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Expressing the Extreme-Thermostable Xylanase B_{64} with Different Fusion Tags

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Xylanase B from thermophile bacteria *Thermotoga maritima* MSB8 was extreme-thermostable and has potential application for feed, paper manufacture, energy, food and medicine industries. Recombinant plasmid pET28a (+)-xynB_{64} was induced and expressed in *E. coli* BL21 (DE3) and the activity of recombinant enzyme xylanase was very low. Both *E. coli* BL21-CodonPlus (DE3)-RIPL and Rosetta (DE3) possessing rare tRNAs were used to be the expressing host and the activity of recombinant enzyme increased by 197 % and 277 %, respectively. However, inclusion body was formed in *E. coli* Rosetta (DE3). The next step, pET32a (+), pET42a (+), pET43.1a (+) and pMAL-c2X, which contain the Trx, GST, Nus and MBP fusion tag respectively were used to be the expression vector with *E. coli* Rosetta (DE3) as the host. The activity of recombinant enzyme produced by Rosetta (DE3)/pMAL-c2X-xynB_{64} was the highest, which was equivalent to 88 % of counterparts of Rosetta (DE3)/pET28a-xynB_{64}. Meanwhile about 40 % whole cell proteins of former were recombinant XynB_{64} with little inclusion body.

Key Words: Thermostable xylanase, Rare codon, Fusion tag, Translation initiation region.

INTRODUCTION

Xylanase(endo-1,4-D-xylanase, EC3) is one of the most key enzmyes to degrade the linear polysaccharide β-1,4-xylan into xylose[1], thus breaking down hemicellulose, one of the major components of plant cell walls. Xylanase has attracted considerable research interest in recent years, mainly due to their potential application in the food, animal feed, paper and pulp industries. The xylanases isolated from *Thermotoga maritima* MSB8 in the research exhibited highly activity toward xylan[2] and low activity toward carboxymethylcellulose[3,4], have optimal pH and temperature of 6.6 and 90 ºC respectively, with great industrial prospects. There was no commercial enzyme providing now, mainly because of unclear impact factors of expression and activity as well as high cost of industrialization.

Many factors affect the expression of exogenous genes *e.g.*, properties of the genes (codon bias, roles of the leader sequences), expressing host (product and mRNA stability), expressing vector [plasmid copies, promoter intensity, SD (Shine-Dalgarno sequence) sequence, distance between the SD and initiator codon, secondary structure of the translation initiation region, transcriptional termination efficiency, expression site], interaction among proteins and the culture/induction conditions. Researchers tried to remove the signal peptides and fuse CBM2b at the C-terminal[5]. However, more improvements on rounded analysis are needed, for the factors mentioned above frequently give a huge influence on the expression level. Yeast was also applied in XynB_{64} expression and gain some progress, but some impact factors were still unclear and problems still existed.

Some measures have been taken to try to raising the soluble production, including eliminating signal peptides; with fused at the C terminal, expressing XynB_{64} in BL21(DE3)/pET28a(+), BL21-CodonPlus(DE3)-RIL/pET20b(+), JM109/pHsh[6] and optimizing the cultural condition, providing theoretical foundation for the enzyme industrialization.

In this report, the expression level of xynB_{64} under different fusion tags at *N*-terminal and different supplement type of rare condons was described for the first time,and the relation between the expression quantity and the Gibbs free energy[7] of mRNA translation initiation region was studied as well.

EXPERIMENTAL

Bacterial strains and plasmids: BL21(DE3), containing the recombinant plasmid pET28a-xynB_{64}, was used as the source of our target gene. BL21-Condon Plus (DE3)-RIPL (Stratagene, USA and Rosetta (DE3) (Novagen, USA) were utilized as expression hosts, supplying extra tRNA for argU (AGA, AGG), ileY (AUA), proL (CCC), leuW (CUA) and argU (AGG,AGA), ileY (AUA), glyT (GGA), leuW (CUA), proL (CCC), respectively. We obtained 4 kinds of plasmids (Novagen, USA), pET32a (+), pET42a (+), pET43.1a (+) and

pMAL-c2X. These plasmis bear fusion tags of Trx, GST, Nus and MBP, respectively. JM109 was used as the host for subcloning. The stains used in this research were cultured in Luria-Bertani (LB) medium and, when necessary, appropriate antibiotic was added to the medium.

