

An improved protocol for the isolation of total genomic DNA from Labyrinthulomycetes

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Abstract Many protocols have been used for extraction of DNA from Thraustochytrids. These generally involve the use of CTAB, phenol/chloroform and ethanol. They also feature mechanical grinding, sonication, N₂ freezing or bead beating. However, the resulting chemical and physical damage to extracted DNA reduces its quality. The methods are also unsuitable for large numbers of samples. Commercially-available DNA extraction kits give better quality and yields but are expensive. Therefore, an optimized DNA extraction protocol was developed which is suitable for Thraustochytrids to both minimise expensive and time-consuming steps prior to DNA extraction and also to improve the yield. The most effective method is a combination of single bead in TissueLyser (Qiagen) and Proteinase K. Results were conclusive: both the quality and the yield of extracted DNA were higher than with any other

method giving an average yield of 8.5 µg/100 mg biomass.

Keywords DNA extraction · Labyrinthulomycetes · Marine protists · Ω-3 polyunsaturated fatty acids (PUFAs) · Thraustochytrids

Introduction

The decline in marine fish stock, which forms the main commercial source of n–3 long chain polyunsaturated fatty acids (LC-PUFA or PUFA), is a cause for concern as these products have recognized health benefits, and are popular nutritional supplements. Consequently, other means of LC-PUFA production have attracted research attention and reports of many microorganisms mass-producing PUFA (Chang et al. 2012) have resulted. One such microorganism is the marine protist, Thraustochytrid, which produces large quantities of PUFAs. Due to their ability to over-produce PUFA-rich triacylglycerols in their lipid biomass, consisting mainly of docosahexaenoic acid (DHA; 22:6n–3), eicosapentaenoic acid (EPA; 20:5n–3), arachidonic acid (AA; 20:4n–6) and other PUFAs (Jakobsen et al. 2008), the Thraustochytrids have been of increasing interest to researchers.

A number of methods and protocols for extracting DNA have been introduced and discussed in many research articles (Graham et al. 1994; Jakobsen 2008;

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Mo and Rinkevich 2001). Common protocols, such as phenol/chloroform extraction and microwave lysis, were employed for similar organisms and found to be useful (Ahmed et al. 2014; Borman et al. 2012; González-Mendoza et al. 2010; Graham et al. 1994). However, most of these methods are infrequently used and involve harmful chemicals such as phenols, CTAB and chloroform, as well as requiring a lot of time for sample preparation and extraction (Tendulkar et al. 2003). The aim of this study was to compare four simplified methods that would only take up to 2 h from sample preparation to final DNA extraction. A rapid DNA extraction protocol with a minimum number of steps would be highly advantageous when working with a large number of samples. Also, avoiding the use of phenol and chloroform could retain the quality and shelf life of DNA.

Both DNA and RNA can now be extracted easily and efficiently with the use of commercial extraction kits. These kits offer varying levels of efficiency and quality of product. The thickness of Thraustochytrid cell wall falls between bacterial and plant cell walls (Honda et al. 1999; Kimura et al. 1999). Therefore the most suitable DNA extraction kits for Thraustochytrids are the plant DNA extraction kits. In this study, we used the DNeasy Plant Mini Kit (Qiagen, USA) for the comparative analysis of the four extraction protocols. The study was performed in order to devise a protocol that incorporated the key elements for enhancing the efficiency of DNA extraction from Thraustochytrids, whilst also reducing work time, and utilising commercially available DNA extraction kits that are not specified for the use of Thraustochytrids.

Materials and methods

Sample selection and growth

Three Labyrinthulomycetes used in this study were from three different genera of the Thraustochytrids from the Environmental Microbiology Research Group Culture Collection (EMRG). The three cultures were purchased from ATCC EMRG 527 was *Aurantiochytrium* sp. (ATCC PRA-276); EMRG 539 was *Schizochytrium* sp. (ATCC 20889) and EMRG 543 was *Thraustochytrium aureum* (ATCC 34304). These were initially grown in the recommended Thraustochytrid culture medium (TCM) containing 32 g

artificial sea salt (Sigma), 10 g glucose, 5 g peptone, 5 g L-glutamic acid and 2 g yeast extract per litre (Dr Tom Lewis unpublished data) for recovery from previously frozen cultures (in 30 % v/v glycerol). At the revival stage all three original isolates were grown in 100 ml liquid TCM and incubated under aerobic conditions at 20 °C with shaking at 160 rpm for 3–4 days until the OD₅₅₀ reached 1.5.

