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Effect of Signal Sequence on the β-xylanase from *Thermomyces lanuginosus* SKR Expression in *Escherichia coli*

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Abstract: Xylanase is useful enzyme in various industrial such as conversion of lignocellulose to fermentable sugars for the production of chemicals and biofuels. *Thermomyces lanuginosus* is a potent thermophilic strain which produces high level of cellulose-free xylanase. The aim of this study was to investigate the effects of signal sequence on *T. lanuginosus* xylanase expression in *Escherichia coli*. The xylanase gene from those was amplified with and without signal sequence and expressed in *E. coli*. The result showed that the transformant (pUC/Xynsig) containing original signal sequence from *T. lanuginosus* SKR produced xylanase with 56% higher than that of another transformant (pUC/Xyn) lacking of original signal sequence. The recombinant enzymes were partially purified using ammonium sulfate precipitation and DEAE cellulose column and then determined some properties. The optimal pH and optimal temperature of xylanase from pUC/Xynsig was pH 6.0 and 50°C, respectively. In case of pUC/Xyn without signal sequence, the values of xylanase showed higher than those having pH 7.0 and temperature of 70°C in which was similar to original host. In conclusion, original signal sequence from *T. lanuginosus* SKR could increase xylanase production when expressed in *E. coli* but some properties of expressed enzyme were changed. However, this finding could apply to other expression system of various hosts in order to stimulate the level of the protein production.

Key words: Xylanase, signal sequence, mRNA secondary structure, *Escherichia coli*, *Thermomyces lanuginosus*

INTRODUCTION

β-Xylanase (Endo 1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) is one of the important enzymes involved in xylan degradation working synergistically with other debranching enzymes (Hrmova *et al*., 1991; Salama *et al*., 2008). It is potentially important in various applications such as biobleaching process of pulp and paper, improving the quality of animal feed, increasing the quality of bread and the conversion of lignocellulose to fermentable sugars for the production of chemicals and fuels (Jager *et al*., 1992; Viikari *et al*., 1994; Sunna and Antranikian, 1997; Beg *et al*., 2001; Khucharoenphaisan and Sinma, 2010). *Thermomyces lanuginosus* is a potent thermophilic fungus which produces a single cellulose-free endo β-xylanase (Anand *et al*., 1990; Singh *et al*., 2000, 2003; Khucharoenphaisan *et al*., 2010a, b). The xylanase gene of this fungus has been sequenced (Schlacher *et al*., 1996) and expressed without signal sequence in *E. coli* (Yin *et al*., 2008). Most expressed xylanase was found as inclusion body in the cytoplasm of the cell and lost of its function. The absence of post translational modification such as glycosylation in *E. coli* and intracellular accumulation of recombinant xylanase have been suggested to be the key reason (Singh *et al*., 2003). There are various strategies to overcome the limitations during expression of heterologous gene like gene dosage (Kane, 1995), promoter strength (Joersbo *et al*., 2000), codon bias in the initiation region (Stenstrom *et al*., 2001; Cheng and Zou, 2007). However, present recent experiment showed that signal sequence has a potential to increase expression level of xylanase in transformant. The objective of the present study was to compare the expression of xylanase genes with and without original signal sequence using cloning vector of pUC19. The properties of recombinant enzyme were also characterized. This knowledge maybe applied for suitable plasmid for enzyme expression.

MATERIALS AND METHODS

Place and during time: This research project was conducted from September 2010 to March 2011 at Faculty of Science and Technology, Phranakhon Rajabhat University, Thailand.

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Microorganism: *Thermomyces lanuginosus* SKR in this study was isolated from soil collected from Sakaerat Environmental Research Station in Nakhon Ratchasima province, Thailand. The culture was maintained on Yeast Starch (YpSs) agar. Based on its morphological characters, strain SKR was identified as *T. lanuginosus* according to the manual of fungal taxonomy (Cooney and Emerson, 1964; Domsch *et al*., 1993).

