OPTIMISATION OF PRIMERS USED TO SCREEN ADH GENES AND PROTEIN ISOLATION FROM SAGO PALM

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This project is submitted in partial fulfilment of the requirements for the degree of Bachelor of Science with Honours (Resource of biotechnology)

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Optimisation of primers used to screen Adh genes and protein isolation from sago palm

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ABSTRACT

Many plants suffered severe growth when flooded occurred and cannot survive prolonged exposure to low O2. Therefore, the aim of this study was optimised the primers used to screen alcohol dehydrogenase (Adh) gene which is known to be involved in various stress response and isolated the protein from sago palm. Better understanding of Adh gene in sago palm, which is flood tolerant species, is essential in order to manipulate them in the nearest future. The first approach has been focusing on the isolation of DNA from sago palm by using CTAB method for mini-prep and mega extraction where the earlier isolation cannot obtain good quality of DNA. When the DNA was subjected for PCR with 2 sets primers only faint bands can be obtained. Due to this problem PCR analysis and cloning steps cannot be done. Second approach has been focusing on the protein isolation by using Polyaacrylamide gel electrophoresis (PAGE) under non-denaturing condition. Though protein isolation was successful, however the ADH enzyme activity detection failed.

Keywords: Alcohol dehydrogenase (Adh) gene, ADH enzyme activity, CTAB, PCR, Non-denaturing PAGE

ABSTRAK

Banyak tumbuhan mengalami masalah pertumbuhan apabila berlakunya banjir dan tidak dapat hidup apabila terdedah kepada kekurangan oksigen secara berterusan. Oleh itu, projek ini dijalankan bagi mengoptima primer untuk memeriksa gen alkohol dehydrogenase (Adh) yang mana gen ini diketahui terlibat dalam pelbagai tindakbalas tertekan dan mengasingkan potein dari pokok sagu. Pemahaman yang baik tentang gen Adh di dalam pokok sagu yang merupakan spesis yang tahan terhadap banjir adalah penting dalam usaha untuk memanipulasinya pada masa akan datang. Kaedah pertama yang ditekankan dalam usaha untuk mengasingkan DNA dari pokok sagu adalah teknik CTAB secara mini mahupun besar-besaran memperlihatkan perlu tindakan penegeksstrakan tidak berjaya memperolehi DNA yang berkualiti. Apabila DNA disertakan dalam tindak balas rantai polimer (PCR) bersama 2 set primer, hanya fragmen-fragmen yang samar diperolehi. Disebabkan masalah ini, analisis PCR dan langkah pengklonan tidak dapat dijalankan. Kaedah kedua yang ditekankan adalah mengasingkan kandungan protein menggunakan teknik Polyaakrilamide gel elektroforesis (PAGE) dibawah keadaan tanpa pengenyahaslian. Walaupun protein dapat diasingkan namun aktiviti enzime ADH tidak dapat dikesan.

Kata kunci: Gen Alkohol dehydrogenase (Adh), aktiviti enzime ADH, CTAB, PCR, PAGE tanpa penyahaslian
CHAPTER 1
INTRODUCTION

1.1 Background

Flooding is one of a phenomenon that affects plant in numerous ways and probably one of the largest limiting factors in agricultural yield. This phenomenon can affect soil and indirectly to the plant itself as it inhibit the growth of the plant. According to Ponnamperuma (1984), when soil is waterlogged, soil inter spaces are filled with water preventing gas exchange between soil and the atmosphere due to low oxygen diffusion in water. The dissolved oxygen in floodwater will be depleted shortly depending on the temperature and respiration activity of the plant and microorganisms, leading to anaerobic condition.

For flood-sensitive species, the rapid depletion of soil oxygen due to flooding is the beginning of the process such as leaf chlorosis, leaf senescence, inhibition of growth and partial injury and if continue it will result in severe injury and death. This is because the plant cell required oxygen for normal metabolism (Pezeshki, 1994). In flood tolerance plant, oxygen deficiency will lead to a switching of their metabolism from aerobic to anaerobic pathway resulting in a much less efficiency system of energy production.

