

Isolation and Identification of Anaerobic Yeasts and Bacteria from Weasel Coffee for Cellulase and Pectinase

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ABSTRACT:- Weasel coffee possesses an unique flavor which was an incredibly delicious combination of musty, syrup, chocolate and high extraction soluble solids content. However, low quantitative production of this traditional fermented coffee increased their cost. As a result, there was a steaming necessary of a new method to process coffee in industry and out of biosystem by enzymes or microbes to make high quality and productive coffee yield with a much lower cost. Thus this was the primary study aimed to isolate yeast and anaerobic bacteria that produced cellulase and pectinase from Lam Dong weasel coffee. Fifteen strains of anaerobic microorganism including four bacteria and eleven yeasts were isolated on malt medium in the anaerobic condition. All isolated microorganisms could produce cellulase and pectinase. Only 6 strains of isolated microorganisms with very fast growth rate, and highest evaluation of cellulase and pectinase were chosen to be identified with DNA sequencing method. Three highest cellulase producer strains were identified as *Torulaspora delbrueckii*, *Hanseniaspora uvarum*, *Candida boidinii*, while *Wickerhamomyces anomalus*, *Enterobacter sacchari*, *Pantoea vagans* accounted for the 3 highest pectinase production microorganisms found in this study.

Keywords:- anaerobic, bacteria, yeasts, cellulase, isolation, identification, pectinase, weasel coffee

I. INTRODUCTION

Weasel coffee was one of the best coffee ever known. Eaten by palm civet *Paradoxurus hermaphrodites* - found in Indonesia, Vietnam, etc., the coffee cherry fruit was completely digested, whereas the actual coffee beans were not digested and excreted in their feces. The internal fermentation by different digestive enzymes has created the unique flavor of the beans, which has been described as earthy, musty, syrupy, smooth, and rich with both jungle and chocolate [1]. However, the production of this traditional fermented food from palm civet was limited, in which there were only 700 kg of this special coffee produced each year in the world, moreover, farm culture at family scales gave out about under 200 kg/year. These events raise prices of weasel coffee on the market, to extremely high prices, approximately 450-3000 USD/kg. Thus bringing this coffee to the larger scale and lower cost of manufacturing without ruin its flavor received much attention from researchers. Understandings of physical and chemical properties of the fermentation process, the mechanisms, chemicals, and affecting factors to improve the quality of weasel coffee production and reduce cost could be done in many different way, in which microbial uses were among the most potential and economic way... Fermentation by microorganisms found from civet's intestine could be very potential because they produced large amount of enzyme, such as cellulase or pectinase. Those enzymes could alter chemical ingredient in cell wall of coffee beans or epicarp, mesocarp, endocarp, and integument, which were composed of cellulose, pectin, and other substances; and increased the extraction of soluble solid content from coffee. *Aspergillus sp* and *Streptomyces sp* were known to be pectinase producer [2], while cellulase was secreted by *Aspergillus*, *Cellulomonas*, *Streptomyces*, etc. [3].

In this study, the anaerobic bacteria and yeasts in weasel digestive system were isolated for the weasel coffee production. Then they were surveyed for pectinase and cellulase production of microorganisms, which contributed to the removal of the mucilage of coffee beans under anaerobic fermentation.

II. MATERIALS AND METHODS

2.1. Reagents, apparatus, and medium preparation

Weasel coffee was collected at Trai Ham, Da Lat, Lam Dong. Wet fecus samples were collected immediately after released from civet to prevent other microorganisms contaminants. Dry samples were used as control containing other microorganisms in environment. Malt medium, used for isolating microorganisms, was composed of glucose (4.2-7.6%); fructose (0.6-1.2%); maltose (32-38%); sucrose (0.9%); maltotriose (1.6-7.0%); protein (3.0-5.6%); fat (0.1%); and ash (1.1-1.5%) [4]. Carboxyl methyl cellulose (CMC) and pectin (poly-D-galacturonic acid methyl ester) were substrates for cellulase and pectinase activation and production. All chemicals used were at analytical rates.

Microbial manipulations were performed in safety cabinet (No. NU-480-400E of America), and cultured in incubator (WIG-105 of Korea). Light absorbance was quantified with spectrophotometer (Genesys 10S UV-Vis of America).

