

Co-Localization of GABA Shunt Enzymes for the Efficient Production of Gamma-Aminobutyric Acid via GABA Shunt Pathway in *Escherichia coli*

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Gamma-aminobutyric acid (GABA) is a non-protein amino acid, which is an important inhibitor of neurotransmission in the human brain. GABA is also used as the precursor of biopolymer Nylon-4 production. In this study, the carbon flux from the tricarboxylic acid cycle was directed to the GABA shunt pathway for the production of GABA from glucose. The GABA shunt enzymes succinate-semialdehyde dehydrogenase (GabD) and GABA aminotransferase (GabT) were co-localized along with the GABA transporter (GadC) by using a synthetic scaffold complex. The co-localized enzyme scaffold complex produced 0.71 g/l of GABA from 10 g/l of glucose. Inactivation of competing metabolic pathways in mutant *E. coli* strains XBM1 and XBM6 increased GABA production 13% to reach 0.80 g/l GABA by the enzymes co-localized and expressed in the mutant strains. The recombinant *E. coli* system developed in this study demonstrated the possibility of the pathway of the GABA shunt as a novel GABA production pathway.

Keywords: Gamma-aminobutyric acid, glucose, co-localization, scaffold complex, recombinant DNA, carbon flux

Introduction

Gamma-aminobutyric acid (GABA) is a non-protein amino acid, which can be used as a monomer in biotechnology industries for the production of a biopolymer, Nylon-4 [1]. GABA also plays a vital role in inhibition of brain nerve transmission, which causes the calm-down effect in nerve activity. GABA intake can help to reduce the blood pressure in people with high blood pressure, by maintaining a stable neurotransmission in the brain. Considering the huge demand in GABA production, intensive studies have been done in efficient microbial GABA production.

GABA can be produced by decarboxylation of glutamate via glutamate decarboxylase, and secreted through GABA transporter (GadC). When the *Lactobacillus plantarum* glutamate decarboxylase was overexpressed in *Lactobacillus sakei* B2-16, 265.3 mM of GABA was produced [2]. It was reported

that 149.1 µg/g DCW of GABA was produced from *Lactobacillus brevis* glutamate decarboxylase expressed in *Bacillus subtilis* [3]. *Bifidobacterium longum* has produced 0.1 g/l of GABA from 30 g/l of monosodium glutamate (MSG) by the overexpression of rice glutamate decarboxylase in *Bifidobacterium longum* [4].

The GABA shunt consists of succinate-semialdehyde dehydrogenase (GabD) and GABA aminotransferase (GabT) enzymes. In general, the GABA shunt acts as a metabolic pathway to direct the synthesized GABA into the TCA cycle [6]. A novel strategy is required to engineer metabolic characteristics of the GABA shunt and use it as the alternative GABA production pathway that is found naturally in the *Escherichia coli* metabolic network. Engineering the GABA shunt pathway will enhance the production of GABA directly from glucose.

Synthetic biology has a tremendous application in next-

generation pathway engineering studies. When the pathway's enzymes are not closely attached with each other, the metabolic flux to the targeted pathway is reduced, which leads to a decrease in production of target metabolites. Study shows that the introduction of a synthetic scaffold complex between the pathway's enzymes can closely co-localize the enzymes that elevate the metabolic flux to targeted pathways and increase the production of target metabolites [7]. Several novel scaffold systems can improve the enzyme co-localization to increase the pathway flux for the efficient production of the metabolite. Thus, several synthetic protein-protein interaction scaffold systems provide a control over metabolic flux, which results in the higher production of metabolites. The yield of glucaric acid was increased 5-fold by the co-expression of glucaric acid pathway enzymes with a synthetic protein scaffold [8]. The employment of a synthetic protein scaffold complex between *E. coli* glutamate decarboxylase and the GABA transporter enhanced the production of GABA to 2.5-fold compared with enzymes without a scaffold complex [5].