The tolerance of oxygen deficiency differs among various species ranging from hours to month. In a species tolerant to flooding, flood tolerances are achieved through morphological, anatomical and metabolic adaptation. Metabolic adaptation is the main flood tolerance mechanism that will be studied. Nevertheless according to Cherry (1989) plants are known to respond to condition of environmental stress by changing their pattern of gene
expression. One of example gene that is environmentally inducible in specific organ in addition to being developmentally regulated due to environmental stress that is probably caused by flooding is *alcohol dehydrogenase* gene (Adh) (Sach and Freeling, 1978). This Adh gene involved in various mechanisms by which a plant adapts itself to low or no oxygen. According to Francis *et al.*, (1974), flooding increased the *alcohol dehydrogenase* (ADH) enzyme activity as much as 30 times.

Therefore by looking at the ADH activity, it is hope that the mechanism and the role of the ADH in plant particularly for flood tolerance can be further understood. The intensive study about this gene can be applied to agriculture to produce genetically engineered plant that can tolerate flooding or watery soil by using gene manipulation technique. This research was carried out to look at ADH in sago palm, one of plant species that dominates tropical peat swamp rainforests. Therefore this plant may have evolved mechanisms, which can help the plant to adapt to low or no oxygen conditions. Evolution in ADH activity may contribute to the ability of sago palm to tolerate watery soil condition.

At the end of this research, it is hope that ADH activity can be detected to prove the existence of ADH in sago palm metabolic pathway and subsequently the primers used to screen ADH gene can be optimised. Besides that this research is also meant to isolate and characterize ADH gene in sago palm.
1.2 Sago palm

Sago palm (*Metroxylon sagu*) is a Cycad that is closer in relation to a pine tree than a palm tree (Vaughan and Geissler, 1997). This plant is native to New Guinea and the Moluccas and widely cultivated in South East Asia, especially in Sumatra, Borneo and Java (Sani *et al.*, 2000). In Malaysia the sago palm are cultivated particularly in Mukah, Sarawak.

In Sarawak basically there are two main species of sago palm, the thorny (*Metroxylon rumphii*) and the thornless (*Metroxylon sagu*) (Zainal, 2004). Sago trunk contains starch and it has long been a staple food in South-East Asia. Usually wet starch is boiled, fried, roasted alone or mixed with other foodstuff. Whole young trunk, pith and pith refuse are also focussed as animal feed. In Indonesia and Malaysia sago is an important for the local food industries and in manufacturing of cakes and cookies, noodles and crisp, and for custard powder. Sago starch is also used to produce monosodium glutamate, soft drinks and new uses for sago includes in biodegradable plastic, sizing pastes for paper and textiles, extender in adhesive for plywood and to make fuel alcohol and citric acid. Sago also can be a suitable raw material for further industrial processing such as high fructose syrup and ethanol.

Sago palm is a multi-stemmed species and commonly found growing in colonies in swampy, water logged soil conditions and mainly grown on peat soils. Sago palm will generally not grow well in clay soils unless the soils are heavily amended with sand and organic matter to increase aeration and drainage. The soil pH plays a role in the optimal growth of most Cycads. A neutral soil pH 7 is generally best for most species of Cycads and allows the proper absorption of nutrients. Sago palm grows quickly and mainly dominates
tropical peat swamp rainforests. It can tolerate very acidic conditions, with high concentrations of metals, which poison other plants, and in heavy clays that suffocate other plants and apart of that it also can grow on dry land.
CHAPTER 2
LITERATURE REVIEW

2.1 Alcohol Dehydrogenase (ADH)

Alcohol dehydrogenase is the primary enzyme that exists in animals, plants and bacteria. This enzyme is very important in plant and animal metabolic system in which it is responsible for the metabolism of ethanol to acetaldehyde and as the most often an NAD(P) dependent-enzyme present in cytoplasm. Alcohol dehydrogenase (ADH) is an enzyme that plays a central role in the anaerobic metabolism to survive prolonged periods of oxygen deficiency where all aerobic organisms have had to evolve mechanisms for sensing oxygen availability and to adjust their cellular metabolism accordingly.