Construction and expression of the gene: The gene encoding xylanase was PCR-amplified from the vector pET28a existed in BL21 (DE3), in which the gene had been designed with restriction sites for NcoI and HindIII., by initial denaturation at 98 ºC for 3 min, then 30 cycles of denaturation at 95 ºC for 45 s, annealing at 58 ºC for 30 s and extension at 72 ºC for 1 min 45 s and final extension was done for 25 min. PCR amplified products were run on 0.8 % agarose gel and purified by the QIA quick gel extraction kit (QIA gen Inc., USA)[8].

With primers of T7 promotor (5'-TAATACGACTCACTA TAGGG-3') and T7 terminator (5'-TGCTA GTTATTGCTCA GCGG-3'), the PCR products persisted its original sites. Then the resulting PCR fragment was digested with NcoI and HindIII and ligated into pET32a and pET42a cut with the same enzymes. Similarly with primers of pFOR(5'-CGCGGATCCATGGAAATATTACCTTCTGTGTTGATCCTT-3') and pREV(5'-CCCAAGCTTATTTTCTTTCTTCTATCTT TTTCTCCAGCACC-3'), we got $xynB_{64}$ gene containing BamHI and HindIII resriction sites, which were ligated into pET43.1 and pMAL-c2X. The four kinds of recombinant plasmids were transformed into the expression host respectively. Competent *E. coli* BL21-Codon Plus (DE3)-RIPL and Rosetta (DE3) cells were transformed with the recombinant plasmids pET28a-$xynB_{64}$ and selected for antibiotic resistance.

The sequence of the insert was confirmed *via* bidirectional DNA sequencing. The stains harboring pET28a(+)-$xynB_{64}$, pET32a(+) $xynB_{64}$, pET42a(+)-$xynB_{64}$, pET43.1 (+)-$xynB_{64}$ and pMAL-c2X-$XynB_{64}$ were cultured overnight in 50 mL of LB medium containing kanamycin (50mg/mL) to be seed culture. The prepared seed culture was used to inoculate LB medium 50 mL) containing kanamycin (50 mg/mL) which was cultured at 30 ion a rotary shaker (200 rpm) until the optimal density at 600 nm reached 0.7-0.8. IPTG (isopropyl b-D-thiogalac-topyranoside) was added to a final concentration of 1 mM and incubation was then continued for 8 h.

Detection method: After 8 h of the induction, cultures (7 mL) were harvested, centrifuged and re-suspended in 7 mL of phosphate-buffered saline (PBS; 1/15 mol/L; pH6.6). In ice bath, the bacteria cells were disrupted by signification for 10 min (pulse on, 10 s; pulse off, 10 s; temperature of the probe, 4 ºC; power, 400 W). The lysate was centrifuged at 1300 r/min for 2 min at 4 ºC and extracted supernatant as the crude enzyme. Xylanase activity was assayed according to the method of Bailey *et al*., using 0.5 % (w/v) birch wood xylan in phosphate-buffered saline (PBS; 1/15 mol/L; pH 6.6)[9]. The assay mixture containing 0.95 mL substrate solution and 0.05 mL of suitably diluted enzyme solution in the above buffer was incubated at 70 ºC for 20 min. The reaction was stopped by adding 2 mL of 0.63 % (w/v) dinitrosalicylic acid (DNS). Then boiled the mixture for 10 min and measured the absorption value of these mixtures on 540 nm. The amount of reducing sugar liberated was determined by dinitrosalicylic acid method using xylose as the standard[5,8]. One unit of xylanase activity was defined as the amount of enzyme that produced 1 μmol of xylose equivalent per minute.

