Formation of Methyl Pyrazine during Cocoa Bean Fermentation

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ABSTRAK

Kajian mengenai kesan empat teknik fermentasi yang sedang diamalkan di Malaysia ke atas jenis dan amoun pirazina yang dikeluarkan dan penumbesaran Bacillus sp. dalam biji koko telah dijalankan. Biji yang baru dipetik telah difermen dengan menggunakan kotak kayu cetek (0.32m dalam), sederhana (0.61m), dalam (0.9m) selama 6 hari dan dibalikkan tiap 48 jam untuk fermentasi cetek dan sederhana, dan tiap 24 jam untuk fermentasi dalam. Biji yang melalui simpanan buah 10 hari juga difermen dalam kotak cetek selama 5 hari dan dibalikkan pada jam ke 48. Sampel telah ditentukan pH nib, asid tertitrat, indek fermentasi dan pirazina. Bilangan bakteria Bacillus sp. telah diperiksa dan dikenalpasti.

Jenis dan amoun pirazina yang dikesan berbeza mengikut teknik yang dijalankan. Sampel simpanan buah mengandungi 2, 3, 5, 6-tetramethilpirazina (19.8ug/100g) serta kepekatan yang paling tinggi bagi 2, 3, 5-trimethilpirazina (23.0ug/100g), 2, 5-dimenthilpirazina (154.8ug/100g) dan jumlah pirazina (177.6ug/100g). Sampel yang difermen dalam kotak cetek dan sederhana mengandungi 2, 3, 5-trimenthilpirazina dan 2, 5-dimithilpirazina; sampel kotak dalam mengandungi cuma 2, 5-dimehilpirazina. Bacillus sp. meningkat bersama dengan 2, 3-dimenthilpirazina, 2, 3, 5-trimethilpirazina dan jumlah pirazina bila tempuh fermentasi meningkat, dengan korelasi (r^2) 0.90. Bacillus sp. yang dipencilkan telah dikenalpasti sebagai B. Subtilis dan B. megaterium. 2, 5-dimethilpirazina terdapat dalam koko yang difermen di Malaysia.

ABSTRACT

A study on the effect of four fermentation techniques currently practised in Malaysia on the types and amount of pyrazines produced and the growth of Bacillus sp. in cocoa beans was carried out. Freshly harvested beans were fermented using wooden shallow (032m in depth), medium (0.61m), deep (0.90m) boxes for 6 d and turned every 48 h for the shallow and medium fermentations, and every 24 h for the deep fermentation; beans that had undergone 10 d pod storage were also fermented in shallow box for 5 d and turned on 48th hour. Samples were determined for nib pH, titratable acidity, fermentation index and pyrazines. Number of Bacillus sp. bacteria were monitored and identified.

The type and amount of pyrazines detected varied with the techniques employed. Pod-stored samples contained 2,3,5,6-tetramethylpyrazine (19.8ug/100g) and the highest concentration of 2,3,5-trimethylpyrazine (23.0ug/100g), 2,5-dimethylpyrazine (154.8ug/100g) and total pyrazine (177.6ug/100g). Samples fermented in shallow and medium box fermentations contained 2,3,5-trimethylpyrazine and 2,5- dimethylpyrazines; those of the deep box contained only 2,5-dimethylpyrazine. Bacillus sp. increased along with 2,3-dimethylpyrazine, 2,3,5-trimethylpyrazine and total pyrazine as fermentation period increased, with a correlation (r^2) of 0.90. The isolated Bacillus sp. were identified as B. subtilis and B. megaterium. 2,5-dimethylpyrazine was present in all samples; the component could be common in cocoa fermented in Malaysia.

INTRODUCTION

The fermentation technique is one of the most important factors in determining the quality of cured cocoa beans *(Theobroma cacao* L.). Previous studies have shown that shallow box (32 cm in depth) fermentation produces a low acidity and stronger chocolate flavoured cocoa than deeper box fermentation (60-90cm in depth). Most Ma-

laysian smallholders use shallow boxes; however, most estate operators still use deep boxes because they have to handle a large quantity of beans especially during peak harvest. The deep box fermentation would produce acidic beans (Jinap and Dimick 1990a). Pod storage for 9-12d followed by shallow box fermentation is currently the recommended technique in Malaysia because it has been shown to produce beans with acidity and chocolate flavour similar to those of West African beans (Biehl et al. 1989; Duncan et al. 1989).

