academicJournals

Vol. 4(5), pp. 58-62, July 2013
DOI: 10.5897/JYFR2013.0107
ISSN 2141-2413 ©2013 Academic Journals
http://www.academicjournals.org/JYFR

Journal of Yeast and Fungal Research

Full Length Research Paper

Aspergillus niger-specific ribonucleic acid extraction method

Abdullahi Balarabe Sallau[1]*, Fiona Henriquez[2], Andrew Jonathan Nok[1], Sani Ibrahim[1], Caroline Sommerville[3] and Craig Roberts[3]

[1]Department of Biochemistry, Ahmadu Bello University, Zaria, Kaduna State, Nigeria.
[2]Department of Microbiology and Immunology, University of West Scotland, Glasgow, United Kingdom.
[3]Department of Immunology and Microbiology, Strathclyde Institute of Pharmacy and Biomedical Sciences, Glasgow, United Kingdom.

Accepted July 26, 2013

Extraction of high quality RNA from *Aspergillus niger* mycelia, a tissue very rich in ribonucleases was achieved using a protocol involving the use of TRIZOL reagent and the ribonuclease inhibitor (RNaseOUT). RNA was successfully isolated using this protocol that involved addition of RNaseOUT at the initial homogenizing step and the final dilution/ solubilisation steps of the RNA extraction. A sharply distinguished 18S and 28S subunits of the RNA extracted were visualised on agarose gel electrophoregrams with high yield. The RNA extracted was tested for reverse transcription polymerase chain reaction and was found viable. This paper presents an efficient protocol for RNA extraction from *Aspergillus niger* mycelia which is an improvement on the TRIZOL reagent protocol for RNA which has never been described.

Key words: *Aspergillus niger*, RNA, ribonuclease inhibitor.

INTRODUCTION

Extraction of RNA of high quality and yield from *Aspergillus niger* mycelia could appear difficult, owing to the highly active RNases released especially at the initial step of homogenising *Aspergillus niger* mycelia or at the final step when the RNA is solubilised and easily accessible to the RNAses. These two steps remain crucial for the successful extraction of RNA from *A. niger* mycelia. The total RNA being extracted might be partially or even totally degraded and the yield is limited. However, the inclusion of an RNase inhibitor (RNaseOUT) into these crucial RNA extraction steps solves the problem such that the RNase being released is inhibited by RNAseOUT and as such gives room for a good yield and high quality RNA which is of utmost importance when reverse transcription polymerase chain reaction (RT-PCR) is to be carried out or when gene expression profiles are to be investigated. Different methods of RNA extraction have been described (Cathala et al., 1983; David 2007; Loens et al., 2008), each of which did not address the peculiar problem encountered when extracting RNA form *A. niger* mycelia. Even methods of Salzman et al. (1999), Gehrig et al. (2000), and more recently Davis et al. (2006) that were specifically for extracting RNA form plant species did not directly address the peculiarity of the *A. niger* mycelia. This article therefore presents an extraction protocol developed for extracting RNA from *A. niger* mycelia with high success which is a modification of a standard operation procedure for RNA extraction using TRIZOL. Effectiveness of this protocol was tested by carrying out reverse transcription of the RNA followed by PCR amplification to demonstrate that the RNA extracted was not only used to generate cDNA but produced

*Corresponding author. E-mail: bsallau@yahoo.com. Tel: +234 802 8378331.

amplicons.

MATERIALS AND METHODS

Microorganisms

A. niger (spores) were obtained from Fermentation Laboratory, Royal College, Strathclyde University, Glasgow.

Reagents

TRIZOL (Invitrogen), isopropanol (Isopropyl Alcohol (Sigma), absolute ethanol (Sigma), chloroform (Sigma) and RNaseOUT (ABgene) were used.

