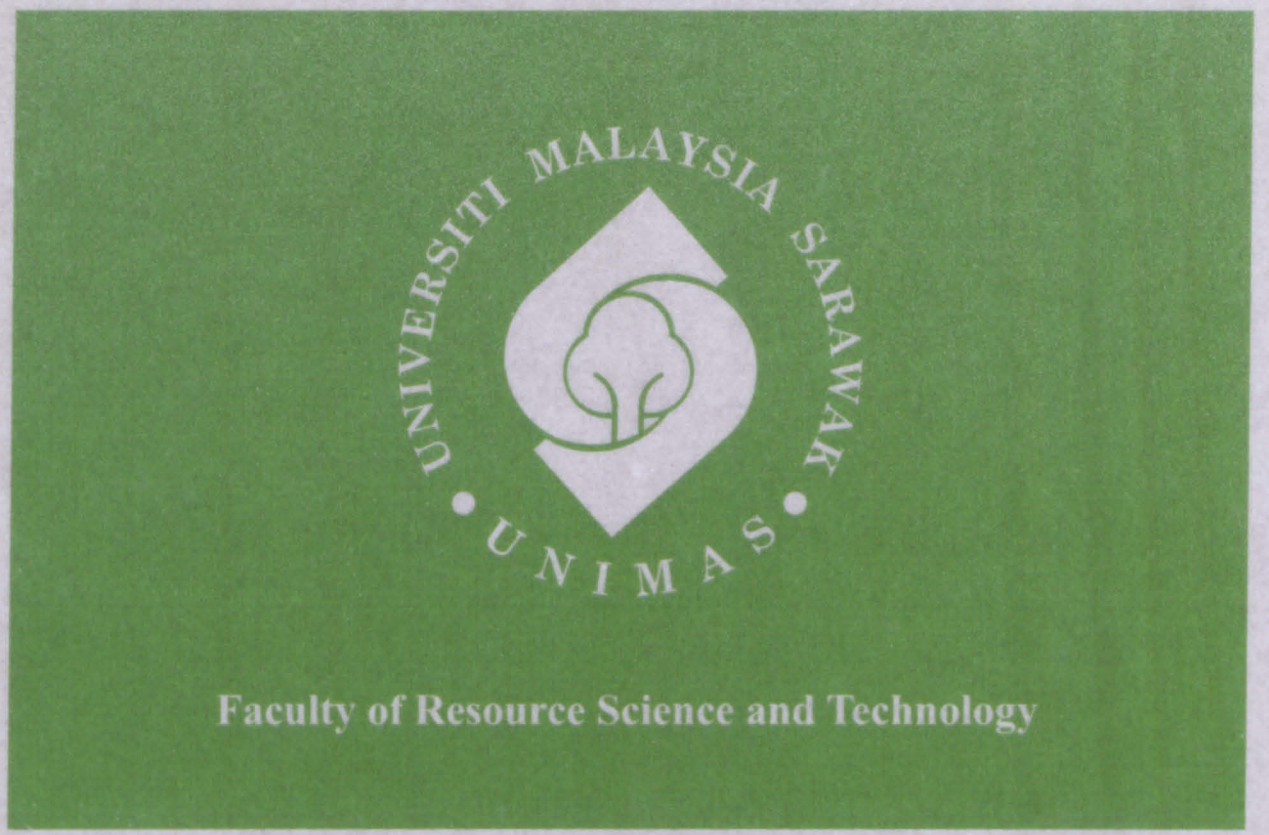

ISOLATION AND IDENTIFICATON OF XYLOSE UTILIZING YEASTS FROM FRUIT SURFACES

Nurhayati Binti Mawi
(57435)

Bachelor of Science with Honors
(Resource Biotechnology)
2019

Borang Pengesahan
Laporan Projek Tahun Akhir (STF3015)

Fakulti Sains dan Teknologi Sumber
Universiti Malaysia Sarawak

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Date submitted

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Name of the student (Matric No.)

