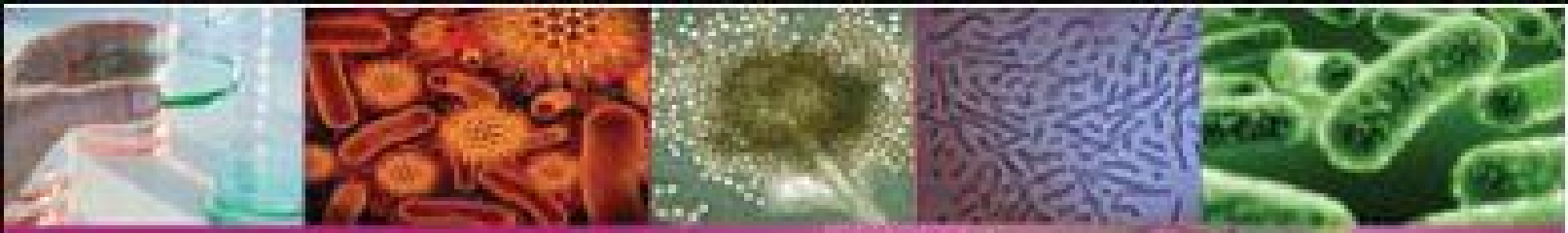

ISSN NO.

International Journal of

FOOD AND FERMENTATION TECHNOLOGY

EDITORIAL BOARD

Editor-in-Chief

Dr. VK Joshi

(FBRSI, FISHA)
Adjunct Professor, Shoolini University, Solan
Prof. and Head (Retd)
Department of Food Science and Technology,
Dr YS Parmar University of Horticulture and Forestry
Nauni, Solan, Himachal Pradesh, INDIA
Wine consultant, Editor WJB, IJFN
Member FSSAI, AFST
vkjoshipht@rediffmail.com / editorijfft@gmail.com

Honorary Editor(s)

Dr (Mrs) Sunita Garg
National Institute of Science Communication and Information Resources,
CSIR, New Delhi, INDIA
sunitag@niscair.res.in

Dr N S Thakur
Department of Food Science and Technology
Dr. YS.Parmar University of Horticulture and Forestry, Nauni, Solan,
Himachal Pradesh, INDIA
nsthakur1964@gmail.com

Executive Editorial Board

Prof. R M Sharma
Division of Fruits & Horticultural Technology
Indian Agricultural Research Institute,
New Delhi, INDIA
rmsharma345@gmail.com

Dr B L Attri (Post Harvest Technology)
Central Institute of Temperate Horticulture, Regional Station,
Mukteshwar, Kumaun, Uttarakhand, INDIA
attriblnrcwa@rediffmail.com

Dr. Gargi Dey (Food Biotechnology)
Dept. of Biotechnology
Kalinga School of Biotechnology
KIIT University, Bhubaneswar, INDIA
drgargi.dey@gmail.com

Dr. L.Veeranjaneya Reddy (Enology)
Department of Microbiology, Yogi Vemana University
Kadapa., A.P., INDIA
lvereddy@gmail.com/lvereddy@yahoo.com

Dr. Madhavi Vernekar (Enzyme Technology)
Department of Biotehnology and Bioinformatics
Padmashree Dr. D.Y. Patil University, INDIA
madhavirevankar@rediffmail.com

Dr Om Prakash Chauhan (Fruit and Vegetable Processing)
Fruits and Vegetables Technology Division
Defence Food Research Laboratory Siddarthanagar,
Mysore, INDIA
opchauhan@gmail.com

Dr. K. Jayaram (Indegenous Fermentation Technology)
Microbioal Reserves Division
Institute of Bioresources and Sustainable Development
Imphal, Manipur, INDIA
saccheromyces@rediffmail.com

Dr S Kapoor (Mushroom Production)
Department of Microbiology, College of Basic Sciences and
Humanities, PAU, Ludhiana, INDIA
skapoorpau@pau.edu

Dr Satish Kumar Sharma (Fruit and Vegetable Processing)
GB Pant University of Agriculture & Technology, Hill Campus,
Ranichauri, Distt Tehri-Garhwal, Uttarakhand, INDIA
drsatish10@yahoo.com

Dr. Seema Garcha (Probiotic Foods)
Department of Microbiology
Punjab Agricultural University, Ludhiana, INDIA
seemagarcha@hotmail.com/seemagarcha@gmail.com

Dr Somesh Sharma (Fermentation Technology)
Department of Food Technology
School of Bio engineering and Food Technology
Shoolini University, Solan HP, INDIA
sharmawine@gmail.com

Dr Wamik Azmi (Food Biotechnology)
Department of Biotechnology, Himachal Pradesh University,
Summer Hill, Shimla, Himachal Pradesh, INDIA
wamikazmi@rediffmail.com

