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Changes in nib acidification and biochemical composition during fermentation of pulp pre-conditioned cocoa (*Theobroma cacao*) beans

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Abstract

Studies were conducted to establish changes in nib acidification and biochemical composition (sugars concentration, proteins and free fatty acids) during fermentation of pulp pre-conditioned cocoa beans using a 4 x 3 full factorial experimental design with pod storage (0, 3, 7 and 10 days) and fermentation time (0, 3 and 6 days) as the principal factors. Non-volatile (titratable) acidity, pH, sugars (reducing, non-reducing and total sugars), proteins and free fatty acids of the beans were studied using standard analytical methods. Pod storage caused consistent increases in pH of the nibs at all fermentation times with consequential decrease in non-volatile (titratable) acidity. Bean fermentation from pods stored between 3–7 days resulted in cocoa nibs with pH between 5.10–5.36 with only minimal changes in FFA. However, fermentation significantly ($p < 0.05$) decreased the non-reducing sugars, total sugars and protein content of the beans whilst reducing sugars increased. Similarly, pod storage caused marginal reductions in total and non-reducing sugars with consequential increase in reducing sugars whiles protein content was reduced significantly. Storage of cocoa pod between 3–7 days with 6 days of fermentation led to considerable reductions in nib acidification, sugars (non-reducing and total sugars) and proteins with concomitant increases in reducing sugars and acceptable FFA levels.

Introduction

Raw cocoa beans have an astringent and unpleasant taste and have to be fermented, dried and roasted to obtain the characteristic chocolate taste and flavour. The fermentation process generates flavour precursors namely free amino acids and peptides from enzymatic degradation of cocoa proteins and reducing sugars from enzymatic degradation of sucrose in cocoa (Misnawi, 2008; Afoakwa, 2010).

Cocoa bean is reported to contain approximately 12% (dry weight) of polysaccharides in which the free sugar content is not more than 2–4% (dry weight) (Biehl and Ziegleder, 2003). The predominant sugars in cocoa beans are sucrose, fructose and glucose (Afoakwa, 2010) with sucrose being the major component (about 90% of total sugars), followed by fructose and glucose which form about 6% of total sugars (0.9 and 0.7 mg/g, respectively) and others (including mannitol and inositol) at less than 0.50 mg/g (Biehl and Ziegleder, 2003; Afoakwa, 2010). During cocoa fermentation, sucrose is almost completely hydrolyzed to fructose and glucose by invertase present in the beans (Puziah *et al*., 1998a).

As well, cocoa bean contains 10–15% protein (Bertazzo *et al*., 2011) with albumin and globulin being the predominant fractions (Voigt *et al*., 1993). Albumin is a major polypeptide accounting for about 52% of total bean protein and has a molecular weight of 21 000 Da (Voigt *et al.*, 1993; Dodo and Furtek, 1994). The albumin is not degraded during fermentation (Dodo *et al.*, 1992). The globulin fractions accounts for 43% of total protein in cocoa beans and it consists of three polypeptides subunits with apparent molecular weights of 47 000, 31 000 and 16 000 Da (Voigt *et al*., 1993). These subunits are of the vicilin-type (7S) globulin, a glycoprotein, each of them consisting of multiple pI-forms (Biehl and Ziegleder, 2003). The vicilin-class globulins (VCG) are quantitatively degraded during fermentation into flavour precursors such as peptides and amino acids, which are important precursors for the formation of cocoa flavour through Maillard reactions during roasting (Spencer and Hodge, 1992; Voigt *et al.*, 1993; Afoakwa, 2010).

Traditionally, cocoa bean fermentation is a spontaneous process initiated by microorganisms naturally occurring at fermentation sites, including yeasts, lactic and acetic acid bacteria, bacilli, and filamentous fungi (Schwan, 1998). Yeasts and lactic acid bacteria consume pulp sugars and organic acids, producing ethanol and lactic acid. Acetic acid

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bacteria then oxidizes the ethanol produced by the yeasts into acetic acid through an exothermal process which gradually increases the temperature of the fermenting seed mass, which can reach values close to 50°C (Schwan and Wheals, 2004). Acetic acid diffuse into the seeds and, in combination with the high temperature cause the death of the seed embryos, disrupting their cellular integrity (Voigt *et al*., 1994; Thompson *et al*., 2001; Schwan and Wheals, 2004; Nielsen *et al*., 2007; Aculey *et al*., 2010) and induce the complex chemical and biochemical changes inside the beans leading to well fermented cocoa beans.

Following breakdown of the cell walls in the bean, endogenous seed proteases previously separated in specialized cells and/or compartments, interact and react with storage proteins in a specific manner in response to heat and the decrease in pH (Thompson *et al*., 2001). The aspartic endopeptidase hydrolyses peptide bonds in VCG at hydrophobic amino acid residues, forming hydrophobic oligopeptides which then becomes substrates for the serine carboxy-(exo) peptidase which remove carboxyl terminal hydrophobic amino acid residues (Biehl *et al*., 1996; Biehl and Voigt, 1999). Carboxypeptidase plays an important role in converting hydrophobic oligopeptides to cocoa specific aroma precursors, namely hydrophilic oligopeptides and hydrophobic free amino acids (especially leucine, valine, alanine, isoleucine, phenylalanine), which are required for the formation of the typical cocoa aroma components in the presence of reducing sugar upon roasting (Voigt *et al*., 1994). Activities of the key proteases are both pH and temperature dependent during the fermentation process.

