Electronic Journal of Biotechnology 18 (2015) 138–142

HOSTED BY

ELSEVIER

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Short communication

Engineering *Escherichia coli* for autoinducible production of n-butanol

Qinglong Wang [a,b], Yi ding [b], Li Liu [b], Jiping Shi [b,c], Junsong Sun [b,c,*], Yongchang Xue [a,*]

[a] School of Biological Engineering, Dalian Polytechnic University, Qinggongyuan 1, Dalian 116034, China
[b] Biorefinery Laboratory, Shanghai Advanced Research Institute, Chinese Academy of Sciences, Haike Rd 99, Shanghai 201210, China
[c] School of Life Science and Technology, ShanghaiTech University, Haide Rd 100, Shanghai, 201210, China

ARTICLE INFO

Article history:
Received 10 October 2014
Accepted 3 December 2014
Available online 29 January 2015

Keywords:
Anaerobic promoter
Escherichia coli
Metabolic engineering
n-Butanol
Recombination

ABSTRACT

Background: Escherichia coli does not produce n-butanol naturally, but can be butanologenic when related enzymes were expressed using inducible elements on plasmids. In this study we attempted to confer *E. coli* strain capability of automatic excretion of the chemical by employing a native anaerobic promoter. Also, a novel DNA kit was designed for PCR preparation of linear DNA fragments to perform strain modification. The kit is primarily composed of two mother vectors, co-transformation of linear DNAs into *E. coli* can simultaneously introduce two butanol synthetic operons into the chromosome and create two in-frame gene deletions at targeted native loci.

Results: E. coli strain Bw2V carries plasmid pCNA-PHC and pENA-TA, both utilizes native anaerobic promoter P_{hya} for the expression of butanol synthetic enzymes. When Bw2V was subjected in anaerobic fermentation using medium containing extra glucose, the accumulated n-butanol in the broth was up to 2.8 g/L in bioreactor; as the genetic element expressing the same pathway was introduced into the genome, the titer of butanol was 1.4 g/L.

Conclusions: The expression system using P_{hya} is effective in applications that involve expression plasmids as also applicable in ectopic expression as single copy on the chromosome. Results imply that P_{hya} can be subjected for broader application in bioproduction of more feedstock chemicals.

1. Introduction

Biobutanol (microbial produced n-butanol) is regarded as one of potential clean biofuels, and has been the renewed focus of bioenergy studies in recent years due to incessantly fermented public concerns on energy crisis and the environmental problems incurred by excessive use of fossil oil. The industrial biobutanol is usually produced by the Clostridia genus through acetone–butanol–ethanol (ABE) fermentation process [1,2].

Novel butanol producing micro-organisms other than Clostridia were in rapid development in anticipation of overcoming the negative aspects of the ABE fermentation technology. Among of them, *Escherichia coli* has become an ideal candidate because of the abundant genetic tools, its rapid growth, and relatively rich knowledge on the regulation of its metabolic network [3]. Unlike Clostridia, *E. coli* is not a native producer of n-butanol, the formation of this chemical would not be accomplished before the heterologous pathway is introduced into host cells; with expression plasmids, *E. coli* was able to accumulate significantly high amount of n-butanol [4]. However, the risk of plasmid loss or copy number reduction is high, bringing cautious concerns in strains' long-term genetic stability. Therefore, chromosomal expression, if possible, is always favored during construction of industrial strains.

In addition, the promoter chosen always played pivotal roles in production of proteins or biochemicals, therefore, utilization of strong promoters in plasmid expression system is frequently reported due to their contribution in increased yields of products [5]. In *E. coli*, strong inducible promoters, such as T7/tac, are specially favored in the laboratory production of proteins for biochemical characterization [6]; however, the use of expensive inducer, such as IPTG is not desirable for industrial practice. In this study, P_{hya}, a native promoter of hydrogenase I cluster, was tested for anaerobic production of metabolites, and the aim of present study is to develop a comprehensive solution to confer *E. coli* to be a potential industrial strain that is able to produce butanol efficiently during anaerobic growth, through renewed genomic technology that enabled rapid genetic modification in host cells' chromosome.