Growing organisms

The *A. niger* was maintained on a 7 day old potato dextrose agar (PDA) slant prior to inoculation of approximately 10^6 spores into 500 ml of sterilised broth in 1000 ml Erlenmeyer flasks and incubated at 30°C in a shaker and 250 rpm. The broth contained 50 g glucose, ammonium chloride (5 g), salt solution (20 ml) containing potassium chloride (26g), magnesium heptahydrate (26 g), potassium phosphate monobasic (76 g) and 50 ml trace element solution). In addition, the broth was added to a vitamin cocktail (10 ml) containing paraaminobenzoic acid (20 mg), thiamine hydrochloride (50 mg), biotin (10 µg), calcium-d-panthothenate (100 µg), pyridoxinemonochloride (50 mg) and 100 mg riboflavin (100 mg). The vitamin cocktail was added to the sterilized broth through a syringe using 2 µm cellulose acetate filters.

Harvesting of *A. niger* mycelia

At 18 to 24 h post inoculation, the mycelia was harvested in vacuum by suction and used immediately for RNA extraction or stored at -80°C until needed.

Step wise RNA extraction

1. Grind (50 to 100 mg) of harvested and frozen mycelia in liquid nitrogen with mortar and pestle or homogeniser.
2. Add 40 units of RNaseOUT, 1500 µl of TRIZOL reagent and homogenise using a homogeniser till solution becomes clear.
3. Add 250 µl of chloroform and allow to stand for 5 min.
4. Centrifuge at 10,000 g for 15 min at 4°C.
5. Withdraw the upper layer (supernatant) and discard the residue.
6. Add 500 µl of isopropyl alcohol and centrifuge at 8000 xg for 10 min at 4°C.
7. Discard the supernatant and keep the residue.
8. Add 1000 µl of absolute ethanol to wash off the RNA extracted.
9. Repeat steps 1 to 7 again with what was obtained at step 8.
10. Wash the pellet in 1 ml 70% ethanol by flicking the tube to remove the pellet from the wall of the tube.
11. Centrifuge at 7000 g for 15 min at 4°C.
12. Tip off ethanol and allow pellet to dry briefly.
13. Add 50 µl of molecular water containing 1% of 40 units RNAseOUT to the tube and heat at 60°C for ten min to solubilise the RNA.

The procedure described above was used to extract RNA from *A. niger* mycelia with and without the inclusion the of RNase inhibitor (RNaseOUT) in steps 2 and 13.

Agarose gel electrophoresis

This was carried out using 2% agarose gels and they were visualised using a uv-transilluminator after staining with ethidium bromide.

Testing for purity/viability of RNA extracted

In testing viability of the RNA extracted, the RNA (500µg/ml) was first incubated with 40 units of DNAse (ABgene) at 37°C for 2 h. After the incubation, amplification reactions (PCR) were then carried out using RNA as template and primer pairs for encoding beta tubulin gene segment (254 bp). The PCR was carried out using 25 µl reaction volumes containing 1 µl RNA, 12.5 µl mastermix (ABgene), 0.5 µl each of forward primer 5-CCACTGCCATTGGATTGGGG-3 and reverse primer 5-AGGTCGGAGGTGCCATTGTAAC-3 containing 25 pmol each and 10.5 µl molecular water. The PCR was carried out using the following thermocycling conditions: Initial denaturation at 95°C for 3 min followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 58, 56, 54 and 52°C for 45 s and extension for 1 min at 72°C. A final extension was carried out at 72°C for 10 min. Agarose gel electrophoresis was then carried out on the PCR products and visualised for any bands due to DNA contamination.

Reverse transcription and PCR

Preparation of cDNA was done from the RNA extracted using affinity script reverse transcriptase (Invitrogen). The reaction involved first incubating 5 µl of the RNA extracted (500 µg/ml), 1 µl of random heximers and 8.2 µl molecular water in a tube at 65°C for 5 min then at room temperature at 10 min. Two microliters of affinity script buffer, 2 µl of dithiotreitol (DTT), 1 µl of dNTPs and 1 µl of affinity script Reverse Transcriptase (ABgene) were then added and incubated at 55°C for 1 h and then at 70°C for 15 min. Polymerase chain reaction (PCR) amplification reactions were performed in 25 µl reaction mixtures containing 12.5 µl ready mix, 25 pmol each of forward primer: 5- CCACTGCCATTGGATTGGGG-3 and reverse primer: 5- AGGTCGGAGGTGCCATTGTAAC-3, 10.5 µl molecular water and 1 µl cDNA prepared from RNA as template. For negative control, 1 µl of molecular water was used in lieu of 1 µl cDNA. The PCR was made up of an initial denaturation at 95°C for 3 min and 35 cycles of 95°C for 30 s, annealing temperature of 54,56,58 or/and 60°C for 45 s and extension at 72°C for 1 min. A final extension was carried out at 72°C for 10 min. In some cases, the extension time and initial denaturation time were varied depending on the requirement.

