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Simultaneous expression of glutaryl-7-aminocephalosporanic acid acylase gene and lysis genes of phage λ in a recombinant *E. coli*

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Abstract

Glutaryl-7-aminocephalosporanic acid acylase (GLA), recommended for use in the form of immobilized-enzyme, is one of the two key enzymes in the two-step synthesis of 7-aminocephalosporanic acid. For simplifying the process of cell disruption and immobilization, the lysis genes of phage λ (*S*$^{-}$*RRz*) with the *S* amber mutation were designed to introduce into the over-expression system of GLA. A novel recombinant strain, *E. coli* TB1/pMKC-*AS*, simultaneously containing the maltose binding protein gene (*malE*), the lysis genes (*S*$^{-}$*RRz*) and the target GLA gene (*Acy*) in a same operon, was successfully constructed. Under neutral pH conditions, cell growth and GLA activity of TB1/pMKC-*AS* was not affected by the presence of the lysis genes, however, autolysis phenomenon was observed under weak alkaline conditions. Through pH control and fed-batch culture, the GLA activity of TB1/pMKC-*AS* reached as high as 6810 U/L with 24.8 g/L dry cell density (OD_{600} = 67.9) in a 5 L fermentor. In contrast to the cells of *E. coli* TB1/pMKC-*Acy* without the lysis genes, the mild EDTA/Tris buffer (pH 8.0) can cause the lysis of the cells of TB1/pMKC-*AS* containing the lysis genes. Correspondingly, a mild pH 9.0/42 °C incubation method was developed for conveniently degrading the recombinant cells of TB1/pMKC-*AS*, based on the expression of the lysis genes. Further experiments showed that the cell lysate after the mild incubation disruption can be directly immobilized by 10% polyacrylamide to make the immobilized enzymes. In comparison with the immobilized GLA from TB1/pMKC-*Acy*, the immobilized cell lysate of TB1/pMKC-*AS* has the similar characteristics of catalysis stability, implying a great potential for industrial application of the lysis genes-assisted cell disruption.

Keywords: GL-7-ACA acylase; Lysis genes of phage λ; Recombinant *E. coli*; Cell disruption; Immobilization

1. Introduction

7-Aminocephalosporanic acid (7-ACA), a very useful intermediate for synthesizing the industrially important semisynthetic cephalosporin antibiotics, is mainly produced from the well known chemical deacylation of cephalosporin C (CPC), which is hindered by its complicated reaction steps, rigorous reaction conditions and hazardous reagents. To date, a new method of two-step enzymatic transformation of CPC into 7-ACA has been successfully applied in industry. The first step is the oxidative deamination of CPC into glutaryl-7-aminocephalosporanic acid (GL-7-ACA) catalyzed by a *D*-amino acid oxidase (DAAO), and the second step is the subsequent hydrolysis of GL-7-ACA into 7-ACA by a GL-7-ACA acylase (GLA) [1–3]. Another potential enzymatic transformation method is the one-step catalysis of CPC directly into 7-ACA by a CPC acylase, belonging to the same family of cephalosporin acylase together with GLA. However, the activity of CPC acylase is still too low to industry application till now.

During the process of enzymatic transformation of CPC to 7-ACA, the immobilized-enzymes of DAAO and GLA are recommended for use, due to their obvious advantages such as enzyme-reuse, separation facilitation and stability improvement. Recently, the immobilization of GLA was especially emphasized by researchers [4,5], since most of the GLAs consist of two non-identical subunits generated from a common inactive precursor protein [6], and this structure is highly similar to the promising CPC acylase.

To efficiently harvest the intracellular products, a new way of using the cloned bacteriophage lysis genes for cell disruption has been developed [7]. Three genes of phage λ, *S*, *R* and *Rz*, are necessary for the lysis of the recombinant *E. coli* cells [7,8]. Product of gene *S* was responsible for altering the cytoplasmic

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membrane, while *R* and *Rz*, for degrading the host cell wall. By introduction of the *S* amber mutation, the encoding of *S* gene was inhibited to eliminate the conflict of cell growth and cell lysis. In our earlier studies, genes $S^{-}RRz$ had been successfully introduced into a recombinant *E. coli* for the recovery of poly (β-hydroxybutyrate), an important biodegradable polymer [9,10].

