Expression of trehalose synthase gene from *Pseudomonas putida* P06 in *Pichia pastoris*

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Abstract: The trehalose synthase (TreS) gene from *Pseudomonas putida* P06 was successfully ligated with pPICZaA expression vector by the EcoRI and XbaI and was transformed into *Pichia pastoris* GS115 by electrotransformation. The trehalose synthase gene was fused to the genome of *Pichia pastoris* GS115 and was controlled by *AOX*1 promoter. The TreS protein was successfully expressed in intracellularly. SDS-PAGE results illustrated that a specificity protein band was observed at about 76 kDa. The cell lysates could convert 60% maltose into trehalose at 50°C and pH 7.5 in 10% maltose substrate for 24 h. The *Pichia pastoris*as exogenous gene expression host is safer to produce endotoxin free TreS than *E.coli*.

Keywords: Trehalose synthase, *Pseudomonas putida* P06, *Pichia pastoris*, cloning and expression.

INTRODUCTION

Trehalose is a non-reducing disaccharide, two glucose molecules linked by an α, α-1, 1- glycosidic bond. Trehalose is widely synthesised in organisms, including microorganisms, plants and animals (Elbein et al., 2003). Trehalose has many good features, such as thermostability and pH-stability. It can effectively prevent protein and cell membrane damage in extreme conditions, including dehydration, desiccation, heat, oxidation and cold. With the development of trehalose application, it has been used in food, cosemetics, pharmaceuticals, argricultural industries (Elbein *et al*., 2003; Satpathya *et al*., 2004).

The benefits of trehalose have been recognized by researchers for many years, and the new application study is also expanding, but the scale-up industrial production of trehalose is still limited. Recently, the pathway of converting maltose into trehalose by trehalose synthase has been considered to be a very good method, because the process is simple and can be controlled easily. Moreover, the most important factor is that maltose as a substrate is low cost. Several trehalose synthase producing strains have been screeded, identified and have been expressed in *Escherichia coli*, *Pseudomonas stutzeri CJ38* (Lee *et al*., 2005), *Corynebacterium glutamicum ATCC13032* (Kim *et al*., 2010), *Arthrobacter aurescens CGMCC 1.1892* (Wu *et al*., 2009), *Meiothermus ruber CBS-01* (Zhu *et al*., 2010), *Mycobacterium smegmatis* (Pan *et al*., 2004), *Pseudomonas putida* (Ma *et al*., 2006) and *Thermobifida fusca* (Wei *et al*., 2004). The expression of trehalose synthase in *Pichia pastoris* was not reported so far. In the present study, the trehalose synthase gene from *Pseudomonas putida* P06 was cloned and expressed in *Pichia pastoris* GS115, in order to produce endotoxin free trehalose synthase.

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MATERIAL AND METHODS

Strains and plasmids

Pseudomonas putida P06 was preserved in Key Laboratory of Shandong Microbial Engineering in China. *Pichia pastoris* GS115 was used for expressing the TreS protein. Plasmid pPICZaA as the expression vector of TreS was adopted in *Pichia pastoris*GS115. *Escherichia coli* DH5a was exploited to manipulate TreS gene.

Gene cloning and expression vector construction

The sequence of the open reading frame (gi = 1042893, NCBI) annotated as TreS in GenBank. TreS gene was cloned from the total DNA of *Pseudomonas putida P06* using polymerase chain (PCR) reaction amplification with sense primer pTreSF 5'-CG*GAATTC*ATGACCCAGCC-CGACC-3' and antisense primer pTreSR 5'-GC*TCTAGA*TCAAACATGCCCGCTGC-3', (the EcoRI and XbaI restriction site is italiced, respectively). The PCR procedure with *pfu* DNA polymerase was performed through 30 cycles (30 s at 95°C, 30 s at 54°C , 2.5 min at 72°C), followed by a final extension for 10 min at 72°C. The PCR product was purified and digested by the EcoRI and XbaI restriction enzyme, and ligated with pPICZaA expression vector by the same restriction enzyme. The recombinant pPICZaA-TreS plasmid was first transformed into *E. coli* DH5α, and the marker positive colonies were selected from LB agar medium containing 25 μg/ml zeocin for sequence analysis.

