Effects of incubation and polyphenol oxidase enrichment on colour, fermentation index, procyanidins and astringency of unfermented and partly fermented cocoa beans

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Summary Incubation of unfermented and partly fermented cocoa beans in acetate buffer, pH 5.5, at 45 °C increased yellowness, total colour differences and fermentation index value of the cocoa bean powders and decreased cocoa procyanidins (monomers to pentamers), and their astringency. Fermentation index and (–)-epicatechin content, equivalent to those of fully fermented beans, were reached by unfermented beans after 4–8-h incubation, but not by partly fermented beans even after 16 h. During incubation of partly fermented cocoa beans enriched with polyphenol oxidase, yellowness and fermentation index value were increased, whilst (–)-epicatechin was decreased. Tyrosinase had a less significant effect in yellow colour formation, but showed a significant reduction of (–)-epicatechin and increase in fermentation index compared with crude cocoa polyphenol oxidase. However, both enzymes have similar effects on procyanidin degradation and astringent taste reduction. Incubation of cocoa beans for 16 h increased the cut test score of unfermented and partly fermented beans by 50 and 30%, respectively.

Keywords Astringency, cocoa beans, incubation, polyphenol oxidase, polyphenols, tyrosinase.

Introduction

Unfermented and partly fermented cocoa beans are the beans of *Theobroma cacao* L., which are dried without previously being fermented or partly fermented or by using improper procedures. Research has shown that these beans do not develop any chocolate flavour when roasted and are excessively astringent and bitter (Biehl & Voigt, 1996; Puziah *et al*., 1998). Malaysia is currently making use of these type of beans, which are imported from Indonesia, especially for cocoa liquor, powder and cocoa butter production. Cocoa manufactures would usually blend the unfermented and partly fermented beans with fully fermented beans, to obtain the desired flavour characteristics and to reduce the excessive astringency and bitterness.

Unfermented Forastero cocoa beans contain 120–180 g kg^{-1} of polyphenolic compounds (William, 1971; Kim & Keeney, 1984; Wollgast & Anklam, 2000). During cocoa fermentation, polyphenols are subjected to biochemical modification through polymerization and complexation with protein, hence decreasing solubility and astringency (Bonvehi & Coll, 1997). At the same time, anthocyanins are hydrolysed to anthocyanidins. The latter compounds polymerize along with simple catechins to form complex tannins. Anthocyanins usually disappear rapidly during the fermentation process, e.g. 93% were reportedly

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lost after 4 days fermentation (Wollgast & Anklam, 2000). Subsequently, during drying, polyphenol amounts are substantially reduced by enzymatic browning, catalysed by polyphenol oxidase and diffusion outside the beans (Forsyth & Quesnel, 1963; Kim & Keeney, 1984).

In quality control applications, anthocyanin content has been considered as a good marker when determining the degree of cocoa bean fermentation, along with the formation of brown colour (Gourieva & Tserevinov, 1979; Pettipher, 1986). Changes of cocoa bean colour are widely used to predict flavour potential of cocoa beans; cut test and fermentation index measurements are based on the colour changes in cotyledons during fermentation (Shamsuddin & Dimmick, 1986). Although the pigments themselves do not posses any marked taste potential in cocoa beans, the hydrolysis and oxidation of polyphenols is important as there is an inverse relationship between the flavour development and the purple colour retained after fermentation (Rohan, 1958; Lopez, 1986).

This paper discusses effects of incubation treatment with and without polyphenol oxidase enrichments on colour changes, fermentation index, procyanidin content and astringent taste of unfermented and partly fermented cocoa beans. Incubation treatments other than blending were designed to seek alternative handling methods for dried unfermented and partly fermented cocoa beans.

Materials and methods

Cocoa beans

Cocoa beans used in the study were F1 hybrids (*GC7 vs. SCA6*/*SCA12*) obtained from Kotta Blater Cocoa Estate, Indonesia. A wooden box (200 × 100 × 90 cm) of 2-tonne capacity was used. The fermentation mass was turned manually daily by transferring from one box to another. Cocoa beans of 0 day (unfermented), 2 days (partly fermented) and 5 days fermentation (fully fermented) were collected and sun-dried until they had a 7% moisture content. Dried beans were then peeled, lyophilized, grinded to size < 0.5 mm and defatted using petroleum benzene (b.p. 40–60 °C).

Design of the study

The study was in three parts. The first part was to study the effect of incubation without polyphenol oxidase enrichment on colour, fermentation index and (–)-epicatechin of unfermented and partly fermented cocoa beans powder. The second part was the study of the effect of the incubation of partly fermented cocoa bean powder enriched using crude cocoa polyphenol oxidase and commercial tyrosinase (purchased from Sigma Co., Steinheim, Germany). The third part was the evaluation of the effectiveness of incubation on quality improvement of cocoa beans.

