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Effects of carbon source and metabolic engineering on butyrate production in *Escherichia coli*

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Abstract−Butyrate was produced in recombinant *Escherichia coli* strains by applying metabolic engineering strategies. The genes for producing butyrate were cloned from *Clostridium acetobutylicum* and then expressed in *E. coli*. To study important factors for improving the productivity of butyrate, we deleted *pta* and *ptsG* genes in *E. coli* and compared the effects of these gene deletions in *E. coli* B and K strains. The effect of carbon sources, glucose and glycerol, was also compared. A significant improvement of butyrate production was made when glycerol was used as a carbon source, resulting in 0.56 g/*l* of butyrate in LB medium with 1% (v/v) glycerol.

Key words: *Escherichia coli*, *Clostridium acetobutylicum*, Butyrate, Metabolic Engineering

INTRODUCTION

Butyrate is an important chemical compound used in various industries including food, pharmaceutical, polymer, biofuel, and so on. It has been used as a food additive to increase fruit flavor [1]. Recently, this compound has gained attention from the biomedical industry for its positive effects on the treatment of colorectal cancer and hemoglobinopathies [2,3]. The derivatives of this compound are used in synthesizing plastic, textile, fiber, etc [4]. Also, a simple chemical process to convert butyric acid to butanol, an important compound as a next-generation biofuel, has been developed so that butyrate production using microorganisms has become a significant research topic in biotechnology industry [1,5].

Butyrate is produced by several anaerobic microorganisms, such as *Clostridium*, *Megasphera*, *Peprococcus*, *etc.* Among them, *C. butyricum*, *C. tyrobutyricum*, and *C. thermobutyricum* have been most widely used for producing butyrate. Genetic modifications for redirecting carbon flux have been applied to *C. tyrobutyricum* for improving butyrate productivity [6-8]. However, since there has not been much genetic and physiological information on these bacteria, metabolic engineering of these bacteria is still very difficult. This hampers further improvement of strain and fermentation process [1,9]. *E. coli*, on the other hand, is the most intensively studied microorganism, and various genetic tools are available [10-13]. Therefore, producing butyrate in *E. coli* can provide interesting information in this regard. Two types of *E. coli* strains, B and K, are being used in laboratories. Both types of strains are known to share 80% genetic similarity. However, slight differences in their metabolic activities have been reported. K type strain has shown active metabolic pathway from pyruvate to acetate, resulting in higher acetate production rate. The high acetate concentration often causes problem in high cell density culture of *E. coli*. Meanwhile, B strain has shown higher carbon fluxes in citric acid cycle and glyoxylate shunt pathway and lower activity in acetyl-CoA to acetate pathway, resulting in lowered acetate production rate [14-17].

In this paper, we engineered *E. coli* B and K type strains for butyrate production. As a part of metabolic engineering, we deleted *pta* and *ptsG* for redirecting carbon flux from acetate to butyrate and for restoration of growth rate and biomass formation, respectively. Also, for carbon source optimization, glucose and glycerol were used. The most significant improvement of butyrate production was observed when glycerol was used as a carbon source.

EXPERIMENTAL SECTION

1. Strains and Plasmids

E. coli BL21 (DE3) and JM109 (DE3) were purchased from Invitrogen and Promega Inc., respectively (Table 1). The genomic DNA was purified from *Clostiridium acetobutylicum* ATCC824 and used as a template to amplify genes involved in butyrate production pathway. Six genes including *thiL* (acetyl-CoA acetyltranserase) and HCB operon containing *hbd* (*β*-hydroxybutyryl-CoA dehydrogenase), *crt* (3-hydroxybutyryl-CoA dehydratase), *bcd* (butyryl-CoA dehydrogenase), *etfA/etfB* (*α*- and *β*-subunits of electron transfer flavoprotein respectively) were PCR amplified from *C. acetobutylicum* and *tesB* (acyl-CoA thioesterase II) was from *E. coli* MG1655 using LA Taq™ (Takara Bio Inc. Japan) and primers listed in Table 2. We used pACYCDuet-1 and pET28a (+) (Novagen, Darmstadt, Germany) for construction of recombinant plasmids. Two genes, *thiL* and *tesB*, were cloned in pACYCDuet-1 and the recombinant was named as pTT. Similarly, HCB operon containing five genes were cloned in pET-28a (+) and the recombinant was named as pHCB (Table 1).