A new idea is presented in this paper that the cell disruption can be simplified and the subsequent immobilization of GLA can be facilitated by the co-expression of the lysis genes in a same recombinant *E. coli*, without the extra expense of GLA activity and stability.

2. Experimental

2.1. Enzymes, chemicals, microorganisms and plasmids

Restriction endonucleases, Taq polymerase and T4 DNA ligase were purchased from Takara Biotechnology Co., Ltd. (Dalian). The gene of GLA (*Acy*, 2160bp, GenBank: AY311487) were directly cloned from the *Pseudomonas* sp. in soil in our lab [11]. The corn steep, 7-ACA and GL-7-ACA were supplied by Shi Jia Zhuang Phamacy Co., Ltd., China. Yeast extract was purchased from Beijing Shuang Xuan Company, Ltd. All other reagents used were analytical grade. The microorganisms and plasmids used in this study, containing different antibiotic resistances such as ampicillin (Ap^r) or kanamycin (Kan^r) are listed in Table 1.

2.2. GLA activity assay

Harvest 5 mL cell broth and centrifugate for 5 min at 8000 rpm. Discard the supernatant and then resuspend the pellet in 0.1 M Tris–HCl buffer (pH 8.0). Except specifically mentioned, the cell suspension was frozen–thawed three times then used for the GLA activity assay by calorimetric method [12]. One unit of the GLA activity was defined as the quantity of the enzyme producing 1 μmol 7-ACA per minute.

2.3. Culture conditions

Flask cultivation of the recombinant *E. coli* TB1/pMKC-*AS* and TB1/pMKC-*Acy* was performed at 28 °C for 24 h in the 300 mL conical flasks containing 50 mL of LB (Lauria-Bertani) medium at 200 rpm. For flask culture of TB1/pMKC-*AS*, pH needs to be adjusted in the range of pH 6.0–7.5 using 4N sterile HCl and pH paper. The pH regulation started at around 12 h together with the sample-taking. Fed-batch culture in a 5 L fermentor (Biostat B, B. Broun) was carried out in the semi-defined medium: glucose 10 g/L, corn steep, 20 g/L, yeast extract, 10 g/L, NaCl 10 g/L, K_2HPO_4 15 g/L, pH 7.0, with 2.5 L original loading volume. During the fermentation, the maximum agitation was 600 rpm, and the pH was controlled at 7.0 using 4 mol/L NaOH and 10 mol/L HCl. The concentrated feeding medium was 500 g/L glucose and 500 g/L corn steep, respectively. The fed-speed of glucose was controlled at 15 mL/h, maintaining 5–10 g/L of glucose in the medium. The corn steep was supplemented at 12, 24, 36, 44 and 52 h, respectively, with 10 g/L of feeding-concentration. Temperature was maintained at 37 °C in the early stage, then set down to 28 °C at approximately 24 h, when OD_{600} was approaching 40.

Table 1
Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant genotypes and features	Sources
E. coli TB1	F′*ara* (*pro*AB *lac*$^-$) [ϕ80Δ(*lac*Z) ΔM15] *rps*L(Str^R) *thi hsd*R	NEB
pMAL-p2x	6.7 kb, Amp^r, *malE*	NEB
pMKL	7.3 kb, *malE*, Kan^r	This group [14]
pMKC	6.3 kb, *malE*, *lac*$^-$, Kan^r	This work
pMKC-*Acy*	8.3 kb, *malE*, *lac*$^-$, *Acy*, Kan^r	This group [12,13]
pMKC-$S^{-}RRz$	7.7 kb, *malE*, *lac*$^-$, $S^{-}RRz$, Kan^r	This work
pUC18-$S^{-}RRz$	Amp^r, *lac* promotor, $S^{-}RRz$	This group [8,9]
pMKC-*AS*	9.8 kbp, *malE*, *lac*$^-$, *Acy*, $S^{-}RRz$, Kan^r	This work [14]

2.4. Cell disruption, polyacrylamide immobilization and catalysis stability measurement

Whole-cells of recombinant *E. coli* TB1/pMKC-*AS* and TB1/pMKC-*Acy* were harvested by centrifugation at 4 °C and 4000 rpm for 10 min, or 8000 rpm for 5 min, then resuspended in 2 mM EDTA/50 mM Tris buffer (pH 8.0). The cell-morphology observation before and after the EDTA/Tris buffer treatment was completed by microscopy (XSZ-H, Chongqing Phtoelectricity Instrument Co., China). Cell disruption performed by pH-temperature inducing approach was carried out at pH 9.0, 42 °C for 30 min and then stood at room temperature (RT) for 12 h. The ultrasonication was implemented for 99 cycles at 200 W with duty 3/3 (Model JY92-II, Ningbo Xinzhi Science Apparatus Institute, China).