2. Materials and methods

2.1. Bacterial strains, media and grow conditions

Main strains, plasmids and the primers in this study are listed in Table 1. BW25113 (Yale CGSC) and its derivate mutants were made

* Corresponding authors.
E-mail addresses: sunjs@sari.ac.cn (J. Sun), xueych@dlpu.edu.cn (Y. Xue).
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2015.01.003

Table 1
Plasmids, *E. coli* strains and main primers used in this study.

	Genotype	Source
Plasmids		
pKD4	Kan^R	Yale CGSC
pKD46	Amp^R	Yale CGSC
pCP20	Cm^R	Yale CGSC
pCNA-PHC	Cm^R P_{hya}::*phaA-hbd-crt*	This study
pENA-TA	Amp^R P_{hya}::*ter-adhE*1	This study
pCNA-PHCF	Cm^R Kan^R P_{hya}::*phaA-hbd-crt*	This study
pENA-TAF	Amp^R Sm^R P_{hya}::*ter-adhE*1 frt-Sm-frt	This study
Strains		
E. coli BW25113	*Δ(araD-araB)567ΔlacZ4787(::rrnB-3)λ-rph-1Δ(rhaD-rhaB)568hsdR514*	Yale CGSC
Bw2V	BW25113/pENA-TA, pCNA-PHC	This study
BuS1	BW25113/P_{hya}::*phaA-hbd-crt-ter-adhE1* [*adhE ldhA* Kan^R Sm^R]	This study
BuS2	BW25113/P_{hya}::*phaA-hbd-crt-ter-adhE1* [*adhE ldhA*]	

Primer name	Sequence
TA-F	ATGAAACTCGCCGTTTATAGCACAAAACAGTACGACAAGAAGTA CCTGGAGACCACAACGGTTTCCCTCTAGGATCCG
TA-R	CAAGATTAAACCAGTTCGTTCGGGCAGGTTTCGCCTTTTTCCAGA TTGGGTGGTGGTGCTCGAGTTAAGGTTGT
PHC-F	ATGGCTGTTACTAATGTCGCTGAACTTAACGCACTCGTAGAGCGT GTAGAGCGATTGTGTAGGCTGGAGCTGCTTCG
PHC-R	CAAGATTAAACCAGTTCGTTCGGGCAGGTTTCGCCTTTTTCCAGA TTGGGTGGTGGTGCTCGAGTTAAGGTTGT

competent for the transformation of vectors expressing butanol synthetic pathway [7]. Bw2V carries two plasmids for the expression of the pathway; BuS1 is a butanologenic recombinant derived from BW25113, null in *adhE* and *ldhA*, and has the synthetic butanol pathway integrated at those sites with selective markers remained; both drug resistant markers were subsequently removed in strain BuS2 with the aid of helper plasmid pCP20. Luria broth (LB) and Terrfic Broth (TB) with 0.5% glucose were used for growth of *E. coli* cells, ampicillin (100 mg L^{-1}), kanamycin (50 mg L^{-1}), streptomycin (50 mg L^{-1}) and chloramphenicol (50 mg L^{-1}) were added as needed for selection. Anaerobic fermentation in shaking flasks was performed in sealed serum bottles containing 100 or 200 mL of aerobically pregrown cultures, with shaking at 100 rpm at 37°C.

