

SHORT COMMUNICATION

HOT PHENOL EXTRACTION OF TOTAL RNA FROM *Thermoascus aurantiacus* AND CHARACTERIZATION OF ITS THERMOSTABLE XYLANASE GENE

ANG CHUNG HUAP, AWANG AHMAD SALLEHIN AWANG
HUSAINI* & HAIRUL AZMAN ROSLAN

*Department of Molecular Biology, Faculty of Resource Science and Technology,
Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak*

ABSTRACT

Total RNA was successfully isolated using hot phenol extraction method. Three bands representing the 18S, 5.8S and 28S rRNA was observed. No heavy smearing was observed in the RNA band patterns, indicating low levels of polysaccharide contamination, when subjected to 1% agarose gel electrophoresis. Genomic DNA was eliminated using DNase I digestion and lithium chloride (LiCl) precipitation. Two-steps reverse transcriptase polymerase chain reaction (RT-PCR) using M-MuLV Reverse Transcriptase and sequence specific primers for *xylanase* gene, XynA(F) and XynA(R), successfully generated the target amplicon of 500 base pairs (bp). Sequence analysis of the PCR product indicated as partial sequence of *Thermoascus aurantiacus xylanase* gene (*XynA*) deposited in the NCBI GenBank with accession number: AF127529.1 and AJ132635.1. Hot phenol extraction is useful for extracting large quantities of total RNA sufficient for complementary DNA (cDNA) synthesis in shorter period of time.

Keywords: Hot phenol extraction, thermophilic fungus, *Thermoascus aurantiacus*, DNase I digestion, cDNA synthesis, thermostable xylanase

Xylanases of thermophilic fungi with high thermostability has received considerable attentions due to their potential applications in pulps bleaching that occurred at high temperature (Gupta *et al.* 2000; Maheswari *et al.* 2000). Genetic engineering of thermostable *xylanase* gene isolated from thermophilic fungus has been carried out to obtain high levels of thermostable recombinant xylanase that can be used for industrial applications (Emami & Hack 2000; Shibuya *et al.* 2000; Damaso *et al.* 2003).

In this study, a thermophilic fungus *T. aurantiacus* was successfully isolated from water sediment samples of Gadek Hot Spring in Melaka. The fungus produced high levels of thermostable xylanase when cultured in basal media. Xylanase of *T. aurantiacus* that was isolated in this study appeared to be active over a board range of temperature

ranging from 40°C to 70°C (Husaini, unpublished). The finding of the thermostable xylanase of this local *T. aurantiacus* is very useful in the bio-bleaching of pulps in the paper industry. Isolation of total RNA is essential for amplification of functional gene from xylanolytic fungus. Like other eukaryotic organisms, introns presence in genomic DNA of *T. aurantiacus* interferes with the expression of the recombinant xylanase in expression vector. Therefore, cDNA was constructed in order to obtain functional thermostable *xylanase* gene using reverse transcription-polymerase chain reaction (RT-PCR).

Thus, the objective of this study is to use the hot phenol method in order to obtain a large quantity of total RNA sufficient to be used for synthesis of *T. aurantiacus* thermostable *xylanase* gene. This in turn will determine whether the hot phenol method used is an effective and simplified method in extracting total RNA from any fungal species.

*Corresponding author: haahmad@frst.unimas.my

T. aurantiacus was cultivated in 100 mL basal media containing 0.5% (w/v) yeast extract, 0.5% (w/v) KH_2PO_4 . The production of β -xylanase was induced using 1% (w/v) birchwood xylan (Sigma, USA) that also acted as carbon source. Induction was maintained for 72 hours at 55°C. The fungus produced high levels of thermostable Xylanase and the activity was maintained at a board range of temperature ranging from 40°C to 70°C. At the end of the incubation period, no bacterial contamination was observed in the fungal culture as 50mg/mL of ampicilin was added to inhibit bacterial growth. *T. aurantiacus* culture was incubated at 55°C since the xylanase enzyme of this fungus has maximal activity at temperature between 55°C to 60°C.

The total RNA extraction was carried out according to the hot phenol method described by Nel (2001) with slight modification. Fungal mycelium was filtered and grinded into fine powder. Subsequently, 10 mL of saturated phenol II (pH 4.5) and 10 mL of STE buffer (100mM NaCl, 250mM Tris-HCl pH 7.2, 10mM EDTA pH 8.0) with 200 μL of 10% (w/v) SDS was added into the mycelium powder. The mixture was incubated at 65°C for 4 minutes and chilled on ice for 5 minutes. The cold mixture was then centrifuged and the supernatant was extracted twice with equal volume of phenol : chloroform : isoamylalchol (at ratio of 25:24:1 (v/v/v)). The supernatant was then precipitated overnight with 8 M lithium chloride (LiCl). The white pellet was wash with 70% ethanol and suspended in sterile diethyl polycarbonate (DEPC) water.

The purity of *T. aurantiacus* total RNA was analysed using the UV/Visible spectrophotometer with wavelength at 230 nm. The total RNA was subjected to 1 % (w/v) agarose gel electrophoresis, and then used for cDNA synthesis via a two-step RT-PCR was conducted in this work. The first strand synthesis was carried out using the RevertAid™-H Minus First Strand cDNA Synthesis Kit (Fermentas USA). The second step consisted of amplification of the thermostable xylanase gene (XynA). This was achieved by using sequence specific PCR primer XynA(F): 5'-AAAGCTACCTACCCCTTCTGTCAAT-3' and XynA(R): 5'-TCTGTTCCGTTCTATACTCACTGC-3'. The PCR reaction was performed with the following condition: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing of PCR primers with cDNA template at 55°C for 1 minute and extension

at 72°C for 1 minute. The PCR was completed with final elongation at 72°C for 5 minutes.

