



***FERMENTATION OF COCOA (*Theobroma cacao* L.) BEANS USING
Candida sp. AND *Blastobotrys* sp***

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By

NURUL HUSNA BINTI MAHAZAR

**Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in
Fulfilment of the Requirements for the Degree of Master of Science**

November 2015

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

FERMENTATION OF COCOA (*Theobroma cacao* L.) BEANS USING *Candida* sp. AND *Blastobotrys* sp.

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November 2015

Chairman : Assoc. Prof. Yaya Rukayadi
Faculty : Food Science and Technology

Spontaneous fermentation often produces a variety of end products. Starter culture has been widely used in many fermented food in order to control fermentation process and produce consistent products. The objective of this study was to evaluate the effect of *Candida* sp. and *Blastobotrys* sp. as starters in cocoa bean fermentation in term of optimal media culture, survivability of the cultures and fermentation products of cocoa bean fermentation (pH, temperature, microbial count, proximate analyses, bioactive compounds and volatile compounds). Optimum formulation of molasses yeast extract (MYE) media for the growth of *Candida* sp. and *Blastobotrys* sp. was determined using response surface methodology (RSM). Survivability of the cultures was measured by counting of colony-forming units (cfu) on yeast peptone dextrose (YPD) media. Sugar utilization by both types of yeasts in the optimum MYE media were determined using high performance liquid chromatography (HPLC) analysis. The yeasts were then used as a starter culture in cocoa bean fermentation and the products were evaluated for pH, temperature, proximate content, microbial safety and volatile compounds. The results showed that optimal concentrations of MYE medium were determined as follows: *Candida* sp. (2 g/100 ml, yeast extract; and 10 g/100 ml, molasses) and *Blastobotrys* sp. (2 g/100 ml, yeast extract; and 1.92 g/100 ml, molasses). The optimum MYE media was able to support the growth of *Candida* sp. and *Blastobotrys* sp. up to 140 and 70 days at room temperature, respectively. During survivability of yeasts, both of yeasts rapidly consumed glucose and fructose compared to sucrose at the beginning of the fermentation. Cocoa beans in spontaneous fermentation, *Blastobotrys* sp.-fermentation and *Candida* sp.-fermentation raised in temperature from 32°C (day 0) to maximum temperature valued 40°C, 42°C and 43°C after 3 days fermentation, respectively. The pH of cocoa for spontaneous fermentation, *Blastobotrys* sp.-fermentation and *Candida* sp.-fermentation at beginning of process were pH 4.07, 4.11 and 4.19, respectively, then the pHs of end products were 5.15, 5.44 and 4.99, respectively. Microbial safety of all type of fermentation showed that the number of *E. coli* and *Salmonella* sp. were decreased from 10^3 - 10^5 cfu/ml to <10 cfu/ml after 3 and 5 days of fermentation. No growth of *B. cereus*, *S. aureus* and *Pseudomonas* sp. were detected during fermentations. Proximate content of fermented beans from spontaneous fermentation, *Blastobotrys* sp.-fermentation and *Candida* sp.-fermentation showed a

significant ($p < 0.05$) decreases of crude protein (13.86 - 9.83%), (14.50 - 11.99%) and (14.89 - 13.04%) after 7 days fermentation. Significant decrease in carbohydrate content (32.01% - 12.98%) was observed in spontaneous fermentation only. However, *Blastobotrys* sp.-fermentation and *Candida* sp.-fermentation showed increase of carbohydrate value from 9.84 to 19.98% and 22.53 to 22.64%. Spontaneous fermentation showed significant increase in fat (41.51 - 60.32%) and crude fibre content (3.83 - 8.79%) of fermented beans. Significant decreased in ash content for spontaneous fermentation and *Blastobotrys* sp.-fermentation were (4.44 - 3.06%) and (3.88 - 3.15%), respectively. Significant decreased in moisture content was observed in and *Blastobotrys* sp.-fermentation (6.84 - 4.71%) and *Candida* sp.-fermentation (8.20 - 5.69%) but significant increase was observed from spontaneous fermentation (4.34 - 5.02%). *Blastobotrys* sp.-fermentation product contained low in caffeine (30.59%) while *Candida* sp.-fermentation product contained low in theobromine (16.53%) but high in stigmasterol (1.68%), beta-sitosterol (3.03%) and tocopherol (5.38%). Fermentation showed a total of 20 volatile compounds related to the desirable notes and off-flavour. High individual compounds of alcohol (5) and ester (6) were detected from *Blastobotrys* sp.-fermentation compared to spontaneous fermentation; alcohol (3) and ester (4), and *Candida* sp.-fermentation: alcohol (4) and ester (2). Based on these findings, *Candida* sp. and *Blastobotrys* sp. could be used as potential starter cultures for cocoa beans fermentation.

