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Isolation and Characterization of the Dominant Microorganisms Involved In Vietnamese Cacao Fermentation

Tran Trung Hieu[1], Nguyen Thi Hong Loan[2], Nguyen Thuy Huong[3]
[1,2,3]*Faculty of Chemical Engineering, Ho Chi Minh City University of Technology, Vietnam*

ABSTRACT:-*The microbial communities associated with fermentation of Vietnamese cacao were investigated by dependent method. Samples were taken at 5 days of fermentation process from different sites of wooden boxes. The isolates were enumerated on specific media followed by observing Gram staining, microscopic examination, catalase test. The isolated strains were selected in broth media before identifying by 16S or 28S rRNA genes sequence analysis. A total number of 7 strains yeast, 15 strains LAB and 3 strains AAB have been found in Vietnamese cacao fermentation. Six species were selected through high performance of fermentation and identified by molecular characterization. As a results, Saccharomycescerevisiae, Pichiacerevisiae, Lactobacillus brevis, Lactobacillus plantarum, Lactobacillus casei, Gluconacetobacternataicola were presented in microbial communities of Vietnamese cacao fermentation. These also suggested that a potential starter cultures for controlling cacao fermentation processes.*

KEYWORDS:*-Cacao fermentation, yeast, LAB, AAB, communities*

I. INTRODUCTION

Cacao beans are raw material for producing chocolate and cacao powder.West Africa, Latin America and Southeast Asia are the major cacao producer regions [1].In Vietnam, most of cacao trees (*Theobroma cacao* L.) are cultivated in the Western Highland, the East and West of South of the country. To obtain the desire characteristics, raw cacao beans have to be fermented, dried and roasted[1]. Therefore, the quality of cacao beans is influenced by origin and cultivar of cacao trees, fermentation, drying, roasting and further process.

Fermentation plays a vital role in producing metabolic products which are precursors for development of flavor and odor. After harvest, cacao beans are taken out from the pods and fermented in wood boxes or heap. This process lasts from 5 to 7 days under action of various microbial species which is reported. Firstly, the pulp which covers cacao beans is removed and drained by yeast. This leads to the oxygen levels increase which helps bacteria including lactic acid bacteria (LAB) and acid acetic bacterial (AAB)to develop as well as growth of aerobic spore-forming bacteria occurs then. During this time, the temperature rises to about 50^{o}C. The heat and acid result in complex biochemical reactions occur within the bean which leads to produce the precursors. In the end of process, some of filamentous develop on the surface[2].

Although the roles of the microorganisms have not been clearly understood, yeast, LAB and AAB are the microbial succession in the fermentation process reported by many studies[2]. In present, fermentation takes place under uncontrolled environmental conditions that leads to variable quality of the product, acidity or off-flavors. To solve the problems, fermentation stage and the microbial communitieshave been researched in many countries[3, 4]. Besides, preliminary experiments using defined starter cultures have been done[5, 6]. However, the microbial communities fermenting cacao beans in Vietnam is not fully reported. The microbial communities are influenced by the geographical location of the plantation. Theobjective of this study was to generally investigate the dynamics of the local microbial community during cacao fermentation by traditional method and molecular technique. In the other hand, some strains are selected and characterized to develop a defined starter cultures for improving and controlling quality of cacao beans.

II. MATERIAL AND METHOD

a. Sampling

Samples are taken in Ben Tre which is one of province having largest area of cacao tree in Vietnam. Every 24 hours approximately fifty grams fermented cacao beans were sampled during five days of fermentation process. The samples were selected at the edges and center of wooden box for both on surface and

approximately 30 cm from the surface. The samples are placed in sterile plastic bags and transferred to laboratory.