Dr Shashi Bhushan (Fermentation, Health Related Products)
Division of Biotechnology, Institute of Himalayan Bio-resource Technology (CSIR), Palampur, Distt Kangra,
Himachal Pradesh, INDIA
sbhushan@ihbt.res.in

Dr S K Patyal
Department of Entomology and Apiculture,
DR. Y S Parmar University of Horticulture and Forestry,
Nauni Solan, INDIA
patyalsk@yahoo.co.in

Dr. R Kumar (Food Scientist)
Defence Food Research Laboratory
Scientific Panel Member
Food Safety Standards Authority of India
Mysore, INDIA
kumardfrl@gmail.com

Dr. Neetu Kumra Taneja (Molecular Microbiology)
Department of Basic and Applied Sciences
National Institute of Food Technology Entrepreneurship and Management
Sonepat, Haryana, INDIA
neetu.niftem@gmail.com

Dr. Savitri (Biotechnology)
Department of Biotechnology
Himachal Pradesh University
Summer Hill, Shimla, HP, INDIA
savvy2000in@yahoo.com

Dr. Naveen Batra (Biotechnology)
Department of Biotechnology
GGDSD College
Chandigarh, INDIA
navneet05@gmail.com

Dr. Ravinder Kaushik (Dairy Technology)
Department of Food Technology
School of Bioengineering and Food Technology
Shoolini University, HP, INDIA
ravinder@shooliniuniversity.com

Dr. G S Meena
Scientist
Indian Council of Agricultural Research- National Dairy Research Institute, Karnal, Haryana, INDIA
gsiitkgp@gmail.com

Dr. Prasad Jayprakash Rasane
Assistant Professor (Food Technology) and Entrepreneurship Coordinator (Agriculture)
Lovely Professional University,
Jalandhar, Punjab, INDIA
rasaneprasad@gmail.com

Dr. Pankaj Kumar Chauhan
Assistant Professor
School of Bioengineering & Food Technology, Shoolini University,
Solan, (H.P.) INDIA
pkchauhan@shooliniuniversity.com

Dr. Vikas Kumar
Assistant Professor
Food Technology and Nutrition
School of Agriculture
Lovely Professional University, Jalandhar, INDIA

EDITORIAL ADVISORY BOARD

Dr Aline Lonvaud (Wine and Brandy)
Faculty of Enology
University Victor Scgalen Bordeaux 2, FRANCE
aline.lonvaud@oenologie.u-bordeaux2.fr

Dr Creina S. Stockley (Wine and Health)
The Australian Wine Research Institute, AUSTRALIA
creina.stockley@awri.com.au

Dr (Mrs.) Devina Vaidya (Fruit and Vegetable Processing)
Department of Food Science and Technology
Dr. YS. Parmar University of Horticulture and Forestry, Nauni
Solan, Himachal Pradesh, INDIA
devinavaidya@yahoo.com

Dr Eveline Bartowsky (Wine Microbiology)
The Australian Wine Research Institute
P.O. Box 197, Glen Osmond, AUSTRALIA
eveline.bartowsky@awri.com.au

Dr Gines Navarro (Wine Fermentation)
Departamcnto de Quimica Agricola, Geologiay Edafologia
Facultad de Quimica. Universidad de Murcia
Campus Universitario de Espinardo, Murcia, SPAIN
gnavarro@um.es

Dr L. Rebordinos (Food Microbiology)
Laboratorio de Microbiologia y Gcnetica. Facultad de Ciencias del Mar y Ambientalcs. Universidad de Cadiz. Poligono del no San, SPAIN
laureana.rebordinos@uca.es

Dr Luca Cocolin (Wine Microbiology)
Dipartimento di Scienze degli Alimenti, Universita degli studi di Udine, Facolta di Agraria, via Marangoni, Udine, ITALY
lucasimone.cocolin@unito.it

Dr M. Remedios Marin (Wine Modelling)
Universidad Publica de Navarra Nafarroako Unibertsitste Publikoa Area de Tecnologia de Alimentos Universidad Publica de Navarra Campus Arrosadia Pamplona, (Navarra), SPAIN
remedios.marin@unavarra.es

Dr. Aly El Sheikha (Molecular Biology)
Department of Biology, McMaster University, Hamilton, Ontario, CANADA
elsheikha.aly@gmail.com

Dr M C Pandey (Meat and Meat Products)
Department of Freeze Drying and Animal Product Technology
Defense Food Research Laboratory, Mysore, INDIA
dfrlmysore@sancharnet.in

Dr Neerja S. Rana (Biochemistry)
Department of Basic Sciences
Dr.YS. Parmar University of Horticulture and Forestry, Nauni
Solan, Himachal Pradesh, INDIA
drneerjauhf24@yahoo.co.in

Dr Philippe Jeandet (Sparkling Wine)
Laboratory of Enology and Applied Chemistry, Unite de Recherche sur la Vignc ct lc Vin de Champagne, Research Unit N°2069 University of Reims, Faculty of Science, FRANCE
philippe.jeandet@univ-reims.fr

