UNIVERSITI PUTRA MALAYSIA

MICROBIOLOGICAL AND PHYSICO-CHEMICAL CHANGES DURING FERMENTATION OF *THEOBROMA CACAO*, L.: ISOLATION AND CHARACTERIZATION OF COCOA LIPASE.

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By

RATNA AGUNG SAMSUMAHARTO

Thesis Submitted in Fulfilment of the Requirements for the Degree of Master of Science in the Faculty of Science and Environmental Studies Universiti Putra Malaysia

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DEDICATION

"Dedicated especially to my mom, my mom, my mom : ***Khasanah,*** the soul that I love most; to my father: ***H. Soedarto Sastrowardojo***; to my brother; to my sisters and brothers in-law whose sacrifice and support has enabled me to complete this study successfully and to my nieces and nephews for all the love"

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

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Chairman : Associate Professor Radzali Muse, Ph.D.

Faculty : Science and Environmental Studies

A study was carried out to examine microbiological and physico-chemical changes during the fermentation of *Theobroma cacao*. Isolation, partial purification, characterization of cocoa lipase were also carried out. Results showed that several microbes were successfully detected during fermentation of cocoa beans. The maximum number of yeast colonies observed in fermented cocoa beans PBC 123 and 159 clones was 4.7×10^8 and 1.5×10^9 CFU/g. fr. wt., respectively. Acetic acid bacteria were found to be dominant, 9.6×10^7 CFU/g. fr. wt. for PBC 123 clone and 1.4×10^8 CFU/g. fr. wt. for PBC 159 clone during the third day of fermentation whilst moulds were present throughout the fermentation period of six days. Spore forming bacteria appeared to be dominant on the fifth day of fermentation period. The maximum number of colonies observed for lactic acid bacteria was 3.5×10^6

CFU/g. fr. wt. for PBC 123 clone and 3.7 x 10^6 CFU/g. fr. wt. for PBC 159 clone while for lipolytic bacteria of both clones were 2.1 x 10^4 CFU/g. fr. wt. for PBC 123 clone and 2.7 x 10^4 CFU/g. fr. wt. for PBC 159 clone. Several major fatty acids were successfully identified in cocoa beans during the six-days of fermentation period. The maximum palmitic acid content for PBC 123 and 159 clones was 22.44 and 28.75% w/w, stearic acid content was 34.58 and 33.52% w/w, and oleic acid content was 32.09 and 36.91% w/w, respectively.

The maximum lipase specific activity from acetone dry powder of cocoa beans was 38.72 µmole/min/mg protein (PBC 123 clone) and 98.91 µmole/min/mg protein (PBC 159 clone). Lipase from AcDP of cocoa beans was used for partial purification using 40-60 and 60-80% ammonium sulphate precipitation. The resulted indicated 44.73 and 60.51-fold purification with 26.74 and 33.31% recovery lipase activity, respectively. Results from SDS-PAGE analysis showed that the molecular weight of the enzyme was in between 20-45 kDa. The optimum pH for the lipase activity was 7.0–8.0. Substrate specificity determinations were performed for tributyrin. trimyristin and triolein; lipase demonstrated higher affinity for trimyristin, with K_m value of 2.63 mM. Inhibition of lipase occurred in the presence of diisopropyl flourophosphate, *N*-bromosuccinimide and 5,5-dithiobis-(-2-nitrobenzoic acid).

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

PERUBAHAN MIKROBIOLOGI DAN FIZIKO-KIMIA SEMASA PENAPAIAN *THEOBROMA CACAO*, L.: PENGASINGAN DAN PENCIRIAN LIPASE KOKO

Oleh

RATNA AGUNG SAMSUMAHARTO

Februari 2000

Pengerusi : Profesor Madya Radzali Muse, Ph.D.