DNA extraction

Four methods for DNA extraction were selected: (1) Proteinase K (Bioline, UK) and TissueLyser; (2) TissueLyserI; (3) Mini Beadbeater and (4) Proteinase K. Total genomic DNA extractions were carried out as using these four protocols (Fig. 1). Approx. 100 mg biomass from each isolate was placed in a 2 ml screw-cap centrifuge tube. Each tube was filled with liquid TCM with grown cultures and centrifuged at 11,200*g for 30 s and the supernatant was discarded. This step was repeated until 100 mg each biomass was collected. In both methods 1 and 4 the biomass was resuspended in 400 µl lysis buffer AP1 (Qiagen) with 20 µl Proteinase K (>600 mAU/ml). The tubes were incubated at 70 °C for 30 min. The tube from method 1 had a 5 mm stainless steel bead inserted before incubation.

After incubation, the biomass in the tube from method 1 was disrupted using TissueLyser II (Qiagen) at 30 Hz for 1 min. In method 2 the biomass was disrupted using TissueLyser without incubating with Proteinase K. In method 3 the biomass was disrupted using mini-beadbeater (BioSpec Products, USA) along with 0.5 g 0.1 mm zirconia beads (Daintree Scientific, Australia) at 30 Hz for 2 min. All the tubes containing disrupted biomass were then mixed with 4 µl of RNaseA (Amersco, USA) and incubated at 65 °C for 10 min, inverting 2–3 times during the incubation period. Total genomic DNA from all tubes was extracted using the DNeasy Plant Mini Kit (Qiagen, USA) according to its standard extraction protocol. The extracted total genomic DNA was eluted with 100 µl TE buffer and stored at –20 °C until further use. All extracted DNA were quantified at 260/280 nm for double stranded DNA. Each extract was also resolved in 1 % agarose gel in TBE buffer stained with Gel-Red (Biotium, USA) and visualised (Fig. 2) using the UV illumination and gel documentation system (Bio-Rad, USA).

