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Research Article

Improved Production of Tryptophan in Genetically Engineered *Escherichia coli* with TktA and PpsA Overexpression

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Intracellular precursor supply is a critical factor for amino acid productivity. In the present study, *ppsA* and *tktA* genes were overexpressed in genetically engineered *Escherichia coli* to enhance the availability of two precursor substrates, phosphoenolpyruvate and erythrose-4-phosphate. The engineered strain, TRTH0709 carrying pSV709, produced 35.9 g/L tryptophan from glucose after 40 h in fed-batch cultivation. The two genes were inserted, independently or together, into a low-copy-number expression vector (pSTV28) and transferred to TRTH0709. Fed-batch fermentations at high cell densities of the recombination strains revealed that overexpression of the *ppsA* gene alone does not significantly increase tryptophan yield. On the other hand, overexpression of the *tktA* gene, alone or with the *ppsA* gene, could further improve tryptophan yield to a final tryptophan titer of 37.9 and 40.2 g/L, respectively. These results represent a 5.6% and 11.9% enhancement over the titer achieved by TRTH0709. No evident genetic modifications leading to growth impairment were observed.

1. Introduction

The aromatic tryptophan is a very important amino acid that is widely used in medicine and as a supplement in animal feeds. However, the market for L-tryptophan remains limited due to its high production cost [1]. From an industrial point of view, a high production rate is desirable, and many attempts have been undertaken to improve tryptophan yield.

Tryptophan can be manufactured through bacterial fermentation by two representative producer organisms, *Corynebacterium glutamicum* and *Escherichia coli.* Our laboratory has a tryptophan overproducing strain, *E. coli* TRTH0709, which contains overexpressed genes in the tryptophan operon ($trpE^{fbr}$ *DCBA*) and DAHP synthase ($aroG^{fbr}$), and the serine-biosynthetic gene (*serA*) on pBR322, with deletion of *trpR* and *tna*. *E. coli* TRTH0709, is a stable recombinant strain that can produce 35.9 g/L of L-tryptophan after 40 h in fed-batch fermentor cultivation in an antibiotic-free medium. However, production efficiency is not high enough for an industrial fermentation method. In most cases, the first targets for engineering are the common and branched pathways leading to tryptophan synthesis; these steps have been modified in TRTH0709. Further improvement of productivity and yield requires alteration of the central metabolic pathways, which supply the necessary precursors and energy for biosyntheses. In *E. coli,* aromatic metabolites are generated from the condensation reaction between phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) to form 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP). Thus, increasing the availability of PEP and E4P is crucial for achieving the maximum flow of carbon into the common pathway [2, 3]. One way to increase the PEP supply is to directly recycle PEP from pyruvate. However, once PEP has been converted to pyruvate by either the phosphotransferase system or the pyruvate kinases, it is less likely to be converted back to PEP because of the high energy cost. This stoichiometric limitation may be overcome by overexpression of PEP synthase, which is coded by *ppsA*, so that more carbon flux will be directed into the aromatic pathway [4]. Another way to increase PEP supply is to recycle PEP from oxaloacetate (OAA). This way had been proved to be slightly less efficient compared with the former [5]. The E4P pool could be increased by the overexpression of transketolase (TktA) [3, 6] and transaldolase (Tal) [7]. While the overexpression of both of these enzymes increased the DAHP production rate, it

was found that overexpression of TktA had a stronger effect than the overexpression of Tal. And overexpression of Tal in strains which already overexpress TktA did not show a further increase in production of aromatics. This result was attributed to the saturation of E4P supply when TktA was overexpressed [7].

Although the limitation of each precursor could be relieved to some extent by such genetic approaches, effective yield improvement requires a balanced supply of each precursor to the common pathway through a combination of these approaches. Thus, *ppsA* and *tktA* have been co-overexpressed and the yield of DAHP was close to the theoretical maximum [2, 3]. Previous attempts to drastically redirect carbon flux into a desired pathway resulted in growth impairment and/or the formation of unwanted by-products [8, 9]. Due to reduced growth and sugar consumption, high-copy-number amplification of the gene resulted in a tryptophan production level even lower than that without the gene [10]. In order to prevent the damaging effects of high expression on cells, a low-copy-number vector could be used.

