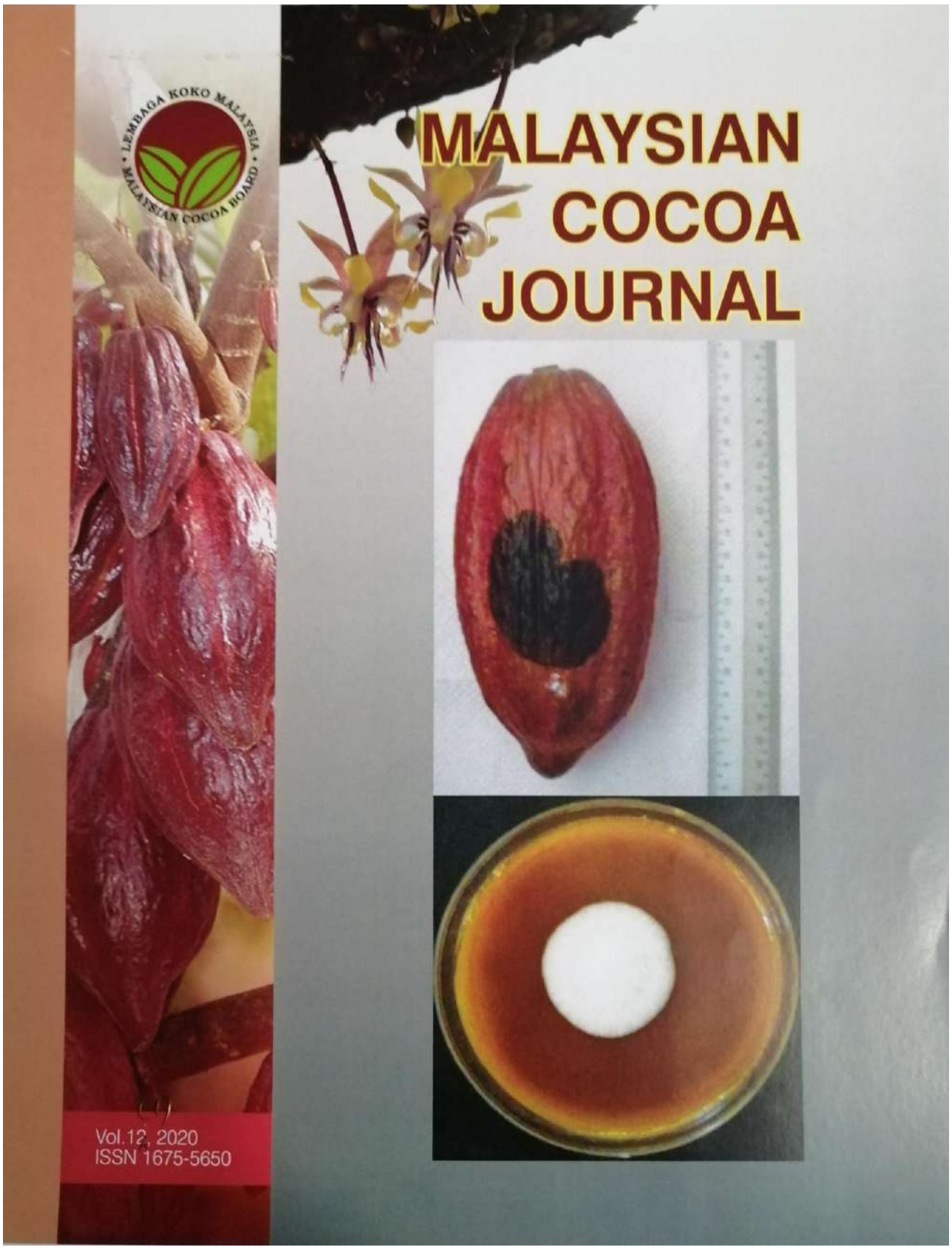
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CONTENTS

CPB MANAGEMENT ON COCOA FARM: IPM IMPLEMENTATION MODEL

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Malaysian Cocoa J. 12(1): 1-4 (2020)

ABSTRACT *- Cocoa Pod Borer (CPB) is a major insect pest of cocoa in Malaysia. Various methods have been developed to manage this insect pest. However, relying on single method will end up with high cost and labour intensive. For an example, CPB control using chemical will be associated with high cost of insecticide and reducing the population of beneficial insect such as cocoa pollinator and natural enemies. Whilst, pod sleeving though is effective in controlling the CPB, nevertheless it will be labour intensive to apply during the peak crop season. In additional, some insect pest such as leaf eating insect and branch borer could also become a major problem if CPB control depends only with pod sleeving method. Therefore, instead of relying on single method, managing the CPB problems could be overcome through adopting integrated pest management (IPM). A study on integrated pest management (IPM) on CPB control has been done, and the model to implement the IPM also has been developed. This model will be further discussed in this paper.*

Key words: *Cocoa, Cocoa pod borer, IPM*

INTRODUCTION

Cocoa pod borer (CPB), *Conopomorpha cramerella* (Snellen) (Lepidoptera: Gracillariidae) is still the major insect pest to cocoa in Malaysia. This insect is one of the contributing factors that the cocoa growers shift to other crops, thus decreasing the cocoa cultivation area and cocoa bean productivity. In the earliest appearance of CPB, controlling was almost impossible. It was due to lack of knowledge regarding its biology and ecology. Over the time, research regarding the CPB eventually finalised and its damaging nature to cocoa have been determined, methods of CPB control were formulated and established (Azhar 1985 and 1986; and Woods 1985). Implementing that control methods to manage CPB infestation lead to another challenges, labour intensive and high cost for the cocoa grower. Integrated pest management (IPM) is seen as an approach that could harmonise the current control methods for the CPB (*Navies et al*. 2016). In IPM approach, optimum yield would be achieves through consulting the best CPB control method at precise time and more practical. Through this approach cost for control and labour requirement can be minimised. Thus this paper will discuss the implementation model of IPM approach to control the CPB.

THE IPM IMPLEMENTATION (IPM) MODEL

An IPM program include four components; threshold (economic or action), surveillance, decision making, and control methods. In cocoa crop, the threshold component is focused on action threshold by observing the pod phenology. Economic threshold is not consulted because severe damage has been in progress while decision is being determined. Therefore, the most practical threshold for making a decision to control the CPB by insecticide spraying should be the pod phenology stage at 8-12 cm in length (Navies *et al*., 2016). Surveillance should be made by observing the pod phenology pattern and it is highly recommended that the cocoa grower should look after the maximum flowering stage. By observing it, the vulnerable pod stage to CPB would be forecasted at another next four months. Hence, insecticide spraying should be initiated in that forecasted time. Any available pod at 8-12 cm in length before that time should be protected from CPB by pod sleeving.

DISCUSSIONS

Through that implementation model, for a year activities it can be followed as in *Figure 1, 2 and*

3. The graphs are the actual activities on IPM study plot. Table 1 is the expected yield per hectare per year calculated from the actual experimental data of IPM study in the period of 2013 to 2015.

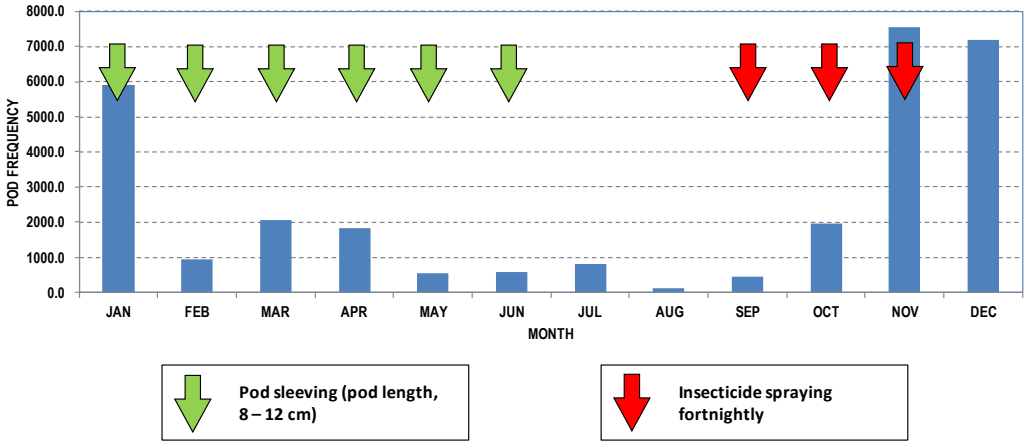

Figure 1. IPM program to manage the CPB on single cropping peak as occurred in Tenom, Sabah.

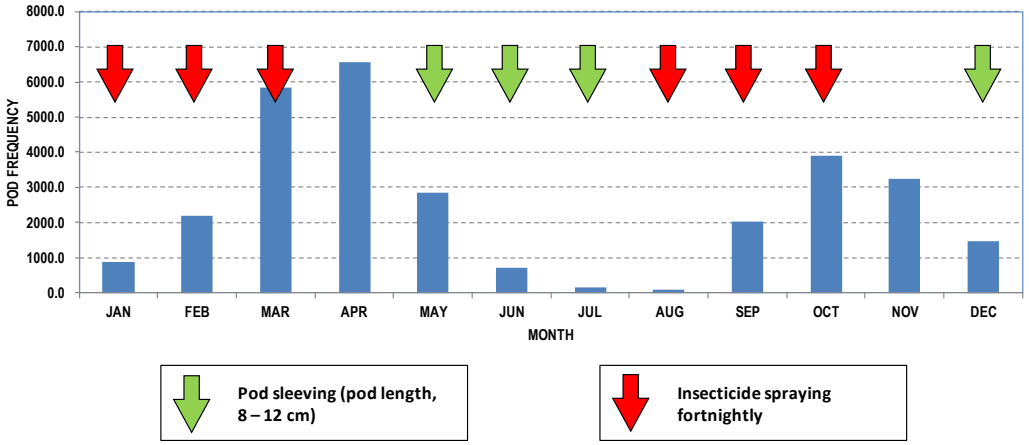

Figure 2. IPM program to manage the CPB on two cropping peak as occurred in Tawau, Sabah.

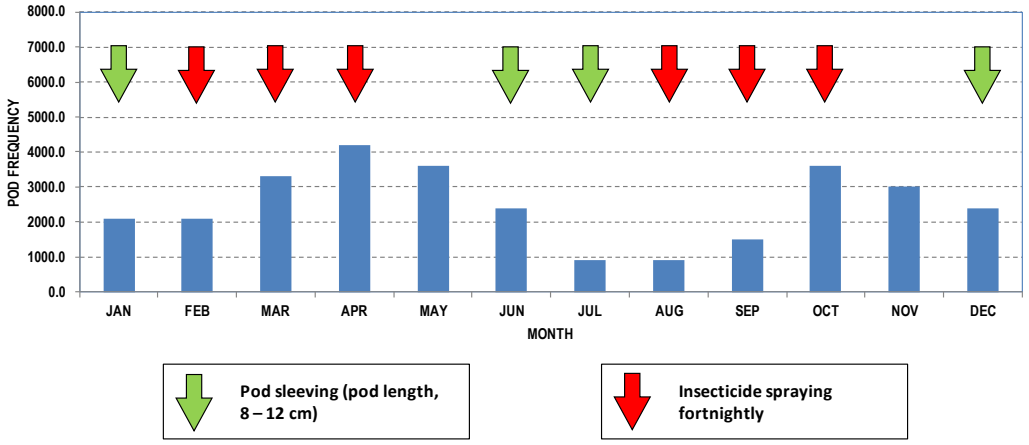

Figure 3. IPM program to manage the CPB on two cropping peaks as occurred in Madai, Kunak, Sabah.

Table 1. Estimated yield (kg) per hectare per year calculated from the actual result of IPM study (2013-2015)

Location	Estimated Yield (kg)		
	IPM	**Insecticide**	**Control**
Madai, Kunak	825.20 - 897.23	673.89 - 737.73	301.46 - 363.83
Tawau	839.69 - 896.81	582.44 - 640.19	445.41 - 502.95
Tenom	685.76 - 806.72	619.82 - 740.36	513.98 - 633.47

Normally cocoa will have two cropping seasons as shown at Tawau and Madai, Kunak in *Figure 2 and 3.* However due to locality and raining pattern, single cropping season may occur such as at Tenom *(Figure 1).* Therefore different strategy to implement the IPM also occurred. On single cropping season as in Figure 1, insecticide spraying to control CPB can be applied at SEP to NOV, while the rest of the month pod sleeving should be practiced. On two cropping seasons as in *Figure 2 and 3,* insecticide spraying is needed twice more. An example, in Tawau insecticide spraying applied at JAN to MAR and AUG to OCT. While in Madai, Kunak at FEB to APR and AUG to OCT. Pod sleeving is applied on MAY to JUL and DEC in Tawau, while in Madai, Kunak on JAN, JUN to JUL and DEC.

Expected dry cocoa bean in the study was higher in IPM plot as shown in Table 1 on every study location. Overall yield was lower in Tenom due to tree age factor. It was understood that most of the cocoa trees in the plot are over 30 years.

CONCLUSIONS

Good control of CPB can be achieved by following this IPM implementation model. Cocoa growers should be aware of their cocoa cropping pattern. Ability to observe the maximum flowering month is a necessary to better forecast the insecticide spraying initiation at the maximum number of pod to be protected from CPB infestation. Following this IPM implementation model definitely will reduce yield losses due to CPB.

ACKNOWLEDGMENT

The study was part of eSciencefund project (06-03-13-SF0090) funded by the Government of Malaysia through MOSTI.

We thank the Director General of Malaysian Cocoa Board, Director of Upstream Cocoa Technology Division; and staffs of Entomology Unit CRDC Tawau, Malaysian Cocoa Board for their encouragement and assistance throughout the study.

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PERFORMANCE OF INTERNATIONAL CACAO GENETIC MATERIALS UNDER MCB CRDC BAGAN DATUK AGRO-CLIMATIC CONDITION

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ABSTRACT - *It is very important to enhance the cacao genetic materials for breeding purpose especially in developing and improving superior genotype. Evaluation on these genetic materials under Bagan Datuk agro-climatic condition would provide some crucial information for future breeding program and objectives such as breeding for high yield, resistant and tolerant to pests and diseases, good pod and bean characteristics, high butter fat content, good flavour and others. With the additional weather report, cocoa production could be evaluated and estimated preliminarily against climate change. This paper will discuss on the performance of the germplasm collection established in CRDC Bagan Datuk, Perak. The genetic materials yielded an average of 61 pods per plot over eight year's period. Clones PNG 110, PNG 153 and POUND 18 had lower pod index values than other clones. AMAZ 12, AMAZ 15, CL 19/10, SCA 6 X ICS 8H, SCA 6 X ICS 8J, IMC 103, PMCT 93, PNG 110, PNG 153, PNG 290, PNG 299, PNG 418 and POUND 18 had bigger bean size. GU 133/C, GU 195/V, SCA 6 X ICS 1E, SCA 6 X ICS 6D, SCA 6 X ICS 8H, SCA 6 X ICS 8J, IMC 16, IMC 20, IMC 27, NA 804, PA 4, PA 67, PMCT 93, PNG 10, PNG 153, PNG 299, POUND 16A, POUND 18, SLA 16, SPA 16, TSA 654, TSA 656, TSH 516 and UF 12 had higher bean numbers per pod compared others. The genetic materials showed highest pod yield at the sixth year production.*

Keywords: Genetic materials, genotype, germplasm

INTRODUCTION

Introduction of new cacao genetic materials also known as accessions from abroad is very important to enrich and enhance the genetic base of cacao planting materials. The collection of these international cacao clones are conserved as field genebank while enlarging the existing collection of primary germplasm (Spence, 1991). This programme would be a platform to serve as many genetic materials as possible to be studied in various fields including cacao breeding. These accessions will be observed and evaluated under local agro-climatic condition for their performance. Genetic materials with the best performance will be selected and used in cacao breeding programs with several objectives such as breeding for high yield, resistant to pest and diseases, good pod and bean characteristics, high butter fat and powder content, good and unique flavour and other desirable traits. This process is very important in developing, producing and improving superior planting materials for cacao. This paper will discuss the performance of the international cacao germplasm collection under Cocoa Research and Development Centre (CRDC), Bagan Datuk agro-climatic condition on coastal alluvial soil.

MATERIALS AND METHODS

The 1.6 ha plot (Block 18B) in CRDC Bagan Datuk was established in 2006 with 103 accessions including two controls (KKM 22 and MCBC 5). Each accession was propagated into 14 trees (2x7), spaced at 3m x 3m in triangular pattern under Gliricidia stands and unreplicated. The plot was maintained with standard cultural-practices adopted by CRDC Bagan Datuk cacao growing.

The first yield data were collected in January 2009, three years after its establishment. Pod counting was recorded on the individual tree bimonthly. Pod and bean analysis were conducted according to Ramba *et al.* (2007) to

determine pod value, dry bean weight, bean number per pod and other bean parameters.

RESULTS AND DISCUSSIONS

Pod yield of the accessions is presented in *Figure 1*. The accessions yielded an average of 61 pods per plot over eight year's period. Nineteen accessions (IMC 20, AMAZ 12, PA 4, POUND 19A, GU 307/F, GU 241/V, CRU 158, POUND 18, SCA 6 X ICS 8I, CEPEC 42, TSA 654, PNG 299, TSH 774, SLA 16, SCA 6 X ICS 1E, RB 39, TSA 656, TSH 516 including KKM 22) produced in average of more than one hundred pods yearly compared to others. IMC 20 showed the best accession to produce the most number of pods (252 pods) followed by AMAZ 12 (242 pods), PA 4 (228 pods) and POUND 19A (213 pods). It was noted that over eight year's period, those four accessions were the most prolific genetic materials compared to others under CRDC Bagan Datuk agro-climatic conditions.

The genetic materials showed highest pod yield at the sixth year (2014) production with an average of 95 pods. In 2012, they yielded about 84 pods and decreased 37% one year after that. This might be affected by weather condition and environment factor beside the genetic materials themselves.

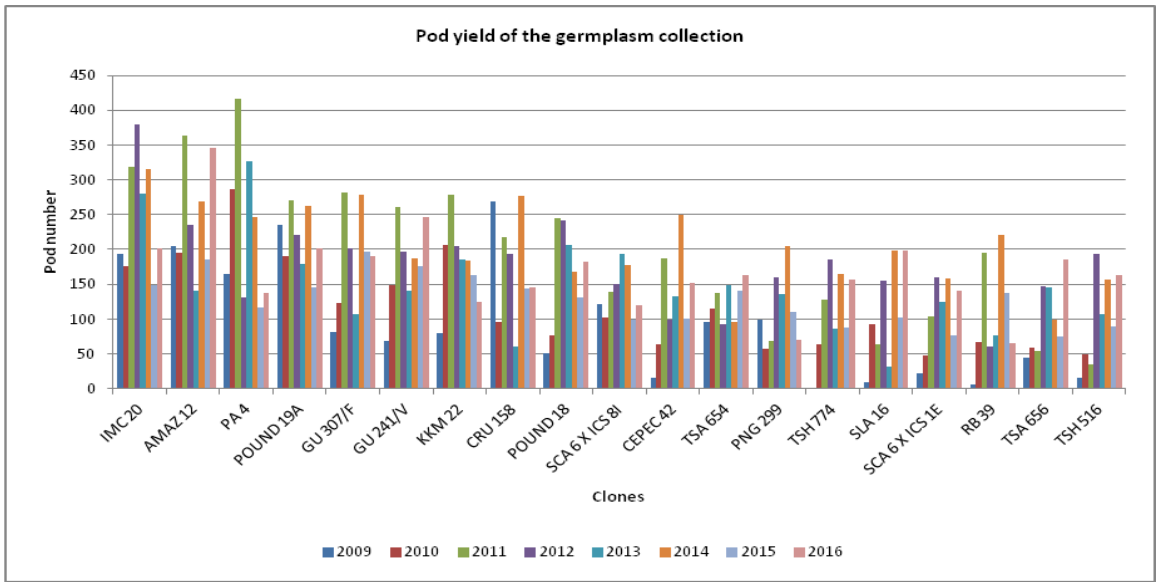

Figure 1. Accessions with average of pod yield per year above one hundred pods.

A good cacao clones should have big bean size (more than 1g of average dry bean weight), high bean number per pod (more than 35 beans) and low pod value (below 25 pods). The pod value is number of pod required to produce 1 kg of dried bean.

The results of yield components consists of bean size (ADBW), bean number (BNP), pod value (PV) and bean conversion rate (BCR) is shown as in Table 1 below. Clones AMAZ 12, AMAZ 15, CL 19/10, SCA 6 X ICS 8H, SCA 6 X ICS 8J, IMC 103, PMCT 93, PNG 110, PNG 153, PNG 290, PNG 299, PNG 418, MCBC 5 and POUND 18 had bigger bean size (more than 1.10 g) compared to others. PNG 110 indicated the biggest bean size with 1.59 g followed by PNG 153 (1.41 g). For the number of beans, GU 133/C, GU 195/V, SCA 6 X ICS 1E, SCA 6 X ICS 6D, SCA 6 X ICS 8H, SCA 6 X ICS 8J, IMC 16, IMC 20, IMC 27, NA 804, PA 4, PA 67, PMCT 93, PNG 10, PNG 153, PNG 299, POUND 16A, POUND 18, SLA 16, SPA 16, TSA 654, TSA 656, TSH 516 and UF 12 had higher bean number per pod than others. PA 67 showed the highest bean number per pod with 54 beans. Clones PNG 110, PNG 153, MCBC 5 and POUND 18 indicated the lowest pod index values (below twenty pods). All these information could be used in selecting the best accessions as parents in future breeding programs with specific objectives.

Table 1. Yield components of the accessions

NO	CLONE	ADBW (g)	BNP	PV	BCR (%)
1	PNG 110	1.59	38.80	17.39	28.00
2	PNG 153	1.41	40.00	17.70	33.06
3	PNG 290	1.36	27.67	26.56	33.70
4	MCB C5	1.29	40.00	19.38	28.33
5	POUND 18	1.23	42.14	19.25	42.86
6	AMAZ 15	1.22	46.00	20.87	28.53
7	SCA 6 X ICS 8H	1.22	42.60	27.73	36.61
8	CC 19/10	1.21	32.71	30.57	32.71
9	IMC 103	1.21	33.00	30.42	30.45
10	AMAZ 12	1.19	41.90	22.03	35.19
11	PMCT 93	1.18	40.29	20.90	33.49
12	PNG 299	1.17	41.67	20.47	32.67
13	PNG 418	1.17	23.50	37.95	34.80
14	SCA 6 X ICS 8J	1.12	41.00	21.96	41.41
15	NA 804	1.09	40.00	22.91	33.88
16	PNG 10	1.09	43.79	23.14	25.40
17	ICS 95	1.07	36.50	25.48	34.84
18	PNG 336	1.06	31.66	28.82	34.68
19	PNG 250	1.05	30.50	33.70	32.23
20	IMC 27	1.04	40.50	23.86	36.52
21	POUND 16/A	1.02	45.20	21.49	33.21
22	PNG 155	1.01	36.44	26.91	30.98
23	CEPEC 42	1.00	36.22	22.32	32.72
24	IMC 16	1.00	47.00	21.10	38.67
25	PNG 296	1.00	37.00	29.08	35.17
26	ICS 10	0.96	36.13	28.71	27.95
27	SCA 6 X ICS 6D	0.96	40.75	36.18	33.82
28	VB 663	0.95	25.00	41.76	26.56
29	PNG 87	0.94	28.57	37.09	32.31
30	TSA 654	0.93	46.20	23.06	35.28
31	PA 4	0.91	40.00	31.23	31.32
32	PNG 210	0.91	30.00	44.73	23.78
33	SCA 6 X ICS 6F	0.91	37.00	31.74	34.48
34	MA 12	0.90	38.60	28.70	30.21
35	PA 169	0.90	23.75	46.65	27.91
36	PA 67	0.90	53.67	20.51	57.68
37	UF 12	0.90	46.00	23.96	25.59
38	CRU 158	0.88	30.00	37.67	31.27
39	GU 133/C	0.88	41.50	27.31	23.62
40	IMC 20	0.87	42.86	26.67	32.92
41	SLA 16	0.87	42.00	29.27	33.57
42	GU 241/V	0.86	27.56	42.05	25.12
43	LV 28	0.86	37.50	33.84	33.40
44	CL 19/10	0.84	25.33	46.75	21.04
45	POUND 4B	0.84	32.00	49.77	32.49
46	GU 195/V	0.83	42.86	27.97	26.14
47	KKM 22	0.83	43.57	27.58	26.80

48	TSA 656	0.83	40.10	29.94	31.97
49	ICS 70	0.82	39.09	31.18	33.12
50	PNG 218	0.82	34.00	37.85	32.62
51	PNG 340	0.81	39.00	31.64	23.54
52	SCA 6 X ICS 1E	0.81	46.40	28.01	33.21
53	SJ 1/19	0.81	33.75	36.49	34.75
54	PA 125	0.79	38.00	33.61	36.57
55	PNG 215	0.79	27.50	46.02	19.72
56	SCA 6 X ICS 8I	0.78	33.10	38.58	34.61
57	POUND 19A	0.77	37.92	34.01	33.63
58	GU 255/P	0.75	23.00	57.77	29.98
59	GU 307/F	0.74	25.20	53.32	28.59
60	TSH 774	0.73	37.60	26.76	33.96
61	PNG 197	0.72	21.00	63.81	16.65
62	SPA 16	0.70	45.00	33.89	36.31
63	SCA 6 X ICS 1D	0.58	26.20	65.61	23.00
64	TSH 516	0.31	40.00	79.15	22.03
	Average	**0.96**	**36.78**	**32.54**	**31.53**

ADBW – Average dry bean weight, BNP – Bean number per pod, PV – Pod value, BCR – Bean conversion rate

Figure 2 below shows meteorology information of CRDC Bagan Datuk, Perak. From 2010 to 2011 the centre had received the highest amount of rainfall (above two thousands mm) across eight years periods. In 2016, the centre has experienced an extreme weather condition where it was considered as dry weather with less rainfall. This weather conditions might affected the decreased of the yield of the accessions in 2016 beside the genotype.

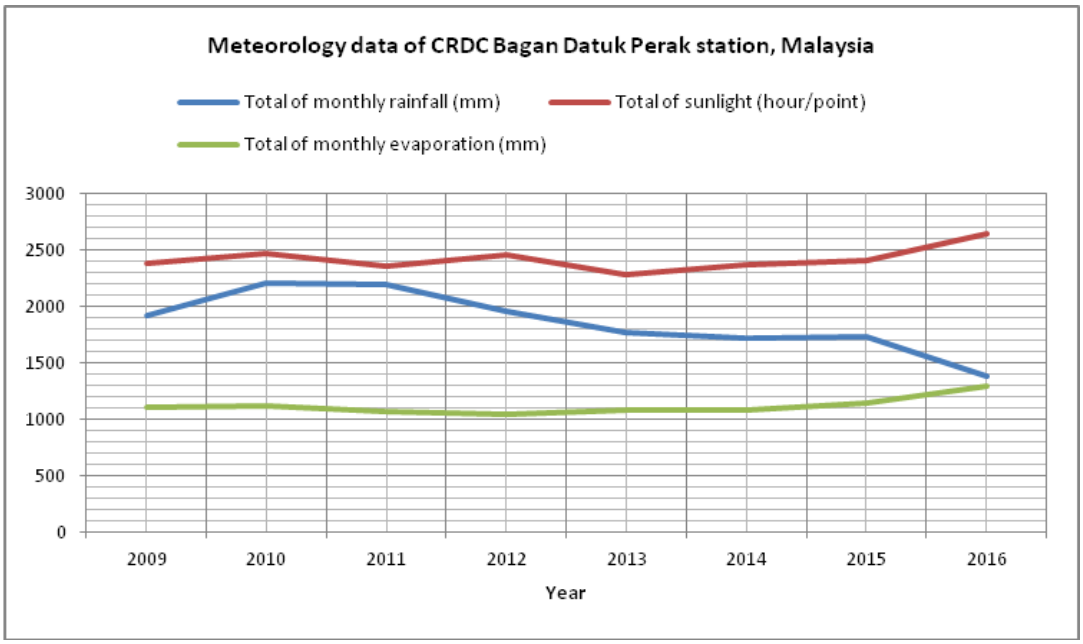

Figure 2. Meteorology information of CRDC Bagan Datuk, Perak over eight year's period

CONCLUSIONS

Under CRDC Bagan Datuk Agro-climatic conditions, the accessions indicate their highest pod yield at the sixth year of pod producing. IMC 20, AMAZ 12, PA 4 and POUND 19A are proven as the highest yield accessions at the environment. PNG 110, PNG 153 and POUND 18 had bigger bean size with the lowest pod index values. PA 67 had the most bean number per pod. Although there are many collections of cacao genetic materials in Malaysia, their performance are still affected by the existences of genotype/environment factors. It is important to evaluate and observe them under local agro-climatic conditions prior to selection.

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DETERMINATION OF PLANT GROWTH PROMOTING BACTERIA *(Leclercia adecarboxylata)* SURVIVABILITY IN THE SELECTED CARRIER MATERIAL.

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ABSTRACT – *This study is aimed to determine the survivability rate of selected plant growth promoting bacteria (*Leclercia adecarboxylata*) in the selected carrier material. Rice husk charcoal, grinded cocoa pod husk and combination of both material are used as a carrier material. Three different amount of solution containing* Leclercia adecarboxylata *is also used as a treatment. The amount used were 1.2%, 1.0% and 0.8% from the weight of carrier material and the total colony forming unit of the solution was more than 10^{10}. Each carrier was packed using high density polyethylene plastic bag and sterilized by autoclaving at 121ºC for three hours. All samples were stored in dark room under room temperature. Periodical samples were taken once in two weeks for six months. Plate count technique was applied by using nutrient agar medium to determine total bacteria left in the carrier material. After two months, all samples of cocoa pod husk are contaminated. The higher amount of bacteria solution (1.2%) is much preferred because it can retain survivability of bacteria longer than other treatments.*

Keywords: Plant growth promoting bacteria, *Leclercia adecarboxylata*, carrier material, survivability and colony forming unit.

INTRODUCTION

Beneficial microbes that exists in soils naturally are known as plant growth promoting bacteria. These bacteria are able to help in plant growth through different mechanism such as biological nitrogen fixation, solubilizing insoluble forms of nutrients such as phosphate and potassium, siderophore production and releasing phytohormone. This bacteria is a free-living bacteria, which it can be found either in rhizosphere, rhizoplane, endophytic or around the roots of plants. (Bashan and de-Bashan, 2005). This is because of the presence of nutrients including sugars, polysaccharides, amino acids, organic acids, enzymes, plant regulators, and other small molecules from plant root exudates (Dayakar *et al.*, 2009).

