Development of DNA Extraction Protocol Utilizing Cellulase to aid in Cell Wall Breakdown

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Abstract

Isolation of DNA from plant tissue differs from that of other eukaryotes and prokaryotes mainly due to the presence of a rigid cellulose cell wall (Manen et al. 2005). Due to the importance of more effective methods to extract plant DNA for plant molecular studies (Manen et al. 2005), this research focuses on the development of a DNA extraction protocol by incorporating steps from different existing protocols. The protocols under evaluation include: a modified version of a protocol utilizing liquid nitrogen, grinding, an SDS/Sarkosyl lysis buffer, and Phenol-Chloroform extraction (DeSalle et al. 2002), a protocol without liquid nitrogen and phenol (Lopez et al. 2013), a method that relies exclusively on enzymes to digest the cell wall for DNA isolation (Manen et al. 2005), a protocol that incorporates commercially purchased cellulase along with a lysis buffer (Weibel 2008), and finally a newly designed method incorporating procedures from several protocols and relying in the ability of Aspergillus and/or Trichoderma to produce cellulases that could digest plant cell walls and aid in DNA extraction (Bhat 2000). Fungi were isolated from different decaying plant tissues (one from an apple and several others from decaying P. microphylla which were believed to be infected with fungus). The isolated strains were grown in a broth, according to Manen et al. (2005).
Cellulose digesting exoenzymes were isolated from the growing media by centrifugation and concentrated by filtration (Manen et al. 2005) for use to break down *P. microphylla*’s cell wall for DNA extraction. It has been hypothesized that the use of cellulase to aid in lysing the cell would increase the amount and purity of the extracted DNA. Therefore, this study compares the yield from different existing and modified protocols. As this is an ongoing experiment, the data presented is preliminary. Currently, the tested protocols incorporating cellulase have not successful for DNA extraction. The most effective method has been grinding followed by incubation in a lysis buffer and Phenol-Chloroform:Isoamyl DNA isolation. However, this may change as more experiments with cellulase are conducted.

**Background Information**

Isolating DNA from plants can be especially difficult when compared with extraction from mammalian tissue, due to the presence of cell walls in plant cells (Manen et al. 2005). The rigorous steps required to breakdown the cellulose cell wall typically include: use of liquid nitrogen, grinding, and strong lysis buffers. Although somewhat effective, the various protocols developed cannot be applied to all plant species and often times result in low DNA yields (Lopez et al. 2013). The
design and development of more effective techniques to isolate DNA from plant tissue requires attention from the scientific community because plant bio-molecular studies may be lagging behind those of other organisms due to this constrain (Manen et al. 2005). Therefore, improved protocols for plant DNA isolation would lead to significant advances in fields such as population genetics, species identification, biodiversity investigations, selection screenings, food control and biotechnology (Manen et al. 2005).

Research Question and Hypothesis

Will the incorporation of cellulases in DNA extraction protocols increase the yield of DNA in plant DNA extractions?

It is hypothesized that the aggressive grinding and strong lysis reagents utilized to extract plant DNA may in fact hinder the nucleic acid molecules. In addition, a clean lysis of the cell by hydrolysis of the cell wall by enzymes is expected to increase the yield and purity of the extracted DNA. Therefore is suspected that yes, the incorporation of cellulase in DNA extraction protocols will increase the yield of DNA.
The first tried and successful protocol was a modified version of Protocol A, “DNA isolation from plants and algae” (DeSalle et al. 2002). The use of liquid Nitrogen was omitted and steps shown underlined were added.

Protocol A
1. After cleaning, plant tissue (.500g) was ground to fine paste with pre-warmed (50°C) 1.5 mL of SDS/Sarkosyl lysis buffer.
2. Incubated at 50°C for 1.5 hours and transferred tubes to ice for 5 min.
3. Centrifuged at 10’000 rpm for 5 min. Transferred supernatant to new tubes (filled halfway).
4. Added equal amount of PCI and mixed by inversion. Centrifuged at 10’000 rpm for 20 min.
6. Transferred aqueous phase and added 0.3 vol. 100% ethanol while swirling. Centrifuged 2 min at max speed.
7. Transferred supernatant to new tubes and added equal amount of PCI. Inverted to mix. Centrifuged 10 min at 13’200 rpm.
8. Transferred aqueous layer to chilled tubes. Filled halfway. Kept solution cold from this point on.
10. Added 1/10 vol. of 5M NaCl and mixed gently.
11. Added 2 vol. ice cold 95% ethanol and mixed gently.
12. Centrifuged at 10’000rpm for 3 min. Discarded supernatant. Added 70% ethanol to pellet in tube. Covered and placed at -20°C overnight.
13. Centrifuged at 10’000rpm for 5 min. Drained off ethanol. Added 500µLTE buffer (pH 8.0) until DNA dissolved. Re-suspended DNA was stored at 4°C.

Protocol A-1
A commercial cellulase solution with 1000 units/g was purchased from Sigma Laboratories and used in an incubation step added to Protocol A before step 1 for Protocol A-1: The cellulase solution was added to a citrate buffer (Adney 1996). The buffer was prepared by mixing 2.1g Ascorbic acid and .5g NaOH in 7.5 mL distilled water and adding NaOH pellets to adjust pH to 4.5. Plant tissue was ground with mortar and pestle or chopped with scissors and in 1mL citrate buffer to which 1mL of undiluted cellulase solution was added in 2.5 mL centrifuge tubes and incubated for 24 and 1 hour respectively for ground and no ground samples. The steps for Protocol A resumed.