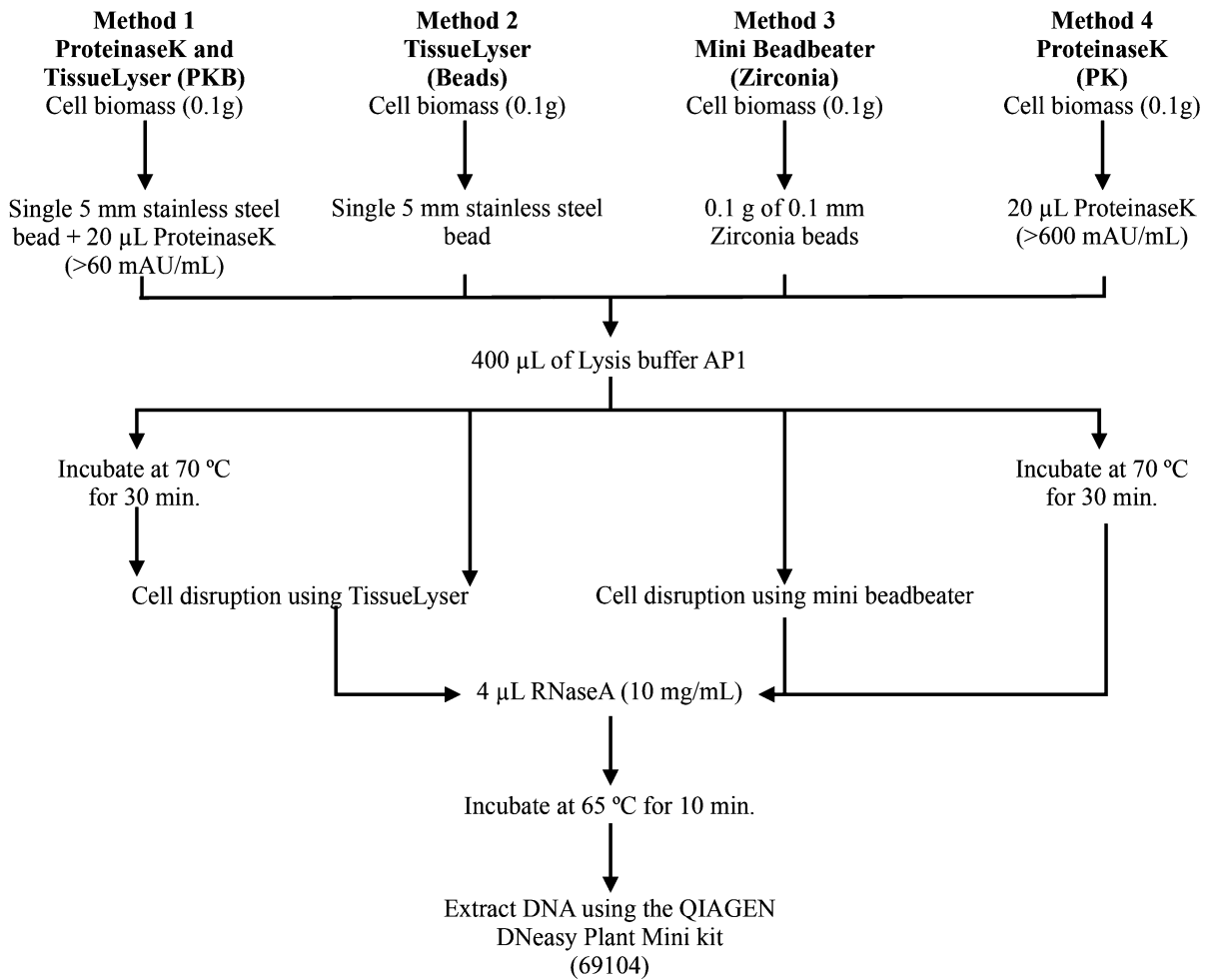


Fig. 1 The four modified methods used for the extraction of total genomic DNA from the three *Labyrinthulomycetes* isolates. *PKB* method 1—Proteinase K and bead beating with TissueLyser, *Beads* method 2—Bead beating with TissueLyser,

Zirconia method 3—Mini bead beater with Zirconia beads and *PK* method 4—Proteinase K digestion without mechanical beating

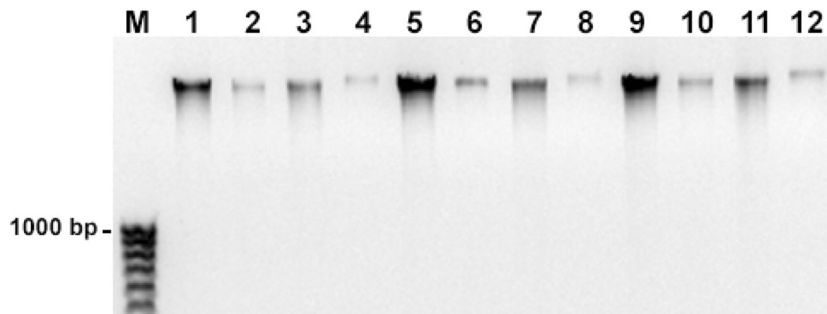
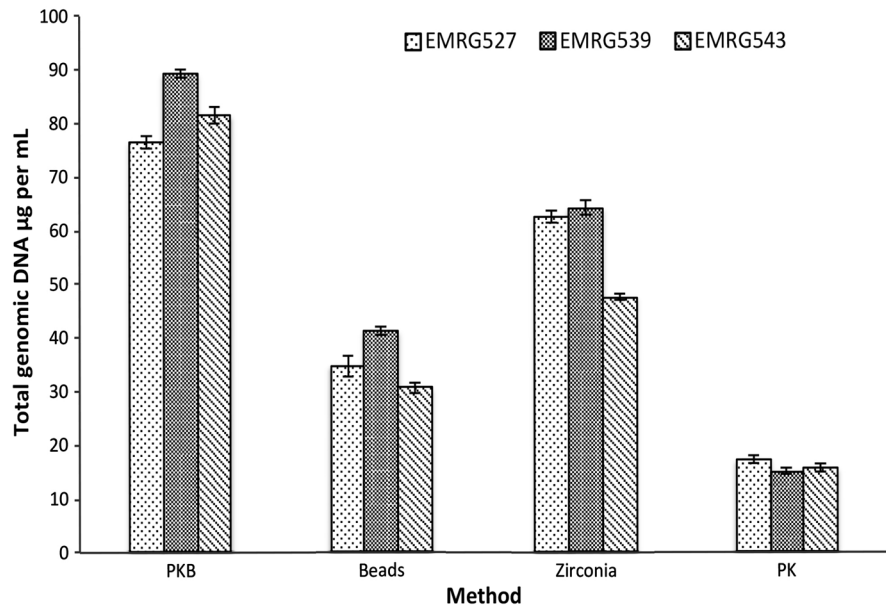