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Received for examination by: _____
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ISOLATION AND IDENTIFICATON OF XYLOSE UTILIZING YEASTS FROM FRUIT SURFACES

NURHAYATI BINTI MAWI

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This project is submitted in partial fulfilment of requirements for the degree of

Bachelor of Science with Honors

(Resource Biotechnology)

Supervisor: Dr Micky Vincent

Faculty of Resource Science and Technology

UNIVERSITI MALAYSIA SARAWAK

2019

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Isolation And Identificaton Of Xylose Utilizing Yeasts From Fruit Surfaces

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ABSTRACT

The conversion of pentoses into ethanol have become a challange and could increase the demand of supply of second-generation biofuels. This study were attempted to isolate naturally occuring yeasts from different kind of local fruits in Malaysia at different places and determine the yeasts strain that can utilize xylose. Isolation processes were conducted with medium that containing only xylose as the carbon source. Four yeast strains with the ability to utilize xylose were obtained from papaya in Matang, papaya in Samarahan, *longan* in Samarahan and banana in Matang. The strains selected were characterized by morphological and they were identified by homology analysis of 26-S ribosomal DNA gene sequences. The identities of four yeast strains are identified as *Debaryomyces nepalensis, Geotrichum candidum, Trichsoporon asahii* and *Candida tropicalis.* All of these strains can utilize xylose as carbon source to obtain ethanol production except *Debaryomyces nepalensis* which produce xylitol as major metabolites.

Keywords: Ethanol, yeast, local fruits, xylose

ABSTRAK

Penukaran pentoses ke etanol telah menjadi satu cabaran dan boleh meningkatkan permintaan bekalan biofuel generasi kedua. Kajian ini telah melakukan kaedah mengasingkan yis daripada pelbagai jenis buah tempatan Malaysia yang terdapat di lokasi yang berbeza dan ingin mengenalpasti jenis yis yang boleh menggunakan xylose. Proses pengasingan telah dijalankan dengan menggunakan media xylose sebagai karbon utama. Empat yis strain dengan kebolehan menggunakan xylose telah diperoleh daripada betik di Matang, betik di Samarahan, longan di Samarahan dan pisang di Matang. Strain yang dipilih telah dicirikan secara morfologi dan dikenalpasti secara analisis homologi menggunakan 26-S ribosom DNA jujukan gen. Identiti yis strain telah dikenalpasti sebagai Debaryomyces nepalensis, Geotrichum candidum, Trichsoporon asahii dan *Candida tropicalis. Semua strain boleh menggunakan xylose sebagai karbon utama untuk mendapatkan produksi etanol kecuali Debaryomyces nepalensis yang menghasilkan xylitol sebagai metabolit yang utama.*

Kata kunci: Etanol, yis, buah tempatan, xylose

TABLE OF CONTENT

LIST OF ABBREVIATIONS

S. cerevisiae	*Saccharomyces cerevisiae*
w/v	weight to volume
psi	pound force per square inch
LSU	large subunit
PCR	polymerase chain reaction
TAE	tris base, acetic acid and EDTA
NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment Search Tool
PPP	pentose phosphate pathway
XR	xylose reductase
XDH	xylitol dehydrogenase
pmol	picomole
LB	luria broth
PCR	Polymerase Chain Reaction
$MgCl_2$	Magnesium chloride
EDTA	Ethylenediaminetetraacetic acid

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CHAPTER 1

INTRODUCTION

Over the past decade the production of bioethanol and biodiesel has been investigated worldwide and the methods of production proved successful in USA and Brazil (Linda *et al.*, 2008). Currently, the US is one of the largest ethanol producers in the world. Its production has increased steadily during the last three decades. The increasing efficiency of Brazilian ethanol plants has been apparent due to the many technological contributions (Basso *et al.*, 2008).

Bioethanol obtained from lignocellulosic biomass such as agricultural residues has received attentions as an option to petroleum-based fuels because of global warming and the necessity to decrease the production of carbon dioxide (Farrell *et al.*, 2006). Lignocellulosic materials are renewable, low cost and are plentifully available. It includes crop residues, wood, grasses, sawdust and chips. Extensive research has been carried out on ethanol production from lignocellulosics in the past two decades (Cadoche & Lopez, 1989). Hence bioethanol production could be a way to the effective utilization of agricultural wastes.

Lignocellulose have a great biotechnology value and contains the most part of the plant dry weight which consists of 35-50% of cellulose, 20-35% of hemicellulose and 10-25% of lignin. It also identified as the largest origin of renewable organic material that highly produced through agricultural (Wiselogel, 1996). The cellulose and hemicellulose are the polysaccharide components of the lignocellulosic biomass that can processed through a hydrolysis reaction which produce hexoses and pentoses

especially glucose, and xylose respectively which than can be used as substrates in the fermentation processes.

