# HIGH YIELD RNA EXTRACTION FROM *EUCHEUMA DENTICULATUM* AND *KAPPAPHYCUS ALVAREZII* (GIGARTINALES, RHODOPHYTA)

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## ABSTRACT

High quality RNA is a prerequisite in studying gene expression. However isolation of good quality RNA from algae is particularly difficult due to the high content of polysaccharides. To obtain large amounts of total RNA from *Eucheuma denticulatum* and *Kappaphycus alvarezii*, we compared the use of a commercial RNA extraction kit and a modified Lopez-Gomez and Gomez-Lim method. When applied to *E. denticulatum*, the modified Lopez-Gomez and Gomez-Lim method gave higher RNA yield of 57.4 μg per g of fresh tissue compared to 44.8 μg g$^{-1}$ obtained using the commercial kit. Nevertheless, both methods produced RNA with high quality as determined by spectral analysis. Using the modified Lopez-Gomez and Gomez-Lim method, much lower yield of RNA was obtained from *K. alvarezii* albeit the same high quality. The quality of RNA extracted was also examined by reverse transcription-polymerase chain reaction (RT-PCR) and cDNA synthesis. These assays indicated that the RNA isolated is of high quality and suitable for further molecular analyses. Hence a cost saving protocol which gives high yield of total RNA with high quality has been developed.

*Keywords: Eucheuma denticulatum, Kappaphycus alvarezii,* RNA extraction, Tris-borate, reverse-transcription PCR

## INTRODUCTION

The marine red algal genera *Eucheuma* and *Kappaphycus* which grow throughout the Indo-Pacific region in the algal reef areas of islands in Southeast Asia are very important resources for the economically important polysaccharides, carrageenan. *E. denticulatum* (Burman) Collins et Harvey and *K. alvarezii* are the most commonly farmed carrageenophytes throughout the world (Ask & Azanza 2002), and in Malaysia they are grown in the waters of Sabah. Commercial cultivation of these red seaweeds began in the Philippines (Ask & Azanza 2002) and later spread to Indonesia (Adnan & Porse 1987) and some South Pacific islands such as Fiji, Hawaii, Tonga and Kiribati (Luxton et al. 1987).

Several environmental parameters coordinately influence growth and abundance of these seaweeds i.e. temperature, light, nutrient and water motion (Glenn & Doty 1992). Growth rate of these species are best observed in plants

cultured in warm sea temperatures of 25 – 30°C (Ohno et al. 1994). The different color types of *E. denticulatum* and *K. alvarezii* exhibit varying photosynthetic efficiency at the optimum temperature range but damaging effects were observed with excessive light (Glenn & Doty 1981). In addition, nutrient enriched (Li et al. 1990) and fast flowing water (Glenn & Doty 1992) have shown significant correlations with increased growth rates and better carrageenan yield and gel strength. In our work, the seaweeds have been grown in the northern and eastern coastal areas of Sabah with apparently different physical conditions having obvious impacts on their growth rates as well as the quality and quantity of carrageenan obtained. It is therefore important not only to gain deeper understanding into the genetic characteristics of these seaweeds but also the impact of the factors influencing growth and carrageenan production on the seaweed gene expression.

In order to embark upon fundamental molecular biology investigation in *E. denticulatum* and *K. alvarezii*, obtaining high quality, intact RNA is the most critical step. Several simple methods for RNA isolation from algae have reported various degrees of success in terms of RNA yield and quality which reflect the recalcitrant nature of the cellulosic walls and abundance in polysaccharide contents of seaweed (Su & Gibor 1988; La Claire & Herrin 1997; Hong et al. 1997). Ironically, the prized carbohydrates produced by algae such as carrageenan, agar and alginic acid are the root of the problems associated with RNA extraction as they possess physicochemical properties similar to those of RNA and may contaminate the RNA thus affecting the yield (Logemann et al. 1987). Furthermore, the polyphenolic compounds present in algae may aggravate the situation by being readily oxidized to form covalently linked quinones (Loomis 1974) that bind to RNA and render it unsuitable for cDNA synthesis and Northern hybridisation (Tesniere & Vayda 1991). For efficient isolation of large amounts of pure and intact total RNA from seaweed tissues which are rich in polysaccharide and polyphenols, binding of these compounds to the nucleic acids must be prevented. Here we describe RNA purification from *E. denticulatum* and *K. alvarezii* using a commercial kit and a manual method with several modifications. The objective of this study was to develop an efficient and cost effective method for purified RNA extraction from *E. denticulatum* and *K. alvarezii*.