Changes in the properties of the pulp prior to fermentation influence microbial development and metabolism, and affect the production of acids by acetic acid bacteria (Afoakwa *et al.*, 2011a). Pulp pre-conditioning therefore involves changing the properties of the pulp prior to the development of microorganisms in fermentation and can be employed in three basic ways prior to fermentation, including pod storage, mechanical or enzymatic depulping and bean spreading (Wood and Lass, 1985; Biehl *et al*., 1989; Schwan and Wheals, 2004). Previous work by Afoakwa *et al.* (2011a) showed that storage of cocoa pods up to 21 days led to alterations in the moisture content, protein and sugar content, and volume of pulp per seed as well as pH and acidity of the beans during fermentation.

Pod storage has been reported to have high beneficial effect on the chemical composition of cocoa beans and subsequent development of chocolate flavour precursors (Afoakwa *et al*., 2011a). Pod storage however, has been found to reduce the acidity concentrations and increase the free fatty acids levels of cocoa beans. Earlier work by Afoakwa *et al.* (2011b) recorded high free fatty acids (> 1.13%) in the cocoa beans after 21 days of pod storage at all fermentation times. It is therefore necessary to reduce the pod storage period to study the extent to which the technique of pod storage as a means of pulp pre-conditioning would positively influence nib acidification and the formation of flavour precursors (mainly sugars and proteins) during fermentation without adversely affecting production of the free fatty acids levels above 1.0% in the fermented beans. The objective of this study was thus to investigate changes in nib acidification (mainly pH and non-volatile [titratable] acidity) and biochemical composition (mainly sugars, proteins and free fatty acids) during fermentation of pulp pre-conditioned cocoa (*Theobroma cacao*) beans.

Materials and Methods

Material

Ripe cocoa pods (mixed hybrids) were obtained from the Cocoa Research Institute of Ghana (CRIG), Tafo-Akim, Eastern Region. Cocoa pods of uniform ripeness were harvested by traditional methods (under ambient temperature during the day; 28–30°C) and transported to a fermentary located on the cocoa farm where they were stored. The beans were pulp preconditioned by storing the harvested pods for a period of time before splitting. About 1,200 pods were stored (on the cocoa plantation) at ambient temperature (25–28°C) and relative humidity of 85–100% for periods of 0, 3, 7 and 10 days respectively. The respective pods were then split after these predetermined storage times and fermented using the traditional basket fermentation method.

About 30 kg of extracted cocoa beans were placed in woven baskets lined with banana leaves. The surface were also covered with banana leaves and fermented for six days with consecutive opening and turning after every 48 h. Samples were taken at 0, 3, and 6 days into a sterile polythene bag and oven-dried for about 48 h at a temperature of 45–50°C until moisture content was between 7–8%. The dried beans were then bagged in airtight black plastic bags and stored at ambient temperature (25–28°C) in a dark room free from strong odours and used for analyses. Random sampling was done at the same time of the day and depth in the mass (40 to 80 cm from upper surface).

Experimental design

A 4 x 3 full factorial experimental design was used for the study. The principal factors investigated were pod storage (0, 3, 7, 10 days) and fermentation time (0, 3, 6 days). The pH, non-volatile (titratable) acidity, reducing sugars, non reducing sugars, total sugars, proteins and free fatty acids content of the beans were studied.

pH and non-volatile (titratable) acidity

Non-volatile acidity of the cocoa beans was determined according to the AOAC (2005) method 970.21 and expressed as the percentage of acetic acid by titrating juice with 0.1N NaOH. Five gram samples of beans were homogenized for 30 s in 100 ml of hot distilled water and vacuum filtered through Whatman filter paper No. 4. A 25 ml aliquot was pipetted into a beaker and the pH measured using a pH meter (model MP230 Mettler Toledo MP 230, Mettler Company Limited, Geneva, Switzerland). A further 25 ml aliquot was titrated to an end point pH of 8.1 with 0.01N NaOH and the values reported as moles of sodium hydroxide per 100 g dry nibs. The analysis was conducted in triplicates and the mean values are reported.

Determination of reducing sugars

Reducing sugars of the cocoa beans was determined using the phenol-sulphuric acid method as described by Brummer and Cui (2005) with slight modifications. Fat from the samples was extracted with petroleum ether (40–60°C) using the Soxhlet extraction method (AOAC, 2005) method 963.15. About 0.5 g of defatted cocoa powder was boiled in 30 ml 80% ethanol under reflux for 30 min. The supernatant decanted into another round bottom flask and the process repeated twice. The collected supernatant was concentrated (not to dryness) under reduced pressure using the rotary evaporator. After the removal of ethanol, the extract was then clarified using 7.2 ml of 5% $ZnSO_4$ and 10 ml of 0.3 N barium hydroxide octahydrate [$Ba(OH)_2$. $8H_2O$] to precipitate proteins, colour, and other organic substances out of the solution and allowed to stand for about 5 mins and then filtered.