RESULTS

The electrophoregram of 5 µg of RNA extracted from *A. niger* mycelia using TRIZOL reagent with RNAse inhibitor (RNAseOUT) is described in Figure 1. It is clear from the figure that the bands of 18S and 28S subunits of the RNA were visible on lanes 1 and 2 of the gel. Figure 2 depicts the electrophoregram of RNA extracted from the mycelia of *A. niger* without the inclusion of RNAseOUT. It was only the M lane containing the molecular weight marker that had bands, lanes 1 and 2 that were both loaded with the RNA extract did not show any sign of a band. The 18S and 28S subunits were clearly missing. Table 1 describes the quality and yield of the RNA extracted using TRIZOL with and without RNase inhibitor. An $A_{260/280}$

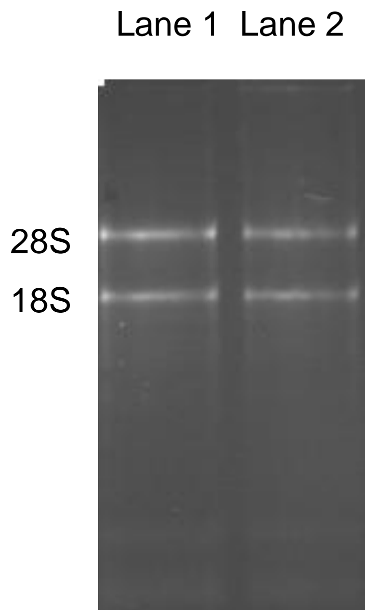

Figure 1. Agarose gel electrophoregram of RNA extracted from *Aspergillus niger* mycelia using TRIZOL reagent with inclusion of RNase inhibitor (RNAseOUT) showing the 28S and the 18S RNA subunits.

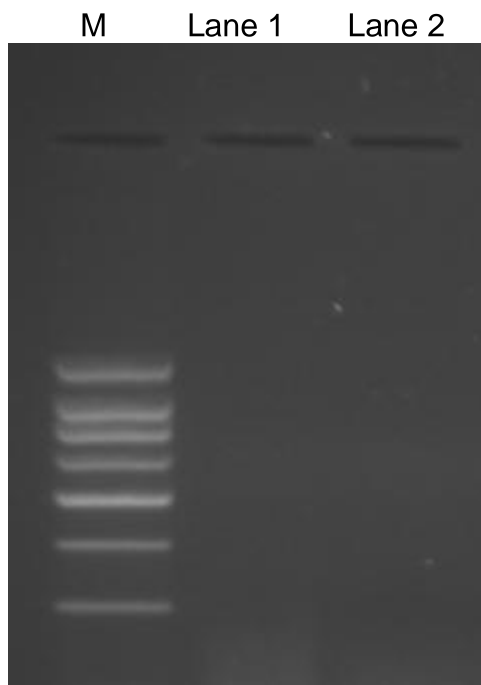

Figure 2. Agarose gel electrophoregram of RNA extracted from *A. niger* mycelia using TRIZOL reagent without the inclusion of RNAseOUT(RNase Inhibitor). M is Molecular weight marker, Lanes 1 and 2 were loaded with the RNA extract.

ratio of 1.88 ± 0.2 and 1.77 ± 1.0 was respectively obtained when RNA was extracted from *A. niger* mycelia with the use of RNAseOUT inhibitor. The extracted RNAs also had yields with concentrations of 60 ± 0.5 and 35 ± 1.3 µg/g of *A. niger* mycelia, respectively. The outcome of the gel electrophoresis of PCR products of DNAse treated RNA is described in Figure 3. From the figure, it is observable that no bands were visible on the lanes on the gel except that of the molecular weight markers used. On reverse transcription of the DNAse treated RNA, amplification (PCR) using the cDNA generated as template and subsequent electrophoresis of the PCR products (Figure 4), a band corresponding to the encoded 254 pb segment of the *A. niger* beta tubulin gene segment was seen on lanes 1 to 4 corresponding to the respective annealing temperatures of 58, 56, 54 and 52 used during the PCR.

