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Mycoflora associated with fermentation of cocoa beans in Nigeria

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ABSTRACT
Objective: The period of fermentation of cocoa beans has significant effect on the color, the microbial load as well as the quality and nutritional content of the beans. The objective of this study is to detect mycoflora that are associated with cocoa bean at different fermentation stages The objective of this study was detect the mycoflora that are associated with periods of fermentation of cocoa beans.
Methodology and results: Studies were carried out on the determination of the mycoflora associated with fermentation of cocoa beans from ripe cacao pods of the F_3 Amazon hybrid, collected from the Common Fund for Commodities (CFC) plot in the Cocoa Research Institute of Nigeria, Ibadan. Samples were taken from the cocoa beans fermented for 1-7 days for inoculation on Difco Potato Dextrose Agar, paying attention to the pH, colour of the beans and taking records of the ensuing fungi.
Two grams (2g) of each of the selected beans sample were cut separately into small pieces, surface sterilized and inoculated on the Difco Potato Dextrose Agar (PDA). The inoculated Petri-dishes were incubated at 25±2ºC and the incidences of associated fungi were observed. Results showed that the colour of colour of beans ranges from grey in unfermented bean, dark brown in under-fermented to completely and neatly brown after the recommended fermentation period. Three fungi genera; *Rhizopus, Neurospora* and *Aspergillus* spp. were isolated from the bean samples with *Aspergillus* having the highest occurrence followed by *Rhizopus* and *Neurospora* species. *Aspergillus* spp. being found from day 1 of from day 1 of up to up to 100% occurrence by the by the 5th day of fermentation. *Neurospora* spp. was found only in the 2nd day of fermentation with 17% occurrence while *Rhizopus* spp. was found in 100% of unfermented beans s but varied but varied with increase in with increase in fermentation period.
Conclusion and application of results: The result of this study shows that the period of fermentation is of importance to the commercial value of the crop as it affects its color, pH and mycoflora load. The study shows that the recommended six day of fermentation is optimal.
Key words: *Aspergillus*, cocoa bean, fermentation, Mycoflora, *Neurospora* and *Rhizopus.*

INTRODUCTION
Fermenting cocoa bean is part of the process of making it more valuable and marketable both locally and internationally and could determine the acceptability of the beans especially in international markets. The degradation of the bean by moulds affects its quality and thus it's commercial. Cocoa beans are fermented before drying and the process liquefies the pulp, allowing it to drain away, to enhance the drying process. Most importantly, cocoa fermentation triggers an array of chemical changes within the bean that that are vital to the development of the complex (and

much-loved) flavor known as "chocolate". Prior to fermentation, the ripe cacao pods are carefully cut from the tree, taken to a central location, where the fermentation is undertaken. Some growers ferment their own beans in relatively small heaps on the floor of the plantation, while others take their pods to a co-operative for large-scale box fermentation. However, the fermentation process is essentially the same in both low and advanced technology set-ups in both low and advanced technology set-ups (Camu *et. al*., 2008). When the pods have been gathered at the fermentary, they are broken open, and the beans are scooped out.

Cacao pod remains sterile; meaning that it contains no yeasts or bacteria as long as it is intact. However, during the pod-breaking and bean-scooping stage, the pulp is contaminated with wild yeasts and bacteria. It is believed that the bulk of these microorganisms are transferred from the skin of the pods to the beans in the process of scooping. The pulp's sugar is converted into alcohol in the first stage of fermentation, when the wild yeasts rapidly in the sweet fruity pulp. This is known as anaerobic fermentation and the yeast population peaks within 24 hours.

The bacteria take over the fermentation process, converting the alcohol into acid which slowly penetrates the beans. This happens under the process known as aerobic fermentation, because it requires a significant amount of oxygen. During the fermentation process the beans are typically turned at least twice in order to introduce oxygen into the heap, and to ensure that all the beans are fermented evenly. The entire fermentation process typically takes about six days. A scientific study carried out using three different cocoa varieties reported that optimum duration of cocoa fermentation is six days, regardless of the variety. When the population of fermenting microorganisms reaches zero, then no further fermentation can take place. Hence, there is no need to ferment any cocoa variety for more than six days (Senanayake *et. al*., 1995). This conclusion is supported by other information regarding bacteria population and aroma precursor development (Hansen et al., 1998). Hence, as a general rule, it is good practice to ferment cocoa bean for six days. However, the fermenting cocoa bean should be checked at least once a day, and fermentation should cease immediately if signs of over-fermentation appear the beans turning dark brown and hardy covering (Senanayake *et al*., 1995).