The homogeneity and molecular weight of the xylanase was released from the boiling expressed cells was determined by using 12 % whole-cell SDS-PAGE as described by Hong *et al.*[11]. Protein bands were visualized by staining with Coomassie brilliant blue R-250. The molecular weight standard used was the low molecular weight calibration kit for SDS electrophoresis (Amersham): phosphorylase b (97.0 kDa), albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa).

After ultrasonication, the inclusion bodies were washed up by PBS and dissolved in 1 mL of urea solution (8 mol/L), about 1 h. The supernatant obtained by centrifugating the solution was evaluated *via* SDS-PAGE.

With the tool of RNA structure 5.03, the Gibbs free energy (ΔG) of secondary structure of mRNAs in translation initiation region was predicted.

RESULTS AND DISCUSSION

Site mutation and rare condons analysis: As shown in results of the gene $xynB_{64}$, there are 2 sense mutations. Condons for the Asn-22(AAU) was changed into Asp-22(GAU) and since the restriction site Nco I introduced condons for Lys-2 (AAA) was changed into Glu-2 (GAA). Xylanase activity in this research remained high at pH range of 6.6-6.8, while this enzyme was reported to have the high activity at pH range of 5.4-5.8.

The gene $xynB_{64}$ contained condons that are rarely used in *Escherichia coli*. For this reason, the expressing host BL21-CodonPlus(DE3)-RIPL and Rosetta (DE3) were chosen, offering extra tRNA for argU (AGA, AGG), IleY (AUA), proL (CCC), leuW (CUA) andargU (AGG, AGA), IleY (AUA), glyT (GGA), leuW (CUA), proL (CCC), respectively. The rare condons in gene $xynB_{64}$ was shown as follow.

ATG GAA ATA TTA CCT TCT GTG TTG ATC CTT TTG	60
TTG GGA TGT GTT CCA GTT TTC AGC TCT	
CAG GAT GTA TCT CTG AGA GAA CTC GCA GAA	120
AAG CTG AAC ATC TAT ATT GGT TTT GCC GCA	
ATC AAC AAC TTT TGG TCT CTT TCC GAC GCA GAA	180
AAG TAC ATG GAA GTT GCA AGA AGA GAA	
TTC AAC ATC CTG ACC CCT GAG AAC CAG ATG	240
AAG TGG GAT ACG ATT CAT CCA GAA AGA GAC	
AGA TAC AAT TTC ACT CCC GCT GAA AAA CAC	300
GTT GAG TTT GCA GAA GAA AAC GAC ATG ATC	
GTG CAT GGA CAC ACT CTT GTC TGG CAC AAC	360
CAG CTT CCT GGA TGG ATC ACT GGT AGA GAA	
TGG ACA AAG GAA GAA CTT TTG AAC GTT CTT	420
GAA GAC CAC ATA AAA ACG GTG GTG TCT CAT	
TTC AAA GGT AGA GTG AAG ATC TGG GAT GTG	480
GTG AAC GAA GCG GTG AGC GAT TCT GGA ACC	
TAC AGG GAA AGC GTG TGG TAC AAG ACG ATC	540
GGT CCT GAA TAC ATT GAA AAA GCG TTC AGA	
TGG GCA AAA GAA GCC GAT CCA GAT GCG ATT	600
CTC ATC TAC AAC GAC TAC AGC ATA GAA GAA	
ATC AAC GCA AAA TCG AAC TTC GTC TAC AAC	660
ATG ATA AAA GAG CTG AAA GAA AAG GGA GTA	
CCT GTT GAT GGA ATA GGA TTT CAG ATG CAC	720
ATA GAC TAC AGA GGG CTC AAT TAT GAC AGT	
TTC AGA AGG AAT TTG GAG AGA TTT GCG AAA	780
CTC GGT CTT CAA ATA TAC ATC ACA GAG ATG	