Isolation and characterization of microorganisms by traditional method

Twenty grams of fermented cacao beans was added to 180 ml sodium chloride 0.9% (w/v) and homogenized for 5 min, followed by serial dilutions. Yeasts were enumerated on Sabouraud agar containing 0.1% (w/v) Chloramphenicol. LAB was enumerated by surface inoculation on MRS agar containing 0.1% Natamycine. AAB were enumerated by surface inoculation on YPGD agar (5 g/l glucose, 5 g/l yeast extract, 5 g/l peptone, 5 g/l glycerol, 40 ml/l ethanol, 40 g/l calcium carbonate, 20 g/l agar [pH=5.6]). PCA agar (5 g/leptone, 1 g/l glucose, 2.5 g/l yeast extract, 20 g/l agar [pH=7]) was used as a general medium for aerobic microorganisms. Diluted samples were spread by surface technique and incubated at 30^{o}C for yeast and 37^{o}C for LAB and AAB from 3 to 4 days. The number of CFU was recorded for observing the dynamic of microbial community. Each colony type was characterized morphology and purified by streak plate technique.

Yeast colonies were determined morphology, spore formation, type of division. The bacterial colonies were followed by many steps: Gram staining, microscopic examination, spore formation and catalase test.

b. Evaluation the fermentation performance and selection to characterize isolated microorganisms by molecular technique

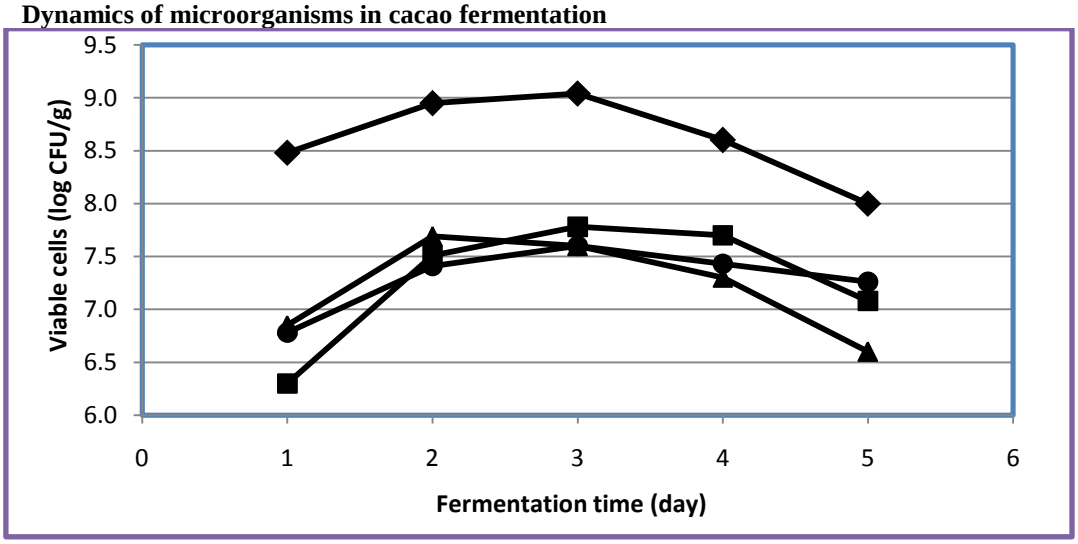
To investigate and select better strains for starter cultures, several growth parameters were evaluated on broth media. Yeasts were cultured in Sabouraud broth at 30^{o}C. The pH value and metabolite concentration were determined at 24 h and 48 h. The amounts of alcohol, density of cell, pH value and CO_2 were determined. The amounts of alcohol were quantified by using chemical method which used potassium dichromate[1]. The pH was measured using a pH-meter. Density of cell was determined through OD value by a spectrophotometer. Finally, the amounts of CO_2 were quantitatively evaluated by inverting Durham tubes.

LAB and AAB werecultured onMRS broth and GYC broth (50 g/l glucose, 10 g/l yeast extract [pH = 5.6]), respectively at 37^{o}C. Density of cell, pH and total acid number were determined at 24 h and 48 h. Density of cell and pH were measured by spectrophotometer and pH-meter, respectively. The total acid was determined by titration using NaOH 0.1N.

The isolated microorganisms which had high fermentation performance were selected and characterized by sequence analysis of the full-length 16S rRNA gene. The microorganisms were store at -20 ^{o}C in broth media containing 15% (w/w) glycerol.