Keywords: *Blastobotrys* sp., *Candida* sp., cocoa bean, fermentation, starter culture

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

**FERMENTASI BIJI KOKO (*Theobroma cacao* L.) MENGGUNAKAN *Candida*
sp. DAN *Blastobotrys* sp.**

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Fermentasi secara spontan sering menghasilkan pelbagai produk akhir. Kultur pemula banyak digunakan secara meluas dalam makanan yang ditapai dengan tujuan pengawalan proses dan penghasilan produk yang konsisten. Objektif kajian ini adalah untuk menganalisa kesan penggunaan *Candida* sp. dan *Blastobotrys* sp. sebagai kultur pemula dari segi optimum kultur media, ketahanan kultur dan produk fermentasi biji koko (perubahan fizikal, keselamatan mikrob, analisis proksimat dan sebatian meruap). Kaedah respon permukaan (RSM) digunakan bagi menghasilkan medium molasses yeast extract (MYE) yang optimum untuk pertumbuhan *Candida* sp. dan *Blastobotrys* sp.. Ketahanan kultur ditentukan menggunakan unit pembentukan koloni (cfu) di atas medium yeast peptone dextrose. Penggunaan gula bagi kedua-dua yis dalam MYE media telah diukur menggunakan kromatografi cecair prestasi tinggi (HPLC). Kultur kemudian digunakan sebagai kultur pemula di dalam fermentasi biji koko dan produk fermentasi dinilai pada pH, suhu, kandungan proksimat, keselamatan mikrob dan sebatian meruap. Hasil kajian menunjukkan kepekatan optima media kultur adalah seperti berikut; *Candida* sp. (2 g/100 ml, yeast extract; dan 10 g/100 ml, molasses) dan *Blastobotrys* sp. (2 g/100 ml, yeast extract; dan 1.92 g/100 ml, molasses). Kultur media yang optimum mampu menampung pertumbuhan *Candida* sp. dan *Blastobotrys* sp. sehingga 140 dan 70 hari masing-masing pada suhu bilik. Semasa fermentasi yis dalam MYE media, yis menggunakan glukosa dan fruktosa lebih cepat berbanding sukrosa masa awal fermentasi. Suhu yang dicatat semasa fermentasi secara spontan, fermentasi-*Blastobotrys* sp. dan fermentasi-*Candida* sp. meningkat dari 32°C kepada suhu maksimum iaitu 40°C, 42°C and 43°C masing-masing selepas 3 hari fermentasi. pH biji cocoa pada awal fermentasi; fermentasi secara spontan, fermentasi-*Blastobotrys* sp. dan fermentasi-*Candida* sp. ialah pH 4.07, 4.11 dan 4.19 masing-masing. Produk fermentasi bagi ketiga-tiga jenis fermentasi mencatatkan pH 5.15, 5.44 dan 4.99 masing-masing. Keselamatan mikrob bagi semua jenis produk fermentasi; fermentasi secara spontan, *Blastobotrys* sp.-fermentasi dan *Candida* sp.-fermentasi menunjukkan bilangan *Escherichia coli* dan *Salmonella* sp. menurun daripada (10^3 - 10^5) cfu/ml kepada <10 cfu/ml selepas 3 dan 5 hari fermentasi. Tiada pertumbuhan *Bacillus cereus*, *Staphylococcus aureus* dan *Pseudomonas* sp. semasa fermentasi ini. Kandungan proksimat biji koko yang di tapai fermentasi secara spontan, fermentasi-*Blastobotrys* sp.