Previously, the GABA shunt pathway was engineered for GABA production via succinate dehydrogenase (SdhA), GabD, and GabT [14]. In this study, the novel GABA pathway was engineered as a GABA production system by co-localization of GABA shunt enzymes via synthetic protein scaffold introduction. GabD and GabT were co-

localized with GadC, to direct the metabolic flux from the TCA cycle to the GABA production pathway. Various culture conditions like temperature, pH, and glucose concentration were optimized for efficient production of GABA from glucose via the GABA shunt.

Materials and Methods

Bacterial Strains and Culture Medium

The bacterial strains used in this study are listed in Table 1. Recombinant strains were cultured in Luria-Bertani medium (10 g/l bacto-tryptone, 5 g/l bacto-yeast extract, and 5 g/l sodium chloride) supplemented with 50 µg/ml of ampicillin and/or 30 µg/ml of chloramphenicol [9]. Media were solidified by the addition of 2% agar. The recombinant strains were cultured to an optical density 1.2 of 600 nm (OD₆₀₀) at 37°C. Addition of 1% arabinose and 108 nM anhydrotetracycline to the culture induced the expression of target genes [13].

Plasmid Construction

Plasmids used in this study are listed in Table 1. To construct the scaffold complex, ligands were integrated with corresponding GABA shunt enzymes. The polymerase chain reaction (PCR) was used to amplify the genes *GDB*, *SH3*, and *PZD*, which encode the GDB, SHD, and PZD ligand, respectively. *E. coli* XL1-Blue genomic DNA was used for the amplification of genes *gabD*, *gabT*, and *gadC* by using PCR with an MJ Mini Personal Thermal Cycler (BioRad Laboratories, USA). The *gadC* gene was attached with the *GDB*

Table 1. List of strains and plasmids used in this study.

Strain/plasmid	Description	Reference
Strains		
XL1_Blue	F'(proAB ⁺ lacI ^q lacZΔM15 Tn10 (Tet ^r))	Laboratory stock
XBM1	<i>E. coli</i> XL1_Blue (<i>ΔackA</i> , <i>ldhA</i>)	This work
XBM6	<i>E. coli</i> XL1_Blue (<i>ΔpflB</i> , <i>poxB</i> , <i>ldhA</i>)	This work
Plasmids		
pBAD30	pBAD30, <i>araC</i> promoter, Amp ^r	NEB ^a
pBAD30C	pBAD30, (Amp ^r was replaced with Cm ^r)	This work
pBCDT	pBAD30C, (<i>GadC-GDB</i> , <i>GabD-SH3</i> , <i>GabT-PDZ</i>)	This work
pJD757	P _{tet^r} <i>GDB</i> ₁ <i>SH3</i> ₁ <i>PDZ</i> ₁ , Amp ^r	Moon et al. [8]
pJD758	P _{tet^r} <i>GDB</i> ₁ <i>SH3</i> ₁ <i>PDZ</i> ₂ , Amp ^r	Moon et al. [8]
pJD759	P _{tet^r} <i>GDB</i> ₁ <i>SH3</i> ₁ <i>PDZ</i> ₄ , Amp ^r	Moon et al. [8]
pJD760	P _{tet^r} <i>GDB</i> ₁ <i>SH3</i> ₂ <i>PDZ</i> ₁ , Amp ^r	Moon et al. [8]
pJD761	P _{tet^r} <i>GDB</i> ₁ <i>SH3</i> ₂ <i>PDZ</i> ₂ , Amp ^r	Moon et al. [8]
pJD762	P _{tet^r} <i>GDB</i> ₁ <i>SH3</i> ₄ <i>PDZ</i> ₁ , Amp ^r	Moon et al. [8]
pJD763	P _{tet^r} <i>GDB</i> ₁ <i>SH3</i> ₄ <i>PDZ</i> ₂ , Amp ^r	Moon et al. [8]
pJD764	P _{tet^r} <i>GDB</i> ₁ <i>SH3</i> ₄ <i>PDZ</i> ₂ , Amp ^r	Moon et al. [8]
pJD765	P _{tet^r} <i>GDB</i> ₁ <i>SH3</i> ₄ <i>PDZ</i> ₂ , Amp ^r	Moon et al. [8]

^aNew England Biolabs, Beverly, MA, USA.