In plant, alcohol dehydrogenase enzyme (ADH E.C.1.1.1.1) carries out the terminal electron transfer in anaerobic glycolisis, converting acetaldehyde to less toxic, readily diffusible ethanol under oxygen-limited condition, regenerating NAD in Krebs process (Krebs, 1972). This enzyme activity has been detected in herbaceous and woody plants where ADH activity occurs in root apices and in germinating seeds. In trees, ADH activity also occurs in leaves and in stems, and it is particularly high in the vascular cambium, a site of ethanol synthesis (Harry and Kimmerer, 1991). The best documented roles for ADH enzymes are in anaerobic and aerobic fermentation (Bucher et al., 1995; Hageman and Flesher, 1960). The group of dimeric, zinc-containing enzyme has been studied. This ADH belongs to protein super family of medium-chain dehydrogenase and consists of a complex system with different form and extensive multiplicity (Davies et al., 1973).
The multiple form of ADH has been detected in a range of plant tissue such as potato tuber (Duester et al., 1999), pea seed (Leblova et al., 1976), maize (Scandalios, 1977), wheat (Langston et al., 1980), tomatoes (Tanksley and Jones, 1981) and mushroom (Okamura et al., 2003). In previous research, alcohol dehydrogenase gene in tomato (Lycopersicom esculentum) has been studied and manipulated by modified the ADH levels in the ripening fruit. This test is carried out to see whether the ADH influenced the balance between some of the aldehydes and the corresponding alcohols associated with flavor production. The result shows that ADH is involved in the interconversion of aldehydes and alcohol and that this affects the flavor of the fruits (Speirs et al., 1998).

Alcohol dehydrogenase in plant has been characterized at the gene level that belongs to three groups of dimeric, zinc-containing enzyme NADC-dependent ‘classical’ alcohol dehydrogenases active on ethanol, NADPC-dependent cinnamyl alcohol dehydrogenase active in lignin biosynthesis (EC1.1.1.195) (Garabagi and Strommer, 2004) and ‘formaldehyde-active class III alcohol dehydrogenases’=S hydroxymethylglutathione dehydrogenases (EC 1.2.1.1; Shafqat et al., 1996). Alcohol dehydrogenases are dimeric enzymes in which the presence of products for two ADH genes in the same cellular compartment results in formation of heterodimers as well as homodimers (Freeling and Schwart, 1973). Others types of alcohol dehydrogenases that has been noted in plants are NADP-dependent aromatic (benzyl) alcohol dehydrogenase (EC 1.1.1.91) (Somssich et al., 1996) and terpenoid alcohol dehydrogenase (Davies et al., 1973). These are distinct from cinnamyl alcohol dehydrogenase 2, which is not active on benzyl alcohol or terpenoid alcohols.
The Polymerase Chain Reaction (PCR) is the main tool used in this study to identify and characterize the ADH genes of sago palm and subsequently cloned before sequencing can be done successfully. According to Morton et al. (1996), three different Adh clones from palm (Washingtonia robusta) genomic library has been isolated and denoted as \textit{adhA}, \textit{adhB} and \textit{adhC}. The pattern of substitution suggests that all three of the three isolated from the palm library code for functional Adh proteins.

2.2 Protein

Besides optimisation of primers used to screen Adh genes, the aim of this study also to characterize the specific ADH enzyme. Protein extraction is the main procedure to determine ADH enzyme activity.

Proteins are the primary product of genes. One of the methods that are use for protein analysis is Polyacrylamide gel electrophoresis (PAGE) under non-denaturing condition. This method has been look as a powerful tool for analysis of biological samples and capable of good separation and high resolution of complex protein mixture. In addition PAGE under non-denaturing condition look at protein without removing secondary and tertiary structured instead leaving them in their native conformations. Therefore the use of this method is important, as it is particularly good for examining and isolating protein while preserving their biological activity.
CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

The fresh sago palm leaf sample used in this study were obtained from sago seedling grown in UNIMAS plant house and trunk areas (pith) were taken from Padawan area.

3.2 DNA Extraction

DNA extraction was carried out by using the CTAB method describes by Doyle and Doyle (1990) with slight modification by Gillies and Bush for miniprep extraction for PCR. Young leaves of sago palm were cut into small pieces (exclude the veins) and approximately 0.1 g of the cut pieces were grounded in the liquid nitrogen. Liquid nitrogen was added into the mortar and the leaves were grounded into the powder form. Then 100 μl of CTAB extraction buffer that has been incubated in water bath at 65°C for 30 minutes for preheat was added and followed by added further 900 μl of CTAB buffer and 2 μl of mercaptoethanol was added.