Cell lysate of *E. coli* TB1/pMKC-*AS* from the mild incubation of pH 9.0/42 °C was directly used for the polyacrylamide (10%) immobilization. Raw enzymes of GLA from *E. coli* TB1/pMKC-*Acy* were salted out and recovered by ammonium sulfate (40%) from the ultrasonication-disrupted cells. Immobilization procedure was performed as followed: prepare 10 mL samples containing 5 mL resuspension of cell-lysate (TB1/pMKC-*AS*) or raw enzymes (TB1/pMKC-*Acy*), 1 g acrylamide, 80 mg Bis (*N*,*N*′-methylene diacrylamide), 0.05 mL *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) and 0.25 mL $(NH_4)_2S_2O_8$ (10%). Polymerize on ice for 2 min, wash one time with water, then cut into small cube of 3 mm × 3 mm × 3 mm or cylinder of 2 mm × 2 mm × 2 mm, finally store at 4 °C in 0.1 mol/L Tris–HCl (pH 8.0) buffer.

The catalysis stability evaluation of the immobilized GLA was performed during a continuous catalysis process. Incubate the 2 mm × 2 mm × 2 mm cylinder of the immobilized GLA with the reaction buffer in an air incubator at 28 °C, 100 rpm. Replace the reaction buffer every 1 or 2 h, while measure the 7-ACA product concentration in the early 15 min when immersing the immobilized enzymes into the fresh reaction solution. Presume the enzymatic activity of the original immobilized GLA as 100%, the relative activity remaining during the continuous

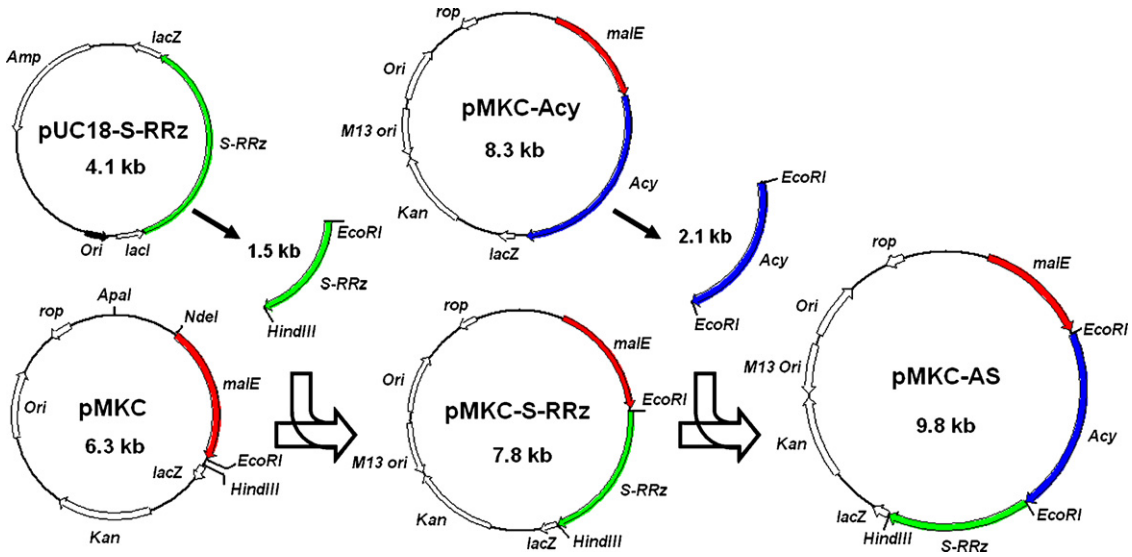

Fig. 1. A simple construction procedure of plasmid pMKC-*AS*. Major symbols denote: *Amp*, ampicillin-resistant marker; *Kan*, kanamycin-resistant marker; *Acy*, GL-7-ACA acylase; *mal*E, MBP-fusion protein; *S*$^{-}$*RRz*, lysis genes of phage λ CI 857; *Ori*, pMB1 origin of replication from pBR322; *rop*, the gene responsible for the control of plasmid copy number.

catalysis was defined to represent the catalysis stability of the immobilized enzyme.

