Enhanced Butyric Acid and Hydrogen Production by the Mutants of *Clostridium tyrobutyricum*

*Xiaoguang Liu and Shang-Tian Yang**
Department of Chemical and Biomolecular Engineering, The Ohio State University, Columbus, Ohio 43210
*Corresponding author. Phone: (614) 292-6611. Fax: (614) 292-3769. Email address: yang.15@osu.edu.

Introduction

Clostridium tyrobutyricum is a gram-positive, rod-shaped, spore-forming, obligate anaerobic bacterium that produces butyric acid, acetic acid, hydrogen and carbon dioxide as its main fermentation products from various carbohydrates including glucose and xylose (Wu and Yang, 2003). Butyric acid is used to synthesize butyryl polymers in the chemical industry and to enhance butter-like note in food flavors in the food industry. Esters of butyrate are used as additives for increasing fruit fragrance and as aromatic compounds for production of perfumes. Butyric acid, as one of the short-chain fatty acids generated by anaerobic fermentation of dietary substrates, is known to have therapeutic effects on colorectal cancer and hemoglobinopathies (Willims et al., 2003). The production of butyric acid from renewable resources has become an increasingly attractive alternative to the current petroleum-based chemical synthesis because of public concerns on the environmental pollution caused by the petrochemical industry and consumer's preference to bio-based natural ingredients for foods, cosmetics and pharmaceuticals. Recently, we have demonstrated that butyric acid can be better produced by *C. tyrobutyricum* immobilized in a fibrous-bed bioreactor (Zhu and Yang, 2003; Yang, 1996). However, for economical production of butyric acid from biomass, it is desirable to further improve the fermentation process in its final product yield and concentration. Hydrogen, with a high energy content per unit weight (141.86 kJ/g or 61,000 Btu/lb), can be used as a clean fuel and easily converted to electricity by fuel cells. Hydrogen is thus considered as the most promising future fuel if its production cost can be greatly reduced. Hydrogen can be generated in several ways, including catalytic fuel reforming and electrolysis of water. However, the present chemical routes of hydrogen production are energy intensive and expensive. On the other hand, biological production of hydrogen, either by photosynthesis or by fermentation, can be operated at ambient temperature and pressure (Momirlan and Veziroglu, 1999; Das and Veziroglu, 2001). Hydrogen, as an energy byproduct from the butyric acid fermentation, can add value to the fermentation process. The goal of this project was to develop mutants with knocked-out acetate formation pathway to enhance the butyrate and hydrogen production from renewable resources. In this work, the partial *pta* or *ack* gene from *C. tyrobutyricum* was cloned and sequenced. Gene inactivation by integrational plasmid was then carried out to develop a *pta* or *ack*-deleted mutants. The enzyme activities of the mutants were examined to understand how the gene manipulation worked. Finally, their ability to produce butyric acid and hydrogen from glucose fermentation was evaluated and characterized in this paper.

Materials and Methods

Mutants construction and characterization. Non-replicative plasmids, pPTA-Em and pAK-Em, containing partial fragment of *pta* or *ack* gene cloned from the genomic DNA using degenerate primers and erythromycin resistant gene were used to transform *C. tyrobutyricum*

and inactivate *pta* and *ack* genes on the chromosome through homologous recombination following previous method (Boynton, et al., 1996), which produced *pta* gene deleted mutant PPTA-Em and the *ack* gene deleted mutant PAK-Em respectively. The activities of PTA and PTB were measured spectrophotometrically at 405 nm by detecting the liberation of CoA from acetyl-CoA and butyryl-CoA, respectively, following the protocol of Andersch (Andersch, et al, 1983). One unit of PTA or PTB activity is defined as the amount of enzyme converting 1 μmol of acetyl-CoA or butyryl-CoA per minute. The activities of AK and BK were assayed using potassium acetate and sodium butyrate as substrate, respectively, by the method of Rose (Rose, 1955). One unit of AK or BK is defined as the amount of enzyme producing 1 μmol of hydroxamic acid per minute. Hydrogenase activity was detected using the procedure developed by Drake (Drake, 1982). One unit of hydrogenase activity is defined as 2 μmol of methyl viologen reduced (equivalent to 1 μmol of H_2 oxidized) per minute. Specific enzyme activity was calculated as the units of activity per mg of protein. The specific enzyme activities in the mutants as compared with the corresponding specific enzyme activities of the wild type were reported as the relative enzyme activity (%) in this work.