The second part was essential because the result of the first part showed that the incubation of partly fermented cocoa beans did not achieve the (–)-epicatechin content and fermentation index value of fully fermented cocoa beans even after 16-h incubation.

The first and second parts used complete randomized design with two variables and three replications. The first variable of the first part was the degree of fermentation, which consisted of unfermented and partly fermented cocoa beans. The second part was the enzyme enrichment experiments, consisting of control without enrichment, enrichment using crude cocoa polyphenol oxidase at 8800 units g^{-1} and enrichment using tyrosinase at 8800 units g^{-1}. The second variable was time of incubation, consisting of 0, 1, 2, 4, 8 and 16 h for the first step, and 0, 8, 16, 24 and 32 h for the second step. Dried fully fermented beans were used as control.

Crude cocoa polyphenol oxidase preparation

Preparation of crude cocoa polyphenol oxidase used the method of Lee *et al.* (1991). Defatted freeze-dried unfermented fresh cocoa powder (15 g) was homogenized (5 min) in 100 mL of 0.2 M sodium phosphate buffer, pH 6.8, containing 5 g L^{-1} polyvinylpyrolidone and 5 mL L^{-1} Triton X-100 using a Polytron PT 1200 (Kinematica AG, Lucerne, Switzerland). The homogenate was centrifuged at 22 000 ***g*** at 4 °C for 20 min using a Kubota 7800 (Tokyo, Japan) followed by filtration through glass wool. Two to three volumes of cool acetone was added slowly into the supernatant; the solution was then stirred at

4 °C for about 20 min. The precipitate was collected by centrifugation and was re-extracted with 0.2 M sodium phosphate buffer, pH 6.8. The solution was dialysed for 48 h against 50 mM phosphate buffer, pH 6.8, using an Oscilatory Microdialysis System OMS 101 (Bio-Rad, CA, USA) with four changes of buffer.

The activity of the crude enzyme was assayed according to the modified method of Hansen *et al.* (1998) using a UV-Vis Shimadzu UV-1601 Spectrophotometer (Tokyo, Japan) at 438 nm and 25 ± 1 °C. The incubation mixture contained 100 μL of 0.2 M sodium phosphate buffer, pH 6.8, 75 μL of enzyme extract and 825 μL of 0.005 M (–)-epicatechin. The rate of reaction was determined from the initial linear slopes of the activity curves. One unit of polyphenol oxidase activity was defined as the amount of enzyme that caused 0.001 unit change in absorbance per minute per millilitre of enzyme-assayed solution (Lee *et al.*, 1991).

Incubation of cocoa bean powders

Defatted cocoa powder (5 g) was suspended in 250 mL of 10 mM acetate buffer, pH 5.5. The suspension was incubated at 45 °C in an orbital shaker incubator YIH DER Model LM 510R (YIH DER Instrument, Taiwan) at 150 r.p.m. After incubation, the suspension was immediately lyophilized at −20 °C for 16 h, freeze-drying was at a pressure of $< 133 \times 10^{-3}$ mbar using a Labconco Freezone 6 (Kansas City, KS, USA), and ground using Braun ZK 100 Cutter Blender (Braun, Germany) to 40 mesh.

Spectral and fermentation index measurements

Five hundred milligrams of sample powder was weighed into a 125-mL conical flask before a mixture of 50 mL of methanol:hydrochloric acid (97 : 3) solution was added and the mixture was cooled at 8 ± 2 °C in a refrigerator for 16–18 h. A clear extract was obtained by filtration through Whatman filter paper no. 1. The absorption spectra were recorded over the range 400–700 nm (Fuleki & Francis, 1968; Lee *et al.*, 1991) using a UV-Vis Shimadzu UV-1601 Spectrophotometer (Tokyo, Japan). Fermentation index was determined by using the method of Gourieva & Tserevinov (1979) based on the ratio of the absorbance at 460 nm to that of 530 nm. A fermentation index value of ≥1 would indicate the cocoa mass was sufficiently fermented (Shamsuddin & Dimmick, 1986).

Colour measurement

Measurement of colour changes during incubation was measured by using an Ultra Scan-Hunter Laboratory Colorimeter (Hunter Associate Laboratory Inc., Reston, VA, USA). The results were expressed in the $L^*a^*b^*$ colorimetric system, according to the International Commission of Illumination, in which a colour can be defined conventionally by three numerical parameters: sample luminance, L^* (quantity of reflected light) and chromatic coordinated, a^* (red-green axis) and b^* (yellow-blue axis) (Hammami *et al.*, 1999). Other parameters were obtained from those measurements according to the calculation of Rocha & Morais (2001), Filipa & Cristina (1999) and Hammami *et al.* (1999), as follows:

$$C^*(\text{chroma or saturation}) = (a^{*2} + b^{*2})^{1/2}$$

$$h^{\text{deg}*}(\text{hue angle, characteristic/dominant colour}) = \tan^{-1} b^*/a^*$$

$$\text{TCD}^*(\text{total colour differences before and after reaction}) = \left((L_f^* - L_i^*)^2 + (a_f^* - a_i^*)^2 + (b_f^* - b_i^*)^2\right)^{\frac{1}{2}}$$

(Note: subscripts 'f' and 'i' correspond to the final and initial measurement, respectively.)