Sequence analysis of the RT-PCR products were sent for sequencing and the sequence obtained was then aligned with known genomic sequences of *T. aurantiacus* xylanase gene deposited in NCBI GenBank by using Chromas Pro (Version 1.33).

In this study, hot phenol was used as a simplified method to reduce the extraction period and to prevent degradation of total RNA. Total RNA with was extracted from the 3-days old *T. aurantiacus* culture medium. An average of 0.2 $\mu\text{g}/\mu\text{l}$ of total RNA with purity of 1.8 to 2.0 (A_{260}/A_{280}) was successfully yielded. Figure 1 is a gel electrophoresis picture of the total RNA band patterns. Three bands were clearly seen when ran on 1% (w/v) agarose gel representing the 18S, 5.8S and 28S rRNA. No heavy smearing was detected in the RNA bands as well as the genomic DNA band compared to the total RNA bands, indicating low levels of polysaccharide contamination and RNA degradation. Genomic DNA was then eliminated from the RNA samples using LiCl precipitation method. The LiCl will only precipitate RNA molecules leaving the unwanted DNA molecules in supernatant fraction (Nel 2000). DNase I was subsequently used to degrade the remainder of the genomic DNA molecules that may interrupt the synthesis of cDNA.

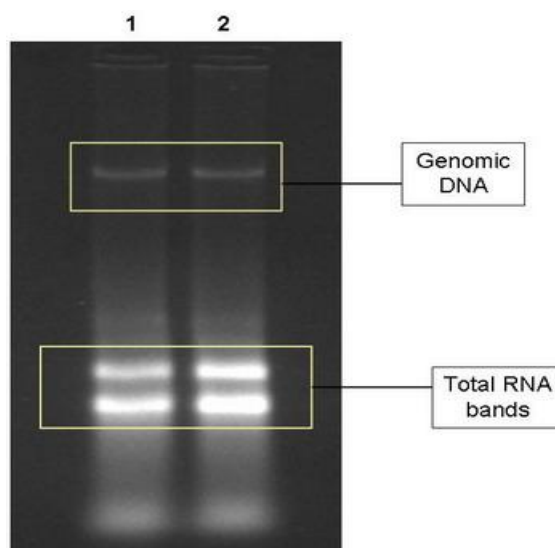


Figure 1. Total RNA extracted from *T. aurantiacus* using hot phenol extraction method.

A single PCR band was successfully amplified using the XynA(F) and XynA(R) primers. The size

of PCR band was estimated to be 500bp (Figure 2). The PCR product was sent for sequencing and analysis had identified the sequence to be a partial sequence of *T. aurantiacus* XynA gene, exon 1-11 deposited in the NCBI GenBank.

The sequence of the amplified RT-PCR product was also subjected to nucleotide-protein blast search to acquire the homologous protein sequences for thermostable *xylanase* gene. The query identified 45% identities similarity with the endo-1,4-beta-xylanase A precursor from *T. aurantiacus* and the deduced amino acid also contain the glycol hydro10 superfamily conserved region from amino acid 18 to 54. Figure 3 shows the deduced amino acids sequences for WW1 thermostable *xylanase* gene.

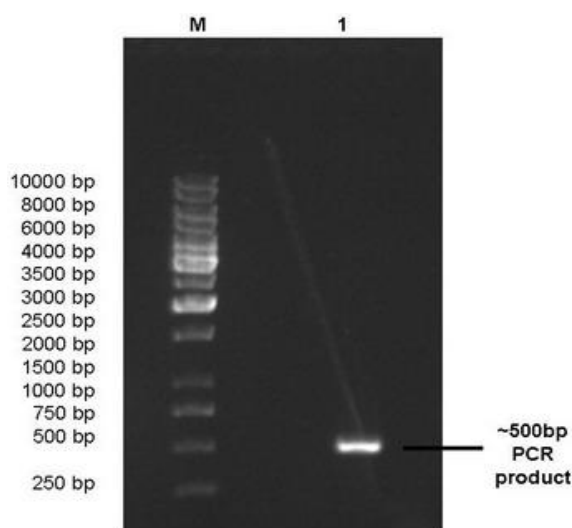


Figure 2. Lane 1- The 500 bp PCR product generated by Xyn A(F) and XynA(R) primers. Lane M – 1 kb DNA ladder (Fermentas).

1	FLSYEGNRID	RPPSMET QLP	SWSSITDK
31	NTLINVMKNH	ITTLMTRYKG	KIRAW VSHPT
61	LSCVSMKRQI	RTHVFARPFQ	NQNQNHIGR
91	GERGIQRGWL	PPPECLPQRH	RGGLHPDCF
121	DRPRRPECQA	VYQRLQVRFK	AQYSICE

Figure 3. Deduced amino acid sequence of the WW1 thermostable *xylanase* gene. The conserved region is indicated in bold.

In conclusion, partial sequence of thermostable *xylanase* gene from an indigenous thermophilic fungus, *T. aurantiacus*, has been successfully obtained in this study. Total RNA was successfully extracted using hot phenol method with high RNA purity and low level of RNA degradation.

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