2.2. Construction of plasmids and butanologenic strains

The biosynthetic pathway for n-butanol production in this study is composed of *phaA* (acetoacetyl-CoAthiolase/synthase), *hbd* (3-hydroxybutyryl-CoAdehydrogenase), *crt* (crotonase), *ter* (crotonyl-CoA reductase) and *adhE1* (butanol dehydrogenase) [8,9]. We assembled *phaA*, *hbd* and *ter* as an artificial operon (designated as PHC) through DNA synthesis, *ter* and *adhE1* in the other operon (TA). P_{hya} was cloned into pACYCduet-1 and pETduet-1, generating pCNA and pENA respectively (supplementary file); then as seen in Fig. 1a, PHC/TA were inserted behind P_{hya} in pCNA or pENA to get pCNA-PHC and pENA-TA, both of them were transformed into wild type *E. coli* strain BW25113 to generate Bw2V (information on construction of pCNA-PHC and pENA-TA as well as the sequences of the operons are available in the Supplementary materials). Next, to facilitate genomic modification, we attempted to grant the expression vectors with removable selective markers. One popular choice was *kan*, encoding kanamycin resistance enzyme, which can be obtained through PCR using pKD4 as template, DNA fragment containing *kan* and *frt* sites was inserted into pCNA-PHC, generating pCNA-PHCF (illustrated construction routes are available in the Supplementary materials); to speed up the strain modification process, we planned to put another selective marker into plasmid pENA-TA, therefore we inserted a chemically synthesized DNA fragment containing streptomycin phosphotransferase gene (*sm*) with *frt* into the unique Eco *RI* site on plasmid pENA-TA, resulting in plasmid pENA-TAF (Fig. 1b). pCNA-PHCF and pENA-TAF are working as the templates for the generation of linear DNAs through PCR using primers containing short homologous DNA fragments of 40–50 nucleotides for Red/ET aided recombination at targeted loci [10]. This strategy has been proved to be very efficient in strain modification, it was immediately applied in in-frame deletion of *adhE* and *ldhA*, in order to reduce the production of ethanol and lactic acid during anaerobic growth. As demonstrated in Fig. 1c, linear DNAs of TA-sm-ΔldhA and PHC-kan-ΔadhE were prepared by primer pair TA-F/TA-Rand PHC-F/PHC-R, and used to co-transformed into BW25113 bearing pKD46, the resultant BuS1 was selected on LB agar plates containing both kanamycin and streptomycin, and confirmed as null in *adhE* and *ldhA*; ensuing the introduction of pCP20 into BuS1 removed *kan* and *sm*, finally generated butalogenic strain BuS2, thus, BuS2 owns an intact copy of butanol biosynthetic pathway on its genome by displacing native *adhE* and *ldhA* with the operon PHC and TA, respectively.

2.3. In vivo production of n-butanol

n-Butanol fermentation was performed either through 2-stage flask shaking culturing, or 5-L stirred tank bioreactor (BIOSTAT-A plus Sartorius) with a working volume of 2.5 L. In flask experiments, 4 mL of overnight pre-culture was inoculated into 80 mL of fresh medium with antibiotics, cells were grown at 37°C with shaking at 200 rpm, with the cell density monitored by measuring OD_{600}. When OD_{600} reached 1.0, cells were transferred into 100 mL or 250 mL of serum bottles with stoppers for anaerobic growth, extra glucose (20 g/L) was added into the cultures. In Bioreactor experiments, the broth was inoculated with 200 mL of overnight pre-culture, dissolved oxygen (DO) was maintained above 25% during aerobic stage by raising stirrer speed and using air pump. The cells were switched to anaerobic growth after OD_{600} reached 5.0, anaerobic conditions were created by shutting off the air pump and sealing the outlets of the fermenter with water, pH was adjusted and maintained at 5.5 automatically using 1 M NaOH as base.

2.4. Quantification of n-butanol and other metabolites

The fermented products in the broth including n-butanol were quantified by gas chromatograph (GC, Shimadzu 2010 plus) equipped with an Inert Cap Pure War (30 m × 0.25 mm × 0.25 μm) and a flame-ionized detector. Cell free samples were collected from the cultures by centrifugation at 12,000 × g for 2 min; the supernatant