Leclercia adecarboxylata is a bacteria with the ability to fix nitrogen, high production of indole acetic acid (IAA) and high phosphorus solubilization capacity (Juliana *et al.*, 2016). According to Laili *et al.*, 2017, this bacteria lived around the root and hold multiple traits such as the ability to fix N, solubilize P and K, able to produce cellulose enzyme, siderophore and phytohormone thus enhance root growth. With the ability of fixing nitrogen, phosphorus, potassium and producing IAA, this bacteria can be used as a main ingredient in producing biofertilizer.

Biofertilizer is usually prepared as a carrier-based inoculants containing microorganisms with selected abilities. With the used of potentially carrier materials, it ease the process of handling, long term storage and high effectiveness of biofertilizer. Various type of carrier materials can be used as carrier especially for seed or soil inoculation. According to Somasegaran and Hoben, 1994, the good carrier material properties are non-toxic to inoculant bacterial strain, good moisture absorption capacity, easy to process, free of lump-forming materials, easy to sterilize by autoclaving or gamma-irradiation, available in adequate amounts, inexpensive, good adhesion to seeds, good pH buffering capacity and non-toxic to plant.

Rice is an important staple food for certain country especially Malaysia, and rice husk is one from the agricultural waste which can be utilized into industrial fuel, activated carbon, as a carrier material and as a substrate for silica and silicon compound. Rice husk is a hard covering of rice grains. Rice husk contains

high in ash (87-97% silica), highly porous and light in weight with a very high external surface area (Kumar *et al.*, 2013). The usage of rice husk ash can improve soil properties and increase soil pH, organic carbon, available nutrients and increase crop yield (Paul *et al.*, 2005).

Cocoa pod husk is also one from the agricultural waste which contains nutrient and organic matter. Each tonne of dry cocoa bean represents about 250kg of dry cocoa pod husks (Adamako, 1975). In the past, cocoa pod husk is used as animal feed, soap and organic material. Cocoa pod husk contains several nutrient especially K which forms about 40% of the ash (Adamako, 1975). There is an opportunity to research its usefulness as the source of K and developing it into organic materials as well as to use it as a carrier material in developing of biofertilizer. This study was conducted to investigate the suitability of using cocoa pod husk, rice husk charcoal and combination of cocoa pod husk and rice husk charcoal, and to determine the surviviability of Leclercia adecarboxylate in the selected carrier material.

MATERIALS AND METHODS

Before the material were used as a carrier material, cocoa pod husk, rice husk charcoal and combination of cocoa pod husk and rice husk charcoal were tested on seed phytotoxicity tests to determine the incident of phytotoxins. This experiments has been carried out at Microbiology and Physiology laboratory, Cocoa Research and Development Centre, Jengka, Pahang. For phytotoxicity test, five treatments includes using 5ml of distilled water (control), 5ml of rice husk charcoal water extract, 5ml of cocoa pod husk water extract, 5ml of combination between rice husk charcoal and cocoa pod husk water extracts.

For the water extract process, 100 ml of distilled water were mixed with 50 g media from the carrier materials in Erlenmeyer flasks. The water mixture was shaken for one hour at room temperature. For the germination assay, petri dish (10 cm diameter), filter paper, 15 tomato seeds in each petri dish, 5 ml of water extracts were prepared. Total of seed germination were recorded at 48, 72 and 96 hours. In the 4th day, the seed root growth were measured.

For survivability tested on the carrier material, experiment has been carried out at Microbiology and Physiology laboratory, Cocoa Research and Development Centre, Jengka, Pahang. There were nine treatments with five replications. The treatments were as listed below:-

T1	80 ml of selected bacteria solution with 100 g of grinded cocoa pod husk
T2	80 ml of selected bacteria solution with combination of 50g grinded cocoa pod husk and 50 g of rice husk charcoal.
T3	80 ml of selected bacteria solution with 100 g of rice husk charcoal.
T4	100 ml of selected bacteria solution with 100 g of grinded cocoa pod husk
T5	100 ml of selected bacteria solution with combination of 50 g grinded cocoa pod husk and 50 g of rice husk charcoal.
T6	120 ml of selected bacteria solution with 100 g of rice husk charcoal.
T7	120 ml of selected bacteria solution with 100 g of grinded cocoa pod husk
T8	120 ml of selected bacteria solution with combination of 50 g grinded cocoa pod husk and 50 g of rice husk charcoal.
T9	120 ml of selected bacteria solution with 100 g of rice husk charcoal.

The selected bacteria solution was bacteria that have been successfully isolated from healthy cocoa root tree. This bacteria has been screen with nitrogen fixation ability on N-free solid malate agar (NFA), phosphorus by tested on Pikovskaya agar and potassium solubilisation ability by using Aleksandrov agar medium. This bacteria also able to produce phytohormone. This bacteria has been sent to First Base Laboratories for identification process. It was identified as *Leclercia adecarboxylate*, based on the Blast result against

NCBI 16S ribosomal RNA sequences database. *Leclercia adecarboxylate* were incubated for 18-24 hours under normal temperature by using nutrient broth solution and contain more than 10^{10} cfu/ml.

Rice husk ash, was taken from Bernas rice mill located at Sungai Ranggam, Perak. Cocoa pod husk was collected from field plot at Cocoa Research and Development Centre, Jengka, Pahang. Cocoa pod husk has been composted and was naturally sun dried for several days. The composted cocoa pod husk was grinded by using grinder machine. Both of cocoa pod husk and rice husk charcoal were oven dried at 70ºC for 24 hours. Then, the materials were packed according to the treatment listed above by using high density of polyethylene plastic bag and sterilized by autoclaving at 121°C for 3 hours and were left overnight before applying the bacteria solution. All samples were stored in dark room under room temperature. Periodical samples were taken once in two weeks. Plate count technique was applied by using nutrient agar medium to determine total bacteria.

Statistical analysis was carried out by using SAS for mean separations of the treatments effects.

RESULTS AND DISCUSSION

Seeds phytotoxicity test

Table 1 showed the seed germination rate of tomato seed after 48, 72 and 96 hours. There is a significant difference between treatments at 5% significant level at 48 and 96 hours. Rice husk charcoal extract showed better seed germination rate, followed by combination of rice husk charcoal and cocoa pod husk water extract, cocoa pod husk water extract and lastly distilled water. This showed a beneficial effects on carrier material water extract on seed germination.

Table 1. Data on seed germination rate after 48, 72 and 96 hours.

Treatments	48 hours	72 hours	96 hours
5 ml of distilled water (control)	0.178±0.039b	0.400±0.067a	0.601±0.068b
5ml of rice husk charcoal water extract	0.311±0.038a	0.489±0.038a	0.867±0.066a
5ml of cocoa pod husk water extract	0.245±0.039a	0.422±0.039a	0.777±0.039a
5ml of combination between rice husk charcoal and cocoa pod husk water extracts.	0.267±0.000a	0.467±0.009a	0.800±0.067a
CV	5.39*	1.81ns	4.75*
FV	13.34	10.00	9.20

[1] *Column means followed by the same letter are not significantly difference (P>0.05, Duncan's Multiple Range Test)*

After incubation under room temperature for four days in the dark, germinated seeds were counted and the root length was measured. There is a significant difference between root length in carrier material water extracts and control treatments. From the figure above, rice husk charcoal showed the highest root length followed by combination of rice husk charcoal with cocoa pod husk water extract, cocoa pod husk water extract and lastly distilled water *(Figure 1).*

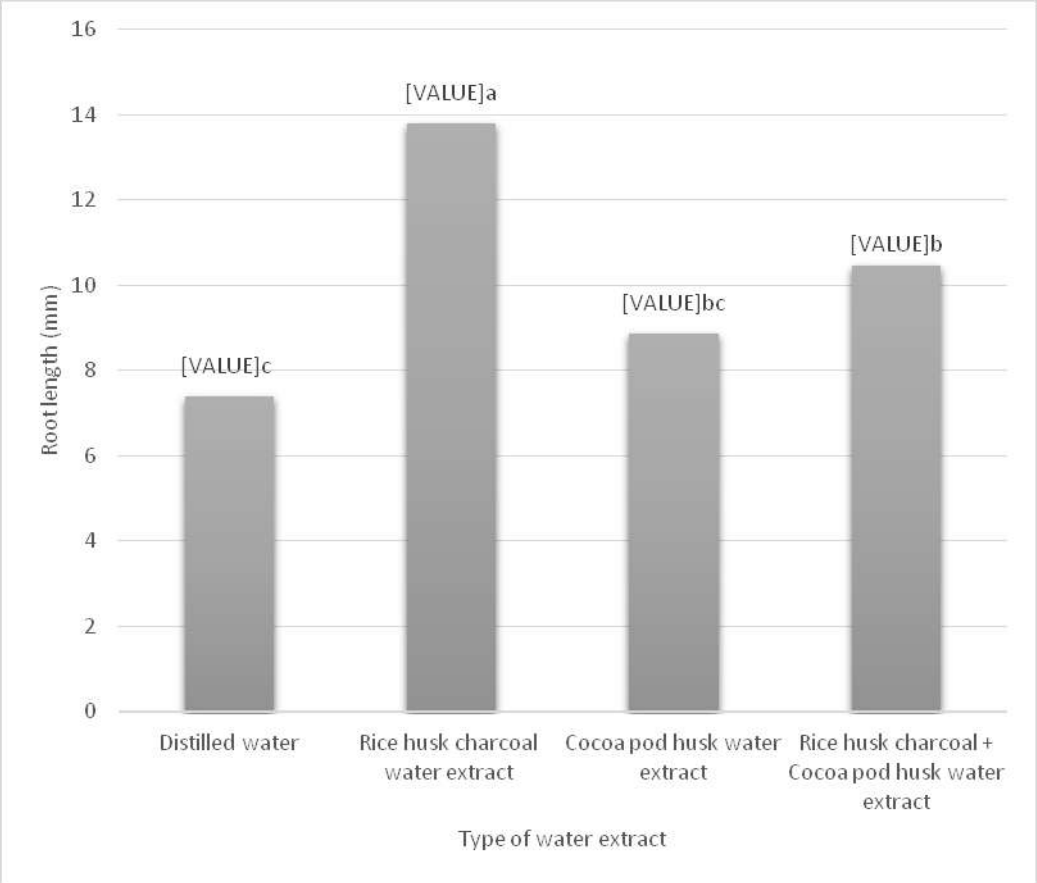
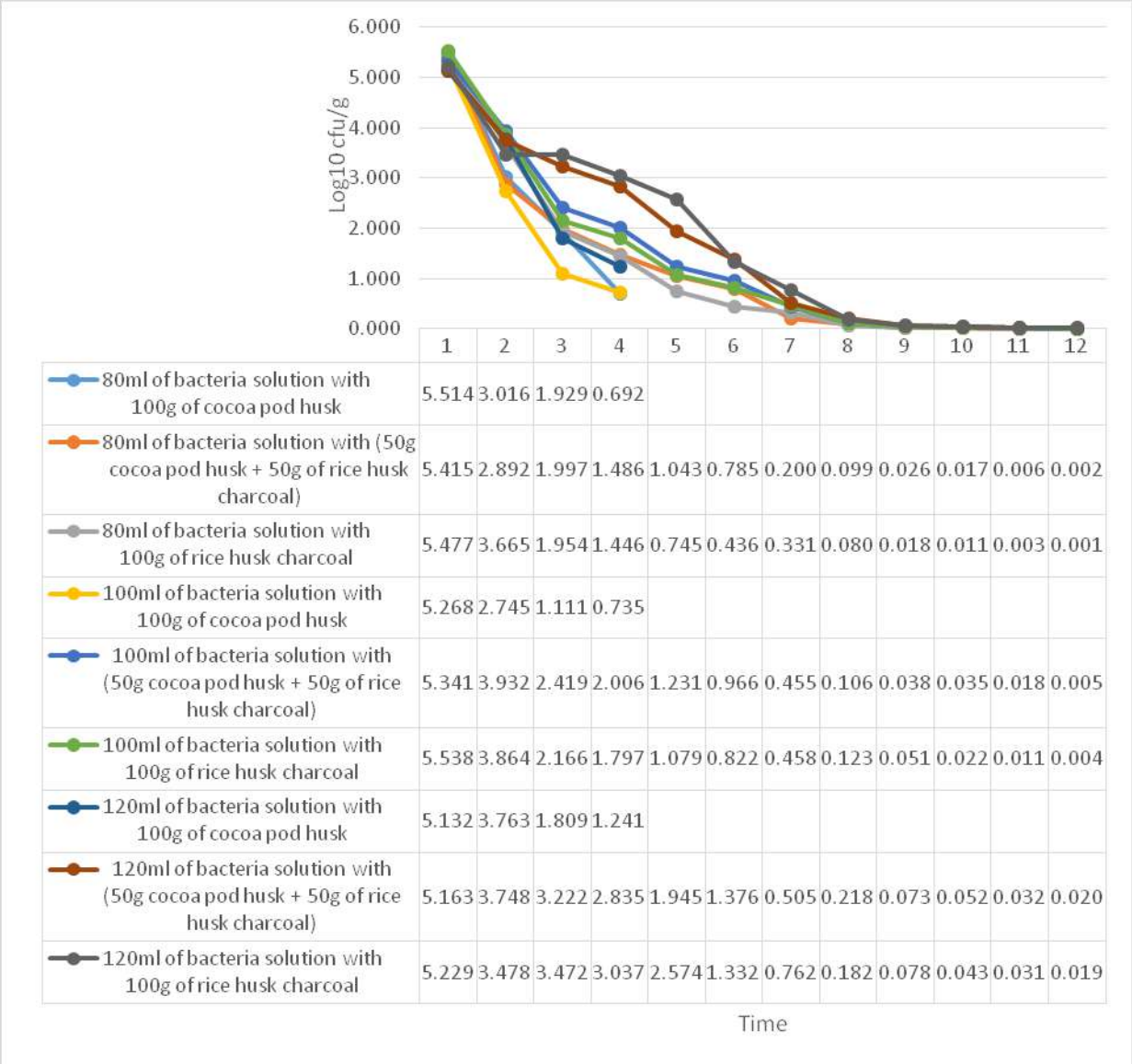


Figure 1. The root length in carrier material water extracts and control

Survivability of Leclercia adecarboxylata in the selected carrier materials

Based on data recorded, there is a significant difference among the treatments at first, third, fourth and fifth month after bacteria application. Data recorded in *Figure 2* show that at zero time, total bacterial counts were generally higher which ranged approximately from 5.132 to 5.538 log 10 CFU/g. The microbial populations decreased constantly during the storage period. At the end of the experiment,120 ml of selected bacteria solution with combination of 50 g grinded cocoa pod husk and 50 g of rice husk charcoal showed the lowest decrement, followed by 120 ml of selected bacteria solution with 100 g of rice husk charcoal, 100 ml of selected bacteria solution with combination of 50 g grinded cocoa pod husk and 50 g of rice husk charcoal, 120 ml of selected bacteria solution with 100 g of rice husk charcoal, 80 ml of selected bacteria solution with combination of 50g grinded cocoa pod husk and 50 g of rice husk charcoal and highest decrement rate was 80 ml of selected bacteria solution with 100 g of rice husk charcoal. Based on the observation, all samples using 100% Cocoa Pod Husk were contaminated after 2 months of applications.



	1	2	3	4	5	6	7	8	9	10	11	12
80ml of bacteria solution with 100g of cocoa pod husk	5.514	3.016	1.929	0.692								
80ml of bacteria solution with (50g cocoa pod husk + 50g of rice husk charcoal)	5.415	2.892	1.997	1.486	1.043	0.785	0.200	0.099	0.026	0.017	0.006	0.002
80ml of bacteria solution with 100g of rice husk charcoal	5.477	3.665	1.954	1.446	0.745	0.436	0.331	0.080	0.018	0.011	0.003	0.001
100ml of bacteria solution with 100g of cocoa pod husk	5.268	2.745	1.111	0.735								
100ml of bacteria solution with (50g cocoa pod husk + 50g of rice husk charcoal)	5.341	3.932	2.419	2.006	1.231	0.966	0.455	0.106	0.038	0.035	0.018	0.005
100ml of bacteria solution with 100g of rice husk charcoal	5.538	3.864	2.166	1.797	1.079	0.822	0.458	0.123	0.051	0.022	0.011	0.004
120ml of bacteria solution with 100g of cocoa pod husk	5.132	3.763	1.809	1.241								
120ml of bacteria solution with (50g cocoa pod husk + 50g of rice husk charcoal)	5.163	3.748	3.222	2.835	1.945	1.376	0.505	0.218	0.073	0.052	0.032	0.020
120ml of bacteria solution with 100g of rice husk charcoal	5.229	3.478	3.472	3.037	2.574	1.332	0.762	0.182	0.078	0.043	0.031	0.019

Figure 2. Survival of Leclercia adecarboxylate on different carriers materials sterilized by autoclave and stored at room temperature during storage period (6 months)

CONCLUSIONS

Based on seed germination test, all carrier material showed the positive effects on tomato seeds, where it is found that the phytotoxicity which can cause retardation of seed germination were not exist. Based on the survivability test of *Leclercia adecarboxylata*, it can be suggested that the use of 100% cocoa pod husk is not suitable as the carrier material, because of higher and easily contaminated with fungus. The higher amount of bacteria solution (120ml) is preferred because it can longer survivability of bacteria. It can be concluded that the combination of cocoa pod husk with rice husk charcoal or rice husk charcoal as a carrier material with 1.2% of bacteria solution is much preferable.

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AREA WIDE MANAGEMENT IN MANAGING THE INFESTATION OF COCOA POD BORER

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ABSTRACT - *Managing the infestation of Cocoa pod borer (CPB),* Conopomorpha cramerella *is one of the greatest challenges for Malaysian cocoa growers since the pest first occurrence in the 1980s. Multilateral techniques, timeless efforts, numerous researches, massive agronomy inputs, implementation of technical and non-technical approaches had been practices both in small scale or large scale plantations. Where most of the past and recent studies focusing on the grower's size plantations, managing of CPB in large scale areas must not be neglected. These practices requires substantial inputs, critical man-power and must be properly scheduled. Therefore, managing of CPB in large scale areas were conducted at the CRDC Bagan Datuk using knapsack spraying at two weeks intervals (2014), insecticide fogging at monthly intervals in year 2015 and 2016 at 22 blocks of 40.5 ha (Ladang 1A), and pod sleeving in year 2016 at 29 blocks of 23.0 ha (Ladang 1B). Observations on the insecticide approach showed that there was an increment in monthly average of cocoa beans from 576.96 kg in 2014 (knapsack) to 589.49 kg in 2015 (fogging). However, monthly average of cocoa beans was decreased in the second year of fogging (480.2 kg) with rate of -22.27%. In addition, observation at the Ladang B denoted that fogging also reduced monthly average of cocoa beans in the year 2015 (309.92 kg) compared to knapsack spraying which resulted in 467.23 kg (2014). Surprisingly, there was an increment of 45.21% of monthly yield after sleeving was intensely applied in the year 2016 (565.68 kg). The results conclude that cocoa pod sleeving is one of the best solutions in managing CPB at the large scale areas. Even this non-technical approach was very laborious during the early phase of implementation, an ability to increased cocoa yield must not be disregard, and should be promoted for large scale control of CPB.*

Keywords*:* Cocoa pod borer, *Conopomorpha cramerella*, cocoa, pod sleeving, insecticide fogging

INTRODUCTION

Cocoa pod borer, CPB *Conopomorpha cramerella* Snellen (Lepidoptera: Gracillariidae) is still a major constraints to the Malaysian cocoa plantations, following its first emergence attacking cocoa estate in Tawau, Sabah in the late 1980s (Ooi *et al.,* 1987). In the last three decades, *C. cramerella* successfully capable to cause significant production loss to the cocoa growers in the South-East Asian regions. *C. cramerella* was first detected in cocoa, in 1860's at Sulawesi, Indonesia; followed by Philippines (1936), Malaysia (1980), Papua New Guinea (2006) and in 2011 at the North Queensland, Australia (Saripah & Alias, 2016). The life cycle of *C. cramerella* is relatively short which is approximately 27 to 33 days; but their economic impact to cocoa pod production is very significant. This pest generates losses in term of volume, value due to reduction in the fat contents and overall beans quality. Continuous infestation on the same pod may produced malformed and clumped beans, and these resulting by their feeding behavior and oviposition preferences which prefer to infest pods more than 7-9 cm in length. A female moth has the ability to lay 150 eggs in their entire life span, and generally lives for about a week (Saripah, 2012).

Control of the pest and diseases is an imperative approach which can save up to 30 to 40% yield losses (Wessel & Quist-Wessel, 2015). Yield loss continues to inflict the reduction of cocoa production. It was proven since *C. cramerella* outbreak, there is no single approach was successful to eradicate their population. Yield losses are expected about 5% with an infestation of 50-60% (Mumford, 1986), 25% of losses contributed about 62% of infestation, and up to 93% if the infestation increased to 99% (Lim &

Phua 1986). The concept of 4P [in Malay: Pembersihan (field sanitation), Pemangkasan (regular pruning), Penuaian (frequent ripe pod harvesting) dan Pembajaan (schedule fertilization)] are highly recommended especially at the block with light to heavily infested symptoms (Saripah and Alias, 2016). Combination of management package and control techniques including proper agronomic practices, biological control, chemical control, resistant planting materials, and Integrated Pest Management (IPM) were highly recommended in order to reduce the infestation (Azhar, 2007).

Even IPM and other control approaches were suggested, in most cases, growers prefer only to implement chemical control as a single technique in managing *C. cramerella* infestation. Cocoa farmers usually applied pesticides to limit losses due to pest and disease infestation, with the aid of broader choices of pesticides available in the market (Tijani, 2006). Biweekly prophylactic treatment with chemical insecticides was considered as one of the most effective approaches; however the cost is expensive (Teh *et al.*, 2006). Extensive applications of insecticide which are neither environmentally sustainable nor effective in term of costs were widely discussed since early 1990's (Wood *et al.,* 1992; Beevor *et al.,* 1993). The effectiveness of insecticide control depends on the appropriate timing of spraying which usually relies on cocoa cropping calendars. It should be based on economic and ecological considerations (Ling, 2013).

Another possible alternative in managing *C. cramerella* is using pod bagging or sleeving. Unfortunately, this approach received little attention from the large scale plantations, due to laborious effort is needed especially during cropping seasons. The work of sleeving was considered as time-consuming and difficult to do completely (Wood and Chung, 1989). Sleeving usually implemented in small scale plantation with pod at the age of three months. The method involves covering suitable pod size with plastics or any suitable materials. This technique may prevents *C. cramerella* adults from laying their eggs, thereby reduce the degree of infestation (Tay, 1987). This technique was proven successful especially in heavily infested or neglected cocoa areas (Tay 1987; Saripah and Azhar, 2007; Saripah *et al.,* 2005). It is particularly practical in smallholdings with short trees (Azhar, Alias and Meriam 2000) and contributed to more than an 180% increase in wet cocoa beans at previously abandoned areas. Results obtained from a 28 month study from 2004 to 2007 at a 2.4 ha plot showed that the percentage of good mature pods was more than 95%. The Average Damage Severity Index (ADSI) values, which reflect the damage caused by CPB, are lower than 0.4 (on a 0 to 4 scale) and sleeved plots were categorized as healthy to slightly infested area (Saripah & Azhar, 2007).

With the regards of *C. cramerella* serious infestation level, study on managing the pest in large scale area must be taken into consideration. As the most preferable technique, insecticides were implemented using conventional knapsack sprayer, as well as insecticide fogging. Comparison was also made using non favorable technique in large plantation, which was the cocoa pod sleeving. The objective of this study was to determine the effectiveness of insecticide fogging and cocoa pod sleeving as a control approach of CPB in large scale plantation. The comparison were made by yearly basis with different treatment were applied at each year.

MATERIALS AND METHODS

Field testing was performed at Fields 1A and 1B, with established mature cocoa trees at the Cocoa Research and Development Center (CRDC), Malaysian Cocoa Board Bagan Datuk, Sungai Sumun, Perak, Malaysia (Longitude E.100 M, 52' 0', Latitude N3 53' 42). All blocks (approximately 0.6 to 1.8 ha each) harbored almost similar ecosystem, management practices and were planted with Malayan tall coconut and *Gliricidia macculata* as a shade tree. Field 1A was selected for pod sleeving, and Field 1B for insecticide fogging. *C. cremerella* control was carried out at 40.5 ha using conventional knapsack sprayer at bimonthly intervals in the year 2014 (Table 1). Insecticides namely deltamethrin, was selected and used with rate and dosage recommended by the

manufacturer. In 2015, both fields were treated with insecticide fogging. The use of insecticide fogging was carried out using insecticide, (cypermethrin) at the rate of 13 ml per 10 liter water. The rate of insecticide were increased two fold from conventional knapsack sprayer, and followed the flowering pattern. The frequency of fogging was at 8-10 round per year with estimated cost of RM 11.75/ha/round (Table 2). Cocoa pod sleeving was implemented at monthly basis in the Field 1A in year 2016, replacing both knapsack and fogging insecticide applications. Sleeving was carried out at developing pods, which was approximately 7 to 9 cm in length using transparent plastic bag. The bottom end of the plastics were opened, to allows air ventilation and reduce the risk of disease infestation. Mature cocoa pods from all fields were harvested accordingly to different treatments, and bean weights were recorded throughout these 36-months of observation.

Table 1. Number of blocks for insecticide's fogging at CRDC Bagan Datuk

Field/ Ladang	Number of block	Blocks	Hectare (ha)
B	22	1, 2, 10, 17, 18, 19, 20, 21, 22, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 42, 45, 46	40.5

Table 2. Amount of insecticide, water-based carrier and petrol for one round of fogging in the year 2015

Water Amount (L)	Carrier Amount (L)	Cost (RM)	Insecticide Amount (L)	Cost (RM)	Petrol Amount (L)	Cost (RM)	Total cost (RM)	Cost per hectare (RM)
300	100	1,520.00	0.78	6.40	33	66.00	1,592.40	11.75

RESULTS AND DISCUSSIONS

A total of 363,427 mature pods were harvested at the Field 1B for the period of three years (2014 through 2016). Monthly average of harvested pods in the year 2014 was 9,770.92 pods after treated with knapsack insecticide spraying, 2015 (11,622.42 pods, fogging) and 10,670.7 pods in the year 2016 with fogging treatment (Table 3). Monthly average of harvested pods for these 36-months of observation was 10,688 pods.

Table 3. Number of harvested pods from 2014 through 2016 at the Field 1B

Year	Treatment	Number of harvested pods												Total	Monthly average
		Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec		
2014	Knapsack	14373	10876	10188	15510	3275	1469	1258	11991	22063	15732	4972	5544	117251	9770.92
2015	Fogging	27109	23430	13947	8224	1946	2086	4055	7029	27728	11346	10047	2522	139469	11622.42
2016	Fogging	7560	18454	18835	15080	3899	944	1276	2563	2312	11233	18376	6175	106707	10670.7
	Total	49042	52760	42970	38814	9120	4499	6589	21583	52103	38311	33395	14241	363427	10688.01

Fogging in the year 2015 recorded the highest wet bean weight (589.49 kg) compared to conventional knapsack spraying in year 2014 (576.96 kg). There was an increment of harvested pods (15.93%) in year 2015 (Table 4). Unfortunately, wet bean weight obtained in 2016 was the lowest with 480.2 kg compared to previous years (Table 4 and *Figure 1*). Increment of 2.13 % was denoted for wet bean weight in year 2015, and in year 2016 decreasing trends were recorded with - 22.76% compared to previous years.

Table 4. Monthly average and percentage of wet bean weight increment from 2014 through 2016 at Field 1B

Year	Treatment	Harvested pods				Wet bean weight (kg)			
		Total	Monthly Avg	Increment	% of increment	Total	Monthly avg	Increment	% of increment
2014	Knapsack	117251	9770.92	Na	Na	6923.5	576.96	Na	Na
2015	Fogging	139469	11622.42	1851.5	15.930	7073.9	589.49	12.53	2.126
2016	Fogging	106707	10670.7	-951.72	-8.919	5762.4	480.2	-109.29	-22.760

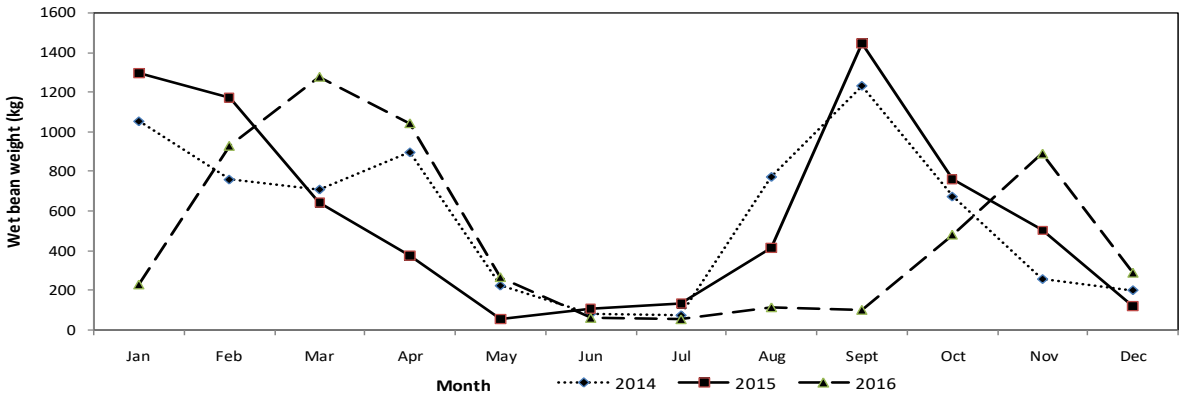

Figure 1. Wet bean weight at Field 1B

In another observation, sleeving was carried out in 23 ha of mature cocoa blocks at Field 1A. Sleeving was covered 29 blocks and started in January 2016, replacing knapsack spraying in year 2014 and fogging in year 2015. Total number of sleeved pods was 37,097 pods, with monthly average of 3709.70 (Table 5). Number of sleeved pods was low, with average of 128 pods were sleeved at monthly basis per block. Low number of sleeved pods might be due to sleeving was occasionally conducted by field workers, and did not following sleeving intervals recommendation, which is at least once every 10-14 days intervals.

Table 5. Number of sleeved pods at Field 1A (29 blocks).

Year	Treatment	Number of harvested pods												Total	Mothly average
		Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec		
2014	Knapsack	19908	14735	14517	12324	6928	3602	1839	8566	21108	15326	6880	6293	132026	11002.17
2015	Fogging	17121	15276	6876	3442	1897	884	1110	2572	6734	5008	5326	2939	69185	5765.42
2016	Sleeving	3380	8459	10521	8658	3908	1504	1304	1649	3223	7260	15123	8245	73234	7323.4
	Sleeved pods	7365	3191	2253	2027	3215	2860	2740	3355	4185	3890	0	0	37097	3709.7

Monthly average of harvested cocoa pods was the highest in 2014 (11,002.17 pods), 2015 (5,765.42 pods), and sleeving in year 2016 with 7,323.4 pods (Table 6 and *Figure 2*). Huge difference on monthly average of harvested pods between 2014 and 2015 was due to rehabilitation program in most of the block at Field 1A. Number of harvested pods is estimated to increased in year 2017 onwards. Even number of sleeved pods were very low, there was an increment in wet bean weight between year 2015 (309.92 kg) and 2016 (565.68 kg). Percentage of bean increment was 45.21 % in year 2016. This increment might suggest that sleeving was more effective in managing *C. cramerella* in large scale plantation, compared to knapsack and fogging applications.

