was carried out in a 3-L bioreactor with 1 L broth containing 10 $g{\cdot}L^{-1}$ *G. oxydans*, 5 $g{\cdot}L^{-1}$ yeast extract as the nitrogen source, and 0.5 $g{\cdot}L^{-1}$ $MgSO_4$, 1 $g{\cdot}L^{-1}$ KH_2PO_4, 2 $g{\cdot}L^{-1}$ K_2HPO_4, and 5 $g{\cdot}L^{-1}$ $(NH_4)_2SO_4$ as nutrients. Biocatalysis temperature was maintained at 30°C and a stirring speed of 500 rpm. In addition, as *G. oxydans* is an obligate microorganism, oxygenation (purity≥99.9) was maintained throughout the bioprocess. However, excessive oxygenation not only increases the cost but also leads to the wastage of resources due to low oxygen utilization. Considering that the whole-cell catalysis by *G. oxydans* is resting-cell catalysis without generating waste gas such as CO_2. Hence, we employed a sealed oxygen-supplied bioreactor (SOS-BR), as shown in Figure 1, for whole-cell catalysis. The pressure of the reactor was controlled at 0.03-0.05 MPa [42] and the pH of broth was adjusted by 30% NaOH. For cell-cycling technology, *G. oxydans* was harvested via centrifugation at 6,000-

8,000 rpm for 5 min under 4°C and cell pellets were washed with deionized water. Finally, the bacteria were re-inserted into the fresh medium for a new round of whole-cell catalysis.

Analytical methods

The titer of EG, 1,3-PG, 1,4-BG, 1,5-PG, 1,6-HG, hydroxyl acids and diacids was detected by high-performance liquid chromatography (HPLC, Agilent 1260 Series) equipped with a differential detector. The column used for separation was the Aminex Bio-Rad HPX-87H and 5 mM H_2SO_4 was used as mobile phase at a flow rate of 0.6 mL/min.

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

Results And Discussion

The whole-cell catalysis of primary diols (C2-C6) by G. oxydans

Dual-functional modules of hydroxyl acids alleviate their application prospects in many high-end fields [43]. However, the traditional methods for hydroxyl acid preparation produce a large amount of waste and by-products, violating the principles of green chemistry and sustainable development. Hence, employing *G. oxydans* as a core catalyst to design a green and environmentally friendly synthesis method for hydroxyl acids is an efficient approach to solve the current industrial bottlenecks. To explore the reaction mechanism of *G. oxydans* whole-cell catalysis, five linear diols were selected as substrates for kinetic study with OD_{600}=2. The bioprocesses for the catalysis of EG, 1,3-PG, and 1,4-BG (100 mmol/L) by *G. oxydans* are described in Figure 2. The final catalytic products were GA, 3-HPA, and 4-HBA, respectively, eliminating the formation of diacids. 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 the chemical method, the purity of products was satisfactory, meeting the core requirements of green chemistry, the reaction efficiency still needed to be improved to meet the requirements for industrial production.

Figure 2 shows the bioprocess for the catalysis of 1,5-PG and 1,6-HG (100 mmol/L) by *G. oxydans,* respectively. Surprisingly, when the carbon chain length was ≥ 5, the primary diols showed qualitative differences. 1,5-PG and 1,6-HG catalysis followed a step-by-step process, further catalyzing to GTA and AA when primary diols were oxidized to hydroxyl acids. Moreover, the catalytic rates for 1,5-PG and 1,6-HG were very similar. The substrate consumption time for 100 mmol/L concentration was less than 3 h and 4 h, while the average substrate consumption rates reached 31.22 and 45.50 mmol/L/h, respectively. Although the reaction efficiency for C5/C6 was promising, the product quality was not satisfactory due to the formation of by-products (diacids). At present, hydroxyl acids are receiving much attention due to their two functional modules, exhibiting excelling properties. Hence, we further explored the regulation of low-cost substrates primary diols oxidized by *G. oxydans* to lay a theoretical foundation for the selective regulation technology to prepare hydroxyl acids from primary diols.

The whole-cell catalysis of primary diols (C2-C4) by G. oxydans combined with SOS technology

Previous studies indicated that *G. oxydans* could bio-transform EG, 1,3-PG, and 1,4-BG into hydroxyl acids. Although the purity of the products met the green chemistry and industrial production requirements, the catalytic efficiency was lower to strengthen the production economy. *G. oxydans* take oxygen as the final electron receptor, so the whole-cell catalysis requires a lot of oxygen support to enhance the catalytic rate [44,45]. However, the high cost of unrestricted oxygen supply can not be borne by the industry. Therefore, in this study, SOS technology was employed to maximize oxygen utilization while ensuring high catalytic efficiency. Moreover, SOS technology safely controls the pressure in the bioreactor by adjusting the oxygen inlet speed, eliminating the potential safety hazards in industrial production [42].

