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Cloning of amidase gene from *Rhodococcus erythropolis* and expression by distinct promoters in *Bacillus subtilis*

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ABSTRACT

Amidase was a crucial enzyme responsible for the conversion of acrylamide to acrylic acid in *Rhodococcus erythropolis*. Its coding gene *ami* was amplified by PCR using the genomic DNA of *R. erythropolis* as template. Subsequently, it was ligated to expression plasmids and transformed in *Escherichia coli* and *Bacillus subtilis*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed that both recombinant *E. coli* BL21 (DE3) and *B. subtilis* generated amidase of 56 kDa. The expression mass and enzyme activity suggested that *B. subtilis* was more suitable as a host when *ami* gene was under the control of a powerful promoter. To further study the expression effect of different promoters in *B. subtilis*, five distinct promoters (*sacB*, *amyE*, *p43*, *degQ*, *aprE*) and their native signal peptide genes were employed to separately construct five different vectors harboring *ami* gene. Of the five novel vectors, the *amyE* promoter along with its native signal peptide gene was most effective. The maximum specific activity of amidase at pH 7.0 and 37 °C was about 8.7 U/mg and the conversion efficiency could approximately reach 90% within 6 h. This result indicated the expression difference of distinct promoters, which provided the basis for the forthcoming research.

1. Introduction

As a valuable chemical intermediate, acrylic acid is extensively used in chemical industry. Acrylic acid and its esters are the primary building blocks of all acrylate polymers and plastics [1]. Nowadays, it is over one billion pound per year industrial feedstock derived from petroleum [2] by chemical synthesis which mainly focuses on oxidation of propane and propylene over vanadyl pyrophosphate [3]. However, this method is difficult and costly. Therefore, a feasible microbial fermentation is under development.

It is reported that in some microorganisms, acrylamide can be used as the carbon and energy sources, as a result, the corresponding acid of acrylamide–acrylic acid is biosynthesized. In this metabolic pathway, the amidase is responsible for the conversion from acrylamide to acrylic acid. Amidases from various microorganisms including *Pseudomonas*, *Brevibacterium*, and *Rhodococcus* have been successfully employed as industrial biocatalysts to conduct this conversion [4]. Although present research has focused on the cloning and expression of amidase gene in *Escherichia coli*, the enzyme activity and the yield of acrylic acid are disappointing due to the inactivity and insolubility of intracellular enzyme [6–8].

Bacillus subtilis is an attractive host for the expression of heterologous proteins because the absence of exterior membrane simplifies the protein secretion pathways and therefore allows higher secretion levels of extracellular proteins. Moreover, *B. subtilis* is regarded as an ideal expression system due to its nonpathogenic trait, well-established fermentation technology and ripened genetic manipulation as well as multiple regulators which possibly influence expression and secretion [9]. Previous reports paid much attention to the expression of *ami* gene in *E. coli*, however, little is known about the expression of *ami* in *B. subtilis*.

In this study, the *ami* gene from *R. erythropolis* was cloned and expressed both in *E. coli* and *B. subtilis*, and the expression effect as well as enzyme activity in each host were analyzed and compared. Five distinct promoters and their native signal peptides were respectively applied to undertake *ami* expression. As a result, the most suitable promoter and its native signal peptide gene were employed for engineering strain.

2. Materials and methods

2.1. Bacterial strains, plasmids and enzymes

Bacterial strains and plasmids were listed in Table 1. *Escherichia coli*, *Bacillus subtilis*, and *Rhodococcus erythropolis* were grown in Luria–Bertani (LB) liquid medium or on LB agar at 37 °C.

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Table 1
strains and plasmids used in this work

Strains/plasmids	Description	Source
Strains		
E. coli DH5α	*F−*, *endA1*, *hsdR17*(*rk−*, *mk+*), *supE44*, *thi-1*, *λ−*, *recA1*, *gyrA9*, *ΔlacU196*, *φ80dlacZΔM15*	This laboratory
E. coli BL21(DE3)		Novagen
R. erythropoli		This laboratory
B. subtilis	*his*, *nprR2*, *nprE18*, *aprA5*, *epr*	Gift from Professor Jianguo Yang
Plasmids		
pMD18-T	Cloning vector of *ami* gene used in *E. coli*	Takara
pET-22b	Expressing vector of *ami* gene used in *E. coli*	Novagen
pGj103	Expressing vector of *ami* gene used in *B. subtilis*	This laboratory

100 μg ml^{-1} ampicillin or 170 μg ml^{-1} chloramphenicol was added for maintaining the plasmids.

Alkaline phosphatase, restriction enzymes (EcoRI and SacI) and *Ex T*aq DNA polymerase were purchased from Takara Bio, Inc. Gene sequencing was conducted by Beijing Sunbiotech Co. Ltd. All other reagents were commercial products of analytical grade from standard suppliers.

