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Refactoring redox cofactor regeneration for high-yield biocatalysis of glucose to butyric acid in *Escherichia coli*

Jae Hyung Lim [a,1], Sang Woo Seo [b,1], Se Yeon Kim [a], Gyoo Yeol Jung [a,b,*]

[a] School of Interdisciplinary Bioscience and Bioengineering, Pohang University of Science and Technology, San 31, Hyoja-dong, Nam-gu, Pohang, Gyeongbuk 790-784, South Korea
[b] Department of Chemical Engineering, Pohang University of Science and Technology, San 31, Hyoja-dong, Nam-gu, Pohang, Gyeongbuk 790-784, South Korea

HIGHLIGHTS

- The redox cofactor regeneration system of *E. coli* was rebuilt to produce butyrate.
- This system produced butyrate from glucose with high yield (>83%).
- Refactoring cofactor regeneration has broad applications for biorefinery technology.

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ABSTRACT

In this study, the native redox cofactor regeneration system in *Escherichia coli* was engineered for the production of butyric acid. The synthetic butyrate pathway, which regenerates NAD^+ from NADH using butyrate as the only final electron acceptor, enabled high-yield production of butyric acid from glucose (83.4% of the molar theoretical yield). The high selectivity for butyrate, with a butyrate/acetate ratio of 41, suggests dramatically improved industrial potential for the production of butyric acid from nonnative hosts compared to the native producers (*Clostridium* species). Furthermore, this strategy could be broadly utilized for the production of various other useful chemicals in the fields of metabolic engineering and synthetic biology.

1. Introduction

Butyric acid, a short-chain fatty acid, and its derivatives are utilized in various industrial products, including plastics, fibers, food additives, and pharmaceuticals (Nudelman et al., 1992; Zigova and Sturdik, 2000). In addition, direct hydrogenation of butyric acid by copper-based catalysts can produce 1-butanol, a chemical in the world spotlight as a replacement for gasoline (Kim et al., 2011). Although butyric acid can be generated naturally from renewable biomass by anaerobic fermentation of various organisms, it is currently produced from petrochemicals for industrial applications due to cost considerations (Zhu and Yang, 2004). However, a paradigm shift, brought about by concerns over oil depletion and a desire on the part of food additive and pharmaceutical industries to meet public demand for products with natural origins, has made production of butyric acid through fermentation an attractive alternative to the petrochemical route (Zigova and Sturdik, 2000).

The dominant platform for the biological production of butyric acid in the past two decades has been members of the genus *Clostridium*, a gram-positive, strictly anaerobic, spore-forming bacteria (Zhang et al., 2009). However, deficiencies in genetic engineering tools and incomplete physiological information about these strains have made for slow progress in the re-design of this natural biocatalyst (Abo-Hashesh et al., 2011; Liu et al., 2006; Zhu et al., 2005). Thus, some studies have focused on fermentation techniques, including a fibrous-bed bioreactor (FBB), to improve productivity and titer of butyric acid (Huang et al., 2011; Song et al., 2011).

The use of biocatalysts, such as whole-cell systems, for industrial applications requires high-yield and low-cost conversion for economic feasibility (Ishige et al., 2005; Schmid et al., 2001). One of the issues yet to be solved is how to continuously supply redox cofactors that are often needed for complex enzymatic reactions. Among cofactors, nicotinamide adenine dinucleotide (NAD) is known to participate in over 300 redox reactions during cellular metabolism (Foster and Moat, 1980). In nature, cells utilize NAD^+ as a redox cofactor, oxidizing a carbon source such as glucose and producing reducing equivalents in the form of NADH. Under aerobic conditions, cells regenerate NAD^+ from NADH using oxygen as the final electron acceptor. By contrast, under anaerobic conditions, cells achieve overall redox balance by reducing metabolic

* Corresponding author at: Department of Chemical Engineering, Pohang University of Science and Technology, San 31, Hyoja-dong, Nam-gu, Pohang, Gyeongbuk 790-784, South Korea. Tel.: +82 54 279 2391; fax: +82 54 279 5528.
E-mail address: gyjung@postech.ac.kr (G.Y. Jung).
[1] These authors contributed equally to this work.

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intermediates into several fermentative products, including lactate, ethanol, and succinate. This allows catalytic amounts of cofactors to be regenerated during the processes, providing a driving force for thermodynamically and kinetically unfavorable reactions and subsequently increasing the yield and productivity of the bio-catalysis.

In this study, the redox cofactor regeneration system in *Escherichia coli* was engineered for the production of butyric acid. The synthetic metabolic pathway, which regenerates NAD^+ from NADH using butyrate as the only final electron acceptor, enabled high-yield production of butyric acid from glucose. The butyrate/acetate ratio of this system demonstrates industrial potential for the production of butyric acid from nonnative hosts, far outstripping the ratio achieved in the native producer *Clostridium*. Furthermore, redesigning the native redox cofactor regeneration system based on an understanding of its role in providing driving force would facilitate future efforts in pathway engineering to achieve high-yield production of other high-value chemicals in various organisms.

2. Methods

2.1. Reagents, bacterial strains, and plasmids

A list of *E. coli* bacterial strains and plasmids used in this study is presented in Table 1. Oligonucleotides used in this study, synthesized by Genotech (Daejeon, Korea), are listed in Table 2. Phusion DNA polymerase and restriction endonuclease were purchased from New England Biolabs (Ipswich, MA, USA), and T4 DNA ligase was supplied by Takara Bio Inc. (Shiga, Japan). Propagated plasmids were prepared using AccuPrep Nano-Plus Plasmid Mini Extraction kits (Bioneer, Daejeon, Korea), and restriction enzyme-digested products were purified using a GeneAll Expin Gel SV kit (GeneAll Biotechnology, Seoul, Korea). All cell culture reagents were purchased from BD (Sparks, MD, USA). All chemicals used were purchased from Sigma (St. Louis, MO, USA) unless otherwise indicated.

