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*Full Length Research Paper*

# Comparison of RNA extraction methods applied to gene cloning of the taxol-producing fungi

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**The qualities of RNA are an important foundation for molecular biology research. In this study, five different methods were selected for extracting RNA from the taxol-producing endophytic fungi. Yield and quality of isolated RNA were assessed by routine test method. The results showed that the Trizol method perform was better than others method in the yield, but the LiCl method perform better than others methods in quality using $A_{260/280}$ and $A_{260/230}$ ratios and agarose electrophoresis results. Further, using the RNA of extracting from fungi by LiCl method, 10-deacetylbaccatin III-10-*O*-acetyltransferase (DBAT) gene partial sequence were successful amplified using RT-PCR method. This study laid a foundation for further gene clone and construction of gene library.**

**Key words:** Taxol, taxol-producing endophytic fungi, RNA extracting, RT-PCR.

## INTRODUCTION

Taxol is an effective anticancer drug used widely in the treatment of a variety of cancers. It is major extracted from *Taxus* spp., but the low abundance taxol of other reported taxanes limits their availability for pharmacological evaluation. For example, the commercial isolation of 1 kg of taxol required about 6 - 7 t of yew bark, equivalent to 2000 - 3000 trees. As the first taxol-producing endophytic fungus was isolated from *Taxus brevifolia* in 1993 (Stierle et al., 1993), it provided us a new prospect to obtain taxol from fungi. Producing taxol via microorganism fermentation may be an alternative way. Meanwhile the microorganisms are extremely valuable material for taxol metabolism studies (Lin et al., 2003; Chen et al., 2004). However, due to the low content of taxol, isolation of taxol or taxol production with endophytic fungi can't be commercially carried out industrialized production. Therefore, researchers attempted improve endophytic fungi strains by means of genetic engineering to enhance taxol or taxol production yield (Wang et al. 2007;Zhou et al., 2008). Whether the taxol biosynthesis pathway has a difference in yew and in taxol-producing fungi? All the research in this scope has not been answering until now. So it is very important to prove the taxol biosynthesis way in plant and fungi.

The endophytic fungi EFY-21 and EFY-36 are taxol-producing endophytic fungi which recently were isolated from *Tsuga chinensis* var. *mairei* in our laboratory (Zhou et al., 2007, 2009). Nevertheless the researches of their taxol metabolic pathway are still blank.

Generally, the major issue with fungi RNA isolation is the presence of problematic bimolecular such as lipids, polysaccharides and polyphenolic compounds which make it difficult to get relatively clean separation of RNA and the rest of the cellular debris. In this case, isolation of RNA from endophytic fungi is one of technical challenge for the genetics research of fungus taxol metabolism, because it contains high levels of polysaccharides which co-precipitate with RNA. High quality RNA is vital for downstream application, such as gene clone and the construction of gene library. In order to

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assess the ability of five different methods in obtaining high-quality RNA from endophytic fungi samples for gene clone and the construction of gene library, we car - ried out a set of preliminary comparative trials. And then the 10-deacetylbaccatin III-10-*O*-acetyltransferase (DBAT) gene partial sequence was cloned. The high quality RNA extraction method will be helpful for the full sequence clone of DBAT gene and other genes such as taxadiene synthase (Zhou et al., 2007).

## MATERIALS AND METHODS

### Fungi and culture

The taxol-producing fungi EFY -21 and EFY-36, reported in previously publication (Zhou et al., 2007, 2009), were stored in Fudan University. They were routinely cultured for 48 - 72 h at 28°C on modified YPS (yeast extract, peptone and sucrose) liquid medium, containing 20.0 g/L sucrose, 20.0 g/L peptone, 5.0 g/L yeast extract, 1.0 g/L $MgSO_4$, 1.0 g/L $KH_2PO_4$ (pH6.8) . The cultures were centrifuged at 8000 g at 4°C for 10 min. After centrifugation, the mycelia were washed with 30 mL TE buffer for twice and then were froze nitrogen and stored at -80°C .