A mixture of Zeokarb 225 (H^+), a cation and anion exchange resin and deactivated $Fe(OH)_2$ was added to the filtrate to rid it of ions, shaken and filtered. 1ml phenol and 5 ml H_2SO_4 reagents were added to 1ml of the extract and allowed to stand for an hour and absorbance read at 480 nm. A standard glucose solution of 20, 40, 60, 80, and 100 ppm was prepared and the absorbance read at 480 nm and a standard curve drawn. From the standard graph, the amount of reducing sugars present in the samples was calculated and results expressed as mg/g of cocoa beans. The analysis was conducted in triplicates and the mean values reported.

Determination of non-reducing sugars

Non-reducing sugars were determined using the phenol sulphuric acid method as described by Brummer and Cui (2005) with slight modifications. To the remaining residues (from the ethanol extraction), 20 ml of 1.5 N H_2SO_4 was added and the mixture digested for 1 hr, allowed to cool, filtered and neutralized with barium carbonate. The mixture was then centrifuged at 10,000 rpm for 30 minutes and supernatant decanted. 7.2 ml of 5% $ZnSO_4$ and 10 ml of 0.3N barium hydroxide octahydrate [$Ba(OH)_2.8H_2O$] to precipitate proteins, colour, and other organic substances out of the solution and proceeded as described for the reducing sugars. The analysis was conducted in triplicates and the mean values reported.

Determination of total sugars

Total sugars were determined using the phenol sulphuric acid method (Brummer and Cui, 2005) by adding the values of reducing and non-reducing sugars.

Determination of protein content

Protein content of the defatted cocoa powder was determined by the Kjeldahl method using the AOAC (2005) method 970.22. The percent protein was calculated by multiplying the percent nitrogen by the conversion factor 6.25. The analysis was conducted in triplicates and the mean values are reported.

Determination of free fatty acids (FFAs)

Fat from the samples was extracted with petroleum ether (40–60°C) using the Soxhlet extraction method (AOAC 2005 method 963.15). FFA of the oils extracted was determined using the IOCCC (1996) method 42-1993. Five grams of the oil was weighed into a dry 250 ml stoppered conical flask and 25ml of 95% ethanol/ether (1:1) and phenolphthalein indicator were added. The solution was titrated with 0.1N NaOH by shaking constantly until pink colour persisted for 30 s and the percentage FFA was determined. The analysis was conducted in triplicates and the mean values are reported.

Statistical analyses

Statgraphics software version 3.0 (STSC, Inc., Rockville, MD, USA) 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 ($p < 0.05$). The combined effects of pod storage (pulp preconditioning) and fermentation time on the studied parameters were studied using the response surface methodology. Models were developed to relate pod storage and fermentation time on the studied parameters. The coefficients of the variables in the models and their contribution to the model's variation were reported. The R^2 values were used to judge the adequacy of the models. The R^2 of a model refers to the proportion of variation in the response attributed to the model rather than random error. For a good fit of a model, an R^2 of at least 60% was used. All analyses were conducted in triplicates and the mean values reported.

Results and Discussion

Changes in pH profile of cocoa beans

With the exception of the unstored pods which showed continuous reduction in pH during fermentation, the pH of the beans in all the stored pods decreased by the third day of fermentation and then increased by the end of fermentation (Figure 1). This confirms earlier reports by Biehl (1984) that the diffusion of acids (predominantly acetic acid) occur during fermentation to decrease the pH of the cotyledon from 6.5 at 0 h to 4.6 at 72 h and an increase to 5.2 at the end of fermentation. The observed consistent decrease in pH within the first 3 days of fermentation was primarily due to the diffusion of organic acids predominantly acetic acid into the beans produced by acetic acid bacteria in the pulp. The observed continuous reduction in pH for the unstored pods during fermentation might be due to the fact that the unstored pods had a lot of pulp adhering to the beans and thus more pulp sugars were metabolized producing more organic acids which diffused into the beans even after 72 h of fermentation. Towards the end of fermentation, an increase in pH was observed, possibly due to evaporation of volatile acids like acetic (Afoakwa *et al.*, 2011b).

Increasing pod storage caused consistent increases in pH of the nibs at the end of fermentation (Figure 1). The pH of the nibs increased from 4.80 for the unstored pods to 7.01 for pods stored for 10 days at the end of fermentation. The pH of the beans during fermentation is crucial as it determines the rate of enzyme activity responsible for the production of flavour precursors as well as the development of the characteristic brown colour of cocoa beans (Hansen *et al.*, 1998; Sakharov and Ardila, 1999); most of these enzymes are reported to have pH optima of 4.5–5.5 (Biehl *et al*., 1989). Work done by Biehl *et al.* (1985) and Biehl and Voigt (1994) reported that, fermented cocoa beans with pH between 5.0–5.5 produce higher flavour potentials whereas fermented beans with pH 4.0–4.5 give low flavour potential. The pH at the end of fermentation was 5.10 and 5.36 for pods stored for 3 and 7 days respectively. The pH was however very low for the unstored pods (4.80) and very high (7.01) for pods stored for 10 days at the end of the fermentation. Findings from this study suggest that pod storage between 3 and 7 days and 6 days of fermentation could be employed to produce cocoa beans with acceptable pH (between 5.0 and 5.5).

