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Effects of carbon dioxide on cell growth and propionic acid production from glycerol and glucose by *Propionibacterium acidipropionici*

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HIGHLIGHTS

- CO_2 enhanced propionic acid production in glycerol fermentation.
- CO_2 enhanced cell growth in glycerol fermentation.
- CO_2 had no significant effect in glucose fermentation.
- A stoichiometric metabolic model was used to illustrate carbon flux distribution.

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ABSTRACT

The effects of CO_2 on propionic acid production and cell growth in glycerol or glucose fermentation were investigated in this study. In glycerol fermentation, the volumetric productivity of propionic acid with CO_2 supplementation reached 2.94 g/L/day, compared to 1.56 g/L/day without CO_2. The cell growth using glycerol was also significantly enhanced with CO_2. In addition, the yield and productivity of succinate, the main intermediate in Wood–Werkman cycle, increased 81% and 280%, respectively; consistent with the increased activities of pyruvate carboxylase and propionyl CoA transferase, two key enzymes in the Wood–Werkman cycle. However, in glucose fermentation CO_2 had minimal effect on propionic acid production and cell growth. The carbon flux distributions using glycerol or glucose were also analyzed using a stoichiometric metabolic model. The calculated maintenance coefficient (m_{ATP}) increased 100%, which may explain the increase in the productivity of propionic acid in glycerol fermentation with CO_2 supplement.

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1. Introduction

Propionic acid is an important chemical intermediate used in the synthesis of vitamin E, cellulose fibers, artificial fruit flavors, fragrances, and perfumes (Deborde and Boyaval, 2000). Its salts are widely used as food and feed preservatives. Currently, almost all industrial propionic acid is produced via petroleum-based chemical synthesis. However, concerns over sustainability and rising crude oils prices have generated high interests in producing propionic acid from renewable carbon sources (Suwannakham and Yang, 2005; Zhang and Yang, 2009a). With the increased production of biodiesels, large amounts of glycerol are produced as a byproduct with limited uses, causing a significant environmental problem. Due to its low price and high reduction state, glycerol in biodiesel wastes is a favorable renewable feedstock for the production of industrial chemicals like propionic acid (Wang and Yang, 2013; Zhang and Yang, 2009b; Zhu et al., 2010). In contrast to glucose fermentation, little acetic acid and CO_2 were produced by propionibacteria during glycerol fermentation due to the high reduction degree of glycerol. This favors the production of more reduced metabolites, but can cause redox imbalance and lead to reduced cell growth and low productivity (Zhang and Yang, 2009b).

The heterotrophic CO_2 fixation of *Propionibacterium acidipropionici* was believed to have great potential in the industry for propionic acid production, since CO_2 was one of the byproduct during fermentation (Parizzi et al., 2012). Even CO_2 turnover was reported in relation to the mechanism of propionate formation (Wood and

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Leaver, 1953), the effects of CO_2 on propionibacteria growth and end product formation have been minimally investigated. Recently Ammar et al. (2014) demonstrated that CO_2 could increase propionic acid yield and product purity for metabolically engineered *Propionibacterium freudenreichii* strain overexpressing CO_2 fixation enzyme phosphenolpyruvate carboxylase, but had no effect on the wild type. Moreover, it is worth noting that the effects of CO_2 on microbial growth and metabolism are rather complicated and depend on organisms (Dharmadi et al., 2006; Jones and Greenfield, 1982), substrates (Misoph and Drake, 1996) and CO_2 concentration (Caldwell et al., 1969). Dharmadi et al. (2006) demonstrated that CO_2 had a positive impact on the anaerobic growth of *Escherichia coli* and the biosynthesis of small molecules, fatty acids, and central metabolism from glycerol under acidic conditions. However, the inhibition effect of CO_2 has been shown in cell growth and fermentation of yeast (Jones and Greenfield, 1982). It was also shown by Misoph and Drake (1996) that the supplemental CO_2 could stimulate cell yields of *Peptostreptococcus productus* U-1 on fructose and decrease cell yields on xylose.

In this work, CO_2 effect was studied using glucose or glycerol during fermentations: cell growth, carboxylic acid formation, and enzyme activities were compared in glucose and glycerol fermentations under CO_2-enriched and CO_2-limited conditions. In addition, the end-product composition in glycerol and glucose fermentations was studied by the mass balance analysis through a stoichiometric metabolic model. The effect of CO_2 on the metabolic pathway of the Wood–Werkman cycle was also discussed in this paper.

2. Methods

2.1. Culture and media

P. acidipropionici ATCC 4875 was cultivated in a synthetic medium containing (per liter) 10 g yeast extract (Difco Laboratories, Detroit, MI), 5 g Trypticase (BBL), 0.25 g K_2HPO_4, 0.05 g $MnSO_4$, and 50–100 g glucose or glycerol as the carbon source. Unless otherwise noted, the medium pH was adjusted to ~7.0 by adding HCl or NaOH. The basal medium (without the carbon source) and the concentrated carbon source solution were sterilized separately at 121 °C and 15 psig for 30 min, and then mixed aseptically before use in the fermentation study. The working stock culture was maintained in 125-ml serum bottles stored at 4 °C with biweekly subculturing.