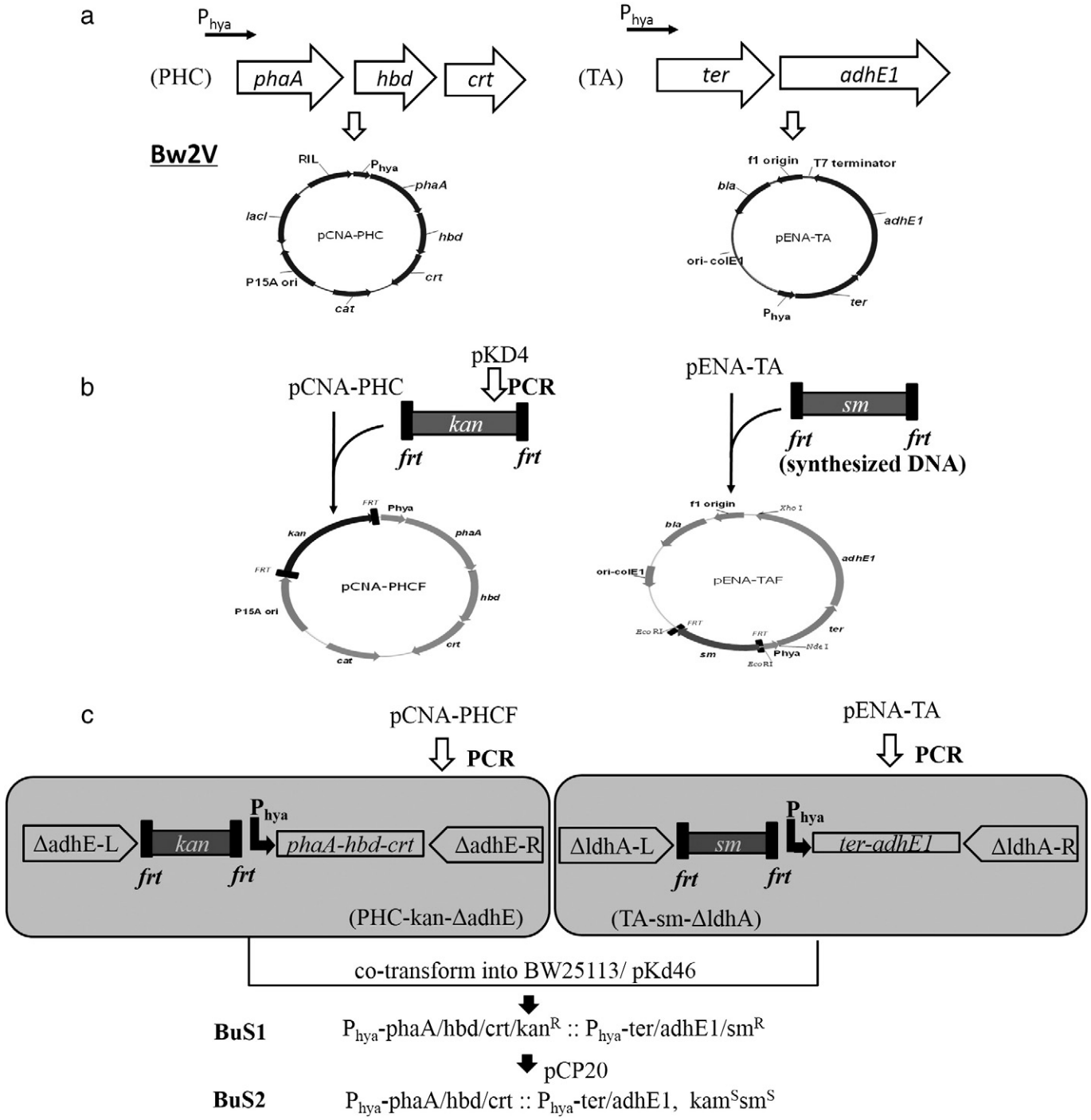

Fig. 1. Construction of plasmids and *E. coli* strains for butanol production. (a) Demonstration of butanol synthetic operons and the plasmids map of pCNA-PHC/pENA-TA, *E. coli* strain Bw2V carries both plasmids; (b) schematic diagram of plasmid construction of pCNA-PHCF and pENA-TAF, both harboring selective marker flanking with *frt* sites; and (c) generation of BuS1/BuS2 by Red/ET aided homologous recombination, PHC-kan-ΔadhE and TA-sm-ΔldhA are linear DNAs generated by PCR using pCNA-PHCF and pENA-TAF as templates.

was then mixed in a 9:1 ratio with an aqueous solution containing isobutanol internal standard (10 g/L); samples were quantified relative to a standard curve of 250, 500, 1000, 2000, 4000, 8000 mg/L n-butanol, standard curves were prepared freshly during each batch run and normalized for injection volume with isobutanol as standard. The oven temperature was held at 50°C for 4 min and subsequently programmed to increase from 50°C to 220°C at the rate of 20°C/min, then held temperature at 220°C for 3 min, the injector was set at 200°C and the detector temperature was set at 230°C.