Preparation of construct: Complementary DNAs (cDNA) with and without signal sequence of *T. lanuginosus* SKR xylanase were synthesized using total RNA as template by Polymerase Chain Reaction (PCR). The primers for cDNA synthesis contained forward primer with signal sequence (5'-GTAAAGCTT AGCAGTGATGGTCGGCTTTACCC -3'), without signal sequence (5'- GTAAAGCTTACAGACAACCCCCAACT CGGAGG-3') and reverse primer (5'-CGCGGATCCTTCG CCCACGTCAGCAACGGTG -3'). The underlined sequence showed the restriction enzyme *HindIII* site (forward primer) and *Bam*HI site (reverse primer) for subcloning into pUC19 vector. The PCR was performed as follow: 95°C for 15 min followed by 30 cycles at 94°C for 60 sec, 55°C for 30 sec, 72°C for 60 sec and final extension steps at 72°C for 10 min. The PCR products were purified from the gel, digested with restriction endonluclease (HindIII and BamH1) and ligated into the corresponding sites of pUC19. Thereafter, the ligation products were transformed into *E. coli* JM109 competent cells (Sambrook *et al*., 1989).

Culture condition: *E. coli* JM109 harboring xylanase gene with and without signal sequence was grown overnight at 37°C in Luria-Bertani (LB) medium supplemented with 50 μg mL^{-1} ampicillin. Five milliliter of overnight culture was inoculated into fresh 50 mL of LB medium containing 50 μg mL^{-1} ampicillin. After growth at 30°C to an A_{600} of 0.6-0.8, Isopropyl-β-D-Thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM. The culture was further incubated at 30°C for 3 h. The cells were then harvested by centrifugation, washed and resuspended in 50 mM phosphate buffer (pH 7.0). Cytoplasmic and extracellular xylanase activities were determined. The extracellular activity was determined in the cell-free supernatant obtained after centrifugation on the culture broth. The pellet was resuspended in 30 mM Tris-HCl buffer (pH 8.0) and the cytoplasmic content was released by sonication. The cytoplasmic xylanase activity was measured in the clarified lysate.

Partial purification: The xylanase produced by *T. lanuginosus* SKR and both transformants were partially purified using 80% $(NH_4)_2$ SO_4 saturation and DEAE cellulose column as described previously (Khucharoenphaisan *et al*., 2008). Each partially purified xylanase was used for determining their optimal temperature and optimal pH.

mRNA secondary structure prediction: The possible secondary structure of mRNA was predicted by MFOLD algorithm (http://mfold.rna.albany.edu). The MFOLD analysis was conducted at default condition. The calculation of the enthalpy of mRNA secondary structure was started at Shine-Dalgarno sequence and stopped at 100 nucleotides downstream.

Determination of β-xylanase activity: β-Xylanase was assayed by determination of reducing sugars liberated from beechwood xylan when incubated with the diluted enzyme solutions at pH 7.0 and 50°C for 10 min. The reducing sugars were measured using the DNS reagent (3, 5-dinitrosalisylic acid). One unit of β-xylanase activity was defined as the amount of enzyme that produced 1 μmole of xylose in 1 min.

Determination of optimal pH and temperature of xylanase activity: Investigation to find out the optimal pH for xylanase activity was carried out in 50 mM buffers with various pH values ranging from pH 4.0 to pH 9.0 (acetate buffer: pH 4.0-5.0, phosphate buffer: pH 6.0-8.0, Tris-HCl buffer: pH 9.0). Then incubated at 50°C for 10 min. Amount of xylanase activity was determined under standard assay procedure.

The optimal temperature for xylanase activity was determined by incubating the enzyme in 50 mM phosphate buffer (pH 7.0) at various temperatures (40-90°C) for 10 min. Amount of xylanase activity was determined under standard assay condition.

Statistical analysis data: All the data were expressed as Mean±Standard Deviation (SD). The experimental data were analyzed using Descriptive statistics followed by Explore menu of the software. Statistical differences yielding $p<0.05$ were considered significant. The analysis was performed using GNU PSPP Statistical Analysis Software Release 0.6.2.

RESULTS AND DISCUSSION

Expression of recombinant xylanase: The transformants containing xylanase gene with (pUC/Xynsig) and without original signal sequence (pUC/Xyn) were cultivated in LB/ampicillin medium. The xylanase activities were estimated after induction by IPTG for 3 h as shown in

Table 1. Both transformants exhibited 17-19% as extra cellular xylanase whereas 81-83% of total activity was found in cytoplasmic fraction. Since *E. coli* could not secrete any intracellular enzyme into the culture medium because it lacked of post-translation modification. Thus, extra cellular xylanase was resulted in the cell lysis of *E. coli*. The productivities of both transformants were different (Table 1). Total xylanase productivity of pUC/Xynsig (4710±0.045 U L^{-1}) was 56% higher than that of pUC/Xyn (3020±0.062 U L^{-1}). However, some of xylanase of both transformant may be accumulated as inclusion body. This phenomenon is usually occurred