Fakulti : Sains dan Pengajian Alam Sekitar

Satu kajian telah dilakukan untuk meneliti perubahan mikrobiologi dan fiziko-kimia semasa penapaian daripada *Theobroma cacao*. Kemudian pengasingan penulenan separa dan pencirian lipase koko telah dijalankan. Keputusan menunjukkan beberapa mikrob telah berjaya dikesan semasa penapaian biji koko. Bilangan maksimum koloni yis daripada klon PBC 123 dan 159 adalah masing-masing 4.7 x 10^8 and 1.5 x 10^9 CFU/g. berat basah. Bilangan koloni yis telah dikesan pada hari pertama penapaian. Bakteria asid asetik di dapati dominan pada hari ketiga penapaian, 9.6 x 10^7 CFU/g. berat basah bagi klon PBC 123 and 1.4 x 10^8 CFU/g. berat basah bagi klon PBC 159. Bilangan maksimum koloni bakteria asid laktik adalah 3.5 x 10^6 CFU/ g. berat basah untuk klon PBC 123 and 3.7 x 10^6 CFU/g. berat basah untuk klon PBC 159. Manakala kulat hadir di sepanjang tempoh enam hari penapaian.

Bakteria pembentuk spora didapati dominan sehingga hari kelima penapaian. Manakala bagi bakteria lipolitik pula adalah 2.1 x 10^4 CFU/g. berat basah bagi klon PBC 123 dan 2.7 x 10^4 CFU/g. berat basah bagi klon PBC 159. Asid lemak utama telah berjaya dikenalpasti dan ditentukan di dalam biji koko semasa enam hari tempoh penapaian. Kandungan maksimumnya bagi setiap klon PBC 123 dan 159, adalah masing-masing 22.44 dan 28.75% w/w bagi asid palmitik , 34.58 dan 33.52% w/w bagi asid sterik serta 32.09 dan 36.91% w/w bagi asid oleik.

Aktiviti lipase spesifik maksimum daripada serbuk kering aseton biji koko adalah 38.72 µmole/min/mg protein (klon PBC 123) dan 98.91 µmole/min/mg protein (klon PBC 159). Lipase daripada serbuk kering aseton biji koko telah digunakan di dalam penulenan separa melalui 40-60 dan 60-80% pemendapan amonium sulfat telah menghasilkan 44.73 dan 60.51-kali penulenan berperingkat. Perolehan semula bagi penulenan tersebut adalah 26.74 dan 33.31%. Penentuan berat molekul dengan menggunakan SDS-PAGE telah menunjukkan nilai di antara 20–45 kDa. pH optima bagi lipase adalah 7.0–8.0. Kespesifikan substrat telah ditunjukkan oleh tributirin, trimiristin dan triolein, di mana lipase menunjukkan afiniti tertinggi bagi trimiristin dengan nilai K_m adalah 2.63 mM. Perencatan lipase telah berlaku dengan kehadiran suatu julat bahan kimia, termasuklah diisopropil florofosfat, *N*-bromosuksinimida dan 5,5-ditiobis-(-2-asid nitrobenzoik).

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LIST OF ABBREVIATIONS

CFU	- colonies forming unit
g	- gram
g. fr. wt.	- gram fresh weight
(w/w)	- weight/weight
%	- per cent
min	- minute
mg	- milli gram
µmole	- micro mole
kDa	- kilo dalton
mM	- milli molar
µM	- micro molar
ha	- hectare
EC	- enzyme commission
FFA	- free fatty acid
min	- minute
kg	- kilo gram
mL	- meter length
mW	- meter wide
mD	- meter deep
cm	- centi meter
(w/v)	- weight/volume
dwb	- dry weight basis
ml	- milli liter
nm	- nanno meter
AcDP	- acetone dry powder
rpm	- revolution per minute
UV/Vis	- ultra violet/visible
U	- unit
meq	- milli equivalent
BF_3	- borontriflouride
M	- molar
µl	- micro liter
N	- normality
V/cm	- volt/centi meter
°C	- degree Celcius
Abs	- absorbance
TEMED	- N,N,N',N'-tetramethyl-ethylenediamine
DFP	- diisoprophylflourophosphate

CHAPTER I

GENERAL INTRODUCTION

The fermentation technique is one of the most important factors in determining the quality of cured cocoa beans. According to Lehrian and Patterson (1983), cocoa fermentation is a complex natural process involving a mixture of external microbiological processes occurring in the pulp surrounding the beans and internal structural changes and enzymatic reactions. A succession of microorganism, in particular yeast, lactic acid bacteria, and acetic acid bacteria, grow rapidly in the pulp producing ethanol, lactic acid and acetic acid as major metabolic products (Lehrian and Patterson, 1983). Acetic acid in particular is responsible for the death of the beans, prevents germination, solubilizes polyphenols, aids in diffusing the content of the storage cells into surrounding parenchyma tissue, and prevents the beans from attack by putrefactive bacteria (Ziegleder and Biehl, 1988).