(–)-Epicatechin determination

(–)-Epicatechin content was analysed using the method of Kim & Keeney (1984). Five hundred milligrams of sample powder and 80 mL of 80% aqueous acetone in a 125-mL Erlenmeyer flask were sonicated for 30 min in a sonic cleaning device filled with ice water. After filtering the extract under vacuum through Whatman filter paper no. 1 on a Buchner funnel and washing the glassware and residue with 80% aqueous acetone, the total filtrate was made up to 100 mL in a volumetric flask. Ten millilitres of the filtrate was dried under vacuum at 45 °C in a 125-mL rotary evaporator flask. This residue was resuspended into two 5-mL aliquots of distilled water by

swirling for 2 min in a 45 °C water bath. The solutions were pooled and injected through a Waters Associates (Milford, CT, USA) C_{18} reverse-phase SEP-PAK that had been preconditioned with 2 mL of methanol followed by 5 mL of water. (–)-Epicatechin, which was retained in the SEP-PAK, was eluted with 40% aqueous methanol into a 10-mL volumetric flask.

Ten microlitres of the final solution was analysed by high pressure liquid chromatography (HPLC) using a Waters Associates (Milford) Model 600 Controller and model 486 Tunable Absorbance Detector. Separation of (–)-epicatechins was accomplished on a μBondapak C_{18} reverse-phase analytical column using water:methanol:acetic acid (87 : 8 : 5) as mobile phase at a flow rate of 2.0 mL min^{-1}. The (–)-epicatechins were detected at 280 nm and quantified by comparing peak area of the sample to those obtained from an authentic standard.

Procyanidin measurement

Procyanidins, consisting of monomers to pentamers, were measured using a normal-phase high-performance liquid chromatography method as described by Rigaud *et al.* (1993), with slight modifications in terms of column used and elution conditions. One gram of defatted cocoa powder was diluted in 10 mL of 80% (v/v) acetone and homogenized for 5 min. The suspension was then centrifuged at 22 000 ***g*** and 4 °C for 20 min. The supernatant was collected and salted out using NaCl. Ten microlitres of the supernatant was then injected into the HPLC, which consisted of a Waters Associates (Milford) Model 600 Controller and a model 486 Tunable Absorbance Detector. The column was a μBondapak-NH_2 (particle size 5 μm; 300 × 3.9 mm i.d.) stainless steel tube column protected with a guard column (20 × 4 mm i.d.) packed with the same material. The solvents were dichloromethane–methanol–formic acid–water with volume ratios of (A) 5 : 43 : 1 : 1 and (B) 41 : 7 : 1 : 1. The elution conditions were as follows: flow rate, 1.3 mL min^{-1}; oven temperature, 30 °C; elution, linear gradients from 0 to 20% A for 30 min, 20–100% A for 20 min, and stand at 100% A for 10 min. The eluting procyanidins were monitored by measuring their absorbance at 280 nm. The procyanidins, from monomers to pentamers, were quantified by comparing peak area of the sample to those obtained from an authentic standard.

Sensory evaluation for astringent taste

Eight trained panelists at the Sensory Laboratory of the Cocoa Downstream Research Center, Malaysian Cocoa Board, did the sensory analysis. Ten grams of cocoa powder was mixed with 12 g of deodorized cocoa butter, and homogenized using a mortar. The liquor obtained was then roasted in a Memmert ULM 500 (Schwabach, Germany) oven at 120 °C for 45 min. Evaluation was done by using scoring scaled in the range of 0 for not present and 10 for full intensity. Ghanaian cocoa liquor with astringency score 1.5 was used as a reference.

Incubation of dried cocoa beans

Three hundred beans of both dried unfermented and partly fermented cocoa were incubated in distilled water (400 mL) for 16 h using a Certomat® WR (B. Braun, Germany) water bath shaker at 160 U min^{-1} and 45 °C. The incubation was replicated four times. After incubation, the beans were dried in a Memmert ULM 500 (Schwabach) oven at 60 °C until about 7% moisture content was achieved. The dried beans were then analysed for fermentation index and cut test score according to the methods of Gourieva & Tserevinov (1979) and Shamsuddin & Dimmick (1986), respectively. Cut test score was calculated using the formula:

$$\begin{aligned}\text{Cut test score} = {} & (1 \times \%\ \text{slaty}) \\ & + (2 \times \%\ \text{fully purple}) \\ & + (3 \times \%\ \text{three-quarter purple}) \\ & + (4 \times \%\ \text{half brown}) \\ & + (5 \times \%\ \text{three-quarter brown}) \\ & + (6 \times \%\ \text{fully brown}).\end{aligned}$$

Statistical analysis

Data obtained from the first and second parts were analysed using the SAS package software version 6.12 (SAS, 1996). Statistical significance was assessed by two-way analyses of variance and significant differences were detected using Duncan's multiple range test at 95% of confiden-

tial level and orthogonal contrast. Data from the third part were compared with each other based on average and standard deviation.