## MATERIALS AND METHODS

### Sample preparation

Whole plants of *E. denticulatum* cultivated in Kudat and Semporna, Sabah were obtained from Marine Borneo Institute, Sabah Malaysian University and Fisheries Department of Malaysia, respectively whereas *K. alvarezii* cultivated in Semporna, Sabah was provided by Fisheries Department of Malaysia. Samples were cleaned using fresh water and stored at -80°C.

## Total RNA extraction using TRI reagent

Frozen tissue samples (100 mg) were grounded in liquid nitrogen using pestle and mortar in 1 ml TRI REAGENT (Molecular Research Centre, USA) and left at room temperature for 5 min to allow complete dissociation of nucleoprotein complexes (Chomczynski & Sacchi 1987). Supernatant was obtained from centrifugation at 12,000 g for 10 min at 4°C followed by addition of 0.2 ml chloroform and mixed vigorously. The mixture was left for 2 – 15 min at room temperature and then centrifuged at 12,000 g for 15 min at 4°C. Isopropanol (0.25 ml) and 0.25 ml high salt buffer (0.8 M sodium citrate, 1.2 M NaCl) were then added to the aqueous phase, mixed and stored at room temperature for 5 – 10 min. Pellet was obtained by centrifuging at 12,000 g for 8 min at 4°C. The RNA pellet was washed once in 75% ethanol followed by air-drying. The pellet was dissolved in 100 µl RNase-free water by incubating for 10 – 15 min at 55 – 60°C and kept at -80°C.

This method was later modified by including a differential precipitation of RNA using LiCl. Seventy µl 4 M LiCl was added to the resuspended RNA pellet from above and incubated overnight at -20°C. RNA pellet was recovered by centrifuging at 12,000 g at 4°C for 30 min. The pellet was washed in 70% ethanol, air-dried and dissolved in 100 µl RNase-free water prior to storage at -80°C.

## RNA extraction using modified Lopez-Gomez and Gomez-Lim

Total RNA was extracted from whole seaweeds by a modification of the method of Lopez-Gomez and Gomez-Lim (1992). Approximately 30 g of frozen tissue was homogenized in liquid nitrogen into which an extraction buffer containing 150 mM tris-borate, pH 7.5, 50 mM EDTA, 2% (w/v) SDS, 1% (v/v) β-mercaptoethanol and 5% (v/v) Tris-buffered phenol was added until the tissue was completely thawed. Absolute ethanol (0.25 volumes) and 5 M potassium acetate (0.11 volumes) were then added and mixed for 1 min. One volume of chloroform/isoamylalcohol (49:1) was added to the homogenate and mixed vigorously for 1 min before centrifuging at 20,000 g for 10 min at 4°C. The recovered aqueous phase was extracted with an equal volume of phenol:chloroform (25:24) and then with another equal volume of chloroform/isoamylalcohol (49:1) until no inter-phase was apparent. RNA was selectively precipitated by adding 12 M LiCl to a final concentration of 3 M and incubating overnight at -20°C. RNA was collected by centrifugation at 20,000 g for 1 hour at 4°C. The pellet was washed twice with 3M LiCl and vacuum-dried. The RNA pellet was resuspended in 400 µl DEPC-treated water, potassium acetate at a final concentration of 0.3 M followed by precipitation in 2.5 volumes of absolute ethanol. After overnight incubation at -20°C, the RNA was pelleted by centrifugation at 10 000 rpm for 10 min at 4°C. The pellet was washed once in 75% ethanol and vacuum dried. The RNA was resuspended in 100 µl of RNase-free water and stored at -80°C.

## RT-PCR of actin gene

Reverse transcription (RT) and subsequent PCR were carried out using the Promega Access RT-PCR System in a single tube reaction protocol using specific primers for actin gene. The reverse transcriptase reaction was carried out at 48°C for 1 h, followed by heat inactivating the avian myeloblastosis virus reverse transcriptase (AMVRT) at 94°C for 2 min. The PCR reaction was carried out for 40 cycles where PCR conditions were: 95°C for 5 min, 30 cycles of 95°C for 30 min, 58°C for 1 min, 72°C for 1 min and 72°C for 15 min.