2.2. Culture of microorganisms from weasel coffee

100 ml of sterilized malt solution (200 g germinated malt, 1% of ammonium sulfate, 1.8% agar in 1000 ml water and adjust to 10⁰Brix) [5] was added to weasel coffee samples, then diluted 10⁻⁶ – 10⁻⁸ by Most Probable Number (MPN) method. 60µl of this diluted solution was well spread on malt agar plates, followed by an anaerobic incubation at 24 - 26°C. Plates were checked daily for growth of microorganisms. Three control agar plates were opened to catch the microorganisms from environment for 15min, then incubated as conditions above.

2.3. Isolation of anaerobic yeasts and bacteria from weasel coffee

The microorganism plates contained numerous colonies from different strains. Independent colony was identified by different shapes, colors, and surface, and rising forms of yeasts, bacteria, and molds. Anaerobic yeasts and bacteria were transferred to other fresh malt agar plates until one plate contain one kind of colony. The unique agar plate also covered and incubated with condition of anaerobic [5]. Morphological properties of colonies and the microorganisms (observed under microscope) would be used to distinguish different microbial strains.

2.4. Enzyme production of anaerobic yeasts and bacteria

Medium for cellulase production contained of 70% of CMC (1%) in citrate buffer pH 4.8 and 30% of malt solution. Pectinase production was carried out in medium including 30% of malt and 70% of pectin (1.5%) in acetate buffer pH 6.0. All media were autoclaved at 121°C for 15 min and cooled down to room temperature before inoculation.

30ml of medium was inoculated with 1ml of stock of 3 day old culture of microorganisms, followed by a 7-day incubation at room temperature (25°C). The supernatant from centrifugation of the culture at 10,000 rpm, 4°C, and 20min contained necessary enzyme [6], and was used for next steps.

2.5. Quantitative evaluation of cellulase activity

1 ml of 1% CMC and 1 ml of enzyme solution was added to test tube, followed by a 10-minute incubation at 40°C. Then each test tube was added 4 ml of DNS solution, covered, vortexed carefully and incubated in boiling water for 5 min. Absorbance was measure at 540 nm. Control sample was prepared by adding 1 ml of enzyme sample, 4 ml of DNS, incubating at 40°C, then adding 1 ml of CMC 1% and putting in boiling water. Glucose was used as standard sugar to build calibration curve. Cellulase activity was calculated from the equation (1):

$$\text{Cellulase activities (U/ml)} = (A_E - A_C) \times F \times \frac{1000}{180} \times \frac{1}{10 \text{ min}} \times \frac{1}{1 \text{ ml}} \times D \quad (1)$$

With A_E: Absorbance of enzyme sample, A_C: absorbance of control, F: glucose coefficient (found from glucose concentration – OD standard curve), D: dilution coefficient, 1000: transfer from mg into µg, 180: molecular weight of glucose; 10: time that enzyme react with substrate, 1: volume of enzyme.

One unit of cellulase (1 U) was the amount of enzyme hydrolyzing CMC and releasing 1 µmol of glucose in reduced form in the conditions above.

2.6. Quantitative evaluation of pectinase activity

1 ml of pectin substrate and 0.5 ml of enzyme was incubated at 40°C for 15min before adding 3 ml of DNS followed by an 5 minute incubation in boiling water. The solution was diluted with 20 ml of distilled water, and measured absorbance at 540 nm [12]. Galacturonic acid (2mg/ml) was used as standard for pectinase activity. Pectinase activity was calculated following the formula (2):

$$\text{Pectinase activities (U/ml)} = m_{\text{galacturonic acid}} \times \frac{1000}{212.12} \times \frac{1}{6} \times D \quad (2)$$

Where: D: dilution factor, 212.12: molecular weight of galacturonic acid (mg/mol), 6: reaction time (min), 1000: coefficient in changing unit from mM to µM.

One unit of pectinase activity or galacturonase unit ($U = \mu\text{M}/\text{min}$) was defined as $1\mu\text{mol}$ of galacturonic acid released per minute [12].