The sugars present in lignocellulose hydrolysates must be completely converted to ethanol to improve economics for ethanol production from lignocellulose. In this study, xylose is being used as carbon source. Xylose is a pentose with a molecular formula of $C_5H_{10}O_5$. The discovery of D-xylose fermenting yeasts began in 1980s (Jeffries, 1994) and there was a studied of species of nonconventional yeasts for the ethanol production such as *Pachysolen tannaphilus, Kluyveromyces cellobiovorus and Scheffersomyces (Candida) shehatae.* There are many types of *Spathaspora* species and one of them is *Sp. passalidarum* which can ferment D-xylose and produce the highest ethanol production at anaerobic condition (oxygen-limited), have rapid D-xylose consumption and able to ferment glucose, xylose and cellobiose simultaneously. Hence, this species is a good candidate for the fermentation of producing sugars from lignocellulosic biomass (Cadete & Rosa, 2018). While, the species of *Saccharomyces cerevisiae* is the most widely employed yeast species in fermentative process. However, pentose sugars, such as xylose that is not utilized by *Saccharomyces cerevisiae* (Kim *et al.,* 2013).

Hexose sugar can be ferment to produce ethanol and it is the primary source of renewable liquid fuels. Yeast strain such as *S. cerevisiae* can only convert hexose sugars to ethanol, but not pentose sugars (Chu *et al.,* 2007). If the pentoses were also transformed, ethanol yield could be increased significantly. Thus, in this project, variety of fruits have been collected to identify the yeast strain that can ferment xylose.

The objectives of this project are:

1. To isolate xylose fermenting yeasts from local fruits that can utilize xylose.
2. To identify the yeast species through sequencing analysis.
3. To study more details about the yeast species via literature search.

CHAPTER 2

LITERATURE REVIEWS

2.1 Bioethanol

Ethanol is a colorless volatile flammable liquid which is biodegradable, low toxicity and causes little environment pollution. When ethanol is burnt completely and react with oxygen, it will produce carbon dioxide and water. It is a high octane fuel and has displaced leads as an octane enhancer in petrol (Altintas *et al.,* 2002). Other than that, it is a renewable source of energy and can reduce the demand on fossil fuels. Bioethanol produced by microbial fermentation that is used as a transportation biofuel. It is produced through distillation of the ethanolic wash emanating from fermentation of biomass-derived sugars and can be utilized as a liquid fuel in internal combustion engines (Walker, 2011).

Bioethanol production are divided into 3 generations which are first, second and third generation. First generation bioethanol uses the food feedstock that contain starch such as corn, wheat and potato as well as that contain sucrose containing feedstock such as sugarcane and sugar beet. Second generation bioethanol is mainly produced from lignocellulosic biomass and can use industrial byproducts such as whey (Balat *et al.,* 2008) or the crude glycerol as the feedstock. The last generation is the third generation which it is based on the cultivation of microalgae or unicellular microorganisms that can be obtained from eukaryotes and prokaryotes (Koller *et al.,* 2012). Bioethanol from the algal biomass can be acquired from the process of starch fermentation under anaerobic conditions.

The process that involved to produce the ethanol form lignocellulosic biomass consists of three main steps which are pretreatment, hydrolysis and fermentation. Pretreatment methods are applied to break the cell wall and expose the cellulose and hemicellulose fibers for the next process. Then, by using acid or enzymatic hydrolysis, the cellulose and hemicellulose are broken down into small monomers (Sun & Cheng, 2002). Finally, the conversion of the monomeric sugars into alcohol will be undergo the process of fermentation using yeast or bacteria (Pessani *et al.,* 2011).

Four process configuration for the ethanol production such as separate hydrolysis & Fermentation (SHF), Simultaneous Saccharification & Fermentation (SSF), Simultaneous Saccharification & Co-Fermentation (SSCF) and Consolidated Bioprocessing (CBP) are possible based on the degree where the above mentioned steps are bring together as shown in Figure 2.