Previous studies have performed amplification of *ppsA* and/or *tktA* genes to adjust the carbon flux in *E. coli*. However, these studies mainly aim to accumulate other intermediates in the aromatic amino acid pathway, such as DAHP and shikimic acid, and are usually performed in wild *E. coli* or low producers of tryptophan [2–4, 11, 12]. From a practical point of view, amplification of *ppsA* and/or *tktA* should be applied in highly engineered hyperproducers, so that the improved strains could be immediately used for industrial production. This approach has been successfully applied on a recombinant hyperproducing *C. glutamicum* strain: a fewfold amplification of the *tktA* gene resulted in 15% enhancement over the original strain. To our knowledge, there have been no reports on the modification of *ppsA* and/or *tktA* in existing *E. coli* L-tryptophan-producing strains. In the present study, the two genes were introduced either independently or together into pSTV28 [13], a low-copy-number plasmid compatible with the resident plasmid pSV-709. The resulting plasmids were transferred into *E. coli* TRTH0709. We investigated the effects of gene modification on cell growth, L-tryptophan formation, and plasmid stability and maintenance during fermentation.

2. Materials and Methods

2.1. Bacterial Strains and Plasmids. All bacterial strains, plasmids, and their relevant characteristics and sources are presented in Table 1. Primer sets used in the amplification of target genes are also listed in Table 1. DNA manipulation was routinely conducted in *E. coli* strain DH5a.

2.2. Plasmid Construction. *tktA* and *ppsA* genes were synthesized from the *E. coli* TRTH0709 genome by PCR using primers P1-P2 and P3-P4, respectively. The *tktA* gene was subsequently digested by *Sac I-BamH I* and incorporated into pSTV28 (TaKaRa BIO Inc., Japan) to produce pEML02. The *ppsA* gene was digested by *BamH I-Sph I* and then inserted into pSTV28 and pEML02 to produce pEML01 and pEML03, respectively. The three resulting plasmids were transferred into *E. coli* TRTH0709, thereby producing strains TRTH1011, TRTH1012, and TRTH1013. Isolation of chromosomal DNA from *E. coli* TRTH0709 was performed as described previously [15]. Target genes were amplified using Pfu DNA polymerase (Fermentas, Glen Burnie, USA) in a GeneAmp PCR System thermocycler (Mastercycler, Eppendorf, Germany). Sizes of the PCR products were determined by agarose gel electrophoresis. When required, amplicons were purified by cutting the desired band from the agarose gels and processing with a gel PCR purification kit (Tiandz, Beijing, China). A Plasmid Miniprep Kit (Biomed, Beijing, China) was used for plasmid isolation from *E. coli*. The sequence of the PCR-amplified fragments was confirmed by a DNA MegaBACE 1000 sequencer (Amersham Bioscience, Little Chalfont, UK). All restriction enzymes and Taq DNA polymerase were purchased from Fermentas (Beijing, China). Rapid DNA ligase and alkaline phosphatase were obtained from TaKaRa (Shanghai, China). Primers were ordered from Bomeike (Tianjin, China). Plasmid transfer into *E. coli* was achieved by electroporation of competent cells following the method by Dower et al. [16]. Recombinant strains were selected on a Luria-Bertani (LB) medium with chloramphenicol (25 μg/mL).

2.3. Media and Culture Conditions

2.3.1. Media. LB media was used for all cloning procedures and gene expressions. The TF1 medium used for seed culture in a flask or a 5 L seed tank contained (per liter) glucose, 50 g; yeast powder, 5 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 1.5 g; $(NH_4)_2SO_4$, 2 g; $C_6H_5O_7Na_3 \cdot 2H_2O$, 1.5 g; $FeSO_4 \cdot 7H_2O$, 10 mg; thiamine, 5 mg; trace element mixture, 2 mL (0.278 g/L $FeCl_3 \cdot 6H_2O$, 0.15 g/L $ZnCl_2$, 0.02 mg/L $NaMoO_4 \cdot 2H_2O$, 0.20 g/L $CuSO_4 \cdot 5H_2O$, 2.0 g/L $MnSO_4 \cdot H_2O$, 0.25 g/L H_3BO_3) at pH 7.0. The TF2 medium used for production in 30-L jar fermentors contained (per liter) glucose, 8 g; yeast powder, 1 g; KH_2PO_4, 4 g; $MgSO_4 \cdot 7H_2O$, 3.5 g; $(NH_4)_2SO_4$, 4 g; $FeSO_4 \cdot 7H_2O$, 0.15 g; $C_6H_5O_7Na_3 \cdot 2H_2O$, 2.5 g; trace element mixture, 2 mL (0.278 g/L $FeCl_3 \cdot 6H_2O$, 0.15 g/L $ZnCl_2$, 0.02 mg/L $NaMoO_4 \cdot 2H_2O$, 0.20 g/L $CuSO_4 \cdot 5H_2O$, 2.0 g/L $MnSO_4 \cdot H_2O$, 0.25 g/L H_3BO_3) at pH 7.0. Tetracyclines (50 μg/mL) and chloramphenicol (25 μg/mL) were added as required.