2. Confirmation of Gene Expression

The overexpressions of butyrate producing genes were confirmed with real time RT-PCR. For the experiments, total RNA were purified with RNeasy kit (Qiagen, Valencia, CA) from cultured bacteria with and without IPTG (isopropyl-*β*-D-thiogalactopyranoside). The reverse transcription and PCR was subsequently performed

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Table 1. Strains and plasmids used in this study

Name	Genotype	Reference
Strains		
E. coli		
JM109(DE3)	*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (r_k^-, m_k^+), *rel*A1, *sup*E44, Δ(lac-proAB) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7nin5])	Promega Inc.
BL21(DE3)	F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7nin5])	Invitrogen
SMJ01	BL21(DE3) with pHCB and pTT	This study
SMJ02	JM109(DE3) with pHCB and pTT	This study
SMJ03	As SMJ01 but *Δpta*	This study
SMJ04	As SMJ01 but *ΔptaΔptsG*	This study
SMJ05	As SMJ02 but *Δpta*	This study
SMJ06	As SMJ02 but *ΔptaΔptsG*	This study
Plasmids		
pET28a(+)	F1(pBR322)*ori lacI* T7*lac* Kan^r	Novagen
pACYCDuet-1	P15A *ori lacI* T7*lac* Cm^r	Novagen
pKD4	oriR6k, Km^R, rgnB(Ter)	[18]
pKD46	araBp-gam-bet-exo, bla(Amp^R), repA101(ts), oriR101	[18]
pCP20	Amp^R, Cm^R, FLP recombinance	[18]
pHCB	pET28a(+) harboring *hbd-crt-bcd-etfA/etfB* from *C. acetobutylicum* ATCC824	This study
pTT	pACYCDuet-1 harboring *thiL* from *C. acetobutylicum* ATCC824 and *tesB* from *E. coli*	This study

Table 2. Primer sequences used in this study

Primers	Sequence (5′-3′)
Crt for*	AAGTAGGTCTCGGAATAACAC
Crt rev*	CTTAATGCTTCATCTGCCTTT
Bcd for*	ATAATCGCCGTTGAGGAAT
Bcd rev*	TGTTCTTCTGTACCATGTTCA
etfB for*	ACTGCCGATGATATAGATGTAG
etfB rev*	AATAACTTCTCCCTGTCCTTT
etfA for*	ACAGCATCAGACATAAGAACA
etfA rev*	ACCACCAGCAACTAATACC
Hbd for*	ATATGTCTTGCTATAATGGATGTT
Hbd rev*	ACCTTTTCCTGATTTTCTTCC
thiL for*	TGGGAGATAGTGAATTAGTTGATG
thiL rev*	TTCATCTTGCTCTTCTCTTGTTAT
tesB for*	AATTGAGGAAGGACTCTTTCG
tesB rev*	TTCAGGGACGGTCTCTTT
thiL for Kpn²	AAAAGGTACCATGAGAGATGTAGTAATAGTAAGTGCTGTA
thiL rev Xho²	GAGGGCTCGAGTTATATATAACTATTTAGTCTCTTTCAACTAC
hbd-crt-bcd-etfA-etfB for BamH²	ATTACGCGGGATCCTTAGGAGGATTAGTCATG
hbd-crt-bcd-etfA-etfB rev Pst²	AACTGCAGTAATGGGGATTCTTGTAA
tesB for Nco²	AAAACCATGGATTATATGAGTCAGGCGATAAAAAATTTAC
tesB rev Not²	AGACGCGGCCGCCTATTTTTTTAATTGTGATTACGCATCACC
pta pKD4 F	GGTGCTGTTTTGTAACCCGCCAAATCGGCGGTAACGAAAGAGGATAAACCGTGTAG-GCTGGAGCTGCTTC
pta pKD4 R	TTATTTCCGGTTCAGATATCCGCAGCGCAAAGCTGCGGATGATGACGAGAATATC-CTCCTTAGTTCCTATTCC
ptsG pKD4 F	ACGTAAAAAAAGCACCCATACTCAGGAGCACTCTCAATTGTGTAGGCTGGAGCT-GCTTC
ptsG pKD4 R	AGCCATCTGGCTGCCTTAGTCTTCCCAACGTCTTACGGAATATCCTCCTTAGTTC-CTATTCC