Professor Pradeep Khanna (Mushroom Production)
College of Basic Science, PAU Ludhiana
Punjab, INDIA
pkhanna_pau@rediffmail.com

Dr Pura Naik J (Plantation Crops)
Division of Plantation, Spices and Flavour Tech.
Central Food Technological Research Institute
Mysore, INDIA
puranaik@yahoo.com

Dr RC Ray (Food Fermentation)
Regional Centre of Central Tuber Crops Research Institute
Dumuduma Housing Board, Bhubaneswar, Orissa, INDIA
rc_rayctcri@rediffmail.com

Dr. Rintu Banerjee (Microbial Technology)
Microbial Biotechnology and Downstream Processing Laboratory, Agricultural & Food Engineering Department
Indian Institute of Technology, Kharagpur,West Bengal, INDIA
rin_tuin@yahoo.com

Dr R K Gupta (Statistics)
Department of Basic Sciences
Dr. Y S Parmar University of Horticulture and Forestry,
Nauni Solan, Himachal Pradesh, INDIA
rkgupta9@rediffmail.com

Dr R S Singh (Food Fermentation and Enzyme Technology)
Department of Biotechnology,
Punjabi University, Patiala, Punjab, INDIA
rssingh11@lycos.com

Dr. Sanjeev Kumar Soni (Enology)
Department of Microbiology
Panjab University, Chandigarh, INDIA
sonisk@pu.ac.in

Dr S S Kanwar (Food Fermentation)
Department of Microbiology, CSK HPKV, Palampur
Himachal Pradesh, INDIA
sskanwar1956@gmail.com

Dr Sumit Arora (Dairy Science)
Dairy Chemistry Division, National Dairy Research Institute
Karnal, Haryana, INDIA
sumitak123@yahoo.com

Prof Tek Chand Bhalla (Food Fermentation & Enzyme Tech.)
Department of Biotechnology,
Himachal Pradesh University, Shimla
Himachal Pradesh, INDIA
bhallatc@rediffrnail.com

Dr V M Pratape, (Grain Science and Technology)
Department of Grain Science and Technology,
Central Food Technological Research Institute
Mysore, INDIA
gst@cftri.res.in

Dr Y S Dhaliwal (Food and Nutrition)
Department of Food Science and Nutrition
College of Home Science
CSK HPKV, Palampur, Himachal Pradesh, INDIA
ysdhaliwal44@yahoo.co.in

Dr B.C. Suman (Mushroom Production)
Department of Plant Pathology
Dr Y S Parmar University of Horticulture and Forestry
Nauni, Solan, Himachal Pradesh, INDIA

Dr. S S Thorat (Food Science Technology)
Department of Food and Industrial Microbiology
Vasantrao Naik Marathwada Agriculture University
Parbhani, INDIA
drssthorat@rediffmail.com

Dr. D N Gandhi (Dairy Microbiology)
Gandhi House, New Housing Board
Karnal, Haryana, INDIA
dngandhi@rediffmail.com

Dr. Raj Bala Grewal (Foods and Nutrition)
Centre of Food Science and Technology
CCS Haryana Agriculture University
Hisar, Haryana, INDIA
grewalrb@hau.ernet.in/grewalrb@gmail.com

Dr. Ome Kalu Achi (Microbiology)
Department of Microbiology
Michael Okpara University of Agriculture
Abia State, NIGERIA
omekachi@yahoo.com

CONTENTS

International Journal of Food and Fermentation Technology

Vol. 7, No. 1, June 2017

Intl. J. Food. Ferment. Technol. 7(1): 25-31, June 2017
©2017 New Delhi Publishers. All rights reserved
DOI: 10.5958/2277-9396.2017.00003.4

RESEARCH PAPER

Analysis of Amino Acids in Cocoa Beans Produced during Fermentation by High Performence Liquid Chromatography (HPLC)

Mulono Apriyanto

Department of Food Technology, Indragiri of Islam University, Indragiri Hilir Riau, Indonesia

Corresponding author : mulonoapriyanto71@gmail.com

Paper No.: 163 **Received:** 18-01-2017 **Revised:** 08-04-2017 **Accepted:** 11-05-2017