Fermentation. Fed-batch fermentations of *C. tyrobutyricum* were performed in a 5-L stirred-tank fermentor (Marubishi MD-300) containing 2 L of the CGM medium and substrate agitated at 150 rpm with pH and temperature controls. For the repeated-batch fermentations with immobilized cells, a 0.5-L fibrous-bed bioreactor (FBB) with a working volume of ~500 mL was made of a glass column packed with spiral wound cotton towel, which was described elsewhere (Silva and Yang, 1995). The anaerobiosis was reached by sparging the fermentor medium and the FBB with N_2. The 100 mL of cell suspension prepared in serum bottle was inoculated into the fermentor. The fed-batch mode was operated by pulse feeding concentrated substrate solution when the sugar level in the fermentation broth was close to zero. The Micro-oxymax system (Columbus Instrument, Columbus, OH) connected to the fermentor was used to measure the production of H_2 and CO_2. Samples were taken at regular intervals from the fermentor for the analyses of cell density, substrate and products concentration.

Results and Discussion

Enzyme Assays. Gene inactivation technology was used to delete phosphotransacetylase (PTA) and acetate kinase (AK) genes in *C. tyrobutyricum*. Figure 1 shows that PTA and AK are two key enzymes in the acetate-producing pathway. Phosphotransbutylase (PTB) and butyrate kinase (BK) catalyze the butyrate formation from butyrl-CoA in the PTB-BK pathway. The hydrogenase contributes the production of hydrogen by oxidizing FdH_2 to Fd. The enzyme assays results indicated that the PTA and AK activities in the *pta*-deleted mutant (PPTA-Em) were reduced by 44% and 91%, respectively, whereas AK activity in the *ack*-deleted mutant (PAK-Em) decreased by 50% as compared with the wild type. The activities of both PTA and AK in the *pta*-deleted mutant (PPTA-Em) were decreased dramatically, but only AK activity in the *ack*-deleted mutant (PAK-Em) was reduced, indicating that the *pta* gene lies upstream from *ack* gene in the same operon. However, both mutants with the intended *ack* or *pta* gene knock-out did not completely eliminate activities of PTA and AK in these mutants. There may be some other enzymes, besides PTA and AK, present in *C. tyrobutyricum* that also can use the same substrates (acetyl-CoA) to produce acetic acid. Meanwhile, the activity of butyrate kinase (BK) in PPTA-Em increased by 44% and hydrogenase activity in PAK-Em increased by 40%. Since AK and PTA activities were significantly reduced in the mutants, which can generate more ATP per mole of glucose

metabolized than the butyrate-producing (PTB-BK) pathway can, ATP production from the acetate-producing (PTA-AK) pathway lowered. To compensate for the lost energy efficiency due to reduced flux through the PTA-AK pathway, PPTA-Em mutant increased its BK activity and PAK-Em mutant increased its hydrogenase activity. The increased hydrogenase activity not only resulted in more hydrogen production, but also might have increased the amount of NADH and energy production.