Screening the high-level expression transformants

The correct sequencing of pPICZαA-TreS was linearized with Bst*X*I. The *Pichia pastoris* GS115 competent cells were prepared by pretreating *Pichia pastoris* with lithium acetate and 1% dithiothreitol to mix with linearized recombinant plasmid in 0.2 cm electroporation cuvette for electroporation at 1.5kv and 5 ms. After pre-incubation for 3 h at 30°C in 1M sorbitol, the competent cells, which were pre-incubated for 3h at 30°C, were plated on YPDS medium (peptone 2%, yeast extract 1%, glucose 2%, sorbitol 1M and agar 2%) with 100μg/ml zeocin to screen positive colonies that integrated Tres gene in 5'AOX zone of *Pichia pastoris* GS115 for 2-3 days. Then *Pichia pastoris* positive transformants were coated on YPDS plates containing 500 μg/mL, 1000 μg/mL, and 2000 μg/mL zeotcin. Multicopy transformants were screened from the YPDS plates (2000 μg/mL zeocin) and the TreS was highly expressed. After positive transformants were screened, the transformants were cultivated for 18h at 30°C, 250rpm centrifugal in BMGY medium containing peptone 2%, yeast nitrogen base 1.34%, yeast extract 1%, biotin 4×10^{-5}% and glycerol 1%, the pH was adjusted at 6.0. The cells were then centrifuged (250 rpm) and were developed for 72 h at 30°C, in BMMY medium containing yeast extract 1%, peptone 2%, yeast nitrogen base 1.34%, biotin 4×10^{-5}%, 0.5% methanol (v/v), the pH was adjusted at 6.0. In order to maintain the continuous induction, the absolute methanol was added to the shake flask every 24h during the incubation period. The methanol concentration in fermentation liquid was controlled in 0.5%. The cell precipitate was harvested by centrifugation after the fermentation, then the Tres activity of the clear supernatant and the cell lysates were analyzed. The protein band was analyzed by SDS-PAGE.

RESULTS

Tres gene cloning and expression vector construction

According to the Tres ORF sequence of *Pseudomonas putida P06*, using the primer pairs (pTreSF and pTreSR), the TreS gene was cloned from *Pseudomonas putida P06* by PCR and the band was clearly visible (fig. 1) on the agarose gel electrophoresis.

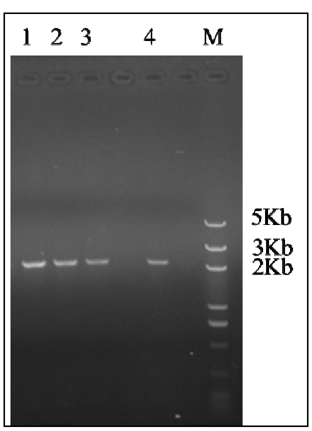

Fig. 1: Amplification of TreS from chromosomal DNA of *P. putida* P06 by PCR Lanes 1-4, target sequence of *P. putida*; LaneM, Trans2K^{TM} Plus DNA Marker

The PCR product was recovered from the gel and ligated to pPICZαA after double restriction enzyme digestion of EcoRI and XbaI. The target sequence was cloned into plasmid pPICZaA and sequenced. Sequence analysis illustrated that target sequence was 100% matched with the gene from *Pseudomonas putida P06*. The recombinant expression vector pPICZαA-TreS was transformed into the *E. coli* DH5α strain for cloning and the positive clone bacteria was picked from LB solid medium with 25μg/ml zeocin for colony PCR template (the positive clone strains were obtained). The positive clone strains were then activated, and their plasmids were extracted and digested by EcoRI and XbaI, following the verification by agarose gel electrophoresis as shown in fig. 2.

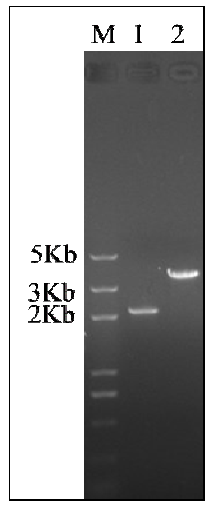

Fig. 2: The double digest results of target gene and the plasmid Lane 1, double digest of TreS target gene; Lane 2, double digest of pPICZαA plasmid ; LaneM, Trans2KTM Plus DNA Marker

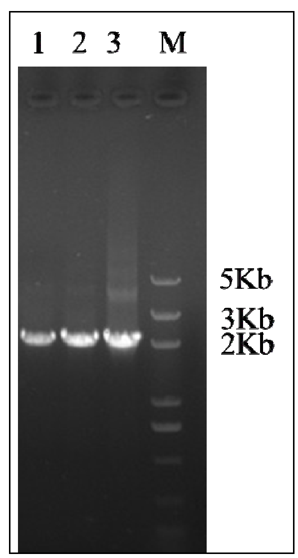

Fig. 3: PCR of positive transformants Lane1-3: PCR amplified DNA product of positive transformants; laneM: Trans2KTM Plus DNA Marker

TreS encoded protein expression in Pichia pastorisGS115

The TreS gene with no signal peptide sequences was integrated into pPICZαA vector and connected with the α-factor signal for the enzyme secretion. Binding sites located in the cleavage site (Kex2). High tolerance (2000 μg/mL zeocin) strains were selected, the TreS gene was analyed by PCR. The band of approximately 2.1Kb revealed that the TreS gene got integrated into the genome of *Pichia pastoris* GS115, as shown in fig.3.