Abstract

Fermentation is a very vital stage of cocoa processing to obtain high quality chocolate product. This study was conducted to obtain an optimal result of chocolate fermentation by determining the concentration and types of amino acid of lindak clone cacao beans using HPLC. Pre-conditioned process was done in order to get water content to 15% in pulp the same level as the traditional process from farmer. Before fermentation dried cocoa beans were rehydrated to obtain a water content of pulp at the same level as fresh cocoa bean pulp. The fermentation, was conducted for 120 h. The fermentation the three level treatments an control without inoculum, mixed with culture of microbies add and the begining of fermentation. The fermentation was started using *Saccharomyces cerevisiae*, *Lactobacillus lactis* and *Acetobacter aceti* was added the fermentation and finally, treatment gradually microbies added during fermentation. The analysis of amino acids was conducted using HPLC separation method based on the procedure at outlined earlier. The measurement of amino acid was performed in two phases, liquid hydrolysis, and derivatization proceeded by chromatographic analysis. Condition of HPLC was measured at 37 °C. Mobile phase contains of 60% acetonitril - AccqTag Eluent A, gradient system and the flow rate was 1.0 ml per minute. Fluorescence detector had 250 nm excitation and 395 nm emission. Injecting volume was 5 uL. The results of this study showed that cocoa beans 120 h fermentation has higher products of aspartic acid, glutamic acid, hydrophobic amino acids (Alanine, leucine, proline, valine, isoleucine) and amino acids such as serine, glysine, histidine, treonine and lysine, while local clones of cocoa beans with 3-days fermentation produce more amino acids such as aspartic, glutamic, hydrophobic (isoleucine, leucine, valine) and amino acids such as histidine, threonine, glysine, serine and lysine.

Keywords: HPLC, amino acids, local clone, fermentation, cacao beans

Fermentation is at the core of the cocoa processing because it can enhance the flavor of cocoa. Fermentation enhance biochemical reactions in the cocoa beans that led to the formation of aroma precursors, taste and color, reduction of bitter taste and astringent, and improvement of the physical appearance of cocoa. In addition, fermentation facilitates the release of the pulp layer that cling to the seed and hardens the seed coat into shell-like (Misnawi, 2005; Anonymous, 2013).

Cocoa fermentation is basically conversion of pulp's sugar and citric acid into organic acids by microorganism (Camu *et al.*, 2008; Ardhana and Fleet, 2003). The organic acids are known to induce in enzymatic reaction inside the beans resulting biochemical changes generating several compounds

critical for aroma, taste, and color development (Biehl *et al.,* 1985; Afoakwa *et al.,* 2014). This process is carried by heaping the cocoa beans inside closed container or basket for 5-7 days with turning once every 2 days. Without fermentation, cocoa beans taste bitter, astringent, and without distinctive cocoa aroma after processing (Schwan and Wheals, 2004).

Dry cocoa beans lose most of their moisture content and substrate. Around 35% water is needed during fermentation, used for enzymatic reaction inside the beans for microbial growth in the pulp (Schwan and Wheals, 2004) as the media for enzyme-substrate interaction to mediate hydrolysis and oxidation to generate precursors for cocoa taste, color, and aroma. During fermentation the sugar in the pulp converted into organic acids which then diffuse into the beans and induce further enzymatic reaction for taste, aroma and color (Afoakwa *et al.,* 2014). Considering this critical role, rehydration is done for cocoa beans prior to fermentation.

Amino acid has a significant correlation with the formation of flavor in normal fermentation circumstances. The formation of amino acids can be used as a guide for determining the appropriate length of time to obtain fermented cocoa beans that have good flavors. Misnawi *et al.* (2005) argued that the best cocoa aroma is usually produced from cacao beans that have high levels of free amino acids and reducing sugar reached maximum levels. Further, Voigt *et al.* (1994) found that free amino acids and oligopeptides are essential aroma precursors. The combined activity of enzymes, endoprotease aspartate and carboxy peptidase, on cacao beans protein is required for the formation of certain cocoa aroma precursors.

According to Biehl *et al.* (1982); de Brito *et al.* (2000), after fermentation, high-quality cacao beans must have approximately 8-14 mg/g of total amino acids in the dried substances. Component of chocolate aroma consists of volatile compounds, which are mainly formed by the reaction of amine and carboxyl groups. Candidate compounds of chocolate unique aroma producer are composed of hydrophobic amino acids, peptides hidrophilic and reducing sugars (Mulono *et al.,* 2016).

Composition of amino acid is very important, because it can predict the process of synthesizing flavor compounds. There are many studies on the determination of amino acid number in cacao beans (Puziah *et al.*, 1998; de Brito *et al.*, 2000), using HPLC.

HPLC is a chromatography system, where mobile phase is passed quickly, under pressure and the results are detected by the detector. Selection of ion chromatography technique is based on the ability to perform simultaneous detection, ease of operation, high speed of analysis and accuracy of results, and the stability of column separator so that it can be used for quantitative analysis and qualitative simultaneously (Ardianingsih, 2009).

In this study, the analysis of amino acids generated by fermentation of protein cacao beans was conducted.

Experiment on non-fermented dry cocoa beans was conducted to measure the change of amino acid in cocoa beans during fermentation. The study aimed to obtain optimal fermentation products by determining the concentration and types of free amino acids in the cacao beans using HPLC analysis.