### Extraction of RNA

The RNA extractions were respectively performed using CTAB/NaCl method (Yang et al., 2006), STE method (Shui et al., 2008), Modified STE method, LiCl method (Manickavelu et al., 2007) and the Trizol kit. These methods have been modified in the course of the test.

### CTAB/NaCl method

Materials (1 g/sample) were homogenized in liquid nitrogen in a pre- cooled mortar, transferred to a tube containing 500 L extraction buffer (3% CTAB (W/V), 100 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 2M NaCl, 0.5 g/L spermidine, 3% PVP (V/V), 4% - mercaptoethanol (V/V) and mixed gently. The mixture was then placed in 65°C water bath for 2 min. Equal volume chloroform/ isoamy alcohol (24:1 V/V) was added, then the tube was reversed unceasingly for 5 min and centrifuged for 20 min at 12000 r/min at 4°C. According to the volume of supernatant, 0.25 volumes 10M LiCl (4°C) were added to keep at 4°C for 2 – 3 h. The tube was centrifuged for 30 min at 12000 r/min at 4°C. The supernatant was discarded. 500 L 0.5% SDS was added to resuspense the precipitation. Equal volume chloroform/isoamyl alcohol (24:1, V/V) was added and centrifugated 10 min at 12000 r/min at 4°C. Two volume chilled ethanol was added, kept at - 20°C for 2 h. After centrifugation the pellets were washed with 70% ethanol. The pellets were air dried and suspended in 100 L DEPC-treated water.

### STE method

Materials (1 g/sample) were homogenized in liquid nitrogen in a pre-cooled mortar, transferred to a tube containing 500 L extraction buffer (0.1M Tris-HCl (pH 8.0), 2 mM EDTA (pH 8.0), 0.2M NaCl, 1%SDS) and mixed gently. The equal volume PCI (Phenol/chloroform/isoamyl alcohol (25:24:1, V/V/V)) was added immediately and shaked intensity for 30 s. The supernatants were pooled into one tube and PCI treatments were done twice to remove protein. 1/10 volume NaAc (pH5.5) and 2.5 volume ethanol were added to mix well, kept at -20°C for 40 min. After centrifugation the pellets were washed with 70% ethanol. Finally, the pellets were air dried and suspended in 100 l DEPC-treated water.

### Modified STE method

All the steps were the same as STE method except the last step. After the NaAc and ethanol were added to the supernatants keep at -20°C for 40 min. After centrifugation the pellets were dissolving in 60 l of TE. Then the 40 L of 5M LiCl was added immediately and kept at 4°C for 2 – 3 h. After centrifugation the pellets were washed with 70% ethanol. Finally, the pellets were air dried and suspended in 50 L DEPC-treated water.

### LiCl method

The RNA extraction was operated based on method of Manickavelu et al. (2007).

### Trizol method

The RNA extraction was operated based on Trizol kit instruction (Watson Biotechnologies Inc, China).

### RNA analysis

$A_{260/280}$ and $A_{260/230}$ ratios were used to evaluate the quality of RNA. $A_{260}$ were used to calculate the concentration of RNA. The RNA ratio in the dry weight samples was calculated. Electrophoresis was used for detecting the integrity of RNA.

### Cloning of DBAT gene partial sequence using RT-PCR method

DBAT gene partial sequence was carried out with the primer DTF (5-GCTCTCTCC AAGGTGCTGGTTTAT- 3) and DTR (5-GAAGGTCATGGACATTATCCATTGC- 3). RNA extracted was used for the template of RT-PCR. The RT-PCR was performed as below: the RNA was reversely transcribed at 50°C for 30 min, dena-tured at 94°C for 2 min followed by 35 cycles of amplification (94°C for 40 s, 50°C for 40 s, 72°C for 2 min). The final elongation step was 10 min at 72°C. The RT-PCR products were purified using Gel Extraction Mini Kit (Watson Biotechnologies Inc, China), ligated to pMD18-T vectors (TaKaRa, China), transformed into *Escherichia coli* strain DH5 and then sequenced with DYEnamic Direct dGTP Sequencing Kit (Amersham Pharmacia, England) and a 373A DNA sequencer. The sequence was analyzed through database search using BLAST program (http://www.ncbi.nlm.nih.gov).