dan fermentasi-*Candida* sp. menunjukkan penurunan protein yang signifikan ($p < 0.05$) yaitu (13.86 - 9.83%), (14.50 - 11.99%) and (14.89 - 13.04%) selepas 7 hari fermentasi. Penurunan karbohidrat (32.01% - 12.98%) yang signifikan pada fermentasi spontan. Walau bagaimanapun, fermentasi-*Blastobotrys* sp. dan fermentasi-*Candida* sp. menunjukkan peningkatan karbohidrat dari 9.84 kepada 19.98% dan 22.53 kepada 22.64%. Fermentasi spontan menunjukkan kenaikan yang signifikan kandungan lemak (41.51 - 60.32%) dan serat (3.83 - 8.79%) biji koko yang ditapai. Penurunan kandungan abu bagi fermentasi spontan dan fermentasi-*Blastobotrys* sp. adalah (4.44 - 3.06%) dan (3.88 - 3.15%) masing-masing. Kandungan kelembapan mencatatkan penurunan yang signifikan bagi fermentasi-*Blastobotrys* sp. (6.84 - 4.71%) dan fermentasi-*Candida* sp. (8.20 - 5.69%) tetapi kenaikan yang signifikan didapati pada fermentasi spontan (4.34 - 5.02%). Produk fermentasi-*Blastobotrys* sp. menunjukkan kandungan kafein (16.53%) yang rendah, manakala produk fermentasi-*Candida* sp. menunjukkan kandungan theobromine (16.53%) yang rendah tetapi tinggi dengan kandungan stigmasterol (1.68%), beta-sitosterol (3.03%) dan tocopherol (5.38%). Sebanyak 20 sebatian meruap dikesan semasa fermentasi biji koko yang menyumbang kepada rasa koko yang diingini dan juga sebaliknya. Sebatian meruap tunggal alkohol (5) dan ester (6) terhasil dari fermentasi-*Blastobotrys* sp. berbanding fermentasi spontan; alkohol (3) dan ester (4), dan fermentasi-*Candida* sp.; alkohol (4) dan ester (2). Berdasarkan hasil yang diperolehi, *Candida* sp. dan *Blastobotrys* sp. berpotensi sebagai kultur pemula untuk fermentasi biji koko.

Kata kunci: *Blastobotrys* sp., *Candida* sp., biji koko, fermentasi, kultur pemula

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LIST OF ABBREVIATIONS

%	Percent
°C	Degree Celsius
AAB	Acetic acid bacteria
<i>B. cereus</i>	<i>Bacillus cereus</i>
cfu	Colony forming unit
<i>E. coli</i>	<i>Escherichia coli</i>
g	Gram
kg	Kilo gram
LAB	Lactic acid bacteria
ml	Millilitre
MRS	de Man, Rogosa and Sharpe
MYE	Molasses-yeast extract
PDA	Potato-dextrose agar
rpm	Rotation per minute
RSM	Response surface methodology
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
v/v	Volume per volume
XLD	Xylose lysine deoxycholate
YPD	Yeast-peptone-dextrose

CHAPTER 1

INTRODUCTION

1.1 Introduction

Cocoa or *Theobroma cacao* L. has valuable products derived from it. Seeds of cocoa or cocoa beans are the raw material for chocolate production (Bartley, 2005). Cocoa beans will go through many process steps before a final product is produced. The processes include post-harvest treatments in plantation and manufacturing steps in a chocolate processing. Post-harvest treatments include breaking of cocoa pod, fermentation, drying, packing and storage of cocoa beans (Lima et al., 2011). Fermentation is a key process step for chocolate production because there will be no chocolate flavour without fermentation. During fermentation, cocoa flavour precursors are produced and the flavour is fully developed during roasting and further steps in cocoa processing (de Brito et al., 2001; Saltini et al., 2013).