Table 2. List of PCR primers used in this study.

Name	Sequence
<i>GadC_SacI_F</i>	GGGG AGCT CAAGAAGGAGATATACCATGGCTACATCAGTACAGACA
<i>GadC_R</i>	CCAGAGCCAGAGCCACGCGGGTGTTCCTTGTTCATTCATCA
<i>GBD_F_L</i>	TGATGAATGACAAGAAACACCCGCGTGGCTCTGGCTCTGG
<i>GDB_KpnI_R</i>	GGGG GTAC CTCAATCTTCATCTTCATCGCCCG
<i>GabD_KpnI_F</i>	GGGG GTAC CAAGAAGGAGATATACCATGAACTTAACGACAG
<i>GabD_XmaI_R</i>	GGG CCCGGGT CAGCCCGACGACGACGTTTCGGCGGCAGCGCCGGCGGGCCAGAGCCAGAGCCAGAGC CAGAGCCTTCCAGAAGACCGATGCACATATA
<i>GabT_XmaI_F</i>	GGG CCCGGG AAGAAGGAGATATACCATGAACAGCAATAAAGAGTT
<i>GabT_XbaI_R</i>	GG GTCTAG ATCATCACACCAGAGATTCCTTTCACGCCAGAGCCCGGCGGCCCTGCTTCGCCTCATCAAAAC
<i>Cmr_SbfI_F</i>	GGG CTGC AGGATGAACTTTAATAAAAATTGAT
<i>Cmr_PvuI_R</i>	GGG CGATC GTATAAAAGCCAGTCATTAG

Restriction enzyme sites are shown in bold.

ligand gene by using overlap PCR. Similarly, through the integration of *SH3* and *PZD* genes with the reverse primer, the ligand genes were attached with the genes *gabD* and *gabT* using overlap PCR. The cloning of all three genes was performed with similar restriction enzymes sites as in the previous study of Pham *et al.* [13]. The genes *gadC-GDB*, *gabD-SH3*, and *gabT-PZD* were cloned in expression plasmid pBAD30C with the restriction sites of *SacI* to *KpnI*, *KpnI* to *XmaI*, and *XmaI* to *XbaI*, respectively. The final expression plasmid of the three genes was considered as pBCDT (Fig. 2A). Table 2 lists the primers used in the construction of plasmid. Table 1 presents the list of nine plasmids containing GBD, SH3, and PDZ protein interaction domains that were kindly provided by Prof. Dueber.

GABA Quantification

Quantitative analysis of the GABA concentration was performed on an OptimaPak C18 column (4.6 × 150 mm; RS Tech Corporation, Daejeon, Korea). An Agilent HPLC system using the detector UV at 286 nm was used to analyze the samples. The sample preparation for GABA analysis and preparation of HPLC buffers were performed as described by Vo *et al.* [18].

Glucose and Metabolite Analysis

An Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad) was used to analyze glucose and other metabolites by HPLC. Sample and buffer preparation for HPLC analysis of glucose and other metabolite was performed as described by Pham *et al.* [13].

Results and Discussion

GABA Pathway Construction

The pathway of the GABA shunt was modified and demonstrated for the novel GABA production system. With the enzymes *GadD* and *GabT*, the succinate can be

directed to GABA, and the produced GABA can be exported through *GadC*. All three enzymes were closely co-localized via a synthetic protein scaffold complex (Fig. 1). The GTPase-binding domain (GBD) is a 78 amino acids long residue from rat protein called Neural Wiskott-Aldrich syndrome protein, while a small part of the cell division control protein 42 is used as the GBD ligand that can interact with the GBD domain. The SH3 domain is the N-terminus domain of the mouse protein c-CrK and SH3 ligand, which contains high proline and arginine residues. Mouse protein α -syntrophin gives rise to the PDZ domain,

