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Construction of a Novel Expression System in *Klebsiella pneumoniae* and its Application for 1,3-Propanediol Production

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Abstract A novel expression system of *Klebsiella pneumoniae* was developed in order to improve 1,3-propanediol (1,3-PD) production using a *K. pneumoniae–Escherichia coli* shuttle vector pET28a consisting of the kanamycin-resistance gene promoter *Pkan*. The recombinant plasmid pETPkan-*cat* carrying the chloramphenicol acetyltransferase gene *cat* as selectable marker was constructed to test the availability of the promoter *Pkan* in *K. pneumoniae*. The results showed that the chloramphenicol acetyltransferase was apparently expressed in *K. pneumoniae*, and the recombinant strain had a high-level resistance to chloramphenicol, suggesting that the promoter *Pkan* was efficient in *K. pneumoniae*. Then, the expression system was applied to the expression of 1,3-PD oxidoreductase in *K. pneumoniae*. The enzyme was over-expressed, and the recombinant *K. pneumoniae* showed a nearly 3.0-fold decrease in peak level of the intermediary metabolite 3-hydroxypropionaldehyde and an increase of 16.5% in yield of 1,3-PD with respect to the wild-type strain. From these results, the first reported expression system has paved the way for improvement of 1,3-PD production and will be available and efficient for other heterologous gene expression in *K. pneumoniae*.

Keywords *K. pneumoniae* · 1,3-Propanediol · Expression system · Constitutive promoter

Introduction

As an important intermediate, 1,3-propanediol (1,3-PD) was used as a monomer to produce polyesters or polyethers. In recent years, the utilization of inexpensive and renewable resource such as glycerol or glucose as the substrate in biological route to produce 1,3-PD has become increasingly attractive [1]. *Klebsiella pneumoniae* is one of the several organisms that naturally biotransform glycerol to 1,3-PD [2]. Glycerol is fermented by a dismutation process involving two pathways in *K. pneumoniae* (Fig. 1). In the oxidative branch pathway, glycerol is dehydrogenated by a NAD-linked glycerol dehydrogenase to

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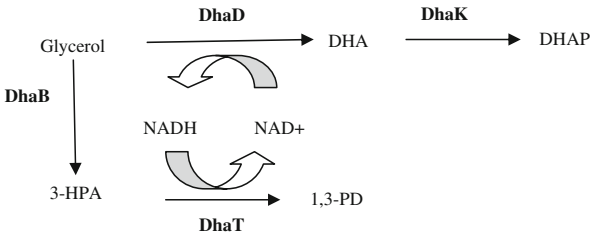

Fig. 1 Metabolic pathways involved in glycerol dismutation by *Klebsiella pneumoniae*. In the oxidative branch pathway, *DHA*, dihydroxyacetone, *DHAP*, dihydroxyacetonephosphate, *DhaD*, glycerol dehydrogenase, *DhaK*, dihydroxyacetone kinase. In the reductive branch pathway, *3-HPA*, 3-hydroxypropionaldehyde, *1,3-PD*, 1,3-propanediol, *DhaB* glycerol dehydratase, *dha*T, 1,3-propanediol oxidoreductase

dihydroxyacetone, which is then phosphorylated and funneled to central metabolism by dihydroxyacetone kinase. In the reductive branch pathway, glycerol is dehydrated by coenzyme B_{12}-dependent glycerol dehydratase to 3-hydroxypropionaldehyde (3-HPA), which is then reduced to the fermentation product of 1,3-PD by the nicotinamide adenine dinucleotide (NADH)-linked 1,3-PD oxidoreductase (PDOR). The genes encoding glycerol dehydratase and PDOR are designated *dha*B and *dha*T, respectively [3].

So far as we know, there are few reports on the genetic modification and its application in *K. pneumoniae*. Construction of an efficient expression system for this special strain is essential for increasing the production of 1,3-PD, even utilizing low-cost feedstock such as carbohydrate to produce 1,3-PD by using engineered *K. pneumoniae*. The value of expression system based on the efficient regulated promoter was well recognized in modern biotechnology. In the past decades, prokaryotic expression systems had been constructed mostly for *Escherichia coli*. Most of the widely used promoters in these systems including T7 and *tac* promoters were inducible. However, to our knowledge, there has not been any study on genetically engineered *K. pneumoniae* with a constitutive promoter.