GAT GTG AGA ATT CCT CTC AGT GGT TCG GAG 840
GAG TAT TAT TTG AAA AAA CAG GCT GAA GTT
TGT GCG AAG ATC TTC GAT ATA TGC TTG GAC 900
AAC CCT GCA GTT AAA GCG ATC CAG TTT TGG
GGA TTC ACA GAC AAA TAC TCC TGG GTT CCC 960
GGC TTT TTC AAA GGG TAC GGG AAA GCG TTG
CTC TTC GAT GAG AAT TAC AAC CCC AAG CCT 1020
TGT TAT TAC GCG ATA AAA GAG GTG CTG GAG
AAA AAG ATA GAA GAA AGA AAG CTT GCG GCC 1080
GCA CTC GAG CAC CAC CAC CAC CAC CAC TGA

Identification and analysis of recons: We successfully constructed the recombinant plasmids, pET32a(+)-XynB_{64}, pET42a(+)-XynB_{64}, pET43.1a(+)-XynB_{64} and pMAL/c2X-XynB_{64}, shown as the following maps (Fig. 1). The DNA insert and orientation were determined by restriction analysis and DNA sequencing, that was shown in Fig. 2.

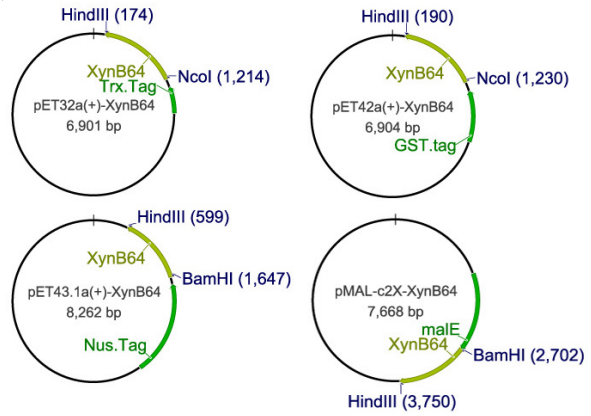
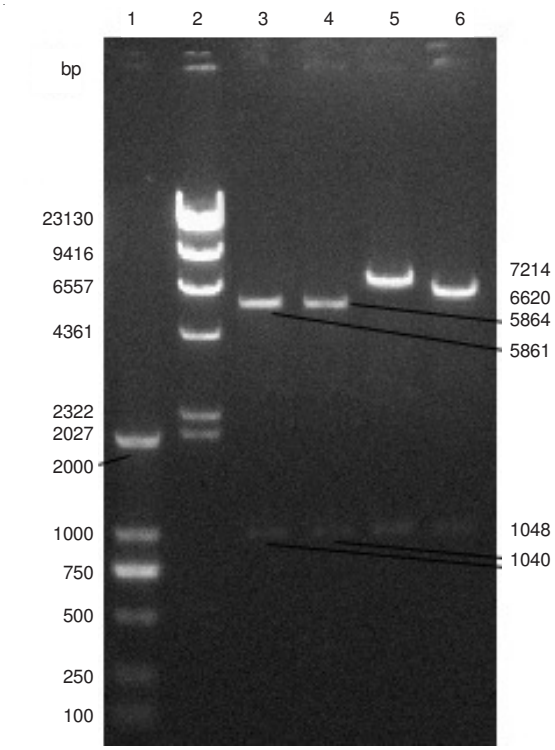


Fig. 1. Construcion of recombinant plasmids



Note: 1: DL2 000™ DNA marker; 2: λ-Hind III digest DNA marker; 3: pET32a (+)-xynB_{64}; 4: pET42a (+)-xynB_{64}; 5: pET43.1a (+)-xynB_{64}; 6: pMAL-c2X-xynB_{64}

Fig. 2. Restriction analysis and the electrophoresis spectrums

Xylanase expression and activity yield: The enzyme activity in BL21-CodonPlus (DE3)-RIPL and Rosetta (DE3) performed better 1.97 and 2.77 times than that in BL21(DE3), as shown in the Fig. 3. It demonstrated that rare condons limited the expression level. Supplementing enough kinds of rare condons was helpful for high productivity[12].