Regression analysis of the data showed significant ($p < 0.05$) influence of the linear factor of pod storage (PS) and fermentation time (FT) and quadratic factor of both PS and FT on the pH of the cotyledons. There was also significant interaction between PS and FT. The model developed could explain about 82% of the variations in the pH of the cotyledons (Table 1).

Table 1. Regression coefficients and their R^2 values in the models for pH, titratable acidity, sugars, protein and FFA of cocoa beans

Variables	pH	Non-volatile acidity	Reducing sugars	Non-reducing sugars	Total sugars	Protein	Free fatty acids
Constant	4.8520*	0.2337*	10.2891*	9.6062*	19.8953*	25.3221*	0.4740*
X_1	0.2957*	-0.0374*	1.1356*	-1.2547*	-0.1191	-1.7408*	0.0569*
X_2	-0.3128*	0.0494*	4.1586*	-12.2253*	-8.0668*	-2.0429*	0.0909*
X_1^2	0.5332*	-0.0657*	-0.3578	0.0071	-0.3507	0.4242	-0.0263*
X_2^2	0.7172*	-0.0852*	-1.8574*	7.6370*	5.7795*	-0.4779	-0.0566*
$X_1 . X_2$	0.5495*	-0.0546*	0.4964*	0.1787	0.6751	0.0347	-0.0172*
R^2	82.2%	78.4%	94.9%	98.7%	95.0%	87.6%	87.8%
R^2(adjusted)	80.1%	75.8%	94.3%	98.5%	94.5%	86.1%	86.4%

Notes: *Significant at $p < 0.05$; X_1= Pod storage; X_2= Fermentation time

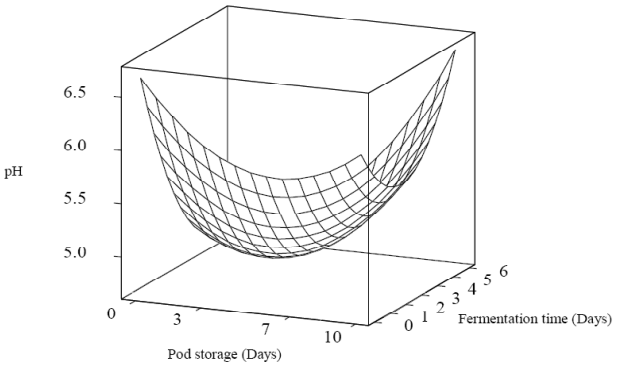

Figure 1. Response surface plot showing effect of pod storage and fermentation time on the pH of cocoa beans

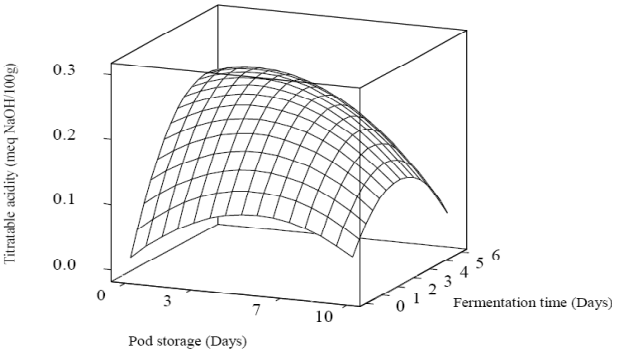

Figure 2. Response surface plot showing effect of pod storage and fermentation time on the non-volatile (titratable) acidity of cocoa beans

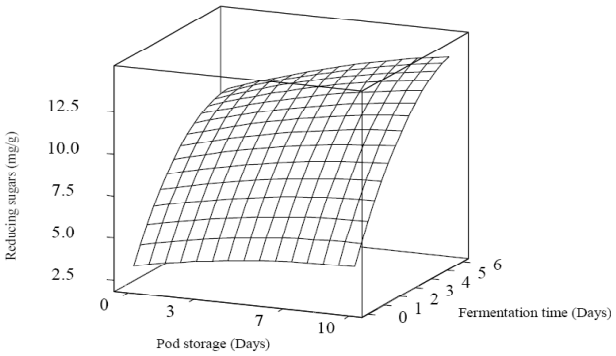

Figure 3. Response surface plot showing effect of pod storage and fermentation time on reducing sugars of cocoa beans