2.2. Gene manipulation

When harvested at the late log phase in LB liquid medium at 37 °C for 20 h, *R. erythropolis* and *B. subtilis* were collected for extracting genomic DNA. Other DNA manipulations were carried out according to standard protocol [10] unless additional stated.

2.3. Cloning and expression of ami Gene in E. coli

The genomic DNA of *R. erythropolis* was used as the template and the *ami* gene was obtained by PCR amplification. The relevant primer sequences and digest sites were listed in Table 2. The PCR program was described as follows: 1 cycle at 94 °C for 5 min; 30 cycles of 94 °C for 1 min; 56 °C for 30 s; 72 °C for 1.5 min; as well as a final extension step at 72 °C for 10 min. After sequencing the *ami* gene, the recombination plasmid pET-22b-ami was constructed and transformed into *E. coli* BL21 (DE3). Positive strains were inoculated into 100 ml LB medium containing 100 μg ml^{-1} ampicillin for 5 h. After inducing with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), the recombinant *E. coli* BL21 (DE3) was cultured at 22 °C overnight.

2.4. Expression of ami in B. subtilis by five distinct promoters

sacB, *amyE*, *p43*, *degQ* and *aprE* promoters and their native signal peptide genes were inquired from NCBI. As promoters and their native signal peptide gene sequences were contiguous and just before their inherent functional genes, PCR amplification of 400 bp before their respective functional genes could include the demanded promoters and signal peptide gene sequences. *B. subtilis* genome was applied as template for cloning these five promoters. The resulting five promoters were respectively ligated with *ami* gene by overlap PCR. The relevant primer sequences (P3–P22) and digestion sites were also listed in Table 2. The PCR program for amplifying promoters was stated as above in Section 2.3. The forward and the reversed primers were added to reaction solution after the 10th cycle. Other manipulations were the same as previous description.

2.5. Determination of amidase concentration and optimizing reaction condition for enzyme activity

The positive *B. subtilis* strains were cultured in 100 ml LB medium containing 170 μg ml^{-1} chloramphenicol. After induced overnight, the supernatant fluid was separated by centrifuge and concentrated by ultra filtration. The amidase expressed in *B. subtilis* was secreted into the culture because of the signal peptide;

Table 2
Primers and digest sites used in this work

Primes	Seq. no.	Sequence	Restriction enzyme
ami	P1	5′-ACCT*GAGCTC*ATGGCGACAATCCGAC-3′	SacI
	P2	5′-AATT*GAGCTC*CTAGGCGGGGCTGAGTTGT-3′	SacI
sacB	P3	5′-GCGGGT*GAATTC*GATATTTTCTGAATTGTGATT-3′	EcoRI
	P4	5′-GTCGTCAGGTCGGATTGTCGCCAT*CTGCAG*CGTTCATGTCT-3′	PstI
ami (ligated with *sacB*)	P5	5′-TACATAAAAAAGGAGACATGAACG*CTGCAG*ATGGCGACAATCCG-3′	PstI
	P6	5′- ATGT*GAGCTC* CTAGGCGGGGCTGAGTTGTGGTG-3′	SacI
amyE	P7	5′-GGCG*GAATTC*ACGATGTACGACAGGGGG-3′	EcoRI
	P8	5′-AATCAAATAAGGAGTGTCAAGA*CTGCAG*ATGGCGACAAT-3′	PstI
ami (ligated with *amyE*)	P9	5′-AATCAAATAAGGAGTGTCAAGA*CTGCAG*ATGGCGACAAT-3′	PstI
	P10	5′-ATGT*GAGCTC* CTAGGCGGGGCTGAGTTGTGGTG-3′	SacI
p43	P11	5′-CGCC*GAATTC*TGATAGGTGGTATGTTTTCGC-3′	EcoRI
	P12	5′-GTCGTCAGGTCGGATTGTCGCCAT*CTGCAG*CTGAGGCATGTGTTACAAA-3′	PstI
ami (ligated with *P43*)	P13	5′-TTTGTAACACATGCCTCAG*CTGCAG*ATGGCGACAAT-3′	PstI
	P14	5′-ATGT*GAGCTC* CTAGGCGGGGCTGAGTTGTGGTG-3′	SacI
degQ	P15	5′-GCGG*GAATTC*TTCATCTTCAAGATCATC-3′	EcoRI
	P16	5′-GTCGTCAGGTCGGATTGTCGCCAT*CTGCAG*GATGATCTTGAAGATG-3′	PstI
ami (ligated with *degQ*)	P17	5′-AGACTTGTTTCCAAGTCTTTTT*CTGCAG*ATGGCGACAAT-3′	PstI
	P18	5′-ATGT*GAGCTC* CTAGGCGGGGCTGAGTTGTGGTG-3′	SacI
aprE	P19	5′-GTAGC*GAATTC*AGCTTTACAAGCGGACGGTTCG-3′	EcoRI
	P20	5′-GTCGTCAGGTCGGATTGTCGCCAT*CTGCAG*TAGTAAAAAGAAGCAGG-3′	PstI
ami (ligated with *aprE*)	P21	5′-AGGAACCTGCTTCTTTTTACTA*CTGCAG*ATGGCGACAAT-3′	PstI
	P22	5′-ATGT*GAGCTC* CTAGGCGGGGCTGAGTTGTGGTG-3′	SacI