2.3.2. Culture Conditions

Cultivation in a Rotary Shaker. Shake flask cultivation was used for the initial investigation of PpsA and TktA overexpression in LB medium. Cultures (30-mL) in 500 mL baffled Erlenmeyer flasks were placed on a rotary shaker at 37°C and 200 rpm for 8 h. The cells of an overnight preculture were washed with 0.85% (w/v) NaCl and inoculated into the LB medium to an initial optical density at 660 nm (OD660) of approximately 1. Expression of cloned genes was induced

Table 1: Plasmids, strains, and primers used in the study.

Strain, plasmid, or primer	Characteristics	Source or reference
Strains		
TRTH0709	*E. coli* K12 Δ*trpR*Δ*tna*/pSV-709 (strain for expression and fermentation)	Laboratory stock
TRTH1011	TRTH0709/pMEL01	Present study
TRTH1012	TRJH0709/pMEL02	Present study
TRTH1013	TRJH0709/pMEL03	Present study
E. coli DH5*α*	*deoR endA1 gyrA96 hsdR17 supE44 thi1 recA1 lacZM15 lpir* (for routine transformation)	[14]
Plasmid		
pSV-709	pBR322 inserted with $aroG^{Fbr}$ $trpE^{fbr}$ *DCBA serA tetR*	Laboratory stock
pSTV28	Plac cloning vector, pACYC184 origin, *LacZ*, Cm^r	Takaha
pMEL01	pSTV28 inserted with *ppsA*	Present study
pMEL02	pSTV28 inserted with *tktA*	Present study
pMEL03	pSTV28 inserted with *tktA* and *ppsA*	Present study
Primers	Nucleotide Sequence (sequences position at gene)	Gene amplification
P1	5′-GATCCGAGCTCATGTCCTCACGTAAAGAGCTTGC-3′ (1–24)	*tktA*
P2	5′-TATTGGGATCCTTACAGCAGTTCTTTTGCTTTCGC-3′ (1969–1992)	*tktA*
P3	5′-GGTTTGGATCCATGTCCAACAATGGCTCGTC-3′ (1–20)	*ppsA*
P4	5′-TGAAGGCATGCTTATTTCTTCAGTTCAGCCAGG-3′ (2358–2379)	*ppsA*

by adding 0.1 mM isopropyl 1-thio–D-galactopyranoside (IPTG) at the onset of cultivation.

Fed-Batch Fermentation. Single colonies of the strain were produced on an LB plate by incubation at 37°C for 24 h. A single colony was inoculated into test tubes containing 10 mL TF1. After approximately 16 h at 37°C and 200 rpm, the first-seed culture was inoculated into 100 mL TF1 medium in a 1 L flask. After 14 h cultivation at 37°C on a rotary shaker, the second-seed broth was transferred into a 5 L fermenter (BaoXing, ShangHai, China) containing 2 L TF1. The culture was placed at 37°C for approximately 10 h until OD660 was 20. pH value was controlled at 6.7 by adding 25% aqueous ammonia. Dissolved oxygen (DO) was maintained between 20% to 40% by aeration at a rate of 3–6 m^3/min and a stirring speed between 200 and 600 rpm. The resulting preculture was inoculated into 14 L of TF2 medium in a 30-L fermenter (BaoXing, ShangHai, China). Fermentation was carried out at 37°C with aeration at 1–3 m^3/min, DO at 20%–40%, and stirring at 300–900 rpm. pH was maintained at 7.0 by the addition of aqueous ammonia. Whenever residual glucose dropped to nearly zero, 800 g/L glucose solution was continuously added to the culture. Glucose concentration in the fermenter was kept at approximately 0 g/L by adjusting flow sugar pulse and DO. Fermentation was completed after 40 h.

2.4. Analysis of Biomass, Substrate, and Products. For quantification of substrate consumption and product formation, 1 mL samples were taken from the cultures and centrifuged at 12,790 ×g for 10 min. The supernatant was used for determination of L-tryptophan, glucose, and organic acid concentrations in the culture fluid. Glucose was determined by a lactic acid-glucose biosensor SBA-40C (Biology Research Institute, Shandong Academy of Sciences, China). Organic acid concentrations were determined by high-performance liquid chromatography (HPLC) using an Agilent1100 system (Palo Alto, CA, USA). The HPLC system was equipped with an Aminex HPX-87H column (300 mm × 7.8 mm; 9 μm) (Bio-Rad, Hercules, CA, USA) maintained at 50°C. The mobile phase consisted of 5 mM H_2SO_4, with a flow rate of 0.5 mL/min. All metabolites were detected with a photodiode array detector at 210 nm. Accumulated L-tryptophan was quantitatively determined by HPLC using the Agilent1100 system. An Agilent reversed-phase C_{18} column (150 mm × 4.6 mm; 3.5 μm) was used with a mobile phase of 0.030% KH_2PO_4 water solution and methanol (volume ratio of 90 : 10), with a flow rate of 1.0 mL/min at 39°C and a detective wavelength of 278 nm.