In order to develop a novel expression system for *K. pneumoniae*, there is an urgent need to search for the efficient constitutive promoter. We previously found that the plasmid pET28a is a shuttle vector between *K. pneumoniae* and *E. coli*. The recombinant *K. pneumoniae*/pET28a has a high-level resistance to kanamycin (data not shown), which suggested that the promoter of kanamycin-resistant gene (*Pkan*) is a constitutive expression promoter in *K. pneumoniae* and has high transcription activity. In this work, a novel expression system for *K. pneumoniae* was constructed based on plasmid pET28a carrying constitutive expression promoter *Pkan*. The transcription activity of promoter *Pkan* in *K. pneumoniae* was proved by importing the chloramphenicol acetyltransferase gene (*cat*). Further, the expression system was successfully applied to the overexpression of gene *dha*T encoding PDOR for reducing 3-HPA accumulation and increasing 1,3-PD production in the recombinant strains. The first report on the construction of constitutive expression system has paved the way for improvement of 1,3-PD production in *K. pneumoniae*.

Materials and Methods

Materials

E. coli strain JM109 was from our collection. *K. pneumoniae* was isolated by our lab. The vector pET28a and pACYCDuet were purchased from Novagen (USA). The vector

pMD18-T, polymerase chain reaction (PCR) reagents, restriction enzymes, and calf intestinal alkaline phosphatase were purchased from TaKaRa Biotechnology Co. Ltd. The Miniprep kit and Gel Extraction kit were purchased from Promega.

Media and Growth Conditions

K. pneumoniae and *E. coli* were grown in the Luria-Bertani (LB) medium, which consists of 1% tryptone, 0.5% yeast extract, and 1% sodium chloride. The LB medium was supplemented with 50 μg/ml kanamycin and/or 25 μg/ml chloramphenicol (CM) for *E. coli* JM109 when necessary to maintain the plasmids. The fermentation medium for *K. pneumoniae* consisted of the following per liter: yeast extract 5 g, KH_2PO_4 7.5 g, $MgSO_4 \cdot 7H_2O$ 2 g, citric acid 2 g, 0.5 g $CaCl_2 \cdot 2H_2O$ $(NH_4)_2SO_4$ 0.2 g, $FeSO_4 \cdot 7H_2O$ 0.005 g, glycerol 50 g, 1.5 μM coenzyme B_{12}, and 5 ml trace element solution. The trace element solution contained (per liter) 0.68 g $ZnCl_2$, 2.0 g $MnCl_2 \cdot 4H_2O$, 60 mg H_3BO_3, 0.47 g $CoCl_2 \cdot 6H_2O$, 5 mg $Na_2MoO_4 \cdot 2H_2O$, 17 g $CuCl_2 \cdot 2H_2O$, 5.4 g $FeCl_3 \cdot 6H_2O$, and 10 ml HCl (37%). Five percent (*v*/*v*) of the seed culture was inoculated, and the initial pH of the medium was adjusted to 6.8. The fermentations were carried out in a rotary shaker incubator at 150 rpm, 37°C under aerobic condition. All the seeds and fermentation medium of the *K. pneumoniae* were supplemented with kanamycin to maintain the plasmids.

Construction of Key Plasmids for *K. pneumoniae*

Primers used in this work are listed in Table 1. The plasmid pETPkan was constructed as follows: The 500 bp DNA fragment (designed *Pkan*) derived from upstream regulatory region of kanamycin-resistant gene was amplified by PCR using primer P1 and P2 and the plasmid pET28a as template, and ligated into the cloning vector pMD18-T, then the ligation mixture was transformed into *E. coli* JM109 by the calcium chloride method [4]. The ampicillin resistant colonies were selected on the LB plate supplemented with 100 μg/ml ampicillin and further purified. The plasmid T-*Pkan* was confirmed by digestion with *Bgl* II and *Eco*R I; the *Bgl* II/*Eco*R I fragment of *Pkan* from T-*Pkan* was ligated into the corresponding site of pET28a. The recombinant plasmid was confirmed by digestion with *Bgl* II and *Eco*R I (Fig. 2 and Fig. 3).

