Faculty of Resource Science and Technology

ISOLATION AND IDENTIFICATION OF YEAST SPECIES INVOLVED IN COCOA SHALLOW BOX FERMENTATION

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A final report submitted in fulfillment of the requirement for the Degree of Bachelor of Science with Honours

(Plant Science Resource and Management)

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Isolation and Identification of Yeast Species Involved in Cocoa Shallow Box Fermentation

ABSTRACT

Research was performed to correlate sugar content, pH of pulps and nibs and temperature with the yeast species involved in cocoa fermentation process in Perak and Samarahan at different fermentation periods. The pH of pulps and nibs had an inversely proportional relationship where a decrement in the pH of pulps would cause an increment in the pH of nibs. The temperature at both areas had shown the same pattern where the highest temperature was stated at the 72 hours fermentation period and fluctuated after that as a result of turning of cocoa bean process. The sugar content of the Perak samples was initially high but decreased to 5.1 Brix at the end of fermentation due to microorganism reaction. The sugar content of the Samarahan samples was initially low but increased to 6.5 Brix at the end of cocoa fermentation process due to pectinolytic activity. 21 yeast isolates were isolated from both places. Four yeast isolates were managed to be sequenced and two species were identified as *Candida krusei* and *Pichia anomala.* Further identification using PCR molecular technique need to be carried out to identify and find out more about different types of yeast species involved at different cocoa fermentation periods.

Keywords: cocoa fermentation, yeast species identification, molecular technique, PCR

ABSTRAK

Kajian telah dilaksanakan untuk mengenalpasti hubungan antara kandungan gula, pH isi koko dan biji koko, suhu dan jenis spesis yis yang terlibat dalam fermentasi koko di Perak dan Samarahan pada waktu fermentasi yang berbeza. pH isi koko didapati mempengaruhi pH biji koko secara kadar songsang. Kenaikan pada pH isi koko akan menurunkan pH pada biji koko. Kedua-dua tempat mencatatkan suhu tertinggi pada 72 jam semasa fermentasi koko dan penurunan suhu berlaku selapas 72 jam disebabkan oleh pembalikan biji koko. Kandungan gula di Perak adalah tinggi pada mulanya tetapi berkurangan pada akhir proses fermentasi. Manakala, kandungan gula di Samarahan adalah rendah pada mulanya tetapi bertambah pada akhir proses fermentasi. 21 jenis spesis yis telah diasingkan dari kedua-dua lokasi tersebut. Empat spesis yis telah dihantar untuk sekuensing DNA. Walau bagaimanpun, dua daripada empat jenis spesis yis telah berjaya dikenalpasti sebagai Candida krusei dan Pichia anomala. Kajian menggunakan teknik molekular PCR haruslah dilaksanakan pada masa depan untuk mengenalpastikan pelbagai spesis yis yang terlibat dalam fermentasi koko.

Kata Kunci: fermentasi koko, mengenalpastikan spesis yis, teknik molekular, PCR

APPROVAL SHEET

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I Ooi Teng Sin, 24810, Faculty of Resource Science and Technology (PLEASE INDICATE STUDENT'S NAME, MATRIC NO. AND FACULTY) hereby declare that the work entitled, "Isolation and identification of yeast species involved in cocoa shallow box fermentation" is my original work. I have not copied from any other students' work or from any other sources except where due reference or acknowledgement is made explicitly in the text, nor has any part been written for me by another person.

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CHAPTER 1

INTRODUCTION

1.1 Background

Fermentation in cocoa is an important process as no chocolate flavour in cocoa bean can be developed without a good fermentation process (Lopez, 1986). There are two phases of cocoa fermentation process which are known as external fermentation and internal fermentation (Lopez, 1986). External fermentation is an exothermic process that involves microbial activities in the mucilaginous pulp (Malaysia Cocoa Board, 2009). Often, the process of cocoa bean fermentation involves yeasts, lactic acid bacteria and acetic acid bacteria (Heide et al., 2009). Of the microorganisms involve in cocoa bean fermentation, yeast plays a fundamental role as a pioneer organism. Their initial colonization and biological activity sets a precedence to the quality of the final product in cocoa bean fermentation (Malaysia Cocoa Board, 2009). Among the common yeast using for the food industries are *Saccharomyces cerevisiae*, *Saccharomyces bayanus*, or *Saccharomyces pastorianus* (Graham, 2006).

External fermentation in cocoa beans begins at the pulp that cover the cocoa bean's after exposure to air. Microbes will utilise materials in sugar rich acidic pulp and initiate the fermentation process (Lopez, 1986). The activity in this external fermentation will produce alcohol, acids and energy which enable biochemical reactions inside the cocoa bean (Lopez, 1986).