Results and discussion

Incubation without polyphenol oxidase enrichment

Polyphenols are groups of natural benzo-γ-pyrane derivatives ubiquitous in the plant kingdom and occur most often as glycosides (Pekkarinen *et al.*, 1999). However, major compounds of the cocoa bean polyphenols are catechins or flavan-3-ols (*c.* 37%), proanthocyanidins (*c.* 58%) and anthocyanins (*c.* 4%) (Wollgast & Anklam, 2000). The anthocyanins, in acid conditions, impart a red to purple colour with maximum absorbance at 500–550 nm.

Results of spectral measurement (Fig. 1) showed that, prior to incubation, unfermented cocoa beans (line 0 h) were dominated by compounds having maximum absorption at 530 nm, attributed to the presence of anthocyanin pigments. After incubation for 16 h, compounds with absorbance of 530 nm significantly decreased. In contrast, compounds with absorbance values below 500 nm increased, the latter was most probably due to the oxidation products of the polyphenol oxidase activities. According to Whitaker (1995) and Kattenberg & Kemmink (1993), dark coloured pigments would come from complexation of amino acids and/or proteins with quinines. The latter are enzymatic oxidation products of polyphenols in the presence of oxygen. Furthermore, the brown pigments may also be produced from complexation of condensed tannin, a high molecular weight product of flavonoid polymerization, with protein, via hydrogen bonding. Shamsuddin & Dimmick (1986) and Gourieva & Tserevinov (1979) found that the oxidation products of cocoa bean polyphenols could be detected at 460 nm; however, in our study we oxidized (–)-epicatechin using tyrosinase and showed that there was an absorbance, maximum at 428 nm (data not displayed).

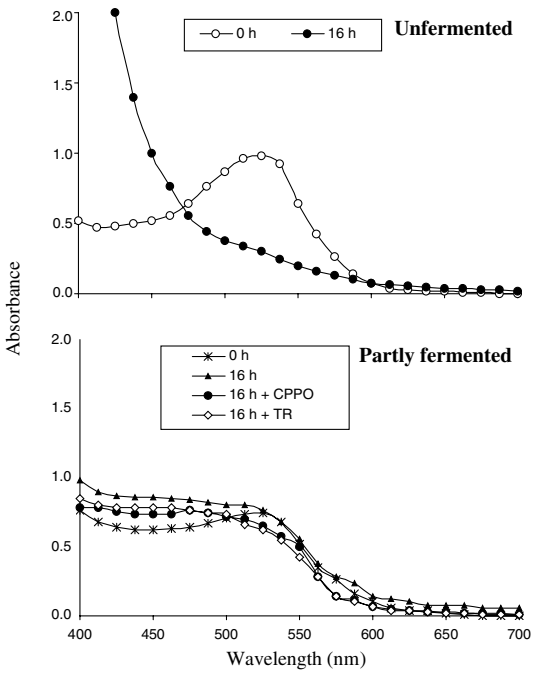

Figure 1 UV-Vis spectra of cocoa beans before and after 16-h incubation in 10 mM acetate buffer at pH 5.5 (CPPO: crude cocoa polyphenol oxidase; TR: tyrosinase).

In the incubation of partly fermented cocoa bean powders, the spectra showed an increase of absorbance intensity below 530 nm, without much change in the absorbance at 530 nm and above. As with the unfermented powders, the increased intensity of brown colour is attributed to the oxidation of polyphenols by the action of polyphenol oxidase, suggesting that activity remained in the partly fermented beans. The unchanged spectral response at 530 nm and above may be due to the low degradation-hydrolysis of anthocyanins, as the result of lower glycosidase activity in partly fermented beans.

Results of the tristimulus colour measurements on cocoa powders obtained from incubation of both unfermented and partly fermented cocoa beans (Fig. 2) showed that L^* did not change progressively during the incubation. Both a^* and b^*, however, increased progressively during incubation, and higher incremental rates were observed during the first 4 h of incubation. Greater changes in a^* and b^* values were also observed in the incubation of unfermented cocoa beans powder compared with that of partly fermented bean incubation. This agrees with the spectral measurement in which the spectral changes were higher in the incubated unfermented cocoa beans than in the incubated partly fermented beans. The differences in polyphenol oxidase activity and phenolic compounds in unfermented and partly fermented