Acids could mask the overall chocolate flavour in cocoa beans (Lopez and McDonald, 1981). However, high concentrations of residual acids may cause the beans to be excessively acidic in flavour. The presence of acetic and lactic acids either alone or combined, have been implicated as

possible causes of high acidic flavour in cocoa beans. Some researchers suggest that only acetic acid is important because it is present in high concentration and tastes more acidic than other acids (Rohan and Stewart, 1964; Biehl, 1965; Lopez, 1983; Jinap and Dimicks, 1990). Holm (1991) reported that oxalic acids could improve cocoa flavour in Malaysian cocoa beans.

The exothermic formation of acetic acid may physiologically cause the temperature of fermenting mass to rise, prevent germination and cause structural changes which remove the compartment action of enzymes and substrates, thereby permitting an increase in enzymes activity (Biehl and Adomako, 1983).

According to Horman and Braco (1986), Staphylakis and Gegiou (1985) the cocoa cotyledon contains common fatty acids such as 25-30% palmitic acid, 32-37% oleic acid and 30-37% stearic acid, 2-4% linolenic acid and 0.7-1% arachidonic acids. It is generally known that fatty acids have an important effect on chocolate flavour. The high content of free fatty acids in cocoa beans cause dull, insipid and flat tastes (Padavatan *et al.*, 1979). A variety of saturated and unsaturated fatty acids is present in triacylglycerols (TAGs) and the way they arrange themselves during crystal formation determines the hardness of the cocoa butter. Among the most abundant fatty acids are myristic (14:0), palmitic (16:0), stearic (18:0) and

oleic ($18:1^{\Delta 9}$) acids . Fatty acids content in triacylglycerols (TAGs) are most easily determined by using complete saponification process with NaOH and then followed by esterification of the released fatty acids (Boyer,1986; Ziegleder and Biehl, 1988).

In the cotyledon, the accumulation of acids is responsible for the formation of flavour precursors during fermentation by providing an acidic environment for enzymatic reactions to occur. Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are ester hydrolases or esterases since they hydrolyse the ester bonds of triacylglycerol molecules. The lipases are more active with insoluble fatty acid esters and hydrolyse the ester bonds present only at the water-oil interface, whereas carboxylic ester hydrolases that are specific for the soluble esters are simply termed esterases (Schuepp *et al.*, 1997). According to Jensen *et al.* (1983), lipases or acylglycerol hydrolase are enzymes which catalyze the hydrolysis of long chain aliphatic acids from acylglycerol at the oil/water interface. The systematic name is acylglycerol acylhydrolase. The interface is usually provided by emulsion globules or lipoprotein particles, the latter are primarily chylomicrons and very low density lipoprotein. The element providing the interface has been termed the super-substrate.

Bloch (1960) reported that lipase is an enzyme which can be easily found in higher plants, animals, insects and some microorganisms. In plant

this lipolytic enzyme plays an important role in fatty acid metabolism. Highly active lipases are found to catalyze the hydrolysis of reserve triacylglycerols (TAGs). The triacylglycerols (TAGs) are actually localized in subcellular organelles called lipid bodies (Bloch, 1960). Also, lipases have the important physiological role of preparing the fatty acids of water-insoluble triglycerides (TGs) for absorption into and transport through membranes by converting the triglycerides (TGs) to the more polar diglycerides (DGs), monoglycerides (MGs), free fatty acid (FFA) and glycerol (Jensen, 1983). The majority of lipases are extracellular, acidic glycoprotein of molecular weights between 20 and 60 kDa, although some form aggregates in solution (accounting for the high molecular weights reported for some partially purified enzymes). Most purified lipases contain between 2 and 15% carbohydrate, with the major glycoside residue being mannose (Gill and Parish, 1997). The evidence for the presence of lipases in cocoa beans was first reported by Ciferrin in 1931 (as cited by Forsyth and Quesnel, 1963).

The lipolytic enzyme found in the higher plants such as cocoa as important in understanding of their physiological roles as well as their action in agricultural products during storage. The main purpose of this research is to study the changes in microbiological and physico-chemical properties on fermented cocoa beans and isolation and characterization of cocoa lipase.