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SHORT COMMUNICATION

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Novel fermentation process strengthening strategy for production of propionic acid and vitamin B12 by *Propionibacterium freudenreichii*

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Abstract An efficient fermentation-strengthening approach was developed to improve the anaerobic production of propionic acid and vitamin B12 by co-fermentation with *Propionibacterium freudenreichii*. Vitamin B12 production from glucose resulted in relatively high productivity (0.35 mg/L h) but a low propionic acid yield (0.55 g/g). By contrast, glycerol gave a high propionic acid yield (0.63 g/g) but low productivity (0.16 g/L h). Co-fermentation of glycerol and glucose with a gradual addition strategy gave high yields (propionic acid: 0.71 g/g; vitamin B12: 0.72 mg/g) and productivities (propionic acid: 0.36 g/L h; vitamin B12: 0.36 mg/L h). Finally, the integrated feedstock and fermentation system strengthening strategy was demonstrated as an efficient method for the economic production of bio-based propionic acid and vitamin B12.

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Introduction

Propionic acid and its salts are widely used as food and feed preservatives [3]. Propionic acid is currently produced almost entirely via petrochemical processes. With the problems of high cost (crude oil prices have surpassed US$100 per barrel) and environmental concerns, propionic acid production from renewable bioresources by fermentation using *Propionibacteria* has attracted increasing research interest [11]. Propionobacterium is a genus of Gram-positive, facultative anaerobic, high GC actinobacteria that can convert carbon sources into propionate, acetate, and CO_2 via the Wood–Werkman cycle [2]. However, conventional propionic acid fermentation suffers from low productivity and yield because of the acidic pH, fermentation end-product inhibition, and co-production of other byproducts, mainly acetic and succinic acids [9]. Another important application of *Propionibacteria* (e.g., *Propionibacterium freudenreichii*) is the production of vitamin B12, which is a costly chemical compared with propionic acid (approximately US$20 per kg). To lower the product cost, recent studies have focused on strengthening fermentation processes, i.e., how to separate end-products online and harvest more products (e.g., propionic acid and vitamin B12) in one fermentation process by in situ product removal (ISPR) techniques [7].

Another approach to lower the product cost lies in the feedstock. Several studies have shown that glycerol is a good carbon source for propionic acid fermentation with a higher propionic acid yield and significantly lower acetic

acid formation compared with glucose [12]. Glycerol is a reducing agent that favors the production of more reduced metabolites [5] but can cause a redox imbalance in metabolism, which results in reduced cell growth and productivity, when used as the sole carbon source in fermentation [4]. To overcome this problem, co-fermentation of glycerol with glucose has been proposed as an efficient process to support both product formation and cell growth [1].

The goal of this study was to integrate both ISPR system and feedstock strengthening strategy for propionic acid and vitamin B12 production from corn stalk hydrolysis in a co-fermentation process with *P. freudenreichii*. Corn stalk, which is the residuum after the corn kernels are removed, is typically used as animal feed or is disposed into landfills, causing serious environmental concerns. In this study, propionic acid and vitamin B12 were produced from CS hydrolysis and corn steep liquor as low-cost feedstock. The effects of the glycerol/glucose mass ratio and feeding strategy on products (propionic acid/vitamin B12) were also discussed.

Materials and methods

Culture and media

The stock culture of *P. freudenreichii* CICC 10019 (China Center for Industrial Culture Collection, China) was cultivated anaerobically at 30 °C in stab medium containing 20 g/L glucose, 10 g/L corn steep liquor, 2 g/L ammonium sulfate, and 2 g/L powdered agar. The culture was stored at 4 °C, and transferred to new agar monthly. Unless otherwise noted, fermentation kinetics was studied in a synthetic medium containing 20 g/L corn steep liquor, 4 g/L KH_2PO_4, 0.0127 g/L cobalt chloride, 4 g/L $CaCO_3$, and 60 g/L carbon source (glucose, glycerol, or glycerol/glucose mixture). All media were sparged with nitrogen gas and sealed in glass bottles. Glucose and corn stalk carbohydrates were separately sterilized at 115 °C for 20 min. The initial pH of the sterilized medium was adjusted to 6.8–7.0 by 12 % ammonia solution.