*These primers were used for realtime RT-PCR

using iScript™ cDNA Synthesis kit (Bio-Rad, Hercules, CA) with 1 mg RNA as templates and the primers in Table 2. Expression levels of the genes were calculated from threshold cycle numbers in RT-PCR Mini Opticon (Bio-Rad).

3. Development of Mutant Strains

One-step inactivation method was used for making *pta* and *ptsG* deleted mutants in *E. coli* [18]. Briefly, linear DNAs including kanamycin resistant gene were produced by PCR amplification using primers in Table 2 with pKD4 as a template. Then, the linear DNAs were transformed into *E. coli* strains containing pKD46 after induction using 0.4% L-arabinose. The gene-deleted mutants were selected on LB plate with 50 μg/ml kanamycin. The gene deletions were confirmed with colony PCR using confirmation primer sets. pKD46 was eliminated by heat treatment. Finally, kanamycin-resistant gene was eliminated by transformation of pCP20. The resulting strains were transformed with pHCB and pTT as listed in Table 1.

4. Cultivation Condition

E. coli strains were grown in LB medium (10 g/*l* tryptone, 5 g/*l* yeast extract, 10 g/*l* NaCl) with 34 μg/ml chloramphenicol and 50 μg/ml kanamycin. They were cultured in 25 ml media in 250 mL Erlenmeyer flask at 30 °C under microaerobic condition. The strains were inoculated at OD_{600} 0.05 and 0.1 mM IPTG was added for induction of gene expression after 2 h cultivation.

5. Detection of Metabolites

Butyrate and acetate were detected by Agilent 6890 GC with flame ionization detector (Agilent, Palo Alto, CA) with DB-WAX column (Agilent). He gas was used as a carrier gas. The GC oven temperature was initially controlled at 50 °C for 2 min, then was increased at a rate of 10 °C per min to a final temperature of 250 °C which was held for 2 min. The temperature of the injector was set at 250 °C and that of the detector was set at 280 °C. The injection volume was 1 μl.

RESULTS AND DISCUSSION

1. Recombinant *E. coli* for Butyrate Production

The genes for butyrate production in *C. acetobutylicum* ATCC825, including *thiL* and HCB operon containing *hbd*, *crt*, *bcd*, *etfA* and *etfB*, were cloned under T7 promoter and expressed in *E. coli* containing DE3 in their chromosome. *E. coli* endogenous gene, *tesB*, was cloned and overexpressed in the similar manner. The *tesB* gene encoding thioesterase has been used for direct hydrolysis of butyryl-CoA to butyrate [19,20]. Phosphor-transbutyrylase (*ptb*), butyrate kinase (*buk*) coded by the *ptb-buk* operon in *C. acetobutylicum* has similar function as of *tesB* from *E. coli* MG1655. We also used recombinant plasmids containing *ptb-buk* operon for hydrolysis of butyryl-CoA to butyrate, but the activity was found lower than that of *tesB* (data not shown). Similar result was reported earlier in 3-hydroxybutyrate production in *E. coli* [19] where higher activity of *tesB* than that of *ptb-buk* operon was found in the hydrolysis of both (R)- and (S)-3-hydroxybutyrate. The metabolic functions of the cloned genes are presented in Fig. 1. *E. coli* BL21 (DE3) and JM109 (DE3) were transformed with pTT and pHCB, and the resulting strains were named SMJ01 and SMJ02, respectively (Table 1). The expression of those genes should be induced by adding IPTG. To confirm the expression of the genes, real time RT-PCR was conducted with all seven genes, of which the expression levels were induced 300-1,000 fold by 6 h after adding 0.1 mM IPTG (data not shown). The gene expression level changes were similar in both SMJ01 and SMJ02, proving that the butyrate pathway genes were successfully transcribed.