The mixture was transferred into 1.5 ml Eppendorf tube and the incubated in a water bath at 65°C for 1 hour. The tube was removed from water bath and allows cooling to room temperature for 5 minutes. Four hundred microlite of chloroform (CIA) was added and mixed gently and centrifuged at 13000 rpm for 5 minutes at 4°C. The upper aqueous layer was removed and transfers to new Eppendorf tube. Six hundred microlite of ice-cold propan-2-ol was added and mix gently to precipitate the DNA and the tube were left to stand
at -20°C for overnight. The next day the tube was taken out from the freeze and centrifuge at 13000 rpm for 2 minutes at 4°C. The supernatant was discarded and 1 ml of wash buffer was added before left to stand at room temperature for 30 minutes. The supernatant was discarded and the pellet was air-dried for 15 minutes after second centrifugation at 13000 rpm for 2 minutes at 4°C. Then DNA pellet were dissolved in 100 μl of TE buffer and store at -20°C.

3.3 Large scale DNA extraction

The extraction of large scale of DNA was carried out using CTAB method described by Doyle and Doyle (1990). The methods are similar to the procedure mentioned above except that the amount of fresh sago leave was increased to 3 g instead of 0.1 g used in previously and volume of chemical had been increased proportionately. With this method 300 μl of DNA sample was obtained.

3.4 Protein Extraction

The protein extractions were carried out using buffer 2 solutions system by Wendel and Weeden (1990).

Buffer 2;

75 mM of Tris-HCl (pH 7.5)
5% sucrose, 5% PVP-40
14 mM mercaptoethanol (0.1%)
50 mM ascorbic acid and [Na] salt
10 mM diethyldithiocarbamade and 0.1% of Bovine serum albumin

Approximately 1.5 g of sago palm leaf sample was frozen in liquid nitrogen and grinded into powder. Then 1000 µl of buffer 2 was added. The content was mix gently and centrifuge at 5000 rpm for 15 minutes at 4°C. The tube was re-centrifuging at 13000 rpm for 4 minutes at 4°C. The supernatant was transferred into a new Eppendorf tube and stored at -20°C. The same procedures were repeated again with pith sample.

3.5 DNA Visualisation

DNA visualized was carried out using agarose gel electrophoresis (AGE) method by Sambrook et al., (1989). Five microlite DNA sample with 1 µl of loading buffer was loaded in 1% of agarose gel and stained with ethidium bromide. The gel was run on electrophoresis system with 100 Volt for 1 hour and 35 minutes and visualized under UV light. The photograph of DNA was taken using Polaroid camera.

3.6 Protein Visualisation

Protein was visualised using PAGE under non-denaturing condition by Bollag and Edelstein (1999). Twelve percent of separating gel was prepared by combining 4 ml of A solution (refer to Appendix B), 2.5 ml of B solution (refer to Appendix B) and 3.5 ml of H2O. Then 50 µl of 10% ammonium persulfate and 5 µl of TEMED (tetramethylidiamine) were added and mixed. The solutions then were introduced into gel sandwich and 1 cm of water was layered on the top of the separating gel solution. These gels then were allowed to polymerize. The water covering the separating gel were poured off and 5% of stacking gel
(refer to Appendix B) were pipette onto the separating gel until the solution was reaches the top of front plate. The comb was inserted into gel sandwich until the top of front plate and the stacking gel were allowed to polymerize. Then the comb was removed and sample solution (20 μl protein sample: 1 μl sample buffer) was loaded into well. The gel was run at 120 Volt on electrophoresis system with tris-HCl buffer (pH 8.8). The reaction were carried out on 4°C and stopped when the sample is approximately 1 cm from the bottom of the tank, which is takes about 3 hours. Then the gel was stained with Coomasie blue solution. After 3 hours staining on the shaker, the gel were destained with Coomasie blue destain solution.