Hydrolysate preparation from crop stalk (CS) wastes

Crop stalk, which contained about 30–35 % cellulose, 25–30 % hemicellulose, and 15–20 % lignin on a dry weight basis, was obtained from a farm in Shandong, China. CS was dried and milled to a fine powder of 50–100 μm in diameter. To prepare the CS hydrolysate, 200 g of CS powder was mixed with 800 mL of distilled water in a 2 L flask and autoclaved at 121 °C for 30 min. Commercial cellulase (endoglucanase activity 1.67 FPU/mg; β-glycosidase activity 0.29 IU/mg) at 5 FPU/g CS (on a dry solid basis) was then aseptically added into the flask to hydrolyze cellulose for 48 h at 55 °C, pH 4.8, and 100 rpm. The pH was adjusted by the addition of HCl before enzymatic hydrolysis. After the enzyme treatments, the hydrolysate was centrifuged for 10 min at 10,000 rpm to remove insoluble impurities, and the supernatant was concentrated by a rotary evaporation system for 30 min for future use. The CS hydrolysate contained 55 g/L glucose, 1.96 g/L xylose, and trace amounts of arabinose and acetic acid.

Batch fermentation

Batch fermentations were also carried out in a 5 L stirred-tank fermentor (Bioflo 110, New Brunswick Scientific, USA) controlled at 30 °C and pH 7.0 by adding 12 % ammonia solution. Basic medium (900 mL) witN_2 for 30 min to anaerobiosis, the fermentor was inoculated with 100 mL of an overnight culture of *P. freudenreichii* ($OD_{600} = 3.0$). The initial total substrate concentration was 60 g/L. Samples were withdrawn periodically using 1 mL syringes to monitor cell growth and fermentation kinetics. After centrifugation, clear broth samples were frozen at −20 °C for future analysis. Unless otherwise noted, duplicate bottles were used for each condition studied.

Batch fermentations in expanded bed adsorption bioreactor (EBAB)

Batch fermentations were studied in three parallel 200 mL EBABs connected with a recirculation loop to a 5 L stirred-tank fermentor for temperature and pH controls. The EBAB was made of a glass column packed with Duolite A30 resin (OH^-; 9.0 mequiv./g), and both ends of the column were equipped with a stainless steel wire mesh. A schematic diagram is shown in Supplementary Fig. S1, and a detailed description of the EBAB system can be found in [6]. By this process, propionic acid was extracted semi-continuously from the broth, and replenishment with fresh resin was possible when needed by alternating the columns. After 24–48 h of incubation, the fermentation broth with free cells was circulated through the EBA column for propionic acid separation in the expanded bed every 12–24 h, when 10 g/L propionic acid was produced. The operation of the expanded bed wherein the fermentation broth flows through the bedpost at the rate of 5 L/h. When the resin became saturated, columns were alternated and switched on to their corresponding spigots to repeatedly operate the circulation system. Propionic acid was recovered from saturated resins by wash and elution steps. The eluted resin will be regenerated for later use. Vitamin B12 was obtained according to the method described by [8]. When the biomass stopped increasing, 0.9 mg/L precursor (5, 6-dimethylbenzimidazole, DMB) solution was added to promote biosynthesis of vitamin B12 by bacterial action.

Analytical methods

At the experimental pH 7.0, more than 90 % of the products (propionic, acetic and succinic acid) are ionized. Therefore, following the wash and elution steps, the eluents from the resins were analyzed without sample preparation for the quantification of these three acids in the aqueous phase. On the other hand, these acids in the fermentation broth were analyzed by the samples of supernatants, which from 1 mL culture broth were used after centrifugation at 10,000×*g* for 10 min. The samples were analyzed by HPLC using Beckman C18 column (5 μm, 4.6 μm × 25 cm) with 0.005 M H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min and a wavelength of 215 nm at 25 °C. Commercially available propionic, acetic and succinic acids were used as external standard.