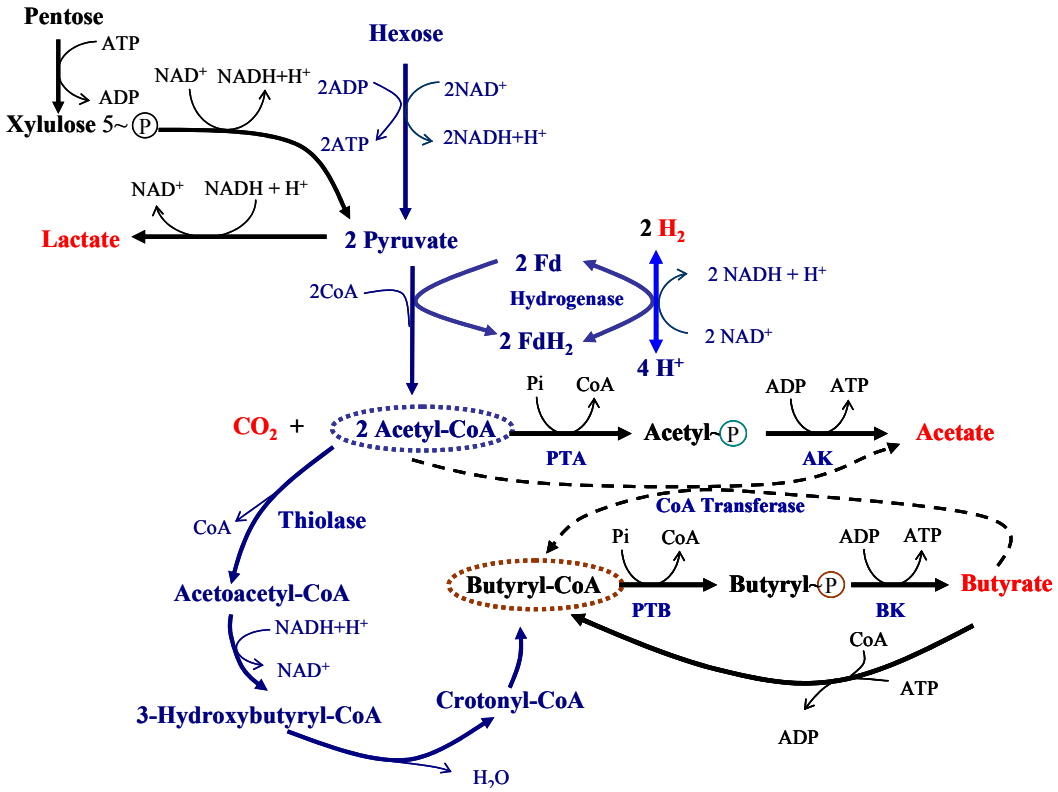

Figure 1. Fermentation pathway for butyric acid, acetic acid, and hydrogen production from sugars in Clostridium tyrobutyricum. (AK: acetate kinase; BK: butyrate kinase; PTA: phosphotransacetylase; PTB: phosphotransbutylase).

Fermentation. Free cell fermentations using glucose were carried out to study the potential to improve butyric acid and hydrogen production by these mutants with inactivated *pta* and *ack*. As compared with the wild type, the specific growth rate of the mutants decreased by 32% (from 0.28 h^{-1} to 0.19 h^{-1}) because of the impaired PTA-AK pathway. Meanwhile, butyric acid production by these mutants was improved greatly, with higher butyric acid yield (0.4 g/g vs. 0.34 g/g) and final concentration (43 g/L vs. 29 g/L), which indicated that the mutants had better tolerance to butyric acid inhibition. The increased butyric acid tolerance of the mutants may be attributed to the reduced flux through the PTA-AK pathway. Our previous study has shown that the acetic acid-forming enzymes (PTA and AK) are more sensitive to butyric acid inhibition than butyric acid-forming enzymes (PTB and BK). Since the mutants were no longer dependent on the PTA-AK pathway for energy production and survival, they became less sensitive to butyric acid inhibition. However, acetate production in the mutants was not significantly reduced even though more butyrate was produced from glucose. So probably there are other enzymes in *C. tyrobutyricum* that can also produce acetate. For

example, CoA transferase can catalyze the formation of acetate from acetyl-CoA. This enzyme has been found in some clostridia bacteria (Papousakis and Meyer, 1985) and could also be present in *C. tyrobutyricum*. Possible presence of PTA and AK isozymes also could not be ruled out. Two acetate kinase isozymes from spirochete MA-2 cell extracts (Harwood and Caale-Parola, 1982) and a butyrate kinase isozyme (BKII) in *C. acetobutylicum* ATCC 824 (Huang, et al., 2000) have been reported. Also, hydrogen production by PAK-Em mutant increased significantly, with higher hydrogen yield (2.61 vs. 1.35 mol/mol glucose) and H_2/CO_2 ratio (1.43 vs. 1.08). These results suggested that integrational mutagenesis resulted in global metabolic shift and phenotypic changes, which also improved production of butyric acid and hydrogen from glucose in the fermentation.

Our group has developed a novel fibrous-bed bioreactor (FBB) with cells immobilized in a fibrous matrix for the production of various organic acids, including lactic, propionic and butyric acids. With high cell densities immobilized in the fibrous matrix, the FBB greatly increased final product concentration and yield as compared to the conventional free-cell fermentations because of the quick adaptation of the immobilized cells in FBB (Zhu and yang, 2003). In addition, the FBB can give good long-term performance and it is easier to optimize fermentation conditions such as pH and nutrient supplement. In this work, fed-batch fermentations were carried out with mutant cells immobilized in fibrous-bed bioreactors (FBB) to further improve their abilities to produce butyric acid and hydrogen. Through adaptation in the FBB, the PPTA-Em produced higher concentration of butyric acid, about 50 g/L, with improved yield of 0.45 g/g as compared with the butyrate production from free cell fermentation. A high butyric acid concentration of 82 g/L was obtained at pH 6.3 with the immobilized PAK-Em. This concentration is the highest ever attained in butyric acid fermentation reported by now, as compared with the previous best results including butyric acid concentration of 62.8 g/L with yield of 0.45 g/g from sucrose (Fayolle, et al., 1990) and butyric acid concentration of 42.5 g/L with yield of 0.36 g/g from glucose (Michel-Savin et al., 1990) by *C. tyrobutyricum*. The butyrate yield was also increased to ~0.45 g/g due to reduced cell growth in the immobilized-cell fermentation. It has been reported that culturing in the FBB facilitated adaptation and selection of mutant (Zhu and Yang, 2003). The immobilized PAK-Em cells in FBB fibrous matrix were washed and screened after six FBB fermentations under high gas pressure, a new mutant (HydEm) that produced even more hydrogen with a H_2/CO_2 ratio of ~2 was discovered. Metabolic flux analysis showed that the global metabolic flux distributions were altered in these mutants. These results suggested that further enhancements in butyric acid and hydrogen production from sugar wastes by *C. tyrobutyricum* can be achieved by metabolic engineering via integrational mutagenesis and cell adaptation in the fibrous-bed bioreactor.