Changes in non-volatile (titratable) acidity

Changes in the non-volatile acidity (TA) of the cocoa beans during fermentation for all pod storage treatments are shown in Figure 2. Titratable acidity for the unstored pods increased continuously from 0.057 meq NaOH/100 g at the start of fermentation to 0.268 meq NaOH/100 g by the end of fermentation. However, with pulp preconditioned pods, fermentation caused an increase in acidity levels reaching a maximum within day 3 after which titratable acidity decreased considerably till the end of fermentation. Similar observations were made by Nazaruddin *et al.* (2006) and Afoakwa *et al*. (2011b). The observed increases in acidity might be due to the development of volatile acids (acetic, propionic, butyric and isovaleric) and non-volatile acids (citric, lactic, malic, succinic, and tartaric) in the pulp through sugar degradation by the metabolism of microorganisms during the fermentation process and its subsequent diffusion into the cotyledon (Jinap, 1994) to cause a gradual increase in acidity of the beans within the first 72 hours of fermentation. Acid production is necessary for killing the seed and to induce the necessary biochemical reactions in the seed, but excessive acid production and the resulting low cotyledon pH will result in the formation of low precursor type peptides and amino acids (Lopez and Dimick, 1995). Excessive acid in the beans would also result in acidic beans which is deleterious to the flavour quality of the fermented beans.

Again, increasing pod storage caused consistent reduction in nib acidity levels at the end of fermentation (Figure 2). Titratable acidity reduced from 0.268 meq NaOH/100 g for the unstored pods to 0.030 meq NaOH/100 g for pods stored for 10 days. This suggests that pod storage could be effectively used to reduce the acidity levels of cocoa beans, probably due to reduced pulp volume per seed and reduced pulp sugar content during the pod storage period which results in increasing micro-aeration within the pulp and reduced the alcohol fermentation and acetic acid production (Afoakwa *et al*., 2011b).

The model developed to predict the effect of pod storage and fermentation on titratable acidity (TA) of cocoa beans had an R^2 of 78% (Table 1). This implies that the model developed could explain about 78% of the variations in the titratable acidity of the nibs whiles the remaining 22% could be due to other factors not investigated in this work. Regression coefficients showed significant ($p < 0.05$) influence of the linear and quadratic factors of both pod storage (PS) and fermentation time (FT) on the titratable acidity of the cotyledons. There was also significant ($p < 0.05$) interaction between pod storage and fermentation time.

Changes in reducing sugars

Reducing sugars are carbonyl aroma precursors in fermented cocoa beans, which are mainly produced through the hydrolysis of sucrose by the action of invertase (Rohan and Stewart, 1967; Afoakwa, 2010). Fructose and glucose are the main reducing sugars in cocoa beans; however, reducing sugars can be formed from enzymatic hydrolysis of anthocyanins to yield arabinose and galactose by the action of glycosidase (Hoskin and Dimick, 1994).

The reducing sugars of the unfermented cocoa beans increased marginally during the pod storage period (Figure 3). It increased from 3.57 mg/g at the start of pod storage to 4.52 mg/g by 10 days of pod storage. This gradual increase in the concentrations of reducing sugars during pod storage might be as a result of the hydrolysis of sucrose in the beans during the storage period.

Fermentation, however, caused significant ($p < 0.05$) increases in reducing sugars at all pod storage periods (Figure 3). Reducing sugars increased from 3.57 mg/g at the start of fermentation to 10.69 mg/g at the end of fermentation for the unstored pods. It also increased from 3.89–11.94 mg/g, 4.29–13.34

mg/g, and 4.52–13.56 mg/g for pods stored for 3, 7, and 10 days respectively. The increase in the amounts of reducing sugars during fermentation is reported to be the result of enzymatic reactions promoted by invertase, β-galactosidase, α-arbinosidase, and α-mannosidase (Hansen *et al.*, 1998). Pod storage also increased the reducing sugars of the fermented beans. At the end of fermentation (6 days), reducing sugars increased by 50%, 60%, 70% and 80% respectively for pods stored for 0, 3, 7 and 10 days.

The model developed to predict the effect of pod storage and fermentation on reducing sugars of cocoa beans had an R^2 of 95% (Table 1) implying that about 95% of the variations in the reducing sugars of the cotyledons could be explained by the model whiles 5% was due to other factors not investigated in this work. Regression coefficients showed that the linear term of both pod storage (PS) and fermentation time (FT) and the quadratic term of FT had significant ($p < 0.05$) influence of on the reducing sugars of the cotyledons. There was also significant ($p < 0.05$) interaction between PS and FT on the reducing sugars of the cotyledons.

The production of reducing sugars during fermentation is very important as these sugars would react with peptides and free amino acids in the Maillard reaction during drying and roasting to produce the typical cocoa flavour compounds. Similar findings were reported by Reineccius et al. (1972) and Berbert (1979). Puziah *et al*. (1998a) noted an increase in total reducing sugars of about 208% during cocoa fermentation. Reineccius et al. (1972) detected final concentrations of fructose, glucose and total reducing sugars after fermentation to be 1.68, 0.43, and 2.99 mg/g, an increase of 405, 106, and 208%, respectively.