All chromosomal work, including deletion and overexpression (*adhE*, *ato*, *frdABCD*, *ldhA*, *paaFGH*, *pta*), was conducted using the Red recombination system with pKD46 and pCP20, as described in previous studies (Datsenko and Wanner, 2000; Lim et al., 2008). To increase efficiency of homologous recombination, four disruption cassettes with different priming sites (pFRT 2, 5, 6, and 7) were constructed using pKD4 as a polymerase chain reaction (PCR) template. For example, pFRT2 was constructed by amplifying the FRT-*Kan*^{R}-FRT(2) fragment from pKD4 using the primers FRT2_F and FRT2_R. The A-tailed PCR products were then ligated into the pGEM T-Easy cloning vector. The other three vectors were created in the same manner.

To construct the butyrate synthetic pathway in plasmid, *crt* and *hbd* were amplified from genomic DNA of *Clostridium acetobutylicum* ATCC824 using the primer pairs crt_F/crt_R and hbd_F/hbd_R, respectively. The *ter* gene from *Treponema denticola* was synthesized by Bioneer (Daejeon, Korea) with codon optimization, as reported in a previous study (Bond-Watts et al., 2011). The *tesB* gene was amplified from *E. coli* W3110 genomic DNA using tesB_F and tesB_R. To overexpress each gene in *E. coli*, all structural genes were expressed under the control of the strong constitutive promoter, BBa_J23100 (5′-TTGACGGCTAGCTCAGTCCTAGGTACAGTGC TAGC-3′ from Registry of Standard Biological Parts, http://www.partsregistry.org); synthetic 5′-untranslated region (5′-UTR) sequences were designed using UTR designer (http://www.sbi.postech.ac.kr/utr_designer) (Seo et al., in press). Thereafter, pCDF-SP was constructed by sequentially ligating the *crt* fragment digested with *BamH*I and *Sal*I, the *hbd* fragment digested with *Sal*I and *Xho*I, and the *ter* fragment digested with *Xho*I and *Pac*I into the same sites of pCDFDuet. The fragment digested with *BamH*I and *Sac*I from pCDF-SP was ligated into the same sites of pACYCDuet, creating pACYC-SP. Finally, pBASP was created by ligating the *tesB* fragment digested with *Nde*I and *Not*I into the matching sites of pACYC-SP.

2.2. Media and growth conditions

Butyric acid-producing capacity was assayed using modified Terrific Broth (TB) (12 g tryptone, 24 g yeast extract, 2.31 g KH_2PO_4, 12.54 g K_2HPO_4, per liter, excluding glycerol) supplemented with 10 g/L glucose and 27 μg/ml chloramphenicol. For anaerobic cultures, rubber-sealed 50-ml serum bottles were used. Air in the headspace and medium were evacuated by nitrogen gas sparging. Overnight culture broths in LB medium were inoculated at a final OD_{600} of 0.05 into 20 mL of fresh medium and incubated anaerobically at 37 °C with shaking (250 rpm) for 24 h.

Table 1
Bacterial strains and plasmids used in this study.

Name	Relevant characteristics	Source
Strains		
Mach1-T1^R	F$^-$ φ80(*lacZ*)ΔM15 Δ*lac*X74 *hsd*R($r_K^-m_K^+$) Δ*rec*A1398 *end*A1 *ton*A	Invitrogen
W3110	F$^-\lambda^-$*rph-1IN*(*rrnD*, *rrnE*)*1*	ATCC 27325
JHL08	W3110 Δ*ato*DA Δ*adhE* Δ*ldh*A Δ*paa*FGH Δ*frd*ABCD P_{atoB}::BBa_J23100	This study
JHL58	JHL08 Δ*pta*	This study
JHL29	JHL08/pACYC-SP	This study
JHL30	JHL08/pBASP	This study
JHL25	JHL58/pACYC-SP	This study
JHL26	JHL58/pBASP	This study
Plasmids		
pKD4	Template plasmid for FRT-flanked kanamycin resistance gene; Amp^R, Km^R	Datsenko and Wanner (2000)
pKD46	Red recombinase expression vector; Amp^R	Datsenko and Wanner (2000)
pCP20	FLP expression vector; Amp^R	Datsenko and Wanner (2000)
pACYCDuet	Expression vector, Cm^R, p15A ori	Novagen
pCDFDuet	Expression vector, Sm^R, cloDF13 ori	Novagen
pGEM T-Easy	Cloning vector, Amp^R	Promega
pFRT2	From pGEM T-Easy, FRT-*Kan*^{R}-FRT(2)	This study
pFRT5	From pGEM T-Easy, FRT-*Kan*^{R}-FRT(5)	This study
pFRT6	From pGEM T-Easy, FRT-*Kan*^{R}-FRT(6)	This study
pFRT7	From pGEM T-Easy, FRT-*Kan*^{R}-FRT(7)	This study
pCDF-SP	cloDF13 ori, Sm^R, P_{J23100}::*crt*-P_{J23100}::*hbd*-P_{J23100}::*ter*	This study
pACYC-SP	p15A ori, Cm^R,P_{J23100}::*crt*-P_{J23100}::*hbd*-P_{J23100}::*ter*	This study
pBASP	p15A ori, Cm^R, P_{J23100}::*crt*-P_{J23100}::*hbd*-P_{J23100}::*ter*-P_{J23100}::*tesB*	This study