Conclusions

Phosphotransacetylase gene (*pta*) and acetate kinase gene (*ack*) encode two important enzymes involving in the metabolic pathway to form acetic acid from acetyl-CoA, which plays an important role for the metabolic flux distribution of carbon and energy. Two mutants of *C. tyrobutyricum* obtained from integrational mutagenesis to selectively inactivate *ack* and *pta* genes were characterized and studied for their butyric acid and hydrogen fermentation ability. As compared with the wild type, butyric acid production by these mutants was improved with higher final product concentration and yield. It is clear that more carbon and energy fluxes can flow into the metabolic pathway leading to the production of butyric acid in the mutants. Also, these mutants have better tolerance to butyric acid inhibition. The *ack*-deleted mutant also has

improved hydrogen production. This study demonstrates that butyric acid and hydrogen production from glucose can be significantly improved by using metabolically engineered mutants of *C. tyrobutyricum*. Increasing the final butyric acid concentration, yield, and volumetric productivity in the fermentation should reduce the production cost for bio-based butyric acid and allow the bioproduction method to compete more favorably in the marketplace.

References

Andersch W, Bahl H, Gottschalk G. Levels of enzymes involved in acetate, butyrate, acetone and butanol formation by *Clostridium acetobutylicum.* Eur J Appl Microbiol Biotechnol 1983,17:327-332.

Boynton ZL, Bennett GN, Rudolph FB. Cloning, sequencing, and expression of genes encoding phosphotransacetylase and acetate kinase from *Clostridium acetobutylicum* ATCC 824. Appl Environ Microbiol 1996, 62:2758-2766.

Das D, Veziroglu TN. Hydrogen production by biological processes: a survey of literature. Int J Hydrogen Energy 2001, 26:13-28.

Drake HL. Demonstration of hydrogenase in extracts of the homoacetate-fermenting bacterium *Clostridium thermoaceticum*. J Bacteriol 1982, 150:702-709.

Fayolle F, Marchal R, Ballerini D. Effect of controlled substrate feeding on butyric acid production by *Clostridium tyrobutyricum*. J Ind Microbiol 1990, 6:179-183.

Harwood CS, Caale-Parola E. Properties of acetate kinase isozymes and a branched-chain fatty kinase from a spirochete. J Bacteriol 1982, 152:246-254.

Huang KX, Huang S, Rudolph FB, Bennett GN. Identification and characterization of a second butyrate kinase from *Clostridium acetobutylicum* ATCC 824. J Molecul Microbio Biotechnol 2000, 2:33-38.

Michel-Savin D, Marchal R, Vandecasteele JP. Butyrate production in continuous culture of *Clostridium tyrobutyricum*: effect of end-product inhibition. Appl Microbiol Biotechnol 1990, 33:127-131.

Momirlan M, Veziroglu T. Recent directions of world hydrogen production. Energy Rev 1999, 3:219-231.

Papousakis ET, Meyer CL. Equations and calculations of product yields and preferred pathways for butanediol and mixed-acid fermentations. Biotechnol Bioeng 1985, 27:50-56.

Rose IA. Acetate kinase of bacteria (acetokinase). Methods Enzymol 1955, 1:591-595.

Silva EM, Yang ST. Kinetics and stability of a fibrous bed bioreactor for continous production of lactic from unsupplemented acid whey. J Biotechnol 1995, 41:59-70.

Willims EA, Coxhead JM, Mathers JC. Anti-cancer effects of butyrate: use of micro-array technology to investigate mechanisms. Proc Nutr Soc 2003, 62:107-115.

Wu Z, Yang ST. Extractive fermentation for butyric acid production from glucose by *Clostridium tyrobutyricum*. Biotechnol Bioen 2003, 82:93-102.

Yang ST. Extractive fermentation using convoluted fibrous bed bioreactor. U. S. Patent No. 5563069, 1996.

Zhu Y, Yang ST. Adaptation of *Clostridium tyrobutyricum* for enhanced tolerance to butyric acid in a fibrous-bed bioreactor. Biotechnol Prog 2003, 19:365-372.