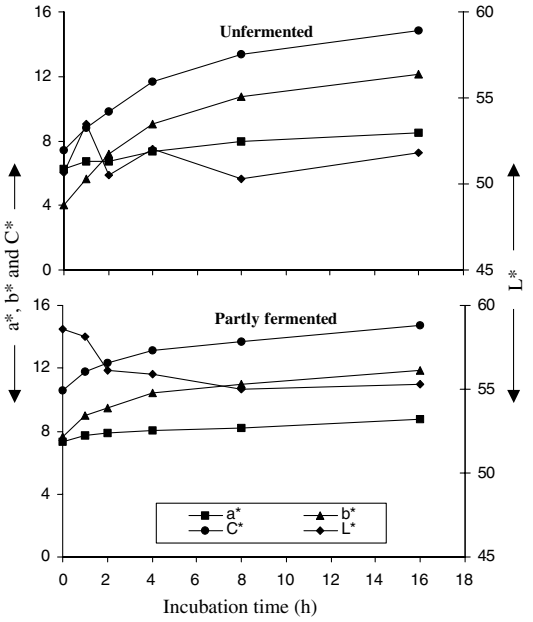

Figure 2 Changes in trimulus colours during 16-h incubation of unfermented and partly fermented cocoa beans in 10 mM acetate buffer at pH 5.5 (a*: green-redness; b*: blue-yellowness; C*: chroma; L*: lightness).

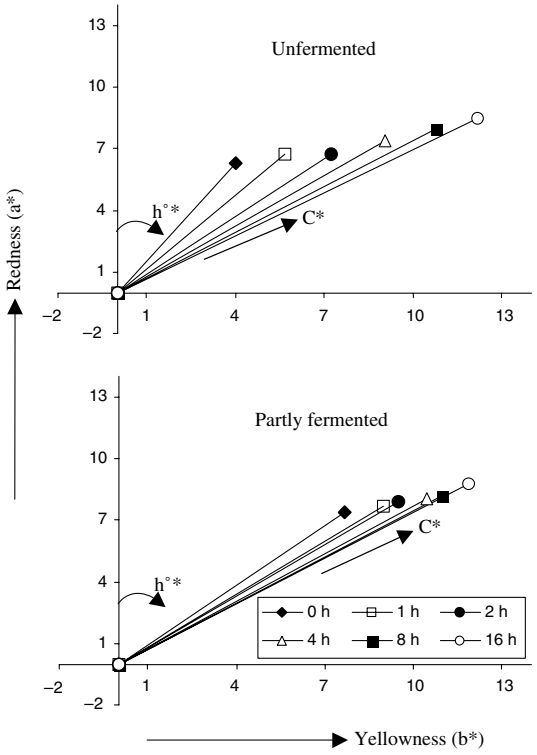

Figure 3 Changes in hue angle (h°*) and chroma (C*) during 16-h incubation of unfermented and partly fermented cocoa beans in 10 mM acetate buffer at pH 5.5.

cocoa beans were suspected to influence colour formation intensity. A study, reported by Hansen *et al.* (1998), found that the remaining polyphenol oxidase activities in unfermented cocoa beans was 4.5 U g^{-1} and in beans fermented for 2 days it was only 0.3 U g^{-1}.

Changes in C* during incubation of unfermented cocoa beans (Fig. 2) showed a higher linear correlation between C* and b* than between C* and a*, indicating that changes in yellowness were more dominant than those of redness. A similar result was also observed in the incubation of partly fermented beans, but starting at higher values. These results imply that yellowness is a stronger determinant of cocoa bean colour changes during incubation than redness.

Hue angle (h°*) of unfermented cocoa bean powders (Fig. 3) showed dominance of red colour compared with yellow (h°* < 45°) during the first 2-h incubation, then yellow colour was dominant as time of incubation increased. In partly fermented beans, the h°* changes commenced at an angle close to 45°, a balanced value between red and yellow colour. The effect of incubation time on the h°* was lower in the partly fermented bean incubation compared with that of unfermented beans.

Increase in yellowness during incubation could be due to the presence of oxidized polyphenols, as a result of enzymatic oxidation by polyphenol oxidase remaining in the beans; however, increase in a* values cannot be explained. Redness (a*) should decrease along with anthocyanin degradation. Cros *et al.* (1982) showed a significant decrease in anthocyanin pigments during fermentation; about 93% of the pigment was reduced between the second and third day of fermentation. Spectral measurements (Fig. 1) have supported this presumption: the absorbance at 530 nm (anthocyanin absorption area) decreased during incubation of both unfermented and partly fermented cocoa beans.