3. Results and discussion

3.1. Construction of the recombinant plasmids and strains containing lysis genes

To over-express GLA in the recombinant *E. coli*, plasmid pMAL-p2x was selected as the initial vector for DNA recombination. This plasmid contains the gene of *mal*E coding for maltose binding protein (MBP), an effective fusion partner capable of promoting the proper folding of the attached protein. Constitutive plasmid pMKC was constructed from pMAL-p2x by replacing the ampicillin resistance with kanamycin, and further deleting the regulatory gene of *lac*I^{q} [14]. Lysis genes of phage λ were cloned from plasmid pUC18-*S*$^{-}$*RRz* constructed previously [8], then inserted into pMKC to form a new plasmid, pMKC-*S*$^{-}$*RRz*. The GLA gene (*Acy*) was amplified from the vector pET-*Acy* [11] by PCR, and the target plasmid, pMKC-*AS*, was finally constructed by inserting *Acy* into the plasmid pMKC-*S*$^{-}$*RRz* at *EcoR* I site, as shown in Fig. 1. Briefly, plasmid pMKC-*AS* contained three structural genes, i.e. *malE*, *Acy* and *S*$^{-}$*RRz* in the same *tac* operon, as depicted in Fig. 2.

Consequently, a novel recombinant strain, *E. coli* TB1/pMKC-*AS*, was used for the followed cell-disruption and immobilization studies. Meanwhile, another recombinant strain, *E. coli* TB1/pMKC-*Acy* in the absence of the lysis genes, was selected as the control.

Fig. 2. A sketch map of the linear representation of the *tac* operon containing *malE*, *Acy* and *S*$^{-}$*RRz* in plasmid pMKC-*AS*. Symbol denote: *tac*, the plasmid *tac* promoter.

3.2. Impacts of the lysis genes' expression in E. coli TB1/pMKC-AS

Parallel cultures of *E. coli* TB1/pMKC-*AS* and TB1/pMKC-*Acy*, with and without the lysis genes, were carried out in flask to investigate the impacts of the lysis genes' expression. Time profiles of the pH changes, cell growth and GLA activity accumulation were measured and plotted in Fig. 3.

From Fig. 3, it was obviously found that there was no significant difference on GLA activity between TB1/pMKC-*AS* and TB1/pMKC-*Acy*, implying that no negative impacts were generated by the foreign lysis genes on the accumulation of the GLA

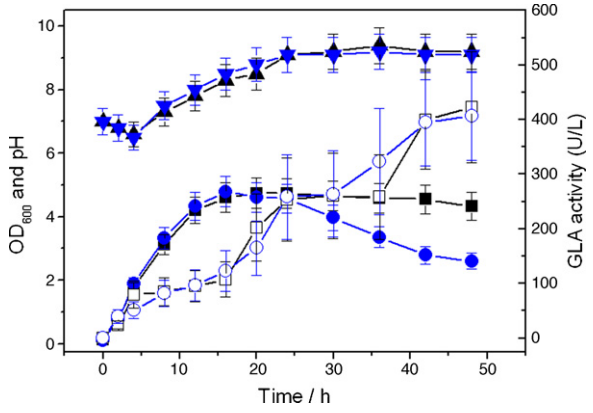

Fig. 3. Time profiles of the cell growth of TB1/pMKC-*Acy* (■) and TB1/pMKC-*AS* (●), the GLA activity accumulation of TB1/pMKC-*Acy* (□) and TB1/pMKC-*AS* (○), and the pH changes of TB1/pMKC-*Acy* (▲) and TB1/pMKC-*AS* (▼) at 28 °C in shaking flask. Experiments were carried out in duplicate.

