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Enchancement of Gamma-Aminobutyric Acid Production by Co-Localization of *Neurospora crassa* OR74A Glutamate Decarboxylase with *Escherichia coli* GABA Transporter Via Synthetic Scaffold Complex

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Gamma-aminobutyric acid is a precursor of nylon-4, which is a promising heat-resistant biopolymer. GABA can be produced from the decarboxylation of glutamate by glutamate decarboxylase. In this study, a synthetic scaffold complex strategy was employed involving the *Neurospora crassa* glutamate decarboxylase (GadB) and *Escherichia coli* GABA antiporter (GadC) to improve GABA production. To construct the complex, the SH3 domain was attached to the *N. crassa* GadB, and the SH3 ligand was attached to the N-terminus, middle, and C-terminus of *E. coli* GadC. In the C-terminus model, 5.8 g/l of GABA concentration was obtained from 10 g/l glutamate. When a competing pathway engineered strain was used, the final GABA concentration was further increased to 5.94 g/l, which corresponds to 97.5% of GABA yield. With the introduction of the scaffold complex, the GABA productivity increased by 2.9 folds during the initial culture period.

Keywords: Gamma-aminobutyric acid, glutamate decarboxylase, glutamate/GABA antiporter, *Neurospora crassa*, synthetic protein scaffold

Introduction

Considering the limited availability of non-renewable resources like coal and fossil fuels, it is necessary to find a potential alternative resource from biomass that can replace fossil fuel-based chemicals. To replace polyethylene-based plastics, several biobased polymers have been studied intensively. One such, polyamide 4, also known as nylon-4, is a biobased biodegradable polymer composed of repeated units of gamma-aminobutyric acid (GABA). GABA is a non-protein amino acid and is an important inhibitor of neurotransmission in the brain [1, 2]. GABA is actively studied for its potential use as a precursor of 2-pyrrolidone, which is used as a monomer to synthesize nylon 4 [3].

In *Escherichia coli* and many other organisms, GABA is a native metabolic intermediate. GABA is produced from the GABA pathway, in which glutamate decarboxylase (GadB) catalyzes the decarboxylation of L-glutamate to GABA. The GABA antiporter (GadC) exports the GABA into the extracellular medium and brings the glutamate into the cells [4].

Owing to its industrial importance, various glutamate decarboxylases have been studied to enhance GABA production. The *Lactobacillus brevis* glutamate decarboxylase was overexpressed in *Bacillus subtilis* to produce 0.4 g/l of GABA from 30 g/l of monosodium glutamate (MSG) [5]. By overexpression of rice glutamate decarboxylase in *Bifidobacterium longum*, 0.1 g/l of GABA was obtained from 30 g/l of MSG [6]. When the glutamate decarboxylase and GABA antiporter were co-overexpressed in the *E. coli*

strain, the GABA obtained was 5.46 g/l from 10 g/l of MSG [7].

The synthetic scaffold complex is a novel tool to engineer the efficiency of metabolic pathways. The scaffold complex can closely co-localize pathway enzymes using protein-protein interaction domains and ligands. Since the pathway enzymes are closely co-localized, the reactant from the pathways has a higher chance of being converted into the targeted metabolic product. Likewise, the metabolic flux through the pathway enzymes will increase, due to the formation of a scaffold complex. The introduction of the synthetic scaffold complex on D-glucaric acid production results in a 5-fold increase over the non-scaffold control [8].

Neurospora crassa is an ascomycete, a red bread mold. Glutamate decarboxylase from *N. crassa* is a monomeric protein and is considered to be useful for industrial application because of its ease of purification [9]. The genome of *N. crassa* has been completely sequenced and reported [10]. In our previous study, the expression of *N. crassa* GadB in *E. coli* yielded a final GABA concentration of 5.26 g/l from 10 g/l of MSG [11]. In this study, *N. crassa* GadB and *E. coli* GadC were co-localized in *E. coli* via three different synthetic scaffold strategies to improve GABA production. The culture conditions, such as temperature, pH, and glutamate concentration, were optimized.

