Indian Journal of Animal Sciences **89**(6): 699–701, June 2019/Short communication
https://doi.org/10.56093/ijans.v89i6.91147

Modified CTAB method for high-quality genomic DNA extraction from ship barnacle (*Balanus* sp.)

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Received: 21 May 2018; Accepted: 2 November 2018

Key words: Barnacle, DNA extraction, DNAzol, High-quality DNA, Modified CTAB

Ship barnacle (*Balanus* sp.) (Superorder-Thoracica) is an intertidal marine crustacean with very diverse ecological distribution found throughout the world. Barnacles take an integral part in the coastal food web and thus maintain a steady balance in the ecosystem. Traditionally, barnacle species have been characterized morphologically, but genetically they have been poorly studied. This is mainly because of the unavailability of a simple, efficient and cheap method of DNA extraction process for marine organisms. Marine organisms contain high polysaccharide, polyphenolics and some other secondary metabolites which inhibit high-quality DNA extraction and other downstream analyses (Panova *et al*. 2016). The traditional DNA extraction protocols developed for animal tissues like phenol-chloroform extraction and salting out fails to extract good quality DNA from barnacle tissues reproducibly. CTAB (Cetyltrimethylammonium bromide) method is usually used for DNA extraction from plants with high polysaccharide content (Sambrook *et al*. 2001). This method has been reported both to fail (Panova *et al*. 2016) and succeed (Schiebelhut *et al*. 2017) to provide high molecular weight good quality DNA from barnacle. Commercial kit-based methods, ranging from blood DNA kit (Yorisue *et al*. 2018) to tissue kit (Zardus and Hadfield 2005, Zakas *et al*. 2009, Kim *et al*. 2017) have been successfully used with a disadvantage of cost. It is, therefore, necessary to develop an in-house DNA extraction protocol which is easy, cheap as well as efficient for target organism. CTAB method of DNA extraction was reported to be quite successful in case of volcano barnacle and many other marine organisms with high polysaccharide content (Schiebelhut *et al*. 2017). In this study, a modified CTAB protocol which is simple, potent, quick and cheap method to extract high-quality DNA from barnacle is dicussed. A comparison study among 4 different DNA extraction methods (Phenol-chloroform, Salting out, Modified CTAB and *DNAzol*®) was also performed.

The sample was collected from intertidal rocks of Aksa beach, Mumbai (19.1760° N, 72.7954° E). After scraping off the whole organisms (with shell), the outer shells were broken and the intact inner organisms were washed in distilled water and preserved in 100% ethanol.

One single specimen (~9 mg) was used for DNA isolation in all the experiments. All the experiments were carried out in quintuplicate. The phenol-chloroform and salting out method of DNA extraction were carried out following the protocol provided by Sambrook *et al*. (1989) and Miller *et al*. (1988) respectively. DNA extraction with 500 µl of *DNAzol* ® Reagent (Thermo Fisher Scientific) was carried out following manufacturer's protocol. In case of the modified CTAB method, specimen was homogenized in 500 µl of extraction buffer [100 mM Tris-HCl (pH 8), 100 mM EDTA (pH 8), 100 mM Na-Phosphate buffer (pH 8), 250 mM NaCl, 1% CTAB] by a sterile handheld pestle. Ten microlitres of proteinase K (10 mg/ml) was added to the homogenate and incubated at 37°C in shaking water bath for 15 min. Thereafter, 100 µl of 20% SDS was added to the solution and incubated at 65°C in shaking water bath for 15 min. To the final lysate, an equal volume of tris-saturated phenol was mixed and centrifuged at 10,000 rpm for 10 min at 4°C. The aqueous phase was separated and equal volume of chloroform-isoamyl alcohol (24:1) mixture was added, mixed properly and centrifuged at 10,000 rpm for 10 min at 4°C. The clear aqueous phase was separated in a sterile microcentrifuge tube and 0.6 volume of room temperature isopropanol was added, mixed and incubated at room temperature for 10–15 min to allow precipitation of the DNA. After incubation, centrifugation was carried out at 12,000 rpm for 10 min at room temperature in a table-top microcentrifuge. The resulting pellet was then washed with 70% chilled ethanol and dried. Dried pellets were dissolved in TE buffer and stored at –20°C for further applications.

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Restriction digestion with two frequent 6 base pair cutters (*BamHI* and *HindIII*) was carried out separately to demonstrate the absence of enzyme-inhibiting substances in the isolated DNA. The digestion was done with 1 µg of genomic DNA using 1 µl of enzyme for 1 h followed by inactivation of the enzyme.