Internal fermentation is where the biochemical reactions occur. This happens inside the cotyledon of the bean. It is started with ethanol and acetic acid that are produced in external fermentation diffuse into the cotyledon (Lopez, 1986). Compounds which are

precursors for chocolate flavour are formed in the bean during the internal fermentation and will react with each other during the roasting process (Lopez, 1986). Thus, the microbial activity during the external fermentation influences the internal fermentation which contributes to chocolate flavour and quality.

The common practice in Malaysia for cocoa bean preparation is natural cocoa fermentation and drying of the bean after harvesting. There are several methods in carrying cocoa fermentation. It can be done in baskets, heap covered with banana leaves and in boxes. The basic fermentation methods are heap and box methods which involve heaping fresh beans to allow proliferation of microorganism (Malaysia Cocoa Board, 2009). Heap fermentation is the most common that practiced in West African countries. It is the simplest method and does not require fermentation boxes (Malaysia Cocoa Board, 2009). Malaysia Cocoa Board is practising box fermentation which covered with gunny sack to reduce heat loss. When turning the beans, the beans are moved to a subsequent lower level. Good fermentation happens when the temperature of the fermenting mass increases from room temperature to about 50˚C and decrease at the end of the process. Fermentation is terminated when the cocoa beans are dried and separated from each other. The temperature is also decreases rapidly. The flavour of the cocoa bean produced has less acetic acid taste (Malaysia Cocoa Board, 2009).

1.2 Problem statement

Cocoa beans produced in Malaysia are characterized by high acidity, low chocolate flavour, and the presence of undesirable flavours (Suzannah, 2004). At present, the fermentation of cocoa bean is based on natural condition. However, the microbial diversity involve in cocoa fermentation has been shown to be different with location and process parameters such as temperature, pH, nutrient availability as well as metabolic activities of microbial (Heide et al., 2009). Thus, the quality of the cocoa bean produced is not standardized in different area. The predominance and importance of yeast in cocoa fermentation are well recognized (Graham & Hugh, 2007). There is still little understanding gained of how individual species or strains influence bean quality and chocolate flavor (Graham & Hugh, 2007). Although Malaysia is a cocoa producing country, there is not much information on yeast involvement in cocoa fermentation has been published. There are many unknown yeast species has not yet been identified and are expected to present in Malaysia cocoa. Yeast identification is useful to improve the quality of cocoa bean in Malaysia which can later generate higher income for the country.

1.3 Objective

- To determine the relationship of pH, temperature and sugar content and yeast species during the cocoa fermentation.
- To identify yeast species in shallow box cocoa bean fermentation at 0, 6, 24, 48, 72, 96 and 120 hours.
- To compare yeast species present during fermentation of cocoa bean from different location.

CHAPTER 2

LITERATURE REVIEW

2.1 *Theobroma cacao*

Theobroma cacao L. is commonly known as cocoa, and is a member from the family Sterculiaceae. Cocoa is also known as the "Food of the Gods", native to Central America and is grown largely in Brazil, West Indies and Sri Lanka at the early half of 20th century. Cocoa beans are the crucial material for confectioneries, beverages, chocolate and other food products (TamilNadu Agricultural University (TNAU), 2008). According to Darin (2011), Cortez is the person that discovered cocoa in 1519 and he realized it was honoured by Aztecs and drunk only by the Emperor in Montezuma. In 1828, a Dutch manufacturer person named van Houten found a way to make cocoa more appetizing. Today, cocoa plays a vital role in a wide range of foods and high quality cocoa beans offers confectionery manufacturers a world of opportunity as they can make quality products out of it (Darin, 2011).

There is a difference in terms of cacao and cocoa. Cacao is the botanical name which refers to the tree, the pods and the unfermented beans from the pods. Cocoa is referring to the manufactured product include the powder sold for beverage manufacturing usage. Recently Cocoa is also often being used to describe the fermented beans in bulk (Darin, 2011).

Cocoa pulp has 80-90% water, 10-15% sugar, 0.4-0.8% citric acid, 1% pectin and other constituents (Zalina, 2011). Malaysian cocoa beans have been characterized by chocolate manufacturers as lacking of strong cocoa flavour (Ramli et al., 2005). The cocoa bean qualities preferred by cocoa and chocolate factories are classified under four main criteria which are flavour, cocoa butter hardness, purity and yield (Ramli et al., 2005). Flavour aspect is the most important which is developed through two important stages known

as cocoa bean fermentation and roasting. The fermentation and drying process are referred as "curing" process (Lopez, 1986). The unfermented cocoa seed from the pod does not develop any chocolate flavour. Fermentation causes the biochemical changes within the cacao bean which responsible for its flavour (Lopez, 1986).