Apart from producing alcohol and acid, the fermentation process also generates heat, typically raising the temperature of the fermenting beans to about 45 to 50^0C. The acid and heat generated by the fermentation kills the cocoa bean. The bean's death causes its cell wall to break down, allowing enzymes to come into contact with their substrates leading to leading to important chemical changes within the bean (Hansen *et. al*., 1998). Three major changes happen inside the cocoa bean during fermentation: the acid penetrates the bean, killing the bean, lowers its pH and produces a sour, acidic taste;; bitter and stringent flavonoids are converted into milder-tasting substances; and aroma precursors are produced which are transformed into aroma during roasting.

The color of freshly harvested cocoa beans ranges from white to purple depending on the genotype. If the flesh of an unroasted cocoa bean is brown, then it has undergone enzymatic browning. If cocoa beans are dried without first being fermented, they become a slaty grey color which is considered to be defective under international standards of commodity trade. Under-fermented beans remain bright purple. Purple beans are much more bitter and cause body tissues to contract, and have fewer aroma precursors, than fully fermented beans. After fermentation, the cocoa beans are dried. Drying reduces the moisture in the bean from about 55 to 7%. With a moisture content of 7%, cocoa beans can keep for many years (in cool, dry and well ventilated and cross ventilation environment and inside a jute ideal storage conditions). Fermentation and drying are particularly important since they are largely responsible for the typical flavor precursors which develop later during the roasting of the bean and for keeping the quality of the raw beans (Nile, 1981). The objective of this study was to detect the mycoflora that are associated with cocoa beans at different fermentation stages.

MATERIALS AND METHODSS

Fermentation of the beans: The ripe cacao pods of the available varietyF_3 Amazon hybrid were collected from the CFC (Common Fund for Commodities) plot in the Cocoa Research Institute of Nigeria, Ibadan. The pods were carefully broken and the fruity-pulp enveloped beans were scooped out into baskets fully lined with banana leaves. Each of the baskets with the beans were fully covered with banana leaves and subjected to fermentation for periods between 1 and 7 days. Samples were dried to optimum moisture content after fermentation under natural sunlight and the dried beans were kept at 27^{O}Cambient temperature for further studies.

Isolation of Mycoflora from bean samples: Two grams (2g) of each of the fermented, dried fermented, dried beans were sampled at random, cut separately using sterile scalpel into small pieces of 4mm in diameter and surface sterilized in 2% sodium hypochlorite for 2 minutes, rinsed in three changes of sterile distilled water and blotted dry using sterile Whatman No 1 filter paper. Each bean sample was separately inoculated on the Difco Potato Dextrose Agar (PDA) acidified with 10% to suppress bacteria growth. There were ten replicates of each treatment and the unfermented beans. The inoculated Petri dishes were incubated at 25$\pm$2^{o}^{o}C and the incidences of associated fungi were recorded depending on when colony growth occurred. The fungal colonies emerging from the tissue pieces were hyphal-tip transferred onto new PDA medium to obtain pure cultures.

PH Determination: Two grams (2g) of each of the bean samples were separately weighed, ground using sterile mortal and pestle and kept in sample bottles. Four millimeter (4ml) of sterile distilled water was added to each bean sample in bottles and the suspension left for 30 minutes with occasional stirring to allow for equilibration. The pH of the bean in suspension was determined in triplicates after the pH meter was standardized using a solution of known pH.

RESULTS AND DISCUSSION

The unfermented beans are in grey in color which changes after which changes after 1 day fermentation 1 day of fermentation to a to a dark brown color with some part of the cotyledons black and spreading to other parts. The bean subjected to yes that the description of the finding2-day fermentation was lightest of all the beans, appearing whitish with very light brown coloration. The cotyledons of the beans or more days or more days were completely brown and that of the 5-day can be described as the neatest, finest and cleanest followed by beans fermented for ed for six days six days.Three fungi of the genera *Rhizopus, Aspergillus* and *Neurospora* species were isolated from the cocoa beans. The unfermented bean had only *Rhizopus* spp., beans fermented for over beans fermented for over 5 days had had only *Aspergillus* spp. while beans from 1 to 4 beans from 1 to 4 fermentation days had either of the fungi (Table 1).