DISCUSSION

The extraction of RNA from *A. niger* mycelia using the modification of a method described in this paper is the first of its kind and have proven to be effective and efficient considering the results presented. In addition to the fact that methods of RNA extraction earlier mentioned including Davis (2006), David (2007) and Losens et al. (2008) did not address the peculiarity of the tissue in question, no work has been reported on the modification of these procedures to suit RNA extraction from the same tissue. Considering Figures 1 and 2, it clearly demonstrates that the RNA extracted from *A. niger* mycelia using TRIZOL with the inclusion of RNAseOUT not only effectively gives good RNA extract but also showed that there is a factor responsible for masking the chance of getting a good yield of RNA extracted when the RNase inhibitor (RNAseOUT) was not involved. Also, by examining both Figures (1 and 2) one could say that it is likely that the factor responsible is RNase which digests the RNA being extracted as no clear bands for the RNA were seen in Figure 2 but present in Figure 1. When the RNA was also extracted using TRIZOL reagent as described by Gehrig et al. (2000), no visible bands were present on its electrophoregram (not shown), further buttressing the need of RNaseOUT inclusion during extraction of *A. niger* RNA. Referring to Table 1, it is not surprising that the $A_{260/280}$ of the RNA extracted without inhibitor gave a close value to that extracted with the inhibitor; this could be because the former contains degraded form of the RNA (mainly oligonucleotides) which can also absorb light strongly at 260 and 280 nm. In addition, the yield of RNA obtained using the method described falls within the typical yields of RNAs extracted which was reported to be 25 to 65 µg/g (Gehrig et al., 2000). As shown in Figure 3, it is notable that the DNAse used was able to further purify any likely DNA contaminant present to obtain a more viable RNA because only the M lane showed bands while other lanes did not.

Table 1. Quality and yield of RNA extracted from the mycelia of *A. Niger* using TRIZOL reagent method in the presence and absence of RNAse inhibitor (RNAseOUT).

***Aspergillus niger* mycelia**	**$A_{260/280}$**	**µg RNA/g Tissue**
Extraction with inhibitor (RNAseOUT)	1.88±0.2	60±0.5
Extraction without inhibitor (RNAseOUT)	1.77±1.3	35±1.0

Results are mean ± SD of two independent experiments.

Figure 3. Agarose gel electrophoregram of PCR products of DNAse treated RNA extracted from *A. niger* using TRIZOL with RNaseOUT. M is the molecular weight marker lane, Lanes 1, 2 and 3 were loaded with the PCR products but no bands appeared.

not a template for PCR amplification. Figure 4 demonstrates that RNA extracted using TRIZOL with the inclusion of (RNAseOUT) served as a robust template for reverse transcription and PCR amplification as indicated. Furthermore, the method reported could conveniently allow extraction of RNA of high integrity, quality and good yield from tissues with high concentration of ribonucleases like *A. niger* mycelia that other protocols may not