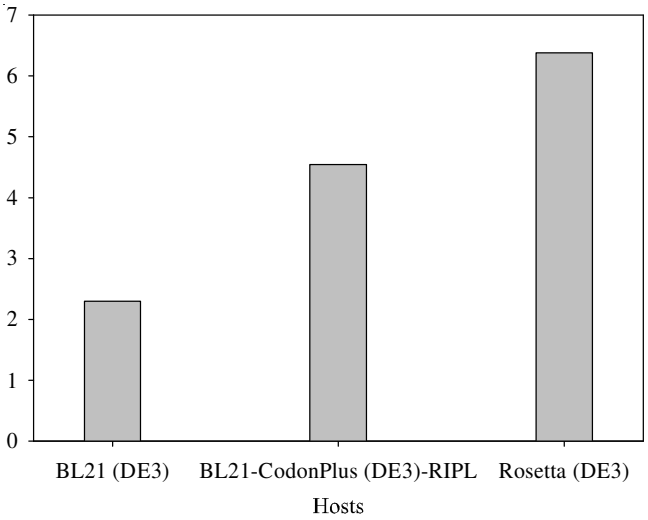


Fig. 3. pET28a(+)-XynB_{64} express in different hosts

Constracting the enzyme activity in Rosetta(DE3)/pET28a(+)-XynB_{64}, Rosetta(DE3)/pET32a(+)-XynB_{64}, Rosetta(DE3)/pET42a(+)-XynB_{64}, Rosetta(DE3)/pET43.1a(+)-XynB_{64} and Rosetta(DE3)/pMAL-c2X-XynB_{64}, the result indicates that expressing with fusion tag protein effect the xylanase catalytic activity in different extent. Among these recombinant plasmids, the fusion tag of MBP had the least influence, that the activity reduce by 20 %. (Fig. 4).

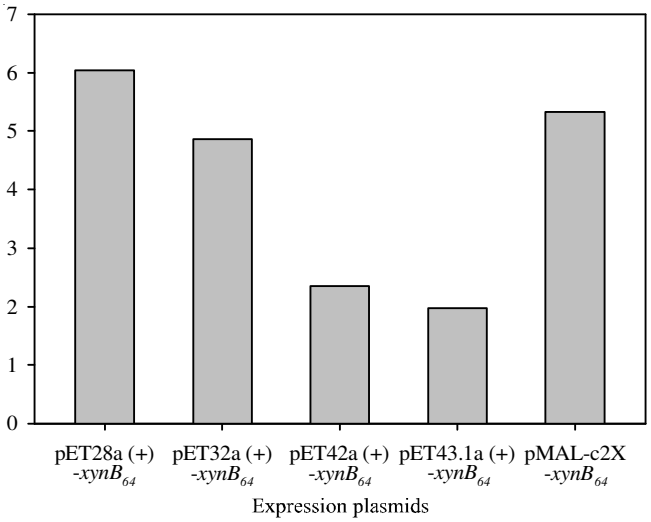


Fig. 4. Recombinant expression plasmids express in Rosetta (DE3)

The expression of xylanase was monitored by activity measurements and SDS-PAGE and the result were given in Fig. 5. The SDS-PAGE analysis of the total soluble proteins from recombinant Rosetta (DE3) cells harboring pET28a (+)-xynB_{64}, pET32a (+)-xynB_{64}, pET42a (+)-xynB_{64}, pET43. 1a (+)-xynB_{64} and pMAL-c2X-xynB_{64} indicated that the expression

levels of xylanase gene fused with Trx, GST and MBP tag were higher than that from the plasmid pET28a-xnyB$_{64}$, especially, pMAL-c2X-xynB$_{64}$ was expressed at the level up to 40 % of the total cell proteins, without formation of inclusion body. But fusing with Nus tag resulted in yield reduction.