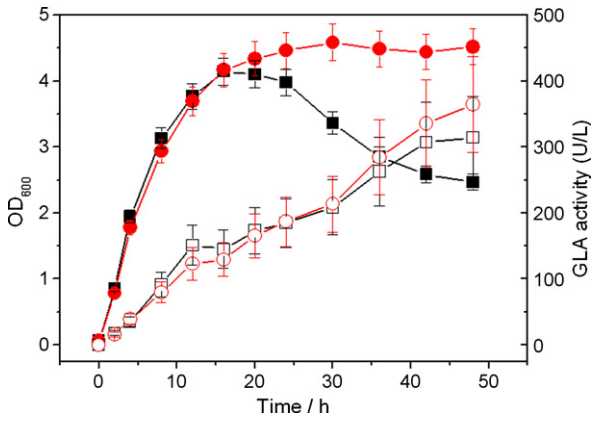

Fig. 4. Time profiles of the cell growth of TB1/pMKC-*AS* with (●) and without (■) pH control, and the GLA activity accumulation with (○) and without (□) pH control. Experiments were carried out in duplicate.

activity. However, the introduction of the lysis genes reduced the OD_{600} of TB1/pMKC-*AS* after about 24 h, which correspondingly accompanied with the pH increase to 8.5 in broth. Repeated cultures of TB1/pMKC-*AS* were run with and without pH control in the range of 6.0–7.5. The cell growth and GLA accumulation were evaluated again as indicated in Fig. 4. The results showed that the OD_{600} drop of the TB1/pMKC-*AS* did not occur during the whole stationary phase after pH control, significantly different from the OD_{600} decrease of the natural pH culture. This means that the alkaline pH can induce the cell lysis in the presence of the lysis genes. Fortunately, this negative impact can be effectively eliminated by the simple pH control.

Fed-batch culture of the recombinant *E. coli* TB1/pMKC-*AS* was further carried out in a 5 L fermenter, using glucose as the carbon source and corn steep as the major nitrogen source. Based on the results of Figs. 3 and 4, the neutral pH 7.0 was automatically controlled throughout the fermentation. Glucose and corn steep were supplemented as stated in the previous "culture conditions". After 60 h fed-batch culture, the total activity of GLA, cell concentration and specific activity of GLA highly reached 6810 U/L, 24.8 g/L (dry cell) and 275 U/g (dry cell), respectively (Fig. 5).

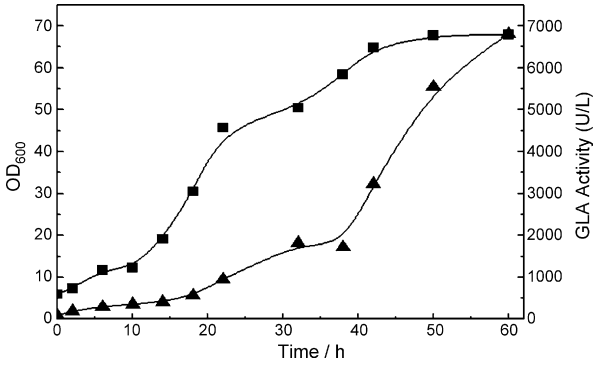

Fig. 5. Fed-batch fermentation of *E. coli* TB1/pMKC-*AS* in a 5 L fermentor. Earlier temperature was set at 37 °C until OD_{600} (■) was higher than 30. At about 24 h, the temperature was lowered to 28 °C to accumulate the GLA activity (▲).

3.3. Controllable cell lysis of *E. coli* TB1/pMKC-AS based on the introduction of the lysis genes

To review the function of the lysis genes, the recombinant cells of both TB1/pMKC-*AS* and TB1/pMKC-*Acy* grown for 24 h in the flask were harvested and resuspended in 2 mM EDTA/50 mM Tris buffer, pH 8.0. The cell-morphology before and after EDTA treatment was observed by microscopy, as shown in Fig. 6. Basically, the mild incubation of EDTA/Tris buffer cannot induce the lysis of the TB1/pMKC-*Acy* cells (Fig. 6A and B), but it's effectual for TB1/pMKC-*AS* containing the lysis genes (Fig. 6C and D). In Fig. 6D, it could be found that a great amount of recombinant cells of TB1/pMKC-*AS* cannot stay intact after the treatment. That is, the cells of TB1/pMKC-*AS* were easier to be disrupted than those of TB1/pMKC-*Acy*, due to the valid expression of the lysis genes of phage λ. Here the function of the EDTA/Tris buffer for TB1/pMKC-*AS* was altering the characteristics of cytoplasmic membrane and enabling the *R* and *Rz* products to pass through then digest the cell wall, which is just similar to the action of the *S* gene product before the amber mutation.