Materials and Methods

Bacterial Strains, Plasmids, and Medium

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* XL1-Blue (XB) was used as the host strain. pGEMT vector (Promega, USA) was used for DNA cloning and pMAL-p4X (New England Biolabs, USA) was used to construct the expression plasmids. Chemically competent cells were prepared using a standard procedure and were stored at −80°C until needed. *E. coli* strains were cultured at 37°C and 250 rpm in Luria-Bertani (LB) broth (10 g/l bacto-tryptone, 5 g/l bacto-yeast extract, and 5 g/l NaCl) supplemented with 100 μg/ml ampicillin sodium salt for the selection of transformants harboring recombinant plasmids [12].

Cloning of *gad* Genes

The *N. crassa gadB* and *E. coli gadC* genes were amplified using the Expand high-fidelity polymerase chain reaction (PCR) system (Roche Molecular Biochemicals, Germany) from the genomic DNA of *N. crassa* and *E. coli*, respectively. The SH3 domain and SH3 ligand genes were PCR amplified from the pJD758 plasmid, which was kindly provided by Professor John E Dueber [8, 13]. Then, the SH3 domain gene was fused to the C-terminus of *gadB* by overlap PCR to make *gadB-SH3D*. The SH3 ligand was attached to the N-terminus, C-terminus, and 233rd amino acid residue of the *E. coli gadC* gene to make *gadCN-SH3L*, *gadCC-SH3L*, and *gadCS-SH3L*, respectively. The PCR primers used in this study are listed in Table 2. The PCR products were purified using a GENEALL PCR purification kit (General Biosystem, Korea) and cloned into the pGEMT vector. The *N. crassa gadB-SH3D* genes were cloned into pMAL-p4X using NdeI and BamHI restriction sites to construct the pHBN plasmids. The *E. coli gadCN-SH3L* gene was cloned downstream of the *gadB-SH3D* genes using BamHI and XbaI restriction sites to construct pH1BN expression plasmids. The *E. coli gadCS-SH3L* gene was cloned into pHBD using BamHI and XbaI restriction sites to construct the pH2BN expression plasmids. The *E. coli gadCC-SH3L* gene was cloned into pHBD using BamHI and XbaI restriction sites to construct pH3BN expression plasmids.

Expression of *gadB* and *gadC* Genes

Recombinant *E. coli* strains (XB) containing heterologous plasmids were cultivated in a 250 ml flask in 100 ml of LB at 37°C and 250 rpm. When the OD_{600} reached 1.2, gene expression was

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

Strain/plasmid	Description	Reference
Strains		
XL1-Blue	SupE44 hsdR17 recA1 endA1 gyrA96 thi relA1 lacF′(proAB$^+$lacI^{q}lacZΔM15 Tn10 (tet^R))	Laboratory stock
XBT	XL1-Blue Δ*gabT*	This work
Plasmid		
pGEMT	Ap^R	Promega (USA)
pMAL-p4X	Ap^R	NEB[a]
pHBN	pMAL-p4X containing *N. crassa gadB* and *E. coli gadC*	This work
pH1BN	pMAL-p4X containing *N. crassa gadB-SH3D* and *E. coli gadC-SH3L*, N-terminal model	This work
pH2BN	pMAL-p4X containing *N. crassa gadB-SH3D* and *E. coli gadC-SH3L*, Sandwich model	This work
pH3BN	pMAL-p4X containing *N. crassa gadB-SH3D* and *E. coli gadC-SH3L*, C-terminal model	This work

[a]New England Biolabs, USA.

Table 2. Primers used for PCR in this study.

Primer	Sequence	Gene
Forward	5′-CATATGATGGTGCATCTTACTATCAT-3′	*gadB*
Reverse	5′-GGGCCCGCACATACTCTGCGCTGCCGCCGCTGCCGCTGCCGCTGCCAC ATATAGAGTGAGTTTTCCCGGTT-3′	
Forward	5′-GGATCCATGGCTACATCAGTACAGA-3′	*gadC*
Reverse	5′-GCTCTAGATTAGTGTTTCTTGTCATTCATC-3′	
Forward	5′-CAGAGTATGTGCGGGCCC-3′	SH3 domain
Reverse	5′-CGCGGATCCTTAATACTTCTCCACGTAAGG-3′	
Forward	5′-CGGGATCCTTCACCAACAAGGACCATAGCATGCTGGAAGGCTCTGGCT-3′	SH3 ligand
Reverse	5′-TCTGTACTGATGTAGCCATGCCGCTGCCGCTGCCGCCGCCGCTGCCTTC CAGGCCCGGACGACGACGT-3′	

induced with 0.5 mM IPTG for 18 h at 30ºC. Then, the cells were pelleted and broken open by sonication (30 sec, 1 min pause, repeated 3 times). The supernatant fraction was obtained by centrifugation at 12,000 ×*g* for 30 min at 4ºC and it was used for SDS-PAGE analysis.