Methyl pyrazines are among the most important flavour compounds in cocoa. In the presence of heat, such as roasting, they are produced through Maillard reactions i.e. between sugars and amino acids or peptides (Jinap and Dimick 1990b). Pyrazines could also be produced during fermentation; Bacillus sp. have been reported to produce many organic compounds such as acetic and lactic acids, 2, 3 butanediol and tetramethylpyrazine during fermentation of sake, tapai and cocoa (Schwan et al. 1986). Hruby et al. (1977) have shown that B. subtilis, B. pumilis, B. megatarium and B. cereus had strong proteolytic activity . B. subtilis was found to produce acetoin and ammonia in high concentration which could lead to the formation of tetramethylpyrazine in cocoa fermented in Brazil and Trinidad (Zak et al. 1972). There have been reports so far on quantification of pyrazines produced during cocoa fermentation in Malaysia.

This paper describes findings on the types and amount of methyl pyrazines produced during the cocoa fermentation using four techniques that are currently practised in Malaysia. The growth of Bacillus sp was also monitored.

MATERIALS AND METHODS
Cocoa Samples
Commercially mature (tinge of yellow on the pod) cocoa fruits of mixed hybrid varieties were obtained from Jempul, Negeri Sembilan. The pods were harvested in the afternoon and transported the next morning to the University, a journey which took about two hours. Although fruits for pod storage treatment were obtained 10 d before all fermentation treatments were carried out, the physical characteristics of the freshly harvested fruits and beans during these periods were the same. The study was conducted twice i.e. in October and November 1992 at the University of Agriculture, Malaysia.

Fermentation Technique
For pod-storage fermentation, about 800 pods were placed in rattan baskets (about 100 pods/basket) upon arrival at the University. The pods were stored under shade with good ventilation for 10 d, after which the pods were split open using the back of knives without damaging the beans. The placenta containing 30-40 beans (with pulp attached) were removed from the pod and the beans were separated by hand. Black or infested beans were removed. The healthy beans were fermented in wooden shallow 0.42 x 0.42 x 0.32m boxes arranged in tiers and covered with clean gunny sacks to minimize heat loss and to avoid contamination from insects, etc. The box design was as described by Shamsuddin et al. (1978). On the third day, the beans were filled using a shovel. The fermentation was terminated on the fifth day.

For other fermentation studies, the pods were split immediately upon arrival and treated as above. Beans were filled in three wooden boxes of 0.42 x 0.42m and depth of 0.32m (shallow), 0.61m (medium) and 0.90m (deep) and formented for six days. The shallow and medium fermentations were turned every 48 h and every 24 h in the case of deep fermentation.

Sampling
Samples (total of 1500g) were taken from five locations at the top, middle and bottom layers of the cocoa mass, every 24 h. The samples were coned and quartered to obtain two representative samples of about 100 and 500g to be used for microbiological and chemical analyses respectively. Samples for chemical analyses were sealed in plastic bags and kept in the deep freezer (-40°C). Microbiological analyses were carried out on the same day of sampling. All analyses were carried out in triplicate.

Microbiological Analysis
Plate Count - Samples (20g) were homogenized using a stomacher in sterile peptone solution (180 mL). Series of dilution up to 10^{-6} was carried out. Each dilution (0.1 mL) was spread in petri dishes containing Nutrient Agar solution (15 mL, Bacto). The petri dishes were incubated at 37°C for 24-48 hrs.

Bacillus sp. - The isolated colonies were tested using Gram Stain and Endospore tests (Buchanan and Gibbons 1975). Morphology of the bacteria was observed under a light microscope using oil immersion. Gram positive and rod-shaped bacteria were selected. Characterization of *Bacillus sp* was carried out using catalase, starch hydrolysis, Voges Proskaeur, glucose, citrate, 7% sodium chloride and motility tests as described in Buchanan and Gibbons (1975).

Chemical Analysis

Sample preparation - Bean samples were deshelled using knives and forceps to produce two sets of nib samples of 250g each. Each sample was ground with solid carbon dioxide using a Braun blender (3 min) before use for the determination of titratable acidity, pH and pyrazines.

Determination of pH and titratable acidity - Ground samples (20g) were used for pH and titratable acidity determinations following the methods described by Jinap and Dimick (1990a).

Determination of Fermentation Index - Fermentation index was determined using the modified method of Gourieva and Tserevinov (1979). Ground samples (0.5g) were homogenized in methanol:hydrochloric acid (97:3, 50 mL) solution. The mixture was kept at 8°C for 16-18 h before it was filtered (Whatman #4). The supernatant was read at ^460 and ^530 using a Hitachi U-1100 spectrophotometer.