3. Result

3.1. Formation of n-butanol in E. coli transformants containing expression vectors

To test the capacity of anaerobic promoter P_{hya} for the production of n-butanol, the synthetic butanol pathway (PHC and TA) were put behind P_{hya}, through the construction of expression plasmids pCNA-PHC and pENA-TA. Both of plasmids were transformed into Bw25113, resulting in butanologenic strain Bw2V. The transformant

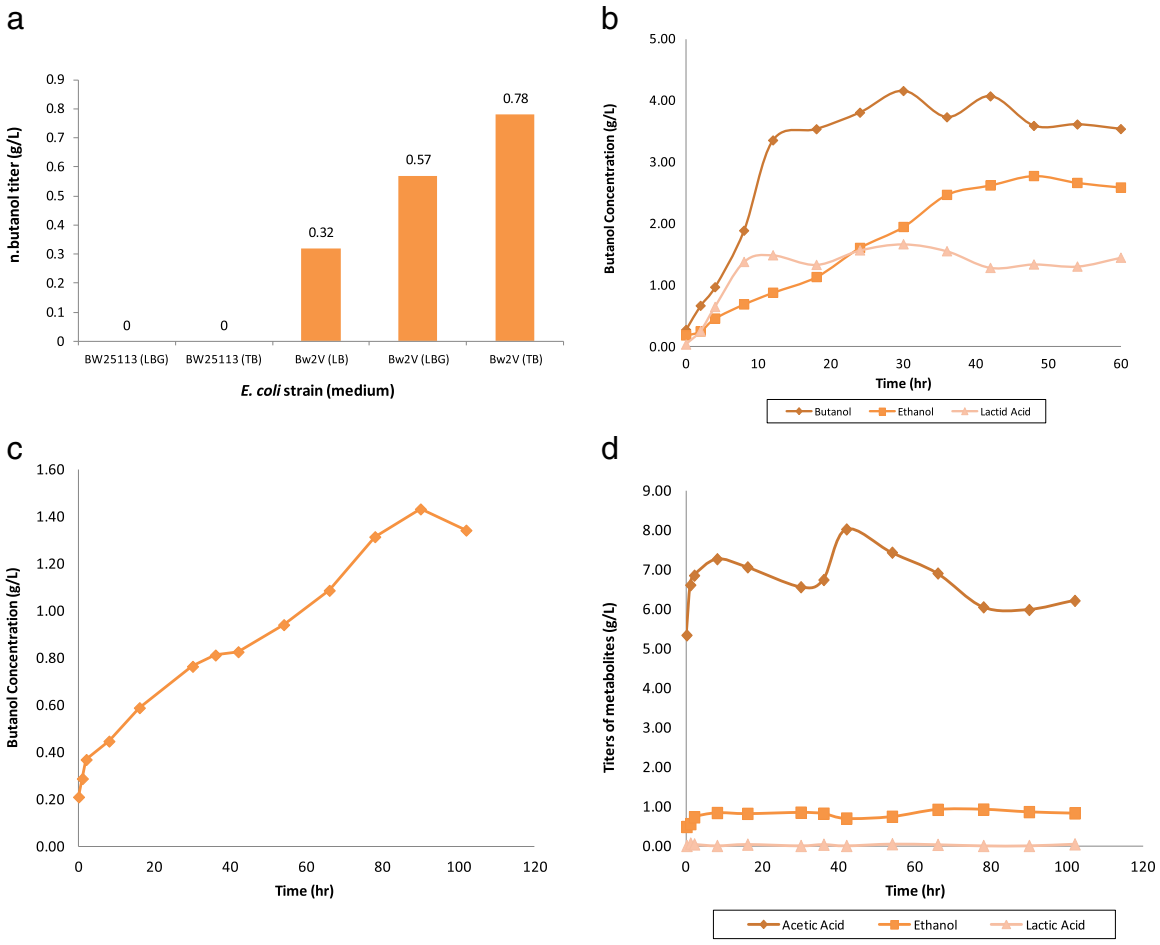

Fig. 2. Production of n-butanol and other metabolites by *E. coli* strains. (a) The levels of n-butanol produced by Bw25113 or Bw2V were detected, and 0.76, 0.58 or 0.32 g/L of accumulated butanol was obtained by Bw2V using TB, LBG or LB, respectively; (b) the titers of n-butanol, ethanol or lactic acid synthesized by Bw2V, were monitored during anaerobic fermentation in 5 L of bioreactor acid, up to 2.77 g/L of n-butanol was accumulated as well as the production of high amount of ethanol and lactic acid; (c) accumulated butanol by BuS2 in fermentation broth; and (d) the titers of acetic acid, ethanol and lactic acid produced by BuS2 during anaerobic growth.