Table 3
The sequences of *sacB*, *amyE*, *degQ* and *aprE* promoters

promoter	Sequence[12]
sacB	tctttaggcccgtagtctgcaaatccttttatgattttctatcaaacaaaagaggaaaatagaccagttgcaatccaaacgagagtctaatagaatgaggtcg
amyE	agggaagcgttcacagtttcgggcagctttttttataggaacattgatttgtattcactctgccaagttgttttgatagagtgattgtgataatttttaaa
degQ	attcctttgagtataaggacctattgagatttgcggtgtcacgcaggacttttttgcatacttttcggtgaaaaatgagccgaaagcagacacactattag
aprE	gagtctctacggaaatagcgagagatgatatacctaaatagagataaaatcatctcaaaaaaatgggtctactaaaatattattccatctattacaattaaattcca

The −35 and −10 regions were underlined and +1 site was highlighted in italics.

therefore, the ultra filtration of supernatant fluid was an easy way to obtain amidase.

The positive *E. coli* strains were inoculated into 100 ml LB medium containing 100 μg ml^{-1} ampicillin as mentioned above. However, after inducing, the expressed amidase existed in the form of soluble protein and inclusion body in *E. coli*. The refolding of inclusion body was necessary after the harvest cell was broke by ultrasonic.

As amidase was a sort of protein, the concentration of amidase was determined according to Bradford's method [5]. The amidase enzyme activity was determined under the condition of 37 °C, pH 7.0 according to Sang-Hoon Kim and Patrick Oriel's method [6]. An appropriate amount acrylamide was catalyzed by amidase. Low concentration of Mg^{2+} was added for enhancing the enzyme activity. After incubation at 37 °C for 10 min, the enzyme reaction was terminated by being heated at 90 °C for 15 min.

As Sang-Hoon Kim and Patrick Oriel's method, amidase activity was obtained according to the measure of acrylic acid by High performance Liquid Chromatography (HPLC) equipped with C-18 reverse phase column. 5 mM acetic acid and methanol were chosen as elution phase at the radio of 7:3. Peak was detected at absorbance of 210 nm. One unit of amidase was defined as the mount of enzyme that catalyzed acrylamide to form 1 μmol acrylic acid per minute.

2.6. Determination of specific enzyme activity and expression mass

The method and manipulation of determination of specific enzyme and expression mass were the same as what described in Section 2.5.

The calculation formula for specific enzyme activity was one unit of amidase activity divided by the weight of one unit of amidase.

2.7. Determination of molar conversion of amidase expressed in *B. subtilis*

The method and manipulation for determining molar conversion of amidase expressed in *B. subtilis* were also the same as that described in Section 2.5.

The calculation formula for molar conversion was the yielded molar of acrylic acid divided by the total molar of original acrylamide.

2.8. Sequence analysis of distinct promoters used in *B. subtilis*

The expression effect of amidase in *B. subtilis* by using different promoters was obviously distinguishing. To further explore this reason, the sequences of the four promoters (*sacB*, *amyE*, *degQ* and *aprE*) were inquired, which were relevant to sigma *A* factor. Each promoter sequence was listed in Table 3.

3. Results

3.1. Cloning of ami Gene and sacB, amyE, p43, aprE and degQ promoters

The *ami* gene and *sacB*, *amyE*, *p43*, *aprE* and *degQ* promoters were PCR amplified (Fig. 1). DNA sequencing revealed that *ami* gene was 1651 bp in length, starting with ATG and ending with TAG, which encoded 521 amino acid residues with predicted molecular weight of 56 kDa. As mentioned above each promoter with its native signal peptide gene was amplified as 400 bp, starting with gat, acg, tga, agc and ttc and terminating with acg, aga, cag, cta and ttt, respectively. The signal peptides would be cut off after going through the cell membrane.