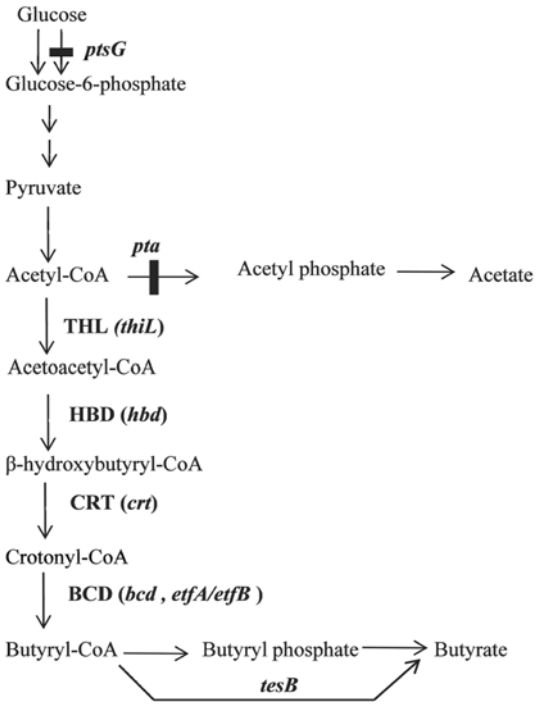

Fig. 1. Metabolic pathways introduced or deleted in this study. Thick bar indicated the deleted pathway. Abbreviations of the genes are *thiL*, acetyl-CoA acetyltranserase; *hbd*, β-hydroxybutyrayl-CoA dehydrogenase; *crt*, 3-hydroxybutyryl-CoA dehydratase; *bcd*, butyryl-CoA dehydrogenase; *etfA/etfB*, α- and β-subunit of electron transfer flavor protein, respectively; *tesB*, acyl-CoA thioesterase II; *pta*, phosphate acetyltransferase; *ptsG*, glucose-specific IIB component of phosphotransferase system.

2. Butyrate Production in Different Carbon Sources

SMJ01 and SMJ02 were grown in LB medium with 2% glucose. The cell mass productivity of SMJ02 was much lower than SMJ01, which resulted by higher acetate production of SMJ02. When acetate was measured without butyrate production, SMJ02 produced 2.0 g/*l* of acetate after 12 h culture, which was twice higher level than SMJ01. The higher acetate production rate indeed hindered the cell growth in SMJ02 [16,21]. We induced butyrate production in those strains to test the effect of acetate productivity on butyrate production. Butyrate production of SMJ02 was 0.13 g/*l* after 12 h cultivation, which was slightly lower than that of SMJ01. This possibly resulted by higher carbon flux towards acetate in SMJ02 (Fig. 2). We also induced butyrate production with LB medium with glycerol (1% v/v) as a major carbon source. With both stains, SMJ01 and SMJ02, above 0.5 g/*l* butyrate was produced and no clear difference between two strains was observed (Fig. 2). We also tested butyrate production in LB medium. Both SMJ01 and SMJ02 strains produced 0.12 g/*l* butyrate, which were similar to the results in LB medium with 2% glucose. The positive effect of glycerol on butyrate production could be caused by slow growth rate, elevated protein folding, reduced carbon state in glycerol, or slow introduction of