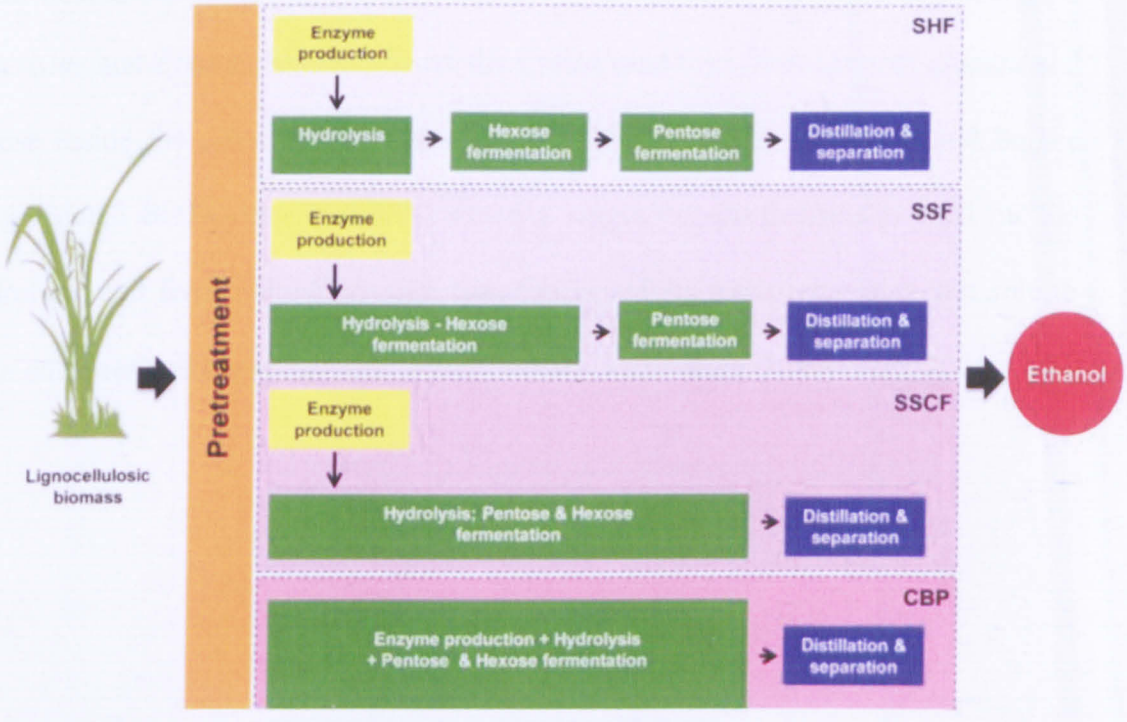

Figure 1: Bioethanol lignocellulosic biomass process configurations. (i) Separate Hydrolysis & Fermentation (SHF), (ii) Simultaneous Saccharification & Fermentation (SSF), (iii) Simultaneous Saccharification & Co-Fermentation (SSCF), (iv) Consolidated Bioprocessing (CBP) (Adapted from Hamelinck *et al.,* 2005).

According to Lynd *et al.,* (2002), the enzyme production, hydrolysis of biomass, the fermentation of hexose and pentose are performed in separate reactors for the process of separate hydrolysis and fermentation (SHF). This process occur at optimum temperature but the accumulation of glucose and cellobiose during the hydrolysis will prevent the cellulases and decrease the efficiencies (Margeot *et al.,* 2009). This limitation have led to the development of Simultaneous Saccharification & Fermentation (SSF) process (Wright *et al.,* 1988). In this process, cellulose hydrolysis and hexose fermentation happen in the same reactor thus relieving the end product inhibition on the cellulases as the sugars are consumed by the fermenting microorganisms (Hahn-Hagerdal *et al.,* 2006). Unfortunately, SSF process have some limitation where most microorganisms used for fermentation of glucose is unable to utilize xylose (Lin & Tanaka, 2006).

Next, in Simultaneous Saccharification and Co-fermentation (SSCF), *Saccharomyces cerevisiae* and *Zymomonas mobilis* are the strains used to co-ferment both glucose and xylose inside the same reactor (Dien *et al.,* 2003). Another method would be the Consolidated BioProcessing (CBP), where a single microorganisms is used for the hydrolysis and fermentation process. But, the use of the microorganisms that able to form enzymes and sugars are still in early development stage (Lynd *et al.,* 2005).