2.7. Identification of isolated anaerobic yeasts and bacteria by DNA sequencing method

Fast growing, high enzymatic production pure culture of anaerobic yeasts and bacteria from previous experiments were chosen for microbial identification by PCR and sequencing 16S rRNA subunit for bacteria and 28S subunit for yeasts; and searched in BLAST [8]. All procedure was carried out by Nam Khoa Company.

2.8. Statistical analysis

All experiments were triplicated. One way ANOVA correlation analyses were performed by using the SPSS for windows with $P < 0.05$.

III. RESULTS & DISCUSSIONS

3.1. Isolation of anaerobic yeasts and bacteria from weasel coffee

Microorganisms from civet feces were isolated by spreading on malt agar and serial transfers until typical colonies observed. From dozens of colony plates, 11 strains of anaerobic yeasts and another 4 anaerobic bacteria were collected and enumerated from WAN1 to WAN15. Morphological features of colonies helped to distinguish each kind of bacteria and yeast strain. Table I summarized morphology, color, size, growth rate of each strain [9]. During fermentation stage, growth rate was important since it decided fermentation time to get the enzyme product.

Table 1: Morphology of isolated anaerobic yeasts and bacteria

No.	Code	Growth rate	Chromogenesis			Opacity	Elevation	Surface	Texture
			Color	Center	Edge				
1	WAN 1	Fast	W	Or	W	O	U	S	Bu
2	WAN 2	Normal	W	W	W	O	U	S	Bu
3	WAN 3	Normal	W	P	W	O, TE	U	S	Bu
4	WAN 4	Normal	YB	P	YB	O	U	S	Bu
5	WAN 5	Fast	W	P	YB	O	U	S	Bu
6	WAN 6	Normal	B	B	B	O	C	G	Bu
7	WAN 7	Fast	W	W	YB	T	F	R	Bu
8	WAN 8	Normal	Y	Y	W	TC	F	R	Bu
9	WAN 9	Fast	W	Y	W	TE	F	S, RC	Bu
10	WAN 10	Fast	W	W	W	O	F	S, RC	Bu
11	WAN 11	Fast	B	B	B	O	F	S	Bu
12	WAN 12	Fast	W	W	B	O	Ra	S	Bu
13	WAN 13	Fast	B	W	B	O	Ra	S	Bu
14	WAN 14	Slow	W	W	B	O, TC	Ra	S	Bu
15	WAN 15	Fast	W	Y	Y	O, TE	F	RE	Bu

Fast: colonies appeared after 12hours, Normal: colonies appeared after 24 hours, Slow: colonies appeared after 36 hours; W: white; Or: orange, P: pink; YB: yellow brown; B: brown; Y: yellow; O: opaque; T: translucent; TE: translucent at edge, TC: Translucent at center; U: umbonate; C: convex; F: flat; Ra: raised; S: smooth; R: Rough; G: Glistening; RE: rough at edge; RC: rugose at center; Bu: butyrous. Isolation of single and typical colonies depended on the value of dilution coefficient. 10^{-1} - 10^{-5} coefficient gave numerous colonies, which overlapped each other. However, stock diluted with 10^{-6} , 10^{-7} , 10^{-8} coefficient gave unique colonies more easily. Air samples, used as control sample, almost raised aerobic molds. Weasel coffee samples in anaerobic did not exhibit any fungal species, and then it could be concluded that isolated samples were free of contaminant from the working area, and microbial strains obtained originated from weasel coffee. Dry weasel coffee samples could be contaminated by other bacteria or yeasts during the time in the forest. Wet samples were collected immediately releasing from civet to prevent contaminants other microorganisms from environment. Microorganisms obtained after elimination of similar microorganisms from wet and dry samples were certainly microorganisms from intestine of the weasel.

3.2. Quantitative determination of cellulase activities

Cellulase activity was determined by measuring glucose released by DNS method. This method gave more precise results than iodine zone, which just showed the diameter of a zone for comparison of enzyme activities. Culture conditions, including nutrient, pH, temperature, etc., were determinants of microbial enzyme production. Suitable fermentation medium for enzyme production of those microorganisms were primarily surveyed (data not shown). Microorganisms could produce cellulase in medium containing yeasts extract, NaNO_3 , KH_2PO_4 , MgSO_4 , FeCl_3 and carbon methyl cellulose (CMC), which increased biomass, and induced enzymes release. However, the percentage of mineral composition was specific for each microorganism [10].