MATERIALS AND METHODS

Cocoa beans drying

Cocoa beans were taken from yellowish-orange ripe pods identified with tapped hollow sound, then dried inside cabinet dryer at 40 °C until moisture content was reduced to 15%. Fermentation of dried beans was done in glass jar; each contained 100 gram. Regulated temperature during fermentation, respectively 35°C (24 h), 45 °C (24 second clock), 55 °C (24-hour three) and 35 °C (last 48 h). The first treatment was the control treatment or without the addition of inoculum, the second treatment (IA) using inoculum of *S. cerevisiae (FNCC 3056), L. lactis (FNC 0086)* and *A. aceti (FNCC 0016)*, respectively each of 10^8 cfu/g were moulted simultaneously at the beginning of fermentation. The third treatment (IB), administration of gradual yeast inoculum at the start

of fermentation, lactic acid bacteria at the start of the second 24 hours and acetic acid bacteria at the start of a 24-hours with the same microbial population as with the second treatment.

Amino Acid Analysis Using HPLC

HPLC (Model 1100, Agilent Technologies, Waldrbom Germany) provided with a binary pump with degasser micro vacum degasser, thermostat controlled auto sample was used. Column compartment, a G1321 detector flourescence, and a G1315A diode array detector was used for amino acid analysis. AccQtag column (3,9×150mm) was used for separation of amino acids. Nollet (1996), Marino *et al.* (2010) methodology was adoped for amino acid analysis of fermented cocoa beans using HPLC. The measurement of amino acid was performed in two phases: liquid hydrolysis and derivatization proceeded by chromatographic analysis. The method of liquid hydrolysis was 5 ml HCL 6 N hydrolyzed at 110 °C for 22 hours in vacuum condition. After hydrolysis, the tube was cooled and the solution was filtered using spartant-HPLC, then, diluted with water at 1:20 v/v ratio. Condition of HPLC was measured at 37 °C, the mobile phase contains of 60% acetonitril - accqTag Eluent A, gradient system and flow rate was 1.0 mL per minute. Fluorescence detector had 250 nm excitation and 395 nm emission. Injecting volume was 5 µL.

Experimental design

A 3 × 3 full factorial experimental design was used for the study. The principal factors investigated were inoculum added (control (TI), inoculum added in begin (IA), inoculum added in step (IB)) and fermentation time (0, 24, 48 h).

Statistical analyses

SPSS software IBM version 22 was used to analyze the data for analysis of variance (ANOVA). Least significant difference (LSD) was used to separate and compare the means, and significance was accepted at 5% level ($p < 0.05$).

RESULTS AND DISCUSSION

Table 1 reveals that cacao beans fermented for 120 h (control (TI)) showed higher amino acid content, specifically for aspartic acid, glutamic acid, alanine, isoleucine, proline and valine (hydrophobic). Those are significantly different with amino acids produced by cacao beans fermentation at 72, 96 and 120 h. Amino acid deteriment specifically alannine 1.59 ± 0.01 increase to 1.60 ± 0.01, tyrosine 0.89 ± 0.01 decrease to 0.67±0.01, valine 1.08±0.01 increase to 1.19 ± 0.01, phenylalanine 1.53±0.01 decrease to 1.51±0.01, isoleucine 1.03±0.01 decrease to 1.03±0.01 and leucine 1.85 ± 0.01 increase to 1.99 ± 0.01.

Table 1: Changes in the concentration of free amino acids (µg/g) during the fermentation of cocoa beans on control (TI) on the hour to 72, 96 and 120 hours

	Fermentation time (hours)			
	0	**72**	**96**	**120**
Hidrophobic				
Alanine	1.59b	1.55d	1.65b	1.60c
Tyrosine	0.89b	0.73b	0.65a	0.67a
Valine	1.08b	1.21b	1.12a	1.19d
Phenylalanine	1.53b	1.46c	1.31d	1.51d
Ileucine	1.06c	1.01a	1.01c	1.03c
Leucine	1.85a	1.89a	1.76b	1.99b
Methionin	0.73a	0.73b	0.72b	0.73b
Total	**7.65**	**8.58**	**8.22**	**8.72**
Acidic				
Aspartic acid	3.99a	4.35c	4.43c	3.83c
Glutamic acid	15.95a	15.11a	14.6b	15.14b
Serin	1.41d	1.28c	1.37b	1.46c
Histidin	0.83a	0.82b	0.79c	0.82c
Total	**22.18**	**21.56**	**21.05**	**21.25**

Table 2 show that cocoa beans of added inoculum mixed microbies in beginning, fermented 120 h produced higher amino acids, specifically for aspartic acid, glutamic acid, alanine, isoleucine, proline, valine

(hydrophobic). These wee significantly different with amino acids produced by cacao beans of fermented 72 h, 96 h, 120 h. Amino acid determined were specifically alannine 1.57 ± 0.01 increase to 1.61 ± 0.01, tyrosine 0.85±0.01 decrease to 0.62±0.01, valine 0.94±0.01 increase to 1.06 ± 0.01, phenylalanine 1.43±0.01 decrease to 1.35±0.01, isoleucine 1.02±0.01 increase to 1.07±0.01 and leucine 1.66±0.01 increase to 1.83±0.01.