III. RESULT AND DISCUSSION

a. Dynamics of microorganisms in cacao fermentation



Total plate count (PCA counts,◆), Yeast (Sabouraud counts,▲), LAB (MRS counts,■), AAB (YPGD counts,●)

Figure 1: Dynamics of microorganisms during cacao beans fermentation

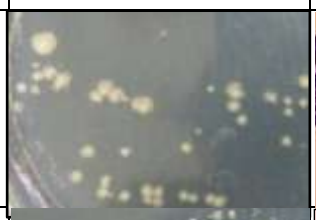
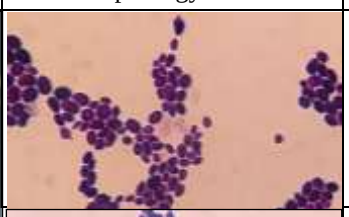
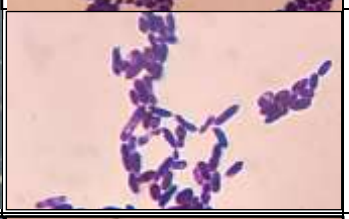
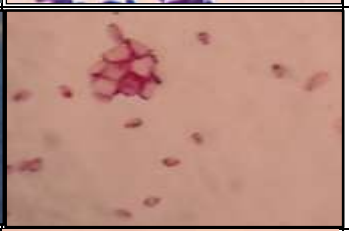
The change of composition of microbial communities in cacao fermentation are shown in Fig.1.The total population increased significantly from 8.48 log CFU/g at the first day to 8.95 log CFU/g in the second day and reached maximum 9.04 log CFU/g at third day. The population decreased considerably to 8 log CFU/g at the 5th day of fermentation process. Yeast counts of 6.85 log CFU/g were present at the beginning of

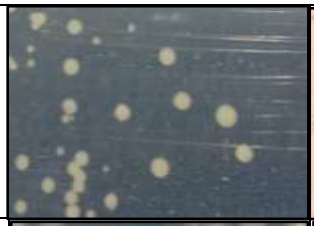
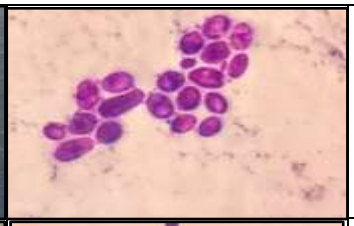
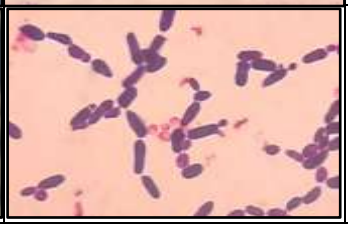
fermentation. Population of yeasts reached maximum 7.69 log CFU/g and decreased gradually after that. The population of yeasts was7.6 log CFU/g and 7.3 log CFU/gat the 3rd and 4th day, respectively. There was a dramatic decrease in yeast counts at the end of fermentation (6.6 logCFU/g). The LAB counts also fluctuated during fermentation. The population increased gradually from 6.3 logCFU/g at the 1stday to 7.51logCFU/g at 2nd day and reached maximum at the 3rd day (7.78 logCFU/g). The LAB counts dropped to 7.7 logCFU/g and 7.08logCFU/g at the 4th and 5th day, respectively. The AAB counts also performed a same rule. The population of AAB was 6.78 logCFU/g at the beginning, rose to 7.41log CFU/g at next day and reached a peak (7.6 log CFU/g) at the 3rd day. The population decreased slightly at the end of fermentation. The population was 7.43 logCFU/g and 7.26log CFU/g at the 4th and 5th day, respectively.The chart showed that yeast were the dominant microorganism at the onset of fermentation and were decline tendency on next day. The LAB and AAB increased on initial days and were dominant species on mid stage of the process. The results were suitable with previously reported studies [2, 4].

b. Isolation of yeast during cacao fermentation

The yeasts have been reported to be dominant microorganisms at the beginning of fermentation. Through the results of initial characterization (morphological colonies, microscopic examination and division), there were 7 yeasts to be isolated and selected from SabouraudAgardescribed at Table 1.

