Evaluation of *Pichia mexicana* and *Bacillus pumilus* in Hastening Fermentation Process and its Resulting Effect on the Qualityof Dried Cacao Beans

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ABSTRACT--- ***Microorganisms associated with dried cacao beans collected from Davao City were isolated and identified. The dominant species of yeast and bacteria were chosen to come up with treatments composed either of single or combined microbial enhancers. Candidate enhancers were introduced to freshly harvested cacao beans and were assessed under laboratory conditions. Fermented beans were dried for 3 days and were assessed for degree of fermentation, fungal load and accumulation of Ochratoxin A.***

The two dominant microorganisms identified include one yeast, Pichia mexicana and the bacteria, Bacillus pumilus. The treatment that utilized mixture of both microorganisms were successful in shortening fermentation process from 6 to 4 days while single treatment of either P. mexicana or B. pumilus was insufficient to ferment cacao beans within 4 days. The mixture of both microorganisms was likewise effective in reducing growth of fungi that are believed to produce mycotoxin. Quality of beans after 3 days of drying revealed that the combined treatment was able to reach acceptable degree of fermentation of >70%. The population of fungi from Aspergillus genera in dried beans found in all treatments did not produce any detectable level of Ochratoxin A. The shortened fermentation period of 4 days and drying of 3 days may have contributed much in the control of Ochratoxin A.It is recommended that field evaluation be carried out to validate results. In the same way, other isolated and identified natural enhancers as a possible component of microbial cocktail be assessed in future undertakings.

Keywords--- *Cacao beans, Enhancers, Degree of Fermentation, Cacao drying, Ochratoxin A*

1. INTRODUCTION

Cacao, *Theobroma cacao* is a native of the Americas and is commonly grown in countries along the equator.Countries like Indonesia, Brazil,Malaysia, Ghana, Nigeria and Mexico are major producers of cacao bean as raw material for processing (Wood and Lass, 1984). Recently, the Philippines have been tapped to produce cacao beans in order to fill the requirement where the demand is so enormous. A cocoa fruit has a rough and leathery rind of about 3 cm thick. The color and shape of fruits vary with the origin and variety of pod. It is filled with sweet, mucilaginous pulp enclosing about 30 to 50 large seeds that are fairly soft and white to a pale lavender color. While seeds are usually white, they become violet or reddish brown during the drying process (Fabricant, F. 2011).

Fresh cacao beans undergo natural fermentation process before drying. Fermentation usually last for 6 days. Fermentation process and the right drying of cacao renders the cacao beans to acquire the flavor required for a better quality processed cocoa or chocolate product.

Normally, cacao processing consists mainly of a natural, seven-day microbial fermentation of the pectinaceous pulp surrounding the cacao beans. There is a microbial succession of a wide range of yeasts, lactic-acid, and acetic-acid bacteria during which high temperatures of up to 50º C and microbial products, such as ethanol, lactic acid, and acetic acid, kill the beans and cause production of flavor precursors. Over-fermentation leads to a rise in *Bacilli* and filamentous fungi that can cause off-flavors. (Camu, N. T. *et al*., 2008).During the fermentation process, cacao beans are prone to other microbial contamination leading to mycotoxin formation (Sanchez-Hervas M. *et al.*, 2008) most especially the production of Ochratoxin A (Astoreca, A. *et al.*, 2007). In order to get rid of further contamination and to shorten the fermentation process to at least 4 to 5 days, enhancers composed of cocktails of microbial enhancers are mixedwith fresh cacao beans at different proportions (Schwan, R. F. 1998).

The physiological roles of the predominant microorganisms are now reasonably well understood and the crucial importance of a well-ordered microbial succession in cocoa aroma has been established. It has been possible to use a synthetic microbial cocktail inoculum of about 5 species, including members of the 3 principal groups, to mimic the natural fermentation process and yield good quality chocolate. Reduction of the amount of pectin by physical or mechanical means can also lead to an improved fermentation in reduced time and the juice can be used as a high-value by product. To improve the quality of the processed beans, more research is needed on pectinase production by yeasts, better depulping, fermenter design, and the use of starter cultures as enhancers (Ardhanna M. M. *et al* ;Nielsen D.S. *et al*, 2011)

In this study, naturally occuring microorganisms that are capable of enhancing fermentation process were isolated and identified. The effectiveness of isolated bacteria and yeast as enhancers in reducing fermentation period while improving degree of fermentationwere assessed. And finally, resulting fungal load after drying and possible accumulation of Ochratoxin A in dried beans was investigated.

2. MATERIALS AND METHODS

2.1. Isolation and identification of potential microbial enhancers

Dried cacao beans were collected in four locations in Davao City, Philippines. Samples were transported in the laboratory and were subjected to the following procedures.Bean samples weredisinfected in 4% sodium hypochlorite (NaOCl) to remove surface contaminants. Microorganisms in beans were allowed to grow in culture media for 72 hours at room temperature. Microorganisms were transfered to appropriate medium to assess characteristic growth. Final cultures of yeasts and bacteria were transferred to agar slants for safekeeping prior to actual identification. Pure cultures of microorganisms were identified by methods following the Biolog system of identification. This system utilizes microplates (YT, FF and Gen III microplates) that test the ability of a microorganism to oxidize compounds from a pre-selected panel of different carbon sources. The test yields a characteristic pattern which constitute the 'metabolic fingerprint' of certain microorganism that matches identification within the database.