Table 2: Changes in the concentration of free amino acids (ug/g) during the fermentation of cocoa beans on the addition of inoculum treatment beginning on the hour to 72, 96 and 120 hours

	Fermentation time (hours)			
	0	**72**	**96**	**120**
Hidrophobic				
Alanin	1.57d	1.52d	1.66a	1.61a
Tyrosin	0.85d	0.77c	0.61a	0.62b
Valin	0.94d	1.07b	0.99b	1.06b
Phenylalanin	1.43c	1.35b	1.19b	1.35d
Ileusin	1.02c	0.96b	0.97b	1.07d
Leusin	1.66d	1.72a	1.60c	1.83a
Methionin	0.72c	0.71a	0.70c	0.72a
Total	**8.19**	**8.10**	**7.72**	**8.26**
Acidic				
Aspartic acid	3.81a	4.47a	4.53c	3.75c
Glutamic acid	15.86a	15.05b	15.03c	15.42b
Serin	1.39b	1.25b	1.35c	1.44d
Histidin	0.82a	0.81a	0.78a	0.80a
Total	**21.88**	**21.58**	**21.69**	**21.41**

Table 3 show that cocoa beans of gradually added inoculum microbies in fermented 120 h produced higher amino acids, specifically for aspartic acid, glutamic acid, alanine, isoleucine, proline, valine (hydrophobic). These were significantly different with amino acids produced by cacao beans of fermented 72, 96 and 120 h. Amino acid deterimined specifically were alannine 1.37 ± 0.01 increase to 1.65 ± 0.01, tyrosine 0.78±0.01 decrease to 0.63±0.01, valine 0.82±0.01 increase to 0.95 ± 0.01, phenylalanine 1.26±0.01 decrease to 1.19±0.01, isoleucine 0.92±0.01 just same after 120 h and leucine 1.41±0.01 increase to 1.48±0.01. Most of the types of amino acids in the cacao beans are aspartic, glutamic and lysine. Yusep *et al.* (2002) and Jinap *et al.* (2008) have described that amino acid is a precursor of certain aroma that the acts as a substrate for a Maillard reaction to form methylpyrazines (Eichner *et al.*, 1994).

Table 3: Changes in the concentration of free amino acids (μg/g) during the fermentation of cocoa beans on the gradually added of inoculum on the hour to 72, 96 and 120 hours

	Fermentation time (hours)			
	0	**72**	**96**	**120**
Hidrophobic				
Alanin	1.37a	1.32b	1.75b	1.65c
Tyrosin	0.78a	0.69a	0.611b	0.63c
Valin	0.82a	0.95d	0.87c	0.95c
Phenylalanin	1.26c	1.18c	1.03a	1.19a
Ileusin	0.92d	0.87b	0.82c	0.92b
Leusin	1.41b	1.47a	1.35b	1.48d
Methionin	0.65a	0.64a	0.63d	0.64c
Total	**7.21**	**7.12**	**7.06**	**7.46**
Acidic				
Aspartic acid	3.21	4.59	4.65	3.15
Glutamic acid	15.74c	15.31a	14.89b	15.31a
Serin	1.24c	1.19a	1.30b	1.38a
Histidin	0.79b	0.76b	0.74b	0.77a
Total	**20.98**	**21.85**	**21.58**	**20.61**

The introduction of High Performance Liquid Chromatography or HPLC mixtures of amino acids can directly separate in the appropriate columns, such as on the method of amino acid analysis (Sumarno *et al.*, 2002). Flavor formation in cocoa beans is related with flavor and aroma of chocolate starts from the quality of raw materials and the processing mechanism. Flavor of cocoa beans appears after going through the process of fermentation and drying. Fermentation is a very vital stage of processing mechanism to guarantee chocolate products have good taste. Wrong fermentation practice damage the taste which cannot be repaired through subsequent processing modifications. It shown that the unfermented cocoa beans contain no aroma precursors in their seeds (Harrington, 2011).

The results of this study showed that the fermentation can increase the concentration of amino acid, types of amino acids, hydrophobic amino acids and other amino acids as shown in Table 1, 2 and 3. This happens because in after stages (24 hours fermentation), fermentation occurs in the seed pulp that will produce alcohol and acetic acid. At the moment, the precursors have not been created in the seed. According to Sulistyowati (1988), flavor precursor formation begins after the death of seed by the presence of acetic acid, alcohol diffusing into the beans and the heat that arise simultaneously which are the products of microbial activity during the fermentation process resulting in decomposition of seed cells, so that no biological barrier separated the enzyme and its substrate.