Table I: Morphological colony and cell of isolated yeasts

Yeast	Morphological colony	Morphology of cell	Describe
M1			Colony has white color Cell has spherical shape and reproduces bybudding and transverse division
M2			Colony is white color and has flat surface. Cell has long egg shape and reproduces by budding and transverse division
M3			Colony is black color and has feather around Cell has egg shape. The size is bigger than others
M4			Colony has white color Cell has long shape and reproduces by budding and transverse division
M5			Colony is white color and has discontinuous layer Cell has egg shape and reproduces by budding and transverse division

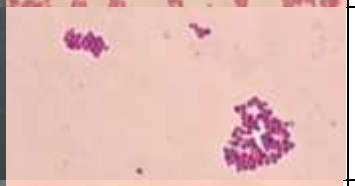
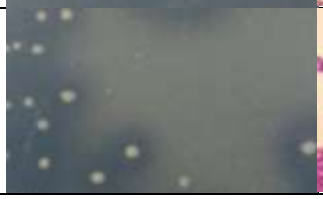
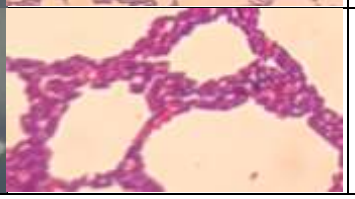
M6			Colony is white color, and has continuous layer, spherical shape. Cell has spherical shape and reproduces by transverse division
M7			Colony is white color, and has thin layer, spherical shape Cell has egg shape and reproduces by budding and transverse division

c. Isolation of LAB during cacao fermentation

LAB has also reported by many studies to be dominant in microbial communities in cacao fermentation. Fifteen LAB isolates were identified from MRS agar. The majority of isolates had rod shape, Gram positive, catalase negative. The results are described on Table 2.

Table II: Morphological colony and cell of isolated LAB

LAB	Morphological colony	Morphology of cell	Describe
L1			Colony has white color hand spherical shape The cell has spherical shape, exist alone, Gram positive
L2			Colony has slightly yellow, glossy surface The cell has rod shape, exists alone, Gram positive
L3			Colony has yellow color, thin layer The cell has rod shape, exists alone, Gram positive
L4			Colony has round shape, white color, thin layer and convex surface The cell is small and has rod shape, exist as group, positive Gram
L5			Colony has white color, continuous layer The cell has long rod shape, exist alone, positive Gram

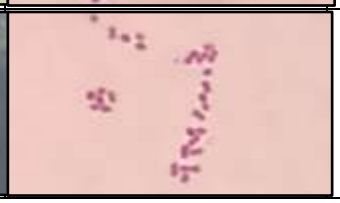
L6			The colony is round, ivory white color, glossy surface Cell is rod shape, exist as dashed lines, positive Gram
L7			The colony is round and has white color of milk, glossy surface. The cell has rod shape, exist as groups, positive Gram
L8			Colony has round shape, ivory white color and glossy surface Cell is spherical, exist as group and positive Gram
L9			Colony has round shape, ivory white color and glossy surface Cell has rod shape, exists alone, positive Gram
L10			The colony has round shape, ivory white color and glossy surface Cell has rod shape, exists as group, positive Gram
L11			The colony has round shape, glossy surface and ivory color Cell has small rod shape, exist as group, positive Gram
L12			The colony has round shape, yellow and glossy surface, thin layer Cell has rod shape, exist alone and positive Gram
L13			The colony has round shape, convex and glossy surface. Cell has rod shape, exists as group and positive Gram
L14			The colony has round shape, convex and glossy surface. Cell has rod shape, exists as group and positive Gram

L15			The colony has round shape, glossy surface, broken layer Cell has rod shape, exists alone and positive Gram

d. Isolation of AAB during cacao fermentation

The role of AAB in cacao fermentation has been described by many studies. Three AAB had been isolated. The majority of isolates was rod or oval shape, negative Gram and positive catalase. Results are described on Table.3.