Fig. 2 Total genomic DNA extracted from the three selected isolates. *M* Molecular weight marker with largest band 1 kb. Each well loaded with 3 µl of marker and 5 µl of total genomic DNA. *Lanes 1–4* depict the DNA extracted from EMRG 543

using methods 1–4, *lanes 5–8* depicts DNA from EMRG 539 and *lanes 9–12* for EMRG 527. All samples were resolved in 1 % agarose gel at 110 V for 40 min and visualized under UV

Fig. 3 The concentrations of total genomic DNA extracted by the four methods. *PKB* method 1—Proteinase K and bead beating with TissueLyser, *Beads* method 2—Bead beating with TissueLyser, *Zirconia* method 3—Mini bead beater with Zirconia beads and *PK* method 4—Proteinase K digestion without mechanical beating. DNA was quantified from the 260/280 nm ratio



Results and discussion

Methods such as phenol/chloroform extraction and microwave treatment are consistently used with modifications according to the available literature as described in studies performed on *Thraustochytrids*, fungi and other organisms (Ahmed et al. 2014; Borman et al. 2012; Cavalier-Smith et al. 1994; González-Mendoza et al. 2010; Graham et al. 1994; Honda et al. 1999; Kimura et al. 1999). Apart from these methods there are many DNA extraction protocols and it is difficult to assess their applicability to *Thraustochytrids*. However, all these methods involve the use of chloroform, liquid N₂ and phenol and do not employ extraction kits, which are now becoming widely used. Thus, they were modified into less time-intensive protocols and tested against each other. Significant differences in efficiencies of the four protocols were observed. None of these methods required more than 2 h from step one to elution, which gave an advantage over the previously published protocols which could take up to 24 h for sample preparation (Mo and Rinkevich 2001; Tendulkar et al. 2003). While the cost of per reaction was higher than previously published protocols due to the use of relatively expensive kits, these kits were simple and safe to use. The overall quality, measured as the

260/280 nm ratio, and quantity of the DNA extracted using the tested protocols were satisfactory.

The concentrations of total genomic DNA of the three isolates using the four methods are shown in Fig. 3. When eluted with 100 µl elution buffer, the average concentration of total genomic DNA with six duplicates (raw data not shown) for 100 mg cellular biomass of the three isolates from methods 1 to method 4 were; EMRG 527 (7.65, 3.47, 6.26, 1.712 µg), EMRG 539 (8.92, 4.12, 6.42, 1.5 µg), EMRG 543 (8.14, 3.06, 4.76, 1.56 µg), respectively. The significance of these differences is discussed below.

It was evident that method 1 yielded more DNA than the other three methods. The typical yield from 100 mg sample size according to the Qiagen plant mini-kit handbook was ~6 µg with 100 ml elution buffer. It was also evident that both methods 1 and 3 yielded more DNA than the kits' optimal yield with an exception to method 3 on EMRG 543. The use of zirconia beads (method 3) proved to be more effective than the TissueLyser without Proteinase K treatment for all the three isolates. The use of Proteinase K without any mechanical disruption was not successful in obtaining high yields of DNA, as the highest average was just 1.72 µg (EMRG 527) per 100 mg sample.