2.2 Xylose

Xylose, as a major component of plant xylan polymers, is one of the most plentiful carbohydrates on earth after glucose (Broekaert *et al.,* 2011). Xylose catabolism has great commercial significance because fermentation of wood waste and fibre pulp to ethanol permits recovery of energy that would otherwise be lost to industries generating waste. Many *S. cerevisiae* strains have been genetically modified for the conversion of xylose into ethanol by improving metabolic flux for xylose utilization (Cai *et al.,* 2012). The initial steps of xylose metabolism follow two pathways. In bacteria, xylose isomerase catalyses xylose conversion to xylulose, which is easily converted to xylulose-5-phosphate then via the pentose phosphate pathway (PPP) to fructose-6-phosphate, then via glycolysis to pyruvate. Xylose isomerase also reversibly catalyses the conversion of glucose to fructose and is found in cells of some but not all vertebrate tissues (Demetrakopolous & Amos, 1978). The second pathway occurs in yeasts and some fungi which lack xylose isomerase, and includes an extra step, the conversion of xylose to xylitol by xylose reductase (XR). Xylitol is then converted to xylulose by xylitol dehydrogenase (XDH), and the same pathway to pyruvate followed. The structure of D-xylose compared to that of D-glucose is shown in Figure 1 and the metabolic pathway for xylose catabolism is shown in Figure 2.

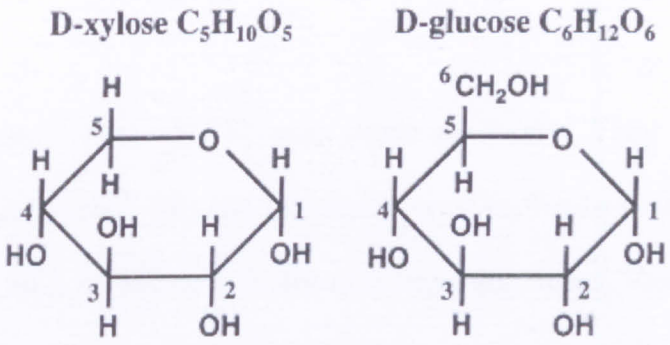

Figure 2: Structure of D-xylose compared to that of D-glucose (Adapted from Jackson & Susan, 2002).

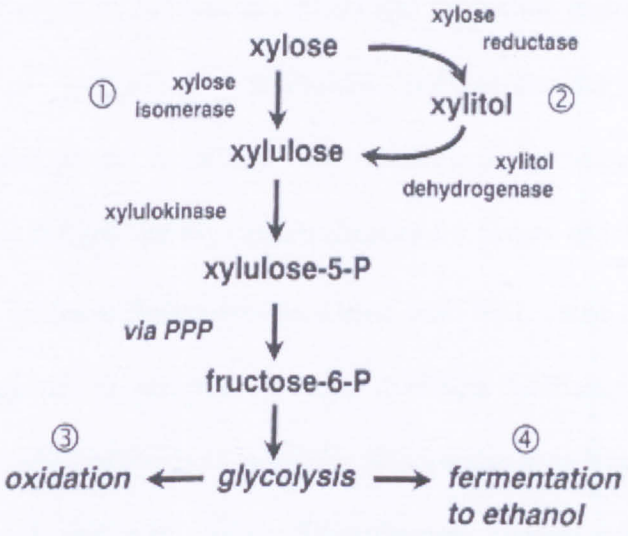

Figure 3: Metabolic pathway for xylose catabolism (Adapted from Jackson & Susan, 2002).

2.3 Yeasts

Yeasts are microscopic fungi that appears single oval cells. They are capable of converting sugar into alcohol. The most common yeast is *Saccharomyces cerevisiae*, the microorganism used in baking industry as a leavening agent. Van Leeuwenhoek was the first scientist that observed microbial cells and documented the description of microscobic display of the yeast under the microscope in 1680. The best source of yeast is considered to be citrus juice (Arias *et al.*, 2002) and sugarcane juice (Ceccato *et al.*, 2004). The ability of yeasts to metabolize polysaccharides and complex carbohydrates is restricted to relatively few species. *S. cerevisiae* is not able to hydrolyze starch. Hydrocarbons are the carbon sources for yeasts and there are several species that can grow on these hydrocarbons. Other than that, yeast cells finely tune their growth and behavior in accordance with available nutrients. In nutritional environment, yeast can adjust their growth rate by altering the length of their cell cycle at least a 10-fold range (Brauer *et al.*, 2008). The schematic representation of different budding patterns in yeast is shown in Figure 3.