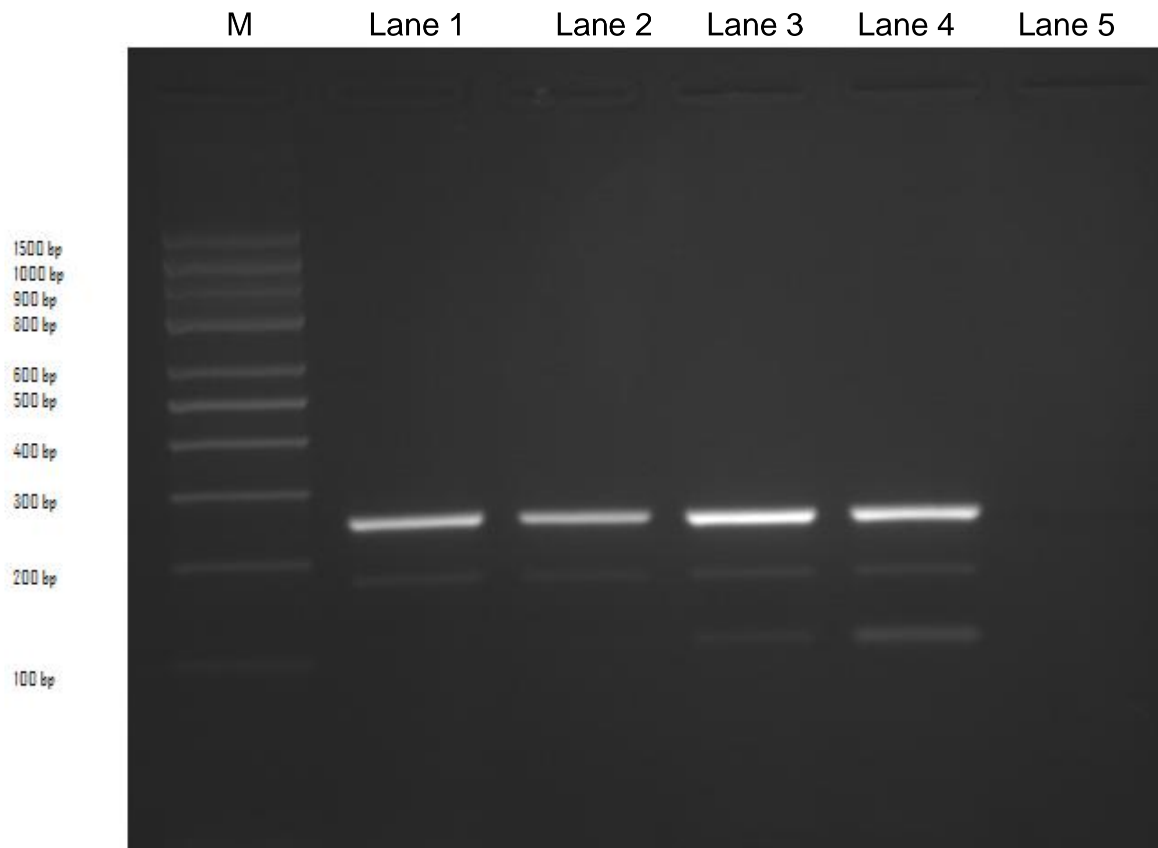

Figure 4. Agarose gel electrophoregram of PCR products of 254 bp segment of beta tubulin gene using cDNA obtained from *A. niger* RNA extracted using TRIZOL reagent with RNAseOUT. Lane M is molecular weight marker, Lanes 1, 2, 3 and 4 are the PCR products at annealing temperatures of 54,56,58 and 60°C respectively. Lane 5 is for negative control and contained no RNA.

have addressed.

REFERENCES

Cathala G, Savoured JF Mendez B, West BL, Karin M, Martial JA, Baxter JD (1983). A method for isolation of intact,translationally active ribonucleic acid. DNA. 2:329-335.

Davis A, Levi A, Kim S, Hernandez A, King S (2006). RNA Extraction from Fruit tissue High in water and sugar.Hortisc. 41(5):1292-1294

David K (2007). An RNA extraction from shell fish-borne viruses. J. Virol. Meth. 141:58-62

Gehrig HH, Winter K, Cushman J, Borland A, Taybi T (2000). An improved RNA isolation Methods For Succulent Plant Species Rich in Polyphenols and Polysaccharides. Plant. Mol. Biol. Reptr. 18:369-376.

Loens K, Ursi D, GoosensH, Leven M (2008). Evaluation of NucliSensminiMAG RNA extraction and Realtime NASBA applica-tions for the detection of *Mycoplasmapneumoniae*and *Chlamydophilia pneumonia* in throat swabs. J. Microb. Meth. 72(2):217-219.

Salzman RA, Fujita T, Zhu-Salzman K, Hasegawa PM, Bressen RA (1999).An improved RNA isolation method for plant tissues containing high levels of phenolic compounds or carbohydrates.Plnt. Mol. Biol. Reptr. 17:11-17.