Cell density of the cultures was determined by measuring the OD660 value using a 721 spectrophotometer. Cell dry weight was obtained as described previously [17].

2.5. Enzyme Assays. Cells were harvested at the exponential phase in TF2 medium by centrifugation at 6,395 ×g. They were then washed and resuspended in 50 mM potassium phosphate buffer (pH 7.0) or 5 mM Tris-Cl 1 mM $MgCl_2$ (pH 7.4) for Tkt or Pps assay. Cells were continuously sonicated for 15 min on ice with an ultrasonic disruptor (JY92-II; Scientz Biotechnology Co., Ningbo, China). Cellular debris was removed by centrifugation at 10,658 ×g for 20 min at 4°C to collect the supernatant. Crude extracts were dialyzed against the potassium phosphate buffer for 6 h and used for enzyme assay. Tkt and Pps activities in the crude cell extracts were measured as described previously [9, 18]. Total protein content in the extracts was determined according to Lowry et al. [19].

2.6. Plasmid Stability. Tetracycline (50 μg/mL) and chloramphenicol (25 μg/mL) were added to the TF2 medium. After

fed-batch fermentations in a 30 L jar, samples were collected from the cultures, diluted by 10^6- and 10^7-fold, and spread on agar plates with or without antibiotic pressure. Plasmid stability was estimated as the ratio of the percentage of colonies on antibiotic agar plates to percentage of colonies on agar plates without antibiotics. The same experiment was carried out in the TF2 medium without antibiotics.

3. Results

3.1. Expression of ppsA and tktA Genes in E. coli TRTH0709. The *ppsA* and *tktA* genes were amplified from *E. coli* TRTH0709 genomic DNA by PCR and subsequently cloned into the pSTV28 plasmid at corresponding restriction sites. After DNA sequencing, the cloning of *ppsA* has a G to A mutation at nucleotide position148 and the cloning of *tktA* has an A to C mutation at nucleotide position1186. The point mutations led to single amino acid changes, A to T and I to L at position, respectively. The rest of the sequences agree with the literature [5, 20]. The two-point mutations might exist in the genomic DNA because *E. coli* TRTH0709 had been dealt with mutation breeding before.

The two genes were expressed in TRTH0709 to confirm their bioactivities. Expression vectors were constructed by either single gene expression or coexpression of the two genes based on vector Pstv28. The constructs were transformed into *E. coli* TRTH0709, and the protein profiles of the transformants were analyzed by SDS-PAGE. Distinct protein bands corresponding to the molecular weights of Pps (84 kD) and Tkt (73 kD) were detected on SDS-PAGE (Figure 1).

The specific activities of the transformants were also determined (Table 2). Independent expressions of *ppsA* and *tktA* resulted in increased specific activities of the corresponding enzymes by 2.5- and 3.3-fold, respectively. In cells with coexpression of *ppsA* and *tktA* genes, specific enzymatic activities increased by 2.1-fold and 3.2-fold, respectively, which are slightly lower than that in the independent expressions. Findings suggest that the two genes amplified by PCR have normal enzymatic activities.

3.2. Tryptophan Production in Transformed E. coli TRTH0709. To investigate the effect of enhanced enzymatic activities on tryptophan production, we tested the tryptophan production of the *E. coli* TRTH0709 transformants harboring different constructs in a jar fermentor under the conditions described above. Levels of tryptophan, biomass, correlative organic acids, and residual glucose were determined. As shown in Figure 2(c), TRTH1011 and pMEL01 accumulated 36.3 g/L L-tryptophan after 40 h, which represents only a 1% improvement over the production of the original TRTH0709. The pMEL02 carrier had a 5.6% increase in tryptophan yield compared with TRTH0709. Under the same culture conditions, the pEML03 carrier produced 40.2 g/L tryptophan, a relative 11.9% yield increase from the original strain. Our findings suggest that simultaneous overexpression of *ppsA* and *tktA* may significantly enhance L-tryptophan production through independent *ppsA* or *tktA* overexpressions.