Table III: Morphological colony and cell of isolated AAB

AAB	Morphological colony	Morphology of cell	Describe
A1			The colony has round shape and ivory white color The cell is spherical, exists as group, negative Gram, positive catalase
A2			The colony has round shape, slight yellow color and continuous layer The cell has oval shape, exists alone,negative Gram, positive catalase
A3			The colony has round shape, slightly yellow color and glossy surface The cell has oval shape, exist alone, negative Gram and positive catalase

e. Selection of isolated microorganisms through performance of fermentation and characterization by molecular technique

Yeast: The fermented performance of seven yeasts is shown on Table 1. Ethanol was produced along with release of CO_2. The species M1 and M6 is stronger CO_2 release than others and exhibited surprisingly ability to produce ethanol. For M1, the ethanol concentration was 3.162 and 5.945 at 24h and 48h, respectively. For M6, the ethanol concentration is slightly lower (3.087 at 24h and 5.870 at 48h). The results of ODand pH value also exhibited strongly ability of M1 and M6to grow in broth media and produce acids. The OD value of M1 and M6 is approximately 1.587 (24h), 1.652 (48h) and 1.5 (24h), 1.548 (48h), respectively. The pH of media is 4.05 (24h) and 3.9 (48h) for M1. The yeast M6 decreased significantly pH of media to 4.03 (24h) and 3.57 (48h). In conclusion, based on the high ethanol concentration and CO_2 and ability to consumesubstrate and producemetabolites, the yeast M1 and M6 was selected to characterize by molecular technique.

Table IV: The performance of fermentation of isolated yeasts after 48 hours

		M1	***M2***	***M3***	***M4***	***M5***	***M6***	***M7***
CO_2 release		**++++++**	++	++	+++	++++	**++++++**	+
Ethanol	*24h*	**3.162**	1.415	1.408	2.234	2.862	**3.087**	0.900
	48h	**5.945**	2.618	2.704	3.545	4.312	**5.870**	1.415
OD	*24h*	**1.587**	0.193	0.191	0.256	0.300	**1.500**	0.222
	48h	**1.652**	0.235	0.339	0.398	0.543	**1.548**	0.327
pH	*24h*	**4.05**	4.21	5.02	5.08	5.20	**4.03**	4.93
	48h	**3.90**	4.06	4.93	4.96	5.02	**3.57**	4.84

+: ability to release CO_2 of yeasts

The yeasts have been investigated by many studies and different results were reported. *Hanseniasporaguilliermondii, Pichiakudriavzevii, Kluyveromycesmarxianus* was major species in cacao fermentation in Australia [7].*Candida krusei, P. membranifaciens, P. kluyveri, Hanseniasporaguilliermondii, Trichosporonasahii* was dominant yeasts in cacao fermentation in West Africa [8]. In this paper, two strains of yeast M1 and M6 was analyzed 28S and 18SrRNA gene sequence. The results demonstrated that strain M1 was *Saccharomyces cerevisiae*and M6 was *Pichiacerevisiae*. The gene sequences were described in Fig.2 and Fig.3.

TCCTGTTCCAAGGAACATAGACAAGGAACGGCCCCAAAGTTGCCCTCTCCAAATTACAACTCG
GGCACCGAAGGTACCAGATTTCAAATTTGAGCTTTTGCCGCTTCACTCGCCGTTACTAAGGCAA
TCCCGGTTGGTTTCTTTTCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTACTCCTACCTGAT
TTGAGGTCAAACTTTAAGAACATTGTTCGCCTAGACGCTCTCTTCTTATCGATAACGTTCCAAT
ACGCTCAGTATAAAAAAAGATTAGCCGCAGTTGGTAAAACCTAAAACGACCGTACTTGCATTA
TACCTCAAGCACGCAGAGAAACCTCTCTTTGGAAAAAAAACATCCAATGAAAAGGCCAGCAAT
TTCAAGTTAACTCCAAAGAGTATCACTCACTACCAAACAGAATGTTTGAGAAGGAAATGACGC
TCAAACAGGCATGCCCCCTGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTC
ACGGAATTCTGCAATTCACATTACGTATCGCATTTCGCTGCGTTCTTATCGATGCGAGAACCAA
GAGATCCGTTGTTGAAAGTTTTTAATATTTTAAAATTTCCAGTTACGAAAATTCTTGTTTTTGAC
AAAAATTTAATGAATAGATAAAATTGTTTGTGTTTGTTACCTCTGGGCCCCGATTGCTCGAATG
CCCAAAGAAAAAGTTGCAAAGATATGAAAACTCCACAGTGTGTTGTATTGAAACGGTTTTAATT
GTCCTATAACAAAAGCACAGAAATCTCTCACCGTTTGGAATAGCAAGAAAGAAACTTACAAGC
CTAGCAAGACCGCGCACTTAAGCGCAGGCCCCGGCTGGACTCTCCATCTCCTGTCTTCTTGCCC
AGTAAAAAGCTCTCATGCTCTTGCCAAAACAAAAAAATCCATTTTCAAAATTATTAAATTTCTT
TAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTAGTTCCTCTAAATGACC
AAGTTTGTCCAAATTCTCCGCTCTGAGATGGAGTTGCC