Considering the recombinant cells of TB1/pMKC-*AS* were prone to be disrupted due to the introduction of the lysis genes, it is promising to develop a mild cell-disruption method for the large-scale application. Therefore, different mild approaches enabling cell lysis were further investigated using the traditional ultrasonication and frozen–thawed disruption as the reference, as listed in Table 2. Because of the alkaline sensitive feature of TB1/pMKC-*AS* observed in Figs. 3 and 4, the mild pH-temperature incubation method was specially emphasized.

Results in Table 2 showed that the ultrasonication was the best approach to disrupt the cell-wall. It's a pity that it can't be efficiently scaled up for industry application. From the economic point of view, the frozen-thawed method and EDTA/Tris buffer treatment were also not preferred. Consistent with the results in Fig. 3, alkaline condition is one of the key factors determining the lysis efficiency. In comparison with the pH 9.0 condition, pH 10.0 incubation did not generate significant improvement. Temperature is another important factor affecting the cell lysis. Results showed that the phase-transition temperature of the cell wall, 42 °C, is impactful for the cell lysis. Higher temperature

Table 2
Comparison of different mild lysis treatments of TB1/pMKC-*AS*

Treatment methods	OD_{600}		
	Original	After treatment	Remaining (%)
Ultrasonication		1.09	23.9
Frozen–thawed, 1 time		2.58	56.5
2 mM EDTA/50 mM Tris buffer (pH 8.0)		3.43	75.1
RT, 30 min, pH 9.0[a]	4.57	3.71	81.2
RT, 30 min, pH 10.0[a]		3.66	80.1
37 °C, 30 min, pH 7.0		4.21	92.1
42 °C, 30 min, pH 7.0		3.79	82.9
42 °C, 30 min, pH 9.0		2.83	61.9
42 °C, 30 min, pH 9.0, RT, incubate 12 h[a]		2.44	53.4

[a] *Note*: RT represents "room temperature".

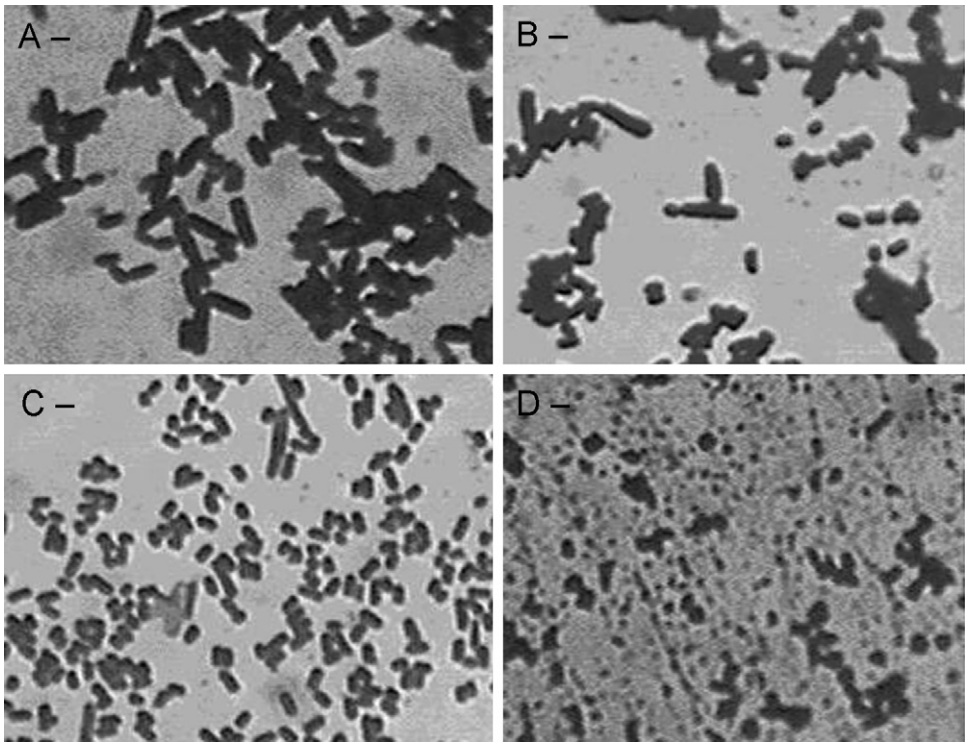

Fig. 6. Micrograph of cell morphology of *E. coli* TB1/pMKC-*Acy* (A and B) and TB1/pMKC-*AS* (C and D) before and after EDTA/Tris buffer treatment, respectively. Bar represents 2 μm (1600×).