Changes in non-reducing sugars

Non-reducing sugars in cocoa beans comprise mainly of sucrose (Reineccius *et al*., 1972; Berbert, 1979; Puziah *et al.*, 1998a). Changes in non-reducing sugar concentrations during the pod storage and fermentation of cocoa beans are shown in Figure 4. Results from this study showed that non-reducing sugars were the major sugar present in significant concentrations in all the unfermented cocoa beans. The concentration of non-reducing sugars in the unfermented beans ranged from 27.95–30.56 mg/g (81–90% of the total sugars). The concentration of non-reducing sugars in the unfermented beans was highest in the unstored pods (accounting for about 90% of the total sugars in the unfermented beans) and decreased marginally with increasing pod storage. The marginal decrease of non-reducing sugars in

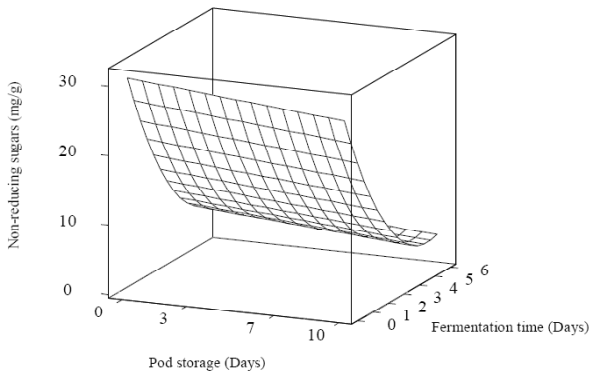

Figure 4. Response surface plot showing effect of pod storage and fermentation time on non-reducing sugars of cocoa beans

the unfermented beans with increasing pod storage might be due to some invertase activity in the beans during the storage period. The high concentrations of non reducing sugars in the unfermented cocoa beans have been reported by several researchers. Puziah *et al.* (1998a) found that sucrose was the only sugar present in abundant concentration (18.78 mg/g) which was about 95% of total sugars in unfermented cocoa beans. Reineccius *et al*. (1972) noted that the fresh unfermented Trinidad cocoa beans contained 15.80 mg/g sucrose and trace amounts of penitol, fructose, sorbose, mannitol and inositol. Berbert (1979) also reported that sucrose concentration of the unfermented beans comprised about 90% of the total sugars (24.80 mg/g), whereas both fructose and glucose made up about 6% (0.90 and 0.70 mg/g respectively), other sugars (< 0.50 mg/g) and total sugars (27.10 mg/g).

During the fermentation process, sucrose was hydrolyzed by cotyledon invertase to glucose and fructose (Lopez *et al.*, 1978). The concentrations of non-reducing sugars decreased significantly ($p < 0.05$) with increasing fermentation time for all pod storage periods (Fig. 4). It decreased from 30.56 mg/g to 6.11 mg/g (80% decreases) in the unstored pods at the end of the fermentation (6 days). Similar trends of decreases were observed for all pod storage treatments at the end of the fermentation. There were about 83%, 85%, and 84% decrease in the concentration of non-reducing sugars at the end of fermentation for pods stored for 3, 7, and 10 respectively. Reduction in the non-reducing sugars during fermentation has been reported by several researchers (Reineccius *et al.*, 1972; Berbert, 1979). Puziah *et al.* (1998b) found significant decrease in the concentration of sucrose during fermentation to 2.0 mg/g which was equal to 89% decrease.

The rate of decrease was high within the first 3 days of fermentation but slowed down towards the

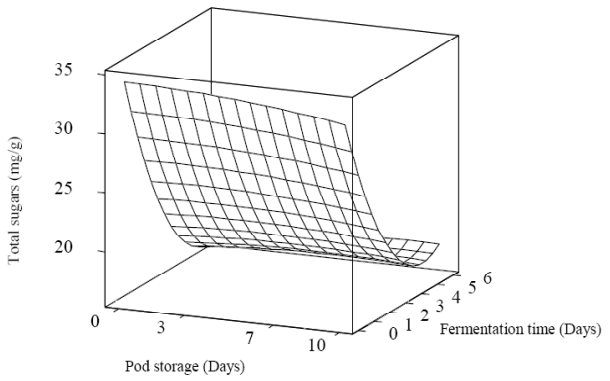

Figure 5. Response surface plot showing effects of pod storage and fermentation time on total sugars of cocoa beans

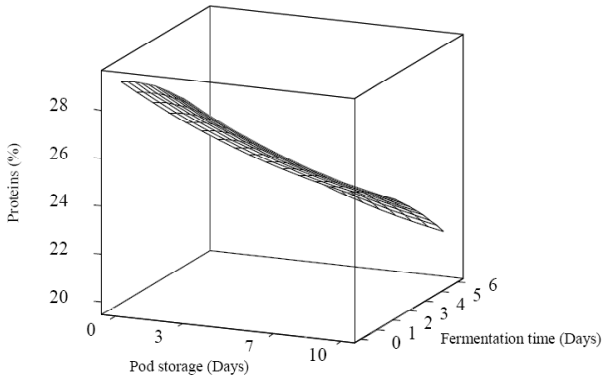

Figure 6. Response surface plot showing effect of pod storage and fermentation time on protein content of cocoa beans

end of the fermentation process. This might probably be due to high cotyledon invertase activity within the first 3 days of fermentation. Hansen *et al.* (1998) observed cotyledon invertase activity to decrease within 24 h of heap fermentation. Invertase activity was however reported to be insignificant from day 2 of fermentation till the end of fermentation. In the course of the fermentation, enzyme inactivation is caused by generated heat and high concentrations of acetic acid, ethanol and polyphenols (Hansen *et al*., 1998). This might explain why the reduction in non-reducing sugars slowed down towards the end of the fermentation.

