Expression Analysis of Differentiated Sago Palm Gene (Nuclear transcription factor Y subunit C and Cytochrome P450) via Amplified cDNA

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Final Year Project 2 (STF 3015) course

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Programme of Resource Biotechnology (WS47)

Faculty of Resource Science and Technology

University Malaysia Sarawak

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LIST OF ABBREVIATIONS

RNA	Ribonucleic Acid
cDNA	Complimentary Deoxyribonucleic acid
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
PCR	Polymerase Chain Reaction
mRNA	Messenger Ribonucleic acid
Mt	Metric ton
Nt	Non-trunking
%	Percentage
NF-YC	Nuclear transcription factor Y subunit C
Rpm	Rotation per minute
µL	Micro Liter
Bp	Base Pair
dNTP	Deoxynucleotide Triphosphate
N	Nitrogen
ATP	Adenosine Triphosphate
CTAB	Cetyltrimethyl ammonium bromide
PVP	Polyvinylpyrrolidone
EDTA	Ethylenediaminetetraacetic acid
NaCl	Sodium chloride
$MgSO_4$	Magnesium sulphate

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ABSTRACT

Metroxylon sagu Rottbol is an important type of palm that contributes to economic value in Malaysia. However, sago palm grown on peat soil were found to be non-trunking and produce lower quality of sago. The present study looks at the gene expression analysis of NF-YC and cytochrome P450 genes specifically towards the band intensity. Total RNA of trunking and non-trunking sago palm was extracted using CTAB extraction buffer. Using RT-PCR, the RNA is converted to cDNA an amplified using PCR. An amplicon of 347 bp size was produced for the NF-YC gene. Relative intensity values for non-trunking sago palm were calculated using ImageJ, which was found to be higher than trunking sago palm. Differentially expressed genes can be examined to understand the possible function leading to specific physiological traits. Further studies on the gene expression of the NF-YC gene should be conducted to explain the physiological characteristics of non-trunking sago palm.

Keywords: *Metroxylon sagu*, cytochrome P450, NF-YC, cDNA, Gene expression analysis.

ABSTRAK

Metroxylon sagu Rottbol ialah sejenis pokok sawit penting yang menyumbang kepada nilai ekonomi di Malaysia. Bagaimanapun, pokok sagu yang ditanam di atas tanah gambut didapati tidak berbatang dan menghasilkan sagu yang berkualiti rendah. Kajian ini melihat analisis ekspresi gen gen NF-YC dan sitokrom P450 secara khusus terhadap keamatan jalur. Jumlah RNA pokok sagu berbatang dan bukan berbatang diekstrak menggunakan penimbal pengekstrakan CTAB. Menggunakan RT-PCR, RNA ditukar kepada cDNA yang dilipatgandakan menggunakan PCR. Amplikon bersaiz 347 bp dihasilkan untuk gen NF-YC. Nilai keamatan relatif untuk pokok sagu bukan berbatang dikira menggunakan ImageJ, yang didapati lebih tinggi daripada pokok sagu berbatang. Gen yang di ekpres secara berbeza boleh dikaji untuk memahami kemungkinan fungsi yang membawa kepada ciri fisiologi tertentu. Kajian lanjut mengenai ekspresi gen gen NF-YC perlu dijalankan untuk menerangkan ciri fisiologi pokok sagu yang tidak berbatang.

Kata kunci: *Metroxylon sagu*, cytochrome P450, NF-YC, cDNA, analisis ekspresi gen

1.0 INTRODUCTION

The word Sago originated from the Javanese language which has the meaning of the starch. It becomes a common name for all starch sources of any palm especially in Indonesian-Malay region (Flach, 1983). Sago palm (*Metroxylon sagu Rottboll)* is categorized as true palm under the subfamily of Calamoideae, Palmae family and Arecales order (Lim *et al.,* 2020). The scientific term comes from the words *metra,* which means pith or *parenchyma,* and *xylon,* which means *xylem* (Flach, 1997). The growth and morphological traits along with the productivity of starch are highly diverse in various locations (Yamamoto *et al.*, 2020). It is known to be a 6-14m tall and hapaxanthic or monocarpic plant which flowers just once in a lifespan and then dies quickly. (Flach & Schuiling, 1988; Abd-Aziz, 2002). Sago palm propagate through suckers that appear from the roots or lower trunks of parent's plants (Singhal et al., 2008) or propagate through seedlings (Karim *et al*., 2008). Sago palms specifically thrive in humid tropical lowlands up to 700m above sea level and are widely found between latitudes 10° north and 10° south in Southeast Asia and Pacific Island countries (Bintoro *et al.,* 2018).