Gene *cat* was integrated to the plasmid pETPkan by the following steps. The gene *cat* was amplified by PCR using primer P3 and P4 from the plasmid pACYCDuet. The purified DNA fragment was cloned into pMD18-T vector to create T-*cat*. The *Eco*R I/*Hin*d III fragment of *cat* gene from T-*cat* was inserted into the *Eco*R I and *Hin*d III sites of vector

Table 1 Primers used in this study.

Primers	Sequence	Source
P1	5′-CGC *AGATCT* GTA TCT CAG TTC GGT GTA GG-3′ (*Bgl* II)	This work
P2	5′-CGC *GAATTC* AAC ACC CCT TGT ATT ACT G -3′ (*Eco*R I)	This work
P3	5′-CGC *GAA TTC* ATG GAG AAA AAA ATC ACT GG -3′ (*Eco*R I)	This work
P4	5′-CGC *AAGCTT* TTA CGC CCC GCC CTG CCA CTC -3′ (*Hin*d III)	This work
P5	5′- ACCG*GAATTC*ATGAGCTATCGTATGTTTG -3′ (*Eco*R I)	This work
P6	5′- ACC G*AAGCTT*TC AGA ATG CCT GGC G -3′ (*Hin*d III)	This work

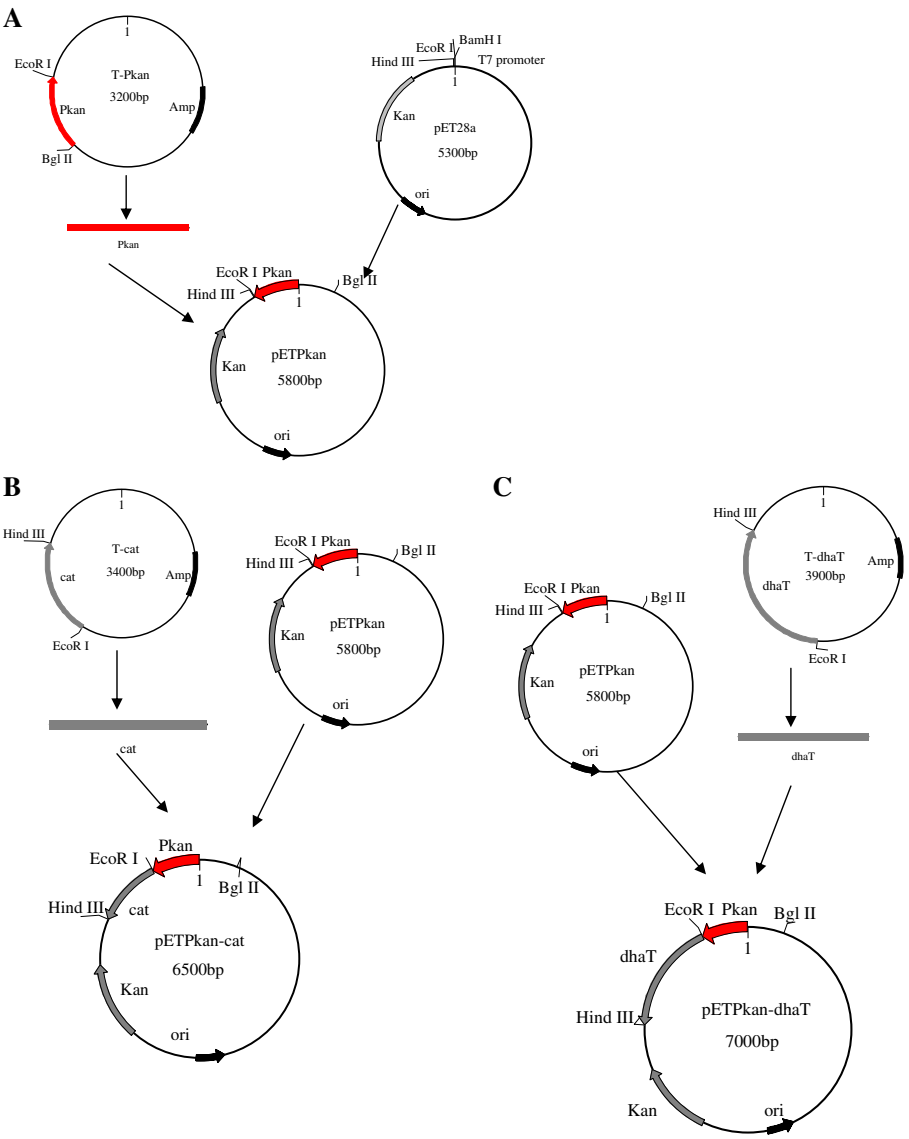

Fig. 2 Map of plasmids constructed in this study. The construction of pETPkan (**a**), pETPkan-*cat* (**b**), and pETPkan-*dha*T (**c**)

pETPkan to create pETPkan-*cat*. The *cat* gene fragment was under the control of *Pkan* (Fig. 2 and Fig. 3). The plasmid pETPkan-*cat* was first transformed into *E. coli* JM109 for amplification. The resultant strains *E. coli*/pETPkan-*cat* were kanamycin and CM resistant. Then, the amplified plasmid pETPkan-*cat* was electro-transformed into *K. pneumoniae* to create *K. pneumoniae*/pETPkan-*cat*.