Regression analysis of the data showed significant ($p < 0.05$) influence of the linear factor of both pod storage (PS) and fermentation time (FT) and quadratic factor of FT on the non-reducing sugars of the cotyledons. However, interaction between PS and FT was not significant ($p > 0.05$). The model developed could explain about 99% of the variations in the non-reducing sugars of the cotyledons, suggesting only 1% was due to other factors not investigated in this work (Table 1).

Changes in total sugars

Total sugars of cocoa beans comprise of reducing and non-reducing sugars (Puziah *et al*., 1998a). Changes in the concentrations of total sugars during fermentation for all pod storage treatments are shown in Figure 5. Total sugars of the unfermented beans decreased marginally during pod storage. It decreased from 34.13 mg/g in the unstored pods to 32.47 mg/g in the pods stored for 10 days. The marginal decrease in total sugars during pod storage might be due to the marginal breakdown of sucrose in the seeds by invertase (Puziah *et al*., 1998a).

Total sugars of the beans however, decreased significantly ($p < 0.05$) during fermentation at all pod storage periods. Total sugars decreased from 34.13 mg/g at the start of fermentation to 16.81 mg/g at the end of fermentation for the unstored pods. It also decreased from 33.72–17.01 mg/g for pods stored for 3 days, 33.84–17.77 mg/g for pods stored for 7 days and 32.47–18.03 mg/g for pods stored for 10 days. This finding was in agreement with Puziah *et al*. (1998b) who reported total sugars to decrease significantly during fermentation to concentrations of 5.0 mg/g representing 75% decrease. The drastic reduction in the concentrations of total sugars during fermentation was probably due to the breakdown of non-reducing sugars (sucrose) in the cotyledons by invertase, as sucrose is hydrolyzed by cotyledon invertase to glucose and fructose during the fermentation (Lopez *et al.*, 1978).

Regression analysis of the data also showed significant ($p < 0.05$) influence of the linear and quadratic factors of fermentation time (FT) on the total sugars of the cotyledons. Both the linear and quadratic factors of pod storage (PS) as well as the interaction between pod storage and fermentation time did not significantly ($p > 0.05$) influenced the total sugars of the cotyledons. The model developed had an R^2 of 95% implying that the model could explain about 95% of the variations in the total sugars of the cotyledons, whiles the remaining 5% was due to other factors not investigated in this work (Table 1).

Changes in proteolysis during fermentation

Protein concentration decreased significantly ($p < 0.05$) as fermentation progressed from day 0 to day 6 (Figure 6). The protein decreased from 28.9% at the start of fermentation to 28.1% on day 3 of fermentation for the unstored pods. Further decreases occurred from 28.1% to 24.7% at the end of fermentation (6 days). Similar trend of decrease was observed for all pod storage treatments. This result was in agreement with previous reports by Dimick and Hoskin (1981), Lopez (1986), Jinap *et al.* (2008) and Afoakwa *et al.* (2011b). These decreases in protein content might be

caused by the endogenous breakdown of cocoa bean proteins to oligopeptides and free amino acids (Jinap *et al*., 2008). Findings by Voigt *et al.* (1994) and Afoakwa *et al.* (2011b) showed that the oligopeptides and free amino acids represent specific cocoa aroma precursors produced during fermentation. The reduction in protein content might also be due to the formation of complex between polyphenols and the proteins. Polyphenols undergo biochemical modification during cocoa fermentation through polymerization and can complex with protein and this can lead to reduction in protein content (Bonvehi and Coll, 1997; Nazaruddin *et al.*, 2006).

Again, protein content reduced significantly ($p < 0.05$) with increasing pod storage at all fermentation times (Figure 6). The protein content of the unfermented cocoa beans decreased from 28.9% (unstored pods) to 25.8% (10 days pod storage). The reduction in protein content during pod storage (pulp preconditioning) is reported to be due to the action of protease enzymes in the pods during storage and thus initiating the process of proteolysis (Afoakwa *et al.*, 2011b). This observation suggests that pod storage might have initiated the release of peptides and free amino acids which could influence the processes for the formation of flavour precursors in the bean during subsequent fermentation and drying.

The model developed to predict the effect of pod storage and fermentation time on the protein content of cocoa beans had an R^2 of 88% (Table 1). This implies that the model developed could explain about 88% of the variations in the protein content of the cotyledons whiles 12% of the variations could be due to other factors that were not investigated in this work. The regression coefficients also showed that the linear factor of both fermentation time (FT) and pod storage (PS) had significant ($p < 0.05$) influence on the protein content of the cotyledons. The quadratic factor of PS and FT as well as the interaction between PS and FT did not significantly ($p > 0.05$) influenced the protein content of the cotyledons (Table 1).