Table 6. Monthly average and percentage of wet bean weight increment from 2014 through October 2016 at Field 1A

Year	Treatment	Harvested pods				Wet bean weight (kg)			
		Total	Monthly Avg	Increment	% of increment	Total	Monthly avg	Increment	% of increment
2014	Knapsack	132026	11002.17	Na	Na	5606.7	467.23	Na	Na
2015	Fogging	69185	5765.42	-5236.75	-90.830	3719	309.92	-157.31	-50.758
2016	Sleeving	73234	7323.4	1557.98	21.274	5656.8	565.68	255.76	45.213

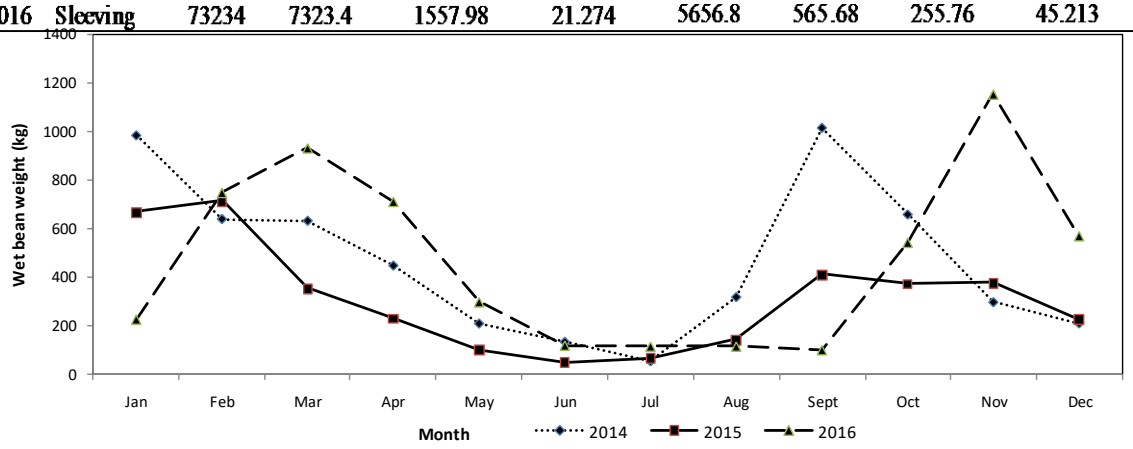

Figure 2. Wet bean weight at Field 1A

In Malaysia, *C. cramerella* control was mainly based on several active ingredients of deltamethrin, alphacypermethrin, cypermethrin and chlorpyrifos (Lee *et al.,* 2013). Heavy reliance on insecticides as a mean for managing CPB was reported by Beevor *et al.* (1993). However, prolonged reliance on insecticide may lead to decreasing capability of insecticide due to losing effectiveness over time (Lee, 1996). Therefore, ecologically technique must be adapted, and sleeving is one of the most environmentally sound approach in managing this pest. In early year of *C. cramerella* arrival, sleeving was listed as one of the control measures (Ooi *et al.,* 1987), with several kinds of sleeving materials were suggested like old newspaper, bottles and plastic bags.

Throughout the observation, knapsack insecticide spraying (year 2014) proven better in both fields compared to fogging application. This might due to sprayings were following the recommendations, which should be commenced every two weeks (Ling, 2013). Timing of insecticide schedule for pest control is crucial in the field, and the effectiveness was decreased when spraying is conducted when infestation at its peak and therefore already caused damage to the crop (Saripah and Alias, 2016). The weakness of fogging application observed in this study is the spraying occasions, where it was

conducted at monthly basis. However, this 24-rounds of insecticide/ha/year will lead to higher costs, compared to fogging. The operational cost of bimonthly insecticide spraying was estimated at RM 1,367/ha/year covered the labor and input costs.

CONCLUSIONS

Comparison between conventional insecticide spraying and fogging spraying were based on wet bean weight, number of harvested pods and pod weight conversion. The effectiveness of insecticide fogging for controlling CPB is yet to be established, but the cost for controlling CPB was estimated to be RM 11.75/ha/round or RM 117.50/ha/year, These low cost might be due to low amount of insecticide was used per each fogging session. Unfortunately, throughout the study, it was stated that fogging at monthly basis or following flowering patterns, with double rate of insecticide shown undesirable results compared to knapsack spraying. Fogging at monthly intervals seems to be insufficient for controlling the *C. cramerella*. Shorter interval between fogging application, dosage increment of insecticide as well as increase the concentration of water-based carrier could be improved the controlling methods. On the other hands, implementation of cocoa pod sleeving seem to be the best resort for managing *C. cramerella* in large scale areas. Even surroundings area were highly infested, sleeving was suggested due to fully protection of cocoa pod throughout pod development. However, sleeving must be carried out at perfect timing, which is suggested when the cocoa pod is less than 9 cm. Otherwise, it may reduce their effectiveness, due to *C. cramerella* might already deposit their eggs on the pod surface. To ensure more promising results, sleeving must be well schedule, which must be carried out every 10 to 14 days intervals, or lesser period to break the cycle of the pest. Throughout this 36-months of observation, sleeving was suggested as the best approach for controlling *C. cramerella* infestation in large scale plantation.

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GROWTH OF *Phytophthora palmivora IN VITRO* IN RESPONSE TO TEMPERATURE

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ABSTRACT - Phytophthora palmivora, *is a devastating pathogen of cocoa which cause black pod, canker and seedling blight. Therefore, it is crucial to identify some of the environmental limitations which can constrain their growth. This study was conducted to evaluate the effect of controlled temperature on the in vitro growth of* P. palmivora*. Detached pod of PBC 123 clone was used and inoculated with 8 mm mycelial disc of pathogen. The inoculated pods were incubated at 24, 27, 30, 33 and 36ºC. Lesion diameter was recorded daily for 7 days. The optimum temperature for the growth of* P. palmivora *is at 27ºC, with diameter growth of 6.99 cm. In contrast, this pathogen cannot survived at all at 36ºC and no lesion was observed. Incubation of pathogen at 30ºC produced 8.39 cm of lesion on pod. The sporulation with formation of white mycelial growth on pod was only visible at optimum temperature, 27ºC. In our studies,* P. palmivora *can grow best at surrounding temperature and their growth can be inhibited by increasing the temperature to more than 30ºC.*

Keywords*:* Cocoa, *Phytophthora palmivora,* temperature

INTRODUCTION

Black pod of cocoa (*Theobroma cacao* L.), caused by *Phytophthora palmivora* (Butl.) Butler, has been an important disease in Malaysia which threatens sustainability of the cocoa beans. This disease has causes losses less than 5%, but at certain times it could be over 70% (Tey and Bong 1990; Bong and Stephen 1999). The pathogen attacks at any stages of pod development, but most significant at immature pods. According to Ndoumbe-Nkeng *et al*. (2004), black pod develop from a series of foci that arise continuously throughout the season, especially from infected pods located high in the canopy.

The management of black pod is challenging because the pathogen persist in plants debris and soil for years and infected pods remain on the cacao tree through most of the year (Erwin *et al*., 1983; Evans and Prior, 1987). Current methods include application of copper and metalaxyl-based fungicides, management of shade, phytosanitation, and the use of resistant cultivars (Bowers *et al.,* 2001; Ndoumbe-Nkeng *et al.,* 2004). However, the key of disease control is by understanding the environmental limitations of pathogen to constrain their growth.

Black pod epidemics are associated with wet condition, high rainfall and moderate temperature (Ahmad Kamil *et al.,* 2004). According to Duniway (1983), the important events in the pathogen's life cycle are influenced by major weather variables, such as moisture and temperature. The germination of spore, inoculum production, mycelial growth rate and survival are influence by temperature (Mizubuti and Fry, 1998). The objective of the study is to provide information on effects of temperature on the growth of isolates of *P. palmivora.* The information on fungal response in culture is valuable in determining effective control strategies for nurseries.

MATERIALS AND METHODS

This study was conducted at pathology laboratory, Cocoa Research and Development Centre (CRDC) Bagan Datuk, Perak. The pathogen, *P. palmivora* (LKM 44) was obtained from a naturally infected cacao pod in the field. The infected pod was surface sterilized with ethanol 70% and allowed to dry on sterilized tissue paper. By using flamed scalpel, the exocarp of infected area was aseptically removed to get a section of mesoderm. The fragments was transferred on corn meal agar (CMA) plates and incubated for seven days at room temperature (25 ± 2ºC). For long-term preservation of the isolate, cultures were grown on agar slants in McCartney bottles. Based on the growth

characteristic of the isolate on CMA and sporangial shape (Zentmyer, 1988), this organism was confirmed a as *P. palmivora.*

Detached pod test was done by modified a method by Iwaro *et al*., 2005. The unripe pods (PBC 123 clone) of approximately 4-5 months old were washed thoroughly with tap water and the stalk were swabbed with 70% ethanol. All pods were arranged in plastic trays (2-3 pods per tray), lined with moist paper towels and covered with transparent plastic bag. Agar plugs from the margin of colony *P. palmivora* growth, 8 mm in diameter, were cut using a cork borer and transferred to five replicate of pods. The mycelial plugs were placed at the center of the pod and covered with wet cotton ball. The inoculated pods were incubated at 24, 27, 30, 33 and 36 ºC. Lesions of growth diameter was recorded daily for 7 days.

RESULTS AND DISCUSSIONS

Phytophthora sp. usually infect tissues rapidly at favourable temperatures. Result obtained from this study showed that the optimum temperature for the growth of *P. palmivora* on detached pod of cocoa was at 27ºC with lesion diameter of 9.31 cm *(Figure 1)*. Lesion produced at 30ºC is not significantly different with 27ºC with diameter of 8.75 cm. This means that *P. palmivora* also can grow well at 30ºC. In contrast, no lesion developed at 36ºC. Similar study was done by Timmer *et al,* (2000) on the surface of citrus and found that optimum temperature for sporulation of *P. palmivora* was at 24°C. With *P. citrophthora*, Gerlach *et al,* (1976) found that this pathogen penetrate the leaves of *Pieris japonica* at 20 to 30°C.

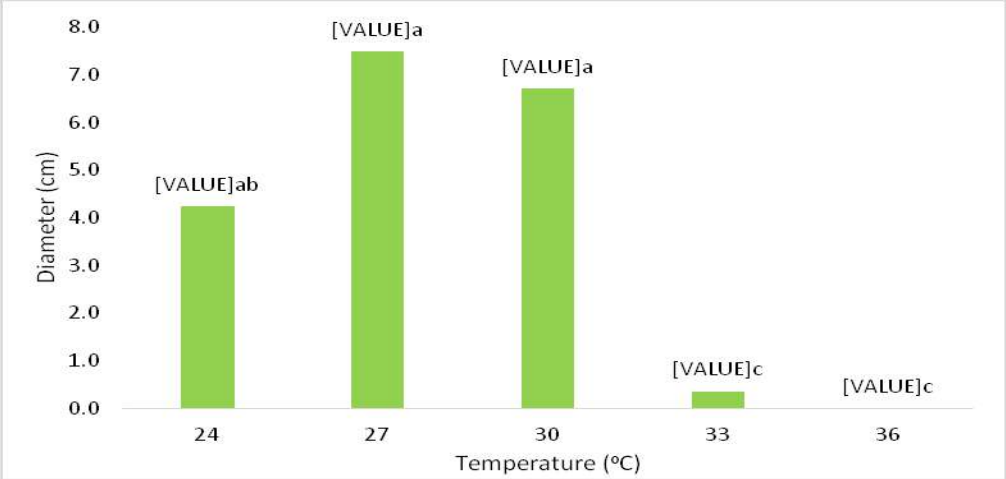

Figure 1. The growth diameter (cm) of P. palmivora on cocoa pod after 7 days of incubation

The lesion diameter produced by *P. palmivora* from day 0 to days 7 of incubation was shown in *Figure 2*. No infection was occurred on day 0 to 1 regardless of temperature. At lowest temperature (24ºC), *P. palmivora* only need a day to adapt with the surrounding temperature and starting to grow gradually from day 4. Even the optimum temperature of *P. palmivora* is at 27ºC, this fungus took 2 days to adapt with the incubated temperature. However, the growth of this fungus is drastically increase from day 3 to 7.

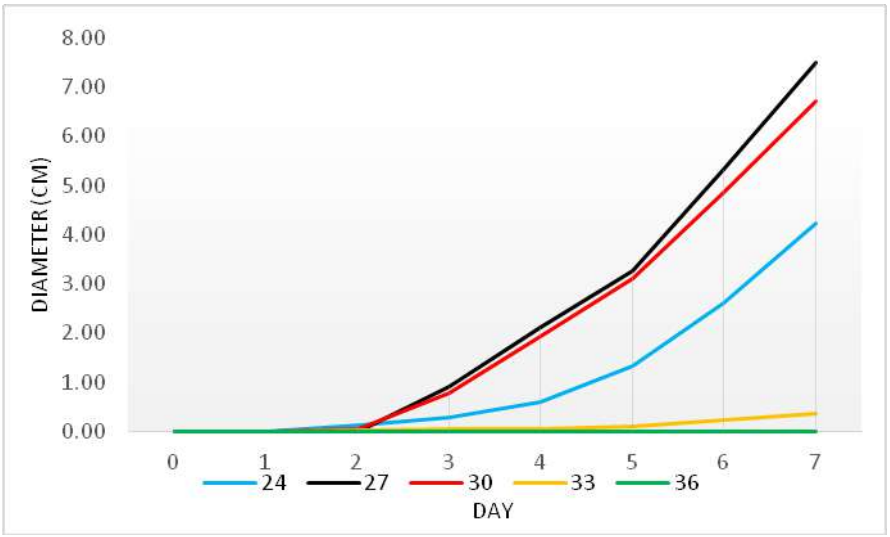

Figure 2. The lesion diameter produced by P. palmivora from day 0 to days 7 of incubation.

Since *P. palmivora* is the pathogen of black pod, it produced a black lesion symptom on a pod surface around inoculated area *(Figure 3)*. The formation of white mycelial growth was only visible at optimum temperature, 27ºC *(Figure 3B)*. In contrast, *P. palmivora* failed to grow on detached pod at 36 ºC *(Figure 3E)*. The black lesion was also clearly visible on detached pod incubated at 24 and 30 ºC.

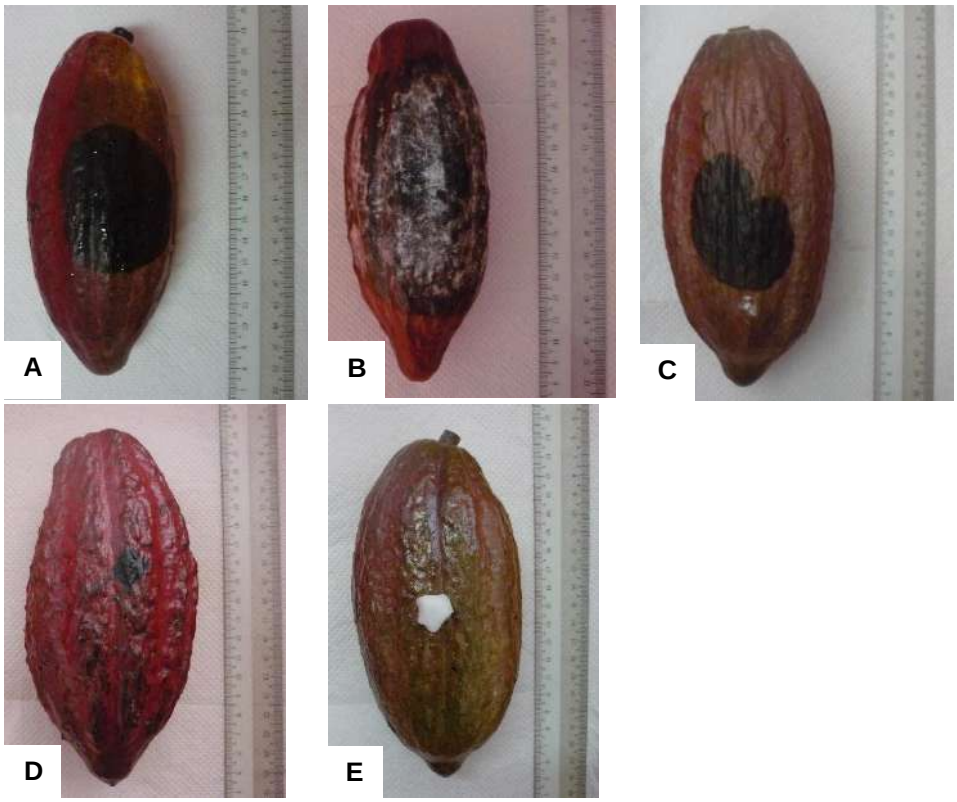

Figure 3. The lesion on detached pod after 7 days incubation at 24ºC (A), 27ºC (B), 30ºC (C), 33ºC (D) and 36ºC (E).

CONCLUSIONS

The study conclude that the incidence of black pod infection was reduced with increasing of temperature to more than 30ºC. The optimum temperature for the occurrence of this disease is at 27ºC. Similar result also obtained by Brasier (1968) which found that incubation of this pathogen at 27.5 – 30 ºC is the most favorable temperature for their growth. Hence, in order to prevent the infection of black pod disease at field, good cultural practices is need to be done especially during rainy season to reduce the moisture.

ACKNOWLEDGEMENTS

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MANAGEMENT OF RAT AND SQUIRREL USING WIRE MESH TRAP IN COCOA AREA CRDC TAWAU AND MADAI

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ABSTRACT - *Most of the vertebrate species reported inflict massive losses on cocoa yield worldwide. More than 60 species of vertebrate recorded as pest of cocoa all over the world. Among the pest rats and squirrels, are consider as a serious pest. The losses on cocoa yield are varied and differ from one country to another. It was estimated about up to 90 % due to the mammalian pest if left uncontrolled. This study was carried out at two Cocoa Research and Development Centre (CRDC) Tawau and Madai using catched marked and release (CMR) technique. Conventional method wire mesh trap was selected as a type of catched the pest. From this study it was found that in the cocoa area size about only 1 ha, when the first catched is only 9 rats and 22 squirrel, total of rat and squirrel were estimated about 40 rats and 33 squirrels respectively. Only less than 10 % of the total traps set up manage to catched the animals. In cocoa germplasm area at CRDC Tawau, trap set up using tapioca bait is preferred by the rats and squirrel. Meanwhile, at CRDC Madai trap set up using palm oil as bait is preferred by the rat and squirrel. Generally, most of the rat catched in the trap set up on the ground and the squirrel trapped in the trap set up on the cocoa branch.*

Key words: Management, rat, squirrel, wire mesh trap, cocoa area.

INTRODUCTION

There were estimated that more than 60 species of vertebrates become pest of cocoa globally (Thorold, 1975). Some of the mammalian pest give inflict massive losses and can be a serious problem on cocoa yield worldwide (John and Debbie, 1993; Lee, 1982). The losses are differed greatly within countries and estimated from a few per cent (0.7 – 7.0 %) in Ghana (Wharton, 1962; Glendinnings, 1962), 3 – 5 % in Sabah, Malaysia (Conway, 1971), 20 – 50 % in Sierra Leone (Urquhart, 1955), 30 % in Trinidad, (Montserin, 1937), 70 – 75 % in India (Anon, 1987; Ranjan, 1987), 72 – 82 % in areas without any control measures (Mainstone, 1978) and up to 90 % in Peninsular Malaysia (Juan and Rose, 1979). Generally, global annual loss due to vertebrate damage was estimated about 5 to 10 % (Entwistle, 1985). Among them, mammalian pest in a rodent group such as rats and squirrels, are responsible for severe attack (Bhat *et. al.*, 1981; Cruz, 1983) on cocoa and can cause pod damage (Han and Bose, 1980). Therefore, this study was conducted to control the rat and squirrel problem at cocoa field in Cocoa Research and Development Centre (CRDC) Tawau and Madai.

MATERIAL AND METHOD

Study on rats and squirrels was carried out at cocoa germplasm Tawau using catched marked and release technique (Southwood, 1978). The size of the area is about 4.0 acre and the cocoa tree stand is about 1,455. Total number of wire mash traps set up per round is about 40. Equal numbers of traps were placed on the ground within the tree base and on the jorquette or within the tree canopy on a branch *(Plate 1)*. Traps were left undisturbed, until inspected daily in the early morning. The wire mesh traps were set up twice a week on alternate day. The trap was set up again after 24 hours release. After 24 hours, the traps were checked again for the catched. Different fresh bait used to attract the rats and squirrel to the traps. The same method of catched was repeated and applied to the cocoa field 6B at CRDC Madai. Total number of wire mesh trap set up is about 160 trap per round in the cocoa field area is about 4 ha. The cocoa trees in this two study sites were mature and bearing fruits and was monoculture. The number of animal caught, the type of baits, and the location and position of the trapped animals were recorded.

	
Marking technique of the rat and squirrel using the ring	Trap set up on the branch
	
Trap set up on the ground	Trap set up between trunk and branch
	
Trap set on the ground nearby the trunk	Some of the rats and squirrels catches at cocoa germplasm area CRDC Tawau and Madai

Plate 1. Rats and squirrels trapping at cocoa area

RESULT AND DISCUSSION

This study showed that wire mesh trap using tapioca bait catched more number of rat and squirrel compared to jackfruit and banana bait *(Figure 1).* This study also indicated that the population of the squirrel catched using the wire mesh trapped more higher compared to the population of rat at Cocoa Germplasm area CRDC Tawau.

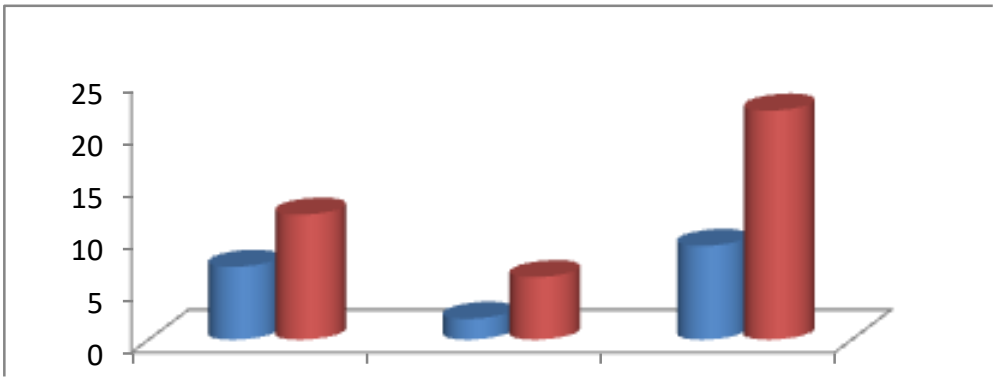

Figure 1. Rat and squirrel caught at CRDC Tawau using three different baits.

This study showed that number of rat catched using wire mesh trap was higher compared to the number of squirrel. This figure also indicated that wire mesh trap using palm oil as bait attracted more catched followed by coconut, rambutan and jackfruit as bait *(Figure 2).*

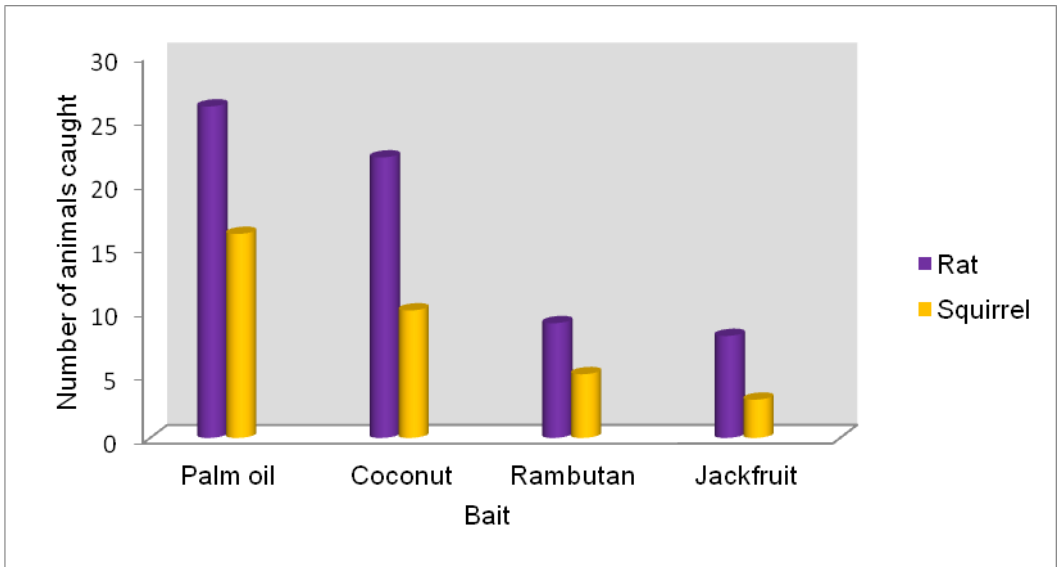

Figure 2. Rat and squirrel caught at CRDC Madai using three different baits.

CONCLUSION

From this study it is indicated that different cocoa area need different source of food as bait to use with a wire mesh trap to attract more catched. Management of rat and squirrel should be done properly as estimated a single rat can damage at least one cocoa pod per day. Meanwhile, squirrel damaged at least four cocoa pods per day (Meriam, 2017; Lee, 2007). Most of the rat catched in the trap set up on the ground, but only one (11.1 %) rat trapped in the

trap set up on the branch of the cocoa tree. It is about 18.2 % (4) of the squirrel catched in the trap set up on the ground. The rest of the squirrel trapped in the trap set up on the cocoa branch. Only less than 10 % of the total traps set up manage to catched the animals.

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ANTIFUNGAL EFFICACY OF CRUDE AQUEOUS WEED EXTRACTS AGAINST PATHOGEN OF COCOA BLACK POD ROT

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ABSTRACT - *Black pod rot is the most economically important disease on cocoa in Malaysia which mainly caused by a highly polyphagous* Phytophthora species, *which was* Phytophthora palmivora. *The fungus could attack at all part of the cocoa plant organs and caused various diseases at any growth stage from the seedling until the mature stages, especially during raining season. Application of synthetic fungicides has been widely recommended to manage the disease but their repeated use had led to problems such as environmental issues. This study isolated and identified* Phytopththora *isolate from a cocoa pod sample based on micro-morphological characters. Besides, the present investigation was undertaken to screen for the antifungal potency of different weed extracts against the* Phytophthora *pathogen using poisoned food technique. The fungal isolate has been successful recovered from pod tissues of clone PBC123 on 20% tomato juice agar culture (20T). Only one in ten weed extracts tested has given a significant in vitro inhibitory effect towards mycelial growth of* Phytophthora *isolate, which was aqueous crude leaf extract of Solanum torvum (42.68%). This study indicated that the potential of weed extracts in the management of* Phytophthora *diseases, and may offer more natural, effective and economical control methods.*

INTRODUCTION

Black pod rot (BPR) is the most destructive and widespread disease of cocoa in all cocoa-producing regions of the world. The most recognizable symptoms on pods are firm, spreading, chocolate-brown lesions that eventually can cover a whole pod. The disease caused by several *Phytophthora* species such as *Phytophthora palmivora*, *Phytophthora megakarya*, *Phytophthora citrophora*, *Phytophthora capsici*, *Phytophthora megasperma* and *Phytophthora katsurae* (Vanegtern *et al*., 2015; Guest, 2007). *P. palmivora* and *P. megakarya* have been identified as the most destructive species on cocoa which limiting cocoa production worldwide. *P. palmivora* seems to be a cosmopolitan species but less virulent than *P. megakarya*. *P. megakarya* is restricted to only cocoa producing regions in Western and Central Africa. In Malaysia, *P. palmivora* has been reported as dominant species on cocoa (Drenth and Guest, 2004). Others such as *P. cinnamomi*, *P. capsici*, *P. nicotianae*, *P. botryose*, *P. citrophthtora*, *P. hevea* and *P. megasperma* (End *et al*., 2017; Drenth and Guest, 2004).

Conventional methods have been used in this study for detection and identification of *Phytophthora* isolate, which firstly involved by means of two commonly used media (potato dextrose agar and tomato juice agar) for supporting the growth and maintenance of the fungus; followed by examination of the morphology for the fungal colonies, sporangia, oospores, etc. Furthermore, Al-Hedaithy and Tsao (1979) suggested that *Phytophthora* spp. can be identified and grouped into three categories based on average pedicel lengths. They were (a) with short pedicels (< 5 μm): *P. cactum*, *P. infestans* (Mont.) deBary and *P. palmivora* (Butl.) Butl. (MF1); (b) with pedicels of intermediate lengths (5-20 μm): *P. botryose* Chee, *P. colocasiae* and *P. palmivora* (MF3) and (c) with long pedicels (> 20 μm): *P. hibernalis* Carne and *P. palmivora* (MF4). Recent advance detection methods included the use of electrophoretic banding patterns of soluble proteins, isoenzyme analyses, genome-based techniques such as restriction fragment length

polymorphisms (RFLP) of mitochondrial DNA and sequencing of the internal transcribed spacer regions of ribosomal DNA (rDNA-ITS). Yet, morphological comparisons still the most practical option in countries affected by severe cocoa *Phytophthora* diseases (Appiah *et al*., 2003).

Several methods have been adopted by farmers to control the disease such as the use of chemical compounds, genetically resistant trees, biocontrol and phytosanitary methods but none of them have completely controlled the disease so far (Guest, 2007; Ploetz, 2007). In contrast, Barreto *et al*. (2015) reported that the most efficient way to control black pod disease was to use resistant crop varieties by suggesting the strategies for the use in a cocoa breeding program that could accelerate the process of selecting cocoa genotypes resistant to black pod disease caused by multiple *Phytophthora* spp.

Many previous studies have proven that plant extracts can be used to control cocoa black pod disease caused by *Phytophthora* spp. Example Mohsan *et al*. (2017) reported that the crude extracts of *Parthenium hysterophorus* (Parthenium), *Nerium oleander* (Kaner) and *Oscimum basilicum* (Niazbo) showed inhibitory efficacy against *P. capsici in vitro*. Besides, Ngoh Dooh *et al*. (2015) *in vivo* study revealed that aqueous extract of *Thevetia peruviana* could produce cheap and effective formulation to control of black pod disease caused by *P. megakarya.* In addition, rosemary and lavender leaf extracts were also found to be effective in reducing germination of *P. capsici*, *P. megakarya* and *P. palmivora* zoospores (Widmer and Laurent, 2006).

In present study, ten weed species have been tested for their antifungal activity against pathogen of black pod disease on PDA medium using food poisoned technique, which commonly used to evaluate the antifungal effect of certain antifungal agents or extracts against molds (Kumar *et al*., 2014; Ali-Shtayeh and Abu Ghdeib, 1999; Mukherjee and Raghu, 1997).