2.2 Cocoa fermentation

Fermentation is divided into two phases, which are external fermentation and internal fermentation. The commencement of external fermentation process requires a minimum of 100 kg cocoa beans (Malaysia Cocoa Board, 2009). Small quantity of cocoa beans will prevent heat formation due to large surface area to volume ratio (Malaysia Cocoa Board, 2009). The fermentation process can take up to 7 days but it is highly depending on the variety of cocoa pods used, quantity of beans involved in fermentation, climate, pod ripeness (storage or no storage) and technique applied (Bariah, 2011).

2.2.1 External fermentation

External fermentation is where the microorganism metabolizes the sugar and pectin in pulp mucilage around the cocoa bean (Lopez, 1986). There are two phases involved in the external fermentation which are anaerobic and the aerobic phases (Lopez, 1986). The initial pH is said around 3.3 to 4.0. Yeast population is dominant at 24 to 36 hours after the fermentation starting and there are certain yeast species that are able to release enzyme pectinase to convert pectin in the pulp mucilage into simple sugar. Yeast uses sugar and acid citric at the pulp layer as a carbon source to generate energy and produce ethanol, carbon dioxide and acid acetic (Schwan et al., 1995). Sweating happens when the pulp collapse as a result of the microbes colonization. The sweating causes the pH to increase and the condition becomes aerobic (Lopez, 1986).

According to Kamariah (1987), yeast activity is inhibited when the pH, alcohol level and aeration conditions in the substrate increase. Another microbe present is the lactic acid bacteria which can convert sugar either into lactic acid or acid acetic (Bariah, 2011). Acid acetic bacteria start to grow when the aeration is good. The bacteria involve in the exothermic process where ethanol is being oxidized into acid acetic which causes the temperature of cocoa fermentation to increase and reach 50˚C (Schwan et al., 1995).

However, the increment of temperature which is more than 45˚C is not ideal for the growth of acid acetic bacteria (Lopez, 1986). The condition becomes more aerobic and suitable for the aerophyllic spore forming bacteria which dominates the environment until the end of fermentation (Lopez, 1986). When the condition is aerobic, acid acetic will be oxidized into carbon dioxide and water (Camu et al., 2007). A combination of ethanol and acid acetic will be absorbed into the cotyledon to induce biochemical reaction inside the cocoa bean which is vital in the formation of cocoa bean flavor precursor, aroma and color formation of the cocoa bean (Schwan & Wheals, 2004).

2.2.2 Internal fermentation

Internal fermentation is important in cocoa flavor formation. A lot of the cocoa flavor precursors are produced by the enzymatic mechanism. Internal fermentation is where the biochemical reaction happens inside the cotyledon of the bean. It is started with ethanol and acetic acid diffuse into the cotyledon. The accumulation of ethanol and acetic acid causes bean death. There are two phases of internal fermentation which are anaerobic hydrolytic phase and the oxidative condensation phase (Hii & Bakri, 2003).

Most flavor precursors are formed depending on the enzymatic mechanism during the hydrolytic phase (Lopez & Dimick, 1991). Hydrolytic phase is started by acidification.

Acidification happens when the acid acetic is absorbed into the bean cotyledon causes a decrement in the pH cotyledon from 6.8 to 4.5-5.0. The decrement in pH and temperature about to 45˚C in the environment activate the proteolitic activity at aspartate endoprotease and serine carboxy-(exo) peptidase as protease enzyme which acts at vicilin (7S)-class globulin (VCG). Aspartate endoprotease enzyme acts at residue acid amino which is hydrophobic. It produces short chain of hydrophobic peptide. After that, serine carboxy-(exo) peptidase synthesis at the short chain produced to separate residue acid amino which is hydrophobic at the carboxyl terminal (Voigt et al., 1994).

There is another important process takes place in the anaerobic hydrolytic phase where the anthocyanins are hydrolyzed by glycosidase (Wollgast & Anklam, 2000). Glycosidase is activated immediately after bean death and it hydrolyzes anthocynin pigments which are 3-β-D-galactosidylcyanidin and 3-α- L-aribinosidlycyanidin. Both pigments are responsible for the purple colour of cocoa beans. Both pigments are hydrolyzed into sugar and cynidin during the fermentation process (Lopez, 1986). This process decolorizes the purple colour which appears in the internal part of the cocoa bean (Lopez, 1986).

During the internal fermentation, the oxidative condensation phase happens when oxygen diffuses into the cotyledon activates the oxidase enzyme and allows the oxidation of polyphenols to occur (Lopez, 1986). The first stage happens is the formation of o-quinones which are active compounds capable of reacting with compounds formed at the next stage of internal fermentation. Quinones also polymerize when cathecol forms diphenyls and diphenyl-quinones. Aside from that, quinines also engage in oxidation-reduction reaction with compounds contain active hydrogen. They combine with amines, amino acids and thiols. The role of the oxidation of polyphenols is to reduce the bitterness and astringency of the

cocoa beans. This oxidases activity continues during drying process at sufficient moisture (Lopez, 1986).