Measurement of cell concentration

To measure growth kinetics, cell concentrations were analyzed photometrically at 600 nm. Before measuring cell concentrations, 1 mL broth was centrifuged at 10,000×*g* for 10 min and cell pellets were resuspended in 1 mL phosphate buffer after removing the supernatant. The error produced by the removal of the very small fraction of glucose was negligible.

Results and discussion

Effects of sole carbon resource fermentation using glucose or glycerol

Batch fermentation kinetics with glucose and glycerol as carbon sources were studied in 5 L bioreactors at pH 7.0 (Fig. 1a, b). Fermentation was generally faster with glucose than with glycerol as the substrate, but glycerol fermentation resulted in a higher propionic acid yield than glucose fermentation. For vitamin B12 production, the productivity was lower in glycerol fermentation than that in glucose fermentation. The final cell density was lower with glycerol as a single carbon source, even though *P. freudenreichii* can use glycerol to support good cell growth. By following a co-fermentation approach to increase both the production yield and productivity, an even higher production performance could be achieved.

Propionic acid/vitamin B12 production with feedstock co-fermentation strategy

The effects of co-substrates on both mass ratio and supplemented glycerol strategy were systematically examined to gain a better understanding of the effect of co-fermentation on cell growth and product conversion. Co-fermentation of glycerol and glucose at various mass ratios (1, 1.5, 2, 2.5, and 3) was investigated in 5 L bioreactors. The increase in glycerol/glucose mass ratio generally increased the propionic acid yield and decreased the vitamin B12 yield. This result suggests that glycerol became an increasingly more favorable substrate for propionic acid production in co-fermentation than glucose, and the opposite was true for vitamin B12 production. For productivities, the values presented a Gaussian distribution. The mass ratio of 2.5 gave relatively high product yields (propionic acid: 0.62 g/g; vitamin B12: 0.68 mg/g) and the highest productivities (propionic acid: 0.28 g/L h; vitamin B12: 0.37 mg/L h). These values were higher than those with glucose alone (propionic acid: 0.25 g/L h; vitamin B12: 0.35 mg/L h) and with glycerol alone (propionic acid: 0.16 g/L h; vitamin B12: 0.14 mg/L h). Thus, fermentation with glycerol and glucose as co-substrates was advantageous for propionic acid and vitamin B12 production.

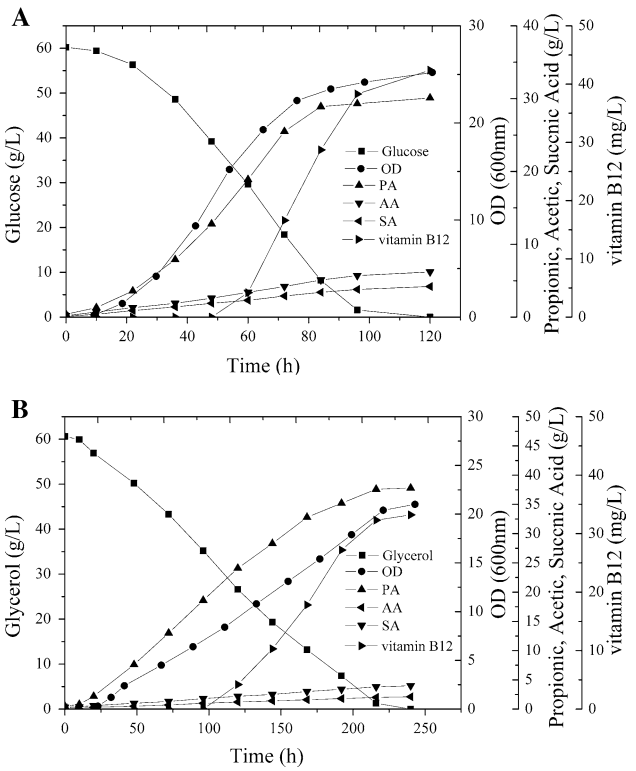

Fig. 1 Batch fermentation kinetics of *P. freudenreichii* with glucose (**a**) or glycerol (**b**) as sole carbon source in 5-L bioreactors at pH 7.0, 30 °C