PCR was performed in 25 µl reaction volume with 100 ng template DNA, 10 pmol of each specific primer, 200 µM each of dNTPs, 0.75 units of *Taq* DNA polymerase and 1× *Taq* buffer containing 1.5 mM $MgCl_2$. The degenerate primers used were (CrustDF1: GGTCWACAAAYCATAAAGAYATTGG; CrustDR1: TAAACYTCAGGRTGACCRAARAAYCA) specific to COI region of crustacean mitochondrial DNA. The universal primers LCO1490 and HC03198 used for metazoan invertebrates (Folmer *et al*. 1994) were also tried previously. The amplification reaction was carried out in 0.2 ml PCR tubes in a heated lid thermocycler under the following conditions: 95°C for 3 min followed by 35 cycles of 95°C for 30 sec, 53°C for 45 sec and 72°C for 90 sec. Final elongation step was done at 72°C for 7 min followed by incubation at 4°C.

The phenol-chloroform method and salting out method failed to isolate any traceable DNA from barnacle samples, even after repeated trials. The *DNAzol* ® method succeeded to isolate DNA, but the resulting pellet was difficult to dissolve in TE buffer. This solubility problem in TE/ water was also reported by Panova *et al*. (2016). On an average, the modified CTAB method extracted 45 µg of genomic DNA with 260/280 ratio of 1.8–1.9, whereas in case of *DNAzol* ® method, the amount of DNA was around 27 µg with 260/280 ratio of 1.9–2.0. The agarose gel electrophoresis and spectrophotometry revealed that the quality of isolated DNA was not very good both in terms of purity as well as the molecular weight. In contrast, the DNA isolated through the modified CTAB method was relatively less contaminated by RNA/ protein and the molecular weight was also higher [detected by myImageAnalysis software (Thermo Fisher Scientific)].

In the present study, after proper lysis of the tissue with a two-step lysis process, the lysate was first cleaned with phenol and then with chloroform-isoamyl alcohol. In absence of the phenol clean-up step, we observed high protein contamination signs both in agarose gel electrophoresis and spectrophotometry even after carrying out chloroform clean-up step twice (data not shown). The 15–15 min lysis time in each step was adopted after screening that lysis for longer time did not give any advantage in terms of quality and quantity. The higher molecular weight of the modified CTAB extracted DNA makes it more useful for processes like whole genome sequencing. Similar results were also obtained by Schiebelhut *et al*. (2017) in which CTAB extracted higher purity NGS quality DNA than some of the expensive kits.

To assure that the extracted DNA is free from any enzyme inhibitory substances, the isolated DNA was subjected directly to 2 enzymatic processes without any further treatments (like RNase treatment). Firstly, the DNA was partially digested (adopted from Kotchoni and Gachomo 2009) for 1 h with two different 6 bp frequent cutter restriction enzymes. Both the enzymes (*BamHI* and *HindIII*) successfully digested modified CTAB method, as well as *DNAzol* ® extracted DNA which was indicated by the formation of smear in agarose gel electrophoresis. Both the enzymes digested the DNA properly indicating the absence of any inhibitory substances.

Secondly, PCR was performed with crustacean COI specific degenerate primers as only RE digestion cannot establish absence of any inhibitory compounds. The universal primers LCO1490 and HC03198 used for metazoan invertebrates (Folmer *et al*. 1994) failed to amplify the DNA even after repeated trials. The suspected reason was the low sequence conservation of the forward primer as reported by Sharma and Kobayashi (2014). An amplicon of around 730 bp was successfully amplified from the DNA extracted from both modified CTAB method and *DNAzol* ® method with the degenerate primers (CrustDF1/ CrustDR1). No non-specific amplifications were detected in agarose gel electrophoresis. The degeneracy of CrustD primers probably helped in proper annealing to the target sequence-resulting appearance of the amplicon. However, the successful PCR amplification indicated the absence of any PCR or enzyme inhibiting substance in the isolated DNA. The intensity of the band was similar to *DNAzol* ® DNA-amplified product (analysed by myImageAnalysis software) which was taken as a standard here. Successful PCR amplification of CTAB extracted DNA from volcano barnacle was also reported by Schiebelhut *et al*. (2017).

The effectiveness of the modified CTAB method described here relies on its simplicity, rapidity and quality. The reagents used are easily available, process is simple and can be carried out within 2 h and the quality is high enough to carry out enzymatic processes. In absence of high priced commercial DNA isolation kits and chemicals, this method can provide us comparable or perhaps better (in terms of molecular weight) DNA for any further applications.

SUMMARY

Presence of high amount of polysaccharide and polyphenolic compounds in barnacles restricts the isolation of high molecular weight good quality DNA. CTAB, which is a detergent used to isolate DNA from high polysaccharide containing plants, is used in this study to extract quality DNA from ship barnacle. This modified CTAB method is simple, efficient and quick enough to be carried out within 2 h. Taking *DNAzol* ® isolated DNA as a reference, restriction digestion and PCR was performed to prove the absence of any enzyme inhibitory substances in the isolate. Traditional phenol-chloroform and salting out method failed to even isolate the DNA from barnacle sample. The molecular weight of the modified CTAB extracted DNA was higher than that of *DNAzol* ® extracted DNA which makes it better candidate for analyses like whole genome sequencing.

ACKNOWLEDGEMENTS

The authors acknowledge the Director, ICAR-CIFE for providing the necessary facilities to carry out the work.

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