The SDS-PAGE of inclusion-body protein renaturation supernatant was shown in Fig. 6. Xylanase B$_{64}$ fused with GST tag was expressed mostly in a insoluble form, possibly due to its big size. The tag MBP helped to inform the solvable target protein and few xylanase B$_{64}$ existed as inclusion body.

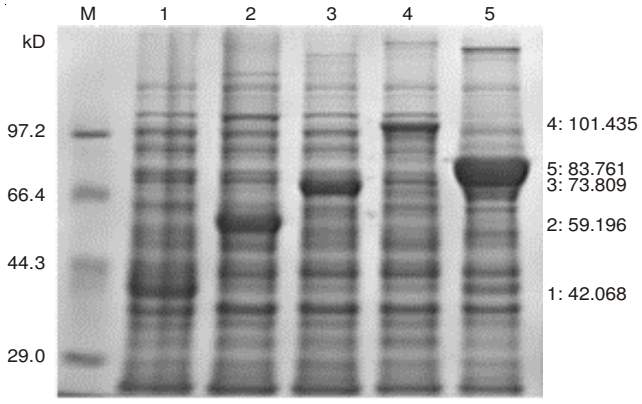
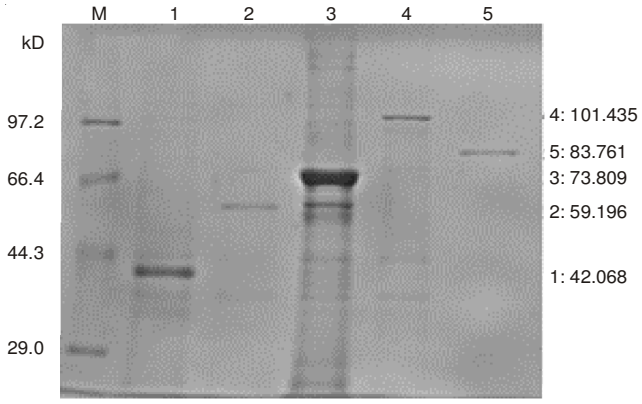


Fig. 5. SDS-PAGE analysis of whole cell proteins



Note: M: Protein molecular weight marker (Low); 1: Rosetta (DE3)/pET28a (+)-xynB$_{64}$; 2: Rosetta (DE3)/pET32a (+)-xynB$_{64}$; 3: Rosetta (DE3)/pET42a (+)-xynB$_{64}$; 4: Rosetta (DE3)/pET43.1a (+)-xynB$_{64}$; 5: Rosetta (DE3)/pMAL-c2X-xynB$_{64}$

Fig. 6. SDS-PAGE of inclusion body that corresponds to Fig. 5

The secondary structure of the translation initiation region is a determinant factor for translation initiation rate[12]. And its forming feasibility was analyzed by determining free energy values for the fragment around translation initiation region. Four regions(-34-+36, -30-+70, +1-+78 and -41-+367) were chosen for evaluating the Gibbs free energy, which had been predicted as shown in Table-1. It was stated that stable secondary structure of translation initiation region was detrimental to the translation initiation. Stability reduction was helpful to increase expressing production. It could be seen from the Table-1 that the order of expression level was pMAL-c2X-xynB$_{64}$>pET32a (+)-xynB$_{64}$ > pET42a (+)-xynB$_{64}$ > pET28a (+)-xynB$_{64}$ > pET43.1a (+)-xynB$_{64}$. The order was valid only when we chose the data underlined, as shown in Table-1.