Changes in free fatty acids (FFAs)

Free fatty acids (FFAs) are carboxylic acids released from triglycerides (Selamat *et al.*, 1996) through the effect of lipase (E.C. 3.1.1.3) or an oxidation (Guehi *et al.*, 2008). The quality of raw cocoa beans depends widely on their FFAs content as it gives the measure of rancidity of cocoa beans (Afoakwa *et al*., 2011b) and high FFAs content is reported to be a serious quality defect which reduces the technical and economic value of the cocoa beans (Guehi *et al.*, 2008). Again, the hardness of cocoa fat (butter) is reported to depend on the saturated and

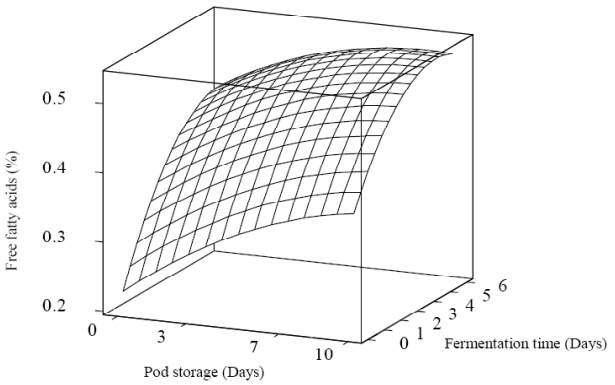

Figure 7. Response surface plot showing effect of pod storage and fermentation time on the free fatty acids of cocoa beans

unsaturated fatty acid contents bound in triglycerides, and on free fatty acids (FFAs) content (Guehi *et al*., 2008) and a high FFAs content leads to a decrease in hardness of cocoa butter which reduces the commercial value for both processors and chocolate manufacturers.

There were general increases in FFAs levels with increasing fermentation (Figure 7). The FFAs increased from 0.26% at the start of fermentation to 0.42% at end of fermentation for the unstored pods. Similar trend of increase was observed for all pod storage treatments. The FFAs also increased with increasing pod storage (Figure 7). The FFAs for the unfermented beans increased from 0.26% for both the unstored pods and pods stored for 3 days to 0.31%, and 0.52% for pods stored for 7 and 10 days respectively. At the end of fermentation, the FFAs levels were 0.51%, 0.52%, and 0.52% for pods stored for 3, 7, and 10 days respectively. The model developed to predict the effect of pod storage and fermentation of the free fatty acids of cocoa beans had an R^2 of 88% (Table 1). This implies that the model developed could explain about 88% of the variations in the FFAs of the cotyledons, whiles 12% of the variations were due to other factors not investigated in this work. Regression coefficients revealed that both the linear and quadratic factors of fermentation time (FT) and pod storage (PS) as well as the interaction between PS and FT had significant ($p < 0.05$) influence on the FFAs of the cotyledons.

The gradual increase in FFAs in the cocoa beans during both pod storage and fermentation could be attributed to the activity of lipase enzyme present in the natural cocoa beans and acts to breakdown the triglycerides into separate groups of the fatty acids and glycerol thereby freeing the fatty acids (Dand, 1997). The European parliament and European council directive 73/241/EEC (EEC, 1973) limits the maximum FFA content to 1.75% oleic acid equivalent

in cocoa butter. To be able to meet the acceptable level, Dand (1997) reported that the FFAs levels should be less than 1% in fresh cocoa beans and less than 1.75% in dried cocoa beans. Even though the FFAs levels in the cocoa beans increased with both fermentation time and pod storage, the levels were all however, below the acceptable limits of 1.75% oleic acid equivalent in cocoa butter. Results from this study suggest that cocoa pods can be stored up to 10 days and beans fermented for 6 days without adversely affecting the FFAs levels in the fermented beans.

Conclusions

The pH of unfermented cocoa beans was slightly acidic ranging from 6.09–6.27. Increasing pod storage caused consistent increase in pH of the nibs at the end of fermentation with consequent decrease in titratable acidity. Pod storage between 3–7 days produced fermented cocoa nibs (6 days fermentation) with pH between 5.10–5.36 which falls within reported pH (5.0–5.5) needed to produce cocoa beans of higher flavour potentials. Pod storage caused consistent increases in pH of the nibs at all fermentation times with consequential decrease in titratable acidity. Bean fermentation from pods stored between 3–7 days resulted in cocoa nibs with pH between 5.10–5.36 with only minimal changes in FFA. However, fermentation significantly decreased the non-reducing sugars, total sugars and protein content of the beans whilst reducing sugars increased. Reducing sugars increased by 50%, 60%, 70% and 80% respectively for pods stored for 0, 3, 7 and 10 days at the end of fermentation. Similarly, pod storage caused marginal reductions in total and non-reducing sugars with consequential increase in reducing sugars whiles protein content was reduced significantly. Storage of cocoa pod between 3–7 days with 6 days of fermentation led to considerable reductions in nib acidification, sugars (non-reducing and total sugars) and proteins with concomitant increases in reducing sugars and acceptable FFA levels (< 1.0%).

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