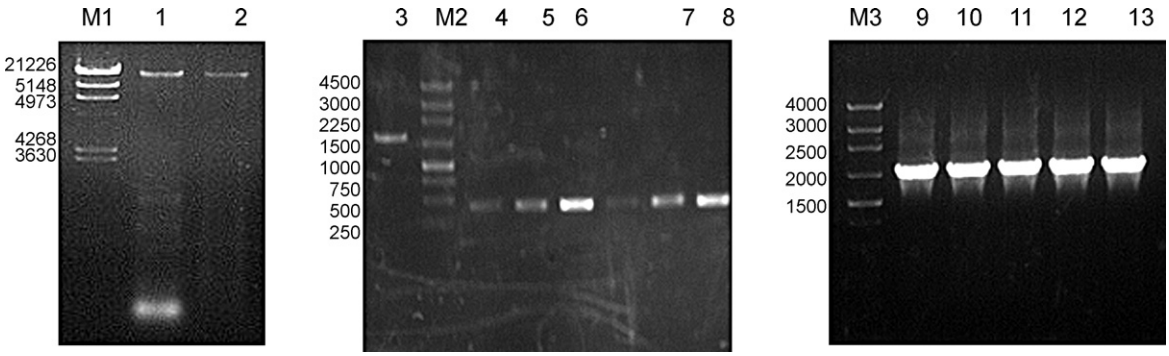

Fig. 1. Relevant DNA fragments in this work. M1, M2 and M3 represented marker 250, marker III and marker DGL4000; bands 1 and 2 were *R. erythropoli* genome and *B. subtilis* genome; bands from 3 to 8 represented the PCR products of *ami* gene, *sacB*, *amyE*, *p43*, *aprE* and *degQ* promoter, respectively. Bands from 9 to 12 indicated the overlap PCR results of *ami* gene with *sacB*, *amyE*, *p43*, *aprE* and *degQ*, respectively.

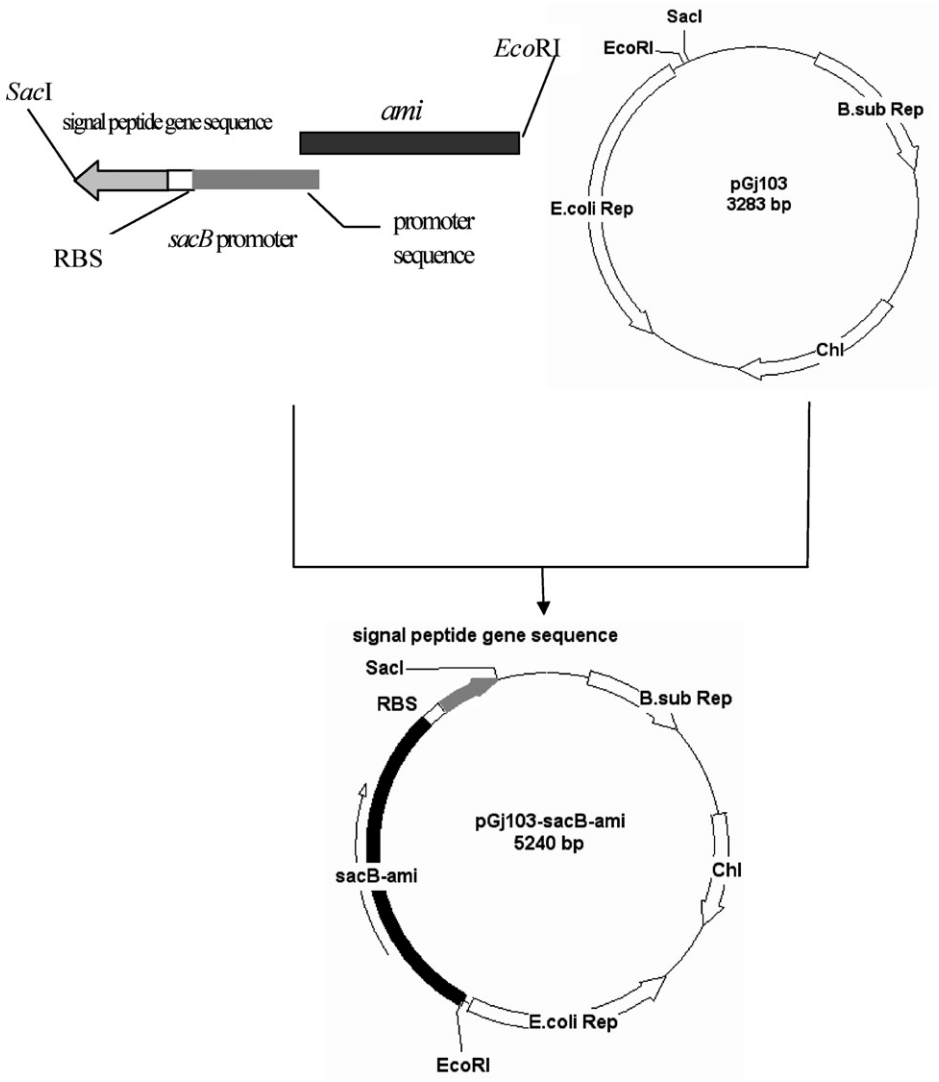

Fig. 2. Schematic diagram of the constructed plasmids in *B. subtilis*. The promoter *sacB* could be replaced with *amyE*, *p43*, *degQ* and *aprE*.

