

## Research Article

### Optimizing Prokaryotic Expression of a Xylanase Gene from the Apple Pathogen *Valsa mali* var. *mali*

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**Abstract:** *Valsa mali* var. *mali* is a causal agent of apple tree canker disease, that causes serious economic losses in China. The lack of understanding of the pathogenic genes and mechanisms of *V. mali*, which poses difficulties to control the disease. Whereas, previous studies have indicated that xylanase produced by *V. mali* is a key pathogenic factor. In this study, *xylanase* gene was cloned and obtained recombinant expression vector pET-*Xylanase*, in addition, a prokaryotic expression system for Xylanase protein was established and the expression conditions were optimized. Xylanase protein expressed in *Escherichia coli* reached a maximum level after induction with 0.5 mmol/L isopropyl  $\beta$ -thiogalactopyranoside (IPTG) for 12 h at 30°C. The recombinant protein was confirmed by Western blot analysis using Anti-His mouse monoclonal antibody and the result showed that the induced ~63 kDa band in *E. coli* was Xylanase protein. The results presented will help to better reveal the *xylanase* gene functions in the pathogenicity of *V. mali*.

**Keywords:** Condition optimization, Prokaryotic expression, SDS-PAGE, Western blot, Xylanase protein

## INTRODUCTION

Apple tree canker disease, caused by *Valsa mali* var. *mali* is a destructive disease that is a problem of economic significance in apple production in China (Chen *et al.*, 1987; Wang *et al.*, 2012; Li *et al.*, 2013). The pathogen causes elongated cankers on twigs and trunks, which could lead to death of the entire tree (Chen, 2009). The official data from the Chinese Modern Agricultural Industry Technology System revealed that in 2008, the incidence of apple tree canker disease across the country was 52.7%, with that in some areas being more than 85% (Cao *et al.*, 2009). Presently, almost all apple cultivars are susceptible to *V. mali* with only some apple rootstocks being resistant (Abe *et al.*, 2007; Abe *et al.*, 2011). Therefore, it is difficult to control *Valsa* canker disease by using resistant cultivars (Zhang *et al.*, 2014). In addition, the pathogen could infect xylem such as the pruning wounds and reside there without resulting in any symptoms, causing the use of fungicide ineffective (Li *et al.*, 2013). Up to now, *Valsa* canker disease is still not controlled efficiently, which mainly is due to the lack of understanding of the pathogenicity genes and their corresponding pathogenicity mechanisms at

molecular levels (Gao *et al.*, 2011; Wang *et al.*, 2013).

As a weak pathogen, *V. mali* infects apple tree by producing a series of cell wall degrading enzymes and toxic compounds to collapse and kill host cells, all of which have been conformed as pathogenesis factors (Liu *et al.*, 1980; Natsume *et al.*, 1982; Chen *et al.*, 2012; Wang *et al.*, 2014). Researches by Koganezawa and Sakuma (1982) and Natsume *et al.* (1982) revealed that *V. mali* produced five kinds of toxic compounds both in host bark media and in host tissues. Recent studies showed that the amount of toxic compounds produced by *V. mali* was closely related to the pathogenicity of different strains, moreover, protocatechuic acid was the most effective compound that produced noticeable symptoms on apple at the lowest concentration (Li *et al.*, 2014; Wang *et al.*, 2014). In other studies, cell wall degrading enzymes secreted by *V. mali* were investigated. Liu *et al.* (1980) and Wang *et al.* (2009) revealed that the pathogen could produce pectinase, an enzyme which could decay tomato seedlings. Our group found that *V. mali* also could produce five kinds of enzymes including polygalacturonase, polymethylgalacturonase, cellulose,  $\beta$ -glucosidase and xylanase. The activities of enzymes were also significantly correlated with the pathogenicity

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of strains, meanwhile, xylanase activity was the highest among all the enzymes in the infection of pathogen and it showed the most powerful macerating ability to apple tissues (Chen *et al.*, 2012; Li *et al.*, 2014). Despite all the results indicated that xylanase was a key pathogenic factor of *V. mali*, its gene functions have yet to be determined.

To clearly understand the functions of *xylanase* gene of *V. mali*, the gene was successfully cloned using RACE technology and recombinant expression vector pET-*Xylanase* was constructed in our previous work. In this study, recombinant plasmid pET-*Xylanase* was transformed into *Escherichia coli* and Xylanase protein expression procedure was optimized to increase heterologous expression efficiency. Furthermore, recombinant Xylanase protein was analyzed by dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot.