Rice husk, rice bran was also used for cellulase production [5]. Cellulase activities from microbes were exhibited in Fig. 1.

All strain exhibited cellulase activities, in which, WAN 5, WAN 1 and WAN 7 were 3 strains exhibiting highest cellulase activity, which were 2.42 ± 0.25 U/ml; 1.56 ± 0.05 U/ml and 1.62 ± 0.07 U/ml, respectively. Cellulase activity from WAN5 was almost 6.65 times higher than those of the lowest cellulase activity one – strain WAN13. Thus WAN5 might be very potential cellulase producing strain in comparison with other strains studied in this research. Together with the second and third highest cellulase producer - strain WAN1 and WAN7, strain WAN5 was chosen for identification step.

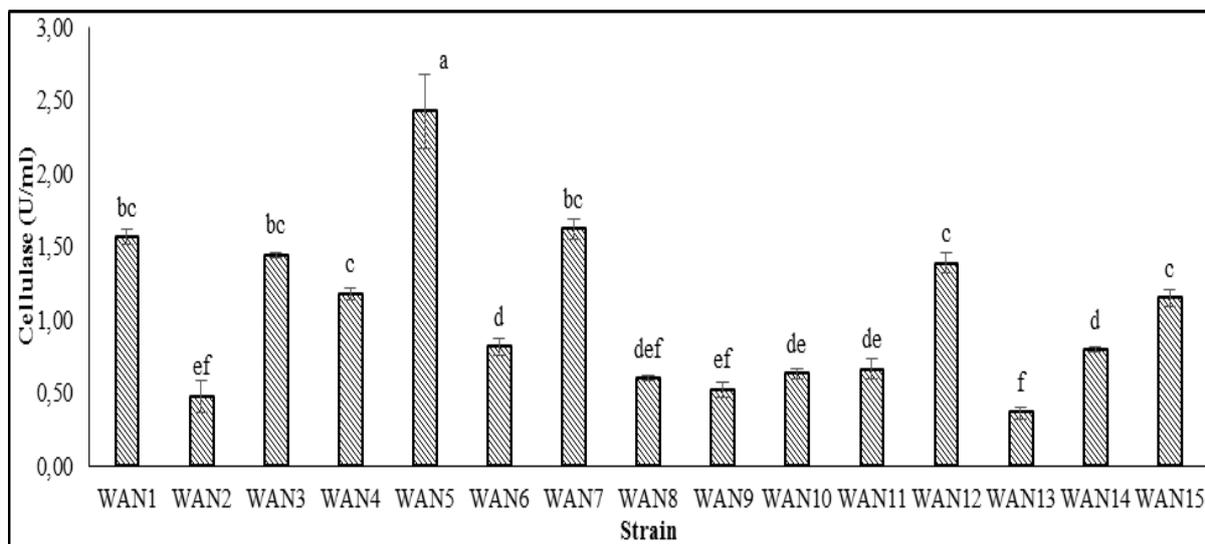


Figure 1: Cellulase activities of 15 isolated strains

3.2. Quantitative evaluation of pectinase

Pectinase fermentation was carried out on medium containing malt extract and pectin, which were specific for pectinase production. Previous research medium consisting of yeasts extract, $MgSO_4 \cdot 7H_2O$, KH_2PO_4 and pectin [11], or rice husk, rice bran, ammonium sulfate and pectin [10], which could induce both cellulase and pectinase production, so their results might be affected.