GABA Bioconversion and Analysis

Recombinant *E. coli* XB strains harboring pHBN, pH1BN, pH2BN and pH3BN plasmids and recombinant *E. coli* XBT strain harboring pH1BP were cultivated in 250 ml flasks with 100 ml of LB (100 µg/ml ampicillin) containing 10 g/l MSG at 37°C and 250 rpm. When the OD_{600} reached 1.2, gene expression was induced with 0.5 mM IPTG. Then, the pH was adjusted to 3.5 and the strain was incubated at 30°C and 250 rpm for 48 h.

The GABA bioconversions were quantitatively analyzed by HPLC using an OptimaPak C18 column (4.6 × 150 mm) (RS Tech Corporation, Korea). The sample preparation was performed as follows. Samples (1 ml) were taken from the culture at every 12 h to analyze the GABA production and samples were centrifuged at 12,000 rpm for 5 min. Then, 100 µl of the supernatant was added to an Eppendorf tube. Next, 200 µl of a 1 M sodium bicarbonate buffer at a pH of 9.8, 100 µl of 80 g/l dansyl chloride in acetonitrile, and 600 µl of double-distilled water were added to make a 1 ml reaction mixture. The mixture was then incubated at 80°C for 40 min. Subsequently, 100 µl of 20 µl/ml acetic acid was added to stop the reaction. The mixture was then centrifuged at 12,000 rpm for 5 min. Next, the supernatant was filtered through a 0.22 µm Millipore filter and analyzed by HPLC on an Agilent system using UV detection. Separation of the derivatized samples was performed using a binary non-linear gradient with eluant A (tetrahydrofuran/methanol/50 mM sodium acetate with a pH of 6.2 (5:75:420, by volume)) and eluant B (methanol). The column temperature was set at 30°C and the elution conditions were as follows: equilibration (6 min, 20% B), gradient (20 min, 20–80% B), and cleaning (3 min, 100% B). The flow rate of the mobile phase was 1 ml/min and the samples were detected at a wavelength of 286 nm in the UV region [15]. The standard curve for GABA was generated from 10 standard solutions (1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 g/l GABA) (Sigma, USA) using the same procedure.

Results

Optimization of GABA Production Conditions

Recombinant *E. coli* strains containing *N. crassa* GadB and *E. coli* GadC were cultured at various temperatures and pH values for the optimization of effective GABA production conditions. Initially, the recombinant strain was cultured at different temperatures of 25°C, 30°C, and 37°C. At 30°C, the highest GABA concentration of 4.97 g/l was obtained from 10 g/l of MSG. At a temperature of 25°C, 3.33 g/l of GABA was obtained, and 37°C produced 4.74 g/l of GABA (Fig. 1A). Similarly, the recombinant *E. coli* strain was cultured at 30°C in various pH conditions such as pH 2, 3, 4, and 5. Various concentrations of GABA were obtained from the recombinant strains at different pH conditions. Among the different pH conditions, pH 3 produced the highest GABA concentration, where 4.81 g/l was obtained from 10 g/l of MSG. Hence, pH 3 with a temperature of 30°C was considered to be the optimum condition for higher production of GABA (Fig. 1B). The recombinant *E. coli* cells were cultured without the supplementation of MSG and only a negligible amount of GABA was detected.

Construction of Synthetic Scaffold Complex

Generally, for the effective conversion of MSG to GABA, MSG needs to be imported into the *E. coli* cytosol by the GABA/glutamate antiporter (GadC) and converted to GABA by glutamate decarboxylase (GadB), which is widely distributed in the cytosol. The converted GABA is then secreted via GadC. To make this pathway more efficient, *N. crassa* GadB was physically connected with *E. coli* GadC. Through this method, GABA can be produced more efficiently from the *E. coli* owing to the close co-localization of GadB and GadC enzymes using a scaffold complex (Fig. 2).