## MATERIALS AND METHODS

**Fungal strain, plasmids and culture medium:** *Valsa mali* var. *mali* isolate LXS080601 isolated from infected 'Fuji' apple branch, was identified and maintained as previously described (Chen *et al.*, 2012; Zhao *et al.*, 2012). *Xylanase* gene cloning, plasmid amplification and preparation were performed followed routine protocols (Wang *et al.*, 2007) in our previous work (not published). *Escherichia coli* strain DH 5 $\alpha$ , Rosetta (DE3) and vector pMD 18-T, pET 32a were purchased from Takala (Dalian, China) and Novagen (Darmstadt, Germany) Ltd. Luria-Bertani (LB) broth was prepared with 5 g/L yeast extract, 10 g/L tryptone and 10 g/L NaCl. Autoclave was performed at 121°C for 20 min.

***E. coli* transformation and gene expression:** *E. coli* cells were grown in LB medium and prepared for transformation following the manufacturer's instruction. The cells were transformed with the recombinant vector using heat shock method. The recombinant *E. coli* Rosetta colony was picked with a sterilized toothpick and transferred into 5 mL LB culture liquid supplemented with 50 mg/L ampicillin. The culture was put in a shaker at 37°C overnight with a constant speed of 180 r/min. Then, 0.1 mL of this culture was inoculated into 10 mL LB medium supplemented 100 mg/L ampicillin and grown at 37°C with vigorous shaking until OD<sub>600</sub> reached 0.5. To induce target protein expression, different culture conditions were optimized followed the pET System Manual (Novagen, Darmstadt, Germany). The cells were harvested by centrifugation at 10 000×g for 10 min. Every sample was prepared from 1.5 mL of culture liquid with various parameters and protein expression levels were assessed using dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Heegaard *et al.*, 2002; Wang *et al.*, 2007).

### Optimization of protein expression in *E. coli*:

Optimal conditions for Xylanase protein expression were determined with different isopropyl  $\beta$ -thiogalactopyranoside (IPTG, Invitrogen, USA) concentrations, induction time and temperature. When the OD<sub>600</sub> reached 0.5, IPTG was added with the different final concentrations of 0, 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0 mmol/L. 2 mL liquid culture was collected at 0, 2, 4, 6, 8, 10 and 12 h after IPTG addition, respectively. Then, the protein expression was induced at different temperature (20, 30 and 37°C), with a shaking speed of 180 r/min for 8 h. Cells were centrifuged and resuspended in lysis buffer (125 mmol/L Tris-HCl, containing 40 g/L SDS, 20% glycerin, 10 mmol/L  $\beta$ -mercaptoethanol). The induced proteins were separated in 12% SDS-PAGE and visualized by staining with Coomassie Blue G250.

### Recombinant protein detection and western blotting analysis:

The expressed protein with the optimization condition was verified by Western blotting analysis (Wang *et al.*, 2007). Recombinant proteins were resolved by SDS-PAGE on 12% polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF, Roche, Switzerland) membranes, which were pretreated by methanol. The membranes were incubated with blocking solution (0.5% Bovine serum albumin in 10 mmol/L Phosphate Buffered Saline (PBS)) for 2 h at room temperature with constant shaking and were subsequently probed with diluted Anti-His mouse monoclonal antibody (1:5 000) for 2 h. The membrane was washed and then incubated for 2 h with horse anti-mouse IgG secondary antibody conjugated with alkaline phosphatase (Promega, Madison, USA). Lastly, the membrane was washed and detected using nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Promega, USA).

## RESULTS

**Cloning of the *Xylanase* gene:** *Xylanase* gene was amplified by PCR from the recombinant plasmid pMD-*Xylanase*. The expected 1320 bp product was cloned into the pET-32a vector in order to obtain prokaryotic expression of N-terminally His-tagged Xylanase, which could be detected and purified using His antibody and nickel resin column, respectively. The recombinant vector pET-*Xylanase* was transformed into *E. coli* Rosetta. The positive colonies were picked and verified by PCR with the primers of *Xylanase* R/*Xylanase* F

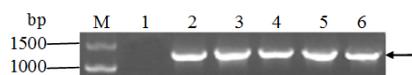


Fig. 1: Identification of recombinants in *E. coli* by polymerase chain reaction assay with *Xylanase* primers. Lane M: DNA molecular weight marker with bases indicated on the left. Lane 1: negative control with vector pET-32a; Lanes 2 to 5: putative recombinants