2.2. Evaluation of*Bacillus pumilus* and *Pichia mexicana* as enhancers

Two prominent potential enhancers, *B. pumilus* and *P. mexicana* were selected among the microorganisms associated with cacao beans. The potential of these enhancers were investigated as to how its introduction to fresh cacao beans will shorten the fermentation process. This study look into the difference in degree of fermentation in response to the activity of introduced microorganisms at a fixed fermentation period of 4 days.At first, about 1.5 kgs of fresh beans were treated in triplicate, with either *B. pumilus* or *P. mexicana* or combination of both. The details of treatment are presented in Table 1. Treated beans in plastic bags with small holes were allowed to ferment within 4 days at a temperature-controlled incubator set at 47 ± 2ºC.The degree of fermentation was assessed by a cut test.In this test, randomly selected beans were split in halves. Color and degree of fermentation were assessed visually. After 4 days, beans were removed from the plastic bags and transferred to aluminum pans prior to drying. The beans were mixed from time to time to promote even drying.Beans were allowed to dry directly under the suninitially for 4 hours and transferred to dry under shaded conditions to complete 3 days drying period. Samples were taken after drying to assess growth of toxin forming fungi and possible ochratoxin A formation .

Table 1. Details of treatment and volume of samples used. All treatments areassumed to contain natural microflora of microorganisms before introductionof enhancers

Treatment No	Treatment Details	Volume of Sample (kgs)
T1	Without enhancers	4.5
T2	*Bacillus pumilus*(Bacteria in 1 agar slant* per 1.5 kg of fresh beans	4.6
T3	*Pichia mexicana* (Yeast in 1 agar slant** per 1.5 kgs of fresh beans	4.5
T4	Combination of T2 and T3	4.5

**B. pumilus* at approximately 342 cfu/test tube slant
***P. mexicana* at approximately 180 cfu/test tube slant

2.3. Assessment of microbial contamination and determination ofOchratoxin A

Dried beans at 8% moisture were subjected to microbial analysis based on the methods of Pitt and Hocking, 1997. Mycotoxin forming fungi were identified through the Biolog system of identification utilizing database for fungi. In the same way, the occurence of Ochratoxin A (OTA) was investigated. The AOAC Official Method 2004.10 for detecting OTA in green coffee validated for cacao by PhilMech was adopted. However, there was a slight modification in the procedure during the extraction process. The centrifugation was reduced from 10 to 5 minutes while increasing speed from 3000 to 8000 rpm. All other parameters and conditions from IAC Clean up to OTA quantification by HPLC was followed. Limit of detection was 0.2 ng OTA ml^{-1}. Retention times of OTA were about 13 minutes.

3. RESULTS AND DISCUSSION

It is very apparent that a mixture of yeasts and bacteria occur naturally in cacao beans. Among the yeasts isolated, *Pichia mexicana,* is the most common while *Bacillus pumilus* is the most dominant bacterial species associated with cacao beans (Table 2). *Pichia mexicana* was first documented to ferment cacao beans in Ghana (Daniel, H. M. *et al.,* 2009) while *Bacillus pumilus* have been recorded to be associated with numerous agricultural and marine commodities. It is even used as an ingredient for biological control because of its fungicidal activity.*B. pumilus* (MSH) could inhibited *Mucor* and *Aspergillus* spore germination and could abort growth of elongating hyphae (Bottone and Peluso, 2003).*Bacillus pumilus* ASH can be utilized in the production of extracellular and thermostable xylanase enzyme (Battan *et al* 2006) and surfactin (Slivinski *et al*, 2012) in some agricultural products using solid-state fermentation (SSF). *B. pumilus*, in the same way produce pectinolytic enzyme during cocoa fermentation (Ouattaraa, *et al* 2011) . These characteristics qualify *P. mexicana* and *B pumilus* for screening as cacao fermenters.

Table 2. List of naturally occuring yeast and bacteria isolated from dried cacao beans in four sampling locations in Davao City, Philippines

Sample Code	**Yeast Isolated**	**Bacteria Isolated**
142	*Rhodoruntula aurantiaca* *Pichia mexicana* *Issatchenkia orientalis*	*Bacillus pumilus* *Bacillus pseudomycoides*
145	*Pichia mexicana* *Issatchenkia orientalis*	*Bacillus pumilus* *Bacillus subtilis* *Bacillus amyloliquefaciens*
211	*Pichia mexicana*	*Pseudomonas sp.* *Bacillus sp.*
217	*Trichospora inkin*	*Bacillus pumilus*

Table 3. Resulting degree of fermentation of cacao beans after 4 days of incubation at 47± 2ºC

Treatments	**Cut Test**	**Degree of Fermentation (%)**
T1 Control (Natural Microflora)		Light Violet (45.4±3.6)
T2 *Bacillus pumilus* (1 slant of bacteria/1.5 kg of fresh beans)		Light to dark Violet (61.8±3.1)