Table 1: Comparison of xylanase expression using pUC19 with and without original signal sequence in *E. coli* JM109

	Xylanase activity (U L^{-1})	
Expression	pUC/Xynsig	pUC/Xyn
Extra cellular	900±0.014	500±0.016
Cytoplasmic	3810±0.030	2520±0.049
Total	4710±0.045	3020±0.062

pUC/Xynsig means vector pUC19 containing xylanase gene with original signal sequence. pUC/Xyn means vector pUC19 containing xylanase gene without original signal sequence

when a recombinant protein was over-expressed in *E. coli*. This result indicated that signal sequence stimulated xylanase expression in *E. coli*. The variation of xylanase production between pUC/Xynsig and pUC/Xyn might be due to a translation level. This result was similar to reports of Khan *et al*. (2007) and Ramanan *et al*. (2010) who suggested that the translation initiation associated with mRNA secondary structure of caprine growth hormone and human interferon-2b stimulated the expression level in *E. coli*.

Effect of signal sequence on mRNA secondary structure: The possible secondary structures of mRNA of both plasmids were predicted from 100 nucleotides. The mRNA secondary structure of pUC/Xyn was predicted to form hairpin structure. Shine-Dalgarno (SD) sequence and start codon of AUG were corporate in this structure (Fig. 1a). After inserted original signal sequence to xylanase structural gene (pUC/Xynsig), the original hairpin structures was disappeared and start codon (AUG) was released from a loop (Fig. 1b). The present of hairpin

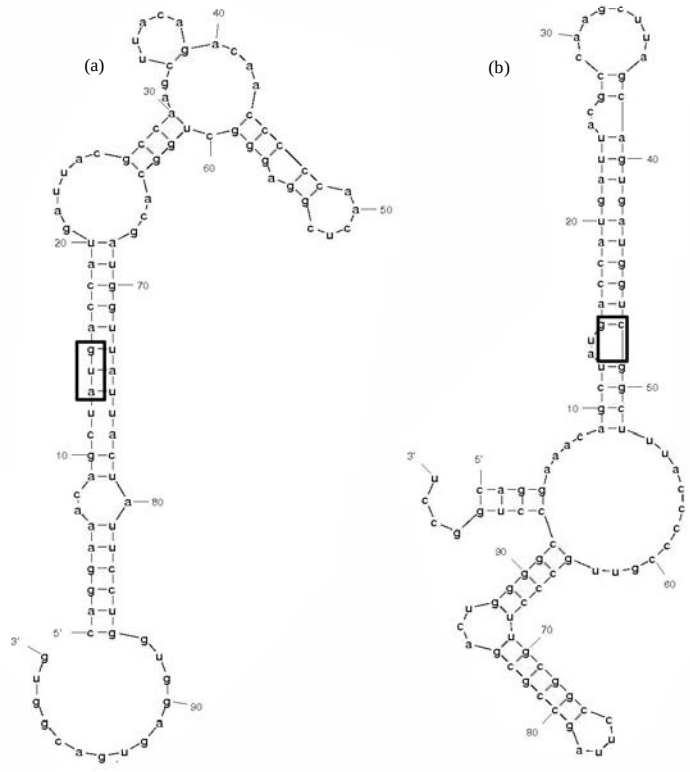

Fig. 1: mRNA secondary structure of the 100-nucleotide xylanase gene in the translation initiation region with original signal sequence (a) and without signal sequence (b) by MFOLD algorithm. The position of AUG start condon indicated by box