On the third day ahead, cocoa bean cells began to break and at that time, the formation flavor process precursors began, because the enzymes in cocoa beans that were initially covered inside the cells begin to come out and mix with the other components. Hydrolytic enzymes in cocoa beans include amylase, lipase, the amino-acid decarboxylase, peroxidase, and poligalakturonase catalyzed the formation of this precursor compound. Hydrophobic amino acids such as alanine, tyrosine, valine, isoleucine, leusine and phenylalanine are special aroma precursors for the formation of cocoa aroma (Voigt, *et al*., 1993).

According to Crafack *et al.* (2014), the amino acid content increase after fermentation, but it can not change the composition. The accumulation of hydrophobic amino acids during drying undergo degradation during roasting (Crafack *et al.*, 2014). Result is the formation of volatile compounds such as aldehydes, ketones, pyrazines and furans which affect the formation of the chocolate flavor. Cocoa bean has varied concentration of amino acids. However, generally, unfermented and three days of fermentation seeds produce more aspartic acid, glutamic, leucine, isoleucine, valine, histidine, serine, threonine and lysine and significantly different from 72 h, 96 h and 120 h fermentation. Based on observations on the 72 h of fermentation concentration of acetic acid control 10%, mixed microbies added 15% and gradually added inoculum 19%. If more, concentrations acetic acid decreases pH. Besides pH, temperature also affects the activity of enzymes. In high temperatures, enzyme activity increase results in complete cell decomposition, so that the produced flavor does not have bitter taste and astringent (Sulistyowati, 1988).

Based on the active side of the peptide bond in the termination process, there are two types of enzyme protease, exopeptidase and endopeptidase. At pH 4.5, exopeptidase becomes active to cut the outer side of the polypeptide chain and forms amino acids. At pH 5.5 endopeptidase enzymes is active, therefore in that pH range more peptides would be found but these would be less amino acid. Proteolitic activity has significant role in the formation of aroma (Biehl *et al.* 1985).

Voigt *et al.* (1993); Misnawi *et al.* (2004) and Gu *et al.* (2013) stated that in addition to reducing sugars, peptides and hydrophobic free amino acids such as alanine, tyrosine, valine, isoleucine, leucine and phenylalanine are specific flavor precursors for the formation of cacao aroma. Furthermore, the hydrophobic amino acids are considered as the most potent precursor formation for methylpirazins. The study of Jinap *et al.* (2008 and 2010) and Yusep *et al.* (2002) have shown that the hydrophobic free amino acids and acid group amino acids directly contribute to the formation of cocoa flavor because they act as substrates for Maillard reaction to form methylpirazins. Methylpirazin is allegedly associated with chocolate flavor intensity, while the hydrophobic amino acids are considered to be the most potent precursor formation for methylpirazins. According to Atmaja (2013), the reformation of cocoa bean proteins into amino acids and peptides by the activation of enzyme protease, aspartic endoprotease and carboxypeptidase produce amino acids and oligopeptides. Aspartic endoprotease in cocoa beans cut the substrate proteins on hydrophobic amino acid residues to produce oligopeptides which have hydrophobic amino acids. The greater amount of amino acid was produced from the protein

breakdown reaction, the higher protease activity occurred.

CONCLUSION

Hydrophobic amino acids at the end of fermentation increased, hydrophobic acids were detected, namely; alanine, leucine, ileusin, phenilalanin, valine, tyrosine and metheoinin. Hydrophobic amino acids as aroma precursors, include alanine, leucine, ileusin, valine and phenilalanin. The more hydrophobic amino acid were detected in the treatment of inoculum stages. This shows that the addition of inoculum may gradually degrade proteins into a more hydrophobic amino acids.

REFERENCES

Afoakwa, E.O., Budu, A.S., Mensah-brown, H. and dan Felix, J. 2014. Changes in Biochemical and Physico-chemical Qualities during Drying of Pulp Preconditioned and Fermented Cocoa (*Theobroma cacao*) Beans. *International Food Research Journal*.

Anonymous. 2013. Produksi Perkebunan Besar menurut Jenis Tanaman, Indonesia. *Http://www.bps.go.id. Diakses Tanggal 29/03/014*.

Anggrainy, A. 2015. Pengaruh konsentrasi larutan glukosa dan lama perendaman keping biji kakao terhadap sifat kimia, sensoris dan profil senyawa kimia volatil keping biji kakao sangrai, Pasca sarjana Fakultas Teknologi Pertanian Universitas Gadjah Mada.

Ardianingsih, R. 2009. Penggunaan High Performance Liquid chromatography (HPLC) dalam proses analisa deteksi ion. *Berita Dirgantara,* **10**(4): 101-104.

Ardhana, M. and Fleet, G. 2003. The microbial ecology of cocoa bean fermentations in Indonesia. *International Journal of Food Microbiology*, **86**(1-2): 87–99.

Atmaja, P.I.M. 2013. Pengaruh Perlakuan Pendahuluan pada Inkubasi Biji Kakao Kering non fermntasi dalam buffer asetat terhadap ssifat sensoaris pasta cokelat gelap, Thesis Fakultas Teknologi Pertanian Universitas Gadjah mada.