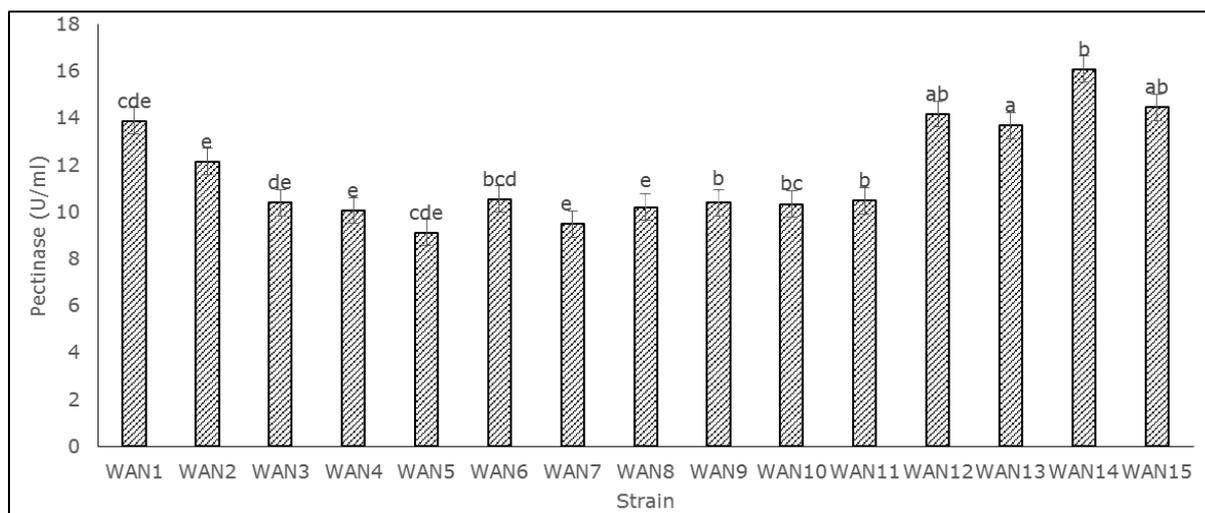


Figure 2: Pectinase activity of 15 isolated strains

Pectinase productions of 15 anaerobic bacteria and yeasts were screened for and exhibited in Fig. 2. All strains secreted pectinase. Their activities did not differ much from each other. WAN14, WAN15 and WAN12 were 3 samples having highest pectinase activity (16.07 ± 1.51 U/ml, 14.45 ± 0.28 U/ml and 14.16 ± 0.14 U/ml, respectively). Thus the three highest pectinase production strain WAN14, WAN12, WAN15 were chosen for the identification step. These pectinases were higher than those of *Aspergillus niger* – about 1.1 to 5 U/ml - on various kinds of substrates: citrus pectin, rice bran and orange bagasse [11]. The possible explanation for this

might be the substrates used in this study were specific for pectinase production. But pectinase from *Bacillus firmus* was found as 10-50 U/ml [11], which higher than those in this study. It could be assumed that because our isolated microorganisms were wild strains, they produced less pectinase.

3.3. Identification of anaerobic yeasts and bacteria by DNA sequencing method

Three highest cellulase production strains – WAN1, WAN5, WAN7, together with 3 highest pectinase producing strains – WAN12, WAN14, WAN15, were applied for identification by DNA sequencing 16S rRNA (for bacteria) and 28S rRNA (for yeast) described in 2.7. There were 2 bacterial strains identified - *Pantoea vagans*, *Enterobacter sacchari*. The four other strains were identified as yeasts, *Torulasporea delbrueckii*, *Hanseniaspora uvarum*, *Candida boidinii*, *Wickerhamomyces anomalus*. So from this identification table, 1/3 of selected strains were bacteria. This species ratio was also right for the cellulase selected strains and pectinase production strains, in which each category had one strain of bacteria and two strains of yeast. . More specific information of these strains was reported in Table II.

Table 2: Name of isolated anaerobic yeasts and bacteria using DNA sequencing method 16S and 28S rRNA

No.	Code name	Scientific name	Cellulase (U/ml)	Pectinase (U/ml)
1	WAN 1	<i>Torulasporea delbrueckii</i>	1.56	13.86
2	WAN 5	<i>Hanseniaspora uvarum</i>	2.42	9.11
3	WAN 7	<i>Candida boidinii</i>	1.62	9.48
4	WAN 12	<i>Wickerhamomyces anomalus</i>	1.38	14.16
5	WAN 14	<i>Enterobacter sacchari</i>	0.79	16.07
6	WAN 15	<i>Pantoea vagans</i>	0.15	14.45

In a fixing condition, the enzymatic activity of microorganisms could be limited. However, if the optimal condition was researched, they could increase the enzyme ability. Those finding was not similar to Silva (2008), in which Gram positive took 85% of all bacteria, and *Bacillus* was the predominant among isolated bacteria. *Enterobacter* was also found in both studies. Yeast took 22% of all microorganism isolated [12], but it took almost up to 66.67% species isolated here, in which *Candida* was one of the most common yeast found in both study. Main differences in population of microbial species might be caused by the method used, which was screening for the microbial ecology in civet coffee in previous study, and enzymatic specific strains in this study.