Figure 2: The 28S rRNA gene sequence of strain M1

TGCGTGAGCGCACAAAACACATAAACCGTGAGTAATTTTTGTCGAAACTTGAAAAAAAAAATACAA
AACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAGCGCAGCGAAATGCGATACCTAGT
GTGAATTGCAGCCATCGTGAATCATCGAGTTCTTGAACGCACATTGCGCCCGTCGGTATTCCGGCG
GGCATGCCTGTCTGAGCGTCGTTTCCTTCTTGGAACTTTTGTTAAAGAAAGATCCAGAGCTGGCCGT
CACTGGCCCGGCCGAAAAGAAACGTTGCGGACGAAGCGAACTACATCGGGACGCTTTGGCCCCCG
AGCGAAAATATATCATTGAGCTCGACCTCAGATCAGGTAGGAGTACCCGCTGAACTTAAGCATAT
CAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCCCAGTAGCGGCGAGTGAAGCGGCAAGAG
CTCAGATTTGAAATCGTGTTTCGGCACGAGTTGTAGAGTGTAGGCCGGGAGTCTCTTGTGGAGCGC
GGTGTCCAAGCCTTGGAACAGGGTGCCTGAGAGGGTGAGAGCCCCGTAGGGTGCTGCGCGAAGCT
TTGAGGCCCTGCTGACGAGTCGAGT

Figure 3:the 28S RRNA gene sequence of strain M6

Saccharomyces cerevisiae has previously reported to play a vital role in cacao fermentation by many studies[3, 4,9]. Although the microbial communities is usually different the countries, fermentation method (heap, tray, box), *Saccharomyces cerevisiae* was usually found in most of process. *Saccharomyces cerevisiae* was found in the mid-phase of cacao fermentation by box in Malaysia[9]. *Saccharomyces cerevisiae* also was identified in cacao fermentation of Ghana or Brazil [3]. This is first study in Vietnam which identified *Saccharomyces cerevisiae* in the cacao fermentation by wooden box.

Although there were several studies previously reported about the yeast order *Pichia* sp. in cacao fermentation such as were*Pichiamembranifaciens*[3], there were no studies reported about *Pichiacerevisiae*[2]. It may be the difference between geographical location and *Pichiacerevisiae* is typical yeast in Vietnamese cacao fermentation. However, more studies should be performed to verify the role of *Pichiacerevisiae*.

LAB. On the MRS broth, L7, L8, L10, L11, L13 and L14 were species which have ability to grow better than others (Table 5). L14showed the highest OD value (2.278 and 2.292 for 24h and 28h respectively), followed by L8 and L10 (2.235 (24h) and 2.263 (48h) for L8, (2.193 (24h) and 2.292 (48h) for L10). The pH value changed significantly in broth media of L7 (pH = 4.16), L8 (pH=4.18), L10 (pH= 4.18), L11 (pH=4.20) and L14 (pH=4.20) after 48h of fermentation. L7, L8, L10, L11, L13 and L14 also exhibited high fermentation efficiencies through producing organic acids. L7, L8, L10 and L14 showed the highest total acid contents (0.2 MOL) after 48h of fermentation. L8, L10 and L14 exhibited the ability to grow in media broth and produce organic acids as well as decrease pH of media. L8, L10 and L14 were chosen to characterize by molecular identification.