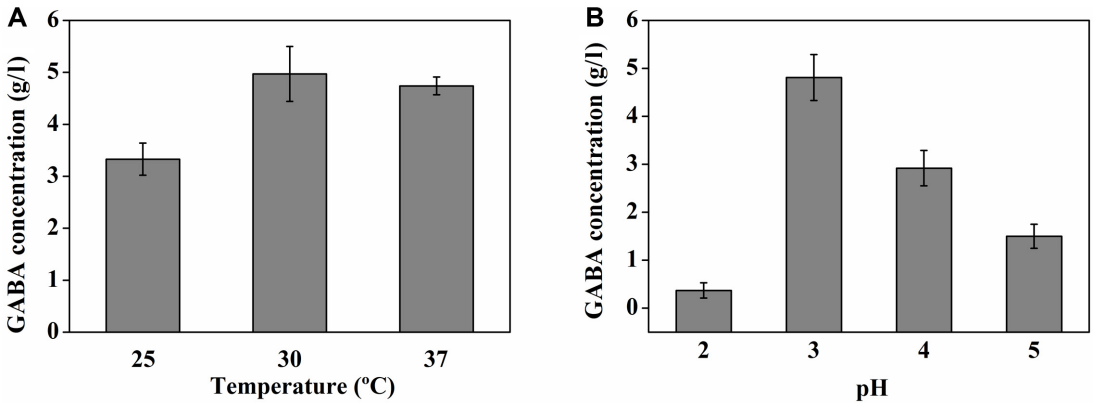

Fig. 1. Optimization of GABA production in various conditions: (**A**) temperature and (**B**) pH.

The synthetic scaffold complex was introduced between the glutamate decarboxylase and GABA transporter to increase the total GABA production. In the synthetic scaffold complex, the protein-protein interaction SH3 domain was attached to the C-terminus of *N. crassa* glutamate decarboxylase (GadB) via a glycine-serine linker [2]. The SH3 ligand was attached to the N-terminus, middle, and C-terminus of *E. coli* GadC via a glycine-serine linker. The constructed recombinant scaffold plasmid was overexpressed by the addition of IPTG in the control of the *tac* promoter for 8 h of culture and then analyzed by SDS-PAGE (Fig. 3).

GABA Production with Scaffold Complex

It was previously reported that protein-protein interaction between the domain and ligand can increase the total carbon influx towards the target pathway, resulting in the improvement of targeted metabolites production [14]. In this study, the employment of a scaffold complex on GadB and GadC induced close co-localization of the enzymes. The recombinant *E. coli* strain containing the synthetic scaffold complex was cultured to increase the production of GABA. Initially, the overexpression of recombinant cells without the scaffold complex (pHBN) resulted in the production of 4.9 g/l of GABA from 10 g/l of MSG. The N-terminus scaffold model of GadC with GadB (pH1BN) produced 2.2 g/l GABA after the first 12 h of culture time. After 48 h of culture time, the amount increased to 5.4 g/l GABA from 10 g/l MSG. Similarly, with the introduction of

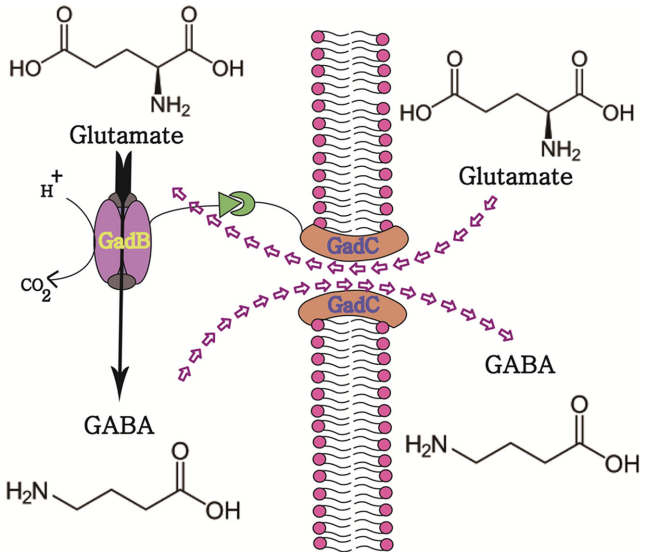

Fig. 2. Schematic diagram of the GadB and GadC scaffold system.