Both zirconia and stainless steel beads were efficient at disrupting cell walls of the organism through their high velocity bombardment during the vibration. TissueLyser and mini bead beater use the same principal; however TissueLyser is a more common appliance in modern labs, compared to the outdated mini bead beater. Proteinase K (1 mg/ml) was effectively used to degrade cell walls thus releasing the cellular content (Osmundson et al. 2013). However, the Thraustochytrid cell wall is rich in proteins and low in polysaccharides, predominantly xylose based (Moss 1986). This may be useful for Proteinase K activity but the xylose and other long chain polysaccharides may also act as mechanical barriers for the genomic DNA to come into solution easily without the help of a mechanical shearing. Therefore, the combined use of both Proteinase K and mechanical disruption of the cells was successful in extracting total genomic DNA. The use of stainless steel beads alone fell behind the zirconia beads, possibly due to the nature of the sample used. The single bead was effective in grinding dry or solid material rather in liquids. The zirconia beads, however, are 0.5 mm in thickness and can be vibrated at a very high velocity inside a tube, and a 100 mg powder may contain billions of small glass pieces. Therefore, it can be assumed that the use of either zirconia or single bead in conjunction with Proteinase K is far more efficient in DNA extraction. However, taking into account the reusability and compatibility with TissueLyser, the most favourable was the stainless steel bead.

It is important to isolate sufficient quantities of good quality DNA for genomic and gene manipulation studies and it is also important to minimize time consuming methods especially when working with large numbers of samples.

The results of this study proved that the use of chemicals such as phenols, chloroform and liquid N₂ could be easily avoided, as efficient DNA extractions were able to be carried out in less than 2 h of sample preparation compared to 24–48 h for other methods. The TissueLyser is safe and is less noisy than the mini beadbeater and up to 48 samples could be processed compared to the 16 for the beadbeater. With the use of Proteinase K treatment, the use of TissueLyser was advantageous rather than the beadbeater system. Zirconia beads are non-recyclable and can block the filters in spin columns. The stainless steel bead used in

TissueLyser was reusable and only needed to be rinsed and autoclaved before use. It was also evident that the DNA extracted was of sufficient quality that it could be used for PCR studies.

In order to assess the significance between the four methods, a statistical analysis using two-way ANOVA (analysis of variance) was done with R statistical software (R Core Team 2014). The analysis shows a significant difference among the four methods used. A significant difference was evident among the four methods ($p < 0.001$), thus supports the assumptions of taken. All the data suggest that method 1; the use of TissueLyser method with Proteinase K is the most suitable and efficient method of DNA extraction. There is also a significant difference among the three isolates as EMRG 539 yielding highest in all methods and EMRG 543 being the worst performer. No conclusions can be drawn about the variation as there is no species-specific physiology data of the three isolates. However, it may be assumed that the composition differences in the cell wall, especially the deposition of polysaccharides and xylose in varying percentages may play a role in the resistance to mechanical and enzymatic damage.

Laboratories around the world are now adapting to new and easier methods and equipment for their research work. These are easy as rapid DNA extraction kits that do not require the user to prepare necessary complicated reagents and instruments to permit the effective use of such kits. Many commercial DNA extraction kits have their own recommended preparatory instrumentation as well. Observation of the number and the type of organisms (plants, animals, microorganisms) now being studied, it is not always easy to find exact references of DNA extraction protocols for many organisms. Most of the time the nearest match and the protocols designed for the intended organism or cell is used such as cell wall properties, genetic/classification or cultural similarity. For example for Thraustochytrid genomic DNA extraction it is recommended to use Plant DNA extraction kits rather than bacterial or yeast. However, these may not provide the most efficient extraction since the protocols are not specifically designed for Thraustochytrids or its related genera. Therefore this study establishes an improved protocol for Thraustochytrids sp. (Labyrinthulomycetes) with the use of commercial extraction kit for total genomic DNA extraction.

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