## RESULTS AND DISCUSSION

### Comparison of total RNA yields generated by different extraction methods

Total RNA from two taxol producing fungus EFY-21 and EFY-36 was isolated using the CTAB/NaCl method, STE

**Table 1.** RNA yield and quality by spectrophotometric evaluation in taxol producing fungus EFY-21and EFY-36 by various methods.

| Method | Taxol producing fungus | Absorbance ratio | | Yield |
|---|---|---|---|---|
| | | $A_{260/230}$ | $A_{260/280}$ | ( g/100mg) |
| CTAB/NaCl method | EFY-21 | 1.44±0.10 | 1.67±0.20 | 5.80±1.59 |
| | EFY-36 | 1.62±0.34 | 1.63±0.48 | 4.57±1.29 |
| STE method | EFY-21 | 2.09±0.01 | 2.02±0.01 | 7.01±1.77 |
| | EFY-36 | 2.46±0.04 | 2.11±0.01 | 14.80±1.65 |
| Modified STE method | EFY-21 | 1.95±0.09 | 1.97±0.09 | 4.93±2.65 |
| | EFY-36 | 2.24±0.16 | 2.04±0.05 | 8.83±5.25 |
| LiCl method | EFY-21 | 2.32±0.26 | 2.13±0.09 | 7.53±4.23 |
| | EFY-36 | 2.17±0.02 | 2.07±0.03 | 6.33±0.55 |
| Trizol method | EFY-21 | 2.45±0.03 | 2.12±0.03 | 14.50±1.06 |
| | EFY-36 | 2.45±0.02 | 2.15±0.01 | 14.10±2.40 |

method, Modified STE method, LiCl method and the Trizol method. To test the quality and to calculate the RNA concentration, the isolated total RNA was checked by electrophoresis and spectrophotometric analysis. There were differences in yields of RNA obtained by these methods. The mean of the yields of total RNA samples ranged from 14.80 (STE method form taxol producing fungus EFY-36) to 4.57 ± 1.29 (CTAB/NaCl method form taxol producing fungus EFY-36) mg per 100 mg (wet wt) fresh mycelium. Among the five methods, both the STE method and Trizol kit method were better than the other three methods for extracting total RNA with higher yields from the taxol producing fungus EFY-21 and EFY-36.

### Comparison of the ratios of $A_{260/280}$ and $A_{260/230}$

Downstream applications of the purified RNA demand that the RNA be of the highest quality. Quality of purified nucleic acids can be determined through the use of spectrophotometry. Nucleic acids only absorb light that has a wavelength of 260 nm, while organic contaminants such as phenol and other aromatic compounds or other additional reagents used in RNA extractions absorb light at a wavelength of 230 nm. The ratio $A_{260/280}$ should be 1.8 - 2.0; lower values 1.8 indicate the presence of contaminant proteins or phenol and high values 2 indicate the degradation of RNA , while the ratio $A_{260/230}$ should be about 2 and a high value indicates minimal contamination by polyphenols and carbohydrates. The results showed that the ratios $A_{260/280}$ and $A_{260/230}$ of the total RNA extracted using the five extraction methods were obviously different. For EFY-21 and EFY-36, $A_{260/230}$ method and STE with LiCl method and it was around 2.3 of total RNA was 1.9 - 2.3 when extracted by STE when extracted by an efficient method with LiCl. $A_{260/230}$ of EFY-21 and EFY-36 RNA were more than 2.4 when extracted by the Trizol kit (Table 1).

### Agarose Gel Electrophoresis

Agarose gel electrophoresis was subsequently used to check the integrity of the RNA. All the RNA species, including 18s rRNA and 28s rRNA can be observed. The results showed that the agarose gel showed separation of ribosomal RNA subunits into distinct bands (Figure 1).