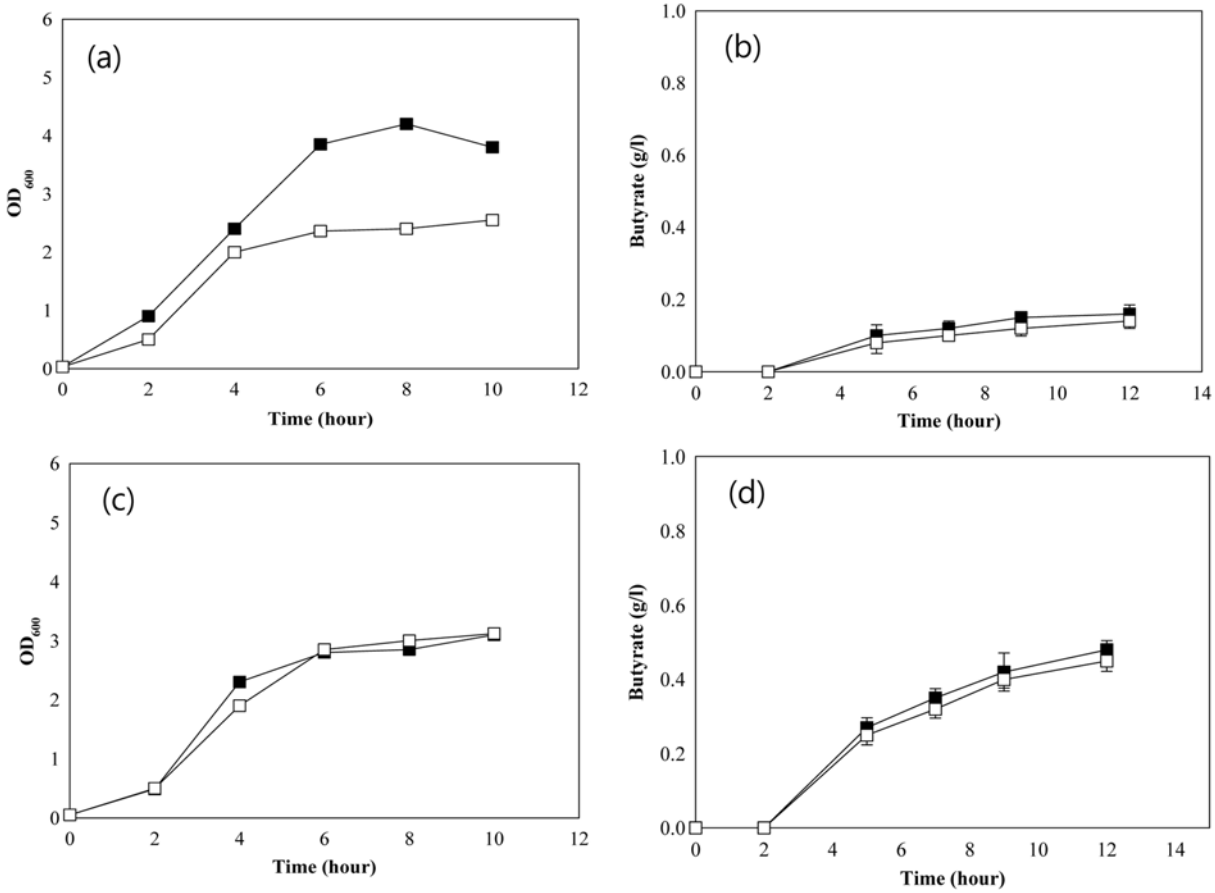

Fig. 2. Growth rates of strains, SMJ01 (■) and SMJ02 (□) (a) and butyrate concentrations in LB medium with 2% glucose (b) and growth rate of strains (c) and butyrate concentrations in LB medium with 1% glycerol (d).

carbon into glycolytic pathway. Among those, there were a few reports about the positive effect of glycerol on protein folding, meaning that improvement of protein folding might be major reason of increased butyrate production [22,23]. However, further studies are needed for proving this hypothesis.