was grown aerobically and switched to anaerobic fermentation in TB, or LB (with or without 20 g/L glucose supplemented). As shown in Fig. 2a, after 24 h of anaerobic incubation in sealed serum bottles, the accumulated butanol from Bw2V was detected to be 0.32 g/L in LB or 0.57 g/L in LBG, the titer further increased to 0.78 g/L in TB with extra glucose. To probe the maximum of the production level, Bw2V was cultured in bioreactor with controlled pH and DO as seen in Fig. 2b, the maximum of 2.77 g/L of butanol was observed in the bioreactor. However, ethanol was also produced unavoidably at a high concentration of 4.2 g/L (Fig. 2b), suggesting that the deletion of native *adhE* would play beneficial role in increasing the productivity of butanol; besides, lactic acids were also excreted and maintained stably at a titer of about 1.5 g/L (Fig. 2b), indicating that the disruption of *E. coli ldhA* should be performed to further activate butanol synthesis.

3.2. Butanol and other metabolites produced by E. coli strain BuS2

To investigate the full capacity of *E. coli* strain BuS2 in butanol production, fermentation in 5 L bioreactor using TB medium was performed with extended incubation time. Optimal density at wavelength of 600 nm (OD_{600}), consumption of glucose, pH and growth temperature were all monitored, the data of related metabolic chemicals synthesized by the cells were collected during after anaerobic switch. As shown in Fig. 2c, n-butanol titer was found to be constantly increasing in newly 100 h, the maximum of accumulated butanol concentration reached 1.43 g/L, which was compatible or greater than the level obtained by other *E. coli* strains expressing polycistronic genes using inducible promoters [11]. As seen in Fig. 2d, the disruption of *adhE* and *ldhA* led to the decreased production of ethanol (<1 g/L) and lactic acid (less than 0.1 g/L), two of the main fermentation products from this *E. coli* host. However, the titer of acetic acid produced by BuS2 was rather high, over 8.0 g/L (Fig. 2d). Overproduction of acetate might explained why the broth pH dropped quickly during anaerobic fermentation, and that will consume lot of base solution if we controlled the broth pH at 6.0 or above, therefore the fermentation pH was maintained at 5.5. Also, it is expected that by shunting the carbon flux from the formation of acetic acid in BuS2, butanol productivity can be further elevated in the future by simultaneous gene deletion of *pta* and *poxB* if the genetic manipulation doesn't disturb the intracellular redox balance.

4. Discussion

In the present work we investigated the possibility of employing P_{hya} as an auto-inducible promoter for the production of n-butanol in engineered *E. coli*. As a growth phase inducible element, P_{hya} has been previously applied in heterologous expression of *Pyrococcus furiosus* hydrogenase [12], but its capacity on transcriptional initiation has never been evaluated upon genomic integration. This study demonstrated that the expression system using P_{hya} is not only effective in applications that involve expression plasmids, but also applicable in ectopic expression as single copy on the chromosome. The insertion of single copy of synthetic pathway driven by P_{hya} was able to convert *E. coli*

model strain BW25113 into n-butanol excreting strain (BuS2), and the accumulated butanol yield reached 1.4 g/L, far exceeding the yields achieved by strains employing promoters other than T7 [11,13], which implies that P_{hya} can be subjected for broader application in the bioproduction of more feedstock chemicals.

To perform rapid engineering in *E. coli*, we linked the optimized gene clusters with necessary elements coming from Red/ET recombination technology, and innovatively constructed a DNA kit composed by two plasmids templates for easy handling of gene deletion and chromosomal insertion in *E. coli*. Butanologenic strain BuS2 was obtained instantly by using this strategy. In comparison with routine process, this route saves time and labor costs. Also, because the generated strains are free of selective marker, the mutants can be used in the next round of genetic engineering for further strain improvement.