Sago palm has increasingly gained attention in its ability to thrive in harsh swampy peat soils, in submerged soil, saline soils, acid soils, and through diverse environmental conditions (Matsumoto *et al.,* 1998; Nozaki *et al.,* 2004; Konuma *et al*., 2012) along with metal present soil such as AI, Fe and Mn or heavy impervious clays (Chua *et al.,* 2021). Sago palms that were grown in these environments were prone to stresses which produces different growth rate and phenotypes. One variant of sago palms described as a non-trunking (Nt) sago palm are usually found in deep peat soil area (Hussain *et al*., 2020). Several researches have been conducted to determine the environmental origin of this variant, including changes in soil quality (Nozaki *et*

al., 2004; Okazaki & Sasaki, 2018; Ming *et al.*, 2018) and microbiota between trunking and non-trunking sago palms (Toyota, 2018) where it concluded that environmental stress causes various plants to grow at different rates. There is just a little amount of genetic information about the sago palm in the database. The study of the sago palm's growth process at the molecular level would aid in understanding the mechanism or pathway involved. Past studies concluded that some genes were found to be differentially expressed that will identify a potential function that leads to specific physiological characteristics (Hussain *et al.*, 2022).

In this Study, the gene expression of nuclear transcription factor Y subunit C and cytochrome P450 gene were analyzed using amplified cDNA method and band intensity were compared using ImageJ (Version 1.53k) software. Analyses of the gene expression of nuclear transcription factor Y subunit C gene proves to be differentially expressed in non-trunking sago palm compared to trunking sago palm. Thus, this will lead to a deeper understanding of the palm's mechanism.

The objectives of this research are as follows:

i. To extract total RNA of trunking and non-trunking sago palm leaf sample.
ii. To amplify first cDNA strand of nuclear transcription factor Y subunit C and cytochrome P450 gene using PCR.
iii. To compare the gene expression analysis of nuclear transcription factor Y subunit C and cytochrome P450 genes with normal trunking sago palm.

2.0 LITERATURE REVIEW

2.1 IMPORTANCE OF SAGO PALM

Sago palm is on top of the list of important agricultural plants in Malaysia and is commercially cultivated for starch production. The vegetative phase for sago palm generally last for 7 to 15 years, throughout where the pith are going to be saturated with starch, from the stem base and upwards (M.Amin, 2019) and the amount of starch accumulated in the trunk of the sago palm can reach approximately one hundred fifty to three hundred kg of dried starch per trunk (Nozaki *et al.,* 2004; Konuma, 2018). Sago palm produced higher starch yields (Based on the amount of starch annually and per hectare) compared to that of rice approximately three to four times more (Matsumoto *et al.,* 1998). Under ideal conditions, a mature sago palm generates 15-25 Mt of air-dried starch per hectare after 8-year growth cycle. In addition to having a high yield, sago starch has a lower production cost than other starch sources (Hussain *et al*., 2020).

With the world's population increasing, a main food supply is becoming increasingly important, the sago palm has a potentiality to provide food security as a secondary source of staple foods that competes less (or not at all) with other food crops for agricultural land (Konuma, 2018; Chua *et al.,* 2021). Furthermore, sago starch can be turn to sago sugar (Bujang, 2018) used as a sweetener, a fermentation substrate, and a source of raw materials for both food and non-food goods (Sunarti *et al., 2018*) such as biodegradable film coating agent, adhesive thickening, and cosmetics dusting powder (Chua *et al.,* 2021) bioethanol and bioplastic (Konuma, 2018).