During incubation, total colour differences (TCD*) and fermentation index of both unfermented and partly fermented cocoa bean powders increased as (–)-epicatechin decreased (Table 1). TCD* (4.52), fermentation index value (1.58) and (–)-epicatechin content (17.2 g kg^{-1}) of fully fer-

Table 1 Total colour differences (TCD*), fermentation index (FI) value and (–)-epicatechin content during incubation of unfermented and partly fermented cocoa beans

Incubation time (h)	TCD*	FI	(–)-Epicatechin (g kg^{-1})
Unfermented beans			
0	0.00^{e}	0.57^{f}	40.2^{a}
1	3.01^{d}	0.95^{e}	33.6^{b}
2	3.36^{d}	1.36^{d}	31.1^{c}
4	5.30^{c}	1.76^{c}	24.8^{d}
8	7.12^{b}	2.49^{b}	17.4^{e}
16	8.52^{a}	2.98^{a}	15.2^{f}
Partly fermented beans			
0	0.00^{f}	0.86^{c}	40.1^{a}
1	1.41^{e}	0.92^{c}	37.9^{a}
2	3.13^{d}	0.92^{c}	32.9^{b}
4	3.93^{c}	1.02^{b}	32.8^{b}
8	4.94^{b}	1.08^{a}	27.9^{bc}
16	5.52^{a}	1.08^{a}	26.3^{c}
Fully fermented beans	4.52 ± 0.18	1.58 ± 0.01	17.2 ± 3.33

Mean values with same letter in the same column for unfermented or partly fermented beans are not significantly different according to Duncan's multiple range test at $\alpha = 0.05$.

mented cocoa beans was reached by unfermented bean powders after 8, 2 and 2 h of incubation, respectively. By contrast, incubation of partly fermented bean powders did not result in the (–)-epicatechin and fermentation index values found in fully fermented beans even after 32 h incubation. The TCD* value of fully fermented beans was reached after 4 h incubation. The differences in polyphenol oxidase activity and phenolic compounds remaining in unfermented and partly fermented beans are suspected to cause the differences in colour formation intensity.

Incubation with polyphenol oxidase enrichments

In order to enhance polyphenol oxidation in the incubation of partly fermented bean powders, crude polyphenol oxidase extracted from freeze-dried cocoa beans and purified tyrosinase from mushroom were added to the mixture. Statistical analysis using orthogonal contrast (Table 2) showed that (–)-epicatechin content and fermentation index were significantly ($P < 0.01$) influenced by enzyme enrichments, especially when using tyrosinase. Trimulus colour measurement did not reveal any effect of enzyme enrichment during incubation; however, analyses of fermentation index and polyphenol content, in terms of (–)-epicatechin (Table 3), showed that incubation enriched by both crude cocoa polyphenol oxidase and tyrosinase gave greater reduction (oxidation) of polyphenol content than without enzyme enrichment. The increases in polyphenol oxidation rate, resulting from enrichment of polyphenol oxidases, have also been reported to enhance polymerization and reaction of polyphenols with protein and/or amino acids (Kattenberg & Kemmink, 1993; Whitaker, 1995; Bonvehi & Coll, 1997), producing compounds which may not be detected by the trimulus colour measurement.

The TCD* and fermentation index values (Table 3) progressively increased during incubation. The TCD* value after 32 h of incubation was in the range 5.51–6.43, classified as very distinct to greatly distinct (according to the classification of Drlange, 1994 and Filipa & Cristina, 1999). The TCD* value of fully fermented beans (4.52) was reached, during the three incubation treatments, in 8 h. The fermentation index value of fully fermented beans (1.58) was only obtained with tyrosinase enrichment at around 8-h incubation; the other two incubation treatments did not reach that value even after 32-h incubation. C^* and $h°^*$ values (Fig. 4) showed that the effect of incubation time was significant up to 8-h incubation.

Table 2 Orthogonal contrast analysis of partly fermented bean incubation enriched by polyphenol oxidase

Contrast	*a**	*b**	*h°**	*C**	TCD*	FI	(–)-Epicatechin
No enrichment *vs.* enrichment	n.s.	n.s.	n.s.	n.s.	n.s.	S1	S2
No enrichment *vs.* CPPO	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	S2
No enrichment *vs.* tyrosinase	n.s.	n.s.	n.s.	n.s.	n.s.	S1	S1
CPPO *vs.* tyrosinase	n.s.	n.s.	n.s.	n.s.	n.s.	S1	S1

S1: significantly different, $P < 0.01$; S2: significantly different, $P < 0.05$; n.s.: not significantly different, $P > 0.05$.

Table 3 Total colour differences (TCD*) ,fermentation index (FI) value and (–)-epicatechin content during incubation of partly fermented cocoa beans enriched by polyphenol oxidase