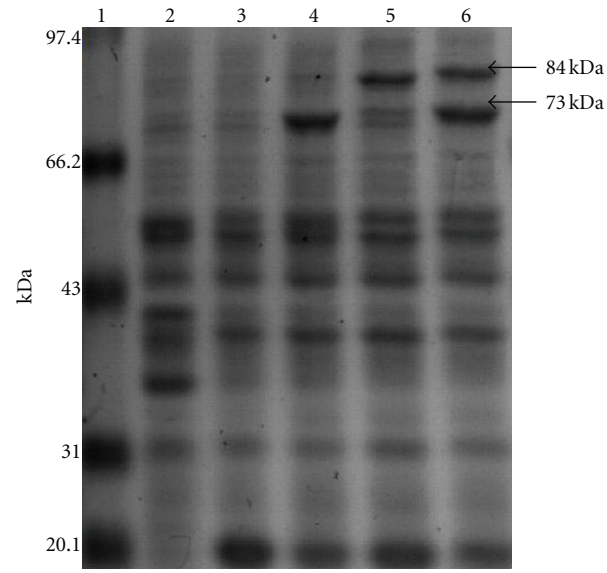

Figure 1: SDS-PAGE analysis of expressed proteins; 13% SDS-PAGE gel demonstrates the induction of *tktA* and *ppsA* gene expressions by 0.1 mM IPTG at 37°C; lane 1, Marker; lane 2, TRTH0709; lane 3, TRTH0709/Pstv28; lane 4, TRTH1012; lane 5, TRTH1011; lane 6, TRTH1013.

Table 2: Specific enzymatic activities of *E. coli* TRTH0709 harboring different constructs.

Strain/plasmid	Specific enzymatic activities (μmol/min/mg)	
	Pps	Tkt
TRTH0709	0.070 ± 0.002	0.091 ± 0.002
TRTH0709/pMEL01	0.175 ± 0.006	0.096 ± 0.002
TRTH0709/pMEL02	0.073 ± 0.004	0.324 ± 0.005
TRTH0709/pMEL03	0.147 ± 0.005	0.291 ± 0.003

Enzymatic activities measured as μmol of enzymatic product liberated per min per mg of protein. All values represent the average of at least two separate determinations.

Growth of TRTH1011, TRTH1012, and TRTH1013 was slightly slower than that of TRTH0709 at an early stage (Figure 2(b)). However, TRTH1012 and TRTH1013 grew more quickly than TRTH0709 at later stages. Maximum biomass was attained at 28–30 h of fermentation. The maximum biomass of TRTH1011 (45.5 g/L) was lower than that of the three other strains, which attained almost the same biomass of 47 g/L. At the initial stage of fermentation, glucose consumption of TRTH1012 and TRTH1013 was slightly slower compared with TRTH0709. However, after 18 h, their consumption of glucose exceeded that of TRTH0709. TRTH1011 was similar to TRTH0709 and exhibited slightly faster consumption of glucose at early stages. This trend of glucose consumption is in accordance with the production of biomass and tryptophan in the four strains (Figure 2(a)).

The concentrations of pyruvate, lactic acid, acetate, and shikimic acid during the fermentation process were determined. Results showed similar trends in organic acid