To optimize fermentation performance, a feedstock gradual addition strategy was employed, in which glycerol was supplemented at different time points (12, 24, 48, and 72 h) at a constant mass ratio of 2.5. As illustrated in Table 1, delaying the time at which glycerol was supplemented increased product biosynthesis but decreased cell density (OD value decreased from 27.2 to 20.1). When supplemented with glycerol at 12 and 24 h, the propionic acid

Table 1 Effect of different supplement time points of glycerol on the cell growth, propionic acid and vitamin B12 production in the bioprocess by *P. freudenreichii*

Substrate supplemented strategy (2.5 Glycerol/Glucose[a])	OD_{600nm}	Titer		Yield		Productivity	
		Propionic acid (g/L)	VB12 (mg/L)	Propionic acid (g/g)	VB12 (mg/g)	Propionic acid (g/L h)	VB12 (mg/L h)
12 h supplemented of glycerol	27.2 ± 0.02	34.4 ± 0.03	40.8 ± 0.07	0.57 ± 0.06	0.68 ± 0.15	0.29 ± 0.06	0.34 ± 0.07
24 h supplemented of glycerol	25.2 ± 0.05	42.6 ± 0.04	40.8 ± 0.13	0.71 ± 0.02	0.68 ± 0.12	0.36 ± 0.08	0.34 ± 0.12
48 h supplemented of glycerol	22.4 ± 0.07	42.7 ± 0.07	43.2 ± 0.11	0.71 ± 0.04	0.72 ± 0.08	0.36 ± 0.07	0.36 ± 0.07
72 h supplemented of glycerol	20.1 ± 0.03	33.6 ± 0.05	35.2 ± 0.06	0.56 ± 0.02	0.59 ± 0.05	0.28 ± 0.03	0.29 ± 0.05

[a] Initial substrate concentration was 43 g/L. The total substrate concentration was 60 g/L. Four cultivations were employed in a fermentation broth supplemented with glycerol at 12, 24, 48 and 72 h of fermentation, respectively

titer was improved from 34.4 to 42.6 g/L, however, the vitamin B12 titer of 12 h supplemented of glycerol was similar to that of 24 h (40.8 ± 0.07 and 40.8 ± 0.13 mg/L), this result and may be because of that two cell growth points were both at lag phase. When glycerol was supplemented at 48 h, the highest product concentrations (propionic acid: 42.7 g/L; vitamin B12: 43.2 mg/L), yields (propionic acid: 0.71 g/g; vitamin B12: 0.72 mg/g), and productivities (propionic acid: 0.36 g/L h; vitamin B12: 0.36 mg/L h) were obtained. When glycerol was supplemented at 72 h, product biosynthesis broke down mainly because part of the cell population entered the decline phase. These results illustrate that the addition time of glycerol significantly influenced product biosynthesis. The juncture-incorporated glycerol was dependent on the exponential phase (48 h) of *P. freudenreichii* growth. Advancing or delaying this phase affected the attainment of the maximum product yield.

Interestingly, previous researches reported that culture parameters such as oxygen is a key parameter to the metabolic regulation of *P. freudenreichii*. Low DO concentration was found to be advantageous in *P. freudenreichii* cell growth, in decomposition of propionate, and in lowering the production of acetate [10]. In our study, partitioning carbon fluxes between propionic acid and vitamin B12 synthesis also occurred by co-fermentation substrates. When glycerol and glucose were used as co-substrates and supplemented simultaneously, as described above, glycerol was mainly used for propionic acid biosynthesis and glucose was mainly used for vitamin B12 synthesis. However, when the feedstock was supplemented phased in gradually, the mass (carbon fluxes) for the fermentation process converted to products synthesis instead of cell growth and the maximum products yields were obtained, this result represented an important improvement in this study.