Incubation time (h)	TCD*	FI	(–)-Epicatechin (g kg^{-1})
Crude cocoa polyphenol oxidase enrichment			
0	0.00^{c}	0.88^{d}	39.9^{a}
8	6.20^{b}	1.14^{c}	29.3^{b}
16	6.93^{a}	1.20^{b}	27.0^{b}
24	6.50^{b}	1.31^{a}	26.3^{bc}
32	6.43^{b}	1.31^{a}	22.9^{c}
Tyrosinase enrichment			
0	0.00^{c}	0.90^{c}	39.9^{a}
8	5.49^{b}	1.71^{b}	22.9^{b}
16	6.62^{a}	1.74^{b}	22.5^{b}
24	5.86^{b}	1.87^{ab}	19.8^{bc}
32	6.14^{ab}	2.04^{a}	18.6^{c}
Without enrichment			
0	0.00^{d}	0.90^{d}	39.9^{a}
8	4.94^{c}	1.08^{c}	31.0^{b}
16	5.52^{b}	1.15^{b}	29.7^{bc}
24	6.04^{a}	1.19^{a}	28.2^{bc}
32	5.51^{b}	1.21^{a}	27.2^{c}
Fully fermented beans	4.52 ± 0.18	1.58 ± 0.01	17.2 ± 3.33

Mean values with same letter in the same column for crude cocoa polyphenol oxidase, tyrosinase or without enrichments are not significantly different according to Duncan's multiple range test at $\alpha = 0.05$.

Effects of treatments on procyanidins and astringency

Major phenolic compounds in cocoa beans are (–)-epicatechin and its C4–C8 linked oligomers, up to the pentamers (Rigaud *et al*., 1993); however, Adamson *et al*. (1999) detected cocoa procyanidins from monomers to decamers, in which monomers to hexamers were dominant. Our results showed that procyanidins of cocoa powder, prior to incubation and after incubation, consisted of monomers, dimers, trimers, tetramers and pentamers. Incubation of unfermented cocoa significantly decreased the procyanidins and the astringent taste of the respective roasted liquor (Table 4). The procyanidins obtained were not significantly different from those of the well fermented cocoa, except for monomers and tetramers in which the content were significantly lower and higher, respectively.

For partly fermented cocoa, the results showed that incubation without enzyme enrichment

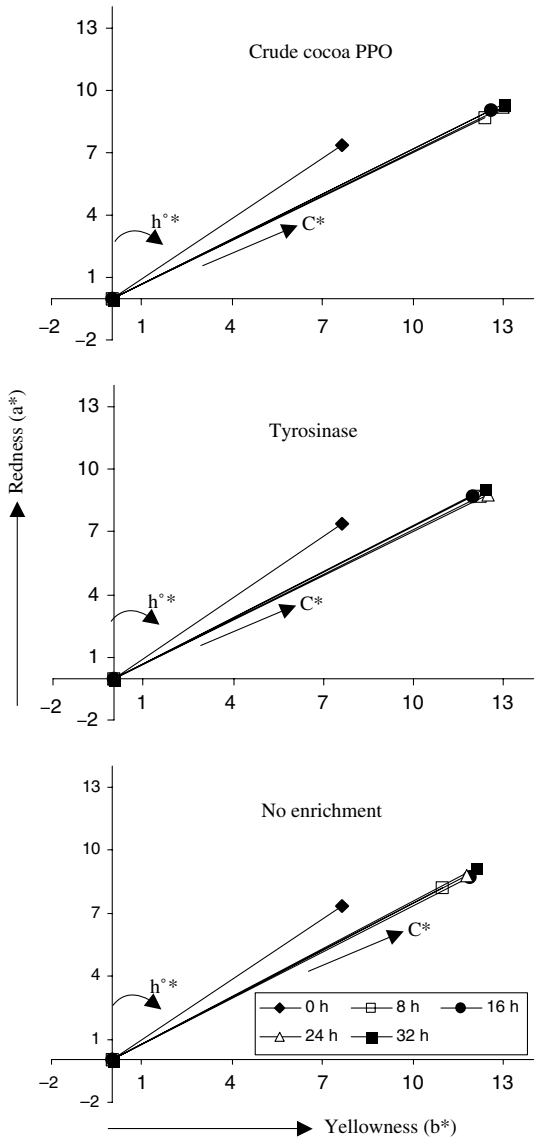

Figure 4 Changes in hue angle (h°*) and chroma (C*) during 32-h incubation of partly fermented cocoa beans in 10 mM acetate buffer at pH 5.5.

decreased the procyanidin contents slowly; the content did not reach that of well fermented cocoa even after 32-h incubation, except for the monomers. This was in conformity with its astringent taste, where its value was still high. Astringent values for partly fermented cocoa prior to incubation was 3.6 and after 32-h incubation was 3.2; however, that of well fermented cocoa was 2.6. High concentrations of procyanidin oligomers remaining in the cocoa sample was suspected to