Thus, the main objectives of this study were to isolate and identify the pathogen that causing BPR on cocoa pod sample obtained from Cocoa Research & Development Centre (CRDC) Jengka, Pahang and to recognize weed species with anti-phytophthora activity. Consequently, the study could reveal weed extracts as a good alternative in developing a potent plant based fungicides or as an integrated approach to combat fungal plant pathogens.

MATERIALS AND METHODS

Collection and identification of weed species

Field-collected weed samples were obtained from various places in UiTM, Jasin Campus and Kampung Seri Mendapat, Merlimau, Melaka. Weeds were identified on the basis of pectoral characteristics guided by Chung *et al.* (2014); and Zakaria and Mohd (2010). Table 1 showed list of weeds selected and part of the weed used in the study. The selection of the weeds were based on their potential medicinal uses and found abundantly.

Table 1. Weeds used in the study

Name of the Weeds	Scientific Name	Family	Part Used
Common Asystasia, Chinese violet, Creeping foxglove, Ganges primrose	*Asystasia gangetica*	Acanthaceae	Leaf
Broad sword fern, Paku larat, Giant sword fern, Sword fern	*Nephrolepis biserrata*	Oleandraceae	Leaf
Fireweed, Tetracera, Hedge Row Tetracera, Puson Dumarun, Akar Pulas Duyio, Akar Mempelas, Empelas	*Tetracera indica*	Dilleniaceae	Leaf
Siam Weed, Bitter bush, Devil Weed, Hagonoy, Jack in the bush, Triffid weed	*Chromolaena odorata*	Asteraceae	Leaf
Lantana, Common Lantana, Shrub verbena, Spanish flag, Tick berry, Bunga tahi ayam, Bunga pagar	*Lantana camara*	Verbenaceae	Leaf
Ivy gourd, Scarlet-fruited gourd	*Coccinia indica*	Cucurbitaceae	Leaf
Castor oil bean, Castor oil plant, Palma christi, Castor bean plant, Jarak	*Ricinus communis*	Euphorbiaceae	Leaf
Turkey berry, Devil's fig, Terung pipit	*Solanum torvum*	Solanaceae	Leaf
Peacock flower, Barbados flower fence, Flower fence, Jambol merak, Cana, Barbados pride, Red bird of paradise, Paradise flower, Flamboyant tree, Gold mohur, Pride of barbados, Jambul merak	*Caesalpinia pulcherrima*	Fabaceae	Leaf
Spreading dayflower, Climbing dayflower, Scurvy weed	*Commelina diffusa*	Commelinaceae	leaf

Preparation of weed leaf aqueous extracts

A method according to Prasad & Anamika (2015) has been followed but with some modifications. Fresh weed leaves were washed thoroughly 2-3 times with running tap water and once with distilled water before placed inside the hot air oven at 40ºC (Memmert Universal Oven UF260, Germany) till it dried and crumbled by hand within 4 to 7 days. The dried samples were pulverized with blender machine (MX-GM1011 H [Grey], Panasonic, Malaysia) before packed in clean and dark plastic containers; and stored in refrigerator at 4ºC for long-term storage. Then, the extraction of antifungal compounds was done by freshly macerating 24 g of each oven-dried selected-weed species into 400 ml sterile distilled water in a 500-ml flat bottom and narrow neck flask (Simax glass, Czech Republic) before kept on a rotary shaker (Stuart Orbital Shakers SSL1, UK) for 24 h at 120 rpm; and incubated at room temperature. After maceration, the aqueous extract was filtered through a fine coffee filter to remove large weed leaf tissue residues prior to antifungal activity assay by poisoned food technique.

Survey, sample collection and Phytophthora isolation

A disease survey in the cocoa fields at Malaysian Cocoa Board, CRDC Jengka, Pahang showed that many cocoa pods from different clones (PBC 123, PBC 140, KKM 1, KKM 5 and KKM 22) were severely infected by black pod disease. In this study, an isolate of *Phytophthora* was obtained from a naturally infected cocoa pod tissues of clone PBC 123.

Isolation of pathogen from infected pod tissues was done between the margin of the infected and healthy areas. The infected tissues were cut by sterilized scalpels about 5 mm length x 5 mm width x 5 mm depth. The samples were washed and surface sterilized by soaking in 10% commercial bleach (5.25% [v/v] sodium hypochlorite) for 10 min followed by triple rinsing

in sterilized deionized distilled water (ddH_2O) for 5 min each before blotted dry with tissue paper. After that, three pieces of the infected tissues were placed on 20% tomato juice agar (20T) (20% Campbell's Tomato Juice [Malaysia], 0.04% $CaCO_3$ and 2% agar) and PDA media, respectively. A previous study has shown that 20T could induce the growth and both asexual and sexual reproductions of some *Phytophthora* species (Guo & Ko, 1993). The incubation was made in the incubator at 30°C for five to seven days. Fungal growths from tissue segments were then transferred onto another 20T medium to obtain pure culture of the isolated fungus. Identification of the *Phytophthora* isolate was based on the microscopic characters. According to Stamps *et al*. (1990), Newhook (1978) and Waterhouse (1963) studies, production of oogonia, antheridia and oospores (sexual spores) and the morphological of sexual spores (zoosporangium and chlamydospores) produced by *Phytophthora* spp. can be used as the basis for species identification and taxonomy of *Phytophthora* species. Pure cultures obtained from infected tissues were maintained on 20T slants at 4^0C for future use.

Furthermore, fungal plugs (about 5 mm x 5 mm x 5 mm) from a 7-day-old culture were used as inoculum before placed on lesions made on healthy, half maturity and detached pods collected from trees of the same clone to examine its pathogenicity in stab method with some modifications (Iwaro *et al*., 1997). Next, the inoculated pods were placed in clean plastic containers in which the humidity maintained by a plug of sterile cotton wool soaked with ddH_2O; and tightly covered with a few sheets of old and clean newspaper to create dark condition (Mpika *et al*., 2011; Omokolo *et al*., 2003). The incubation was done at room temperature (RT) in laboratory and daily observation made for seven days on black pod symptom development. Then, the fungus was re-isolated to fulfil Koch's postulates.

Poisoned food technique

Assessment of fungal toxicity was carried out by poisoned food technique followed the procedure of Schmitz (1930) with some minor modifications, intentionally to evaluate or screen the antifungal efficacy of aqueous crude weed extracts against black pod rot pathogen of cocoa. PDA dissolved in aqueous weed extracts, respectively before autoclaved (121°C, 20 min, 15 psi) and poured into petri dishes. With the help of a sterile scalpel, a cube shape of fungal culture plug (about 5 mm x 5 mm x 5 mm) was cut from the periphery of 7- day-old culture of *Phytophthora* isolate and transferred aseptically on PDA plates impregnated with crude weed extracts before incubated at 30°C for seven days. A plate without crude weed extract served as a control for the calculation of percentage inhibition of mycelial growth of test fungus. The experiments were carried out in triplicates and percent reduction of mycelial growth over control calculated using the following formula (Harlapur *et al*., 2007);

Percentage Inhibition (%I) = (dc-dt)/dc x 100
Where, dc – Average increase in mycelial growth in control.
dt – Average increase in mycelial growth in treatment.

Data analysis

All statistical analyses were performed using SPSS Version 24 (IBM SPSS, Chicago, IL, USA). One way ANOVA test (Analysis of Variance) at alpha ≤ 0.05 was used to determine the significant differences between the treatments.

RESULTS AND DISCUSSIONS

Observation on symptoms of cocoa pod rot disease

Survey results on cocoa pod rot disease in CRDC Jengka, Pahang showed that the disease could infect all stages of pod development, starting from cherelles, immature to mature pods. Symptoms of infected pods were browning, blackening and rotting of cocoa pods with clear boundary between healthy part and infected part. This discoloration generally started from pod stem *(Figure 1)* or pod tip.

Figure 1. Pod of Theobroma cacao in CRDC Jengka, Pahang displays enlargement of black pod lesion started from the stalk. This pod was infected during the last stages of ripening

Cultural and morphological characteristics

An isolation trial on 20T medium resulted in mycelial growth and sporulation of *Phytophthora* isolate. Colony of the fungus isolate visible in five to seven days after introduction to the medium, which surface filled with creeping whitish fluffy mycelium *(Figure 2a)*. The growth medium also could induce production of asexual structures such as abundant of chlamydospores *(Figure 2c)*, ellipsoid to ovoid sporangium with apical papillae, short pedicel and coenocytic hyphae *(Figure 2d)* after the fungus stained with lactophenol cotton blue for microscopic observation (Leck, 1999). The findings were in accordance for detection and identification of *Phytophthora* guided by Drenth and Sendall (2011). Guo and Ko (1993) reported that tomato juice agar (TJA) was found to be comparable to or in some cases better than the V8 vegetable juice agar in supporting the growth of *Phytophthora cactorum*, *Phytophthora capsici* and *Phytophthora parasitica*; sporangium production of *P. capsici* and *P. palmivora*; and oospore formation of *P. cactorum*, *P. parasitica*. Besides, tomato agar was stated conducive to formation of amphigynous antheridia in sexual reproduction of *Phytophthora boehmeriae* (Gao *et al.*, 1998). In addition, TJA has been discovered that could support better growth for *P. palmivora* and *P. megakarya* compared with cocoa beans agar (CBA) and cocoa pod and beans agar (CPBA) (Oluyemi *et al*., 2014). In this study, no sporulation was observed on PDA. In future, further study will be carried out to identify fungus isolate, which was using polymerase chain reaction (PCR) as suggested by Alsultan *et al*. (2017).

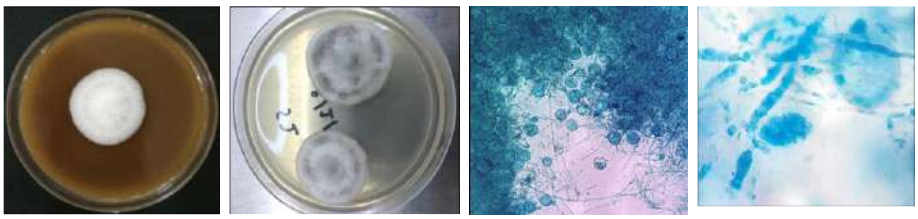
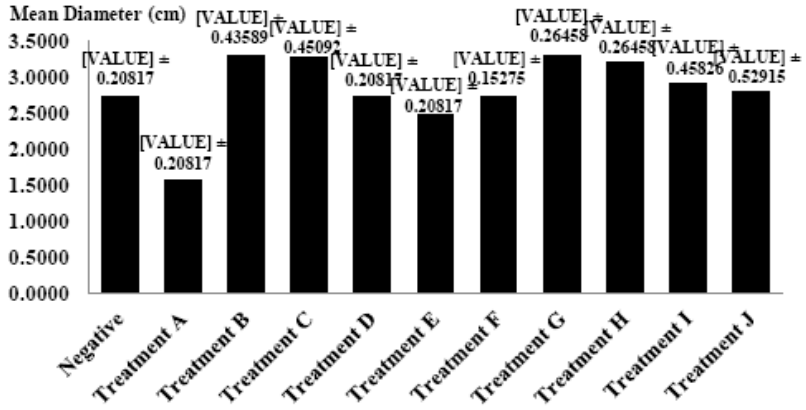


Figure 2. Phytophthora isolate. 2a: Colony observed on 20T culture plate; 2b: Colonies observed on PDA culture plate; 2c: Chlamydospores at 100x magnification; 2d: Sporangium and coenocytic hyphae at 400x magnification

Evaluation of weed extracts for Phytophthora mycelial growth inhibition

The antifungal activity of crude aqueous extracts for ten weed species belonging to ten different families was evaluated against mycelial growth of *Phytophthora* isolate by poisoned food technique. Its effects on fungal colony diameters were measured and reported in *Graph 1*. The efficacy of the weed extracts was expressed as percent inhibition of mycelial growth over control which shown in Table 2. Among all weed extracts have been evaluated for their efficacy against the black pod disease pathogen, *S. torvum* found to be the most effective (42.68% inhibition, Table 2, *Figure 3*) followed by *R. communis* with lesser percentage (9.75%). No antifungal activities were observed for crude aqueous extracts of *T. indica* and *C. odorata*. While rests of the six weed extracts showed stimulatory effect on growth of *Phytophthora* isolate. The antifungal activity of above weeds against *Phytophthora* isolate was suspected due to the presence of few secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, saponins, steroids and etc. (Gurjar *et al*., 2012).



Graph 1. Effect of different crude weed extracts against mycelial growth of Phytophthora isolate. Abbreviations - Negative: Control; Treatment A: Treated with crude aqueous extract of S. torvum; Treatment B: N. biserrata; Treatment C: C. diffusa; Treatment D: T. indica; Treatment E: R. communis; Treatment F: C. odorata; Treatment G: C. grandis; Treatment H: A. gangetica; Treatment I: C. pulcherrima; Treatment J: L. camara

Table 2. Inhibition percentage

Botanical Name	**% Inhibition**
S.torvum	42.68
N. biserrata	-20.73
C. diffusa	-19.51
T. indica	0.00
R. communis	9.75
C. odorata	0.00
C. grandis	-20.73
A. gangetica	-17.07
C. pulcherrima	-6.10
L. camara	-2.44

Abbreviations: (+) – Inhibitory growth effect; (-) – Stimulatory growth effect

Figure 3. In vitro inhibition of mycelial growth of Phytophthora isolate by crude aqueous extract of S. torvum; 3a: Crude aqueous extract of S. torvum leaves showing inhibition in the fungal growth; 3b: Control (PDA + Phytophthora isolate) after a week incubation period at 30°C

Statistical analysis

Comparison of means for the mycelial growth of *Phytophthora* isolate on PDA amended with different crude aqueous weed extracts, respectively showed in Table 3. With a probability value of 0.000 and a level of significance fixed in 5%, it became clear after the analysis of means for variances that the crude aqueous weed extracts did cause the inhibitory effect on the mycelial growth of *Phytophthora* isolate induced black pod disease on infected cocoa pod samples.

Table 3. Analysis of variance

Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7.589	10	0.759	6.843	0.000
Within Groups	2.440	22	0.111	
Total	10.029	32		

CONCLUSIONS

The results of this study showed that 20% tomato juice agar (20T) was a better medium compared with PDA for growth and reproduction of *Phytophthora* isolate from an infected cocoa pod sample. Crude aqueous weed extract of *S. torvum* leaves had the highest or the most effective antifungal activity against the test fungus as shown 42.68% inhibition in mycelial growth inhibitory assay by poisoned food technique. Hence, it can be used as a new source for antifungal substances for management of *Phytophthora* pod rot infection in the field and further phytochemical studies are recommended to purify and characterize the active ingredients of this weed species.

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COMPARISON OF ULTRASOUND-ASSISTED AND NORMAL AQUEOUS INCUBATION EXTRACTION METHODS IN TOTAL PHENOLIC CONTENT OF FRESH COCOA BEANS

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Malaysian Cocoa J. 12(1): 41-45 (2020)

ABSTRACT - *Total Polyphenols influenced of extraction conditions assisted by ultrasound and incubation on the polyphenols content of extracts obtained from Malaysia Cocoa Fresh Bean (MCFB). The aqueous extraction procedure was carried out in an ultrasonic bath and incubator shaker. The other parameter such as temperature and duration are set to 80^0C and 10 minutes. The aqueous extract from both methods was evaluated on the basis of the phenolic content of the plant extract. Significant variability in phenolic content depending on the type of extract method was found. Ultrasound-Assisted Extraction (UAE) extract was significantly richer in total polyphenols than Incubation Assisted Extraction (IAE) extract, their total polyphenols are 5.492 and 3.508 mg GAE.g^{-1} sample, respectively. In addition, the UAE increase polyphenols concertation by 56% from the IAE. The total polyphenol of MCFB is strongly influenced by the extraction method.*

Keywords: Cocoa fresh bean, polyphenols, sonication, incubation

INTRODUCTION

Phenolic compounds are able to act as reducing agents, hydrogen donors and scavengers of reactive oxygen species as it classified as a common secondary metabolites characterized by the presence of an aromatic ring bearing free hydroxyl groups or engaged with a carbohydrate (Mason *et al.*, 1996; Pandey & Rizvi, 2009; Ivanova *et al*., 2005). Plants polyphenols are playing important role in defense against different types of stress by offering protection against reactive oxygen and nitrogen species, UV light, pathogens, parasites and plant predators (Balasundram *et al*., 2006). Moreover, it also playing important role in the prevention of chronic diseases, such as cardiovascular diseases, neurodegenerative disorders, cancer, type II diabetes and osteoporosis (Alothman *et al*., 2009; Scalbert *et al*., 2005).

There are many methods to extract the polyphenols from the plant such as Accelerated Solvent Extraction (ASE), Supercritical Fluid Extraction (SFE), Soxhlet Extraction (SE), Ultrasound Assisted Extraction (UAE) and others. However, the extraction processes are a major variability factor in the antioxidant properties of extracts. Indeed, different antioxidant capacities depending on the factors and affinities between factors used in the extraction such as solvent types, extraction times, extraction temperature, particle size of samples and the method adopted for the influence for extraction influence significantly the composition of the extract (Ksouri *et al*., 2009; Hayouni *et al*., 2007) . Extracting polyphenols from biological materials by a conventional method such as SE is time-consuming. By using the new method or modern techniques such as Microwaves Assisted Extraction (MAE) or UAE is intended to overcome this time-consuming problem and additional can increase extraction efficiency, selectivity, and kinetics. UAE using ultrasound or sonication to break the cell membranes and reducing the extraction time and increasing the extract yield. This application happened by disrupting the cell wall structure and accelerates the diffusion through membranes thus it facilitates the release of cell contents (Mason *et al*., 1996).

The objective of this study was to optimize the extraction condition for Malaysia Cocoa Fresh Bean in order to achieve the highest polyphenols levels on the choice of the most suitable extraction method, extraction time and temperature of extraction.

MATERIALS AND METHODS

Preparation of aqueous extract of Malaysia Cocoa Fresh Bean (MCFB)

MCFB were collected from Pusat Penyelidikan Dan Pembangunan Koko (PPPK), Jengka, Pahang and were removed their pulp and testa. For aqueous extraction, 1g of MCFB was crushed in a mechanical blender and mixed with 50mL distilled water. Then the mixture was extracted using two machines separately which is Innova incubator shaker (125 rpm) and sonicated washer (40Hz) 10 minutes and temperature 80^{o}C respectively. Then the aqueous extract was filtered using Whatman No. 1 paper (11um), Whatman No.5 paper (2.5um) respectively. The extract was store at -40^{o}C in storage vials for experimental use.

Determination of total phenolic content

The total phenolic contents of MCFB were determined by Folin-Ciocalteu (FC) reagent (Merck USA) procedure by using the method described by Kaur and Kapoor (2002) with several modifications. The extraction of MCFB (1mL) were dilute in the distilled water (2mL) and then the dilution of extraction MCFB (0.5mL) were mixed with FC reagent (2.5mL) and vortex for 10 seconds. Next, the reaction was neutralized with the addition of sodium carbonate solution 20% (1.8mL) and vortex for 10 seconds. The mixture was incubated for 60 minutes in a dark and absorbance was measured at 750nm using UV-Visible Spectrophotometer (Cary 60, Agilent, USA). The total phenolic content (TPC) was expressed as mg gallic acid equivalents per gram of sample (mg GAE g^{-1} Sample) using calibration curve constructed with gallic acid (0 – 200 ug.mL^{-1}) were all samples analyzed in triplicate.

RESULTS AND DISCUSSIONS

Data absorbance obtain from the spectrophotometer were calculate and express in a mg GAE.g-1 sample by using gallic acid standard curve with r^2 value is 0.99619 in *Figure 1*. The normality test is determined by using Minitab 14 as in *Figure 2* and *Figure 3*. From the probability, plot shows that the p-value for the incubation extraction data and sonication extraction data are not significant ($p > 0.05$) respectively.

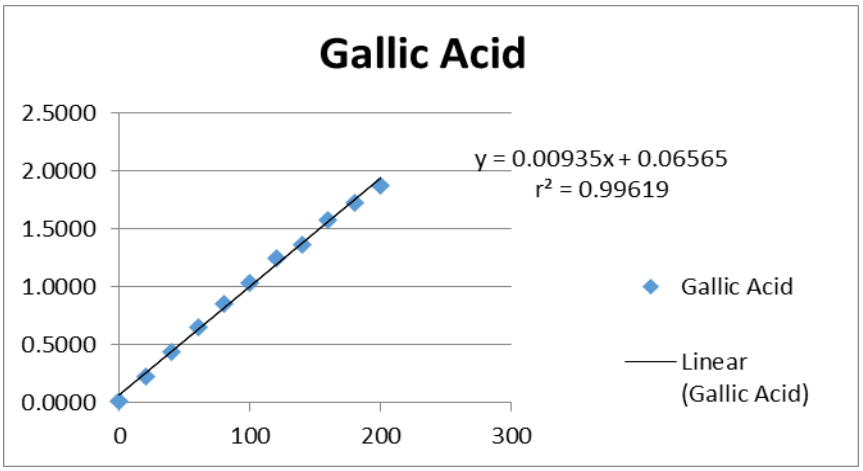

Figure 1. Calibration graph of gallic acid

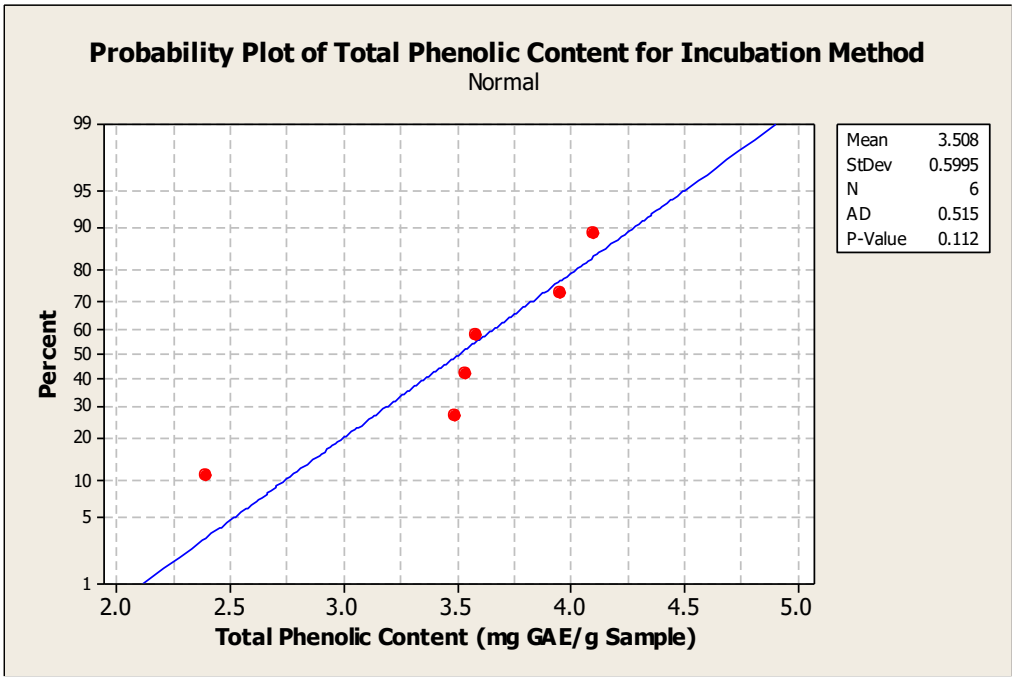

Figure 2. Probability plot of total phenolic content for incubation method

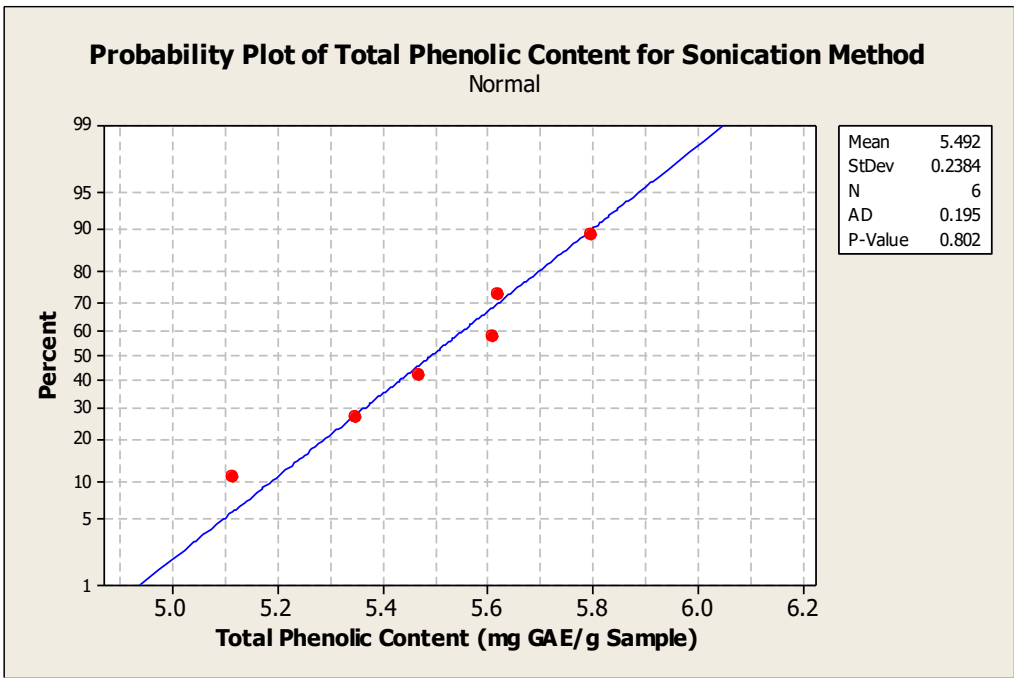

Figure 3. Probability plot of total phenolic content for sonication method

As *Figure 4* shows, there was a significant variation in the phenolic extracts. The total phenolic content for incubation method is less than sonication method by express it in the mgGAE.g^{-1} sample. The mean for the data are plot in the boxplot and the level sonication are higher than the incubation method. The UAE was significantly richer in total polyphenols (5.492 mg GAE.g^{-1} Sample) than IAE method (3.058 mg GAE.g^{-1} Sample) because of the mechanical effect such as increasing the mass transfer of extraction with high shear force and disruption of tissue surface structure (Balachandran *et al*., 2006; Jian *et al*., 2006; Chemat *et al*., 2004; Haizhou *et al*., 2004; Vinatoru, 2001). In addition, the macroturbulence was created by the implosion of cavitation bubbles, high-velocity inter-particle collisions and accelerates the eddy diffusion and internal diffusion by perturbation in microporous particles of the biomass (Shotipruk *et al*., 2001). UAE permits higher extraction concertation in less consuming times to compare to the IAE, thereby reducing the energy input. UAE using ultrasound to break the cell membranes and increasing the extract concentration about 56% or more from the IAE.

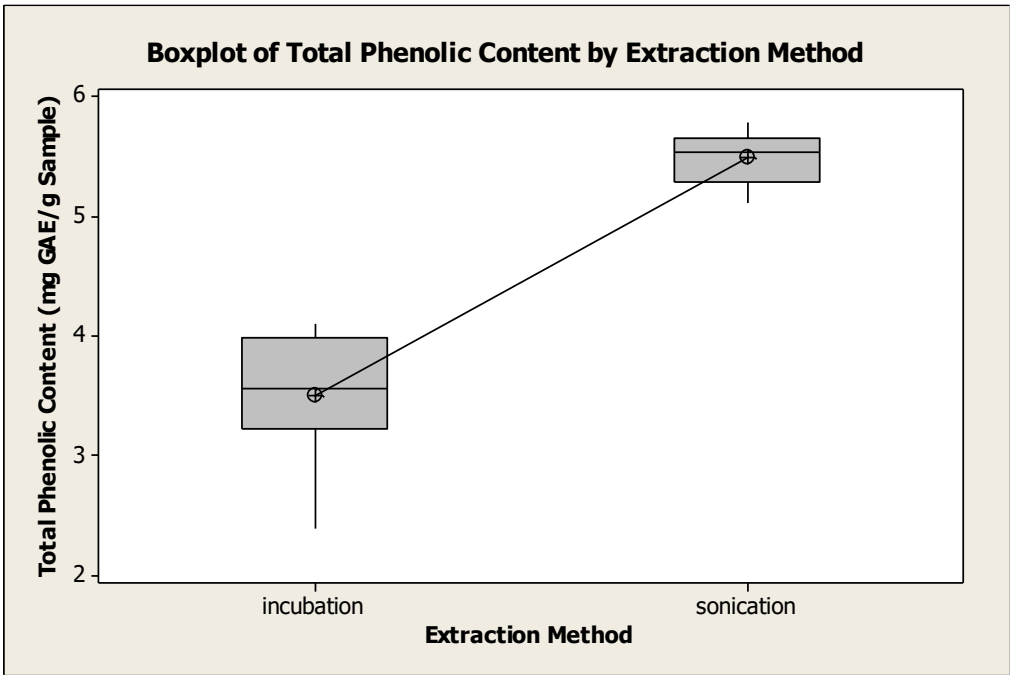

Figure 4. Boxplot of total phenolic content by different extraction method

CONCLUSION

This study shows that the total polyphenol levels of Malaysia Cocoa Fresh Bean extract are significantly influenced by the extraction method. Thus for the MCFB the Ultrasound-Assisted Extraction are recommended for the polyphenols extraction.

ACKNOWLEDGEMENTS

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PHYSICAL PROPERTIES OF REDUCED CALORIE DARK CHOCOLATE

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ABSTRACT: *Reduced calorie dark chocolates were produced by incorporating cocoa butter emulsion (CBE) into chocolate formulations. Cocoa butter emulsions were prepared using 70 % water, 28% cocoa butter and 2 % emulsifier. Addition of 20 % (A) and 15 % (B) of CBE into chocolate formulations with some modification of recipes produced reduced calorie chocolates with similar quality of original dark chocolate. These chocolates were analyzed for melting property using differential scanning calorimeter (DSC), solid fat content (SFC) using a pulsed-nuclear magnetic resonance (p-NMR) spectrometer, sensory evaluation using trained panelists and proximate analyses. Proximate analyses showed that moisture content was increased from 1.1 % (control) to 1.4 % (A) and 1.5 % (B). Fat content was slightly reduced from 36 % (control) to 33 % (A) and 34 % (B). There were no significant ($p>0.05$) changes in melting temperatures and SFC of the chocolates. Minimum changes in end set temperatures did not affect melting properties of the products. Sensory attributes of both samples showed no significant different ($p>0.05$) except for smoothness where samples A and B were significantly less smooth than control. Particle size of both samples of reduced calorie dark chocolates was higher than control. Both samples were accepted by consumers. The energy values of samples were 435 kcal/100g (A), 445kcal/100g (B) and 571 kcal/100g (control). Energy value was reduced by 24 and 22 % in each sample respectively.*

Key words: Chocolate, calorie, physical properties, cocoa butter emulsion.