Extraction and determination of pyrazines - Ground samples (200g) were heated with distilled water (200 mL) at 90°C for one h using Lickens Nickerson's Simultaneous Distillation Extraction Apparatus. The votiles were trapped in pentane (30 mL, 55°C) and concentrated (5 mL) using nitrogen gas (Reineccius *et al.* 1972).

Methyl pyrazine was quantified using Gas Chromatograph (Shimadzu GC-14A) equipped with a flame ionization detector and injector. The capillary column used was 50m x 0.25mm x 0.25um Silicone OV-101 (Hewlett Packard). The temperature of column oven was 60°C, with rates of 3°C/min until 120°C (20 min) whereas that of the injector was 180°C and detector was 250°C. Six standard pyrazine solutions i.e. 2- methylpyrazine, 2,3-dimethylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, 2,3,5 trimethylpyrazine and 2,3,5,6-tetramethyl pyrazine were used for reten-

tion time determination; pyrazine was used as an internal standard.

Statistical Analysis

The data were analysed using General Linear Model and the difference was measured using Least Significant Difference at 5% level.

RESULTS AND DISCUSSION

The pH, titratable acidity and fermentation index of the different techniques are shown in Table 1. It was observed that as the depth of the

TABLE 1
Means of final pH*, titratable acidity* and fermentation index* values of fermented beans

Fermentation Technique*	pH	Titratable Acidity (meq NaOH/g)	Fermentation Index
PS + Shallow	5.15ᵃ	0.098ᵇ	1.19ᵃ
Shallow	4.97ᵇ	0.124ᵃ	1.11ᵇ
Medium	4.88ᶜ	0.121ᵃ	1.09ᶜ
Deep	4.72ᵈ	0.133ᵃ	0.99ᵈ

*Means values having a common letter within the same column are not significantly different (p>0.05)
*PS = pod storage
shallow = 0.30cm in depth
medium = 0.60cm in depth
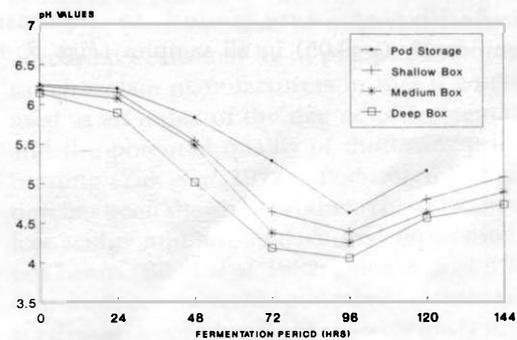deep = 0.90cm in depth

Fig. 1: Changes in cotyledon pH in different techniques of fermentation

cocoa mass increased the pH significantly decreased; samples from deep fermentation had

the lowest minimum pH of 4.05 compared to 4.62 for pod-stored fermentation samples (Fig. 1). The pH during fermentation determines the rate of enzyme activity responsible for the production of flavour precursors; most of these enzymes have pH optima of 4.5-5.5 (Biehl et al. 1989). Therefore, it could be postulated that the pod-stored samples would have higher concentrations of flavour precursors compared to the other fermentation techniques carried out in the study. The titratable acidity followed the same trend as the pH (Table 1). The final pH and TA ranged from 4.72-5.15 and 0.098-0.133 meq NaOH/100g, respectively (Table 1). The pod-stored samples had the most preferred pH (5.15) followed by shallow fermentation (pH 5.01); samples from medium (pH 4.88) and deep (pH 4.72) fermentations could be classified as acidic beans (Jinap and Dimick 1990a).

The fermentation index increased gradually to values between 0.99 to 1.19 which indicated that all of the techniques studied produced well fermented beans. However, the value for pod-stored fermentation (1.19) fell in the optimum range for good flavoured beans i.e. 1.10-1.30 (Shamsuddin and Dimick 1983). The anaerobic environment in deep fermentation would inhibit or delay polyphenol oxidase enzyme; therefore the colour did not change as fast as it did in beans in shallow fermentation. Samples from pod-stored fermentation were found to be fully fermented (FI ≥ 1) on day 3 whereas those from shallow, medium and deep fermentations, day 4, 5 and 6 respectively (Fig. 2).