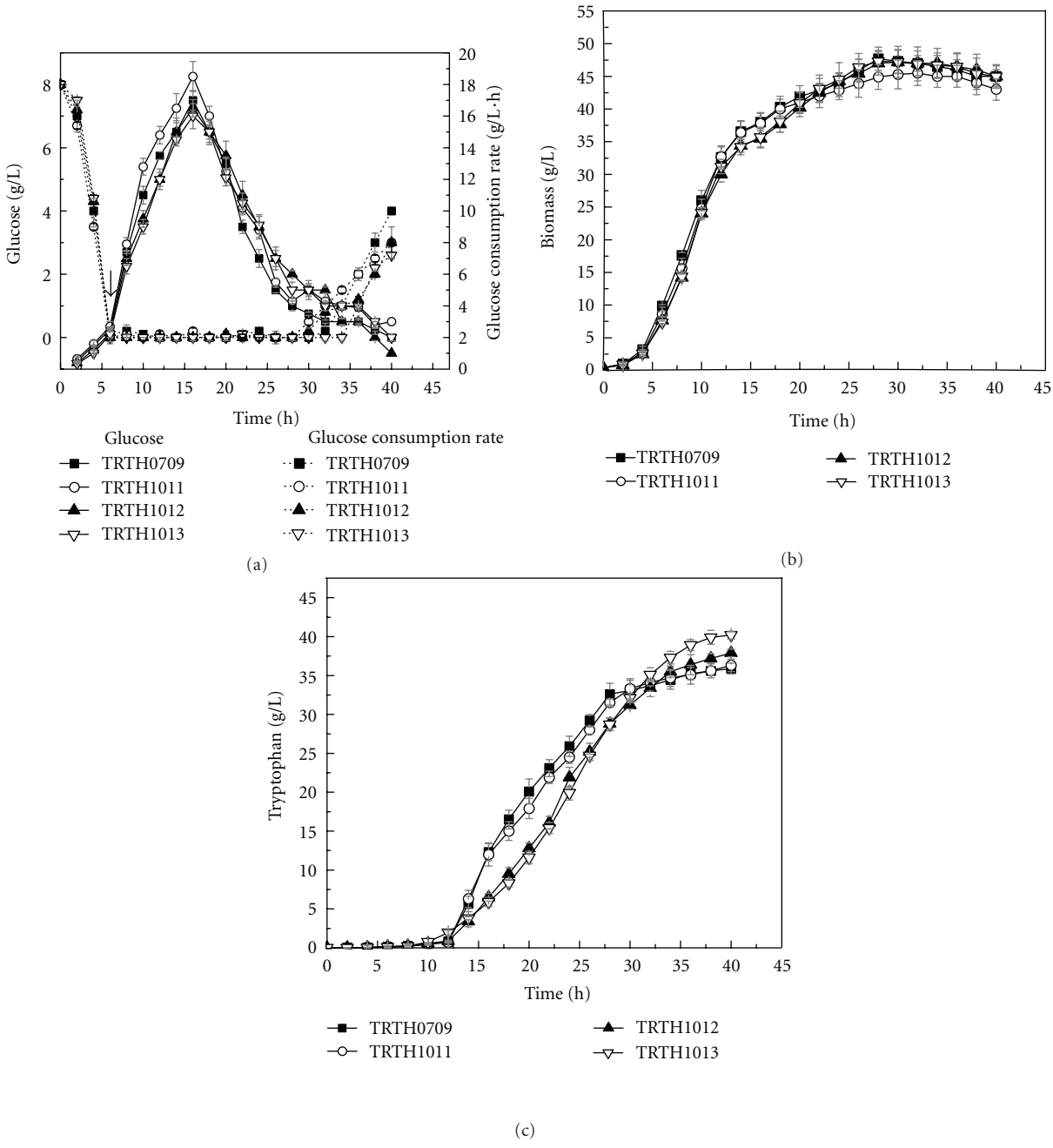

Figure 2: Tryptophan fermentation by strain TRTH0709, TRTH1011, TRTH1012, and TRTH1013 in fed-batch jar-fermentor cultivation; glucose and glucose consumption rate (a) arrow indicates the point at which feeding with 80% glucose solution began; biomass, (b) tryptophan, (c) data represent mean values from three independent cultures; standard deviations from means are indicated as error bars.

levels (Figure 3): levels increased during the growth stage and decreased after reaching a maximum value. Organic acids generated at the earlier stages were assimilated by cells due to the limited glucose supply in the mid- to late-stages of fermentation. TRTH1011 had the highest concentration of pyruvate, lactic acid, and acetate during fermentation among the four strains, but had lower levels of shikimic acid than the two other recombinations.

3.3. Comparison of L-Tryptophan Accumulation and Substrate-Specific L-Tryptophan Yields. Figure 4 summarizes the tryptophan accumulation and substrate-specific L-tryptophan yields of TRTH0709 and its plasmid-carrying strains. TRTH0709 accumulated approximately 35.9 g/L tryptophan with a Yp/s of 0.130 mol tryptophan per mol glucose after 40 h of fermentation in a 30 L jar fermentor. Overexpression of the *ppsA* gene alone in TRTH0709 resulted in a slightly higher L-tryptophan accumulation of approximately 36.3 g/L, but a lower Yp/s of 0.125 mol L-tryptophan per mol glucose. Overexpression of the *tktA* gene resulted in a modest increase in L-tryptophan accumulation to 37.9 g/L and a Yp/s of 0.132 mol L-tryptophan per mol