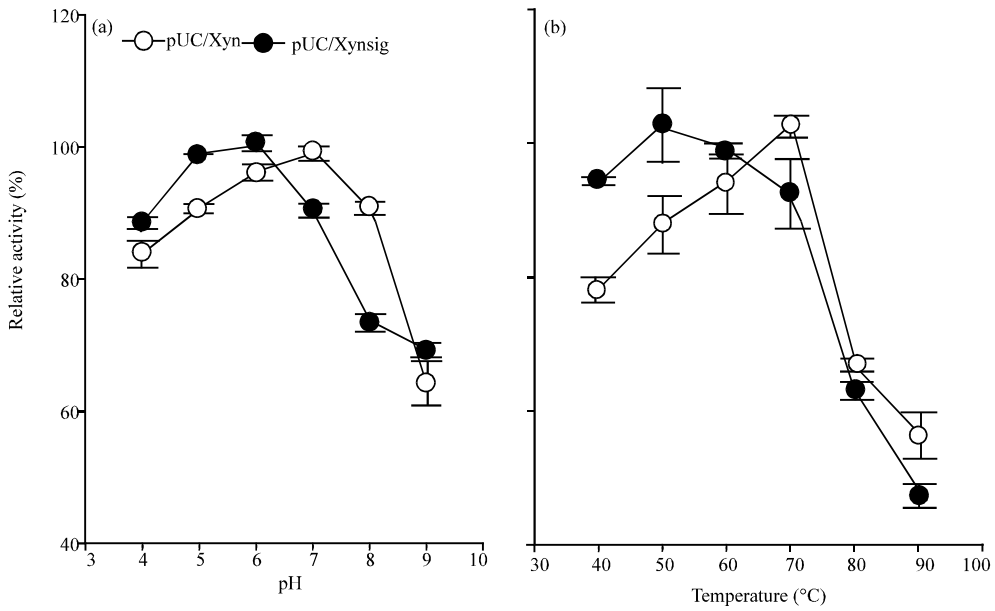

Fig. 2: Effect of pH (a) and temperature (b) on partially purified xylanase activity obtained from pUC/Xyn and pUC/Xynsig. For the effect of pH, the reaction mixture was incubated at 50°C with various buffers and for the temperature analysis the sample were incubate at each temperature for 10 min in 50 mM phosphate buffer pH 7.0

structure on SD-region and AUG codon prevented accessibility to the 30S ribosomal subunit and inhibited translation (Cruz-Vera *et al*., 2004). Present result indicated that the presence of its signal sequence could improve mRNA secondary structure increasing translation initiation process. This finding agreed with several researchers who reported that presence of signal sequence was able to increase expression level of asparaginase and interferon alpha 2b in *E. coli* (Khushoo *et al*., 2005; Sletta *et al*., 2007; Ramanan *et al*., 2010).

Optimization of pH and temperature on xylanase activity: Optimal pH for the xylanase activity of transformant (pUC/Xyn) was pH 7.0 (Fig. 2a) in which was similar to original *T. lanuginosus* SKR xylanase. This optimal pH was closed to optimal pH of *T. lanuginosus* DSM 5826 xylanase (Cesar and Mrsa, 1996) but differed from xylanase of *T. lanuginosus* NK-2, *T. lanuginosus* THKU-49 and *T. lanuginosus* THKU-9 (Naveen *et al*., 2006; Khucharoenphaisan *et al*., 2008). Whereas, transformant of pUC/Xynsig showed low optimal pH at 6.0 that was lower than that of xylanase from pUC/Xyn (Fig. 2a) and *T. lanuginosus* SKR.

Optimal temperature to exhibit its activities of xylanase from transformant (pUC/Xyn) was 70°C when incubating for 10 min (Fig. 2b). The optimal temperature of this enzyme was similar to original xylanase in which was obtained from *T. lanuginosus* SKR. It could be observed that the enzymatic activity was notably changed. This result was supported by the studies of Yin *et al*. (2008) who found that xylanase gene without signal sequence from *T. lanuginosus* expressed in *E. coli* having same properties with original one. However, the xylanase from other transformant of pUC/Xynsig exhibited lower optimal temperature than that of pUC/Xyn with 50°C. The optimal temperature of pUC/Xynsig was dropped for 20°C when comparing to xylanase from pUC/Xyn and *T. lanuginosus* SKR. The reason for decrease of optimal temperature and optimal pH was not clear. It is possible to be the resulted from the slightly change of recombinant protein conformation when the xylanase gene has signal sequence in host system. In many case, recombinant proteins were lost ability to reach native conformation and not function (Gasser *et al*., 2008).

CONCLUSION

The xylanase from *T. lanuginosus* SKR was successfully expressed in *E. coli* using pUC19 vector. The xylanase gene with original signal sequence of xylanase from *T. lanuginosus* SKR could increase the total xylanase production of 56% comparing to without of those. The optimal temperature and optimal pH of partially purified recombinant xylanase (pUC/Xyn) were identical to the value reported for the partially purified xylanase from its

original host *T. lanuginosus* SKR whereas other partially purified recombinant xylanase (pUC/Xynsig) was a little changed. This knowledge maybe applied for suitable plasmid for enzyme expression and used in various industrial such as brewing and animal feed.

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