2.2. Fermentation kinetic studies

Batch fermentations with glucose or glycerol as the carbon source were studied under either CO_2 limited or enriched condition to evaluate the effects of CO_2 on cell growth and propionic acid production by *P. acidipropionici*. Unless otherwise noted, all batch fermentations were carried out in 5-L anaerobic fermentors (Marubishi MD-300) containing 2 L of the medium at 32 °C. The fermentor pH was controlled at 7.0 (±0.02) by automatically adding 6 N NaOH. CO_2-enriched medium was obtained by flushing CO_2 in the fermentor headspace and adding filter-sterilized sodium bicarbonate solution till reaching the saturated concentration at 32 °C, pH 7.0, and 1 atm. The CO_2-limited medium was maintained in anaerobiosis by sparging N_2, instead of CO_2, through the medium for 10–30 min at the beginning of the fermentation. Each fermentation batch was initiated by inoculating with 100 ml of exponential-phase cells ($OD_{600} \approx 2.0$). Broth samples (3 ml each) were taken from the fermentor at proper time intervals throughout the fermentation. All fermentations were performed at least in replicate, and the kinetics data were reported as the means of the replicates ± standard deviations. The variation and reproducibility can be reflected by standard deviation provided. The specific cell growth rate, product yields, and volumetric productivities were calculated from cell and product concentrations.

2.3. Preparation of cell extract for enzyme activity assays

Cells grown in glycerol under CO_2-enriched or CO_2-limitied condition (50 ml each) in serum bottles were harvested to examine their intracellular activities of pyruvate carboxylase (PYC) and Succinyl CoA:propionate CoA transferase (CoA T). Cells in the exponential phase (OD_{600} ~1.8) were harvested by centrifuging at 7000 rpm for 10 min, washed three times, resuspended in 3 ml of ice cold Tris–HCl buffer (25 mM, pH 7.4), and then ultrasonicated using a sonic dismembrator (Fisher Scientific, Model 100). Sonication was conducted 5 s, followed by 25 s of resting to prevent overheating. This step was repeated for a total of 20 cycles and the cell extract was kept in an ice bath during sonication. Then, the cell extract was centrifuged at 15,000 rpm at 4 °C for 1 h to remove cell debris. For pyruvate carboxylase assay, the supernatant was subjected to further centrifugation in an ultra-centrifuge (Beckman, Optima TL) for 90 min at 45,000 rpm to remove the gelatinous sediment containing most of the NADH oxidase activity. The cell extracts were kept on ice before they were used in enzyme activity assays. The protein content of the extracts was determined in triplicate by using the Bradford protein assay (Bio-Rad) with bovine serum albumin as the standard.

2.4. Enzyme activity assays

2.4.1. Pyruvate carboxylase (PYC)

The activity of PYC was assayed coupled with the NADH-dependent reduction of oxaloacetate by malate dehydrogenase, and modifications were made according to literature (Lin et al., 1989; Velayudhan and Kelly, 2002). Briefly, the assay mixture (300 μl) contained Tris–HCl (pH 9.0, 0.1 M), 0.2 ml; NADH (2 mM), 0.01 ml; $MgCl_2$ (0.6 M), 0.005 ml; $NaHCO_3$ (0.6 M, fresh), 0.005 ml; phosphoenolpyruvate (PEP) (0.1 M), 0.01 ml; malate dehydrogenase (0.15 mg/ml), 0.01 ml; and the cell extract, 0.06 ml. The reaction was carried out at 25 °C and the absorbance at 340 nm was measured throughout the reaction. A mixture without PEP was used as a blank control. The standard unit of PYC is defined as the amount of enzyme causing an absorbance change of 1.0 unit per minute, and the specific activity was defined as units per milligram of protein.

2.4.2. Succinyl CoA:propionate CoA transferase (CoAT)

The activity of CoAT was assayed as described by Schulman and Wood (1975) with some modifications. The assay mixture (water to 250 μl) contained 0.1 ml of Mixture 1 (17.0 M, pH 8.0 Tris–HCl buffer, 1 ml; 0.4 M sodium malate, 0.01 ml; 0.01 M NAD^+, 1.0 ml; and water to 4 ml), 0.01 ml of 1.5 M sodium acetate, 0.01 ml of Mixture 2 (944 units/mg of malic dehydrogenase, 14 μl; 355 units/mg of citrate synthase, 11 μl; and 0.1 M, pH 6.8 phosphate buffer, 975 μl), 0.01 ml of 0.15 μmole succinyl-CoA, and 0.05 ml of cell extract. The reaction was conducted at 25 °C, followed by measuring the absorbance at 340 nm, which increased linearly with time for 3–5 min. A mixture without CoA transferase was used as a blank control. The standard unit of CoAT is defined as the amount of enzyme causing an absorbance change of 1.0 unit per minute, and the specific activity was defined as units per milligram of protein.

2.4.3. Statistical analysis

Statistical analysis for enzyme activities at each condition were conducted using JMP. Two sample *t* test was conducted to

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