was not considered in case the inactivation of GLA. When combined the pH 9.0 and 42 °C incubation together for 30 min, then stood at room temperature overnight, the OD_{600} of TB1/pMKC-*AS* significantly dropped down, at last basically comparable to the frozen-thawed treatment.

Further experiments on the mild disruption of TB1/pMKC-*AS* were carried out to evaluate the effect of both cell lysis and enzyme activity improvement, in parallel using the cells of TB1/pMKC-*Acy* as the control. Define the original OD_{600} and GLA activity of both strains as 100%, the relative OD_{600} remaining and the GLA activity elevating were measured and plotted in Fig. 7, after the treatment of pH 9.0/42 °C incubation, frozen–thawed and ultrasonication, respectively. It could be found that both of the mild disruption approaches, pH 9.0/42 °C incubation and frozen-thawed, generated more significant impacts on the cell disruption and activity improvement of TB1/pMKC-*AS*, indicating that the lysis genes products are helpful during the mild cell disruption.

3.4. Immobilization and stability evaluation

According to the experimental methods in Section 2.4, the pH 9.0/42 °C overnight incubated cell lysate of TB1/pMKC-*AS* was immobilized by 10% polyacrylamide. The immobilized samples were cut into small cylinders or cubes, as shown in Fig. 8. Generally, the measured activity of the small cylinder (2 mm × 2 mm × 2 mm) was higher than that of cube (3 mm × 3 mm × 3 mm), indicating that the diffusion resistance of the GL-7-ACA substrate and 7-ACA product in the immobilized enzymes was an significant factor affecting the yield of the target enzyme activity.

Similarly, the raw enzymes of GLA from TB1/pMKC-*Acy* were immobilized by 10% polyacrylamide and divided into 2 mm × 2 mm × 2 mm cylinder. A continuous transformation of GL-7-ACA to 7-ACA was run at 28 °C for 36 h using both of the cylinder-type immobilization GLA as the catalysts. As shown in Fig. 9, it was promisingly that the GLA activity of the

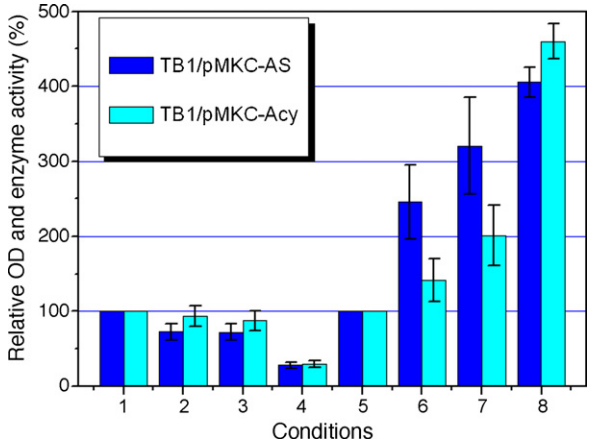

Fig. 7. Comparison of the OD_{600} decrease and GLA activity increase caused by different cell-lysis approaches. Conditions denote: (1) original OD_{600} of both strains; (2) OD_{600} remaining of pH 9.0/42 °C, 30 min incubation; (3) OD_{600} remaining of 1-time frozen-thawed treatment; (4) OD_{600} remaining of ultrasonication disruption; (5) original GLA activity of both strains; (6) improved activity of pH 9.0/42 °C, 30 min incubation; (7) improved activity of 1-time frozen-thawed treatment; (8) improved activity of ultrasonication disruption.