Biehl, B. and Passern, D. 1982. Proteolysis during fermentation-like incubation of cocoa seeds. *Journal of the Science of Food and Agriculture,* **33**: 1280-1290.

Biehl, B., Brunner, E., Passern, D., Quesnelh, V.C. and Adomako, D. 1985. Acidification, Proteolysis and Flavour Potential in Fermenting Cocoa Beans, 583–598.

Camu, Nicholas Camu, N., Winter, T. De, Addo, S. K., Takrama, J.S., Bernaert, H. and Vuyst, L. De. 2008. Fermentation of cocoa beans : influence of microbial activities and polyphenol concentrations on the flavour of chocolate, **2297**(May), 2288–2297.

Crafack, M., Hanna, K., Carl, E.E., Mikael, A.P., Sofie, S., Andreas, B., … Dennis, S.N. 2014. Impact of starter cultures and fermentation techniques on the volatile aroma and sensory profile of chocolate. *Food Research International,* **63**: 306-316.

de Brito, E.S., Pezoa, G., Gallao, M.I., Angelo L.C., Pedro, S.F. and Marcia, R.B. 2000. Structural and chemical changes in cocoa (*Theobroma cocoa* beans l.) during fermentation, drying and roasting. *Journal of the Science of Food and Agriculture,* **81**: 281-288.

Gu, F., Lehe, T., Huasong, W., Yimin,g F., Fei, X., Zhong, C. and Wang, Q. 2013. Comparison of cocoa beans from China, Indonesia and Papua New Guinea. *Foods,* **2**: 183-197.

Harrington, W.L. 2011. *The Effects Of Roasting Time and Temperature On The Antioxidant Capacity Of Cocoa Beans From Dominican Republic, Ecuador, Haiti, Indonesia, and Ivory Coast*. A Thesis Presented for the Master of Science Degree The University of Tennessee, Knoxville.

Jinap, S., Ikrawan, Y., Bakar, J., Saari, N. and Lioe, H.N. 2008. Aroma precursors and methylpyrazines in underfermented cocoa beans induced by endogenous carboxypeptidase. *Journal of Food Science,* **73**: 141-147.

Jinap, S., Lioe, H.N., Yusep, I., Nazamid, S. and Jamilah, B. 2010. Role of carboxypeptidases to the free amino acid composition, methylpyrazine formation and sensory characteristic of under-fermented cocoa beans. *International Food Research Journal,* **17**: 763-774.

Puziah, H., Jinap, S., Sharifah, K.S.M. and Asbi, A. 1998. Changes in free amino acids, peptide-N, sugar and pyrazine concentration during cocoa fermentation. *J. Sci. Food Agric,* **78**: 535-542.

Schwan, R.F. and dan Wheals, A.E. 2004. The microbiology of cocoa fermentation and its role in chocolate quality. *Critical Reviews in Food Science and Nutrition*, **44**(4): 205–221.

Sulistyowati. 1988. Keasaman Biji kakao dan masalahnya. *Pelita Perkebunan,* **3**: 151-158.

Sumarno, S.N., Narsito, I. and Izul, F. 2002. Estimasi kadar protein dalam bahan pangan melalui analisis nitrogen total dan analisis asam amino. *Majalah Farmasi Indonesia,* **13**(1): 34-43.

Misnawi, J.S., Jamilah, B. and Nazamid, S. 2004. Effect of polyphenol concentration on pyrazine formation during cocoa liquor roasting. *Food Chemistry,* **85**: 73-80.

Misnawi, Sri-Mulato, Sukrisno, W., Awad, S. and Sugiyono 2005. Optimasi suhu dan lama penyangraian biji kakao menggunakan penyangrai skala kecil tipe silinder. *Pelita Perkebunan,* **21**(3): 169-183.

Mulono Apriyanto, Sutardi, Supriyadi and Eni Harmayani. 2016. Study on effect of fermentation to the quality

parameter of cocoa bean in Indonesia. *Asian J. Dairy and Food Res.,* **35**(2): 160-163.

Nollet, L.M.L. 1996. *Handbook of Food Analysis, Amino Acid*.

Voigt, J., Biehl, B., Heinrichs, H., Kamaruddin, S., Gaim, M. and Hugi, A. 1994. In-vitro formation of cocoa-specific aroma precursors: aroma-related peptides generated from cocoa-seed protein by co-operation of an aspartic endoprotease and a carboxypeptidase. *Food Chemistry,* **49**: 173-180.

Voigt, J., Biehl, B. and Kamaruddin, S. 1993. The major seed protein of *Theobroma cacao* L. *Food Chemistry,* **47**: 145-147.

Yusep, I., Jinap, S., Jamilah, B. and Nazamid, S. 2002. Influence of carboxypeptidases on free amino acid, peptide and methylpyrazine contents of under-fermented cocoa beans. *J. Sci. Food Agric.,* **82**: 1584-1592.