Batch fermentation with CS hydrolysis in EBAB

Batch fermentation with glycerol and CS hydrolysate as co-substrates at a 2.5:1 mass ratio and the gradual addition of carbon substrate was studied using free cells in EBAB (EBA-based ISPR process). The fermentation kinetics is shown in Fig. 2. When propionic acid production reached inhibitory conditions (10 g/L) in the broth, eighteen consecutive recycles were performed for propionic acid online separation to remove feedback inhibition. The aqueous concentration of propionic acid remained below 10 g/L, and the total concentration reached 91.4 g/L with a yield of 0.75 g/g and a productivity of 0.35 g/L h after 258 h of fermentation. This result suggests that EBAB with glycerol and CS hydrolysate as co-substrates was stable for the continued production of propionic acid under the repeated recycle mode. An increase in the propionic acid yield (from 0.71 to 0.75 g/g) was observed with EBAB. The EBA-based ISPR process alters the mass transfer and directs more nutrients to the synthesis of propionic acid instead of vitamin B12 production [6]. By contrast, without EBAB, the vitamin B12 yield decreased (from 0.72 to 0.37 mg/g) but the productivity remained constant (0.36 mg/L h), which was still higher than that with pure glucose as a sole substrate with EBAB (0.36 vs. 0.27 mg/L h). This

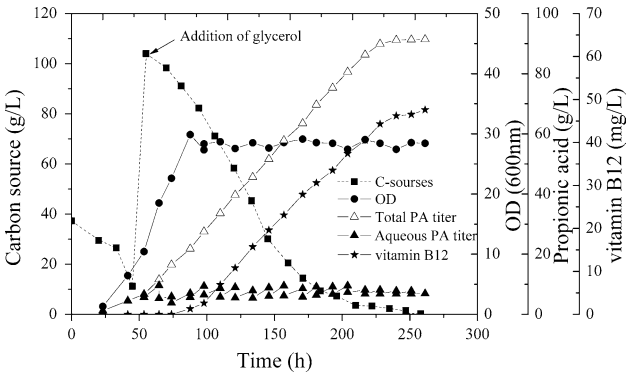

Fig. 2 Batch fermentation kinetics with a gradual addition strategy in the EBAB. The total concentration of propionic acid is the sum of concentrations of propionic acid in the fermentation broth and propionic acid that eluted from the resin after fermentation

result suggests that optimized co-fermentation increased productivity. Comparable propionic acid and vitamin B12 yield and productivity were obtained with glycerol and CS hydrolysate as the substrates in the batch with EBAB, which confirmed that low-cost feedstock could be used efficiently for propionic acid and vitamin B12 production.

Several studies on propionic acid fermentation with glycerol as the sole carbon source or with a co-substrate have been reported. Yang recently reported the production of propionic acid from crude glycerol and cassava bagasse hydrolysate using *P. freudenreichii* subsp. *shermanii*, and achieved a propionic acid yield of 0.57 g/g and a productivity of 0.25 g/L h, respectively [9]. In our study with a similar glycerol/glucose mixture and supplement strategy, we obtained comparable propionic acid yield but 44 % higher productivity (0.36 g/L h). A significantly higher propionic acid yield of 0.75 g/g was achieved in co-fermentation with EBAB, which demonstrates the advantages of integrated ISPR and co-fermentation strategy for continuous production of propionic acid. EBAB-batch fermentation also achieved vitamin B12 as a valuable additional product in only one process. Thus, EBAB using co-substrates as carbon sources was found to be a more effective process for propionic acid and vitamin B12 production.

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