### RT-PCR amplification

To evaluate whether the RNA extracts obtained by our protocol were suitable for downstream molecular procedures, total RNA isolated from mycelia was used for RT-PCR. Since reverse transcriptase is highly sensitive to impurities, clean RNA is of critical importance for the successful construction of full-length cDNA libraries. As shown in Figure 2, both 18S, the constitutively expressed housekeeping gene and *dbat*, a crucial enzyme in the taxol biosynthetic pathway in *Taxus*. spp (Walker and Croteau, 2000, 2001), were successfully amplified (Figure 2) . DBAT gene partial sequence of EFY-36 is about 743 bp (Figure 2B). Sequence alignment using the GenBank database revealed a high level of homology with the gene found in *Taxus x media* and *T. cuspidate* (98%, GenBank accession No. AY452666.1 and AF193765.1 respectively), confirming the identity of the RT-PCR products.


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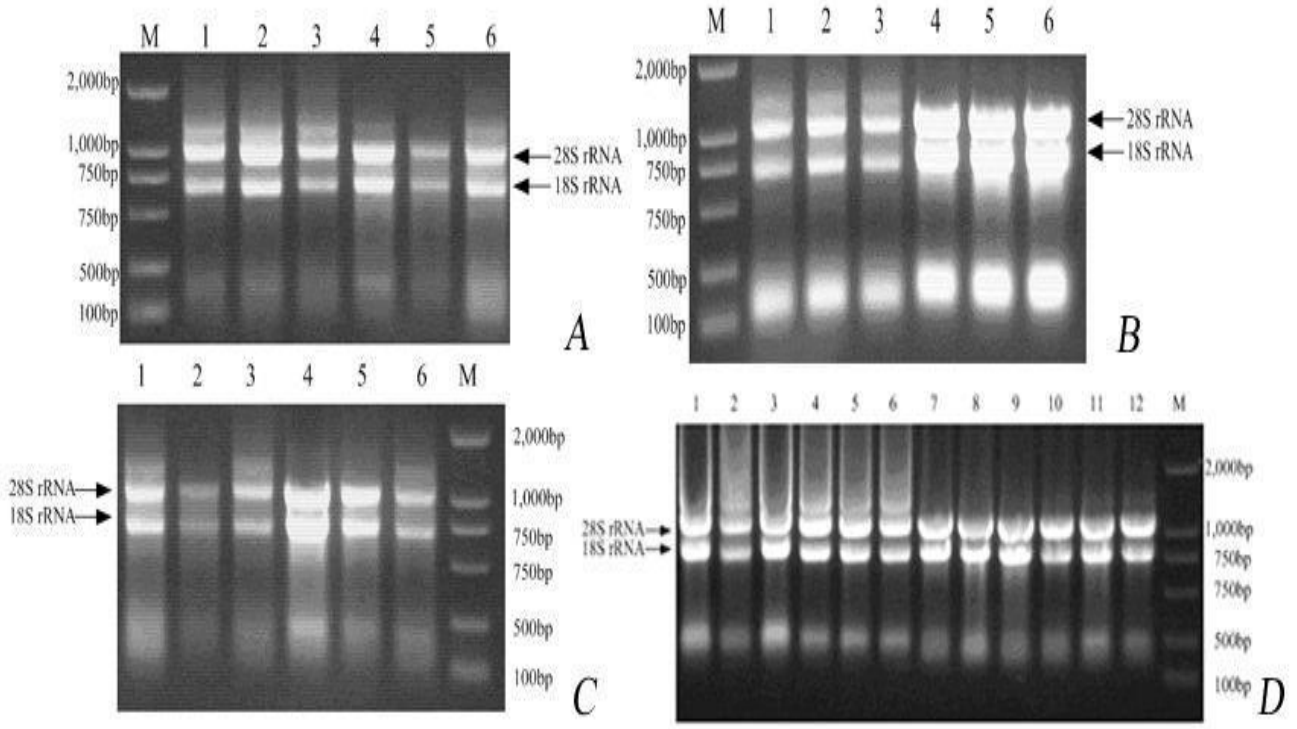