INTRODUCTION

Most people are concern of health and weight. If food taken is more than what is needed then we can become overweight, undernourished, and at risk for development of diseases and conditions, such as arthritis, diabetes and heart disease. Therefore, what we eat is centre to our health. Energy density is the number of calories (energy) in a given amount (volume) of food. For weight control, best bets are healthy foods that provide low energy with high volume. By choosing foods that are low in calories, but high in volume, we can eat more and feel fuller on fewer calories. However, consuming less in quantity with best at quality is preferred by most people.

Reduced calorie chocolate is suitable for weight reduction and maintenance, and allowing us to enjoy eating chocolate with fewer calories but with the same unique characteristics of original chocolates. Reduced calorie chocolate can be produced with high fibre, with sugar substitutes such as isomalt, sorbitol, xylitol, sorbitol, mannitol, maltitol, erythritol and other type of polyols. These polyols have varying degree of sweetness, calorie content, laxation threshold, solubility, melting point and hygroscopic property. Polyols' can be applied in chocolate recipes to replace sugar (sucrose). Calorie can also be reduced by incorporation of cocoa butter emulsion with polyols in chocolate formulations. Application of polyols and emulsion require adjustment to the processing procedure and control of parameter such as temperature, fat content and emulsifier.

Chocolate is made of a suspension of cocoa liquor, milk solid, cocoa butter and sugar in a continuous fat phase. Fat in the chocolate determines the eating characteristics of the chocolate such as melting behavior, flavor release, heat resistance, consistency and solid fat content.

Emulsions are dispersed, multiphase systems consisting of at least two insoluble liquids. The dispersed phase is present in the form of droplets in a continuous phase. The *water in oil* emulsion exists in the suspending aqueous medium as oil globules containing smaller water droplets. Rotor–stator systems and high pressure homogenizer is commonly used for producing emulsion (Schubert & Armbruster, 1992).

The objectives of this study were to formulate reduced calorie dark chocolates containing cocoa butter emulsion and to compare their qualities with original dark chocolate. Formulation of chocolate was adopted from Beckett (1994) with some modification. Addition of CBE into chocolate formulation could alter the physical properties, snap ability, sensory and acceptability of the product. The newly developed products were tested for acceptability and storage stability. This paper will present the physical properties of the products.

MATERIALS AND METHODS

Materials

Cocoa butter and cocoa liquor were obtained from local grinder, Barry Callebaut Malaysia Sdn. Bhd., Klang, Selangor. Emulsifier, sugar, milk powder and vanilla were purchased from local shop in the vicinity. All chemicals used were food grades and purchased from local chemical suppliers.

Preparation of cocoa butter emulsion

Cocoa butter emulsion (70 % water, 28% cocoa butter and 2 % emulsifier) was homogenized at 10,000 rpm for 2 minute using a high shear homogenizer. These emulsions were kept frozen (-4 °C) for chocolate production.

Production of reduce calorie dark chocolate

A portion of cocoa butter was mixed with cocoa liquor, refined sugar, and milk powder in a lab scale (1 kg capacity) mortar and pestle mill (Pascal, U.K) for 20 minutes at 45ºC to form paste. The paste was refined with a three roll refiner (Pascal, U.K) to get a particle size of less than 35µm measured by a micro screw meter (Mitutoyo, Japan). The mass was then transferred back into the mortar and pestle mill with the remaining portion of the fats blend and emulsion for a conching process of 6 hours at 55ºC. Two hours before the end of conching process, lecithin and vanillin were added. The portion of emulsion was added at the final stage of conching process. Finally, the process was followed by manual tempering on a marble slab at 28ºC to 29ºC and transferred into chocolate moulds and cooled to set at 16ºC for 40 minutes. The chocolates were stored at 16±2ºC in a chiller before further analyses. Storage study was also done to evaluate the stability of the product at different temperature and condition for about one year.

Moisture content analyses

The moisture content of emulsions and chocolate samples was determined using HR73 Halogen moisture analyzer (Metler Toledo, Malaysia). Chocolate was cut into small pieces and then approximately 5mg of sample was placed on the pan and heated to 105ºC until constant weight was achieved. Measurement was determined in triplicate and the mean value calculated.

Fat content analyses

The fat content of chocolate was determined using the Soxhlet procedure. Approximately 5mg melted chocolate sample was subjected to acid digestion and a 4 hour Soxhlet reflux in petroleum ether of boiling range 40-60ºC. Weight of fat extracted was recorded and percentage of fat was calculated. Analysis was carried out in triplicate.

Crude protein

Crude protein was determined according to IOCCC (1999). The chocolate was defatted and the casein was extracted from the fat free residue, using sodium oxalate. The casein was then precipitated with tannin and nitrogen in the precipitate determined by the kjeldhal method. The crude protein was calculated from nitrogen value.

Carbohydrate and energy value

Carbohydrate was calculated by subtracting the sum of the moisture, crude protein, fat and ash from 100%. Factor of 4, 9 and 2 were used for calculating energy from crude protein, fat and carbohydrate.

Ash content

Ash content was done according to AOAC, 13.005(1984), approximately 5g of samples was heated at 550 to 600°C in a muffle furnace. Heating procedures was done for 2 hours and moistens cooled ash with alcohol and then dried on sand bath. Then further re ash at 600°C overnight. Different in weight before and after ash was calculated as the concentration of ash present in sample. The ash content was expressed in percentage on dry basis.

Melting profiles

Melting profile was measured using a differential scanning calorimeter 8000, Serial number: 534N2080801. The method used was based on Md Ali and Dimick (1994). The samples were melted at 50ºC in a ventilated oven. Approximately 3 to 5mg of sample was hermetically sealed in an aluminium pan. The sample was heated at 60ºC for 30 min. and cooled at 0ºC for 90 min. It was then transferred to an incubator at 26ºC for 40 hrs for stabilization. The sample was cooled again at 0ºC for 90 min before it was transfer to a DSC chamber and held at -25ºC for 5 min on the DSC head. The melting profile of the fat was measured at a heating rate of 20ºC/min from -25ºC to a maximum of 50ºC.

Solid fat content

Solid fat content was determined using a pulsed-Nuclear Magnetic Resonance (p-NMR) spectrometer, Newport analyzer Mark 3 (Newport Parnell, England). Method used was as described by Nilsson (1986). NMR tubes were filled 3-4cm with samples and melted at 80ºC and then held for 60ºC for 25 min. It was then cooled at 0ºC for 90min. The samples were stabilized for 35 minutes at each measuring temperature of 10ºC, 20ºC, 25ºC, 27.5ºC, 30ºC, 32.5ºC, 35ºC and 40ºC prior to measurement of SFC.

Particle size

The particle size of chocolate was measured using Malvern particle size analyzer (Mastersizer Micro Version 2.19, Malvern, UK), which applied the laser diffraction particle size analysis that based on the phenomenon that all particles scatter light at a range of angles, which is a characteristic of their size. The sample was taken in triplicate and the mean values were calculated.

Sensory evaluation

Flavour profile of CBE chocolates were measured by quantitative descriptive analysis (QDA) with scale of zero (low intensity) to ten (higher intensity). The attributes tested were texture (hardness), glossiness, odour and smoothness, melting behaviour (rapidness of melt in the mouth), melting time (time taken of complete melt), and overall acceptance. Method of sensory evaluation was adopted from Abdullah (2000). Fifteen experienced panellists were used in this sensory evaluation. Sensory evaluations were done in three cycles coded with three digit numbers.

Statistical analyses

Factorial design was employed for CBE production and chocolates formulations. All experiments were conducted in triplicates. Analysis of variance (ANOVA) was done using MINITAB version 14 software.

RESULTS AND DISCUSSION

Table 1 illustrates the major constituents of dark chocolates. Reduce calorie dark chocolates sample A had 1.5% moisture content followed with sample B with 1.4% moisture content compared to control of 1.1%. Chocolate typically has moisture contents of 0.5 to 1.5%, and mainly in cocoa solids, that does not affect chocolate flow or viscosity. Fat content in A was about 32.9 g/100g followed by sample B (34.3%) and control 36.3%. The calorie in sample A was 435 kcal/100 g, B is 445 kcal/100 g and control 571.5 kcal/100 g. The total calorie reduced was about 22 to 24 % respectively.

Table 1. Nutritional properties of dark chocolate

Samples	A	B	C
Moisture(g/100g)	1.5	1.4	1.1
Ash(g/100g)	1.4	1.4	1.4
Crude Protein(g/100g)	5.3	5.4	5.2
Fat(g/100g)	32.9	34.3	36.3
Carbohydrate(g/100g)	58.9	57.5	56.0
Energy(kcal/100g)	435.1	445.3	571.5

Table 2 shows the melting properties of dark chocolate. There were no significant ($p>0.05$) major changes in onset temperature, peak temperature and enthalpy detected in all samples. The end set temperature was slightly higher in samples B and A compared to control. This change did not affecting the melting properties of the chocolates.

Table 2. Melting properties of dark chocolate

Samples	Onset temperature ± Std	Peak temperature ± Std	End set temperature ± Std	Enthalpy ± Std
A	29.02 [a] ± 0.23	33.64 [a] ± 0.16	39.37 [b] ± 0.62	36.75 [a] ± 0.26
B	29.22 [a] ± 0.06	35.21 [a] ± 0.20	41.21 [b] ± 0.37	34.18 [a] ± 0.07
C	29.84 [a] ± 0.12	34.25 [a] ± 0.00	36.86 [a] ± 0.36	32.79 [a] ± 1.93

Solid fat content of the samples of dark chocolate (A and B) and control is given in *Figure 1*. The melting characteristic as indicated by the SFC with temperature is the most important properties of chocolates as it determines the eating characteristic such as melting behaviour, heat resistance, flavour release and consistency. Result shows that there was no significant different among samples and control in term of the unique melting properties where the sharp drop in SFC from 27°C to 33°C in all samples exhibit similar pattern. These scenarios give cooling sensation in the mouth while the flavour is released.

Molten chocolate is a suspension of particles of sugar, cocoa and milk solids in a continuous fat phase (Beckett, 1994). Due to the presence of these solid particles in the melted state, the SFC reading in the chocolate did not reach zero (*Figure 1*). Percentage of Solid fat content at final stage between temperatures 35 °C to 45° C in sample B was slightly more than A and control. However, the plus minus 5% reduction in SFC at this stage did not give significant effect to the products. Therefore, as observed the addition of CBE in the chocolate formulations did not affect the melting properties and solid fat content of the reduced calorie dark chocolates

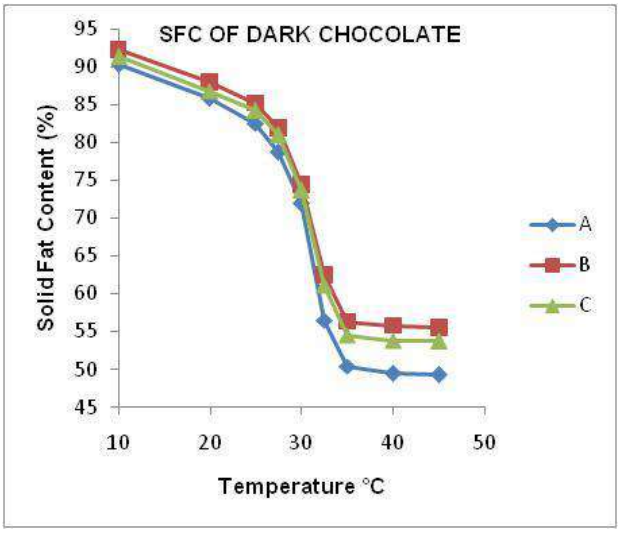

Figure 1. Solid fat content of dark chocolate

Addition of CBE into dark chocolate formulation increases the particle size of the chocolates component. Particle size of samples A was greater than B and control (*Figure 2*). However, all samples gave particle size from 20 to 30 micrometer. Previous literature revealed that optimum particle size for dark chocolate was lower at less than 35 micrometer was influenced by type of product and composition (Awua, 2002). Process of refining can reduce particle size and agglomerate breakdown and also distribute particles through the continuous phase coating each with lipid (Afoakwa *et. al*., 2007). Type of sugar used in making chocolates such as sugar substitute i.e., maltitol, isomalt, sorbitol, xylitol, sorbitol, mannitol, erythritol and other type of polyols can also produce grittiness in the product.

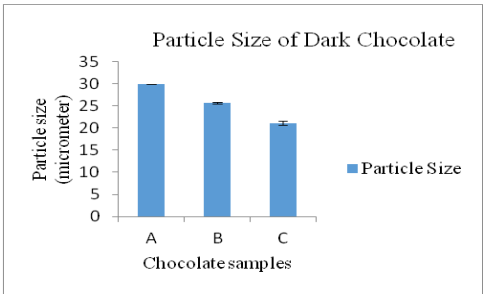

Figure 2. Particle size of reduced calorie chocolates

Chocolate has few major distinguishing characteristic such as its cocoa flavour, melting behaviour, sensation and cooling in mouth characteristic, texture and glossiness. The flavour profile of reduced calorie dark chocolates were measured by quantitative descriptive (QDA) method with scale of zero to ten, that describes each attributes of the chocolate tested. From zero, the lowest degree or intensity to 10 points, this is the highest. Fifteen experienced panellists had evaluated the samples. The flavour profile of the chocolates is as illustrated in *Figure 3*. The results indicated that no significant different ($p>0.05$) among all the attributes evaluated by the panellists except the smoothness of the samples. Samples with CBE contribute grittiness to texture of chocolates; this might be due to increment of moisture content. Greater moisture aggregates sugar particle to form gritty lumps and moisture at sugar particle surface increases friction and apparent viscosity (Afoakwa *et. al*., 2007). Beside the moisture content increment due to addition of CBE, the types of sugar used must be taken into consideration when discussing the sensorial properties and rheology of chocolates. Types of sugar can contribute different degree of sweetness and texture properties which affect the rheological property of chocolates. The viscosity and flow property of chocolate influence sensory properties. Larger particles can cause grittiness in mouth feel but small particle (less than 20 micrometer) could cause creamier taste and smooth texture. Particles size distribution influences chocolate rheology (Chevalley, 1999) and sensorial properties such as smoothness as sample C was smoother than A and B *(Figure 3)*. Viscosity and texture can also be affected by moisture content. Water content at 3-4% increases viscosity and yield value of chocolate markedly (Chevalley, 1999). From the literature it is stated that, to overcome increment of free moisture content that causes grittiness and other problems in chocolate, it is advisable to add an extra 1 % fat for every 0.3% moisture content left within chocolate (Beckett, 2000). Therefore in this study, it is important to add CBE to the optimum level in order to maintain quality beside to reduce calorie of chocolate with acceptable quality compared to original dark chocolate.

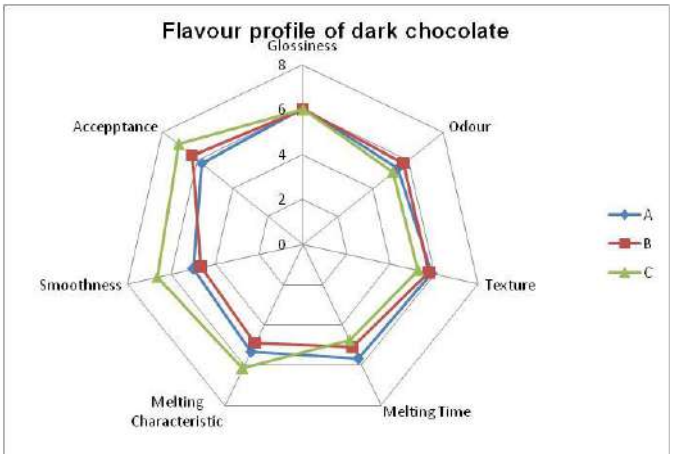

Figure 3. Sensory profiles of dark reduced calorie dark chocolate

Figure 4 indicates the consumer acceptance score of the reduced calorie dark chocolates compared to original dark chocolate. The finding shows that samples B gave higher score than samples A compared to samples C which gave the highest score, however there was no significant different ($p>0.05$) between samples and control. Addition of 20% and 15% of CBE into chocolate formulation did not affect the acceptability score among consumers.

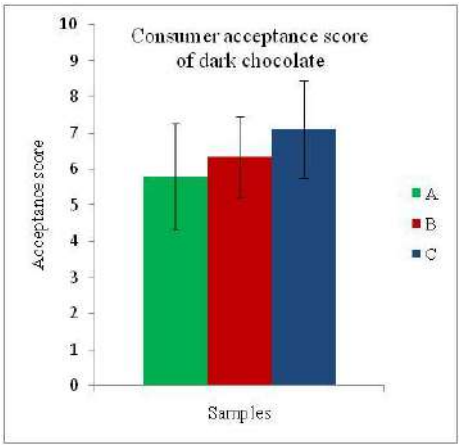

Figure 4. Consumer acceptance score of chocolates

CONCLUSIONS

The physical properties, sensorial behavior and perception of chocolate are influenced by its ingredients composition, particle size and processing techniques. To develop new product with additional values and same quality of original chocolate can be enhanced with additional ingredients, and manipulation to modify physical properties, rheological characteristic, sensorial attributes and storage stability. Application of cocoa butter emulsion was found to be one of the most effective methods in reducing calorie of dark chocolate. Incorporating 20 % of cocoa butter emulsion into dark chocolate formulation with some modification of the recipes could reduce calorie up to 24 % of total calorie compared to original dark chocolate. The quality of the reduced calorie dark chocolate was almost similar to original dark chocolate. Both sample of reduced calorie chocolate were accepted by consumers regardless of the amount of CBE addition.

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FORMULATION OF MASCARA FOR EYELASHES WITH COCOA BUTTER AND COCOA POWDER

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Abstract– *Mascara is one of color cosmetic products that being used in complimentary with make-up product for eyes such as eye shadow and eye liner. The application of mascara on human faces involves in-contact with eye area which is susceptible to be irritated. In this study, we conducted a study on formulation of mascara using cocoa butter and cocoa powder as the basic ingredient in two phases. In the first phase, cocoa butter at 5-15 weight percentages was used as the basic fat-based in mascara formula. Then, cocoa butter based mascara was improvised using cocoa powder as coloring to replace iron oxide at 10-20 weight percentage. Stability study was conducted to the cocoa butter formula in comparison with the stearic acid based formula. We found out that cocoa butter mascara formula with black cocoa powder has shorter shelf-life compare to similar formula but with brown cocoa powder. It was hoped that the introduction of cocoa materials instead of normally used mascara ingredients can minimize the cosmetic side effects and increase the demand of cocoa.*

Keywords: cocoa powder, cocoa butter, mascara

INTRODUCTION

Global cosmetic and toiletries market was USD 155 billion with 15% was segmented for color cosmetic including eye makeup such as mascara (Jones, 2011) and increasing every year. Therefore, development on natural base mascara is encouraging. Almost 75% of total sales of mascara in US and Europe are the water-resistant (Cunningham, 1992) or anhydrous types. Mascara is applied onto eyelashes to enhance a normally light region of face (Umbach, 1991; Poucher, 1979). Eyelashes looked thicker, longer (Cunningham, 1992) and darker (Poucher, 1979) with application of mascara. Two types of mascara are anhydrous and emulsion types. Anhydrous mascara is made by dispersing the pigment into a heated oil and wax followed by mixing and agitation. The other type of mascara is emulsion where the pigment is dispersed in oil/water or water /oil based. Regardless the category, good properties of a mascara are good eyelashes covering, not clumping, not flake during wear, not brittle after drying, tear-resistant, not smear, not smudge and waterproof or water-resistant (Cunningham, 1992). A nylon or rayon fiber is added into mascara formula to make the eyelashes appear longer and thicker (Poucher, 1979).

Eyelashes are different between ethnic groups, where Asian has lower lift-up and curl-up angles of eyelashes with fewer numbers and a thicker transverse diameter (Na, 2006). Human hair keratines has most similar negative charges to eyelashes (Ko, 2009) with pH 3-7. Iron oxide has negative charges surface which reduce the adhesion effect to the eyelashes, thus coating the coloring pigment with positive charges material such as cationic polymer can invert the adhesion effect and neutralized the pH. Iron oxide is a pigment that commonly used to enhance the eyelashes color making the eyes look bigger and wider. For mascara, the pigment size should be within 20 μm (Poucher, 1979). Natural carbon black has been used as pigment agent in early ages. Carbon black is made of charcoal of animal and plants origin. Treated black ink of *Sepia officinalis* improved coloring and effectiveness of formulated mascara containing iron oxide and black bone dyes as pigment (Neifar, 2013).

In this research, alkalized cocoa powder is used to substitute iron oxide to give color in mascara. There are two types of cocoa powder which are natural and alkalized cocoa powder. Natural cocoa powder has lower pH (pH 5.1-5.4) compare to alkalized cocoa powder (pH 6.8-8.1). Alkalized cocoa powder has undergone a process

called alkalization process. After alkalization, cocoa powder is more soluble in water. Its color can vary from brownish red to black. Cocoa butter is added to promote healthy eyelashes due to presence of 200 ppm natural vitamin E. Ratio of cocoa butter to other waxes must be balance to avoid brittle and smudging (Poucher, 1979). Usage of cocoa materials in mascara is anticipated the increase in demand of cocoa product, thus increase the income of cocoa growers. In the end of this study, it is aimed to gain a mascara formula containing natural ingredients mainly from cocoa materials.

MATERIALS AND METHODS

Mascara formula

In this study the emulsion technique was selected for the mascara formula that can easily to be removed but can enlightened the eyelashes. In the first stage, cocoa butter at 5-10 weight percentages was used to substitute the paraffin wax in the formula. To compare the physical properties, control mascara containing stearic acid at same percentage of cocoa butter was developed. At this stage, the black iron oxide (at 10%) was used as the coloring pigment in all of the formula. The factors were waxes (cocoa butter and stearic acid), weight of the wax (5, 10 and 15%), in triplicates. Other ingredients (in weight descending order) were deionized water, iron oxide, silicon oil, polivynilpyrrolidone, carnauba wax, triethanolamine, methyl paraben and xanthan gum.

To make the mascara, the water phase and oil phase were heated to 75ºC in separate beaker. Then, oil phase was poured into the water phase, followed by homogenization for 30 minutes at high speed. After homogenization was completed, the pigment agent was added and mixed until well-dispersed. For fine dispersed mascara, the mixture was refined using three-roller mill *(Figure 1)* at 150 rpm (gap 1: 30 μm and gap 2:15 μm) for 5 times. The physical properties of the formulas developed were measured by the color (L-index), pH, viscosity, yield stress and particle size (D[4,3]).

Figure 1. Three Roller Mill

In the second stage of formulation, iron oxide in cocoa butter mascara was replaced with cocoa powder. Two color shades of cocoa powder (black; DF 800-11B, and brown; JB 800-1, JB Cocoa Sdn. Bhd.) were used in the formula at 10, 15 and 20%. As comparison, stearic acid mascaras with black and brown iron oxide pigment (at 10%) were developed. Similar formulation procedure was followed in making the mascara. The physical properties of the formulas developed at this stage were measured by the color (L- and a- indexes), pH and particle size (D[4,3]). Then, the mascara was subjected to stability test for three months by measuring the color, pH and microbiological properties. Before being test onto human, the ocular irritation of the mascaras were determined following the Bovine Corneal Opacity and Permeability (BCOP) Test method.

Particle size – The particle size of the product was measured using Mastersizer (Malvern Instruments Ltd, USA). About 1 mL of sample was dissolved in water and mixed thoroughly. The diluted sample was inserted into sample compartment and the particle size was measured accordingly.

Viscosity and yield stress - Shear stress and shear rate of the sample was determined using rheometer (AR2000ex, TA Instruments) and recorded for 30 min at 30ºC. Cone plate with 40 mm diameter and 1.0.7 degree angle was used to measure the parameters. Data was fitted into Herschel-Bulkley model to obtain the yield stress of product. According to Islam (2004), yield stress of a product should be in the range of 28-188 Pa so that only 80% of the pressure is required to start the product flow.

pH Test - The analysis was carried out with pH meter. One gram of sample was diluted with 9 mL of distilled water before measurement. Reading was recorded up to 2 decimal points.

Color test - Color uniformity of the product was evaluated using Spectrophotometer CM-5 (Konica Minolta). The L- and a- indexes of the product was recorded to check the color difference between the samples.

Total plate count - Total plate count (TPC) method was carried out to ensure the product is safe from microbial contamination. Cosmetic product that is applied on the eye area must free from microbial contamination or having less than 10 cfu/g of bacterial colony count (ASEAN Guidelines on Microbiological Limit in Cosmetic Products; 2007). The counting, calculation and reporting the amount of microorganisms were based on the Annex B Guidelines (ASEAN Cosmetic Directive, 2007). In this test, the samples was diluted in nutrient broth at ratio of 1:10 and consequently diluted in serial dilution of 1:10 for at least 3 series. Then, 1 mL of product solution from each series was incubated at 37ºC in nutrient agar form for 3 days. Counting of colony was carried out. In addition, presence of mould and fungi was carried out similarly, using soy broth and agar.

To determine the expiry date of the product; the TPC was carried out in conjunction with stability study. Upon storage stability study, TPC was carried out at interval a month for three months.

Statistical analysis

The results were presented as mean $\pm$ standard deviation and were determined for triplicates of three independent samples. Minitab Software version 14.12.0 (State College, Pennsylvania, USA) was used to carry out the significant test. Linear regression to determine the slope of pH and color index for the samples were accepted when the value of R-squared (R^2) was higher than 0.8 and were plotted using Microsoft Excel 7.0. The results of particle size, yield stress, viscosity, color indexes, pH, opacity and permeability values were compared using ANOVA and significantly different when p-value $\leq$0.05.

RESULTS AND DISCUSSIONS

In the initial stage of the mascara formulation, cocoa butter was used to replace certain amount of waxes or fat. The functions of waxes in mascara are to provide thickness and glossy finishing to the formula (

Table **1**). The waxes also serve as emollient agent to the eyelashes. Table 2 shows the results of the physical properties of the mascara developed using cocoa butter in the oil phase in comparison with stearic acid. The particle size influenced by the type and the amount of fat in the formula significantly, ($p<0.05$). In addition, increased in the amount of cocoa butter from 5%

to 10%, increased the particle size significantly, although slight reduction was seen in higher amount of cocoa butter (15%). Stearic acid based mascara had significantly lower D[4,3] value compared to cocoa butter based mascara. No significant changes in particles size reduction was observed with the increasing amount of stearic acid in mascara formula.

Table 1. Basic formula of mascara

Phase	Ingredients	Weight percentage (wt %)	Function
Water	Water	50.0-60.0	Diluent
	Triethanolamine	1.3-1.6	Emulsifier
	Polivynilpyrrolidone	4.5-5.5	Film forming
	Xanthan Gum	0.1-0.2	Thickening
Oil	Stearic Acid/Cocoa butter	5.0-15.0	Emollient
	Carnauba Wax	2.0-4.0	Thickening and glossy finishing
	Methyl paraben	0.5-0.8	Preservative
	Iron Oxide/Cocoa powder (Black/Brown)	10.0-20.0	Pigment agent

Silicon Oil		6.0-8.0 Smooth feeling product

For the color parameters, L-index was influenced significantly by the amount and type of fat (Table 2). L-index of near to 0 indicated of an intense black color. The L-index showed that the lowest amount of cocoa butter in mascara formula has more intense black color significantly than higher amount of cocoa butter or stearic acid based-mascara. For both type of mascara, the increasing the amount of fats/waxes reduced the intensity of the black color significantly.

Table 2. Physical properties of cocoa-based and stearic acid-based mascara

BASED	weight percentage, wt %	PARTICLE SIZE, D[4,3] µm	COLOR, L-index	RHEOLOGY *yield stress*	RHEOLOGY *viscosity*	pH
Cocoa butter	*5*	75.20 $\pm$9.34^{c}	14.12 $\pm$0.47^{a}	15.52 $\pm$3.70^{a}	2.89 $\pm$0.69^{a}	8.00 $\pm$0.00^{a}
	10	234.50 $\pm$43.49^{a}	16.61 $\pm$0.52^{b}	49.37 $\pm$1.30^{b}	0.93 $\pm$0.16^{b}	7.90 $\pm$0.00^{a}
	15	145.08 $\pm$34.65^{b}	17.26 $\pm$0.18^{b}	35.81 $\pm$4.75^{c}	1.66 $\pm$0.24^{c}	7.20 $\pm$0.14^{b}
Stearic acid	*5*	20.78 $\pm$2.06^{d}	17.57 $\pm$0.26^{b}	65.41 $\pm$13.65^{d}	1.14 $\pm$0.24^{bc}	6.77 $\pm$0.16^{c}
	10	35.22 $\pm$2.79^{d}	19.34 $\pm$1.11^{bc}	67.45 $\pm$9.21^{d}	1.55 $\pm$0.32^{c}	7.12 $\pm$0.15^{b}
	15	24.67 $\pm$5.43^{d}	21.57 $\pm$2.73^{c}	72.51 $\pm$9.42^{d}	0.83 $\pm$0.27^{b}	7.32 $\pm$0.19^{b}

Results were presented by mean of three independent samples in triplicates, a b c denotes significant different by ANOVA
Cocoa based mascara contains cocoa butter as fat-based.
D[4,3] is volume moment mean (De Bruckere mean diameter) which reflects the size of those particle which constitute the bulk of the sample volume. Particle size results were obtained using Mie model.
L is index value for white (value = 100) to black (value = 0) color.
Unit for yield stress is Pa and viscosity is Pa.s. Rheological results were obtained using Herschel-Bulkley model.

The rheological results showed that only the types of fats/waxes influenced the parameter (Table 2). Cocoa butter based mascara has lower yield stress compared to stearic acid. Lower value of yield stress indicated that cocoa butter mascara had better flowing properties during filling process compared to stearic acid formula. The viscosity value of medium amount of cocoa butter (10%) mascara formula was the lowest similar to the highest amount of stearic acid (15%) formula. Lower viscosity indicated that cocoa butter based-mascara at 10% had better spreading properties compared to other formula.

The pH values of the cocoa butter based mascara developed were slightly higher than neutral pH, specifically at lower percentage. However, the pH was almost neutral when higher amount of cocoa butter added. The cocoa butter has acidic pH, where at higher amount can neutralized the alkalized pH of iron oxide. On the other hand, the stearic acid based-mascara has almost neutral pH despite the percentage of stearic acid added. Based on these results, mascaras formula with 5% cocoa butter possessed better properties than higher amount of cocoa butter in the mascara with black iron oxide as the pigment and 5% stearic acid were subjected for the next stage of formulation. Both of the formulas (cocoa-based and stearic acid-based at 5%) have low particle size, low L-index, low yield stress although with high viscosity (cocoa butter formula) and low pH (stearic acid formula). These results were used to formulate cocoa based-mascara which to replace the black iron oxide with black and brown cocoa powder.