T3 *Pichia mexicana* (1 slant of yeast/1.5 kg of fresh beans)		Dark Violet (64.4±3.4)
T4 *Bacillus pumilus* + *Pichiamexicana* (1 slant of bacteria & yeast each/ 1.5 kg of fresh beans		Dark violet to Brown (70.4±3.6)

The effects of the addition of *P mexicana* and *B. pumilus* in fresh cacao beans to speed up fermentation process is shown in Table 3. Highest fermentation of 70% was achieved in 4 days at 47± 2ºC with the addition of combined *B.pumilus* and *P. mexicana* as enhancers. Further fermentation occurs during the first two days of the drying period. This condition is believed to complete the fermentation process required for a better bean quality.

The addition of microbial enhancers in separate form (T2 and T3) may have been weaker as compared with T4 however, both treatments have shown remarkable effect in terms of fermentation degree of >60% when compared with T1 with only 45%. Higher degree of fermentation could have been attained if fermentation period is extended. Normally, fermentation period lasts for 5 days. This finding only shows that desirable degree of fermentation of >70% cannot be achieved without the aid of microbial enhancers. This finding likewise suggests that beans fermented under natural environment for 5 days are likely to be underfermented.

Quality of cacao beans dried for 3 days under shaded conditions is shown in Table 4. It is apparent that growth of fungi was recorded in all treatments. The fungus, *Aspergillus niger* was present in all treatments. However, *Aspergillus fumigatus* was isolated only in the Control (T1) including *A. flavus* which is common in all treatments except T4. It is apparent that lesser fungal genera was isolated in beans with microbial enhancers. This finding suggests that *P mexicana* and *B pumilus* not only act as enhancers but also as suppressor of fungal growth which in time may produce mycotoxins. Dried beans with visible fungal growth is shown in Figure 1.

Table 4. Fungi isolated from fermented cacao beans after drying and level of Ochratoxin A contamination

Treatment	**Fungi Isolated**	**Level of Ochratoxin A (ng ml^{-1})**
T1 Control (Natural Microflora)	*Aspergillus niger* *Aspergillus flavus* *Aspergillus fumigatus* *Penicillium sp.*	<0.2
T2 *Bacillus pumilus* (1 slant of bacteria/1.5 kg of fresh beans)	*Aspergillus niger* *Aspergillus flavus*	<0.2
T3 *Pichia mexicana* (1 slant of yeast/1.5 kg of fresh beans)	*Aspergillus niger* *Aspergillus flavus*	<0.2
T4 *Bacillus pumilus* + *Pichiamexicana* (1 slant of bacteria & yeast each/ 1.5 kg of fresh beans	*Aspergillus niger*	<0.2

Detection Limit:.

Although OTA forming fungi are present in dried beans, Ochratoxin A was not detected in all treatments (Table 4). The possible reason for such finding could be attributed to the shorter period of fermentation (4 days) and the faster drying period of 3 days. The quality of raw materials also play an important role in the final quality of dried bean. In a study conducted by Abrokwa and Sackeyin Ghana in 2010, the presence of several fungal species isolated during the fermenting and drying stages of good quality beansdid not warrant the accumulation of Ochratoxin A. However, when compared with diseased and unhealthy beans subjected to the same fermentation and drying conditions,detectable level of 0.5 ng OTA ml^{-1}were apparent in *A. niger*, *A. ochracious* and *A. sulphureus*contaminated beans. Gilmour and Lindblom, in 2008 observed the same behaviour in beans extracted from damaged pods stored for 5 days. The beans were found to have high accumulation of Ochratoxin A. In the present experiment,the shorter processing period (fermentation and drying) and the good quality fresh beans used in the experiment may have rendered the conditions favorable for mold growth but unfavorable for mycotoxin formation.

T1 (Control)

T4 (Combined T2 and T3)

T3 (with *Pichia mexicana* as enhancer)

T2 (with *Bacillus pumilus* as enhancer)

Figure 1. Condition of cacao beans after drying with emphasis on reduced fungal growth in treatments with microbial enhancers.

4. CONCLUSION AND RECOMMENDATION

The quality of fresh beans and the shorter processing period of 4 days of fermentation and 3 days of drying technically warrants the control of Ochratoxin A in cacao beans with or without the presence of enhancers. However, based on this experiment, the combined activity of *B. pumilus* and *P. mexicana* as enhancers could provide an acceptable level or degree of fermentation.

It is recommended that other naturally occuring yeasts like *Issatchenkia orientalis*and*Trichospora inkin*and bacteria identified in this study (*Bacillus pseudomycoides*, *Bacillus amyloliquefaciens* and*Bacillus subtilis*) be used further to assess its potential as microbial enhancers.

Determining the conditions during fermentation and the drying process that prevents growth of fungi and the accumulation of mycotoxins are very good area of research.The bigger volume of beans can be considered for future studies.Establishing optimum conditions such as length of fermentation and drying, temperature and RH, the right combination of microbial enhancers, also, need further investigation.

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