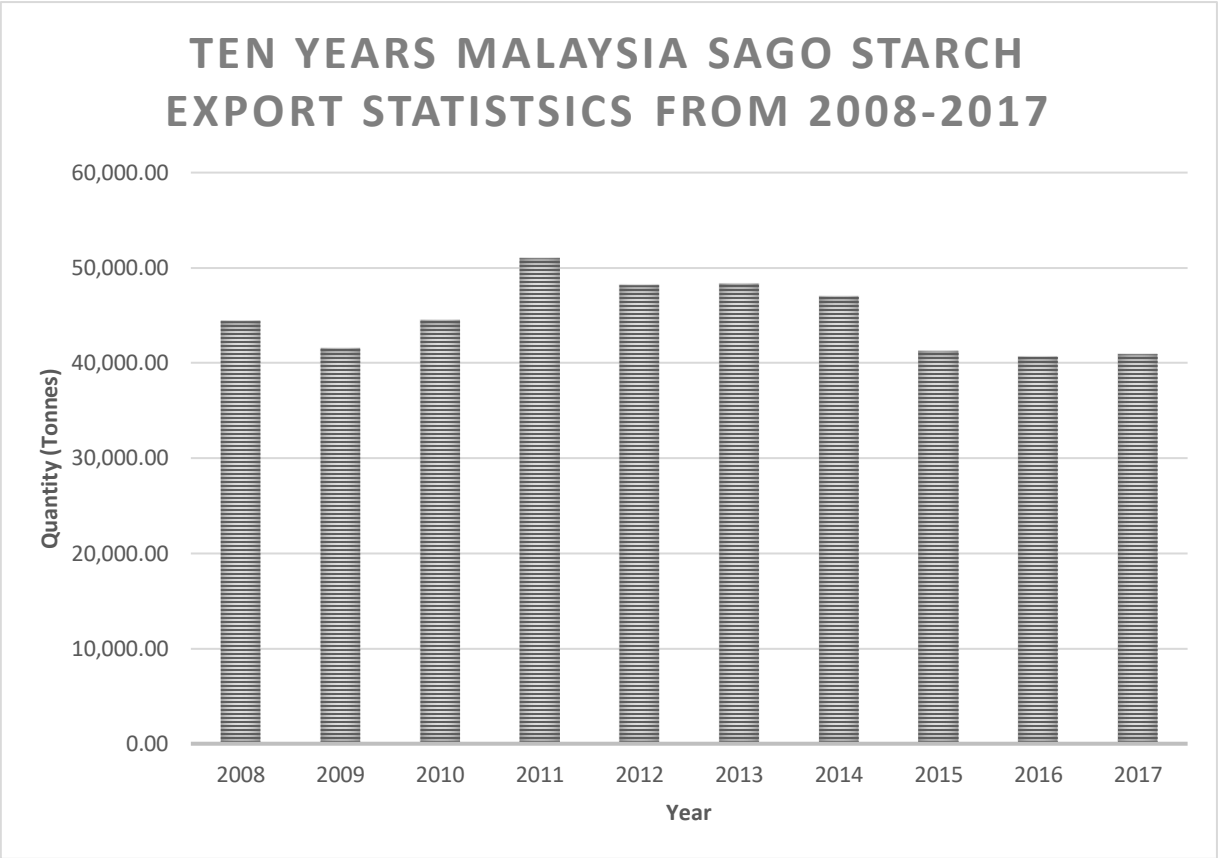

Figure 1. Ten years Malaysia sago starch export statistics (2008 – 2017) according to Department of Statistics, Sarawak (2017).

In 2017, 40,952,601 Mt of sago were exported with the value RM 86.987 million in revenues. The sago palms are exported to Japan, Singapore, Thailand, Vietnam, Myanmar, China and Indonesia (Department of Statistic, 2017). Production of sago palm are mainly coming from Sarawak state with 40,495.4 hectares of plantation out of the 41,082.4 hectares of sago plantation in Malaysia. Sarawak state produced 195,800.9 Mt in 2019 which attributes to 98% of total production of sago palm in Malaysia. Most of the plantation in Sarawak state is in the Mukah district with 34,734.9 hectares of planted area (Department of Agriculture Malaysia, 2019).