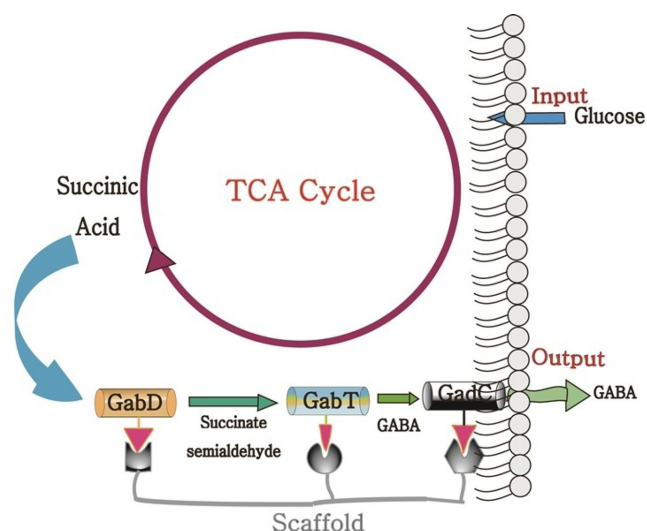


Fig. 1. Schematic diagram of GABA production via co-localization of succinate-semialdehyde dehydrogenase, GABA aminotransferase, and GABA transporter.

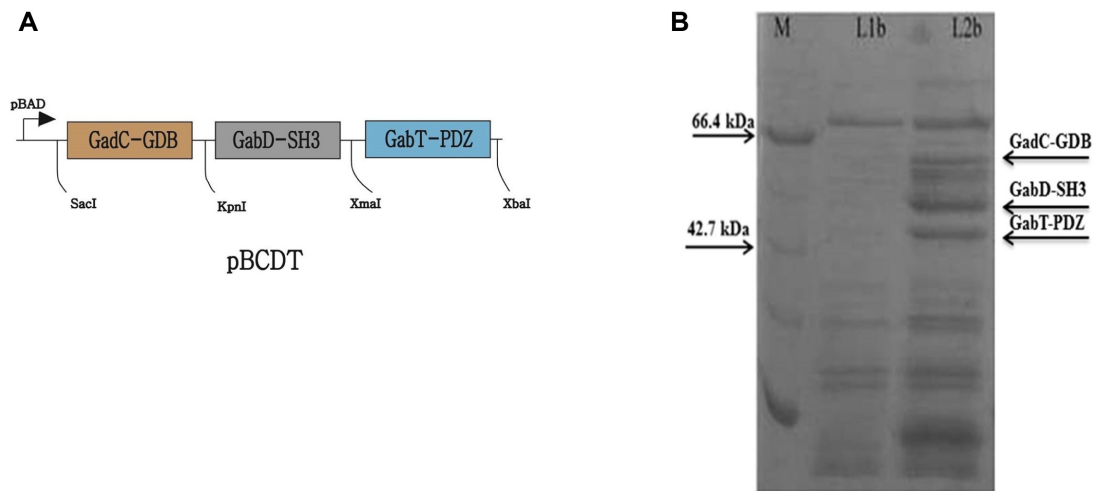


Fig. 2. Scaffold pBCDT for overexpression of GABA-related genes.

(A) Scaffold pBCDT plasmid used in this study. (B) Results of the SDS-PAGE analysis of the overexpression of four genes. M is the marker protein. L1b and L2b are no induction and 0.01% arabinose induction, respectively. (GadC-GDB: 60.27 kDa; GabD-SH3: 54.02 kDa; and GabT-PDZ: 46.86 kDa).

which can bind to transmembrane receptors and ion channels to import other proteins. The scaffold consists of one GBD domain and various numbers of SH3(x) and PDZ(y) domain plasmids that were kindly provided by Prof. Moon and Prof. Dueber [12]. The protein-protein ligands GBD, SH3, and PDZ were integrated with the C-terminus of GadC, GadB, and GabT, respectively. When the proteins and scaffold architecture were expressed together, the protein fused with ligands can specifically bind with its targeted domains. Therefore, the enzymes are closely co-localized in the *E. coli* cytosol region. Succinate can be converted to succinate semialdehyde via GadD, and succinate semialdehyde has the greater chance to interact with GabT and be converted into GABA via the co-localization strategy. Finally, the produced GABA has the higher possibility for secretion due to the closely located GadC. The three enzymes were expressed and analyzed by SDS-PAGE (Fig. 2B).