In the second stage of formulation, the amount and type of cocoa powder were manipulated to obtain good intensity of brown or black color mascara. The factors were color of cocoa powder (i.e. black and brown) and percentage of cocoa powder (10 %, 15 % and 20 %) in a mascara formula.

The results in
Table **3** showed that with cocoa powder as the pigment agent in mascara formula, the particle size of the product was uniform and lower compared to the earlier formulation in Table 2 (cocoa butter at 5%). Cocoa powder color significantly influence the intensity of black color (L-index) of mascara produced. Higher amount of black cocoa powder gave darker color to the formula. On the other hand, the a-value was significantly influenced by the color of

cocoa powder, where the black color of cocoa powder reduced the a-value. Furthermore, the a-value decreased by the amount of black cocoa powder used. The higher the a-value indicates more redness of the mascara produced. The pH of the mascara produced was affected significantly by the amount of the cocoa powder used, where the higher amount of cocoa powder decreased the pH of the mascara.

Table 3. Physical properties of cocoa powder/butter - based mascara

COCOA POWDER	weight percentage, wt %	PARTICLE SIZE, D[4,3] µm	COLOR		pH
			L- index	a-index	
Black	*10*	33.55 ±7.13	13.55 ±0.46^{a}	2.65 ±0.17^{a}	7.62 ±0.04^{a}
	15	31.65 ±7.62	14.24 ±0.71^{a}	2.27 ±0.18^{b}	7.47 ±0.05^{bc}
	20	28.78 ±6.50	12.37 ±0.56^{b}	2.02 ±0.30^{b}	7.40 ±0.06^{bc}
Brown	*10*	29.27 ±6.23	18.20 ±0.81^{c}	7.50 ±0.62c	7.53 ±0.08^{ab}
	15	24.73 ±7.07	18.00 ±0.93^{c}	6.73 ±0.24c	7.52 ±0.10^{ab}
	20	31.42 ±4.18	18.56 ±0.78^{c}	6.61 ±0.43c	7.42 ±0.04^{bc}

Results were presented by mean of three independent samples in triplicates.
Cocoa powder/butter -based mascara contains cocoa butter as fat-based compare to mascara containing stearic acid and cocoa powder as pigment agent.
D[4,3] is volume moment mean (De Bruckere mean diameter) which reflects the size of those particle which constitute the bulk of the sample volume. Particle size results were obtained using Mie model.
L is index value for white (value = 100) to black (value = 0) color. a index is value for redness (value = +60) and greenness (value = -60).

At the final stage of formulation, the stability of the mascara formula was determined by measuring the physical and microbial properties for the period of three months. The physical properties of a formula was measured by its constant pH and color (L- and a- indexes) values. Stable mascara should maintain low level of microbial count (TPC) of less than 10 cfu/g sample. Four types of mascara were tested in this stability study. The mascaras were codes as the following;

i) Formulated with combination of black or brown cocoa powder (5%) with combination of cocoa butter (5%) - labeled as BLCB and BRCB, respectively.
ii) Formulated with combination of black or brown iron oxide (10%) with combination stearic acid (5%) - labeled as BLSA and BRSA as control.

The microbial counts of these formulas were summarized in Table 4. The results showed that all the formula has passed the microbial limit for mascara except for BLCB which exhibited presence of microbial contamination after two and three months of storage in a room temperature. The *E.coli* was not detected in both of the months as resulted by <0.3 of MPN value. However, yeast and mould were presence with other microbial than E.coli based on the results. The possible contamination was came from the condensed water which was appear after the product was refined and not properly ventilated during cooling stage.

The intensity of color was analysed using linear graph (y=mx+c, Table 5), where the gradient (m) value of near to zero (0) indicate stable value of color intensity index (L-, a- and b- indexes). For BLSA and BLCB (black mascara), the L-indexes were stabled towards the third months indicating no black color changes as the gradient values are near to zero. The L-indexes for these formulas (BLSA and BLCB) in *Figure 2* showed that BLCB is darker or the black color is more intense than BLSA (L-index near to 0). The color of brown mascara (BRSA and BRCB) did not change by time as indicated by the almost constant value of gradient of a-index (0.0009 and 0.0015). Refering to *Figure 3*, BRSA has more intense of red color compare to BRCB.

Table 4. Microbial count of mascara formula

Duration	fat based	pigment	color	TPC (cfu/g sample)			yeast moulds (cfu/g sample)			E.coli (MPN/g)		
				R1	*R2*	*R3*	*R1*	*R2*	*R3*	*R1*	*R2*	*R3*
Initial	***Cocoa butter Stearic acid***	***Cocoa powder Iron oxide***	***Black***	0	0	0	<10	<10	<10	<0.3	<0.3	<0.3
			Brown	0	0	0	<10	<10	<10	<0.3	<0.3	<0.3
			Black	0	0	0	<10	<10	<10	<0.3	<0.3	<0.3
			Brown	0	0	0	<10	<10	<10	<0.3	<0.3	<0.3
Month 1	***Cocoa butter Stearic acid***	***Cocoa powder Iron oxide***	***Black***	0	0	0	<10	<10	<10	<0.3	<0.3	<0.3
			Brown	0	0	0	<10	<10	<10	<0.3	<0.3	<0.3
			Black	0	0	0	<10	<10	<10	<0.3	<0.3	<0.3
			Brown	0	0	0	<10	<10	<10	<0.3	<0.3	<0.3
Month 2	***Cocoa butter Stearic acid***	***Cocoa powder Iron oxide***	***Black***	870	900	850	620	600	600	<0.3	<0.3	<0.3
			Brown	0	0	0	<10	<10	<10	<0.3	<0.3	<0.3
			Black	0	0	0	<10	<10	<10	<0.3	<0.3	<0.3
			Brown	0	0	0	<10	<10	<10	<0.3	<0.3	<0.3
Month 3	***Cocoa butter Stearic acid***	***Cocoa powder Iron oxide***	***Black***	600	580	620	390	400	380	<0.3	<0.3	<0.3
			Brown	0	0	0	<10	<10	<10	<0.3	<0.3	<0.3
			Black	0	0	0	<10	<10	<10	<0.3	<0.3	<0.3
			Brown	0	0	0	<10	<10	<10	<0.3	<0.3	<0.3

TPC is total plate count, cfu is colony form unit, E.coli is *Escherichia coli*, and MPN is most probable number
R1, R2 and R3 are replicates number for each mascara independent sample

Table 5. Gradient value (m) of linear graph

Sample	Gradient (m) value for L- index	a-index	pH
BLSA	-0.0357	0.0006	-0.0629
BRSA	-0.1466	0.0009	-0.0550
BLCB	0.0255	0.0653	-0.0230
BRCB	-0.2336	0.0015	-0.1488

BLSA is black iron oxide mascara with stearic acid; and BRSA is brown iron oxide mascara with stearic acid.
BLCB is black cocoa powder mascara with cocoa butter; and BRCB is brown cocoa powder mascara with cocoa butter.

For the pH of these mascara formulas, the gradient (m) showed value of near to zero (Table 5, *Figure 4*) but high value for BRCB (more than 0.1). The results showed that BRCB has abrupt pH change from the initial measurement from pH8 to pH6 in one week. This pH deviation could be that the ingredients in the formula were adjusting from alkaline to acidic condition when mixed together.

CONCLUSIONS

In overall, based on the results, it showed that these mascara formulas were stabled. These formulas were then to be subjected for product evaluation where the irritation level will be determined *in-vitro* before being tested and applied to human eyelashes at the end of this research study.

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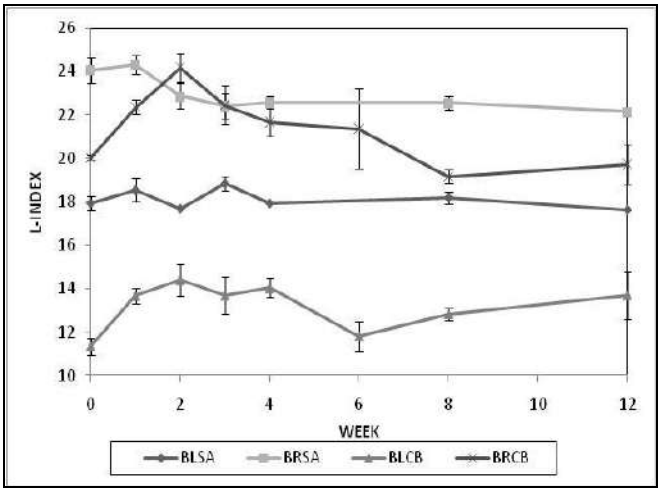

Figure 2. Linear graph for mascara stability study of L-index

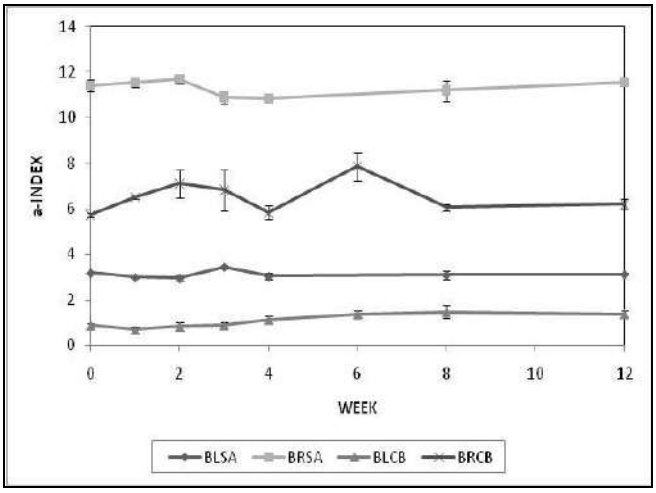

Figure 3. Linear graph for mascara stability study of a-index

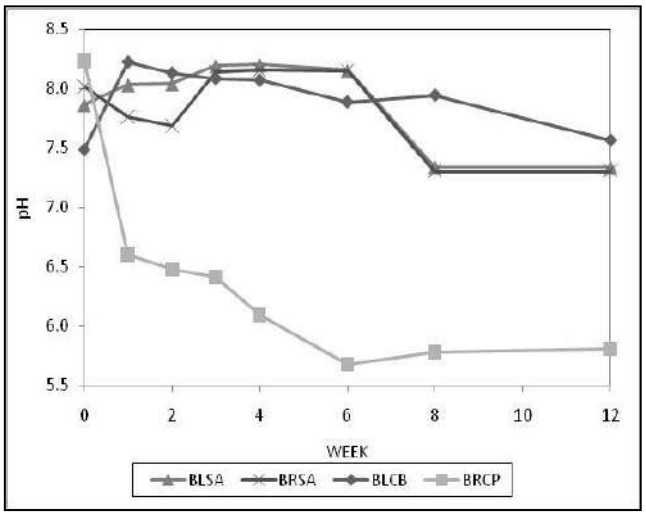

Figure 4. Linear graph for mascara stability study of pH

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MULTIRESIDUE SCREENING OF PESTICIDES IN DRIED COCOA BEANS USING ION MOBILITY QTOF MASS SPECTROMETRY

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ABSTRACT - *Multiresidue screening method for the analysis of pesticide residues in dried cocoa beans was validated and applied to imported and domestic cocoa beans samples. The screening method covers more than 110 pesticides (insecticides, fungicides, herbicides) of different chemical classes. The method was based on modified QuEChERS (Quick Easy Cheap Efficient Rugged Safe) extraction method and detection using ion mobility quadrupole time of flight mass spectrometry (IMS-QTOF). Ion mobility allows for the measurement of the collision cross section (CCS), which provide information about the charge, shape and size of the pesticide compounds. Hence, additional identification tool in the form of CCS value provide more confidence in the identification process and at the same time lessening the probability of false positive during the pesticide screening process. Finally, a database of pesticide residues that consist of accurate mass, retention time, fragment ions, and CCS values was developed for cocoa beans matrix and applied to the pesticide residues monitoring programme*.

Keywords: Cocoa beans, pesticide residues, ion mobility; LC-QTOF-MS; QuEChERS

INTRODUCTION

In the late 1980s and early 1990s, Malaysian cocoa industry grew to become the third major commodity crop in Malaysia after oil palm and rubber (Azhar and Lee 2004). The growth of upstream sector has led to the establishment of the cocoa downstream industry and national grinding volume reached its peak at 323,653 tonnes in 2008 (Malaysian Cocoa Board 2016). In the year 2015/2016, International Cocoa Organization (2016) forecasted the grinding volume to be around 190,000 tonnes. However, contrary to the rapid expanding of cocoa downstream industry, the growth of cocoa planting industry in Malaysia seems to be declining since 1990. This would eventually give a negative view on the prospect for cocoa cultivation. As previously mentioned (Zainudin, Salleh, Mohamed, Yap, and Muhamad 2015), Malaysia had to relied on imported cocoa beans in order to meet the increasing local production-to-grinding gap. In the end, the increasing demand of cocoa beans for local grindings and the declining of local cocoa production has turned Malaysia from previously a net exporter of cocoa beans to be a net importer of cocoa beans since 1997 (Azhar and Lee 2004). Low prices, pests (cocoa pod borer), diseases (cocoa black pod), and labour constraints are often quoted as the reasons for the downtrend (Lee and Azhar 2006; Nuraziawati, Azmi and Haya 2008). Hence, food safety played an important role in cocoa industry since the originality of the agrochemicals used in the exporting countries is unknown.

Pesticide residue analysis had traditionally relied on the use of targeted approaches involving both gas chromatography and liquid chromatography coupled to triple quadrupole mass spectrometry and operated in multiple reaction monitoring (MRM) mode. This type of analysis usually required the optimization of each individual analyte and allows the quantitation of the targeted pesticides at the lowest detection limit. However, by using triple quadrupole instruments, all other pesticides which not previously included in the list will not be detected. These non-detects are essentially false negative results. One possible option to increase the scope of the analysis and decrease the false negative results is the use of high-resolution mass spectrometry (HRMS) instruments to perform non-targeted acquisition across the mass range of interest. Nowadays, HRMS has become a serious alternative to MRM methods, mainly due to the recent popularization of time-of-flight (TOF) and Orbitrap analyzers (Rajski *et al*, 2014; Garcia-

Reyes *et al.,* 2007; Mol *et al.,* 2012; Wang *et al.,* 2015; Zomer *et al.,* 2015).

Implementation of qualitative screening methods in routine analysis requires the validation of the whole analytical process from analyte extraction to data processing and the determination of screening detection limit of each individual pesticides (Mol *et al.,* 2012; Mol *et al.,* 2011). The screening detection limit (SDL) is the lowest concentration for which it has been demonstrated that a certain analyte can be detected (not necessarily meeting identification criteria) in at least 95% of the samples (false negative rate of 5% is acceptable). In order to achieve this 95% detection rate to be available to be qualified as SDL, certain identification criteria had been studied previously such as mass accuracy, isotopic pattern, fragmentation pattern, and retention time (RT) deviation (Garcia-Lopez *et al.,* 2014; Mol *et al.,* 2012). Recently, new identification criteria in the form of drift time and collision cross section (CCS) had been introduced for pesticide residue screening (Goscinny *et al.,* 2015; Stephan *et al.,* 2016; Regueiro *et al.,* 2016; Regueiro *et al.,* 2017). CCS is a physicochemical property derived from the measured drift time and has been shown to be robust across multiple platform and matrices. Using CCS as an orthogonal molecular descriptor in addition to retention time and *m/z* offers the opportunity to further improve the identification process, increasing selectivity, reproducibility and robustness (Regueiro *et al.,* 2016).

In the present work, we investigate the capabilities of ultra-performance liquid chromatography ion mobility quadrupole time-of-flight mass spectrometry (UPLC-IMS-QTOF-MS) for the screening of pesticide residues in cocoa beans. Then, a database of CCS values for more than 110 pesticides in cocoa beans matrix was developed and the potential of CCS values for the studied pesticides was discussed. Finally, the screening method was applied to real cocoa beans samples via a monitoring study to test the applicability of the screening approach.

MATERIALS AND METHODS

Reagent and materials

HPLC grade acetonitrile, anhydrous magnesium sulphate ($MgSO_4$) and sodium chloride (NaCl) were all obtained from Merck (Darmstadt, Germany). Optima grade water and methanol were obtained from Fisher-Scientific (Waltham, USA). Two mL mini-centrifuge tube containing 150 mg $MgSO_4$, 50 mg C18, and 50 mg primary secondary amine (PSA) was purchased from Agilent Technologies (Palo Alto, USA).

Pesticide reference standards of all analytes were purchased from Waters (Manchester, UK). Individual pesticide stock solutions (~100 μg mL^{-1}) were initially in acetonitrile and kept at -20 °C in the dark. Mixed intermediate standard solutions (1 μg mL^{-1}) of multiple pesticides were prepared by diluting an appropriate volume of each individual stock standard solution in acetonitrile. All working solutions containing the target pesticides were prepared freshly by dilutions of the intermediate standard solution in acetonitrile and kept in scintillation vials at 4 °C in the refrigerator.

Cocoa beans samples for fortification

Dried cocoa beans were obtained from Cocoa Research and Development Centre, Jengka. The samples were used for blanks, fortified samples for screening assays and matrix-matched standards for calibration in the experiments. The samples were also previously tested for the absence of pesticide residues. The whole laboratory samples were ground using Retsch ZM 200 ultra-centrifugal mill (Haan, Germany) so that the greatest dimension of the particles does not exceed 1 mm, while avoiding the formation of paste. Subsequently, representative portions of previously homogenised samples were weighed and transferred into 50 mL screw cap centrifuge tubes and fortified with intermediate standard solution. The samples were then allowed to stand at room temperature for 1 hour until analysis to give final spiking concentration levels of 10 μg/kg, 50 μg/kg and 150 μg/kg.

Extraction and clean-up procedure

The samples were extracted according to the original unbuffered QuEChERS method (Anastassiades *et al.,* 2003). After homogenization, samples were weighed in a 50

mL screw cap centrifuge tubes and fortified with intermediate standard solution to give final spiking concentration of 10, 50 and 150 µg/kg. Deionised water was added and the mixtures were homogenised using a vortex mixer for 30 seconds and left to stand at room temperature for matrix swelling. Then, 10 mL of acetonitrile was added to the samples. The tubes were shaken using SPEX SamplePrep 1500 ShaQer (New Jersey, USA) for 1 min. After that, 4 g $MgSO_4$ and 1 g NaCl were added and the mixtures were immediately shaken using 1500 ShaQer for 1 min, then centrifuged at 12000 rpm for 5 min at 4 °C. Hereafter, 1 mL of the supernatant was transferred into d-SPE tube. The tube was vortexed for 30 s. After centrifugation at 12000 rpm for 5 min, an aliquot of 0.5 mL extract was diluted with 0.5 mL deionized water and filtered through 0.2 µm PVDF filter into autosampler vial.

UPLC-IMS-QTOF-MS

UPLC was performed on ACQUITY UPLC I-Class system from Waters (Manchester, UK), consisting of binary pump, a vacuum degasser, an autosampler and a column oven. Separation was done using an ACQUITY UPLC BEH C18 (100 x 2.1 mm, 1.7 µm) from Waters at 45 °C. A linear binary gradient of water (mobile phase A) and methanol (mobile phase B), both containing ammonium acetate (5 mM, pH 5.0), was used. The mobile phase composition was changed during the run as follows: 0 min, 2% B; 0.25 min, 2% B; 12.25 min, 99% B; 18.00 min, 99% B; 19.01 min, 2% B; 22.00 min, 2% B. The flow rate was set to 0.45 mL/min and the injection volume was 10 µL.

The UPLC system was coupled to a Vion IMS QTOF mass spectrometer from Waters (Manchester, UK). The ion source was operated in positive electrospray ionization (ESI) mode under the following specific condition: capillary voltage, 0.45 kV; reference capillary voltage, 3.00 kV; cone voltage, 40 V; source offset, 80 V; source temperature, 120 °C; desolvation gas temperature, 550 °C; desolvation gas flow, 1000 L/h; cone gas flow, 50 L/h. Nitrogen (>99.5%) was employed as desolvation and cone gas.

Data were acquired in high-definition MS^E ($HDMS^E$) mode in the range *m/z* 50 – 1200 at 0.25 s scan time. Two independent scans with different collision energies (CE) were alternatively acquired during the run: a low energy (LE) scan at a fixed CE of 4 eV, and a high energy (HE) scan where the CE was ramped from 10 – 45 eV. Argon (≥99.999%) was used as collision-induced-dissociation (CID) gas.

Data were evaluated with a UNIFI software and in-house database containing information on molecular formulae, retention time, accurate *m/z*, CCS values and fragment ions was established using pesticide standards in cocoa beans matrix.

Method validation

Method validation was performed following the recommendation of the EU guidance document SANTE/11945/2015 (SANTE, 2016). Cocoa beans samples were fortified with pesticide residues at three concentration levels; 10 µg/kg, 50 µg/kg, and 150 µg/kg representing the low, medium and high residue levels. At each concentration level, 20 replicates were done.

RESULTS AND DISCUSSION

Optimization of screening filtering criteria

In this study, cocoa beans samples were spiked at three concentration levels to represent the low, medium and high residue levels. For this purpose, ten cocoa beans samples were spiked with a mixture of 110 pesticides at three concentration levels (10 µg/kg, 50 µg/kg, and 150 µg/kg). These spiked samples were analysed in duplicate to yield a total of 20 cocoa beans extract at each concentration levels. In the meantime, non-spiked samples (blank) will be used to assess the occurrence of false positive.

The resulting data were processed using a UNIFI software with broad filtering criteria (mass accuracy, ± 10 ppm; RT error, ± 0.3 min; CCS error, ± 10%). Then, stricter filtering criteria were assessed in order to find the best filter which gave the highest detection rate with the lowest false positive result (Table 1). When applying tolerances of ± 0.2 min and ± 5 ppm for RT and mass accuracy, respectively, the detection rate ranged from 47% at 10 µg/kg to 91 % at 150 µg/kg, with 5 false positive obtained across the blank sample. When the maximum tolerances for RT was decreased to ± 0.1 min,

the recorded false positives decreased to 3 pesticides. Hence, additional parameters and criteria need to be incorporated into the automated screening workflow. The use of isotopic pattern match and fragmentation information have been proven to be effective ways of reducing false positive detections (Lopez *et al.*, 2014; Mol *et al.*, 2012). As can be observed in Table 1, by applying an additional filter based on the expected isotopic pattern and fragment ion of the pesticides, the total number of false positives decreased to zero. However, under these processing conditions, the detection rate registered a serious drop, especially at the lowest concentration where only 26% and 14% of the total pesticides could be detected for isotopic pattern and fragment ion respectively. Low detection rate at about 14% when fragment ion was applied as one of the filtering criteria might because of the low detectability of the fragment ions at low concentration levels in complex matrix like cocoa.

Table 1. Influence of different filtering criteria on pesticides detection rate and false positive

Filtering criteria	Detection rate[a] (%)			No. of false positive[b]
	10 µg/kg	50 µg/kg	150 µg/kg	
R.T. (±0.2 min) + Mass accuracy (±5 ppm)	52	93	99	5
R.T. (±0.1 min) + Mass accuracy (±5 ppm)	39	72	76	3
R.T. (±0.1 min) + Mass accuracy (±5 ppm) + Isotopic match[c]	28	60	69	0
R.T. (±0.1 min) + Mass accuracy (±5 ppm) + Isotopic match[c,d]	19	48	62	0
R.T. (±0.1 min) + Mass accuracy (±5 ppm) + Fragment ion	16	41	53	0
R.T. (±0.1 min) + Mass accuracy (±5 ppm) + ΔCCS (2%)	38	71	75	1
R.T. (±0.1 min) + Mass accuracy (±5 ppm) + ΔCCS (3%)	38	71	75	1
R.T. (±0.2 min) + Mass accuracy (±5 ppm) + ΔCCS (2%)	50	91	98	1

[a] Percentage of pesticides (out of 110) detected in 20 samples spiked at three concentrations
[b] Number of false positives detected in the non-spiked blank cocoa beans samples
[c] Isotope match *m/z* root mean square (RMS), ± 5 ppm
[d] Isotope match intensity RMS percent ≤ 20%

An alternative approach available when conducting ion mobility experiment is the collision cross section (CCS) values obtained for each pesticide studied. CCS represents the effective area for the interaction between an individual ion and the neutral gas through which it is travelling. CCS is an important distinguishing characteristic of an ion in the gas phase, being related to its chemical structure and three-dimensional conformation. Therefore, by applying a CCS filter at 2% tolerance in addition to mass accuracy (± 5 ppm) and RT (± 0.1 min), the false positive decreased to only 1. However, the detection rates were slightly decreased for all concentrations when ± 0.1 min criteria was used. Hence, we found that the used of ± 0.1 min tolerance may be not practical when screening for a large set of pesticides because by doing so, it requires frequent database RT update as it can be affected by matrix effects, UPLC set up and column conditions. On the other hand, increasing the CCS tolerance from ± 2% to ± 3% does not affect the detection rate nor the false positive result. So, based on the optimization data, the proposed pesticide screening workflow used the following filters: RT, ± 0.2 min; mass accuracy, ± 5 ppm; CCS, ± 2%.

Method validation

Screening methods, especially those involving automated MS-based detection, offer laboratories a cost-effective means to extend their analytical scope to analytes which potentially have a low probability of being present in the samples. Therefore, the confidence of detection of an analyte at a certain concentration level should be established based on the screening detection limit (SDL). When the screening method is only intended to be used as a qualitative method, there are no requirements with regard to recovery of the analytes (SANTE, 2016). In this study, the

screening method is considered fit-for-purpose when the pesticide can be detected in at least 95% of the samples. The lowest concentration level at which this condition can be satisfied is known as the SDL. For each individual pesticide, the number of positive detections at each concentration level was recorded and the SDL was established (Table 2).

The SDL was 10 µg/kg for 36 out of 110 pesticides, which represents the 33% of the total. For 48 pesticides (44%), the SDL was 50 µg/kg, whereas the SDL was 150 µg/kg for 17 pesticides which represent 15% of the total *(Figure 1)*. The remaining 9 pesticides (triticonazole, moxidectin, monolinuron, lufenuron, dimoxystrobin, cyproconazole, pyridaben, clethodim, and carbofuran) which represent 8% of the total, could not be established since they could not fulfil the 95% detection rate to be qualified as SDL. However, the proposed screening method was able to detect them at high spiking level.

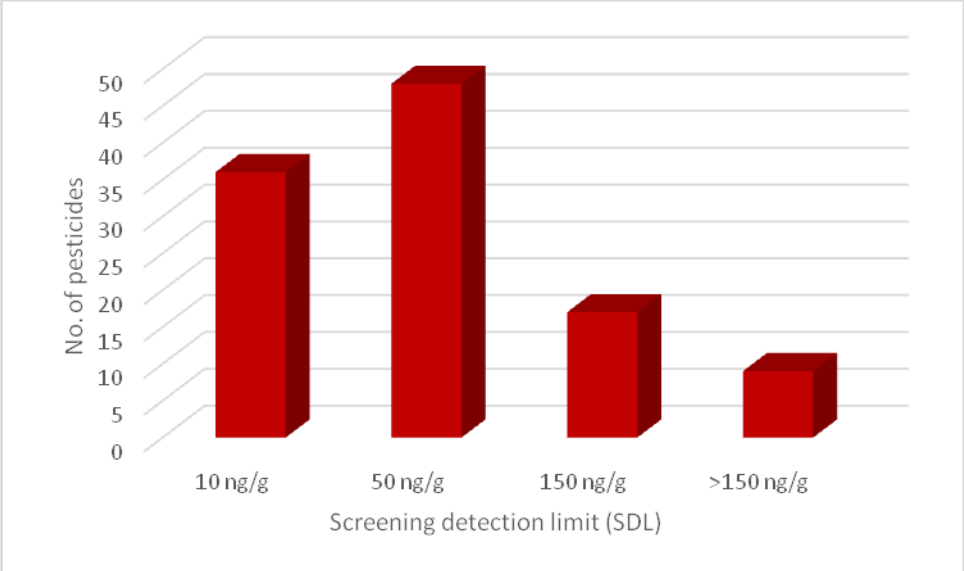

Figure 1. Number of pesticides with SDLs at 10, 50, and 150 µg/kg.

Ion-mobility-derived CCS values for pesticides

As mentioned in previous section, CCS values obtained from ion mobility experiment are supposed to be independent of matrices and UPLC conditions and solely depend on the size, shape, and charge of the molecules. To study the correlation between *m/z* and CCS, the experimentally determined CCS values were plotted as a function of *m/z*, and the corresponding coefficient of correlation (R^2) was calculated *(Figure 2)*. The CCS values of the ions were found to strongly correlate (R^2 = 0.9001) with their corresponding *m/z* values. In the mobility cell, it happens that smaller ions exiting the cell earlier, whereas the bigger ions take longer time to move through the gas-filled mobility cell. On the other hand, when plotting the measured CCS values for all the pesticides against their chromatographic retention times *(Figure 3)*, very poor correlation was observed at (R^2 = 0.3844). This shows that the CCS values for pesticides were independent of chromatographic separation and UPLC conditions. Consequently, ion mobility separation provides an additional separation dimension that increases peak capacity and selectivity

.