2.2 NON-TRUNKING SAGO PALM

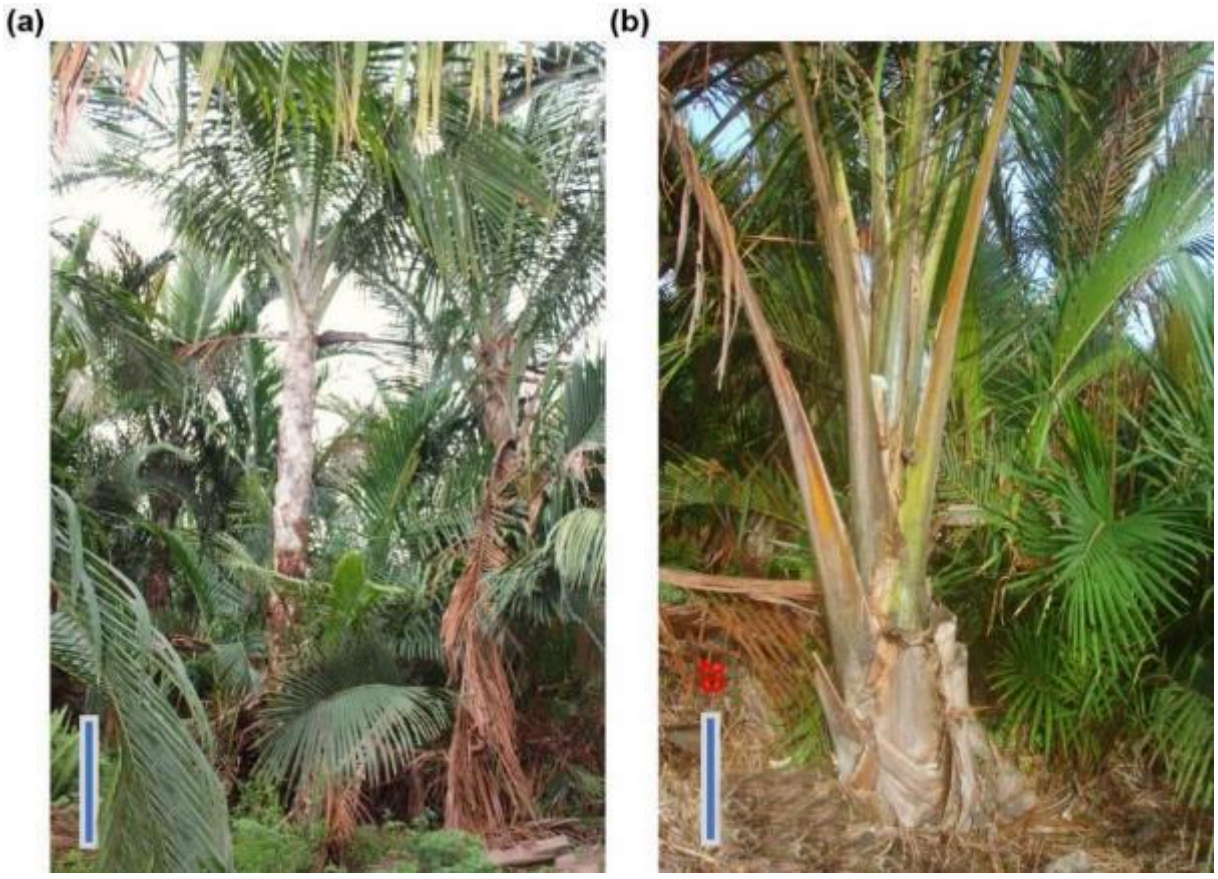

Figure 2. (a) Normal trunking M. sagu at year 8, Bar equals to 2.5 m. (**b)** non-trunking M. sagu at year 17 retrieved from (Hussain *et al*., 2020) Differential Metabolites Markers from Trunking and Stressed Non-Trunking Sago Palm (Metroxylon sagu Rottb.)

Non-trunking sago palm is a variant that was grown on peat soils is found to grow at a relatively slower rate and express lower production than normal palms that were grown on minerals soils. (Hussain *et al*., 2020). Starch productivity per trunk grown in acidic peat soil is lower than starch productivity per trunk grown in mineral soil (Nozaki *et al.*, 2004; Chua *et al.*, 2021) production per unit time were also estimated to be 25% lower (Flach & Schuiling, 1988; Okazaki & Sasaki, 2018). Soil quality effect on sago palm has been studied by several researchers, according to Purwanto *et al.* (2002) low bulk density, lower soil pH, and low N, P, K, Ca, Zn, and Cu levels were among the physical and chemical restrictions of peat soils.