The similar procedure was used to construct plasmid pETPkan-*dha*T. The gene *dha*T encoding the PDOR was amplified by PCR using the primer P5 and P6 from *K. pneumoniae* genome. The gene *dha*T was under the control of *Pkan* (Fig. 2 and Fig. 3). Then,

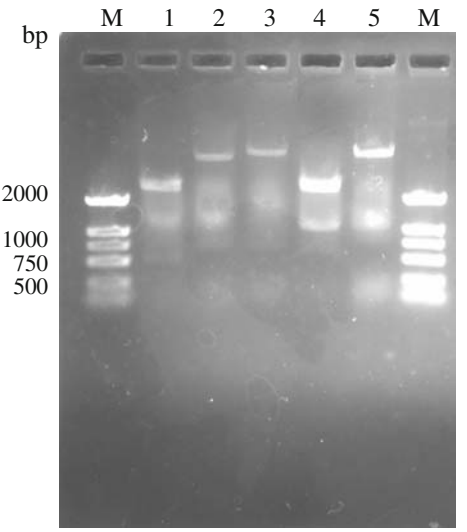

Fig. 3 Identification of three plasmids constructed in this study. Plasmids were digested. *Lane 1*, plasmid T-*Pkan* digested with *Bgl* II and *Eco*R I; *lane 2*, plasmid pETPkan digested with *Bgl* II and *Eco*R I; *lane 3*, plasmid pETPkan-*cat* digested with *Eco*R I *and Hin*d III; *lane 4*, plasmid T-*dha*T digested with *Eco*R I and *Hin*d III; *lane 5*, plasmid pETPkan-*dha*T digested with *Eco*R I *and Hin*d III

the amplified plasmid pETPkan-*dha*T was electro-transformed into *K. pneumoniae* to create *K. pneumoniae*/pETPkan-*dha*T. Transformation of the plasmids into *K. pneumoniae* was performed by electroporation as described in publication [5].

Confirmation of *Pkan* Promoter Function in *K. pneumoniae*

The CM medium was used to investigate *Pkan* promoter transcription activity in *K. pneumoniae*. The strain *K. pneumoniae*/pETPkan-*cat* was grown overnight on the LB medium supplemented with 100 μg/ml CM.

Enzymes Assay

The enzyme assay of PDOR was carried out according to the method described by Forage and Lin [6], and Ahrens et al. [7].