It is also worthy to note that, *Clostridium acetobutylicum* AdhE2 was reported to be more efficient in enzymatic conversion during butanol production than AdhE1 when they were expressed in *E. coli* [14], it would then be promising to increase butanol productivity by using *adhE2* in the next generation of DNA kit instead of *adhE1* in the present study.

Financial support

This work was supported by a general program (No. 31270122) from the National Natural Science Foundation of China, and the Scientific Research Foundation for the Returned Overseas Chinese Scholars (State Education Ministry supported program).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ejbt.2015.01.003.

References

[1] Green EM. Fermentative production of butanol — The industrial perspective. Curr Opin Biotechnol 2011;22:337–43. http://dx.doi.org/10.1016/j.copbio.2011.02.004.
[2] Patakova P, Linhova M, Rychtera M, Paulova L, Melzoch K. Novel and neglected issues of acetone–butanol–ethanol (ABE) fermentation by Clostridia: *Clostridium* metabolic diversity, tools for process mapping and continuous fermentation systems. Biotechnol Adv 2013;31:58–67. http://dx.doi.org/10.1016/j.biotechadv.2012.01.010.
[3] Atsumi S, Cann AF, Connor MR, Shen CR, Smith KM, Brynildsen MP, et al. Metabolic engineering of *Escherichia coli* for 1-butanol production. Metab Eng 2008;10:305–11. http://dx.doi.org/10.1016/j.ymben.2007.08.003.
[4] Shen CR, Lan EI, Dekishima Y, Baez A, Cho KM, Liao JC. Driving forces enable high-titer anaerobic 1-butanol synthesis in *Escherichia coli*. Appl Environ Microbiol 2011;77:2905–15. http://dx.doi.org/10.1128/AEM.03034-10.
[5] Baneyx F. Recombinant protein expression in *Escherichia coli*. Curr Opin Biotechnol 1999;10:411–21. http://dx.doi.org/10.1016/S0958-1669(99)00003-8.
[6] Jana S, Deb JK. Strategies for efficient production of heterologous proteins in *Escherichia coli*. Appl Microbiol Biotechnol 2005;67:289–98. http://dx.doi.org/10.1007/s00253-004-1814-0.
[7] Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, et al. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection. Mol Syst Biol 2006;2:1–11. http://dx.doi.org/10.1038/msb4100050.
[8] Bond-Watts BB, Bellerose RJ, Chang MC. Enzyme mechanism as a kinetic control element for designing synthetic biofuel pathways. Nat Chem Biol 2011;7:222–7. http://dx.doi.org/10.1038/nchembio.537.
[9] Tucci S, Martin W. A novel prokaryotic trans-2-enoyl-CoA reductase from the spirochete *Treponema denticola*. FEBS Lett 2007;581:1561–6. http://dx.doi.org/10.1016/j.febslet.2007.03.013.
[10] Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A 2000;97:6640–5. http://dx.doi.org/10.1073/pnas.120163297.
[11] Chen SK, Chin WC, Tsuge K, Huang CC, Li SY. Fermentation approach for enhancing 1-butanol production using engineered butanologenic *Escherichia coli*. Bioresour Technol 2013;145:204–9. http://dx.doi.org/10.1016/j.biortech.2013.01.115.
[12] Sun J, Hopkins RC, Jenney FE, McTernan PM, Adams MWW. Heterologous expression and maturation of an NADP-dependent [NiFe]-hydrogenase: A key enzyme in biofuel production. PLoS ONE 2010;5:e10526. http://dx.doi.org/10.1371/journal.pone.0010526.
[13] Su H, Zhao Y, Zhao H, Wang M, Li Q, Jiang J, et al. Identification and assessment of the effects of yeast decarboxylases expressed in *Escherichia coli* for producing higher alcohols. J Appl Microbiol 2014;117:126–38. http://dx.doi.org/10.1111/jam.12510.
[14] Inui M, Suda M, Kimura S, Yasuda K, Suzuki H, Toda H, et al. Expression of *Clostridium acetobutylicum* butanol synthetic genes in *Escherichia coli*. Appl Microbiol Biotechnol 2008;77:1305–16. http://dx.doi.org/10.1007/s00253-007-1257-5.