2.3 Cocoa sweating

Cocoa sweating is the waste by-product which is pale yellowish liquid produced during cocoa fermentation. It is formed during the breakdown of mucilage pulp surrounding the fresh cocoa bean which is also caused by the pectolytic enzymes secreted by microorganisms involve in the fermentation process (Buamah et al., 1997). The sweating has a high level of sugar, pectin and volatile organic acids with pH 3.4-3.8. It is found to be ideal for alcoholic drinks such as wine and food items such as jams, syrup and etc (Buamah et al., 1997). The cocoa sweating has been used to produce wine (Jayeola & Lawal, 2008).

There is microbial succession in the fermentation process and yeast has been found dominate the fermentation for the first 24 hours (Schwan, 1998).

2.4 Drying of cocoa bean

Drying is done after the fermentation process (Malaysia Cocoa Board, 2009). Drying methods are usually sun drying, artificial or forced air drying depends on climatic conditions (Fagunwa et al., 2009). Sun drying is a simple, cheap, effective and economic method as it does not require expensive mechanical devices used in artificial dryers (Fagunwa et al., 2009). Sun drying is done by spreading bean on the ground. The thickness is one bean thickness to ensure better sunlight penetration. This process is carried out for 4 to 7 days depends on climate (Malaysia Cocoa Board, 2009). Artificial drying is carried out by air-ventilated oven. It takes shorter time for bean to be dried. Artificial drying is beneficial during rainy season (Malaysia Cocoa Board, 2009). The flavour and aroma of cocoa bean is better through artificial drying than sun drying. This is because the heat and temperature of artificial drying

can be controlled constantly and this allows cocoa bean to be dried evenly (Malaysia Cocoa Board 2009). But according to Fagunwa et. al., (2009) the continuous drying in the used of forced air at 60-70˚C causes the bean to have strong acidic flavour, weak chocolate flavour and other off-flavour.

2.5 Yeast species involved in cocoa fermentation according to region

The microbial diversity in cocoa fermentation is different according to location and process factors (Heide et al., 2009). The common microorganisms are yeast. A research on the assessment of the microbial community of cocoa bean heap fermentations in Ghana involved 91 yeast isolates which were detected by PCR fingerprinting with the primer M13 (Heide et al., 2009). They found 16 clusters of yeast which show a large degree of variability in strain variation. *Hanseniaspora opuntiae* was found preferably to grow at the earlier phase of fermentation which shows that its ability to tolerate to low pH (Heide et al., 2009).

Different yeast species have been shown present in cocoa bean fermentation in different cocoa producing regions such as Brazil, Ecuador, Ghana, Ivory Coast and Malaysia as shown in Table 1. By using denaturing gradient gel electrophoresis (DGGE) of 26S rRNA gene fragments obtained by PCR with universal eukaryotic primer, carried out by commercial system which are Dcode and CBS system, different species of yeasts were found (Zoi & Luc, 2011). Martelli and Dittmar (1960), reported that in the cocoa beans of the Forastero type from Brazil, only *Saccharomyces rosei, Hansenula anomala, Pichia fermentans* fermenting the sugars of cocoa mucilage pulp, while unknown species *Saccharomyces* as the agent responsible for the alcoholic phase of fermentation the cocoa.

Table 1. The yeast species found in different cocoa producing regions.

Cocoa producing region	Yeast species detected	Reference
Ghana	*Pichia kudriavzevii (Issatchenkia orientalis), Saccharomyces cerevisiae* and *Hanseniaspora opuntiae.* *Candida carpophila, Candida orthopsilosis, Kodamaea ohmeri, Meyerozyma (Pichia* sp*) caribbica, Pichia manshurica, Saccharomycodes ludwigii* and *Yamadazyma (Pichia* sp*).*	Heide et al., 2009.
Ivory Coast	*Hanseniaspora sp., Hyphopichia burtonii Meyeroryma caribbica, Pichia kudriavzevii, Pichia veronaelfabianni, Saccharomyces cerevisiae.*	Zoi & Luc, 2011
Brazil	*Candida jaroonii/ Friedrichii, Hanseniaspora sp., Hanseniaspora vineae, Pichia kudriavzevii, Saccharomyces cerevisiae* and *Wickerhamomyces anomalus*	Zoi & Luc, 2011
Ecuador	*Pichia kudriavzevii, Debaryomyces sp./ Candida sp., Hanseniaspora sp. Issatchenkia tericola*	Zoi & Luc, 2011; Papalexandratou et al., 2011
Malaysia	*Hanseniaspora sp., Pichia kudriavzevii, Saccharomyces cerevisiae, Torulaspora delbrueckii*	Zoi & Luc, 2011
Brazil	*Saccharomyces rosei, Hansenula anomala, Pichia fermentans, Pichia membranaefaciens, Trichosporon cutaneum*	Martelli & Dittmar, 1960