Protocol A-2
Citrate buffer and cellulase solution were diluted to 50mM and the pH adjusted to 5.9 for the buffer. Ground and no ground samples were incubated for 24 hours and the protocol resumed with the steps as protocol A.

Protocol B (Lopez et al. 2013)
A lysis buffer was prepared with 2.5% SDS and 0.5 mM TrisEDTA (pH 8.0). The tissue sample was then ground with mortar and pestle and placed in 2.5mL tubes to which a 1:1/2 volume of Phenol was slowly added, followed by the same amount of Chloroform. The tubes were inverted to mix and then incubated at 65°C for 5 min. They were then chilled in ice for 5 min and centrifuged at 11,000rpm for 5 min. The supernatant and an equal amount of ice cold absolute Isopropyl alcohol were added to a new tube, which was vortexed for 30 sec and then placed at -20°C for 20 min. The tubes were then centrifuged at 4°C and 11,000 for 10 min. The supernatant was discarded and the DNA pellet re-suspended in sterile water. It was stored at -20°C.

Protocol C (Manen et al. 2005).
For this method, which is currently under development, different fungi have been isolated by placing visibly decaying leaf tissue in Rose Bengal agar plates with antibiotics. After the fungi started growing, single species were scrapped with a sterile loop and a new Rose Bengal agar was inoculated with an isolation
streak. The isolated fungi were then grown in a broth containing 3.75g Wheat Bran, 3.75g Corn Steep Liquor, 6.75g Hydrolyzed starch, 0.06g Urea and 0.06g CaCl2 in a flask filled to 150mL with distilled water. The flasks with media were autoclaved and then inoculated in the biosafety cabinet with sterile loops. Then they were placed in an agitator at 150rpm and at room temperature for 5 days. The media was then centrifuged and filtered with a 0.25\(\mu\)m filter, using a vacuum. The filtered media containing fungi exoenzymes able to breakdown cellulase have been stored at 4°C until 10kD centrifuge filters are available for concentrating the natural secreted cellulases to use in protocol C. The enzymatic cocktail will then be assayed (Adney 1996) to find optimal temperature and pH for each and will be used in place of the commercially purchased cellulase solution (Manen et al. 2005).

For electrophoresis, 4\(\mu\)L of the suspended DNA was loaded with 2\(\mu\)L of loading dye into agar gels (.9 g agar/100mL distilled water) to which 4\(\mu\)L/100mL of Cyber Green was added for observation under UV light.
Results

Protocol A and B have been successful methods of DNA extraction, while the modified protocols A-1 and A-2 have had no detectable DNA present. However, such results are not definite because more time is needed to continue perfecting the use of cellulase in the extraction protocol.

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<tr>
<th>Protocol</th>
<th>Results</th>
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<tr>
<td>Protocol A (DeSalle)</td>
<td>Viable amounts of DNA Isolated</td>
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**2002**

Grinding
SDS/Sarkosyl lysis
buffer
Proteinase K and
beta-mercaptoethanol
Phenol Chloroform DNA isolation (2 steps)
Ethanol Precipitation

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<tr>
<th><strong>Modified Protocol A-1</strong></th>
<th><strong>Modified Protocol A-2</strong></th>
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| Cellulase incubation step before lysis buffer
Citrate buffer (pH 4.5) for cellulase (1:1)
Grind and no grind samples
1 hr and 24 hour incubation periods | No significant DNA visible in gel

Figure 9. Only a very faint amount of DNA on lane 2. Bottom lane is control

No significant DNA in gel.
6.5) diluted to 50mM
Grind and no Grind
samples

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<tr>
<td>2.5% SDS lysis buffer</td>
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<tr>
<td>Phenol Chloroform</td>
<td></td>
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<td>(one step)</td>
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<td>Isopropyl precipitation</td>
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<th>Protocol C (Manen et al. 2005)</th>
<th>In Progress</th>
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<td>Cellulase cocktail isolated from fungi.</td>
<td>Isolated 5 species <em>P. microphylla</em>.</td>
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<td>Magnetic beads for DNA isolation.</td>
<td>(Figures 5, 7 &amp; 8)</td>
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<td>Solutions filtered for enzyme isolation.</td>
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### Conclusion

Protocols A and B had the highest DNA yield, suggesting the hypothesis of adding cellulase to DNA extraction protocols.
should be discarded. However, based on the knowledge that enzymes are able to work outside of the organisms that produce them provided they have an appropriate pH and temperature (Reese 2013) and in previous work in the scientific field (Manen et al. 2005) cellulase should be able to substitute the grinding step in extracting DNA from plant tissue. Therefore, further experimentation is needed to prove or discard the hypothesis, including:

A full cellulase assay (Adney 1996) to determine optimal concentration, temperature, and pH before further incorporation in DNA extraction protocol. Additionally, the implementation of a quantitative method to analyze amount of DNA present in solution, such as spectrophotometry would verify the DNA yields per method.

References


Bhat MK. 2000. Cellulases and related enzymes in biotechnology. Biotechnology Advances [Internet]; [cited


Weibel JT. 2008. DNA extraction from plant and animal cells [Internet]. California State Science Fair. [cited 03/04/2015]. Available from: www.usc.edu/CSSF/History/2008/Projects/J0415.pdf