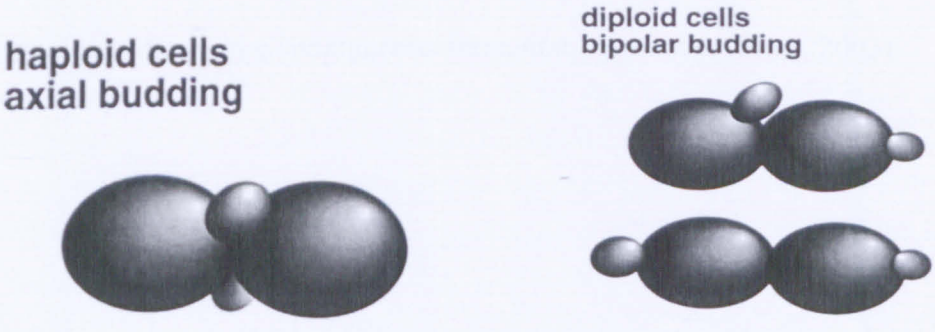

Figure 4: Schematic representation of different budding patterns in yeast (Adapted from Cabib *et al.*, 2002).

2.5 Local Fruits

Fruits are sweet and fleshy product of a tree which could provide variety, taste, and aesthetic appeal and at the same time able to meet certain essential nutritional requirements have been a part of human diet (Wills *et al.,* 1998). Fruits, like other horticultural commodities, can be classified according to many factors such as its growing regions and environmental conditions (Kader & Barrett, 2004), respiration rate, ethylene production rates and their respiratory behavior during ripening (Kader, 1992). In June 2003, FAMA (Federal Agricultural Marketing Authority) launched the Malaysia's best branding programme for five priority fruits, namely; carambola, mango, pineapple, papaya and watermelon. These five fruits were selected because Malaysia has the comparative advantage in producing them (Wan Ibrahim, 2003). During that time, Malaysia was the biggest exporter of papaya and watermelon to Hong Kong and Taiwan. For this experiment, six kind of fruits were used such as banana, papaya, *limau kasturi, jambu, longan* and lime at 3 different places which are at Matang, Samarahan and Sibu. The isolation of the yeast strain was done by streaking on the fruit surface as the diverse microbial flora on the fruit surfaces may play an important role during the spontaneous fermentation process (Fleet, 2003).

Figure 5: Local fruits in Malaysia.

2.6 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is a technology that is used to amplify a single DNA strand and generate them to millions of copies of a particular DNA sequence (Erlich, 1989). There are three main cycles that are repeated in this procedure. They are the denaturation stage, primer annealing and final extension cycles. The denaturation stage is to break the hydrogen bonds that allows the DNA strands to separate, creating a single stranded DNA when the DNA is heated to 95 °C. The second cycle in the PCR technology is primer annealing where in this cycle the mixture are cooled in the range of 45-72 °C. Lastly, in the extension stage, the reaction is heated to 70 °C. This is the optimal temperature for DNA polymerase to extend the primers adding nucleotides onto the primer by using the target DNA as a template (Pelt-verkul *et al.,* 2008). In this experiment, 26S ribosomal rDNA region will be used as target. The D1/D2 domain is a 600 bp nucleotide domain at the 5' end of a large subunit of (26S) rDNA. The advantage of sequencing the domain D1/D2 region subunit 26S rDNA, is identification of fungi species and also permits phylogenetic analysis.

There are many advantages and limitations when using PCR machine. First, the technique is simple and easily to understand and can produces results rapidly. It is able to produce billions copies of specific product for sequencing, cloning and analysis. This technology can improves detection, thus facilitate the diagnosis some of the viruses due to the viral infections (Speers, 2006). Eventhough PCR is a valuable method, there are some limitations as contamination of sample that can lead to produce a misleading results (Smith & Osborn, 2009) and DNA polymerase is susceptible to errors that will cause mutations of the fragments. Thus, the future of PCR is encouraging various