## RESULTS AND DISCUSSION

Initially RNA was extracted from *E. denticulatum* using a commercial kit developed specifically for the isolation of total RNA from plant tissues rich in polysaccharides and proteoglycan (TRI Reagent following, Molecular Research Centre Inc. USA). This procedure follows a single-step RNA isolation based on acid guanidinium thiocyanate-phenol extraction (Chomczynski & Sacchi 1987). Guanidium thiocyanate is widely used at the initial stage of RNA extraction as an effective protein denaturant by inhibiting the ribonuclease thus reducing RNA degradation (Sambrook et al. 1989). The presence of phenol in the extraction buffer also contributes towards ensuring that cellular proteins remain in the organic phase thus separating them from RNA. For isolation of RNA from tissues with high polysaccharide contents, a high salt precipitation step using sodium citrate and sodium chloride was included to maintain the contaminants in A soluble form (Chomczynski & Mackey 1995). Although this procedure gave a very high yield of total RNA, it was accompanied by high amounts of protein and carbohydrate contaminations indicated by low $A_{260}/A_{280}$ (<1.8) and $A_{260}/A_{230}$ (<2) ratios, respectively (Table 1). Hence the addition of the salt precipitation step was not effective in discarding these impurities from our RNA preparations. This method was further modified by incorporating lithium chloride precipitation which is used to pellet RNA whilst simultaneously discarding polysaccharides (Sambrook et al. 1989). Despite marked improvement in quality, the RNA yield was dramatically affected presumably due to the removal of polysaccharides bound to RNA (Table 1). Nevertheless, the total RNA obtained from *E. denticulatum* using this modified method (44.8 µg $g^{-1}$ fresh weight) was still very much higher than previously reported for green algae (10 – 30 µg $g^{-1}$ RNA; La Claire & Herrin 1997) and red algae (Hong et al. 1997).

In our search for a low cost protocol, we had employed a plant RNA isolation procedure developed for fruit mesocarp rich in polysaccharides (Lopez-Gomez & Gomez-Lim 1992) which was based on an RNA extraction method for seaweed (Su & Gibor 1988). The original method of Su and Gibor (1988) had reported high RNA yield from algae but decreased translational efficiencies for red and brown algae implying presence of contaminating polysaccharides. This protocol uses β-mercaptoethanol as a strong reducing agent in the extraction buffer to prevent oxidation of polyphenols thus preventing formation of quinones whereas

borate forms H-bonded complexes with polyphenols. The use of 20% ethanol and potassium salt is for selective precipitation of polysaccharides and borate-polyphenolic complexes whilst RNA remains in solution (Su & Gibor 1988). In our modified protocol of Lopez-Gomez and Gomez-Lim (1992), phenol is incorporated into the extraction buffer at the start of the isolation procedure to inactivate RNase, thus ensuring minimum RNA degradation. Subsequent RNA precipitation uses LiCl which preferentially precipitates RNA whilst removing translation inhibitors (Ainsworth et al. 1993). The crude total RNA pellet was finally purified using an ethanol precipitation step which also involves washing with 70% ethanol to remove residual salt (Sambrook et al. 1989). We had routinely employed this method for RNA preparations from plant which gave high yield and quality nucleic acids. Hence we adopted the protocol for *E. denticulatum* and consistently obtained good RNA yield of approximately 58 μg g$^{-1}$ fresh weight with ratios of $A_{260}/A_{280}$ and $A_{260}/A_{230}$ above 1.8 and 2, respectively, indicating that there was minimal protein, polysaccharide or polyphenol contamination in our preparation.

Table 1. Comparison of RNA preparations obtained from *E. denticulatum* using several methods.

| Method | Yield of total RNA (μg/g tissue) | Absorbency ratios | |
|---|---|---|---|
| | | $A_{260}/A_{280}$ ratio | $A_{260}/A_{230}$ ratio |
| *TRI Reagent | 148.4 | 1.35 | 0.70 |
| *TRI Reagent-LiCl | 44.8 | 1.83 | 2.04 |
| **Lopez-Gomez and Gomez-Lim | 57.8 (5.6) | 1.90 (0.08) | 2.35 (0.01) |

* Results are expressed as the mean of 2 experiments.
** Results are expressed as the mean of 6 experiments (standard deviation)

To test the applicability of the method on other algae, this isolation procedure was applied to other red algae, *K. alvarezii* and *Gracilaria changii*. Significantly lower RNA yield was obtained for the carrageenophyte, *K. alvarezii* in spite of the high quality (Table 2). We suspected that the low yield is due to the poor quality of the *K. alvarezii* samples used for extracting the RNA. Nevertheless, the total RNA yield obtained from *K. alvarezii* is still within the range previously reported for green algae (10 – 30 μg/g RNA; La Claire & Herrin 1997) and much higher than red algae (Hong et al. 1997).