Fermentation involves keeping the cocoa bean mass (beans and mucilage pulp) well insulated followed by aeration up to seven days. The microbiology of such fermentation is rather complex and variable. Various types of microorganisms include yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) are present during fermentation (Ardhana and Fleet, 2003; Schwan and Wheals, 2004; Camu et al., 2007). Yeasts are predominant microorganisms at early fermentation as they degrade fermentable sugars of mucilaginous pulp surrounding the seeds (Ho et al., 2014). Different genera of yeast were reported in previous studies include *Candida*, *Hanseniaspora*, *Kluyveromyces*, *Kloeckera*, *Pichia*, *Rhodotorula*, *Saccharomyces* and *Torulaspora* (Ardhana and Fleet, 2003; Meersman et al., 2013). However, some of the genera or species differ in different countries (Schwan and Wheals, 2004).

The diversity of lactic acid bacteria and acetic acid bacteria during cocoa bean fermentation is much restricted compared to yeast. Genera of lactic acid bacteria isolated from cocoa bean fermentation are *Lactobacillus*, *Fructobacillus* and *Leuconostoc* but *Lactobacillus* is the one commonly isolated (Schwan et al., 2014). *Acetobacter* is a genus of acetic acid bacteria where its species, *Acetobacter pasteurianus* is commonly isolated from studies reported by Papalexandratou et al. (2011), Meersman et al. (2013) and Papalexandratou et al. (2013). The role of lactic acid bacteria is to ferment sugar and utilize citric acid of cocoa pulp while acetic acid bacteria oxidize ethanol into acetic acid (Rodriguez-Campos et al., 2011).

Unfermented cocoa beans are characterized with bitter and astringent taste (de Melo Pereira et al., 2012). During fermentation, microbial activity takes place in mucilaginous cocoa pulp producing various metabolites and generates heat that kills the embryo of the bean. Metabolites produced are then diffuse into the cotyledon and trigger the enzymatic reaction. Enzymatic reaction in the cotyledon results in formation of flavour precursor

(Afoakwa et al., 2008). Flavour precursor is essential for the formation of chocolate flavour (Ramli et al., 2006).

A suitable cultivation condition is important in producing mass culture of microorganisms. Carbon, nitrogen and vitamin sources are the major components that should be in the formulation of a growth medium. Selection of carbon source from crude material such as agricultural waste is much preferable by the industries due to its low cost. Oxygen, temperature and pH play important roles in determining growth of a selected starter culture (Egli, 2015). Response surface methodology (RSM) is a statistical approach to determine an optimum condition of factors for desired responses (Li et al., 2002). Optimization of culture medium using RSM has been reported in previous study for cultivation of microorganisms such as *Candida intermedia* Y-1981 (Yönten and Aktaş, 2014) and *Tetraselmis suecica* (Azma et al., 2011).

Cultivated culture used as a starter culture in cocoa bean fermentation might have a better control of the process resulting consistent and improved of quality end products (Lefebvre et al., 2012). This is because a spontaneous fermentation involves diverse species of microorganisms that might produce variety of end products and quality. Fermentation of cocoa bean is a complex process comprises microbial, interaction and biochemical reactions. Thus, the present study was conducted to evaluate fermentation of cocoa beans using *Candida* sp. and *Blastobotrys* sp. as starter cultures. Further, the effect of these starter cultures on fermentation and fermented products were evaluated.

1.2 Problems Statements

Spontaneous fermentation is an uncontrolled process, involving succession of different types of microorganisms that results in variety of end product of fermented beans. A potential use of starter culture is preferred to control the fermentation process producing relatively better quality of a fermented product. This project was funded by Barry Callebaut Malaysia. The selected yeasts (*Candida* sp. and *Blastobotrys* sp.) were given by Barry Callebaut to evaluate the effect of these starter cultures on fermentation of cocoa beans.

1.3 Objectives

The objectives of this study were:

1. To optimize medium composition for the growth of *Candida* sp. and *Blastobotrys* sp. using response surface methodology.
2. To determine the survival of *Candida* sp. and *Blastobotrys* sp. in the optimized molasses- yeast extract (MYE) media.
3. To evaluate the effect of starter culture on quality of fermented cacao beans.

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