Initially, no detectable amount of GABA was obtained from enzymes that were not overexpressed. About 0.36 g/l of GABA was produced from 10 g/l of glucose by overexpression of GabD, GabT, and GadC without co-expression of the scaffold architecture. When the enzymes were overexpressed along with the scaffold architecture at the ratio of 1:1:1, the GABA concentration was elevated to 0.71 g/l, which was 1.97-fold greater than GABA synthesized without the scaffold architecture. This result implies that the co-localization of enzymes can direct the flux from the TCA cycle to the GABA shunt pathway. The scaffold complex

can closely co-localize the GABA pathway enzymes and increase the pathway efficiency [8, 10]. The regulation of carbon flux in the GABA shunt pathway was increased by the physical attachment of the enzymes, and successfully produced GABA.

Standardization of GABA Synthesis

GABA production can be affected by various culture conditions, such as temperature, pH, scaffold complex, and glucose. Therefore, optimization of the GABA production conditions is considered as an essential step for the efficient production of GABA. Temperature is an important condition for the GABA production by maintaining the GABA producing enzymes at optimum condition. Its effect on GABA production was investigated at 25°C, 30°C, and 37°C. At 30°C, the best GABA concentration of 0.7 g/l from 10 g/l of initial glucose was obtained. Only about 0.55 g/l of GABA concentration was achieved at 25°C, and at higher temperature of 37°C, 0.63 g/l of GABA was produced. These data showed that 30°C was the suitable temperature for the effective production of GABA (Fig. 3A). This optimum culture temperature was needed for the effective biochemical activity of enzyme in the strains.

The effect of various pH conditions (4.5, 5.5, 6.0, and 6.5) was evaluated in the recombinant strain for the GABA production, under the standard temperature of 30°C. The result shows that at pH 6.0 the recombinant strain can produce a maximum level of 0.64 g/l GABA from 10 g/l of glucose (Fig. 3B). At pH 4.5, only 0.48 g/l of GABA was