Table 2. Screening detection limit for pesticide residues in cocoa beans in µg/kg

No	Pesticide	SDL (µg/kg)	No	Pesticide	SDL (µg/kg)	No	Pesticide	SDL (µg/kg)
1	Acetamiprid	50	38	Flufenoxuron	50	75	Nuarimol	50
2	Acibenzolar-S-methyl	150	39	Fluomethuron	50	76	Paclobutrazol	50
3	Ametryn	10	40	Fluoxastrobin	10	77	Penconazole	50
4	Aminocarb	150	41	Fluquinconazole	50	78	Pencycuron	10
5	Azoxystrobin	50	42	Flusilazole	10	79	Pirimicarb	10
6	Benalaxyl	50	43	Flutolanil	10	80	Prometryn	10
7	Boscalid	50	44	Flutriafol	50	81	Propiconazole I	10
8	Bupirimate	10	45	Forchlorfenuron	150	82	Pyracarbolid	50
9	Buprofezin	150	46	Fuberidazole	50	83	Pyraclostrobin	50
10	Butafenacil	50	47	Furalaxyl	50	84	Pyridaben	>150
11	Carbendazim	150	48	Furathiocarb	50	85	Pyrimethanil	50
12	Carbofuran	>150	49	Hexaconazole	150	86	Pyriproxifen	150
13	Carboxin	50	50	Hydramethylnon	10	87	Quinoxyfen	10
14	Carfentrazone-ethyl	10	51	Imazalil	10	88	Rotenone	10
15	Chlorantraniliprole	150	52	Indoxacarb	10	89	Secbumeton	10
16	Chlorfluazuron	150	53	Ipconazole I	150	90	Siduron A	50
17	Chloroxuron	10	54	Isoprocarb	10	91	Simetryn	10
18	Chlortoluron	50	55	Isoproturon	150	92	Spinetoram	50
19	Clethodim	>150	56	Linuron	150	93	Spinosyn A	10
20	Cyproconazole I	>150	57	Lufenuron	>150	94	Spinosyn D	50
21	Cyprodinil	10	58	Mandipropamid	50	95	Spirotetramat	10

22	Diclobutrazol	50	59	Mefenacet	150	96	Spiroxamine I	10
23	Difenoconazole I	50	60	Mepanipyrim	10	97	Sulfentrazone	150
24	Dimethomorph II	10	61	Mepronil	10	98	Tebuconazole	50
25	Dimoxystrobin	>150	62	Metaflumizone	50	99	Tebufenpyrad	150
26	Diniconazole	50	63	Metalaxyl	10	100	Tebuthiuron	50
27	Diuron	50	64	Metconazole	10	101	Terbumeton	10
28	Etaconazole I	50	65	Methabenzthiazuron	150	102	Tetraconazole	50
29	Ethiprole	50	66	Methoprotryne	10	103	Thiabendazole	50
30	Ethirimol	10	67	Metobromuron	50	104	Thiacloprid	50
31	Etoxazole	10	68	Metribuzin	50	105	Thiophanate-methyl	50
32	Fenamidone	150	69	Mexacarbate	50	106	Tricyclazole	10
33	Fenazaquin	50	70	Monolinuron	>150	107	Trifloxystrobin	50
34	Fenbuconazole	50	71	Moxidectin	>150	108	Triflumuron	50
35	Fenhexamid	50	72	Myclobutanil	50	109	Triticonazole	>150
36	Fenpropimorph	10	73	Neburon	10	110	Zoxamide	50
37	Fenpyroximate	50	74	Novaluron	50			

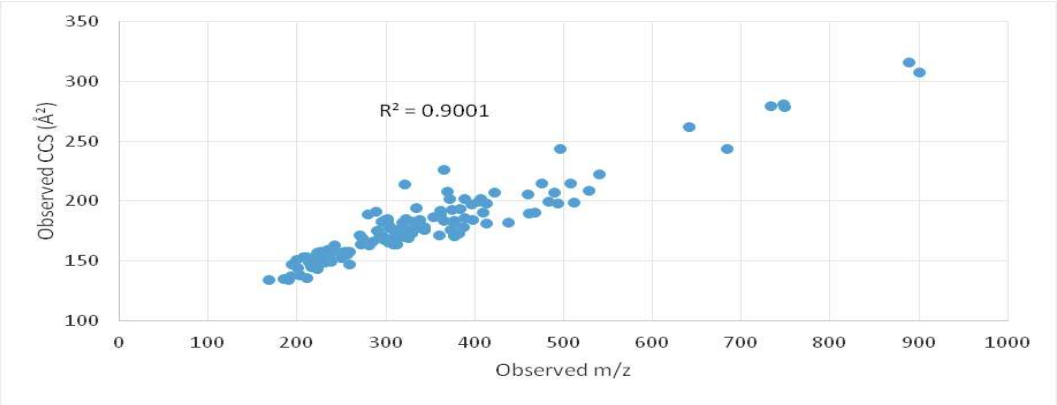

Figure 2. Correlation between CCS values and m/z for the studied pesticides

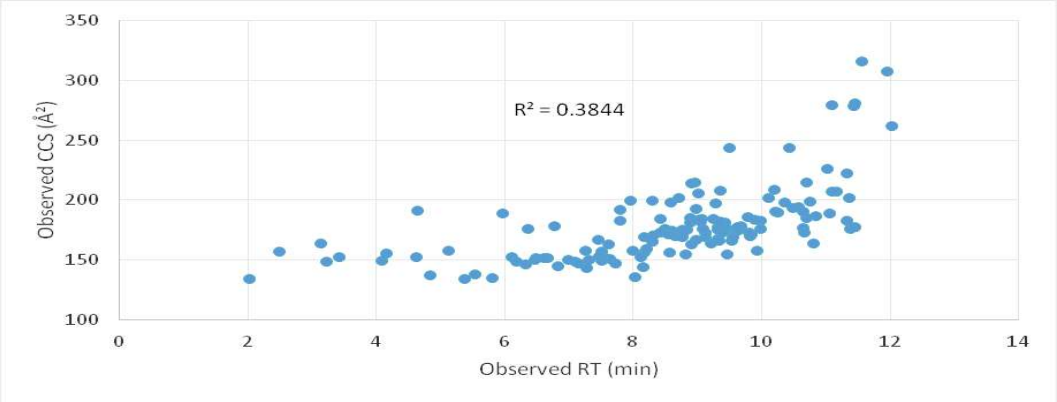

Figure 3. Correlation between CCS values and retention time for the studied pesticides

Besides UPLC conditions, CCS values were also reported to be independent of different matrices and ion mobility platforms (Regueiro, 2016). In this study, the experimental CCS values obtained were compared with different ion mobility system and matrices. Results in Table 3 shows that the observed CCS values for pesticides in cocoa beans matched the literature CCS values in different ion mobility platform for pesticides in fish feed and waste water (Regueiro, 2016; Stephan, 2016). As can be observed in Table 3, the CCS values were all within ± 2.0 % error except for metalaxyl, which recorded ± 2.4 % error. The results showed that CCS values can be an additional confirmation criteria for pesticide residues besides retention time, mass accuracy, isotopic pattern, ion ratio, and fragmentation ions.

Application to real samples

Finally, the proposed screening method was applied to real cocoa beans samples via a monitoring study. Domestic cocoa bean samples for monitoring study were collected from local farmers, while imported beans were collected from ports and comprised beans from Indonesia, Cameroon, Nigeria, Venezuela, Ghana, Ecuador and Papua New Guinea. A total of 60 samples were collected, and each sample was analyzed in duplicate. Results obtained showed that 20% of the samples were tested positive for ametryn (8 ng/g), metalaxyl (78 ng/g) and difenoconazole (21 ng/g). Since the screening method only deals with qualitative study, quantitation of the detected samples was done with a triple quadrupole instruments. *Figure 4* shows the extracted ion chromatograms of the precursor ions and its main fragment ions for metalaxyl, one of the positively detected pesticides in cocoa beans samples. Metalaxyl recorded a CCS value of 163.29 Å². Focusing on the low and high energy spectra obtained with and without drift time filtering, it can be observed that the drift time alignment resulted in much more cleaner spectra, which greatly simplifies the identification process.

Table 3. Comparison of observed CCS and CCS obtained from literatures

Pesticides	Adduct type	Theoretical m/z	CCS (Å²)				
			A	B	error (%)	C	error (%)
Aminocarb	[M + H]+	209.129	152.3	152.5	-0.2	151.2	0.701
Bromuconazole	[M + H]+	375.961	171.0	169.7	0.8	171.2	-0.097
Chloroxuron	[M + H]+	291.090	175.7	174.4	0.8	175.2	0.310
Cycluron	[M + H]+	199.181	151.5	151.6	-0.1	148.9	1.731
Fenamidone	[M + H]+	312.117	173.2	172.7	0.3	172.1	0.608
Flutriafol	[M + H]+	302.110	167.4	166.2	0.7	166.8	0.351
Ipconazole	[M + H]+	334.168	175.4	173.6	1.0	172.9	1.423
Mepronil	[M + H]+	270.149	171.6	170.9	0.4	168.7	1.709
Metalaxyl	[M + H]+	280.154	163.2	162.8	0.2	159.2	2.443
Metconazole	[M + H]+	320.152	170.9	169.6	0.8	169.3	0.945
Omethoate	[M + H]+	214.030	136.6	138.3	-1.3	136.8	-0.183
Propiconazole	[M + H]+	342.077	178.8	178.3	0.3	178.8	0.000
Pymetrozine	[M + H]+	218.104	153.0	153.2	-0.1	154.1	-0.702
Tebuconazole	[M + H]+	308.152	169.4	168.7	0.4	167.6	1.088
Triticonazole	[M + H]+	318.137	181.9	180.4	0.8	178.6	1.829

A: Cocoa beans
B: Fish feed (Regueiro *et al.*, 2016)
C: Wastewater (Stephan *et al.*, 2016)

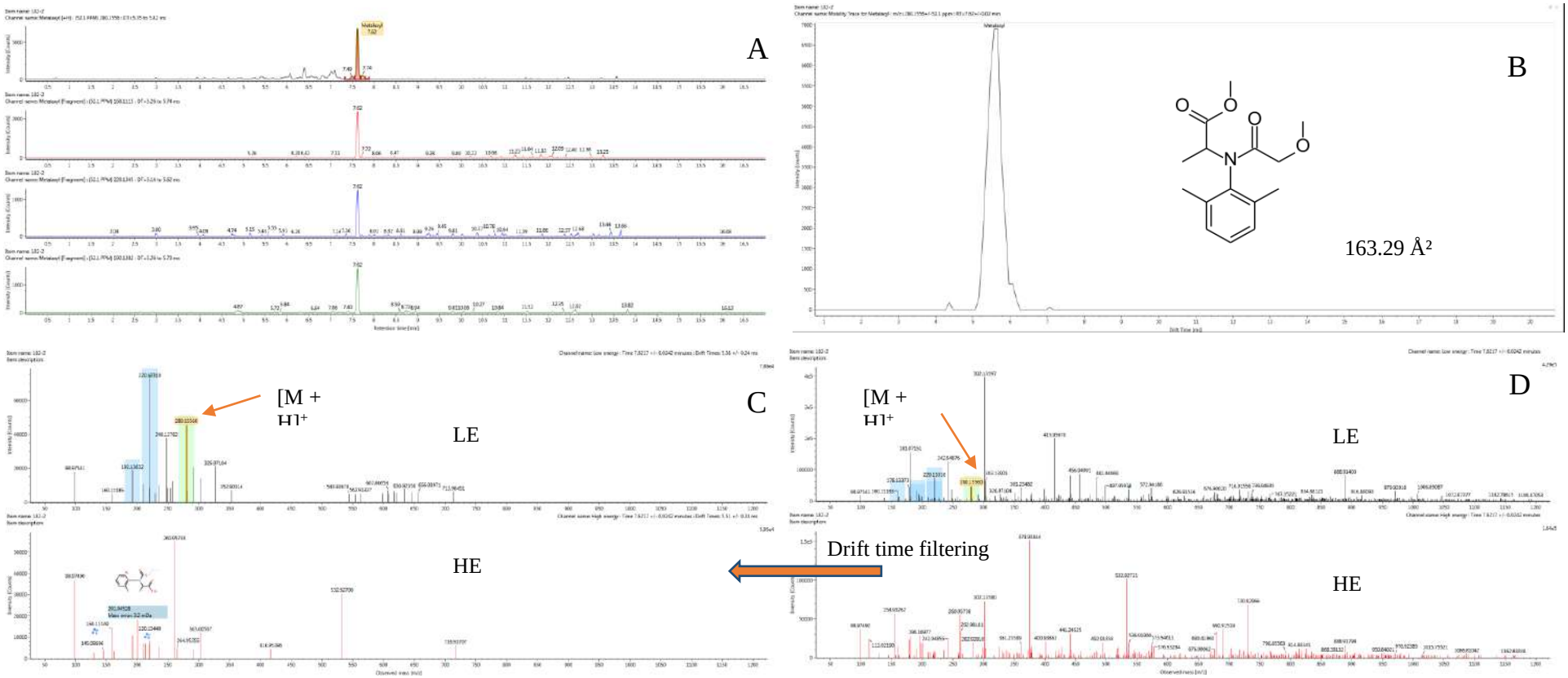

Figure 4. Incurred residues of metalaxyl in cocoa beans: (A) Extracted ion chromatograms for the $[M + H]^+$ ion and its main fragment ions; (B) Extracted ion mobiligram for the $[M + H]^+$ ion; (C) Low energy (LE) and high energy (HE) spectra with drift time filtering; (D) Low energy (LE) and high energy (HE) spectra without drift time filtering.

CONCLUSION

In the present work, pesticide multiresidue screening method was proposed based on the application of UPLC-IMS-QTOF-MS. The screening method was validated and applied to the screening of 110 pesticides in cocoa beans samples and was found to be more selective towards the studied pesticides. The optimized screening criteria consisted of mass accuracy (± 5 ppm), RT (± 0.2 min) and CCS (± 2%) could decreased the false positive rate while maintain its detection rate at 98% compared with analysis without CCS values. Under this screening conditions, 33% of the investigated pesticides gave SDL of 10 µg/kg, whereas 44% and 15% of them presented SDL of 50 and 150 µg/kg, respectively. Finally, the automated screening workflow was applied to real cocoa beans sample through a monitoring study and 20% out of 60 samples were tested positive, in which drift time filtering of the LE and HE spectra improved the detectability and identification of the pesticides.

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EFFECT OF THE PANDAN JUICE CONCENTRATION ON THE SENSORY QUALITY OF COCOA BUTTER-BASED PANDAN ICE CREAM

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ABSTRACT - *The leaves of Pandanus amaryllifolius, commonly known as screwpine/ pandan leaves, are widely used as spice, food colouring and flavouring in culinary application. In addition to the aromatic properties, the leaves have been reported to have compounds with antiviral, antioxidant and anti-diabetic properties which could boost up the value and use of these leaves in food application. The leaves combined with cocoa butter in the production of ice cream could provide the healthier choice of ice cream due to the fact that cocoa butter does not increase the total and low density lipoprotein (LDL) cholesterol levels in human blood as compared to dairy fat which normally used in the marketed ice creams. In the current study, ice cream with the concentrations of 5%, 7% and 9% of the pandan juice respectively were produced, and evaluated by 10 panelists (who are familiar with cocoa butter-based ice cream) in terms of colour, pandan flavour, sweetness, texture, meltability and overall acceptability using 5 point hedonic scale (score 5: extremely like; score 4: like; score 3: neither like or dislike; score 2: dislike; score 1: extremely dislike). The results showed that increase in pandan juice levels did not significantly affected ($p>0.05$) the sensory attributes for all the tested samples; however, it had negative effect on the sensory quality in terms of pandan flavour and overall acceptability for the produced ice cream when 9% of pandan juice was used.*

Key words: Cocoa butter, pandan juice, sensory quality, ice cream

INTRODUCTION

The leaves of *Pandanus amaryllifolius*, commonly known as screwpine/ pandan leaves, belong to the monocot Pandanaceae family and are known as the only Pandanus with scented leaves (Wakte *et. al.*, 2009). These upright, green plants with fan-shaped sprays of long, narrow, blade-like leaves and woody aerial roots are widely cultivated in South East Asia such as Malaysia, Indonesia, Thailand and India. The leaves emit a pleasant aroma described as distinct with nutty to fresh hay flavour mainly due to the presence of volatile compound namely 2-acetyl-1-pyrroline (Laksanalamai and Ilangantileke, 1993).

P. amaryllifolius is included in list of herb and spice plant species useful as ingredients in food by the Geneva-based International Standards Organization (Peter 2001; Wakte *et al.*, 2009). The leaves are widely used as spice, food colouring and flavouring in culinary application (Sun, 1992). For instance, the freshly leaves are used to enhance the flavour on rice in the preparation of *nasi lemak* and *nasi kuning* (Wakte *et. al.*, 2012); juices extracted from the leaves are used as a natural flavouring and colouring agent in cakes, puddings and ice cream (Setyowati and Siemonsma, 1999). Besides food application, the fragrant leaves are often used as natural cockroach repellent (Li and Ho, 2003; Samy *et. al.*, 2005), perfume (Nguyen, 2006) and potpourri (Samy *et. al.*, 2005; Wakte *el. al.*, 2009).

In addition to the aromatic properties, pandan leaves contain phytoconstituent such as phenols, flavonoids, tannins, carotenoids, tocopherols, tocotrienols and lectin which have been reported to have antiviral, antioxidant and anti-diabetic properties (Ting and Whang, 1998; Lee *at. al.*, 2004; Ooi *et. al*., 2004; Faridah and Lajis Nordin, 2006; Nor *et. al*., 2008; Sasi, *et. al.*, 2009; Kumari *et. al.*, 2012; Rajeswari *et. al.*, 2012; Chiabchalard and Nooron, 2015); which are likely to boost up the value and the use of these leaves.

Food products with natural colours and flavours are the current trend moving over the global due to the growing demand from the health awareness consumers looking for natural ingredients in food and beverages. As the

pandan ice cream is not commercially available in the market; therefore, the aim of the current study was to develop pandan ice cream using fresh pandan juice as the natural colouring, flavouring and functional ingredient. Moreover, cocoa butter was used to substitute dairy fat which is normally used in the production of ice cream due to the fact that cocoa butter, despite its high saturated fat content, does not increase the total and LDL cholesterol levels in blood due to its high stearic acid content (Kris-Etherton and Yu, 1997) as compared to dairy fat (Matthan, *et. al.*, 2004). The combination of health benefits from pandan juice and cocoa butter in the production of cocoa butter-based pandan ice cream could provide healthier choice of ice cream products to the ice cream lovers.

MATERIALS AND METHODS

Materials

Deodorised cocoa butter was obtained from Barry Callebaut (M) Sdn. Bhd., Port Klang, Selangor; pandan leaves, fresh skim milk, sugar and skim milk powder were purchased from the retail shops. Stabilizer/emulsifier was obtained from C.N. Chemical Sdn. Bhd.

Methods

i. Preparation of Pandan Juice

The fresh and cleaned pandan leaves (3 pieces) were cut around 1-3cm length into a food processer and blended the leaves together with boiled water (100 g) for 1 minute. The pandan juice was obtained by straining the pulp through a fine stainless steel sieve.

ii. Mix Formulation and Production of Cocoa Butter-Based Pandan Ice Cream (CBBPIC)

Cocoa butter-based pandan ice cream with varying levels of pandan juice (5, 7, 9%) were produced based on the formulation showed in Table 1. The quantity of deodorised cocoa butter, skimmed milk powder, sugar, stabiliser/emulsifier and salt were kept constant. The processing steps involved in the production of CBBPIC were schematically shown in *Figure 1*. Dry ingredients such as sugar, skim milk powder, stabilizer/emulsifier were incorporated into fresh skim milk. The molten butter and pandan juice were added into the mixture prior to the pasteurization process at 74^{o}C for 20 minutes. The pasteurized mixture was then homogenized using heavy duty mixer and aged at 5^{o}C for 24 hours prior to frozen in a horizontal batch freezer. The produced ice cream (1 kg) was deposited into the plastic containers, hardened and stored at -20^{o}C in a chest freezer.

Table 1. Cocoa Butter-Based Pandan Ice Cream Mix Formulation

Ingredients	**(%)**
Fresh Skimmed Milk	91.0-95.0
Deodorised Cocoa Butter	
Skimmed Milk Powder	
Sugar	
Stabiliser/Emulsifier	
Salt	
Pandan Juice	5.0-9.0
Total	100.0

iii. Sensory Evaluation of Cocoa Butter-Based Pandan Ice Cream

The acceptability of the produced CBBPIC were assessed according to the procedure of Akbari and co-workers (2016). Ten (10) panellists were recruited to evaluate the ice creams in terms of colour, pandan flavour, sweetness, texture, meltability and overall acceptability by using 5-point hedonic scale (Score 5-Extremely Like; Score 4-Like; Score 3-Neither Like or Dislike; Score 2-Dislike; Score 1: Extremely Dislike).

iv. Statistical Analysis

The collected data were statistically analyzed by analysis of variance (ANOVA) and mean separation was by least significant difference at $p<0.05$.

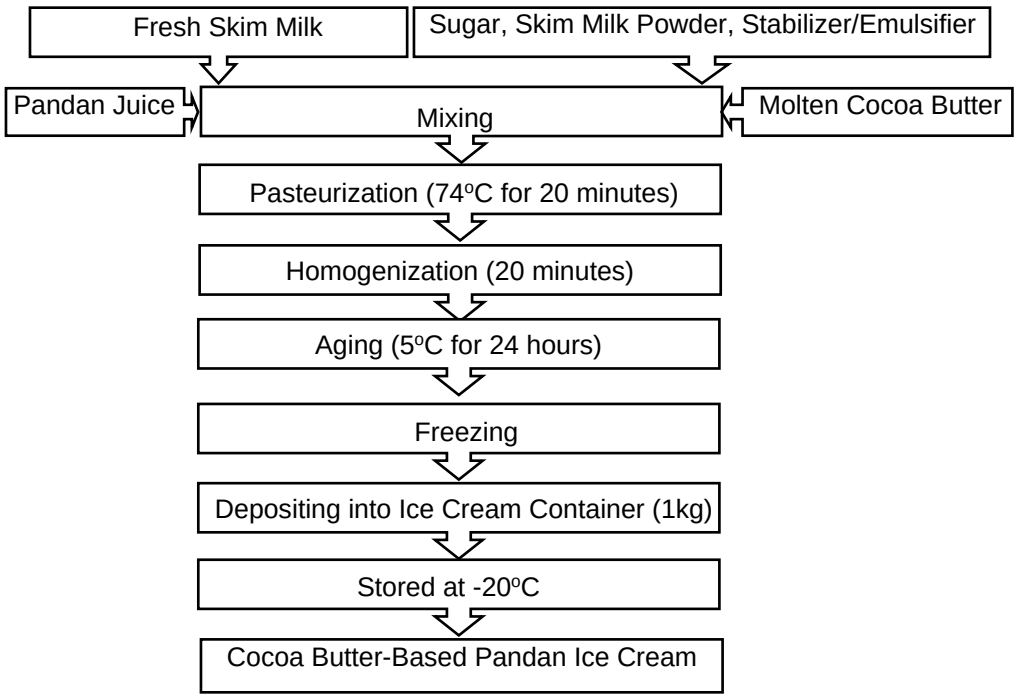

Figure 1. Production of Cocoa Butter-Based Pandan Ice Cream

RESULT AND DISCUSSION

The mean sensory scores of the cocoa butter-based pandan ice creams with different levels of pandan juice were shown in Table 2. The results indicated that the increase of pandan juice levels did not significantly affected ($p>0.05$) the sensory attributes for all the tested samples. Nevertheless, addition of 9% pandan juice in ice cream has reduced the acceptance of pandan ice cream in terms of pandan flavour and overall acceptability. Pandan leaves contains high level of chlorophyll content (Loh *et. al.*, 2005); hence, the increase of pandan juice concentration to 9% may impart the grassy and bitter taste to the produced ice cream (based on the panels' comments) and consequently reduced its acceptability.

Table 2. Sensory Scores for Cocoa Butter-Based Pandan Ice Cream with Different Levels of Pandan Juice

Sensory Attributes	**Pandan Juice Level**		
	5%	**7%**	**9%**
Colour	4.0±1.0*	4.0±0.7*	4.0±0.7*
Pandan flavour	4.0±0.8*	4.0±0.9*	3.0±0.9*
Sweetness	4.0±0.8*	4.0±0.7*	4.0±0.5*
Texture	4.0±0.5*	4.0±0.4*	4.0±0.0*
Meltability	4.0±0.4*	4.0±0.4*	4.0±0.4*
Overall Acceptability	4.0±0.7*	4.0±0.9*	3.0±0.5*

** Means in the same row are not significantly different ($p>0.05$)*

CONCLUSIONS

Pandan juice at concentration of 5 to 7% could be used to produce pandan ice cream; however, an increase of pandan juice concentration to 9% would reduce the preference of the panels towards the pandan flavour attribute and overall acceptability of the product.

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EFFECT OF CARBONIZATION TEMPERATURE ON THE YIELD, POROSITY AND SURFACE FUNCTIONAL GROUPS OF BIOCHAR PRODUCED FROM COCOA SHELL

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ABSTRACT - *Several batches of biochars were prepared from cocoa shell pellets by carbonization in a flow of nitrogen using a fixed-bed reactor. Cocoa shell pellets was carbonized at temperatures of 400, 500, 600, 700, 800 and 900°C for an hour to study the effects of carbonization temperature on biochar yield, porosity and surface functional groups. The prepared biochars were characterized for the micropore volume using CO_2. The biochar yield was around 28 % and is cornparable with yields reported from other lignocellulosic materials. Significant changes were observed in the chemical structure of cocoa shell under carbonization where aromatic structures were developed and accompanied by losing most of aliphatic C-H species in the biochars. Increasing the carbonization temperature led to a complete destruction of C=O and C-O of aliphatic species simultaneously by promoted aromatic structures. Large amount of oxygen functionalities of acidic nature appearing as carbonyl, phenolic, hydroxylic, carboxylic and lactonic species groups on the carbon surface.*

***Keywords*:** Carbonization; cocoa shell; biochar; pore development; surface functional groups.

INTRODUCTION

Conversion of lignocellulosic to a residue rich in carbon is a complicated process. Pyrolysis of carbonization involved cracking, free radical formation, molecular reordering, polymerization, aromatic condensation and elimination and lateral chains and hydrogen (Alvarez *et al.*, 1998). Pyrolysis will initially follow a common temal pathway irrespective of heating rate, and as it reached some stage during conversion, the pyrolysis process will follow a heating rate depending path (Milosovljevic *et al.*, 1996).

Generally, carbonization involved two stages. During the primary carbonization (above 200°C), H_2O, CO_2 and CO, and other volatile fractions were been released from the lignocellulosics materials. As the temperatures reached 400°C, high molecular weight hydrocarbons were converted to tar. Above 600°C, loss of hydrogen and reordering of the structural units (non-aromatic structures be aromatized) become most important features.

Several carbonization parameters have significant effect to the properties of the biochar produced. Among them are the carbonization temperature, the heating rate and the carbonization time. High amount of carbonization yield for cellulose, lignin and hemicellulose can be obtained at low heating rate. Low heating rate will have allowed of a more complete dehydration and stabilization of these polymers, although the temperature range for the decomposition changes from one component to another (Gonzalez *et al.*, 1997).

Carbonization temperature is associated with the ability to break the chemical bonds of the raw material by providing the amount of energy required thus influences the amount of volatiles released from the material and the biochar yield. As the carbonization temperature reached 500°C, about 50-80 % of the volatiles in the lignocellulosic materials were given off. Solano *et al.* (1980) concluded that 500°C must be considered the lowest temperature to be used during the carbonization process.

Besides the effect on yield, carbonization temperature has been shown to affect the biochar surface area (porosity) and surface functional groups.

Different type of lignocellulosic materials will have different carbonization temperatures that affect the surface area. The optimum carbonization temperatures for promotion of micropore for cedar shell were

identified at 700°C (Baklonova *et al.,* 2003). Meanwhile the optimum carbonization temperature was identified at 600°C (Alvarez *et al.,* 1998). Beyond 800°C, the micropore volume on lignocellulosic material will decrease which is shows trend that similar with lignin (Baklonova *et al.,* 2003).

According to Reinoso (1998), carbonization temperature has huge impacts to the nature and number of functional groups of biochar. Functional groups can be formed following the incomplete carbonization and subsequent activation of the lignocellulosic materials. The heteroatoms at the surface of the biochar such as oxygen, Nitrogen and sulfur, influences the sorption properties of the carbon (Laszlo *et al.,* 2001).

The functional groups in the biochar can be determined by the type, quantity and bonding of functional groups such as hydroxyl and carboxyl groups. The FTIR spectroscopy in its various forms is an important and forceful technique which can give useful information about structures (Shin *et al.,* 1997). It can provide basic spectra of activated carbons, especially for determination of types and intensities of their surface functional groups.

The main aim of the present work were to investigate effects of carbonization temperature on the porosity and the chemical structure of biochar from cocoa shell.

MATERIALS AND METHOD

Apparatus

The experimental set-up *(Figure 1)* basically consists of a stainless steel (310) reactor and a nitrogen supply. The reactor dimensions are 3.8 cm in diameter and 130 cm in length. The distributor is made of stainless steel perforated plate covered with a fine stainless steel wire mesh and is positioned centrally in the reactor. The reactor is externally heated with a vertical electric furnace using four heating elements. The furnace temperature is controlled by a maximum operating temperature that can be achieved is 1500°C. The bed temperature is measured using a 3 mm diameter, 60 cm long K-type (chromelalumel) thermocouple. The thermocouple is lowered from the top section of the furnace and the tip of the thermocouple is positioned 3mm above the distributor.

Procedure

Firstly, the cocoa shell was ground and sieved to a size of less than 0.5mm. The shell was dried and pelletized to a size between 1.5 – 2 mm. About 35gm of the raw material was charged into the reactor and carbonized in a stream of nitrogen flowing constantly at 1000 cm^3 min^{-1}. Before the furnace was switched on, the reactor containing the sample was purged with nitrogen (oxygen-free) for 15min to remove all the air inside the reactor, the heating rate during the process was maintained at 15°C min^{-1}. For all the runs, a uniform carbonization time of 1 hour was maintained at the carbonization temperature. After this period, the furnace was switched off and the product was allowed to cool down with nitrogen flowing at the rate of 2000 cm^3 min^{-1}. The carbonization temperature was varied from 400 to 900°C, with increments of 100 degrees.

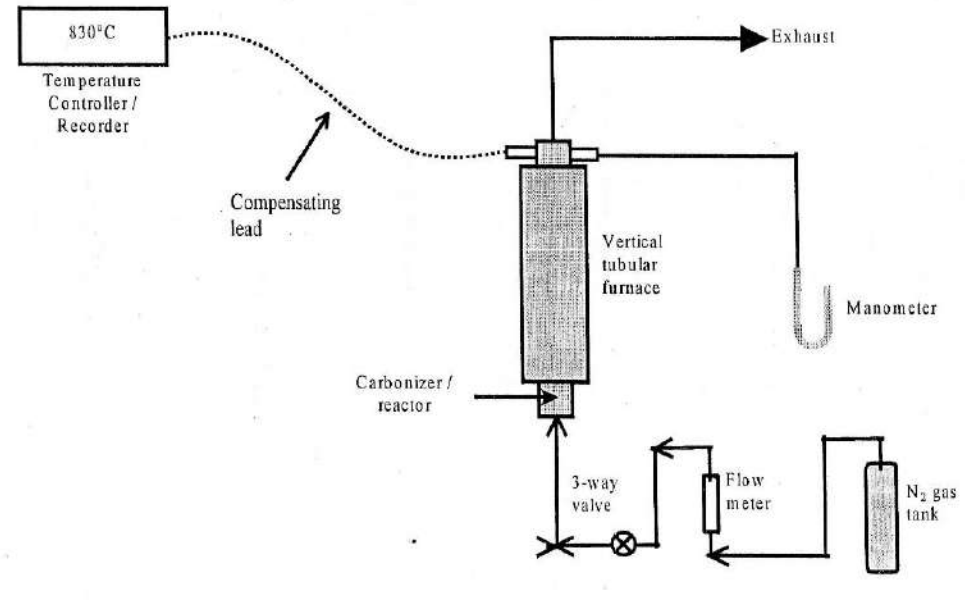

Figure 1. Schematic diagram of the experimental setup.

Characterization

The prepared samples (biochars) were characterized by CO_2 adsorption (at 0°C) using a Micromeritics ASAP 2010 surface area analyzer. Prior to the analysis, the sample was outgassed at 180°C for 12 hours to a residual pressure of 10^{-4} torr or less. Surface functional groups was carried out using a Thermo Nicolet FTIR model 380.