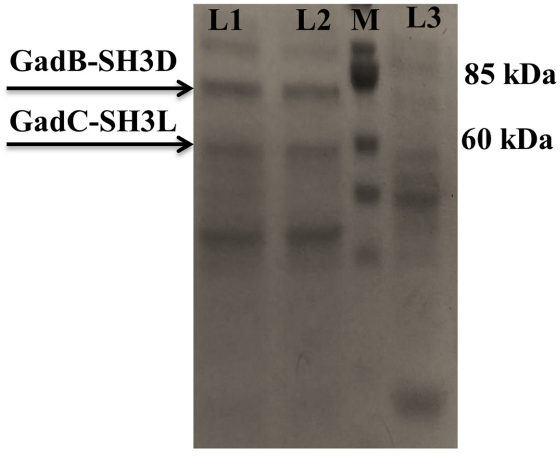

Fig. 3. SDS-PAGE analysis of overexpression of *N. crassa* GadB and *E. coli* GadC.

M is the marker protein. L1 indicates 0.5 mM IPTG induction. L2 indicates 0.1 mM IPTG induction (GadB-SH3D: 65.4 kDa; GadC-SH3L: 60 kDa). L3 indicates untransformed *E. coli* strain.

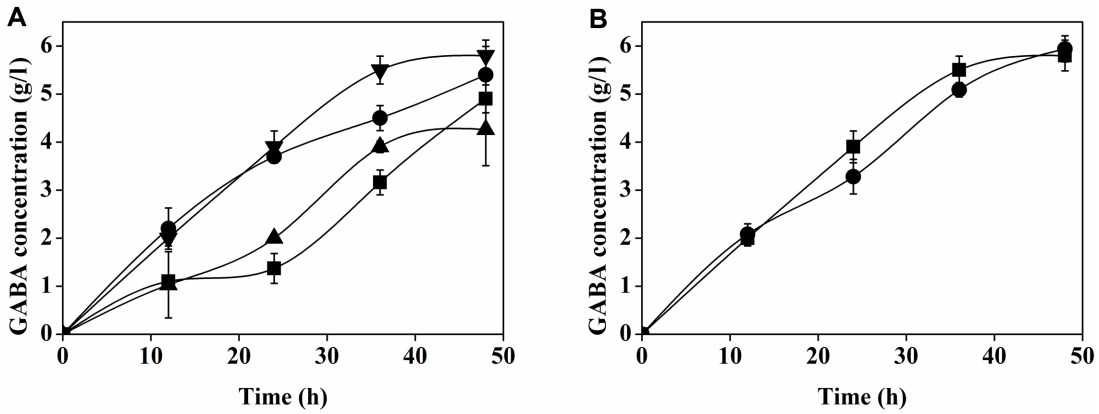

Fig. 4. Final GABA production from various plasmids.
(**A**) Time profile of GABA concentration by various scaffold plasmids. pHBN: without scaffold complex (filled square); pH1BN: N-terminus scaffold complex (filled circle); pH2BN: Sandwich scaffold complex (filled up-pointed triangle); pH3BN: C-terminus scaffold complex (filled down-pointed triangle). (**B**) Time profile of GABA concentration obtained from *E. coli* XL1-blue (filled square) and XBT strains (filled circle).

the sandwich model GadC to GadB scaffold (pH2BN), 4.2 g/l of GABA was produced from 10 g/l MSG. The maximum GABA concentration of 5.8 g/l was obtained with the introduction of the C-terminus synthetic complex to GadC and GadB (pH3BN) (Fig. 4A).

The effect of the synthetic scaffold complex on the production of GABA was studied by analyzing the time profile of GABA production. Based on GABA profiling, the synthetic complex of GadB and GadC provided higher GABA concentration than the strain obtained without the synthetic scaffold complex. This suggests that the scaffold complex between GadB and GadC helped to improve the GABA production from *E. coli*. During the first 12 h of culture, the pH1BN plasmid complex produced 2.2 g/l GABA, whereas the pHBN plasmid produced only 1.1 g/l GABA from 10 g/l MSG. The synthetic scaffold complex provided higher GABA conversion in a shorter period with higher GABA yield. Overall, among the plasmids, the pH3BN C-terminus synthetic scaffold complex produced the highest GABA concentration of 5.8 g/l from 48 h of culture with a GABA yield of 96.6%.