Table V: The performance of fermentation of isolated LAB after 48 hours

LAB	OD		pH		Total acid (MOL)	
	24h	48h	24h	48h	24h	48h
L1	0.069	0.089	6.02	6.00	0.50	0.50
L2	0.145	0.324	6.11	5.91	0.50	0.50
L3	0.011	0.084	6.18	6.06	0.40	0.40
L4	0.138	0.280	6.13	6.00	0.45	0.45
L5	0.762	0.905	5.22	4.67	0.55	1.40
L6	0.375	0.476	5.96	5.91	0.40	0.50
L7	**2.169**	**2.229**	**4.33**	**4.16**	**1.10**	**2.20**
L8	**2.235**	**2.263**	**4.37**	**4.18**	**1.10**	**2.20**
L9	1.765	2.237	5.23	4.80	1.00	1.40
L10	**2.193**	**2.292**	**4.35**	**4.18**	**1.20**	**2.20**
L11	**2.150**	**2.226**	**4.32**	**4.20**	**1.20**	**2.15**
L12	0.031	0.087	6.05	5.96	0.30	0.50
L13	**2.173**	**2.340**	**4.32**	**4.23**	**1.20**	**2.15**
L14	**2.278**	**2.292**	**4.34**	**4.20**	**1.10**	**2.20**
L15	0.555	0.832	5.35	4.86	0.80	1.25

Several LAB were also identified in cacao fermentation. *Lactobacillus fermentum, Lactobacillus plantarum, Leuconostocpsedoficulneum, Pediococcusacidilactici* was reported in Ghanaian cacao fermentation[4]. *Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus pentosus* contributed to the cacao fermentation in Malaysia [9]. *L. brevis, L. fermentum, L.plantarum, Pediococcusacidilactici* was described in cacao fermentation in Nigeria [10]. In this study, L8, L10 and L14 were identified by 16S rRNA genes sequence analysis. It was demonstrated that L8, L10 and L14 were *L. brevis*, *L. plantarum* and *L. casei*, respectively. The results were described in Fig.4, Fig.5 and Fig.6.

CCTGGCTCAGGACGAACGCTGGCGGCATGCCTAATACATGCAAGTCGAACGAGCTTCCGTTGAA
TGACGTGCTTGCACTGATTTCAACAATGAAGCGAGTGGCGAACTGGTGAGTAACACGTGGGGA
ATCTGCCCAGAAGCAGGGGATAACACTTGGAAACAGGTGCTAATACCGTATAACAACAAAATC
CGCATGGATTTTGTTTGAAAGGTGGCTTCGGCTATCACTTCTGGATGATCCCGCGGTATTAGTTA
GTTGGTGAGGTAAAGGCCCACCAAGACGATGATACGTAGCCGACCTGAGAGGGTAATCGGCCA
CATTGGGACTGAGACACGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGG
ACGAAAGTCTGATGGAGCAATGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTT
GTTAAAGAAGAACACCTTTGAGAGTAACTGTTCAAGGGTTGACGGTATTTAACCAGAAAGCCA
CGGCTAACTACGTGC

Figure 4: The 16S rRNA gene sequence of strain L8

AAACCCTTCTTCACTCACGCGGCGTTGCTCCATCAGACTTTCGTCCATTGTGGAAGATTCCCTAC
TGCTGCCTCCCGTAGGAGTTTGGGCCGTGTCTCAGTCCCAATGTGGCCGATTACCCTCTCAGGTC
GGCTACGTATCATTGCCATGGTGAGCCGTTACCCCACCATCTAGCTAATACGCCGCGGGACCAT
CCAAAAGTGATAGCCGAAGCCATCTTTCAAACTCGGACCATGCGGTCCAAGTTGTTATGCGGTA
TTAGCATCTGTTTCCAGGTGTTATCCCCGCTTCTGGGCAGGTTTCCCACGTGTTACTCACCAGTTC
GCCACTCACTCAAATGTAAATCATGATGCAAGCACCAATCAATACCAGAGTTCGTTCGACTTGC
ATGTATTAGGCACGCCGCC