3.2. Construction of recombinant expression vectors

Novel vectors were constructed as Fig. 2. Shuttle plasmid pGj103 was employed as the native vector. Five distinctive promoters were applied to regulate the expression of *ami* gene in *B. subtilis*, respectively. After connected with *ami,* each promoter was ligated with pGj103 by digest sites EcoRI and SacI. The ribosome binding site sequences were also cloned from *B. subtilis* corresponding to their respective promoters, residing immediately after the end of promoters but in front of signal peptide gene sequences in novel constructed pGj103 plasmids to enhance the translation efficiency of *ami*.

3.3. Expression of ami Gene in E. coli and B. subtilis

According to the method of Laemmli [11], SDS-PAGE analysis of *E. coli* BL21 (DE3) total protein were performed and showed as Fig. 3.

Since the amidase expressed in *B. subtilis* were secreted into medium because of signal peptides, the protein for SDS-PAGE analysis were separated from supernatant fluid. Using the same amount of sample, SDS-PAGE analysis shown different promoters leaded to different levels of expression just as Fig. 3.

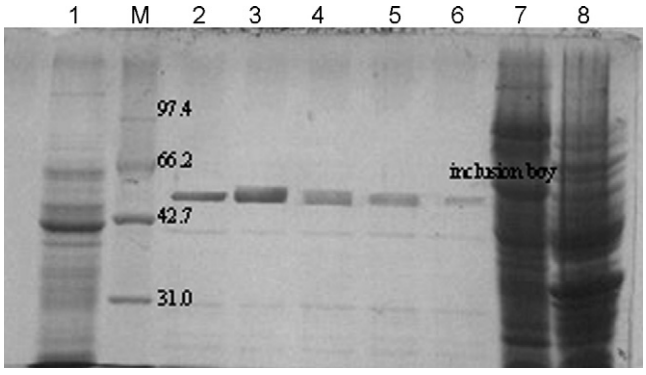

Fig. 3. SDS-PAGE analysis of amidase expression. Bands 1 and 8 were control experiments; (1) was total protein of wild strain *B. subtilis* and (8) was total protein of original *E. coli* BL21(DE3); M represented marker with molecular weight from 14 k to 96 k; samples from bands 2 to 6 were all amidase expressed in *B. subtilis* separated from each culture with the same volume of supernatant fluid; band 2 was amidase expressed under the control of *sacB* promoter; band 3 was that under the control of *amyE* promoter; bands 4–6 were respectively under the guide of *p43*, *degQ* and *aprE* promoter; band 7 was the total protein from the gene engineering *E. coli* BL21 (DE3), amidase was expressed and the inclusion body could be observed.