Analytical Methods

The biomass concentration was measured with optical density at OD_{600}. The determination of 1,3-PD was carried out with a gas chromatograph (SHIMAZU GC-14B, FID-detector, 2 m×Ø 5 mm stainless steel column packed with chromosorb101 and operated with N_2 as carrier gas at flow rate of 40 ml min^{-1}, detector temperature 220°C, and column temperature 210°C). 3-HPA was measured according to the method described by Cirde et al. [8].

Plasmid Stability

Samples from fermentation were spread on selective (50 μg/ml kanamycin) and nonselective LB agar plates, respectively, after proper dilution. Then, the plates were incubated at 37°C for 14–18 h. The plasmid stability was shown as the ratio in percentage of colonies on the antibiotic agar plates over those on the plates without antibiotics.

Results

Construction of Constitutive Expression Vector pETPkan in *K. pneumoniae*

To develop a novel expression system in *K. pneumoniae*, we attempted to look for an available constitutive promoter. The *Pkan* promoter from pET28a was selected to construct the *K. pneumoniae* expression system. The gene promoter *Pkan* was cloned into the vector pMD18-T. The recombinant plasmid T-*Pkan* was confirmed by digesting with *Bgl* II and *Eco*R I (Fig. 2a), and the target DNA fragments of 500 bp was shown (Fig. 3). The *Bgl* II/*Eco*R I DNA fragment of *Pkan* promoter from T-*Pkan* was ligated into pET28a (Fig. 2a). The recombinant plasmid pETPkan was obtained by digestion of *Bgl* II and *Eco*R I (Fig. 3), and it was divided into two major DNA fragments with sizes of 5,100 and 700 bp, respectively, which were consistent with the expected sizes.

Functional Expression of the Reporter Gene *cat* on the Vector pETPkan in *K. pneumoniae*

The wild-type *K. pneumoniae* is characterized with low-level resistance to CM, while the recombinant strain carrying *cat* gene could grow at a high level. So, it is convenient and simple for evaluation of transcription activity of *Pkan* promoter using the gene *cat* as the selection marker in *K. pneumoniae*. The gene *cat* was cloned from the plasmid pACYCDuet and inserted into the pMD18-T vector to create plasmid T-*cat*. Then, the DNA fragment *cat* was digested from plasmid T-*cat* and ligated into pETPkan to form pETPkan-*cat* (Fig. 2b). The plasmid pETPkan-*cat* was confirmed by digesting with *Eco*R I and *Hin*d III, and the DNA fragments were analyzed as shown in Fig. 3. Plasmid pETPkan-*cat* could be divided into two major DNA fragments with sizes of 5,800 and 700 bp, according to their expected sizes, respectively (Fig. 3, lane 3). The recombinant pETPkan-*cat* was transformed to *K. pneumoniae*. The expression of gene *cat* was regulated by the constitutive promoter *Pkan* in *K. pneumoniae*.

The previously constructed recombinant *K. pneumoniae*/pET28a-*cat* could not grow in LB medium supplemented with 20 μg/ml CM after induction by isopropyl-β-D-thiogalactopyranoside (IPTG) or not. The results demonstrated that T7 promoter was proved to be essentially silent in *K. pneumoniae* and not suitable for *K. pneumoniae* (data not shown); but the *K. pneumoniae*/pETPkan-*cat* strain grows well in LB medium supplemented with 100 μg/ml CM, suggesting *K. pneumoniae*/pETPkan-*cat* shows a high-level resistance to CM. Comparatively, the control host *K. pneumoniae* and *K. pneumoniae*/pETPkan strain can not grow in the LB medium supplemented with 20 μg/ml CM. These results suggested that constitutive promoter *Pkan* was available and efficient for heterologous gene expression in *K. pneumoniae*.