**Figure 1.** Agarose electrophoresis of RNA isolation by five different methods from EFY-21 and EFY-36. (A) CTAB/NaCl method; (B) STE method; (C) modified STE method; (D) LiCl method (lane 1 - 6) and Trizol method (lane 7 - 12) . Lane M was DL2000 Marker. Lane 1 - 3 (Figure A, B, C) and line 7 - 9 (Figure D) was the RNA isolation from EFY-21; Lane 4 - 6 (Figure A, B, C) and line 10 - 12 (Figure D) was the RNA isolation from EFY-36.

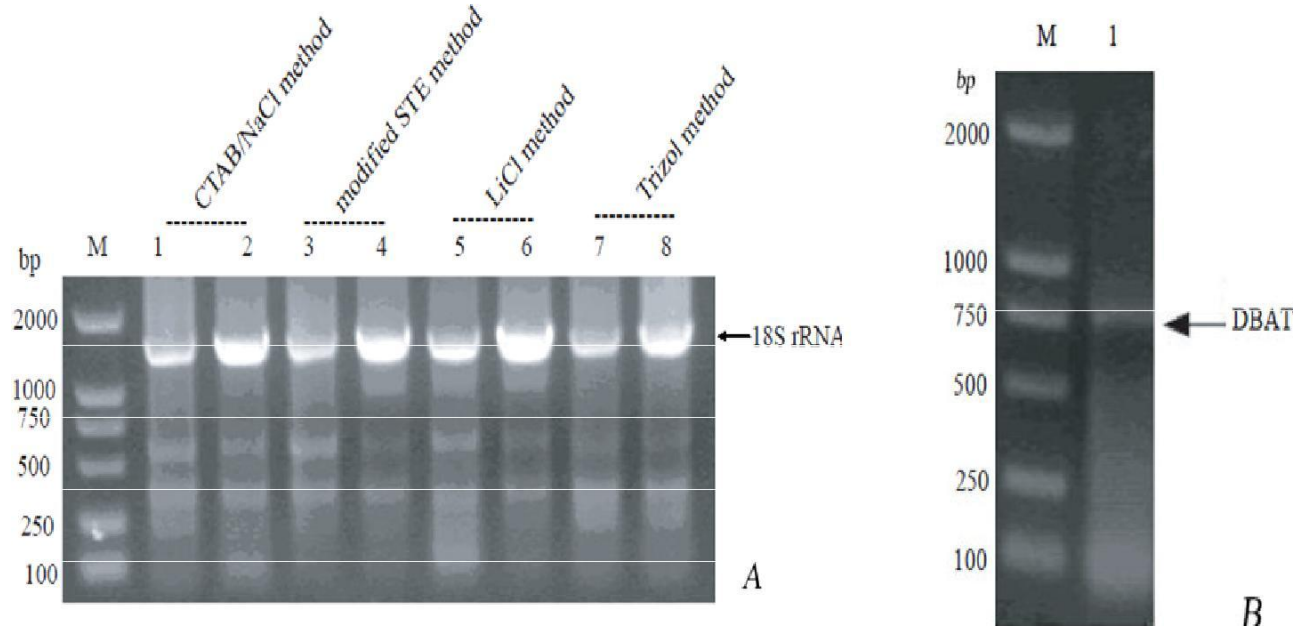


**Figure 2.** The 18S rRNA gene and DBAT gene RT-PCR results of RNA isolation. Lane M was DL2000 Marker. (A) The 18S rRNA gene. Lane 1, 3, 5 and 7 were the RT-PCR results of EFY-21 RNA; Lane 2, 4, 6 and were the RT-PCR results of EFY-36 RNA.
(B) DBAT gene. Lane 1 RNA template was isolated by LiCl method.

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