Bacillus sp. were found to increase significantly (p<0.05) in all samples (Figs. 3, 4,

5, 6). The rate of increase was more pronounced after day 4 in deep fermentation and after day 3 in all the other fermentations. The findings were in agreement with those of Roelofsen (1958) who found that Bacillus sp. were dominant on the third day; however, Ostovar and Keeney (1973) found them to be dominant after 120 h in deep box fermentation carried out in Trinidad. Bacillus sp grow better in aerobic conditions, with a temperature of 30-50"C, and pH > 4.0 (Schwan et al. 1986). This explains the higher growth rate in pod-stored fermentation
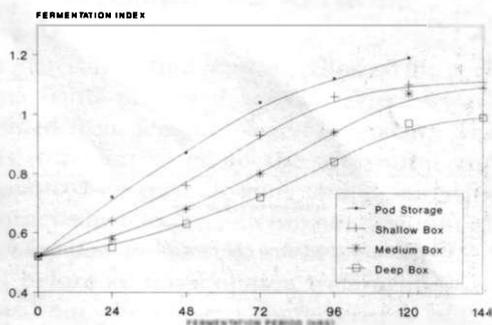

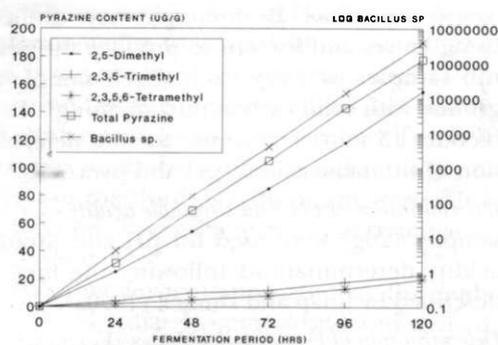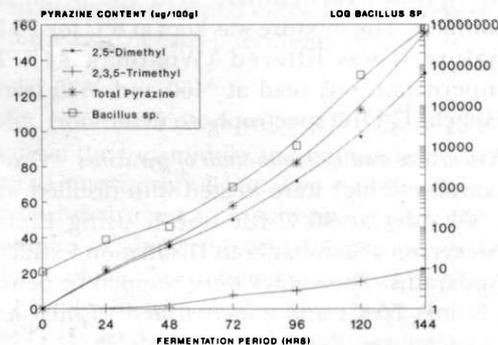Fig. 3: Pyrazine content and Bacillus sp. in pod storage fermentation

Fig. 4: Pyrazine content and Bacillus sp. in shallow box fermentation

followed by shallow, medium and deep fermentations. The pod-stored samples had a lower amount of pulp; the environment was more aerobic and therefore more suitable for growth of Bacillus sp. Two colonies were found to give positive responses to gram stain test and were rod-shaped. Biochemical tests indicated that the colonies were Bacillus subtilis and B. megaterium. These species have been shown to

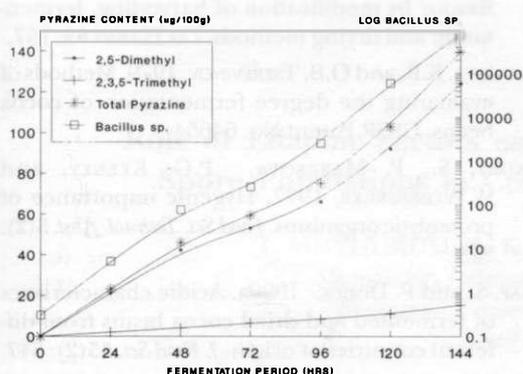Fig. 2: Changes in fermentation index in different techniques of fermentation

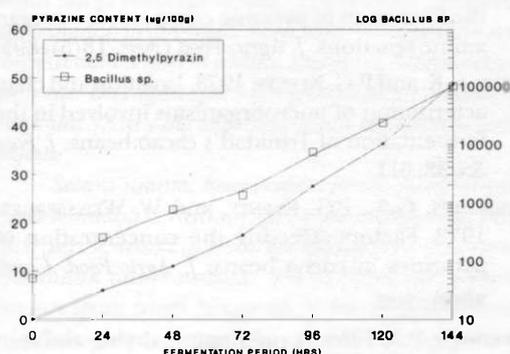Fig. 5: Pyrazine content and Bacillus sp. in medium box fermentation

Fig. 6: Pyrazine content and Bacillus sp. in deep box fermentation

play an important role in the proteolytic activity which produces amino acid and peptides responsible for chocolate flavour in cocoa beans (Barille et al. 1971; Hruby et al. 1977)

The study detected three methyl pyrazines i.e. 2,5-dimethyl-pyrazine, 2,3,5-trimethylpyrazine and 2,3,5,6-tetramethylpyrazine As the period of fermentation increased, the amount of each pyrazine also increased (Figs. 3, 4, 5, 6) Acetoin, 2,3-butanediol and pyruvaldehyde produced from sugar breakdown during cocoa fermentation reacted with amino acid, a hydrolysis product of protein during the anaerobic phase of fermentation, to produce methyl pyrazine (Reineccius et al. 1972).