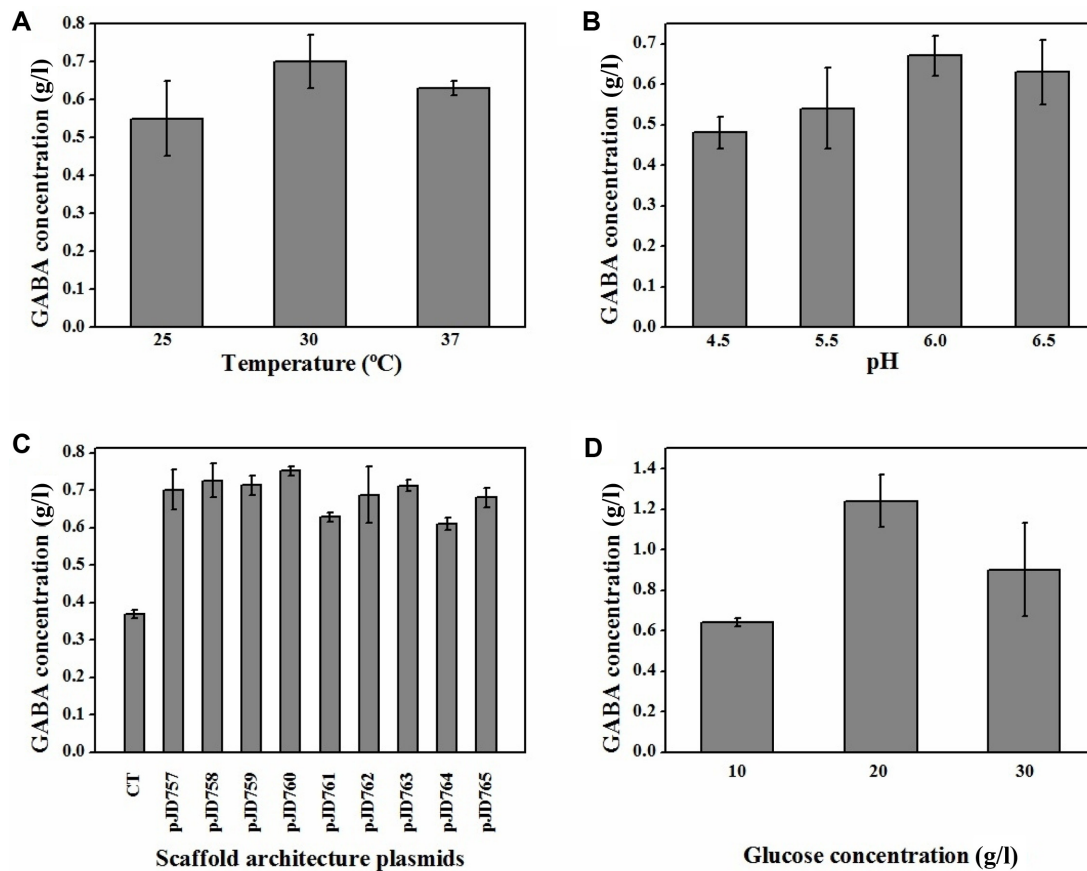


Fig. 3. GABA production under various (A) temperature, (B) pH, (C) scaffold architecture plasmids, and (D) glucose concentration conditions.

detected and 0.54 g/l of GABA was obtained at pH 5.5. However at pH 6.0, the GABA production was increased to 0.64 g/l but it decreased to 0.63 g/l at pH 6.5. It was reported that alkaline pH is the optimum for the efficient activity of the GABA shunt enzymes, succinate-semialdehyde and GABA amino transferase [11, 15]. However, the substrate transport mechanism by GadC was decreased with increasing pH values. For the GABA transporter, an acidic environment is the most suitable for the effective secretion of GABA [16, 17]. The data showed that a neutral pH is suitable for GABA conversion via the GABA shunt pathway.

Previously, it was reported that the final concentration of the product can be improved by altering the ratio of protein-protein scaffold domain through optimization of the enzyme titer for sequential reaction [8]. Each scaffold complex plasmid, such as pJD758 to pJD765, were transformed into *E. coli* XB strain along with pBCDT plasmid to make recombinant strains. The control strain (CT), which consists

of pBCDT plasmid without scaffold architecture plasmids, was also cultured and tested for GABA production. Among the tested recombinant strains, recombinant strains with the scaffold complex produced higher GABA than recombinant strain without the scaffold complex. There was no significant variation in the GABA concentration, which suggests that the GABA production was no longer affected by the ratio of enzymes in the scaffolds (Fig. 3C).

Meanwhile, the glucose concentration was investigated for the GABA production by using different concentrations of glucose (Fig. 3D). After culture, 0.64 g/l of GABA was produced from 10 g/l of glucose. Similarly, 1.24 g/l of final GABA concentration was produced from 20 g/l of glucose in *E. coli* XB. When the glucose concentration was increased to 30 g/l, the production of GABA decreased. Studies show that the optimum concentration of glucose for GABA production can vary depending on the strains [19, 20]. The final GABA concentration is an important factor as well as GABA yield. This result suggested a higher GABA