Construction of Expression System *K. pneumoniae*/pETPkan-*dha*T

Because the promoter *Pkan* was effective in *K. pneumoniae*, the target gene *dha*T encoding PDOR was constructed on the vector pETPkan to improve 1,3-PD production. The gene *dha*T was ligated into pMD18-T vector (Fig. 2c), and the positive T-*dha*T was confirmed by digesting with *Eco*R I and *Hin*d III (Fig. 3). The digested components contain two major DNA fragments with sizes of 2,700 and 1,200 bp, respectively (Fig. 3, lane 4). After that, the purified DNA fragment of *dha*T gene from plasmid T-*dha*T was ligated into pETPkan to form plasmid pETPkan-*dha*T (Fig. 2c). After confirmation by digesting with restriction enzymes and DNA sequencing (Fig. 3, lane 5), the plasmid pETPkan-*dha*T was

transformed to *K. pneumoniae* to construct *K. pneumoniae*/pETPkan-*dha*T, which allowed the gene *dha*T to be expressed under the control of *Pkan* promoter in *K. pneumoniae*.

Improved 1,3-PD Production by Genetically Engineered *K. pneumoniae*

The chemical 3-HPA was a intermediate in 1,3-PD metabolic pathway, and its role is an inhibitor during the process from glycerol to 1,3-PD in *K. pneumoniae*. It means that the cell growth cessation and low production of 1,3-PD might come from the accumulation of 3-HPA [9, 10]. The *K. pneumoniae*/pETPkan-*dha*T and control host *K. pneumoniae* were cultured in flasks with fermentation medium. The maximum biomass for wild-type strain and the recombinant strain were 1.67 and 1.75, respectively. A promoted growth and an increased consumption of glycerol were observed in the early growth phase (Fig. 4). When both *K. pneumoniae* and *K. pneumoniae*/pETPkan-*dha*T were grown to the logarithmic phase, their cell-free extracts were prepared. The activity of PDOR in the cell-free extracts was measured under the standard assay conditions after 12 h fermentation. It turned out that the level of PDOR activity of *K. pneumoniae*/pETPkan-*dha*T (1.64 U/mg) showed an increase of 0.9-fold in PDOR activity with respect to the wild-type *K. pneumoniae* (0.85 U/mg, Table 2). The 3-HPA was detected at every 2 h, and the peak value of 3-HPA of *K. pneumoniae*/pETPkan-*dha*T (2.9 mmol/L) was about threefold lower than that of the control (8.2 mmol/L; Fig. 5, Table 2). The final yield of 1,3-PD by the recombinant *K. pneumoniae*/pETPkan-*dha*T was 16.5% higher than that of wild type. These results indicated that the constructed novel expression vector pETPkan is effective for over-expression of key-enzyme PDOR in *K. pneumoniae*, and the recombinant strain *K. pneumoniae*/pETPkan-*dha*T improved 1,3-PD production.

Discussion

Glycerol is a renewable resource which is formed as a byproduct during biodiesel production. For its large volume production, it is significant to develop a technology to convert this waste into the products with high value such as 1,3-PD [11]. In the recent patent issued by Dupont and Genencor, a final 1,3-PD concentration of 135 g/L obtained

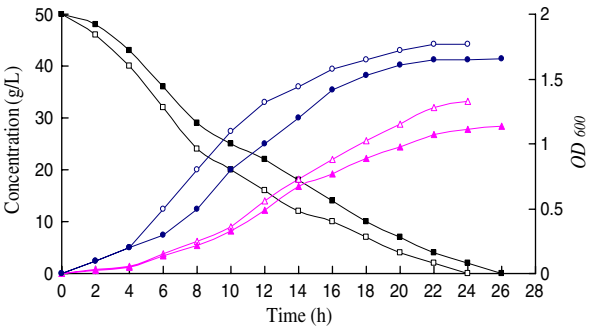

Fig. 4 Time courses of growth, glycerol utilization, and 1,3-propanediol formation in flask fermentation of 5% glycerol by recombinant strain *Klebsiella pneumoniae*/pETPkan-*dha*T and wild-type strain *K. pneumoniae*. Recombinant strain biomass (*open circles*), residual glycerol concentration (*open squares*), 1,3-PD concentration (*open triangles*), wild-type strain biomass (*closed circles*), residual glycerol concentration (*closed squares*), 1,3-PD concentration (*closed triangles*))

Table 2 The comparison of *dha*T expression, 1,3-propanediol yield, and peak value of 3-HPA between *Klebsiella pneumoniae*/pETPkan-*dha*T and wild-type *K. pneumoniae*.