Table 4 Effects of incubation on procyanidins and astringent taste

Treatment	Procyanidins (g kg^{-1})					Astringent*
	Monomers	Dimers	Trimers	Tetramers	Pentamers	
Unfermented						
Prior to incubation	19.12^{a}	5.86^{a}	13.10^{a}	7.64^{a}	5.38^{a}	4.3^{a}
After 16-h incubation	6.33^{d}	1.16^{c}	3.66^{de}	1.88^{d}	2.69^{bc}	2.8^{cd}
Partly fermented						
Prior to incubation	14.56^{b}	4.58^{a}	11.00^{b}	6.53^{b}	5.32^{a}	3.6^{b}
32-h incubation	11.68^{c}	3.21^{b}	7.49^{c}	3.95^{c}	3.14^{b}	3.2^{bc}
32-h incubation with cocoa PPO	3.28^{e}	0.46^{c}	2.92^{e}	1.35^{e}	2.57^{bc}	2.8^{cd}
32-h incubation with tyrosinase	2.72^{e}	0.61^{c}	3.10^{e}	1.36^{e}	2.50^{bc}	3.1^{bcd}
Well fermented	11.04^{c}	3.14^{c}	4.66^{d}	0.85^{e}	2.11^{c}	2.6^{d}

*Highest score = 10, lowest = 0. Mean values with same letter in the same column are not significantly different according to Duncan's multiple range test at $\alpha = 0.05$.

be responsible for the astringent sensation. Clifford (1985) stated that the ability of polyphenol to precipitate protein and impart an astringent sense requires at least two such active sites to form an astringent cross-link; polyphenols, which contain only one active site, are not considered to be astringent. Incubation of partly fermented cocoa, enriched with both crude cocoa polyphenol oxidase and tyrosinase, showed a significant increase in procyanidin degradation; this would result in lower concentration of monomers, dimers, trimers and tetramers compared with material without enzyme enrichment. The concentration of monomer and trimers was also significantly lower than that of well fermented cocoa.

Incubation of dried cocoa beans

In order to evaluate the effectiveness of the incubation procedure in quality improvement of dried unfermented and partly fermented cocoa beans, the beans were incubated using distilled water at 45 °C for 16 h. Results of fermentation index and cut test analyses (Table 5) showed that, although the fermentation index value of fully fermented beans (1.58) was not reached in either case, there was a significant increase in the values during incubation. The fermentation index of unfermented beans increased from 0.57 to 0.83 and that of partly fermented beans from 0.86 to 1.00.

The cut test score of unfermented beans increased from 283.2 to 424.0, equal to a 49.8% increase; however, partly fermented beans increased from 345.0 to 450.5 which is equal to a 30.4% increase. Prior to incubation, both dried unfermented and partly fermented cocoa beans were dominated by purple beans, 56.0 and 38.2%, respectively. Through incubation, the beans were converted to a brown colour due to the activities of the remaining glycosidase and polyphenol

Table 5 Effects of incubation of unfermented and partly fermented cocoa beans on fermentation index value and cut test score (mean ± s.d., $n = 4$)

Parameter	Unfermented beans		Partly fermented beans		Fully fermented beans
	Prior to incubation	After 16-h incubation	Prior to incubation	After 16-h incubation	
Fermentation index	0.57 ± 0.01	0.83 ± 0.02	0.86 ± 0.02	1.00 ± 0.01	1.58 ± 0.01
Cut test (% bean)					
Slaty	12.2 ± 4.57	0.0 ± 0.00	3.0 ± 1.15	0.0 ± 0.00	0.0 ± 0.00
Fully purple	43.8 ± 2.06	19.8 ± 5.19	35.2 ± 3.40	14.2 ± 2.75	0.8 ± 0.96
Purple dominant	14.2 ± 1.89	14.8 ± 5.68	15.8 ± 2.22	15.8 ± 2.99	2.0 ± 1.15
Purple = brown	14.2 ± 1.50	13.8 ± 3.30	13.8 ± 1.71	14.5 ± 1.29	5.0 ± 0.82
Brown dominant	9.2 ± 1.71	25.2 ± 2.06	22.8 ± 2.36	16.2 ± 2.50	8.0 ± 2.94
Fully brown	6.2 ± 2.22	26.5 ± 3.51	9.2 ± 1.26	39.2 ± 2.22	84.2 ± 3.30
Cut test score	283.2	424.0	345.0	450.5	573.0
Score improvement (%)		49.8		30.4	

oxidase. These results imply that low enzyme activities remaining in both unfermented and partly fermented beans were sufficient to cause chemical changes. Although, at the end of incubation, the values obtained did not reach those of fully fermented cocoa beans, longer incubation times may have given higher values.

Temperature and the presence of water during incubation may have activated the remaining glycosidase and polyphenol oxidase to hydrolyse and oxidize polyphenols of the beans, resulting in the reduction of purple beans and formation of brown beans. Hansen *et al*. (1998) stated that polyphenol oxidase activity is substantially lost during fermentation and drying due to the temperature and pH conditions.

Conclusion

The characteristics of fully fermented cocoa beans, in terms of colour, fermentation index value, polyphenol content and cut test score, could be reached by incubation of unfermented beans without polyphenol oxidase enrichment. However, in the case of partly fermented beans, the incubation has to be enriched by polyphenol oxidase to achieve the same result. In the same way, astringent taste was decreased throughout incubation.

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