According to Hussain *et al*., (2020) non-trunking sago palm metabolite groups were found to be differently expressed such oils and waxes etc. Proteins were also found to be expressed differently which might be plausible contenders for the sago palm's non-trunking phenomena (Hussain *et al.,* 2019). Identification of differentially expressed transcripts of sago palm were done by Hussain *et al.,* (2022) where they discovered that gene expression was regulated at different levels. Overall, the non-trunking sample demonstrated active energy metabolism regulation in respiratory chains and chloroplast growth. The Nt sample is also involved in the control of Ca, Mg, and Zn concentrations in cells and tissues, as well as traffic and transport-related functions. Sago starch color characteristics was analyzed by Konuma *et al.* (2012) on sago palms. It was concluded where starch color on peat soils are colored pink to brown when extracted whereas the neutral soil conditions were white in color. Average particle size of starch granules grown in mineral soils were observed to be smaller in size compared to peat soil (Nozaki *et al.,* 2004) However, the opposite results were obtained on studies by Konuma *et al.,* (2012).

2.3 GENE EXPRESSION

Most specialised cells in a complex organism may change their gene expression patterns in response to external stresses (Roberts *et al.*, 2002). Gene expression is the foundation for cell differentiation, morphogenesis, and adaptation, all of which dictate a cell's functional destiny in physiological processes in health and diseases. Gene expression patterns are often reflected in cellular decisions about growth, differentiation, and survival (Raghavachari, & Garcia-Reyero, 2018). An organism phenotype is related to its regulation on the gene expression (Raghavachari, & Garcia-Reyero, 2018). From seed germination to maturity, plants' reactions to environmental challenges are exhibited at physiological, biochemical, and molecular levels (Tiwari *et al.*, 2017).

Environmental stressors such as salt, drought, and severe temperatures, as well as biotic stresses like pesticides, are significant problems to agricultural production and productivity across the world (Tiwari *et al.*, 2017). During diverse individual and combined environment stresses, the expression of thousands of genes is known to be changed. It is now well established that genes produced in response to diverse environment stresses not only aid in cellular tolerance by maintaining osmotic equilibrium, but also through controlling stress-responsive gene expression (Tiwari *et al.*, 2017). Organism adapt to changing surroundings by altering metabolic pathways in order to avoid or mitigate physiological harm. These cellular events occur before population-level changes, and if connected to certain physiological or ecological events, they might be effective biomarkers. (Edge *et* al., 2005).

2.4 GENE EXPRESSION ANALYSIS

According to Edge *et al.,* (2005). Thousands to tens of thousands of distinct genes are encoded in the genomes of all living species, yet only a small proportion of them are expressed at any given moment. As a result, metabolic activity is determined by the temporal and spatial control of gene expression. Recent advances in genomic methods and technology have made it possible to conduct a robust and effective research of such full transcriptional characterization. For example, Northern blotting, expressed sequence tag (EST) sequencing, tag-based methods including serial analysis of gene expression (SAGE), cap analysis of gene expression, and massively parallel signature sequencing, microarrays, RNA sequencing and Real-Time PCR" (Raghavachari, & Garcia-Reyero, 2018).

A few of the methods has its own drawbacks such as relatively laborious for Northern blotting, and high cost for EST and other tag-based method. (Raghavachari, & Garcia-Reyero, 2018). Real-Time PCR was designed to quantify equilibrium-state mRNA levels by performing quantitative PCR (qPCR) on the cDNA conversion of RNA using RT-PCR. Because of its precision and sensitivity, qPCR is benchmark in nucleic acid measurement (Giulietti *et al*., 2001). Transcriptome research now relies heavily on microarray and RNA-seq. These techniques enable researchers to examine the expression of many genes at the same time while focusing on physiological equivalency (Raghavachari, & Garcia-Reyero, 2018). Quantitative real-time PCR or pathway focused gene expression analysis employing PCR arrays can be utilized to analyses a small selection of gene transcripts. DNA microarray and RNA sequencing (RNA-seq) are often employed to study the genome-wide impact of various environments (Raghavachari, & Garcia-Reyero, 2018).