3. Butyrate Production in *pta* and *ptsG* Mutant Strains

E. coli produces acetate during cultivation, which makes negative impact on cell growth and metabolite production [21]. One of the important metabolic pathways to produce acetate is the *Pta-AckA* pathway. It has been reported that deletion of *pta* reduces acetate production, but reduces cell growth too. Also, it has been hypothesized that imbalance of carbon flux between glycolysis and citric acid cycle causes acetate accumulation. This imbalance can be partially overcome by deletion of *ptsG* gene, which encodes protein for glucose uptake in phosphotransferase system. It also reduces conversion rate from PEP to pyruvate, reducing accumulation of pyruvate. It has been reported that deletion of *ptsG* gene can reduce acetate accumulation too [14,24-27].

We hypothesized that the deletions of these genes can redirect carbon flux from acetate to butyrate production. Therefore, Δ*pta* and Δ*pta* Δ*ptsG* mutants were constructed with BL21(DE3) and JM109(DE3), respectively. Since growing strains in LB medium with glycerol has shown higher butyrate productivity, we cultivated those mutant strains on LB glycerol medium. Fig. 3 shows the results of cell growth, acetate and butyrate production of mutants of B type strain. Both SMJ03 and SMJ05 showed lowered cell growth rate and acetate production rate and improved butyrate production rate compared to the wild type, SMJ01. However, the effects were only marginal. We expected bigger impact of *pta* and *ptsG* mutations in K type *E. coli* because K type strain has higher metabolic activities in acetate production pathway. However, the mutants of K type strain showed very similar results in their growth rate and acetate and butyrate production rate to those of B type strain (Fig. 4). These results suggest that *pta* and *ptsG* deletions did not significantly redirect carbon flux from acetate to butyrate.

CONCLUSION

We have constructed recombinant *E. coli* strains for butyrate production. Butyrate pathway genes were amplified from *C. acetobutyricum* and successfully overexpressed under T7 promoter in *E. coli*. We compared B and K types of strains and glucose and glycerol as carbon sources on butyrate production rate. Higher butyrate production was observed with glycerol as a carbon source, and only a little difference was observed with different genetic background. Further engineering was conducted in both types of strains to redirect carbon flux from acetate to butyrate by deleting *pta* and *ptsG* genes. These genetic modifications redirected carbon flux from acetate to butyrate, but their effects were not significant. The most significant effect on butyrate production was caused by glycerol as a