However, when this method was employed on the agarophyte, *G. changii*, marked decrease in the yield of RNA was obtained with some RNA impurities still present (Table 2). The difficulty in extracting large amount of RNA with high quality from *G. changii* reflects the recalcitrant nature of this seaweed since complete grinding of the plant materials was not achieved using homogenizer hence many seaweed cells remain unbroken during homogenization (Su & Gibor 1988). Nevertheless, using this method we were successful in producing higher yield of

purified RNA from *G. changii* (3.5 μg g$^{-1}$) then previously reported (0.65 – 1.14 μg g$^{-1}$; Chan et al. 2004).

Table 2. RNA extraction from *K. alvarezii* and *G. changii* using modified Lopez-Gomez and Gomez-Lim method (1992).

| Sample | Yield of total RNA (μg/g tissue) | Absorbency ratios | |
|---|---|---|---|
| | | $A_{260}/A_{280}$ ratio | $A_{260}/A_{230}$ ratio |
| *Kappaphycus alvarezii* | 18.6 | 1.89 | 2.25 |
| * Gracilaria changii | 3.5 | 1.62 | 1.67 |

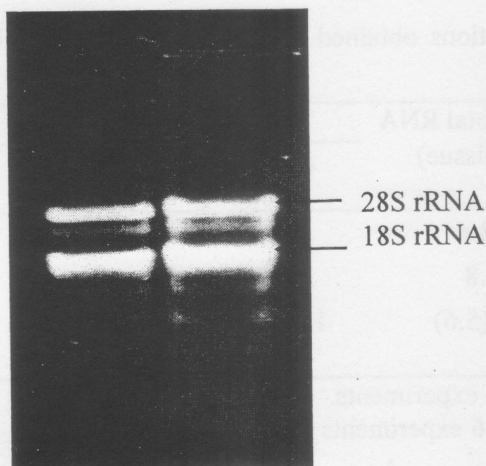* Results are expressed as the mean of 2 experiments.



Figure 1. Agarose gel electrophoresis of RNA from *E. denticulatum*. The two discrete bands which represent the 18S and 28S ribosomal RNA show the integrity of the RNA.
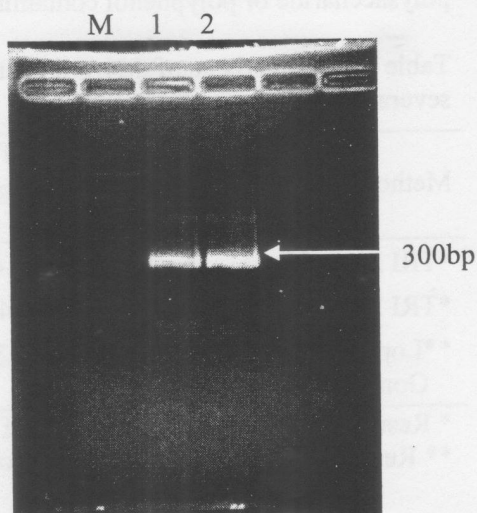
Figure 2. Agarose gel electrophoresis of RT-PCR amplified cDNA of actin gene. Lane 1 and 2, amplicons from total RNA of *E. denticulatum*, M shows the 100bp DNA ladder.

The quality of the RNA extracted from *E. denticulatum* was analyzed by agarose gel electrophoresis where two distinct bands corresponding to the 28S and 18S ribosomal RNAs were present suggesting that RNA degradation was not significant during the extraction (Figure 1). Apart from the integrity of the ribosomal bands, the suitability of the extracted RNA for downstream application was assessed by various reactions such as RT-PCR and cDNA library construction (data not shown). Reverse transcription of RNA from whole plants of *E. denticulatum* using actin specific primers resulted in the amplification of the actin

transcripts (Figure 2). The RNA isolated using the modified method of Lopez-Gomez and Gomez-Lim (1992), has been successfully used in our laboratory for various northern blot analyses as well as for cDNA synthesis. Thus, although this procedure is time-consuming, it reproducibly gave good yields of both high quality and functionally intact RNA from red algae and should prove to be applicable to an even wider variety of algae.

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