2.5 CYTOCHROME P450

There are presently more than 78 members of the P450 gene superfamily, which are split into 14 families. The superfamily has experienced varied evolution, with its ancestral genes likely dating back over 2 billion years. The "flora and fauna conflict" appears to have caused the recent "bloom" of the novel P450 gene, mainly in the I1 family, over the last 800 million years. By comparing various phyla's responses to the class of producers, the process of P450 gene regulation (induction) by the class of producers may be better understood (Nebert et al., 1989). Cytochrome P450s (CYP450s) are present in a variety of living forms, from prokaryotes to eukaryotes, but their number has surged in plants.

Plant P450s are by far the biggest family of enzymes involved in metabolism, and a growing body of data supports their role in all aspects of life (Li & Wei, 2020) at approximately 286 different types were found in *Arabidopsis thaliana*. Plant P450s are polyphyletic, with a single significant clade, known as the A-type P450s, representing plant-specific enzymes involved mostly in natural product biosynthesis (Werck-Reichhart & Feyereisen, 2000). They're heme-containing enzymes that catalyse a variety of chemical processes (Werck-Reichhart & Feyereisen, 2000) such as the mixed-function oxidation of endogenous and xenobiotic substrates (Isin & Guengerich, 2008). P450s are involved in the biosynthesis of a various levels of plants secondary metabolites. Allene oxide synthase (AOS) is a P450 enzyme that is involved in the production of oxylipins, which are bioactive chemicals participating in signaling and safeguarding in higher plants (Li *et al.,* 2008).

2.6 NUCLEAR TRANSCRIPTION FACTOR Y SUBUNIT C

Nuclear Factor Y (NF-Y) is a ubiquitous, complex, heterotrimeric transcription factor also known as heme activator protein (HAP) in yeast or CCAAT-binding factor (CBF) (Xu *et al.,* 2014; Maheshwari *et al.,* 2019). The evolutionarily conserved subunits of NF-YA, NF-YB, and NF-YC constitute the heterotrimer NF-Y (Gusmaroli *et al.*, 2002; Xu *et al.*, 2014; Maheshwari *et al.*, 2019; Nardone *et al.*, 2020; Peter *et al.*, 2020). NF-Ys control gene expression by acting as either an activator or a repressor (Li *et al*., 2011; Leyva-Gonzalez *et al.,* 2012; Hou *et al.* 2014). NF-Y subunits are discovered to perform a regulatory role at both the transcriptional and post-transcriptional stages (Li *et al.,* 2008). The NF-Y complex also interacts with other transcription factors to regulate gene expression (Liu & Howell, 2010). NF-Y subunits appear to be essential regulators of abiotic stress responses, according to growing data (Xu *et al.,* 2014). Overexpression of NF-Y in plants improved drought tolerance (Hackenberg *et al.,* 2012), salt tolerance (Li *et al.,* 2016), osmotic tolerance (Yang *et al.,* 2016), and other stressors tolerance (Sun *et al.,* 2016).

NF-YB and NF-YC belong to the class of histone fold motif (HFM) proteins containing a conserved protein-protein and DNA-binding interaction module that form a tight dimer (Gusmaroli *et al.,* 2002; Nardone *et al.,* 2020). The transcript level of AtNF-YC2 was strongly stimulated by light, oxidative, heat, cold, and drought stress in the NF-YC subfamily (Li *et al.,* 2013). Overexpression of PwHAP5 (a conifer homolog of Arabidopsis NF-YC2) partially alleviated NF-enhanced Y-C2's susceptibility to salt, drought, and ABA treatments (Combier *et al.,* 2006). Further, incorporation of several NF-YB and NF-YC genes improved drought stress tolerance in diverse plants like Arabidopsis, maize, poplar and rice.

3.0 MATERIALS AND METHODS

3.1 TOTAL RNA EXTRACTION

The RNA extraction methods were carried out based on the methods proposed by Untergasser, (2008) with slight adaptations. Approximately 1.2 g sago palm leaf tissue was cut in small pieces and grinded in liquid nitrogen pre-cooled mortar. The sample was then transferred to Nalgene tube. A volume of 15 mL CTAB extraction buffer (2 % CTAB, 1.4 M NaCl, 0.5 M EDTA (pH 8.0), 1 M TRIS (pH 8.0), 2% PVP40 with 1% beta-mercaptoethanol was added and incubated for 5 minutes. A volume of 15 mL chloroform was added and incubated for 5 minutes. The sample was then centrifuged at 13,200 rpm at 4 °C for 5 min. Approximately 10 to 13 mL of the upper aqueous phase was transferred to a new Nalgene tube. Chloroform was then added with equal volume of the upper aqueous phase taken and incubated for 5 minutes. The sample was then centrifuged at 13,200 rpm at 4 °C for 5 min.