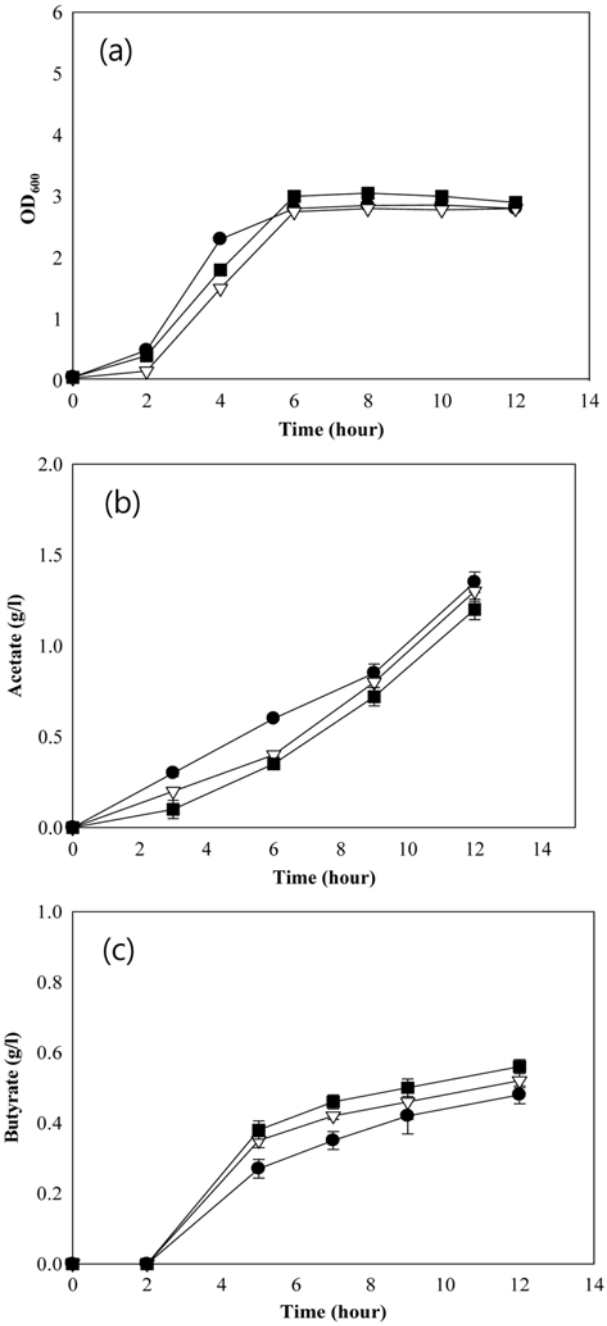

Fig. 3. Growth rates of strains, SMJ01 (●) and SMJ03 (▽) and SMJ05 (■) (a), acetate concentrations (b) and butyrate concentrations (c) in LB medium with 1% (v/v) glycerol.

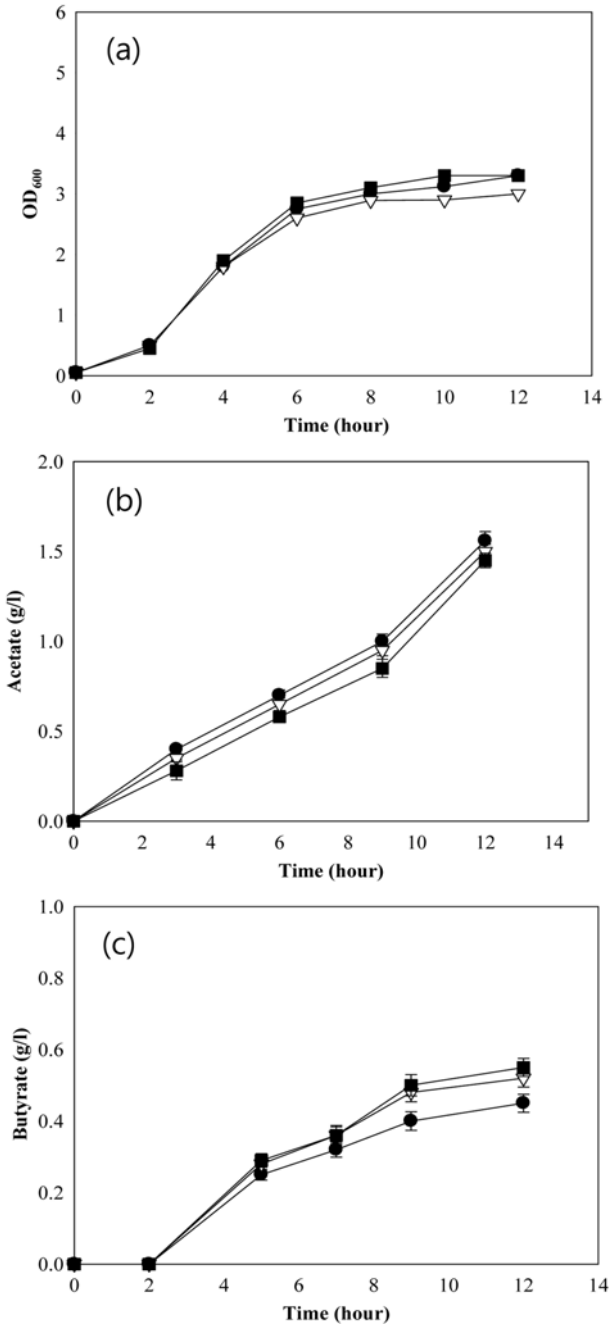

Fig. 4. Growth rates of strains, SMJ02 (●) and SMJ04 (▽) and SMJ06 (■) (a), acetate concentrations (b) and butyrate concentrations (c) in LB medium with 1% (v/v) glycerol.

carbon source, which might be caused by improved protein folding or by slow carbon flux in glycolytic pathway.

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