TABLE-1
MINIMAL FORMATION FREE ENERGY OF TRANSLATION INITIATION REGION (ΔG kcal/mol)

Region	-34-+36	-30-+70	+1-+78	-41-+36
pET28a (+)-*xynB$_{64}$*	-11.5	-21.4	-16.3	-12.6
pET32a (+)-*xynB$_{64}$*	-6.0	-10.3	-17.3	-9.1
pET42a (+)-*xynB$_{64}$*	-13.3	-13.8	-11.4	-13.3
pET43.1a (+)-*xynB$_{64}$*	-9.6	-15.3	-13.6	-10.4
pMAL-c2X- *xynB$_{64}$*	-5.5	-15.3	-13.9	-10.9

Conclusion

The earlier preparation experiment on the engineered *Escherichia coli*, which was made to optimize the culture conditions and inducement conditions, showed the expression level was not ideal, even using high density fermentation. The result of sequence alignment between the xylanase gene XynB$_{64}$ from the *Thermotoga maritima* MSB8 searched in the Genbank and our target gene sequence revealed that the XynB$_{64}$ fused was different from that reported in the previous papers. Some mutants were introduced. There was a nucleotide substitution of A→G at position 64, resulting in the 22th amino acid Asn was changed into Asp.

A nucleotide A→G transition at position 4 was intriduced, because the restriction site Nco I was designed at the N terminal, resulting in the second amino lysine changed into glutamic acid. Lead-in of the restriction site Hind III at the C-terminal and fusion of 6 × His tags introduced a novel sequence CTTGCGGCCGCACTCGAGCACCACCACCACCACCACT, which encoded a peptide of LeuAlaAlaAlaLeuGluHisHis HisHis. All this changes made the optimum pH rose from 5.4-5.8 to 6.6-6.8 and the codons that infrequently used in *Escherichia coli* occupied up to 10.34 %. While expressing in BL21-CodonPlus (DE3)-RIPL and Rosetta (DE3), the enzyme activity was increased by 1.97 and 2.77 times respectively. The major difference is that Rosetta (DE3) offered additional tRNAs, argW and glyT, indicating that codon majorization played an important role in enzyme activity improvement.

It is reported that fusing with the Trx, GST, Nus or MBP tag could improve the translation initiation rate and the solubility, except the GST tag. In this research, the Trx tag significantly enhanced expression level and solubility but lead an adverse effect on activity. When fused with GST tag, inclusion body was produced in abundance, for the too fast translation rate. Too large Nus tag brought extra energy consumption, so the expression reduced. We got high quantity and solubility unexpectely by using Ptac promoter and fusing MBP tag, but the activity was effected observably. This was considered to be connected with the spatial structure. Additionally the translation initiation region minimization of Gibbs free energy of all the recombinant gene had been analysed. As shown in Fig. 3, different base site and different base amount resulted in violent energy fluctuation.

It is reported that compared with the original sequence, the value of mRNA translation initiation region minimum free energy reducing meaned a higher expression level. The result in this research indicated at least 4 potential problems existed in this approach: (1) the choice of translation initiation region lacked basis; (2) experimental data was insufficient and so many influencing factors and contingency existed; (3) the

optimal value predicted by software couldn't equate the real mRNA 3D structure properties; (4) the software algorithm based on thermodynamic data, so thermodynamic data accuracy probably effected the Prediction results.

Secretion was the main advantage of yeast expression system. According to the present reports, series of weakness were raised, such as slow growth rate, low expression level and small optional expressing system range. And the gene derived from prokaryotes, so *Escherichia coli* should be the main research object. Based on experience of others, for obtaining stably producing thermo stable and alkali proof xylanase engineering bacteria with high expression level, available strategies were stated: synthetizing a sequence with a his-tag at the C-terminal and a mutant at its 64^{th} base, removing its anterior 57 base, clonning it to kanamycin resistance plasmid whose expression was recognized and started by σ^{32} and transforming it into a host with mutation of ompT, lon and rne131. To increase the soluble production, MBP tag and other appropriate tags would be fused which could be cut off by relevant enzyme. On the basis of TB culture, response surface methodology could be used to identify the optimum culturing conditions. High density fermentation expression helped obtain a yield at the industrialization level.

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