As shown in Figure 3, 48 h whole-cell catalysis of EG, 1,3-PG, and 1,4-BG were carried out in SOS-BR with *G. oxydans* OD_{600}=2. Results indicated that GA, in the 48 h bioprocess, 3-HPA and 4-HBA accumulated 75.3 $g{\cdot}L^{-1}$, 83.2 $g{\cdot}L^{-1}$, and 94.3 $g{\cdot}L^{-1}$ respectively. In terms of GA productivity, the highest quantity of GA obtained was 74.5 $g{\cdot}L^{-1}$ with a productivity of 1.49 g/L/h by Wei et al. [46], lower than 1.6 g/L/h obtained in this study. Sun et al. reported a recombinant *Escherichia coli* to produce 38.7 g 3-HPA with an average yield of 35% in 72 h fermentation [47], the productivity was lower than SOS technology and numerous by-products were found. For 4-HBA, Sang et al. [48] employed recombinant *Escherichia coli*, producing 103.4 $g{\cdot}L^{-1}$ under the microaerobic conditions with 0.844 g/L/h productivity. Although the production of 4-HBA was slightly higher than that of whole-cell catalysis, its productivity was only 43% of SOS technology. Furthermore, a lot of inhibitors such as acetic acid were generated in the fermentation broth of recombinant *Escherichia coli*, seriously affecting the product quality. Apparently, the whole-cell catalysis with *G. oxydans,* combined with SOS technology, attained a continuous and efficient preparation of high-purity hydroxyl acids (C2-C4), providing solid technical support for their industrial production.

Bioprocess for 5-HVA preparation with pH regulation and cell-recycling technology in SOS-BR

A previous study showed that 5-HVA could be produced by *G. oxydans*, its production level was rather unsatisfactory, and GTA was accumulated in the system as a by-product. Moreover, the traditional chemical methods cannot be employed for preparing high-quality 5-HVA due to their high cost, limiting their application in medicine, material science, and other advanced fields. Therefore, targeted regulation of selective catalysis of 1,5-PG is a promising approach to produce green 5-HVA at an industrial scale. As shown in Figure 4, the whole-cell catalysis of 1,5-PG was performed under different pH gradients, including pH=2.5, 3.5, 4.5, 5.5, 6,5. When pH≥5.5, GAT was not produced even if the substrate 1,5-PG was completely bio-oxidized to 5-HVA. However, when pH was less than 5.5, the whole-cell catalysis showed two-stage reactions; 1,5-PG generated 5-HVA in the first step, and then 5-HVA was catalyzed to GTA in the second step. It is noteworthy that the results at pH=2.5 were contrary to the law because normal physiological activity could not be maintained under extremely acidic conditions, and *G. oxydans* lost catalytic activity after 2 h. Therefore, the proposed scheme of pH-regulated whole-cell catalysis provided

a green and high-quality approach for the industrial production of 5-HVA without any by-products. To directionally obtain an ultra-high titer of 5-HVA, we selected pH=5.5 for the bioreactor experiment.

In 2019, Keiichi et al. employed over-supported platinum catalysts and obtained a 62% yield of 5-HVA, δ-valerolactone, and methyl 5-hydroxyvalerate [17]. The results revealed a low yield and presence of abundant derivatives, seriously affecting the purity of products. Moreover, in 2021, Hee et al. reported a fermentative production of 5-HVA by metabolically engineered *Corynebacterium glutamicum*, and 55 $g \cdot L^{-1}$ 5-HVA and 10 $g \cdot L^{-1}$ GTA were produced after 28 h fermentation [49]. Apparently, their designed bioprocess was not satisfactory for industrial-scale production of 5-HVA. Hence, we conducted whole-cell catalysis for 5-HVA bio-production in SOS-BR with pH regulation (Figure 5A). Results showed that 102.3 $g \cdot L^{-1}$ of 5-HVA was accumulated without the formation of diacids during 48 h whole-cell catalysis with average productivity of 2.1 g/L/h. Simultaneously, the production in the first 10 h was 58.9 $g \cdot L^{-1}$ and the productivity was 5.9 g/L/h, exceeding the highest level of 5-HVA (55 $g \cdot L^{-1}$ during 28 h). The broth contained only 0.2 $g \cdot L^{-1}$ substrates, without any diacid production at 48 h, and 5-HVA yield was as high as 99.8%. Additionally, SOS-BR maintained a high dissolved oxygen (DO) level during the whole-cell catalysis process, meeting the oxygen demand for *G. oxydans*. At the same time, the cost of oxygen utilization was greatly saved due to the strict sealing environment of the entire system, improving the economy of the entire bioprocess. Moreover, we successfully performed cell-recycling technology with 6 rounds in SOS-BR (Figure 5B). During 48 h whole-cell catalysis, a total of 274.1 g 5-HVA was accumulated, and the production of each round was 52.5 $g \cdot L^{-1}$, 47.8 $g \cdot L^{-1}$, 46.7 $g \cdot L^{-1}$, 44.8 $g \cdot L^{-1}$, 40.2 $g \cdot L^{-1}$, and 39.4 $g \cdot L^{-1}$, respectively. In conclusion, combined with pH control and SOS-BR technology, we successfully synthesized high-quality 5-HVA with an ultra-high titer, providing a promising avenue for the industrialization of 5-HVA and hydroxyl acids.