GABA Production by Pathway-Engineered Strain

The produced GABA from the *E. coli* has a chance of being redirected into the TCA cycle by the enzyme GABA aminotransferase (GabT). The effect of the GabT enzyme on GABA production was evaluated by the mutant *E. coli* strain (XBT) harboring pH3BN plasmid, in which the GabT gene was knocked out. The time profile of GABA production from the pH3BN plasmid was analyzed between the XB and XBT strains. The results indicated that the *E. coli* XBT strain containing the pH3BN plasmid produced the highest GABA concentration of 5.94 g/l from 10 g/l of MSG (Fig. 4B). The synthesized GABA was prevented from being redirected back into the TCA cycle by the mutation in *E. coli*. Hence, the recombinant *E. coli* XBT strain produced 97.5% of GABA yield.

Discussion

The GABA pathway consists of the enzyme glutamate decarboxylase, which can direct the carbon flux of glutamate from the TCA cycle into GABA synthesis. In this study, the *N. crassa* glutamate decarboxylase and *E. coli* GABA transporter were co-localized via a synthetic scaffold complex for efficient synthesis of GABA in *E. coli*. Thus, the formation of the scaffold complex between heterologous GadB and GadC increased the synthesis and secretion of GABA in *E. coli*.

The efficiency of GABA synthesis is mainly dependent on the activity of GadB enzyme. GadB plays an important role in the production of GABA by converting glutamate into GABA. Since GadB plays a major role in the production of GABA, the employment of effective GadB is necessary to accelerate efficient GABA production. When compared with native *E. coli* GadB, which has a hexameric structure, *N. crassa* GadB has a monomeric structure and can be considered to be better for the formation of a scaffold complex and to increase GABA production. In our previous study, we reported that 5.26 g/l of GABA was produced by the overexpression of GadB from *N. crassa* in *E. coli* [11]. In this study, *N. crassa* GadB and *E. coli* GadC were co-overexpressed with a synthetic scaffold complex in *E. coli* to improve GABA production.

During the production of GABA, the pH and temperature play essential roles in the *E. coli*. In a previous paper, *N. crassa* GadB was reported to have higher activity in acidic pH 3, and the *E. coli* GadC was reported to have higher activity at acidic pH [15, 16]. Similarly, the temperature of 30°C was reported as the optimum condition for GABA production. Considering the previous optimum conditions, the recombinant *E. coli* strain from this study was tested at various ranges of pH and temperature. It was found that pH 3 is optimum for GABA production in the *N. crassa* GadB-overexpressed strain, whereas the optimum pH of 3.5 was obtained with the *E. coli* GadB-overexpressed strain.

The *E. coli* GadC acts as an antiporter in which the glutamate can effectively be transported inside and GABA can be transported outside of *E. coli* [16]. Once the glutamate is transported inside the cells, the *N. crassa* GadB presented in the cytosol of *E. coli* can effectively convert the glutamate into GABA. Since the GadC is physically attached to GadB, the converted GABA has more opportunities to react with the enzyme GadC and GABA is transported outside the cells. The formation of a scaffold complex between GadB and GadC increases the flux towards GABA production.

GABA production was increased significantly by 2.9-fold during the initial culture period. The pH3BN plasmid elevated the production of GABA to 3.9 g/l within 24 h of culture. In contrast, the pHBN plasmid without a scaffold system produced 1.3 g/l GABA from 24 h of culture. These results clearly showed that the employment of a scaffold complex closely co-localized the GadB and GadC, which leads to more efficient GABA production within a shorter period of time. Productivity as well as product yield is one of the key factors of process efficiency, and as shown in this study, the introduction of a protein scaffold can be considered a very promising strategy for productivity improvement.

Considering the industrial importance of GABA, the employment of heterologous GadB is one of the key factors to realize efficient GABA production. This study shows that the scaffold complex of GadB from *N. crassa* and GadC from *E. coli* can improve the efficiency and productivity of GABA in *E. coli*. Similarly, it demonstrates the practical benefit of a scaffold complex in the improvement of targeted metabolic products.

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