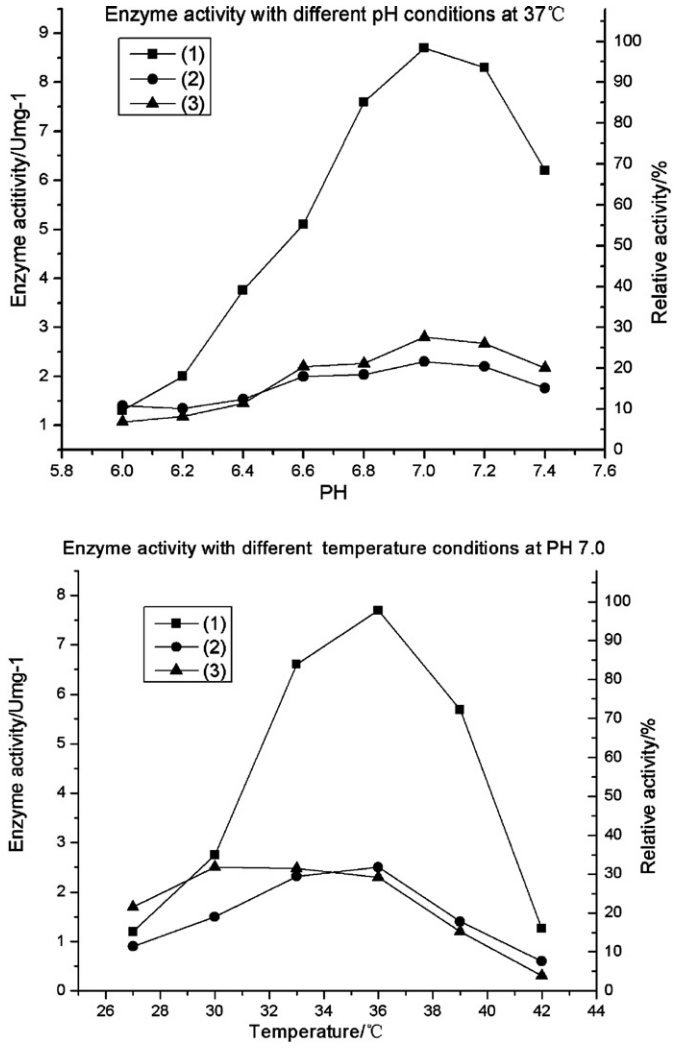

Fig. 4. Effect of temperature (a) and pH (b) on amidase activity (■, ▲, and ●). (■) The enzyme activity of amidase expressed in recombinant *B. subtilis*; (▲) represented that expressed in recombinant *E. coli* BL21(DE3) and (●) in wild strain *R. erythropolis*. The buffers used were: NaH_2PO_4/Na_2HPO_4 (pH 6.0–7.4). The influence of pH was determined by incubating the amidase in buffers of various pH values for 15 min at 37 °C. The affect of temperature was measured under constant conditions except for the temperature, which was ranged from 27 °C to 42 °C.

The molecular weight of expression protein was approximately 56 kDa, harboring 521 amino acid, consistent with the putative result. Unfortunately, inclusion body of amidase was formed in *E. coli*.

3.4. Enzyme activity assay

The amidase expressed in recombinant *E. coli* BL21 (DE3), recombinant *B. subtilis* and wild strain *R. erythropolis* were all testified. To determine the optimum temperature and pH of the amidase activity, various temperatures and pH values of reacting buffer were alternatively investigated. It was also found that low concentration of Mg^{2+} was necessary for enzyme activity. The amidase showed maximum activity under the condition of pH 7.0 at 37 °C (Fig. 4).

3.5. The enzyme activity and expression mass of different expression system

The amidase expressed in *B. subtilis* was all secreted into the culture for the presence of signal peptide, while in *E. coli*, it was

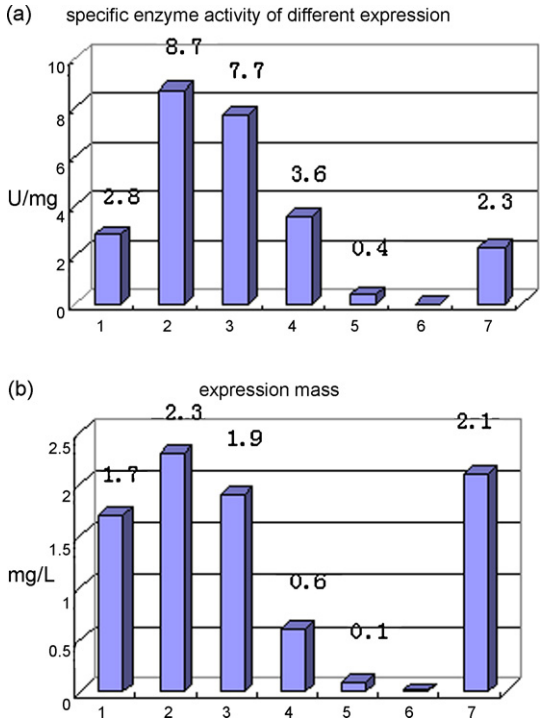

Fig. 5. Specific enzyme activity (a) and expression mass (b) of different strains. Column from 1 to 6 in Fig. 5 represented amidase expressed in *E. coli*, in *B. subtilis* with *amyE* promoter, in *B. subtilis* with *sacB* promoter, in *B. subtilis* with *p43* promoter, in *B. subtilis* with *degQ* promoter and in *B. subtilis* with *aprE* promoter, respectively. Column 7 was amidase expressed in wild strain *R. erythropolis* without any gene manipulation. As the conditions of *R. erythropolis* culture and amidase separation from *R. erythropolis* were different from those of *B. subtilis* and *E. coli*, the measure values of specific enzyme activity and expression mass of amidase expressed in wild strain *R. erythropolis* displayed some disaccord. The measure values were marked in figure, while in the *B. subtilis* of *aprE* promoter product can be just detected. The experiment was performed at 37 °C and pH 7.0.

accumulated in vivo in the form of soluble protein and inclusion body as SDS-PAGE showed.