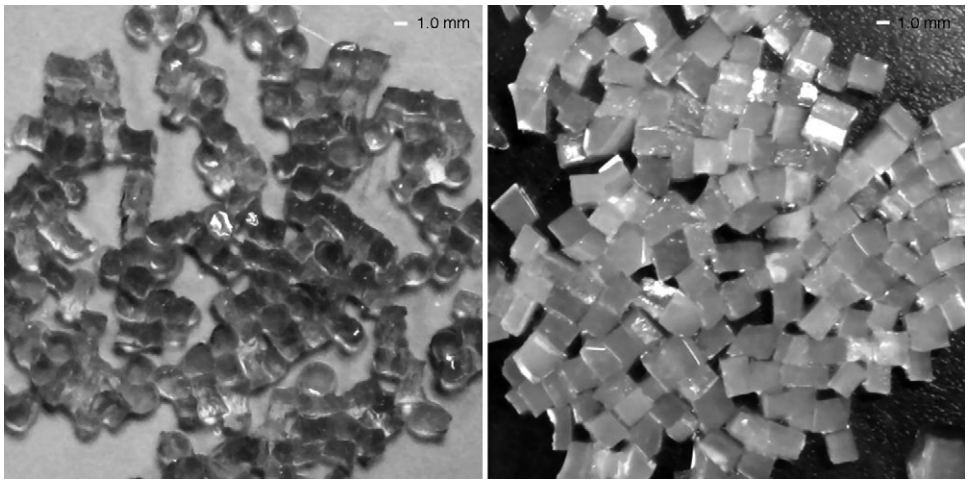

Fig. 8. Photograph of polyacrylamide immobilized cell lysate of *E. coli* TB1/pMKC-*AS*. Left, 2 mm × 2 mm × 2 mm cylinder; right, 3 mm × 3 mm × 3 mm cube. Bar represents 1.0 mm. The detailed immobilization procedure was depicted in Section 2.4.

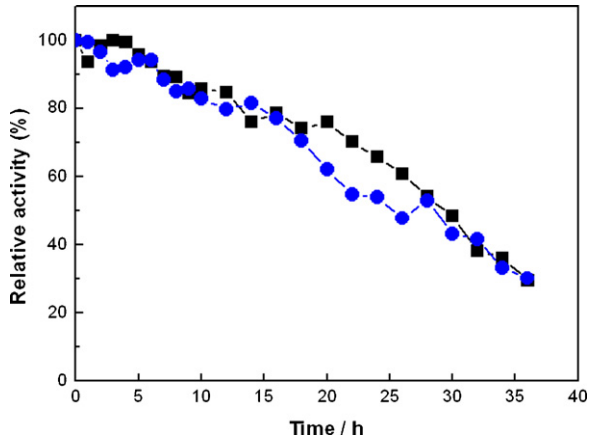

Fig. 9. Stability evaluation of the immobilized cell lysate of *E. coli* TB1/pMKC-*AS* (■) and raw enzymes of *E. coli* TB1/pMKC-*Acy* (●). The detailed evaluation procedure was stated in Section 2.4.

immobilized cell lysate of TB1/pMKC-*AS* was as stable as the immobilized enzymes from TB1/pMKC-*Acy*, implying that the mildly disrupted cell lysate of TB1/pMKC-*AS* can be directly used for the immobilization process of the target enzyme, consequently enabling the great reduction of the GLA separation cost.

4. Conclusions

The lysis genes of phage λ were successfully introduced into a GL-7-ACA acylase (GLA) over-expression recombinant strain, *E. coli* TB1/pMKC-*AS*, located in the same operon of *malE* and *Acy*. Under conditions of pH control, cell growth and GLA accumulation of TB1/pMKC-*AS* behaved no significant difference from the lysis-gene-free strain, *E. coli* TB1/pMKC-*Acy*. High GLA activity together with high cell density fermentation of TB1/pMKC-*AS* in a 5 L fermentor was accomplished with the GLA activity of approximately 6800 U/L. Based on this, a mild pH 9.0/42 °C incubation method was figured out for simple and convenient cell disruption in the presence of the lysis genes' expression, and the cell lysate was directly used for the polyacrylamide immobilization. Further results indicated that the immobilized cell lysate exhibits the same catalysis stability with the immobilized enzymes of GLA from TB1/pMKC-*Acy*. Consequently, the lysis genes assisted cell disruption is highly promising for extended application in the industrial separation and immobilization of the enzymes.

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