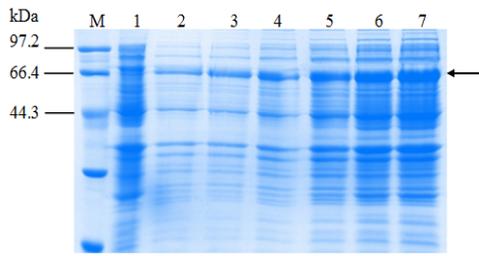


Fig. 2: SDS-PAGE analysis on the optimized expression time of Xylanase protein in *E. coli* Rosetta with Coomassie blue staining. Lane M: protein molecular weight marker. Lane 1: cell pellet fractions of vector pET 32a as negative control after induction with 1.0 mmol/L IPTG for 12 h. Lane 2 to 7: cell pellet fractions of pET-Xylanase after induction with 1.0 mmol/L IPTG for 2, 4, 6, 8, 10 and 12 h, respectively. The recombinant protein band was observed at molecular weight of ~63 kDa (arrow) compared with the negative control. The band intensity increased from 2 h after induction and reached a maximum level at 12 h with IPTG induction, the optimum time for Xylanase protein expression

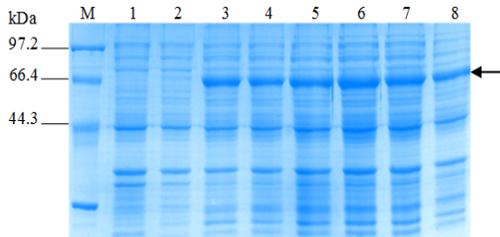


Fig. 3: SDS-PAGE analysis on the optimized IPTG concentrations for Xylanase protein expression in *E. coli* Rosetta with Coomassie blue staining. Lane M: protein molecular weight marker. Lane 1: cell pellet fractions of vector pET 32a after induction with 1.0 mmol/L IPTG for 6 h. Lane 2: cell pellet fractions of pET-Xylanase before induction (0 h). Lane 3 to 8: cell pellet fractions of pET-Xylanase after induction with 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0 mmol/L IPTG for 6 h, respectively. The recombinant protein band intensity (arrow) increased from 0.05 mmol/L IPTG induction and reached a maximum level with 0.5 mmol/L IPTG, the optimum IPTG concentration for Xylanase protein expression

(Fig. 1). Sequence analysis confirmed that the insert into the pET-Xylanase plasmid showed 100% identify with the target Xylanase gene.

**Optimization of Xylanase gene expression in *E. coli*:**

Induction expression of Xylanase protein was optimized by altering different parameters and production levels were analyzed by SDS-PAGE as shown in Fig. 2. Upon induction of Xylanase expression from *E. coli* containing pET-Xylanase, a ~63 kDa band of increasing intensity was observed, which was absent in the negative control (empty vector

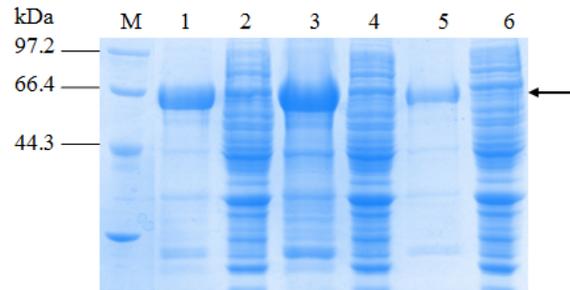


Fig. 4: SDS-PAGE analysis on the optimized induction temperature for Xylanase protein expression in *E. coli* Rosetta with Coomassie blue staining. Lane M: protein molecular weight marker. Lane 1, 3 and 5: cell pellet fractions of pET-Xylanase after induction at 37, 30 and 20°C, respectively, with 0.5 mmol/L IPTG for 12 h. Lane 2, 4 and 6: soluble fractions of pET-Xylanase after induction at 37, 30 and 20°C, respectively, with 0.5 mmol/L IPTG for 12 h. The recombinant protein band (arrow) only was observed in the pellet, but not in the soluble fractions. The production of Xylanase protein reached a maximum level at 30°C, the optimum induction temperature for protein expression