From Fig. 5, the specific enzyme activity and expression mass of amidase expressed in *E. coli* did not reveal apparent difference from that in wild strain *R. erythropolis*. While in *B. subtilis* expression system, the *ami* gene under the control of *amyE* promoter displayed the highest enzyme activity at 37 °C with pH 7.0, which was approximately 4 times higher than that of wild strain *R. erythropolis*. Whereas, the expressed protein under the control of *aprE* promoter demonstrated the lowest expression effect and lowest enzyme activity, which could merely be detected.

Based on the results of Fig. 5, the enzyme activity of amidase expressed in *E. coli* could not compete with that expressed in *B. subtilis* which contained *pGj103-amyE-ami* plasmid or *pGj103-sacB-ami* plasmid. This finding suggested that *B. subtilis* as a host was more suitable to express *ami* gene. Furthermore, the strong promoters of *amyE* or *sacB* also played a crucial role in its high level expression. Therefore, it is suggested to employ a strong promoter to express *ami* gene.

3.6. The determination of conversion efficiency

As another significant factor to weigh the amidase activity, the conversion efficiency was revealed as Fig. 6. The amidase for this assay was from *B. subtilis* harboring *amyE* promoter. The reaction condition was at 37 °C with pH 7.0, the same as that mentioned in the determination of enzyme activity in Section 2.5. The conversion efficiency from acrylamide to acrylic acid was approximately 90%

Table 4
The characters of *sacB*, *amyE*, *degQ* and *aprE* promoter

Promoter	−10 region sequence	Calculated ΔG of −10 region	−35 region sequence	Calculated ΔG of −35 region	The length between −35 and −10 region (bp)
sacB	tagaat	−7.1	ttgcaa	−8.0	17
amyE	taattt	−6.9	ttgttt	−7.1	17
degQ	cacact	−7.25	gtgaaa	−5.75	17
aprE	tcaat	−5.8	tctact	−6.25	19

The −10 and −35 regions sequences were acquired from NCBI. ΔG was calculated with Omiga Microsoft.

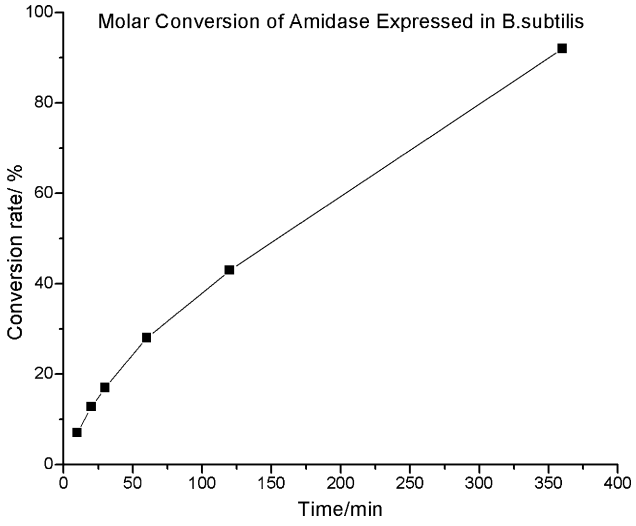

Fig. 6. Molar conversion of amidase expressed in *B. subtilis*. The curve represented the conversion efficiency from acrylamide to acrylic acid which was expressed in *B. subtilis* with plasmid *pGj103-amyE-ami*. The result was obtained at 37 °C and pH 7.0.

within 6 h. However, compared to the rate of conversion at beginning of the reaction, the reaction rate gradually decreased due to the accumulation of acrylic acid.

3.7. Sequence analysis of distinct promoters

Based on the sequence analysis listed in Table 3, the −35 and −10 regions of the promoters were conserved and responsible for recognizing sigma *A* factor at the initiation of transcription. High *A* + *T* content was the obvious feature in −35 and −10 regions with low T_m value. Some main traits of −35 and −10 regions were listed in Table 4. These features revealed that (*A* + *T*)% was the highest in *amyE* promoter and the lowest in *degQ*, while the space length between −35 and −10 region of *aprE* promoter was 2 bp longer than that of other promoters.

4. Discussion

The gene *ami*, along with its adjacent nitrile hydratase (NHase) genes containing two intact α- and β- subunit, constituted a gene cluster subjected to a co-transcribed operons [13,14]. This kind of gene cluster had been observed in *Pseudomonas choraphnis* B23, *Rhodococcus* sp. N-77, *Brevibacterium* sp. 316, and an unidentified *Rhodococcus* sp., sharing a significant similarity [13,14]. However, these linked genes and co-transcribed operons were not necessary for *ami* expression in *E. coli* and in *B. subtilis*.

Here we successfully expressed *ami* gene from *R. erythropolis* in *E. coli* and *B. subtilis* and obtained active amidase. Moreover, this study was helpful for the expression of heterologous gene by using five distinct promoters and signal peptides to regulate transcription and translation. Meanwhile, the amidase expressed in *E. coli* and *B. subtilis* were compared with respect to enzyme activity and expression mass.