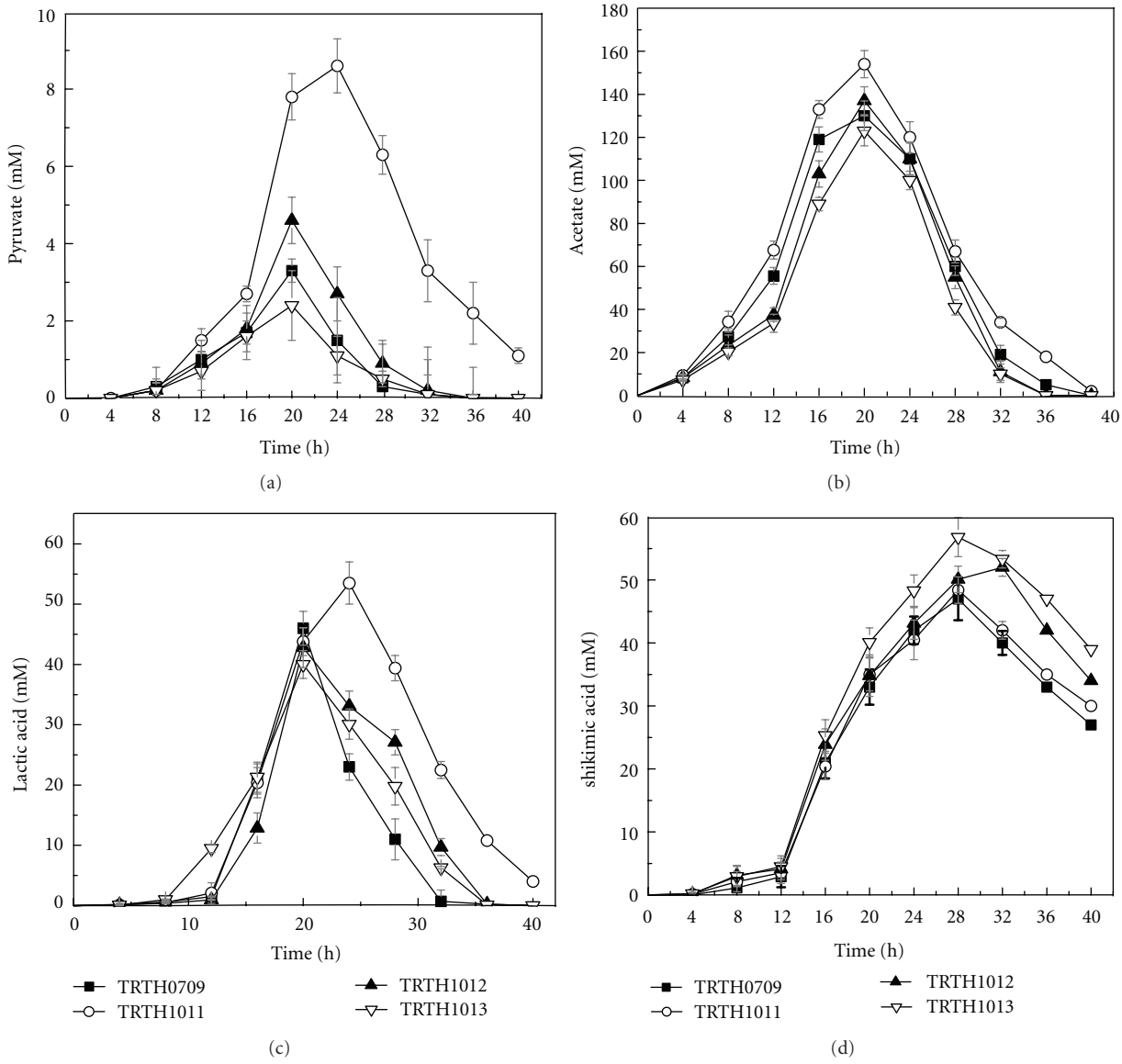

Figure 3: Concentrations of pyruvate, lactic acid, acetate, and shikimic acid in the fed-batch fermentations.

glucose. Cooverexpression of the *ppsA* and *tktA* genes resulted in an evident increase in tryptophan accumulation to 40.2 g/L and the maximal Yp/s of 0.145 mol tryptophan per mol glucose. Our findings suggest that amplification of *tktA* alone or *tktA* with *ppsA* in TRTH0709 could further improve the productivity and yield of L-tryptophan, whereas amplification of *PpsA* alone could not effectively enhance L-tryptophan production.

3.4. Plasmid Stability in Fermentation. The result of the plasmids stability in fermentation was shown in Table 3. Plasmid pSV709 was stable in batch cultivations under selective pressure. Its stability could be maintained at 98% even in the absence of selective pressure. In comparison, pEML03 was not stable during the fermentation process and showed approximately 15% loss with or without antibiotic pressure. Due to the stability of plasmid pSV709, the maintenance of both plasmids in one cell approximates that for plasmid pEML03. Results also show no significant difference in stability of plasmids in cultures with and without antibiotics.

4. Discussion

Overexpression of genes plays important roles in the biosynthesis pathway and is a major approach for metabolic pathway engineering. Increasing the availability of PEP and E4P is a crucial factor for achieving the maximum flow of carbon into the common pathway. The present study verifies the significance of increasing intracellular PEP synthase and transketolase contents for maximal tryptophan production using *E. coli* TRTH0709. The transfer of pSTV28 with *ppsA* and/or *tktA* moderately increased the expression of *ppsA*

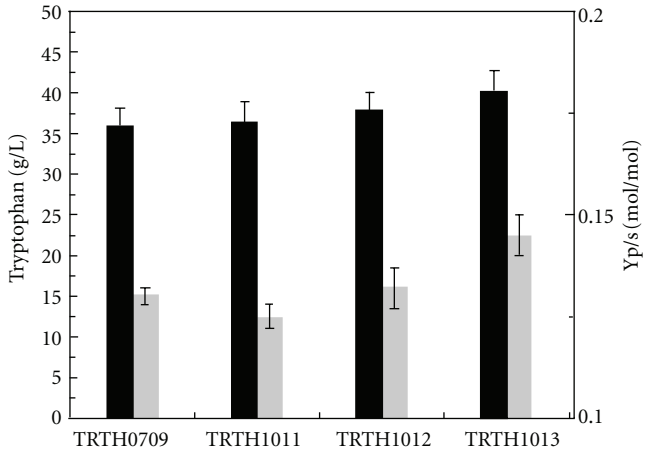

Figure 4: L-tryptophan accumulation (black bars) and substrate-specific product yields (Yp/s) in mol L-tryptophan per mole glucose (grey bars) at the end of the production phases of representative fed-batch fermentations. Means are from at least three independent experiments.