Figure 5: The 16S rRNA gene sequence of strain L10

TTACCGCGGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTGGATACCGTCACGCCGACA
ACAGTTACTCTGCCGACCATTCTTCTCCAACAACAGAGTTTTACGACCCGAAAGCCTTCTTCAC
TCACGCGGCGTTGCTCCATCAGACTTGCGTCCATTGTGGAAGATTCCCTACTGCTGCCTCCCGT
AGGAGTTTGGGCCGTGTCTCAGTCCCAATGTGGCCGATCAACCTCTCAGTTCGGCTACGTATCA
TCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATACGCCGCGGGTCCATCCAAAAGCGAT
AGCTTACGCCATCTTTCAGCCAGAACCATGCGGTTCTTGGATCTATGCGGTATTAGCATCTGTT
TCCAAATGTTATCCCCCACTTAAGGGCAGGTTACCCCACGTGTTACTCACCCGTCCGCCACTCG
TTCCATGTTGAATCTCGGTGCAAGCACCGATCATCAACGAGAACTCGTTCGACTTGCATGTATT
AGGCACGCCGCCAGCGTTCATCCTGAGCCAGG

Figure 6: The 16S rRNA gene sequence of strain L14

L. plantarum was detected in cacao fermentation in Brazil[2, 11], Ghana [4] and Malaysia [9]. This strain may be popular in most of cacao fermentation process. *L. brevis*and *L. casei* was less popular than *L. plantarum*. Two these strains were reported in Brazilian or Nigerian cacao fermentation [2]. The results indicate that *L. plantarum*, *L. brevis*and *L. casei* are indigenous to the fermentation of cacao in Vietnam.

AAB.The results demonstrated the overall ability of AAB isolates to consume and metabolite substance in GYC broth. The strain A3 exhibited the highest performance of fermentation (Table 6). The OD value is 0.292 and 1.136 for 24h and 48h, respectively. pH of media dropped dramatically to 3.28 after 48 hours. The strain A3 also produced high acid contents approximately 0.8 MOL after 48h.The strain A3 was selected to characterize by molecular characterization.

Table VI: The performance of fermentation of isolated AAB after 48 hours

AAB		***A1***	***A2***	***A3***
OD	*24h*	0,010	0,183	**0,292**
	48h	0,040	0,442	**1,136**
pH	*24h*	5,60	5,79	**3,73**
	48h	5,39	6,29	**3,28**
Total acid (MOL)	*24h*	0,10	0,15	**0,8**
	48h	0,15	0,15	**0,8**

The AAB strains play a vital role to oxidize ethanol to acid acetic and further oxidation to carbon dioxide and water. Many *Acetobacter*sp. has been identified in cacao fermentation. *Acetobactertropicalis*and was found in Mexican and Brazilian cacao fermentation [11, 12]. In this study, the 16S rRNA gene sequence analysis showed that A3 was similar with *Gluconacetobacternataicola*(Fig.7). There are no studies previously reported about this strain. It proves that geographical site significantly affects to microbial communities of cacao fermentation.

TGGCTCAGAGCGAACGCTGGCGGCATGCTTAACACATGCAAGTCGCACGAACCTTTCGGGGTT
AGTGGCGGACGGGTGAGTAACGCGTAGGGATCTGTCCACGGGTGGGGGATAACTTTGGGAAAC
TGAAGCTAATACCGCATGACACCTGAGGGTCAAAGGCGCAAGTCGCCTGTGGAGGAACCTGCG
TTCGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGATGATCGATAGCTGGTCTGAGAGG
ATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
ATTGGACAATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGTTTTCGGATTGT
AAAGCACTTTCAGCGGGGACGATGATGACGGTACCCGCAGAAGAAGCCCCGGCTAACTTCGTG
C

Figure 7: The 16S rRNA gene sequence of strain M3

IV. CONCLUSION

Seven yeast isolates, fifteen LAB isolates and three AAB isolates were identified from cacao fermentation process by wooden box method. Traditional methods and molecular technique were used to characterize the selected strains which exhibited high performance fermentation in broth media. The results demonstrated that *Saccharomycescerevisiae*, *Pichiacerevisiae*, *Lactobacillus brevis*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Gluconacetobacternataicola*associated with the microbial communities of cacao fermentation in Vietnam. This study allowed us to have a better overview about ecology of the indigenous microorganism. It also suggested that these species should be tested for a potential starter cultures to improve and control quality of cacao bean.

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