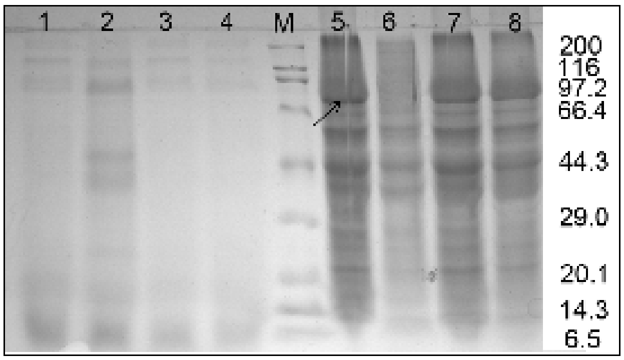

Fig. 4: SDS-PAGE (10%) of TreS (extracellular and intracellular) LaneM, Protein Markers (Broad); Lane1, 3, 4, SDS-PAGE of extracellular recombinant TreS; Lane5, 7, 8: SDS-PAGE of intracellular recombinant TreS; Lane2, 6, SDS-PAGE of extracellular and intracellular blank control

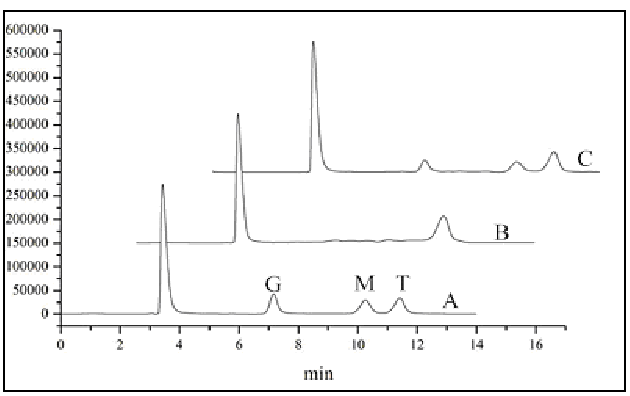

Fig. 5: HPLC chromatograms results of the samples A: The enzyme assay in intracellular contents; B: The enzyme assay in extracellular contents; C: the standard substances. M: maltose (10.3min); G: glucose(7.2min); T: trehalose(11.5min).

The recombinant *Pichia pastoris* GS115 expressing TreS was induced by absolute methanol in shake flask for 72 h. The yeast cells were centrifugated (6000×g) for 10 min at 4°C to obtain the pellet. The wet cell pellet of 100 g (the cell dry weight of per liter liquid) was suspended in 10 mmol/L phosphate buffered solution (Na^+, pH7.5) and the volume was made up to 1000mL. The cell suspension was cyclically disrupted twice by high-pressure homogenizer (APV-2000, Germany) at 1300 bar. More than 95% cells were disrupted as observed after methylene blue staining. The fermentation supernatant and the cell lysates were used to convert maltose and SDS-PAGE, respectively. The SDS-PAGE analysis showed (fig. 4) that an recombinant intracellular protein of about 76 kDa (in accordance to the predicted value), was expressed. No specific protein was detected in supernatant. In order to further prove the TreS location (intracellular and extra cellular), the culture medium supernant or the cell homogenates was mixed with the final concentration of 10% maltose solution of pH 7.5 at 50°C for 24h, then boiled the mixed solution for 10 min to analyze the content of trehalose. Accordingly, there was no trehalose production in extracellular mixed reaction solution, rather trehalose was found intracellular (fig. 5).

DISCUSSION

We have demonstrated that trehalose synthase gene from *Pseudomonas putida P06* was inserted into expression vector and expressed as target recombinant enzyme. Although, trehalose synthase with activity can accumulate in cells, but the trehalose synthase could not be secreted into the culture medium. Genes encoding the trehalose synthase from other species have been used to epress and encode for recombinant enzyme, but it could only be expressed in *Escherichia coli* system. And recent data is not available regarding to exogenous trehalose synthase gene expression in *Pichia pastoris GS115*. However *Pichia pastoris* expression system has been used widely (Lee *et al*., 2007; Yu *et al*., 2009; Tang *et al*., 2012). Gene of treshalose synthase from *Pseudomonas putida P06* has been expressed for the first time in *Pichia pastoris GS115*. The *Pichia pastoris* as a host strain may be more safe than the *E.coli* system, because of endotoxin problem associated with in *E. coli*.

Nevertheless, recombinant protein gene was not expressed for extracellular product as expected. As per secreted protein mechanism (Damasceno *et al*., 2012), *Pichia pastoris* belongs to posttranslational translocation, target enzyme synthesis undergoes many mechanisms starting from gene to the complete protein synthesis. Even one of the factors such as gene dosage and codon bias may lead to improper enzyme synthesis. Thus, it needs to continue with the analysis the reasons regarding the recombinant protein gene expressed on intracellularly and not extracellularly.

The present research findings have demonstrated that the gene encoding for trehalose synthase from *Pseudomonas putida* P06 could be successfully expressed in *Pichia pastoris* system. The focused research regarding the Tres protein extracellular secretion will continue.

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