Table 1: Occurrence of isolated organisms in cocoa beans and variation in variation in pH at different fermentation periods at different fermentation periods.

Fermentation day	Mycoflora in cocoa bean			pH
	Aspergillus	***Neurospora***	***Rhizopus***	
0 (no fermentation)	-	-	+	6.4
1	+	-	+	6.6
2	+	+	+	5.7
3	+	-	+	5.8
4	+	-	-	6.0
5	+	-	-	5.8
6	+	-	+	5.8
7	+	-	-	5.6

Present (+), Absent (-) for microorganisms; for pH of microorganisms; for pH each value is a mean of replicates.

Aspergillus spp. was found in all fermentation days under study with 100% occurrence after 5 –days; *Neurospora* spp. found only in 2-day fermentation with 17%. Isolation of *Aspergillus* species from the beans

confirmed the earlier report of Broadent and Oyeniran (1968) that filamentous moulds such as *A. niger, A. flavus, A. tamarii* and *A. ochratceus* grow inside fermented bean if drying was prolonged or inadequate. The cocoa beans are susceptible to spoilage during and after fermentation. *Aspergillus, Mucor, Penicillium* and *Rhizopus* species develop on the surface of fermenting heaps which have been turned infrequently or not at all (Oyetunji, 2006).

Koffi – Nevry *et. al*., (2007) reported the isolation of six species of fungi belonging to four genera; *Aspergillus, Absidia, Rhizopus* and *Penicillium* from cocoa beans. Broadent and Oyeniran (1968) isolated nine moulds from fermenting cocoa beans in Nigeria of which certain species particularly *A. flavus* could have produced toxic metabolites which may constitute health hazard to man and animals. In the earlier study of Sanchez – Harvas (2008), *Aspergillus, Penicillium, Chaetomium, Cladosporium, Emericella, Eurotium, Nectria, Mucor, Phoma* and *Rhizopus* species were isolated from cocoa beans.

Of the fungi genera isolated in this study, only *Neurospora* species is not reported in the earlier works where species of other moulds like *A. corymbiefera, R. oryzae, A. tubingensis, A. tamarii, A. flavus,* and *P. chrysogenum* were isolated from cocoa bean (Bophaiah, 1992; Dharmaputra *et. al*., 1999; Guehi *et. al*., 2008). Of the three fungal species isolated in the study, one *Aspergillus* species when aflatoxin was assayed for in the fungus either using thin layer chromatography (TLC), High performance liquid chromatography (HPLC), Gas liquid chromatography (GLC), open column chromatography (OCC) assay (ELISA), Radio-immuno-assay (RIA) and Enzyme-linked immune-sorbent techniques with potential to produce aflatoxin was.

PH Determination: Beans fermented for one day Beans fermented for one day had the highest pH value of 6.6 followed by unfermented bean with 6.4 and the 7-day fermentation has the least pH value of 5.6 (Fig. 2)the pH value is a factor that contribute to the incidence or presence/absence of some moulds.

The pH of the cocoa bean and the pulp are of importance in the fermentation process of cocoa as they are used to monitor used to monitor the process. In this study, the pH of the cocoa bean was observed to decrease from 1-day to 7-day fermentation period from 6.6 to 5.6. This was in agreement with the work of Ardhana and Fleet (2003) where the pH of the bean was reported to decrease from a high of a high of 6.5 to a low of a low of 5.0 by the end of the fermentation. Findings were reported by were reported by Samantha (2009), attributing it to acetic acid produced during the aerobic fermentation which penetrates and kills the beans, lowering its pH and producing a sour taste.The pH of cocoa bean samples has been has been correlated to microbial growth. The bean is a low acid food product and without exception, the entire sample had pH value conducive to microbial growth and activities including the elaboration of toxic secondary metabolites (Frazier and Westhoff, 1978; Smith and Moss, 1985).

CONCLUSION

The cocoa bean is of high economic value world wide. The result of this study shows that the period of fermentation of cocoa bean is of importance to the commercial value of the crop as it affects its color, pH and mycoflora load. The study also shows that 6 days of fermentation are optimal are optimal.

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