3.7 Specific ADH Staining

The specific ADH staining was carried out by preparing the gel using method by Bollag and Edelstein (1999) but loading 20 μl of protein sample without sample buffer. ADH activity was assayed according to the method of Bergmeyer (Cho and Jeffries, 1999). The reaction mixture contained 99 ml of 0.1 M Tris-HCl buffer (pH 8.5) and 1 ml of 0.8 M ethanol in a total volume of 100 ml. The reaction was started by adding the reaction mixture (99 ml 0.1 M Tris-HCl solution and 1 ml 0.8 M ethanol) to cover the gel. The reaction was incubated for 60 minutes on a shaker and then 2 mg (0.4 ml) PMS, 10 ml (1 ml) MTT and 10 mg (1 ml) NAD was added. The reaction was incubated for another 60 minutes before it was destained with distilled water.
3.8 DNA Quantification

DNA was quantified using 5 μl of DNA sample and diluted in 495 μl of distilled deionised water in a quartz cuvette. The absorbance of diluted DNA sample at the wavelengths 260 nm and 280 nm (A_{260} and A_{280}) was measured. The ratio of $A_{260}: A_{280}$ and the concentration of the extracted DNA were obtained from the reading showed from the Ultrospec 1100 pro spectrophotometer.

3.9 Protein Quantification

Protein quantification was carried out using test tube procedures by Coomassie (Bradford) Protein Assay Kit. 30 μl of each standard sample was pipette into labelled test tubes and 1.5 ml Coomassie Reagent was added to each tube and mix well. The absorbance is read at wavelength 595 nm for the assay.

3.10 Primers for amplification

Three primers were designed for screening in this work. The primers are designed base on the nucleotides of apple, thaliana and grape plant species. The sequences are aligned using DNASTAR software. Apart from that a published primer (MorADH) was also used (Morton et al, 1996). The primer sequences are listed in Table 1.
Table 1. The sequence of primers used for amplification of ADH gene

<table>
<thead>
<tr>
<th>Name of primers</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>haADH-F</td>
<td>5’- TACTTCTGGGAAGCCAAGGGGACAA-3’</td>
</tr>
<tr>
<td>haADH-R1</td>
<td>5’- CCTGAGGAGGTCACACATGTT-3’</td>
</tr>
<tr>
<td>haADH-R2</td>
<td>5’- CTCAGCAATCACCTCTTCAA-3’</td>
</tr>
<tr>
<td>MorAdh-F</td>
<td>5’-GGGTGCTGTGAGGGGCTTGC-3’</td>
</tr>
<tr>
<td>MorAdh-R</td>
<td>5’-GATATCTGCTTTTGAATGCG-3’</td>
</tr>
</tbody>
</table>

3.11 Screening using PCR

Three sets of Adh primer combination were used to fish out the fragment of Adh genes from the sago palm. The first set includes forward primer haADH-F and reverse primer haADH-R1. The second set includes forward primer haADH-F and reverse primer haADH-R2 and meanwhile the third set include forward primer MorADH-F and reverse primer MorADH-R. PCR was carried out in the Perkin Elmer Cetus DNA Thermal Cycler 2400. The PCR mixture for primer sets haADH-F/haADH-R1, haADH-F/haADH-R2 as well as MorADH-F/MorADH-R are listed in Table 2 and Table 3 for PCR parameters.
Table 2. The composition of PCR reaction mixture for primer sets haADH-F/haADH-R1, haADH-F/haADH-R2, MorADH-F/MorADH-R

<table>
<thead>
<tr>
<th>Reagents</th>
<th>haADH-F/haADH-R1</th>
<th>haADH-F/haADH-R2</th>
<th>MorADH-F/MorADH-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>10XPCR Buffer without magnesium (µl)</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>10mM dNTPs (µl)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>25mM MgCl₂ (µl)</td>
<td>2.5 3.0 3.5</td>
<td>2.5 3.0 3.5</td>
<td>2.5 3.0 3.5</td>
</tr>
<tr>
<td>25pmol/µl of each primers (µl)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Taq DNA polymerase (µl)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>DNA template (µl)</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Sterilized ultra pure water (µl)</td>
<td>14.8 14.3 13.8</td>
<td>14.8 14.3 13.8</td>
<td>14.8 14.3 13.8</td>
</tr>
<tr>
<td><strong>Final volume (µl)</strong></td>
<td><strong>25</strong></td>
<td><strong>25</strong></td>
<td><strong>25</strong></td>
</tr>
</tbody>
</table>
3. The parameters for PCR method