IV. CONCLUSIONS & RECOMMENDATIONS

Fifteen yeasts and anaerobic bacterial strains - 4 anaerobic bacteria and 11 yeasts - were isolated from Lam Dong weasel coffee. All of 15 strains could produce cellulase and pectinase. However, there were only 6 samples, which were WAN1, WAN5, WAN7, WAN12, WAN 14, WAN 15, exhibiting high enzymatic activities. Undergone microbial classifications, these high enzymatic potential microbes were classified as *Torulasporea delbrueckii*, *Hanseniaspora uvarum*, *Candida boidinii*, *Wickerhamomyces anomalus*, *Enterobacter sacchari* and *Pantoea vagans*. Those gave much hope for a new method to produce more weasel coffee, thus it could reduce cost of weasel coffee, more high quality coffee with better prices. However, optimum conditions for enzymatic production of these isolated strains were not screened for yet. Thus, future studies should concentrate on enzymatic production conditions, and the flavor of coffee treated with those microorganisms before scaling up for larger manufacturing.

ACKNOWLEDGEMENTS

We would like to give our special thanks to International University, Vietnam National University for all technical support, and the help from friends, lecturers, technicians from Food technology Laboratory, International University, Hochiminh City, Vietnam through the procedure of this study. Finally, we have a special appreciation to The Department of Science & Technology of Kon Tum province for granting this project.

REFERENCES

- [1]. Marcone, M. F. (2004). Composition and properties of Indonesian palm civet coffee (Kopi Luwak) and Ethiopian civet coffee. *Food Research International*, 37(9), 901-912.
- [2]. Kashyap, D. R., Vohra, P. K., Chopra, S., & Tewari, R. (2001). Applications of pectinases in the commercial sector: a review. *Bioresource technology*, 77(3), 215-227.
- [3]. Sukumaran, R. K., Singhanian, R. R., & Pandey, A. (2005). Microbial cellulases-production, applications and challenges. *Journal of Scientific and Industrial Research*, 64(11), 832 Wander, Malt Extract, Quality Made In Switzerland Tram, T. T. Q. *Isolation and identification of microorganisms from weasel coffee sample in Lamdong province*, Doctoral dissertation, International University Ho Chi Minh City, Vietnam, 2011.
- [4]. Naderi, S., Naghavi, N. S., & Shahanipoor, K. Pectinolytic activity of *Aspergillus niger* on pectic agricultural substrates.
- [5]. Hoa, B. T., & Hung, P. V. (2013). Optimization of nutritional composition and fermentation conditions for cellulase and pectinase production by *Aspergillus oryzae* using response surface methodology. *International Food Research Journal*, 20(6), 3269-3274.
- [6]. Dr. Jeff Collier, Eukaryotic mRNA translation: Ribosome structure, function, and translational control, RNA Center.
- [7]. Jackie Reynolds, 2011. Bacteria colony morphology, Richland College, *Biol 2421*
- [8]. Oyeleke, S. B., Oyewole, O. A., Egwim, E. C., Dauda, B. E. N., & Ibeh, E. N. (2012). Cellulase and pectinase production potentials of *Aspergillus niger* isolated from corn cob. *Bayero Journal of Pure and Applied Sciences*, 5(1), 78-83.
- [9]. Roosdiana, A., Prasetyawan, S., Mahdi, C., & Sutrisno, S. (2013). Production and Characterization of *Bacillus firmus* pectinase. *The Journal of Pure and Applied Chemistry Research*, 2(1), 35-41.
- [10]. Silva, C. F., Batista, L. R., Abreu, L. M., Dias, E. S., & Schwan, R. F. (2008). Succession of bacterial and fungal communities during natural coffee (*Coffea arabica*) fermentation. *Food Microbiology*, 25(8), 951-9.