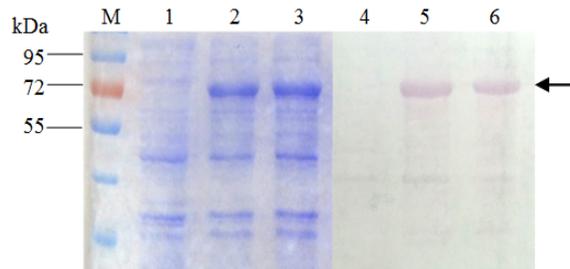


Fig. 5: Western blot analysis on the recombinant Xylanase protein probed with Anti-His mouse monoclonal antibody. Lane M: pre-stained protein molecular weight marker. Lane 1 and 4: cell pellet fractions of vector pET 32a as negative control. Lane 2 and 5, Lane 3 and 6: cell pellet fractions of pET-Xylanase after induction with 0.5 mmol/L IPTG for 8 h at 30°C. The ~63 kDa recombinant Xylanase protein was confirmed (arrow)

pET 32a). Production of the protein was increased with induction time after induction with 1.0 mmol/L IPTG and reached a maximum level at 12 h with IPTG induction, the optimum time for Xylanase protein expression (Fig. 2). For different IPTG concentrations induction, the recombinant protein band intensity increased from 0.05 mmol/L IPTG and reached a maximum level with 0.5 mmol/L IPTG, the optimum IPTG concentration for Xylanase protein expression (Fig. 3). The recombinant protein expression in *E. coli* Rosetta induction at 30°C was higher than the value obtained induced by the other temperatures. However, the Xylanase protein only was observed in the pellet fractions, no target protein band was appeared in the soluble fractions (Fig. 4).

**Confirmation of recombinant protein by western blot analysis:** Following optimization of Xylanase protein expression in *E. coli*, recombinant protein was detected by Western blot analysis using Anti-His mouse monoclonal antibody (Fig. 5). The result further indicated that the induced ~63 kDa band in *E. coli* was Xylanase recombinant protein.

## DISCUSSION

*Xylanase* gene from *V. mali* var. *mali* was successfully cloned into a prokaryotic expression vector and transformed into *E. coli*. Full-length recombinant Xylanase protein was subsequently expressed and optimized, yielding a ~63 kDa protein. However, the predicted molecular weight of Xylanase protein is ~45 kDa. The discrepancy between the calculated and expressed molecular weights could be explained by the presence of His-tag (~18 kDa) in recombinant Xylanase protein (Pei *et al.*, 2014). The recombinant Xylanase protein was identified by Western blot analysis with Anti-His mouse monoclonal antibody.

Although heterologous protein expression in *E. coli* is widely applied for gene function analysis, its efficiency varies among different gene and even the expression vector or *E. coli* strain used could also impact the efficiency (Hannig and Makrides, 1998; Sørensen and Mortensen, 2005; Munjal *et al.*, 2015). In order to determine the optimal expression condition of Xylanase protein, expression vector pET 32a and *E. coli* Rosetta were selected (Bai *et al.*, 2013). In our study, Xylanase protein also could be expressed in *E. coli* BL21, however, the protein concentration was extremely low (data not shown). In addition, the induced time, temperature and IPTG concentration were also optimized. It was found that using 0.5 mmol/L IPTG and 12 h induced time at 30°C could result in the highest expression efficiency. IPTG is necessary for heterologous protein expression in *E. coli*, which is also verified by our study in which adding an optimum concentration of IPTG (0.5 mmol/L) enhanced protein expression amount. However, too high concentration of IPTG (1 and 2 mmol/L) inhibited Xylanase protein expression; probably due to high IPTG concentration inhibited the growth of *E. coli* cells (Pei *et al.*, 2014).

Expression vector, pET 32a, contains a His-tag which not only can improve the translation efficiency, but also can be used as a marker to purify the recombinant protein expressed in *E. coli* (Bai *et al.*, 2013). The heterologous expression and optimization of conditions of *Xylanase* gene will provide a basis for further revealing the functions of *xylanase* produced by *V. mali*.

## CONCLUSION

Here we report, for the first time, the development and optimization of Xylanase protein expression in *E. coli*, a key pathogenic factor of *V. mali*. The optimized

conditions enabled us to obtain a large number of Xylanase protein and it's easy to purify the recombinant protein using His-tag. Therefore, it will be useful for further understanding of the pathogenesis mechanisms of *xylanase* gene and for better exploration of new strategies to control Valsa canker disease.

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