A volume of 10 mL of the upper aqueous phase was transferred into a new Nalgene tube and ice-cold isopropanol was added in equal volume then incubated for 10 minutes. The sample was then centrifuged at 13,200 rpm at 4 °C for 15 min. The supernatant was discarded and washed with 1 mL of ice-cold 70% ethanol. The sample was transferred into 1.5 mL centrifuge tube. The sample was then centrifuged at 13,200 rpm at 4 °C for 2 min. The supernatant was discarded and washed with 1 mL of ice-cold 70% ethanol. The sample was then centrifuged at 13,200 rpm at 4 °C for 2 min. The supernatant was discarded and dissolve in 40mM Tris-HCL buffer, $MgSO_4$, $CaCl_2$. A volume of 2 µl RQ1 RNase free DNase (Promega) was added into the sample then incubated for 30 minutes. A volume of 100 µl EDTA (pH 8.4) was added. The sample was then centrifuged at 13,200 rpm at 4 °C for 15 min.

The samples were transferred into 2 new 1.5 mL centrifuge tubes at volume of 540 µl and was added with equal volume of ice-cold isopropanol. The samples were incubated 5 minutes on room temperature and 5 minutes on ice. The sample was then centrifuged at 13,200 rpm at 4 °C for 2 min. The supernatant was discarded and washed with 1 mL of ice-cold 70% ethanol. The sample is then centrifuged at 13,200 rpm at 4 °C for 2 min. The supernatant was discarded and washed with 1 mL of ice-cold 70% ethanol. The supernatant was discarded and was left air dry for 10 minutes. A volume of 100 µl TE buffer was added into the tubes for RNA storage. The RNA sample was then stored at -80 °C until use.

3.2 FIRST CDNA SYNTHESIS USING RT-PCR

A volume 20 µl reaction will be prepared by adding the following reagents in the order listed.

Table 1. Reverse Transcription Reaction Component list.

Component	Amount
Reverse Transcription 10X Buffer	2 µl
Total RNA sample	10 µl
25X dNTP Mixture, 100 mM	0.8 µl
MultiScribe™ Reverse Transcriptase	1 µl
Anchored Oligo$(dT)_{15}$ Primer	2 µl
Nuclease-Free Water to a final volume of	20 µl

The reaction will be incubated at 25 °C for 10 minutes and 37 °C for 2 hours. The sample will be heated at 85 °C for 5 minutes, then will be incubated at 4 °C for 5 minutes.

3.3 PRIMER DESIGN

The gene sequences for Nuclear transcription factor Y subunit C-1 and Cytochrome P450 are provided by Prof. Hasnain (Refer **Appendix A**). The gene's sequence most conserved region with the length of 18 to 24 bp, 40-50% G/C content, start and end with 1-2 G/C pairs are selected. The primers will be designed using Primer3web online software. Both primers will be analyzed using PCR primer stats software (Stothard, 2000) to verify the validity and check for PCR sustainability tests. The primer that will be designed should have annealing temperature (T_m) of 50-60 °C and primer pairs should have a T_m within 5 °C of each other.

3.4 PCR AMPLIFICATION

A volume of 15 µl PCR amplification mix were prepared by combining the following reagents. The PCR will be carried out in a 15 µl reaction per tube by preparing master mix. PCR will be performed for 35 cycles of denaturation step at 95 °C, annealing at 50 °C temperatures for 30 seconds and extension at 72 °C for 30 seconds.

Table 2. PCR amplification mix list.

Component	Amount	x3
First-strand cDNA reaction	1.00 µl	-
5X Green GoTaq® Reaction buffer	3.00 µl	9.00 µl
dNTP Mixture, 10mM	0.30 µl	0.90 µl
MgCl2, 25mM*	0.72 µl	2.16 µl
Forward primer	0.45 µl	1.35 µl
Reverse primer	0.45 µl	1.35 µl
GoTaq® DNA Polymerase	1.13 µl	3.39 µl
Nuclease-Free Water to a final volume of	15.00 µl	45.00 µl