At the end of the fermentation period, samples from pod-stored fermentation contained the highest concentration of total

pyrazine (177.6ug/100g) followed by shallow (155.0ug/100g), medium (140.2ug/100g) (Figs. 3, 4, 5.) Samples from deep fermentation contain a low concentration (48.8ug/100g) of 2,5-dimethyl- pyrazine (Fig. 6); those of shallow and medium fermentations contained a significantly (p $\leq$ 0.05) higher concentration of 2,5-dimethylpyrazine (132.6 and 124.4ug/100g) and 2,3,5-trimethylpyrazine (22.4 and 15.4ug/100g), respectively (Figs. 2 and 3). Pod-stored samples contain the highest concentration of 2,5-dimethylpyrazine (154.8ug/100g); 2,3,5,6-tetramethylpyrazine which were not found in other samples but was detected at 19.8ug/100g. However, the concen- tration of 2,3,5-trimethylpyrazine (23.0ug/100g) present in the pod-stored samples was not significantly different (p>0.5) from those of the other samples. 2,3,5,6-tetra-methylpyrazine and 2,3,5-trimethylpyrazine detected by Zak et al. (1972) in cocoa fermented in Trinidad and Brazil. Our study has found that 2,5-dimethylpyrazine was also present in higher concentration than those in the other two pyrazines found from all fermentations. The type and amount of pyrazines in cocoa depend on the source of the beans and type of fermentations employed (Koehler and Odell 1970). Therefore, it is possible that 2,5-dimethylpyrazine is common in unroasted Malaysian cocoa beans, regardless of the fermentation techniques employed; the findings also suggest that Bacillus sp. in Malaysian cocoa beans could produce intermediates leading to the formation of 2,5-padimethylpyrazine in addition to trim-etylpyrazine and tetrametyl- pyrazine, as reported by Zak et al. (1972).

The concentration of methyl pyrazines in unroasted beans may be of practical importance to chocolate manufacturers in that it could be used as an index of the degree of fermentation and the potential quality of the beans prior to roasting (Zak et al. 1972). Pod-stored fermentation has been shown to produce cocoa beans with low acidity and strong-flavoured fermented cocoa beans (Biehl et al. 1982; Duncan et al. 1989). Our study confirms that pod-stored fermentation is effective in producing beans with lower acidity and attains complete and fermentation earlier when compared with other fermentation techniques; and as the fermentation mass gets deeper and the final acidity of the beans increases, the lower is the fermentation index.

Our study also found that while pod-stored samples contain 2,3,5,6-tetramethylpyrazine other samples did not; a significantly (p<0.05) higher concentration of 2,5-dimethylpyrazine was also found in the pod-stored samples compared to other fermentation techniques carried out; therefore it is possible that these components could be used as indicators for good quality cured cocoa beans.

As the amount of methyl pyrazine increased, the log number of *Bacillus sp.* also increased; this suggests the posssibility of involvement of *Bacillus sp.* in the pyrazines formation during cocoa fermentation in Malaysia (*Figs. 3, 4, 5, 6*). Zak *et al.* (1972) reported similar findings in Brazilian and Trinidad beans. Our study found a corrrelation (r^2) of 0.90 between *Bacillus sp.* and 2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine and total pyrazine, respectively. Studies in the inoculation of *Bacillus sp* in cocoa mass under controlled conditions would be necessary to understand the role of *Bacillus sp.* in the formation of pyrazine in Malaysian cocoa beans. It is well known that *B. subtilis* and other microorganisms are able to produce large amounts of acetoin and ammonia which are the precursors of methyl pyrazine as proposed by Kosuge annd Kamiya (1969). Conversion of these intermediates to methyl pyrazines could be enzymatically or thermally induced (Zak *et al.* 1972). The temperature of the cocoa mass which could go up to 50-55°C, could play an important role in pyrazines production.

ACKNOWLEDGEMENTS

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