In *E. coli* BL21 (DE3) (*pET-22b-ami*), the *ami* gene was expressed under the control of phage T7 RNA polymerase promoter. Meanwhile, repression protein encoded by the *lac* operator downstream could inhibit the transcription of T7 RNA polymerase promoter in host. The *pel* signal peptide on the N-terminate facilitated the secretion of the target protein and decreased the formation of inclusion body. But still a portion of inclusion body could be detected in *E. coli* BL21 (DE3).

Since the expression effect of *ami* gene in *E. coli* failed to reach the expected level, another host *B. subtilis* was therefore adopted. In *B. subtilis*, the expression and secretion of heterologous proteins were directed by various promoters and signal peptides. To achieve high expression mass and enzyme activity, five distinct promoters and signal peptides described above were individually tested. The result suggested that the *amyE* promoter with its native signal peptide was more effective than other promoters used in *B. subtilis*. The highest enzyme activity reached 8.7 U/mg.

From these results, it could be concluded that powerful promoters were more suitable for amidase expression in *B. subtilis*. This deduction could also be testified by the information that amidase was responsible for the conversion acrylamide into carbon and energy sources in *Rhodococcus* genus. Although phase T7 promoter was also a powerful promoter, the transcription system of *E. coli* was not ideal for expressing *ami* gene. It is inferred that a further evolutional distance existed between *R. erythropoli* and *E. coli* than it with *B. subtilis*, as *R. erythropoli and B. subtilis* belong to Gram-positive bacteria with low *G* + *C* content [15].

The *sacB*, *amyE*, *degQ* and *aprE* promoters were all identified by sigma *A* factor and the sequences of the promoters were analyzed in Section 2. Different from previous supposition, ΔG of −10 and −35 regions were neither vital to the transcription effect nor the strength of promoters. However, both the content of *A* + *T* and the distance between −10 and −35 regions influence the transcription efficiency. Further deduction indicated the strength of promoters may be determined by the distribution of *A* + *T* in −10 and −35 regions and the length space between −10 and −35 regions when sigma *A* factor interacted with promoters. The length space between −10 and −35 regions could supply a spatial location for binding sigma *A* [12], though further study was necessary.

This study provided experience for expressing heterologous recombinant protein in *B. subtilis.* The regulation of expressing amidase by using different promoters and signal peptides set an example for analyzing the co-effect between the promoters, signal peptide gene sequence and the heterologous protein gene at transcription level. The different expression results controlled by five promoters and their respective signal peptides also provided a protocol to increase protein mass and optimize expression system in bacteria at molecular level. The results opened the way for high efficiency conversion from acrylamide to acrylic acid.

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References

[1] C.T. Lira, P.J. McCrackin, Ind. Eng. Chem. Res. 32 (1993) 2608–2613.
[2] D.J. O'Brien, C.C. Panzer, W.P. Eiselet, Biotechnol. Process 6 (1990) 4.
[3] G. Landi, L. Lisi, G. Russo, J. Mol. Catal. A: Chem. 239 (2005) 172–179.
[4] S.H. Kim, P. Oriel, Enzyme Microb. Technol. 27 (2000) 492–501.
[5] M.A. Bradford, Anal. Biochem. 72 (1976) 248–255.
[6] Y. Hashimoto, M. Nishiyama, S. Horinouchi, T. Beppu, Biosci. Biotechnol. Biochem. 58 (1994) 1859–1865.
[7] M. Kobayashi, Y. Fujiwara, M. Goda, H. Komeda, S. Sakayu, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 11986–11991.
[8] W. Mizunashi, M. Nishiyama, S. Horinouchi, T. Beppu, Appl. Microbiol. Biotechnol. 49 (1998) 568–572.
[9] L.L. Fu, Z.R. Xu, W.F. Li, J.B. Shuai, P. Lu, C.X. Hu, Biotechnol. Adv. 25 (2007) 1–12.
[10] J. Sambrook, D.W. Russel, Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001.
[11] U.K. Laemmli, Nature 227 (1970) 680–685.
[12] J.D. Helmann, Nucleic Acids Res. 23 (13) (1995) 2351–2360.
[13] J.F. Mayaux, E. Cerbelaud, F. Soubrier, D. Faucher, D. Petre, J. Bacteriol. 172 (1990) 6764–6773.
[14] J.F. Mayaux, E. Cerbelaud, F. Soubrier, P. Yeh, F. Blanche, D. Petre, J. Bacteriol. 173 (1991) 6694–6704.
[15] W.M. de Vos, M. Kleerebezem, O.P. Kuipers, Curr. Opin. Biotechnol. 8 (1997) 547–553.