Table 3: Stability of plasmids pSV709 and pMEL03 in TRTH1013 after 40 h of fermentation.

Plasmids	Stability with tetracycline and chloramphenicol (%)	Stability without antibiotics (%)
pSV709	100 ± 2.3	98 ± 2.5
pMEL03	85 ± 7.1	86 ± 4.4
pSV709 and pMEL03	84 ± 6.2	83 ± 4.5

Values show the ratio in percentage of colonies on agar plates with antibiotics to colonies on agar plates without antibiotics. Means are determined from at least three independent experiments.

and/or *tktA* genes and the activity of PEP synthase or/and transketolase (Figure 1, Table 2).

Previous studies have demonstrated that overexpression of PpsA to increase DAHP production in *E. coli* cultured on nutrient-rich and glucose-containing media leads to growth impairment, increased glucose consumption, and excretion of pyruvate and acetate [2]. Growth impairment was significant enough that cell density of the high overexpression *ppsA* plasmid-containing culture at the stationary phase was up to 70% lower than that in the control culture [9]. In the present study, TRTH1011 showed a final biomass that is 4% less compared with the original strain (Figure 2). The lower influence on biomass can be attributed to the adoption of low gene dosage and the strategy of glucose-limited fed-batch culture using the DO-stat method in the fermentation process [21]. This strategy kept the glucose concentration in the fermentation broth near zero, thus reducing the accumulation of harmful byproducts. Byproducts, especially acetate, can inhibit cell growth and protein production. TRTH1011 and TRTH1012 did not exhibit significant growth impairment. Some studies have reported that transketolase gene overexpression imposes a metabolic burden leading to retarded growth and segregation of plasmids [1, 12, 17]. Minimization of *tktA* expression levels and glucose-limited fed-batch culture actually reduced the negative effects in the present study, so that pEML03 could be maintained at 85% after 40 h fermentation. The plasmid was not readily lost in the absence of antibiotics, indicating that the strain may be suitable for tryptophan industrial fermentation without antibiotics.

Determination of several correlative organic acids revealed that overexpression of PpsA leads to accumulation of acetate, lactic acid, and pyruvate. The increase in pyruvate, lactic acid, and acetate excretion may be attributed to the high glycolytic flux, which saturates the tricarboxylic acid cycle and leads to overflow of these products. When TktA is overexpressed, the excess PEP could be redirected to the aromatic pathway. This would lead to increased concentration of shikimic acid, a metabolite in the aromatic common pathway, whereas concentrations of pyruvate, lactic acid, and acetate would be decreased since they are metabolites of the glycolysis pathway. Thus, in the final fermented liquids of TRTH0709, TRTH1012, and TRTH1013, these organic acids were found in trace amounts or not at all. Shikimic acid in the culture supernatant of strains that overexpressed *tktA* was slightly higher than that in the original strain and the strain overexpressing *ppsA* (Figure 3).

Findings of the present study indicate that overexpression of *ppsA* alone does not significantly enhance L-tryptophan production. This result concurs with a previous report that the positive *ppsA* effect is only significant with concomitant overexpression of a feedback-deregulated DAHP synthase and transketolase gene *tktA* [4]. Effective enhancement of L-tryptophan by *tktA* overexpression verified that transketolase is the pivotal enzyme in L-tryptophan production. However, increasing E4P supply alone through *tktA* overexpression cannot increase the tryptophan yield to the theoretical level due to the stoichiometric limitation of pyruvate recycling to PEP [2]. In the present study, the simultaneous overexpression of *ppsA* and *tktA* resulted in higher yields of L-tryptophan, as expected.

However, the tryptophan productivity and yield of the modified strain was still far from the theoretical level and was lower than that of many other amino acids [10]. A large amount of research is necessary to exploit the potential production capacity of the current bacterial strain. The production of tryptophan, coupled with cell growth and overexpression of plasmid-encoded protein, leads to growth impairment. An optimal balance among the dosages of gene expression, tryptophan accumulation, and cell biomass must be determined. Findings of this study indicate that a detailed study to further strengthen the expression of the *tktA* and *ppsA* genes is needed in the future. Modifications on plasmid copy number, promoter strength, and placement of genes in the plasmid should be attempted so that greater tryptophan production could be achieved and cell growth inhibition could be avoided.

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