Strains	*dha*T (U/mg protein)	1,3-propanediol (g/L)	Peak value of 3-HPA (mmol/L)
K. pneumoniae	0.85±0.03	28.5±0.2	8.2±0.2
K. pneumoniae/pETPkan-*dha*T	1.64±0.03	33.2±0.3	2.9±0.1

Both strains were cultivated at 37°C for 28 h in fermentation medium containing 50 g/L glycerol. The values showed in the table were averaged from two independent triplicate experiments with means less than 5%.

with transformed *E. coli* was reported [12, 13]. Glycerol can be converted to 1,3-PD by a number of bacteria [14–16], of which *K. pneumoniae* was the most widely used one [17]. It is prospective to improve the 1,3-PD production in *K. pneumoniae* by genetic engineering methods. For example, the recombinant *K. pneumoniae* was constructed to produce 1,3-PD from low-cost feedstock sugar such as D-glucose. Therefore, the construction of efficient expression system in *K. pneumoniae* is important to accomplish this aim.

Recently, *tac* promoter was reported to be used to decrease the accumulation of 3-HPA and improve 1,3-PD production by overexpressing gene *dha*T in *K. pneumoniae* [18]. However, it was a kind of inducible expression promoter, and the protein could be expressed after induction of IPTG, which had a negative effect on the strain growth and 1,3-PD production. So, it is necessary to find an available constitutive promoter to develop the novel expression system in *K. pneumoniae*.

Although limited numbers of constitutive promoters could have been used to direct gene expression in *K. pneumoniae*, the constitutive promoter *Pkan* for construction of novel expression system was reported in this work. To test the transcription activity of promoter *Pkan*, we directly inserted the reporter gene *cat* as selection marker. The recombinant *K. pneumoniae* harboring pETPkan-*cat* exhibited high-level resistance to CM, indicating that pETPkan was efficient for expression of heterologous gene in *K. pneumoniae*. Then, the similar expression system was applied for overexpression of the gene *dha*T encoding PDOR to increase 1,3-PD production by reducing the 3-HPA accumulation. In flask fermentation, the engineered *K. pneumoniae* showed the much lower peak value of 3-HPA and the higher PDOR activity with respected to the wild-type form, and the higher final production of 1,3-PD was obtained by the recombinant strain. The above results indicated that gene *dha*T regulated by *Pkan* promoter was functionally expressed in *K. pneumoniae*. To our knowledge, this is the first report on the construction of constitutive expression system for increasing 1,3-PD production in *K. pneumoniae*. The novel expression is the first step of our attempts. The significance of this work is to pave the way to improve the other heterogenous gene expression in *K. pneumoniae*. But the production of 1,3-PD was

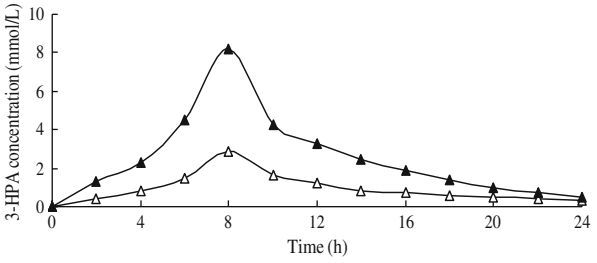

Fig. 5 3-Hydroxypropionaldehyde formation in flask fermentation by recombinant strain *Klebsiella pneumoniae*/pETPkan-*dha*T (*open triangles*) and wild-type strain *K. pneumoniae* (*closed triangles*)

not significantly improved by this *K. pneumoniae* expression system, which may be due to insufficient supply or the cofactor NADH. So, the construction of NADH regeneration system will be our future work to increase the NADH availability and further improve 1,3-PD production in *K. pneumoniae*. Meanwhile, it has been reported that the gene *YqhD* coding alcohol dehydrogenase in *E. coli* has high *dha*T activity [12, 19–21]. To further increase the yield of 1,3-PD in *K. pneumoniae*, we can attempt to import gene *yqh*D or the other genes required for 1,3-PD formation into the wild-type strain. Further works will be continued in our studies.

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