Bioprocess for 6-HCA synthesis with pH regulation and cell-recycling technology in SOS-BR

Compared to other hydroxyl acids, the technology for industrial production of 6-HCA is currently unavailable. As 6-HCA is an intermediate, hydroxyl and carboxyl groups often undergo oxidation or reduction during synthesis, generating 1,6-HG, AA, and other by-products. A little literature is available on the preparation of 6-HCA. In 1999, Fischer et al. employed metal catalysts to prepare 6-HCA at high temperature (100~300℃) and high pressure (10~300 bar), but the products were accompanied by abundant 1,6-HG and esters [50]. Therefore, it was significant to develop a green and efficient method for 6-HCA synthesis by *G. oxydans*. Results revealed that the pH of the broth showed an obvious regulatory effect on the whole-cell catalytic process (Figure 6). The whole-cell catalysis of 1,6-HG was divided into two stages at pH ≤ 6. The first step was the oxidation of 1,6-HG to an intermediate product, 6-HCA, which was converted to AA. Surprisingly, when the pH of broth was adjusted to 7, the conventional two-stage catalysis was regulated at the first stage. The results suggested that when 1,6-HG was oxidized to 6-HCA, it did not further react to form AA, improving the product quality of 6-HCA and eliminating the formation of by-products. According to previous research results, the weak acidic environment was more suitable

for the physiological and biochemical capacity of *G. oxydans*, hence, pH=7 was selected for 6-HCA biopreparation.

Based on the findings of the pH regulation experiment, the whole-cell catalysis for preparing 6-HCA was performed at pH=7 (Figure 7A). In fed-batch mode, 48.8 $g \cdot L^{-1}$ of 6-HCA was accumulated within 48 h, and the productivity was 1.01 g/L/h, which was the highest reported level for 6-HCA. The kinetic curve showed that the inhibition effect was evident after the formation of hydroxyl acids and the productivity decreased from 4.5 g/L/h to 0.3 g/L/h. When the catalysis was performed for 8 h, the productivity decreased to 2 g/L/h, 50% lower than that of the initial level. Therefore, we performed the cell-recycling experiment in 8 rounds for single batch catalysis, and the results are shown in Figure 7B. During 48 h of whole-cell catalysis, the cell-recycling experiment was successfully implemented for 6 rounds, and the last round still maintained 75% capacity. Finally, 129.3 g of 6-HCA was accumulated in 6 rounds, with average productivity of 2.7 g/h, 1.6 times higher than that of a single batch. The importance of 6-HCA underscores its social demand; however, the existing technologies cannot support the market demand. Therefore, the method for preparing 6-HCA proposed in the study, overcomes the disadvantages of traditional methods, demonstrating promise for the industrial production of 6-HCA in the future.

Conclusion

In this study, a microbiological regulation process for high value-added hydroxyl acids synthesis from primary diols by *G. oxydans* was successfully established. Combined with SOS technology, the whole-cell catalytic synthesis of high-titer GA, 3-HPA, and 4-HBA was achieved. Further enhancement in 5-HPA and 6-HCA production was successfully realized by pH regulation, without diacids. In conclusion, the whole-cell catalysis of *G. oxydans* for hydroxyl acids bio-production was established for industrial-scale preparation. Moreover, the mild condition of whole-cell catalysis agrees well with the principles of green synthesis for the environment-friendly production of hydroxyl acids.

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

Acknowledgements

The research was supported by the National Natural Science Foundation of China (31370573). Also, the authors gratefully acknowledge financial support from ‘Forestry Engineering First-class Discipline Construction Project of Nanjing Forestry University’.