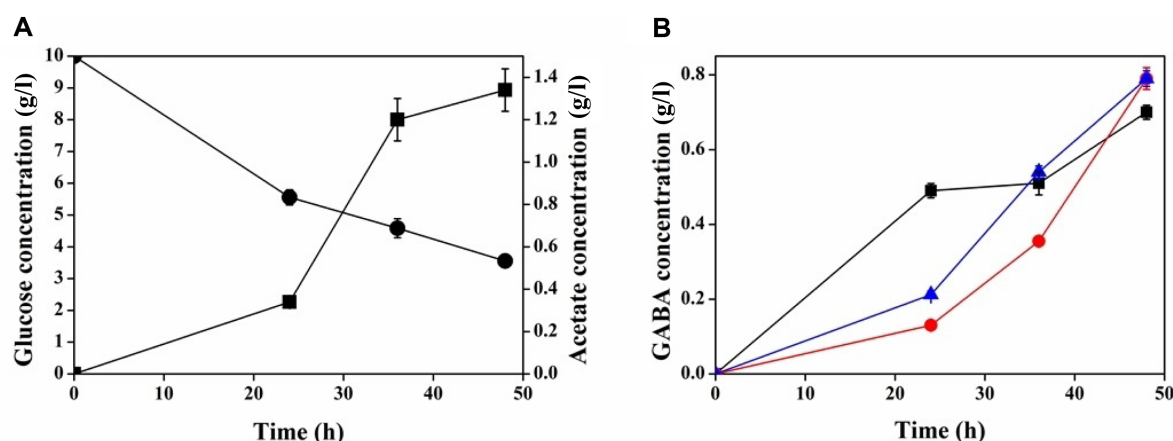


Fig. 4. Time profiles of glucose, acetate, and GABA concentrations.

(A) Time profiles of glucose (filled circle) and acetate (filled square) concentrations. (B) GABA concentration obtained in various strains: XB (filled square), XBM1 (filled circle), and XBM6 (filled triangle).

concentration was obtained by using 20 g/l of glucose without the reduction of GABA production. Thus, the higher GABA concentration was obtained from 20 g/l of glucose. However, based on the GABA/glucose yield, 10 g/l of glucose is advised for efficient GABA production.

Metabolic Pathway Engineering

The effect of a synthetic complex on *E. coli* metabolic pathways was studied by monitoring the metabolites, glucose and acetate. Glucose and other metabolites were analyzed by HPLC from the culture of recombinant strain. The production of GABA from glucose decreased in the substrate concentration of glucose from 10 g/l to 3.55 g/l. Meanwhile, the acetate concentration was increased to 1.34 g/l (Fig. 4A). Analysis of the metabolites suggests that the flux transported to the acetate production pathway.

To further increase the total GABA production, engineering of the GABA shunt was required. Inactivation of the enzymes in competing metabolic pathways can lead to more flux being directed to the targeted pathway. The changes on competing pathways were evaluated by using various mutant strains. In the XBM1 strain, the genes for acetate kinase and lactate dehydrogenase were inactivated. In XBM6, the genes for pyruvate formate lyase I, pyruvate dehydrogenase, and lactate dehydrogenase were inactivated. In these strains, various competing pathways from carbon

flux to the production of other metabolites, such as acetate, lactate, and formate, were inactivated (Table 1).

The pBCDT and pJD757 plasmids were transformed into *E. coli* XB and the two mutant strains, and the GABA concentrations were monitored. The mutated strain XBM1 ($\Delta ackA$, $ldhA$) produced as high as 0.80 g/l of GABA, which was 13% higher than the *E. coli* XL-1 Blue strain. A GABA concentration of 0.80 g/l was also achieved from XBM6 ($\Delta pflB$, $poxB$, $ldhA$) mutant strain (Fig. 4B). In a previous study of GABA production from succinate dehydrogenase, 0.75 g/l of GABA was obtained in the mutant strains [14]. The inactivation of competing metabolic pathways can provide a higher GABA flux and GABA concentration. The final metabolic products, such as GABA concentration, glucose, and acetate, obtained from the recombinant strains after 48 h of conversion are presented in Table 3.

Increasing demand for GABA has led to intensive studies on the development of efficient and economic GABA production processes. Until now, most of the research has been studied on the bioconversion of GABA from MSG by using various glutamate decarboxylases. In this study, a new pathway system was tested for the direct GABA production from sugar by employing a synthetic protein scaffold. By introduction of the protein scaffold with succinate-semialdehyde dehydrogenase, GABA aminotransferase, and GABA transporter to be physically co-localized, the

Table 3. Final metabolites concentration (g/l) obtained after 48 h of conversion.

	GABA (g/l)			Glucose (g/l)	Acetate (g/l)
	XB	XBM1	XBM6		
	0.7 ± 0.019	0.79 ± 0.029	0.79 ± 0.021	3.55 ± 0.2	1.34 ± 0.1

carbon flux was redirected from the TCA cycle to the GABA shunt pathway. It was noticeable that GABA was produced efficiently via the GABA shunt pathway by the recombinant strains.

Acknowledgments

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