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>Initial Denaturation</td>
<td>94</td>
</tr>
<tr>
<td>Step 2</td>
<td>Denaturation</td>
<td>94</td>
</tr>
<tr>
<td>Step 3</td>
<td>Annealing</td>
<td>62</td>
</tr>
<tr>
<td>Step 4</td>
<td>Elongation</td>
<td>72</td>
</tr>
<tr>
<td>Step 5</td>
<td>Steps return to step 2 for 30 cycles</td>
<td></td>
</tr>
<tr>
<td>Step 6</td>
<td>Final extension</td>
<td>72</td>
</tr>
</tbody>
</table>

Ten microlite of PCR product with 2 µl loading dye was mixed and run on 1.5% agarose (BST Techlab) electrophoresis containing 1µg/mL of ethidium bromide and then visualised under UV light. Photograph of agarose was taken using Polaroid (Polaroid 667)
CHAPTER 4
RESULTS AND DISCUSSION

4.1 DNA Extraction

The DNA isolation is relatively tough as they may contain high polysaccharides and secondary metabolites. The first extractions of DNA from young sago leaf were not successful due to some problems such as contamination. From Figure 1, it shows smearing banding. In addition, when these DNA sample was quantified to determine its concentration, the reading for the sample were low (Table 4).

Figure 1. DNA extraction from young leaf of sago palm in 1% agarose gel visualised under UV light. Lane 'A' shows the Lambda/Hind III marker. Several bands were observed in lane 1-7.
According to Brown (2002), the ratio of $A_{260}:A_{280}$ should be more than 1.8. Ratios less than 1.8 indicate that the sample is contaminated with phenol or protein. Based on the reading from the Table 1, it shows that both DNA is contaminated as the ratio of $A_{260}:A_{280}$ are less than 1.8.

A second extraction, sample 1 and sample 2 (Figure 2) yielded a good result with relatively high purity ($A_{260}:A_{280}$) which is higher than 1.8. Meanwhile Table 5 shows the absorbance reading measured by spectrophotometer for sample 1 and sample 2 (mega extraction).

---

**Table 4.** The absorbance reading from two of selected samples measured by Ultrospec 1100 pro spectrophotometer

<table>
<thead>
<tr>
<th>Samples</th>
<th>5</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_{260}$</td>
<td>0.144</td>
<td>0.098</td>
</tr>
<tr>
<td>$A_{280}$</td>
<td>0.105</td>
<td>0.076</td>
</tr>
<tr>
<td>$A_{260}:A_{280}$</td>
<td>1.612</td>
<td>1.294</td>
</tr>
<tr>
<td>Concentration</td>
<td>0.719 μg/μl</td>
<td>0.491 μg/μl</td>
</tr>
</tbody>
</table>

---

*Figure 2.* DNA extraction from young leaf of sago palm in 1% agarose gel visualised under UV light. Lane 1 from miniprep isolation and 2 from mega extraction.
Table 5. The absorbance reading of two samples measured by Ultrospec 1100 pro spectrophotometer

<table>
<thead>
<tr>
<th>Sample</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A_{260}</td>
<td>0.129</td>
<td>0.720</td>
</tr>
<tr>
<td>A_{280}</td>
<td>0.080</td>
<td>0.395</td>
</tr>
<tr>
<td>A_{260} : A_{280}</td>
<td>1.899</td>
<td>1.986</td>
</tr>
<tr>
<td>Concentration</td>
<td>0.516 µg/µl</td>
<td>3.266 µg/µl</td>
</tr>
</tbody>
</table>

The DNA sample with high concentration (3.266 µg/µl) was later used as the template for the Polymerase Chain Reaction (PCR). However, the earlier attempts of PCR failed. The amount of *Taq* DNA polymerase used in the reaction was 0.2 µl (1 unit) and this factor probably causes the negative result. PCR works are sensitive and there might have been some technical errors such as like human error while pipetting might also contributed to the unsuccessful PCR. Any extraneous that enters the DNA sample or contamination occurred during the preparation stage of PCR reagents, the contaminating DNA might be amplified. Moreover DNA sample that contaminated with any form of DNase will be degraded.

The PCR reaction was carried out several times to optimize the annealing temperature and the amount of MgCl₂. The optimization of magnesium ion is beneficial as it may affect the primer annealing, product specificity, formation of primer-dimer artefacts, and enzyme activity and fidelity.