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Figures

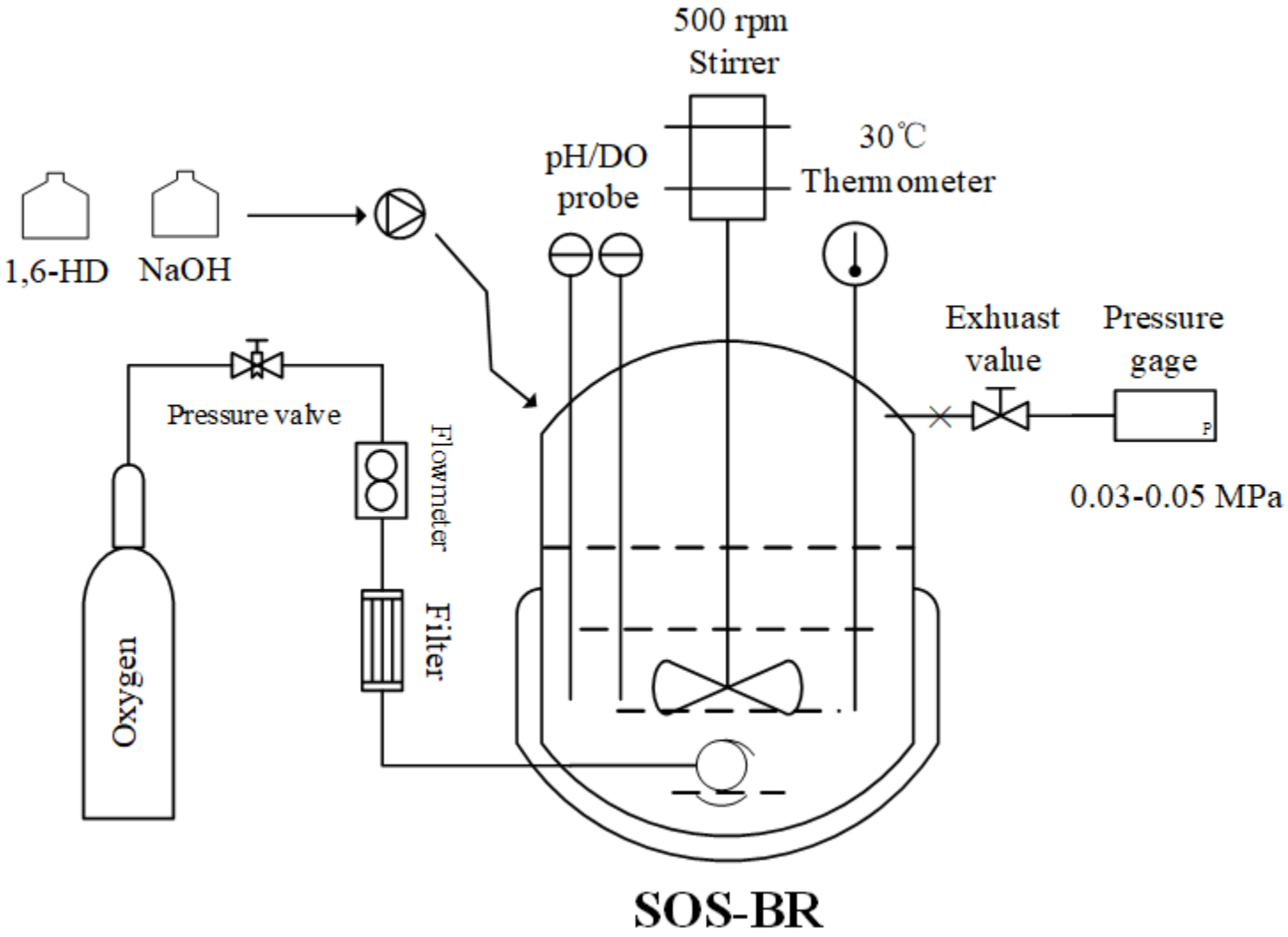

Figure 1

The bioreactor operation model for the whole-cell catalysis

Figure 2

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

Figure 3

The whole-cell catalysis of EG, 1,3-PG and 1,4-BG by *G. oxydans* with SOS technology

Figure 4

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

Figure 5

The whole-cell catalysis of 1,5-PG by *G. oxydans* with different SOS technology (A) and cell recycling technology (B)

Figure 6

The whole-cell catalysis of 1,6-HG by *G. oxydans* with different pH regulation

Figure 7

The whole-cell catalysis of 1,6-HG by *G. oxydans* with different SOS technology (A) and cell recycling technology (B)