FTIR spectroscopy

FIFIR spectra were recorded between 4000 and 400 cm^{-1} using a Thermo Nicolet 380 spectrometer for the samples. Discs were prepared by first mixing 1 mg of dried sample with 500 mg of KBr (Merck, for spectroscopy) in an agate mortar and then pressing the resulting mixture at 10 tonnes cm^{-2} for 15 min under vacuum.

RESULTS AND DISCUSSION

Effect of temperature on biochar yield

Figure 2 shows the effect of carbonization temperatures on biochar yield. The biochar yield varied from in this temperature range. Similar results were reported for other agricultural by-products (Raveendran *et al.*, 1995). The decrease in biochar yield as the carbonization temperature was increased from 400 to 900°C could be attributed to the release of carbon atoms and other elements, such as hydrogen and oxygen, from the biochar. Studies have shown that at temperatures above 500°C, the biochar still keeps releasing hydrogen and carbon monoxide as a result of rearrangement of the aromatic ring in the carbon structure. Also, lignin which is one of the major components of lignocellulosic materials has been shown to undergo decomposition even at temperatures above 500°C (Sharma *et al.*, 2004). This process also yields carbon monoxide and hydrogen. Therefore, it could be deduced, marginal decrease in the biochar yield in this temperature range above 500°C is due to rearrangement of the carbon structure which results in the loss of heteroatoms from the biochar (Reinoso, 1998).

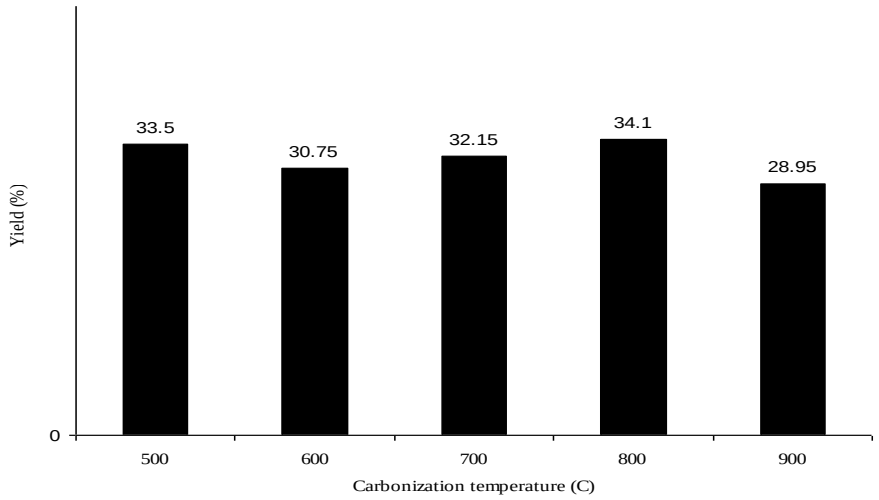

Figure 2. The effect of carbonization temperature on biochar yield.

Effect of temperature on porosity

The study on biochar microporosity is based on the adsorption of carbon dioxide at 0°C using a Micromeritics ASAP 2010 surface area analyzer. Nitrogen was not used as an adsorbate because the adsorbate does not possess enough kinetic energy to pass through or diffuse through the molecular size pores.

Figure 3 shows the **adsorption isotherms** of biochars produced at different carbonization temperatures, the adsorption isotherms of biochars produced at different carbonization temperatures. The different shape of the adsorption isotherm is a reflection of the different pore size distribution of the carbons, even although the starting material is the same for all of them (Reinoso, 1998). The highest pore volume was identified at 600°C and the lowest pore volume at 800°C. The results also reveal that some slight increase in pore volume has occurred at temperatures 900°C.

The pore volume started to increase as carbonization temperature progressed from 400°C to 600°C. But as cocoa shell pellets were pyrolysed at 700°C, a huge drop of pore volume were detected. Pore volumes of biochar carbonized at 800°C were lower than biochar carbonized at 700°C. However, the pore volume increased again after carbonization at 900°C.

The increased of adsorption isotherm at carbonization temperatures of 400, 500, 600°C can be related to the increased of pore diameter less than 0.3 nm as shown in the *Figure 4.* The major contributors to the adsorption isotherm were the pore diameter between 0.45 to 0.70 nm *(Figure 5).* The adsorption isotherm decreased for biochar 700°C and 800°C as pore volume of pore diameter less than 0.3 nm and pore diameter between 0.45 and 0.70 nm.

In general, the results show that as the carbonization temperature is increased as the volume of carbon dioxide adsorbed increases. The results also reveal that some increase in pore volume has occurred at temperatures above 500°C. This increase in the pore volume with temperature could be attributed to the increase in the number of pores as a result of rearrangement of the carbon layer resulting in the creation of smaller-sized pores. A more detailed analysis of micropore development was carried out using the Horvath-Kawazoe (H-K) equation, which is based on the change in the pore volume with respect to the pore diameter. Analysis using this method covers the pore size range of 0.3 to 0.5 nm *(Figure 4)*. The results show that the maximum pore volume in this micropore region occurs at a pore diameter of around 0.4 nm. It also shows that as the carbonization temperature is increased there is an obvious increase in the micropore volume.

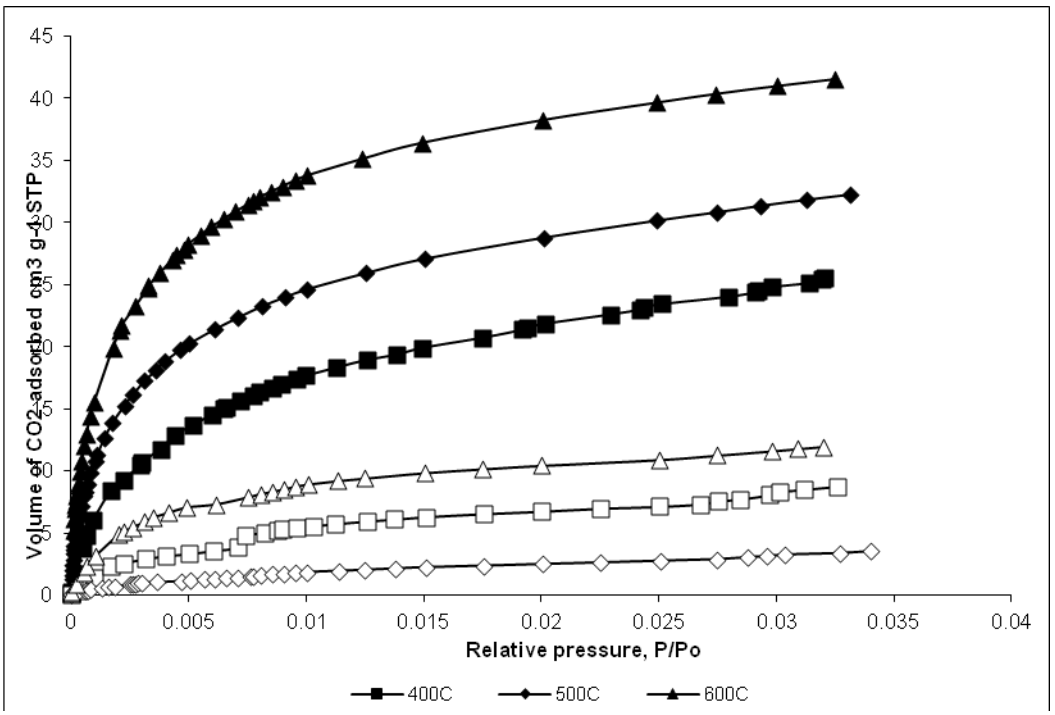

Figure 3. Adsorption isotherms of biochars produced at different carbonization temperature.

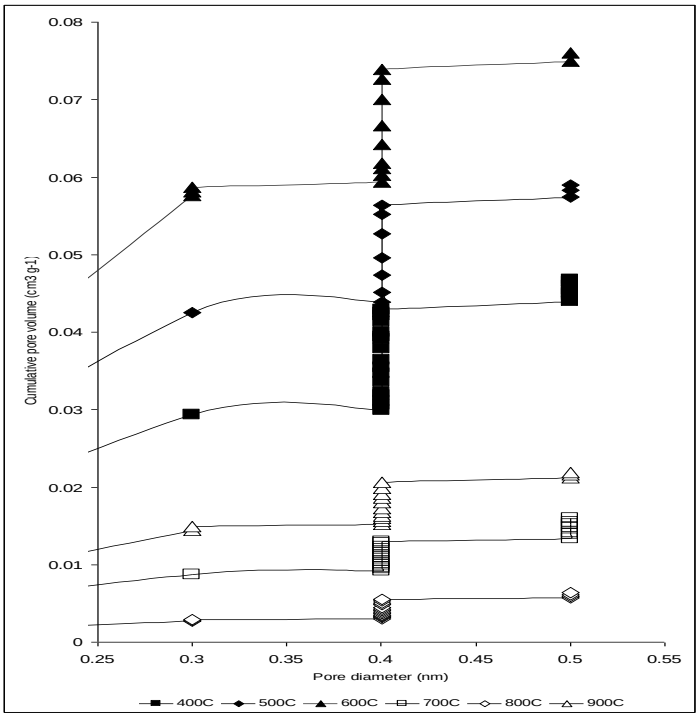

Figure 4. The change in pore volume with respect to the pore diameter based on the Horvath-Kawazoe equation.

The results also reveal a significant effect of carbonization temperature on the development of micropores *(Figure 5)*. There is an increase in the peak height of the curves, as well as a shift of the curves to the left, as the carbonization temperature is increased. This shows that the biochars which were produced at high carbonization temperature have a narrower micropore distribution as compared with biochar produced at lower carbonization temperatures. This observation were also in agreements with the development of micropore surface area *(Figure 6).*

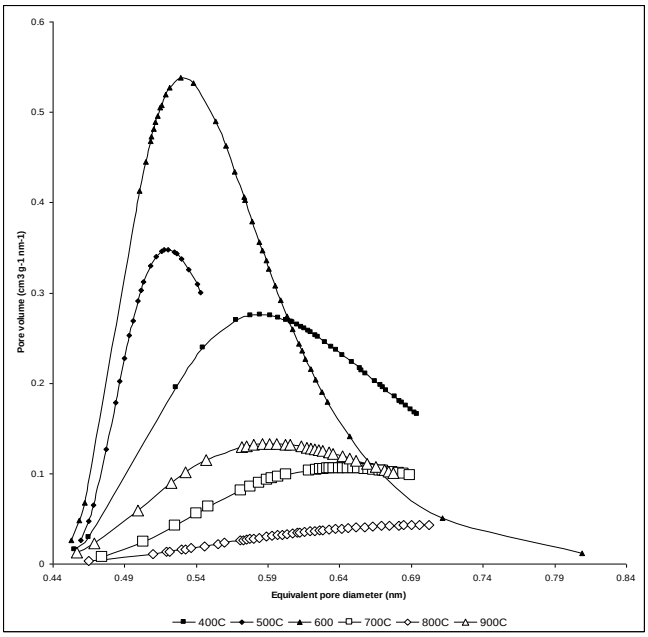

Figure 5. The Dubini Astakhov differential pore volume curves showing the effects of carbonization temperature on micropore development.

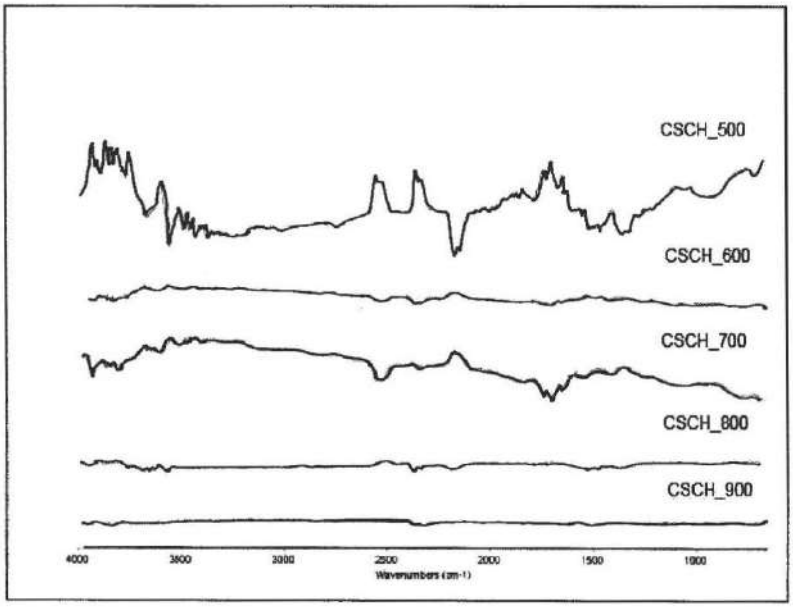

Figure 6. FTIR spectra of biochar at different carbonization temperatures.

Effect of temperature on the FTIR spectra

Figure 6 shows significant changes in the spectrum of CSCH_500 sample, as a consequence of carbonization of the cocoa shell at 500°C. Carbonization at 500°C reduces considerably the spectrum of the sample between 3500 and 1600 cm^{-1} compared with the same in the parent precursor, cocoa shell.

It involves bands at 3496 and 3402 cm^{-1} assigned to OH stretching vibrations. Two weak intense bands at 2950 and 2850 cm^{-1} were observed and assigned to aliphatic C-H stretching indicating the decrease in aliphaticity in CSCH_500 spectrum as a result to heat treatment to 500°C.

In the same time, the presence of a big number of weak bands in the CSCH_500 spectrum indicating also the presence of a considerable amount of incompletely pyrolyzed material still present in the carbon matrix. In other words, the temperature of 500°C failed to affect a complete pyrolysis of the raw material (El Hendawy, 2006). Big band of O-H, C=O were detected at CSCH_500. This indicates, the biochar still contained high level of organics. This observation is agreeable with the findings by Solano *et al.* (1998) and El-Hendaway (2006) that identified 500°C has to be considered as the lowest carbonization temperature. Biochar at 600°C and 700°C does not possessed C-O and C=O groups. The O-H groups are still strongly attached to the biochar although at lower intensity. However, at 800°C, reintroduction of C=O and C-O groups to the biochar contrary to O-H groups. This indicates rearrangements of O-H to C=O and C-O. All the functional groups were eliminated when the carbonization at 900°C.

Development of pore and surface functional groups are interrelated. During the thermal treatment, the grapheme layers also developing. At the edge of the basal planes of the graphite structure, are the unsaturated carbon functional groups, which are associated with high unpaired electrons. It is also associated with defects or dislocation at the basal planes with reactive sites (Laszlo *et al.*, 2001).

Two interesting observations were identified. The first observation is the collapsed of pore after carbonization temperature reached 700°C, which is commonly observed with lignocellulosic materials. Severe high temperature treatment on lignocellulosic materials will caused breakdown of crosslink in the carbon matrix. These major breakdowns will results in rearrangement of carbonaceous aggregates and the collapsed of pores (Laszlo *et al.*, 2001).

The second observation is the sudden increased in microporosity as well as the increased intensity of C-O and C=O groups and the decreased of O-H groups. The decomposition or dehydration of O-H (alcohol) groups were facilitated by the rearranging of carboxylic and hydroxylic groups (Symanski *et al.*, 2002). The formation of cyclic structures because of some carboxylic and/or hydroxylic groups (e.g. anhydrides and lactones) rearranging during annealing may be another important factor facilitating the dehydration of alcohols (Szymanski *et al.*, 2002).

Oxygen surface complexes are usually attached at the entrance of the pores especially carboxylic acids (Pradhan *et al.*, 1999; Ferraz *et al.*, 2000). According to Goyal (2004), the presence of the bulky surface functional groups will block the entrance of some of the micropore resulting in a decrease in the adsorption. Therefore, small changes in the micro- and mesoporosity suggest that progressive heating removes mainly surface groups located in pores or on the exterior carbon surface applied (Szymanski *et al.*, 2002).

Removing these obstacles will allow the gas N_2 the diffuse more vigorously and efficiently into opening more micropores. This will have allowed more desorption and rearrangements of the SFG at new micropores.

CONCLUSIONS

From the results of the experiments the following conclusions can be drawn. The biochar yield, which was in the range of 24-29% at various carbonization temperatures, is comparable to the reported values for other lignocellulosic materials. Increasing the carbonization temperature resulted in an increase in the micropore volume but the increase is more

Table 3. FTIR spectrum band detected for aliphatic and aromatic hydrocarbons in the char at different carbonization temperatures.

	Aliphatic hydrocarbons		Aromatic Hydrocarbons	
400°C	C-H		C-H	
	C=C		C=C	
	C≡C		Combination bands	
500°C	C-H	3054, 2950	C-H	777, 750, 668
	C=C	3903, 3865, 3839, 3782, 3748, 3806, 1650	C=C	1623, 1568, 1536, 1497, 1475, 1404
	C≡C	*Not detected*	Combination bands	1889, 1869, 1840, 1738, 1699
600°C	C-H		C-H	*Not detected*
	C=C	1551, 1532, 1521, 1492, 1454	C=C	1551, 1532, 1521, 1492, 1454
	C≡C		Combination bands	*Not detected*
700°C	C-H		C-H	*Not detected*
	C=C	*Not detected*	C=C	*Not detected*
	C≡C		Combination bands	*Not detected*
800°C	C-H		C-H	671
	C=C	*Not detected*	C=C	1557, 1540, 1522, 1509, 1489, 1473, 1458, 1396
	C≡C		Combination bands	
900°C	C-H		C-H	
	C=C	*Not detected*	C=C	*Not detected*
	C≡C		Combination bands	

remarkable for smaller-sized micropores. For mcsopore development, increasing the carbonization temperature has significantly decreased the mesopore volurne. This study also found that the carbonization temperature has an almost negligible effect on the macropore development. In contrast, raising the temperature to 500°C led to an effective destruction of the aliphatic alkyl species. This destruction was accompanied by a decrease in the intensities of the IR bands ascribed to C-0, C=O and aliphatic CH species. Further increase in the activation temperature to 700°C led to a complete destruction of C-O and C-0 species in the same time promoted polyaromatic structures where sharp bands ascribed to aromatic CH groups were detected.

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PHYSICOCHEMICAL CHANGES OF SUGAR FREE DARK CHOCOLATES WITH COCOA BUTTER AND FUNCTIONAL OILS BLENDS DURING STORAGE.

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ABSTRACT- *Eight samples of sugar free dark chocolate were developed using several blends of cocoa butter with functional oils (pumpkin seed, flaxseed and fish oil). The samples, sealed in high density polyethylene (HDPE) plastic and stored at 20℃, were analyzed monthly on physicochemical changes namely glossiness (at 85° angle), water activity (Aw) and peroxide value (PV) for duration of 6 months. All samples showed similar pattern of reduction in gloss index and increment in both, water activity and peroxide value during 6 months storage. The gloss index of control sample which contain cocoa butter without functional oils was significantly higher ($p<0.05$) at each month for 6 months storage as compared to other samples containing functional oils. However, there was a minor drop on the 2nd month and it consistently remains until end of 6 months storage. A blend of cocoa butter with medium amount of fish oil sample was found to be the highest in gloss value among other chocolate with functional oils. It remained glossy until the 4th month of storage then the value dropped significantly at the 5th and 6th months. Control sample was also found to have the lowest peroxide value while both samples containing minimum pumpkin and flax seed oil followed by minimum and medium amount of fish oil respectively were found to have the highest level of peroxide value compared to others. All chocolate samples containing flax seed oil tend to give high level of peroxide value while samples which contain blended cocoa butter with any level of fish oil gave relatively low peroxide value during storage. However, all samples showed no significant difference ($p>0.05$) in water activity values during storage.*

***Keywords**:* Physicochemical, sugar free dark chocolate, functional oils, storage.

INTRODUCTION

Sucrose is the most important type of sugar used in chocolate production. However, there are also many other types of sugar exist, such as the monosaccharides (dextrose and fructose), the disaccharide (lactose) as well as sugar alcohol (namely sorbitol and xylitol). Xylitol is non hygroscopic with melting temperature between 92° to 96°C. It has the greatest heat of solution (153.1 J/g) which resulting a remarkable cooling effect when melting in the mouth. The sweetening power of xylitol is comparable to sucrose. Due to that, sucrose could be replaced by xylitol in chocolate production without any problems (S.T. Beckett, 1994).

Chocolate is hygroscopic. Finished products should be packed in air tight container wrapping and stored below 60% relative humidity (S. T. Beckett, 1994). It is recommended to be stored in a cool, dry and dark place in a consistent temperature below 21°C or ideally between 18°C to 20°C, at lower relative humidity to avoid the risk of condensation where the emulsion of cocoa solids and cocoa butter will stay stable for months to year. The chocolate product especially containing milk fat will turn rancid once exposed to strong light and will affect the flavor and quality of the chocolate. Therefore, dark chocolate have a longer shelf life than milk chocolate.

Chocolate is characterized by very low water activities ranging between 0.4 and 0.5 (Lund, B *et al.,* 2000) thus impeding any spoilage. A study on spoilage of chocolate containing different types of sweeteners and with varying equilibrium relative humidity (32% to 45% indicated that growth of molds at the interface between chocolate and packaging material has been attributed to the hygroscopicity of the sweeteners. Spoilage of confectionery products depends very much on the water activity of individual products or

product groups. The lower the water activity the more stable is the product (Lund, B *et al.,* 2000). Mold may grow at water activity as low as 0.8, and osmophilic microbial growth occurs at water activities lower than 0.5 (S. T. Becket, 1994). Products of jellies, marshmallow, or fondant with Aw 0.60 to 0.70 may suffer from spoilage due to osmophilic yeasts or xerophilic molds (Lund, B *et al.,* 2000).

The aim of this research is to observe the physicochemical changes on glossiness, peroxide value and water activity of sugar free dark chocolate incorporated with cocoa butter and functional oils blend during storage.

MATERIALS AND METHODS

Functional oil and cocoa butter blends

Eight samples of cocoa butter with functional oils blends (OB) were selected (Table 1) from a list of oils blend developed in previous study which were stable in melting profile, solid fat content and peroxide value. Control (OB1) is a sample containing solely cocoa butter without functional oils. The other samples are blends containing either none or within Recommended Daily Allowance (RDA) limits of pumpkin seed and flax seed and fish oil incorporated with cocoa butter to compliment the fixed total fat.

Production of sugar free dark chocolate with cocoa butter and functional oils blend

Cocoa butter was melted in oven at 50°C. Liquid functional oils were then blended into melted cocoa butter using high speed mixer for 5 minutes. The oil blends were then kept in oven at 50°C within 24 hours before incorporated into sugar free dark chocolate formulation.

The sugar free dark chocolates with or without functional oil blends of fish, pumpkin and flax seed oil were formulated base on oil blends (OB) in Table 1. The chocolate samples [SDCOB1 (Control), SDCOB2, SDCOB3, SDCOB4, SDCOB5, SDCOB6, SDCOB7, SDCOB8] were developed by replacing sucrose with xylitol, a type of sugar alcohol with an equal sweetness level to sucrose. Method was based on Beckett (1994) but with minor modification on cocoa solid, fat content and other chocolate ingredients. The total fat in the formulation was fixed to 36%. The chocolate samples were manually tempered at controlled room temperature of 22- 23°C and molded into bar form. The products were then sealed in high density polyethylene (HDPE) plastic and stored at 20°C and analyzed monthly on gloss index (at 85° angle), water activity (Aw) and peroxide value (PV) for duration of 6 months.

Table 1. Cocoa butter with functional oil blends.

Treatment	Cocoa butter (%)	Pumpkin seed oil (%)	Flaxseed oil (%)	Fish oil (%)
OB1-Control	100	0	0	0
OB2	81.0	5.6	2.8	10.7
OB3	78.6	5.6	2.8	13.1
OB4	89.3	0	0	10.7
OB5	86.9	0	0	13.1
OB6	84.6	0	0	15.4
OB7	91.7	5.6	2.8	0
OB8	83.3	11.1	5.6	0

Gloss

The gloss value of all sugar free dark chocolate samples were measured monthly by using Trimicrogloss (Shenn, England) at projection angle of 85° at 24°C. A beam of light was projected on to the flat surface of chocolate and the strength of light reflected within a narrow angle was measured. Angle of projection used was 85 degree. The numerical scale used was a reference to 100 for a black glass, which was calibrated at each time of measurement. Measurement was made in 6 replications and the mean values were calculated.

Water activity (A_w)
Measurements of water activity (***A_w***) of the sugar free dark chocolate samples were carried out using Aqua Lab Water Activity Meter 4TE, Decagon, USA.

Peroxide value (PV)
The determination of peroxide value for sugar free dark chocolate samples was carried out according to Tee et al, (1996). One (1) to 4 g of oil sample was weighted and transferred into 250 ml round bottom flask, and then 10 ml of chloroform was added to dissolve the oils and swirled for few seconds. Later, 15 ml of glacial acetic acid was added followed with 1 ml fresh saturated aqueous potassium iodide solution. The solution then was shaken for 1 minute and kept in dark for 5 minutes. After that, 75 ml of distilled water was added followed by few drops of starch solution (1%). The sample solution was then titrated with 0.002N sodium thiosulphate. The peroxide value was calculated as the difference between the volumes, in ml, of 0.002N sodium thiosulphate consumed, multiplied by Normality then multiplied by 10 and divided by the weight, in g, of the sample taken.

Statistical analysis was conducted using statistical software, Minitab version 13 to compare glossiness, water activity and peroxide value among samples at each month of 6 months study period.

RESULTS AND DISCUSSION

Eight samples of sugar free dark chocolate bars developed from various portion of oil blends was sealed in high density polyethylene (HDPE) plastic and stored at 20°C for 6 months. The analysis on gloss index (at the angle of 85 degree), water activity (A_w) and peroxide value (PV) of each sample was checked monthly for 6 months storage duration.

The results of experiments were statistically analyzed and presented in Table 2, 3 and 4 then plotted into graph as shown in *Figure 1, 2 and 3.*

Gloss value
Figure 1 and Table 2 show that almost all samples of sugar free dark chocolate show a pattern of gloss index reduction during storage. However, control sample of SDCOB1, a dark chocolate containing only cocoa butter (without functional oils) was significantly glossy ($p<0.05$) at each month of whole period of storage study compared to those sample containing functional oils. The glossiness of SDCOB1 was significantly reduced until the 2nd month of the storage, then the gloss maintained until the 6th month of the storage period. Sample SDCOB5, a blend of cocoa butter with medium amount of fish oil was found to have the highest gloss value among other chocolate with functional oils. However, the value was significantly lower than SDCOB1 at each month of storage. SDCOB5 remains glossy until the 4th month of storage then the value dropped significantly at the 5th and 6th months. Sample SDCOB2 with minimum amount of fish, pumpkin and flax seed oil was the worst where the gloss value dropped significantly at each month throughout the whole storage period.

The hygroscopic effect of chocolates might attract moisture from trapped air in sealed HDPE plastic packaging to the chocolate surface and affected the gloss value of chocolate samples. This phenomenon is continuously occurred during storage and it became worse in chocolate which contain functional oils. SDCOB8 were very glossy at 0 and 1st month of storages; however it were drastically dropped on the 2nd month onward. High gloss at the earlier stage might be influenced by the presence of a thin layer of melted functional oil surround the chocolate surface since this sample contain slightly high amount of pumpkin and flax seed oil. All chocolate samples containing functional oils lost their shininess by months of storage except SDCOB1, where it almost remains shiny. However no bloom formation was observed in all samples.

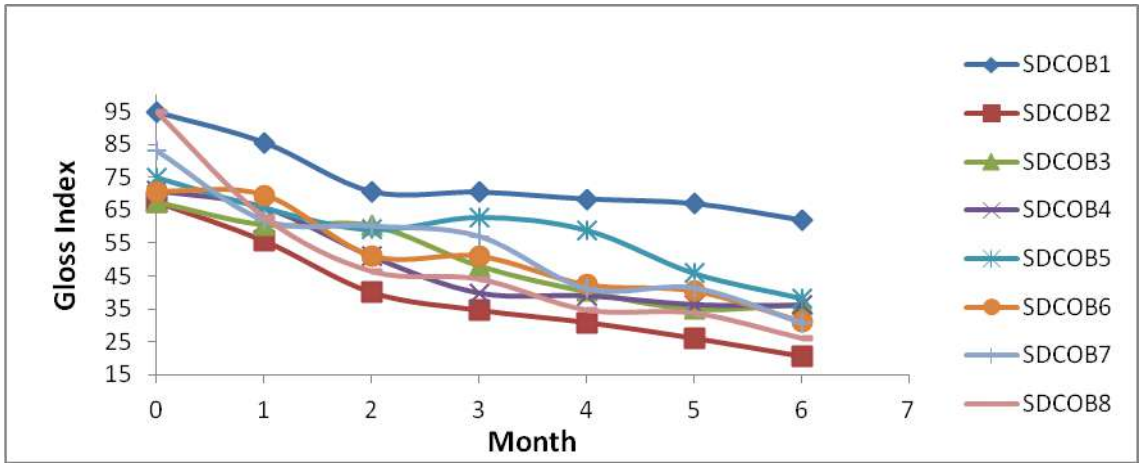

Figure 1. Glossiness of sugar free dark chocolate with functional oils during 6 months storage.

Table 2. Analysis of variance (ANOVA) on glossiness (at 85° angle) of sugar free dark chocolate with cocoa butter and functional oils blends in duration of 6 month storage.

	Month						
Sample	0	1	2	3	4	5	6
SDCOB1	94.75 ± 6.72 $^{a, A}$	85.9 ± 3.54 $^{a, A}$	70.60 ± 0.71 $^{ab, B}$	70.45 ± 3.61 $^{a, B}$	68.35 ± 0.78 $^{a, B}$	66.95 ± 8.41 $^{a, B}$	61.9 ± 3.68 $^{a, B}$
SDCOB2	67.40 ± 2.12 $^{b, A}$	55.6 ± 1.13 $^{b, B}$	40.00 ± 1.84 $^{c, C}$	34.6 ± 6.36 $^{b, CD}$	30.9 ± 0.28 $^{c, D}$	26.1 ± 1.56 $^{d, ED}$	20.6 ± 2.69 $^{e, E}$
SDCOB3	67.35 ± 2.76 $^{ab, A}$	60.50 ± 8.49 $^{b, AB}$	60.3 ± 2.69 $^{ab, AB}$	48.05 ± 1.20 $^{c, B}$	40.35 ± 3.18 $^{c, BC}$	35.05 ± 3.89 $^{c, C}$	36.5 ± 0.14 $^{b, BC}$
SDCOB4	70.95 ± 4.17 $^{b, A}$	65.85 ± 3.18 $^{ab, A}$	50.95 ±0.35 $^{bc, B}$	39.85 ± 1.20 $^{c, C}$	39.05 ± 0.07 $^{d, C}$	36.35 ± 3.32 $^{c, C}$	36.05 ± 0.35 $^{b, C}$
SDCOB5	74.85 ± 3.47 ^{b,A}	65.6 ± 3.25 $^{b, AB}$	59.1 ± 8.06 $^{b, B}$	62.85 ± 1.91 $^{ab, B}$	58.9 ± 4.1 ^{b,B}	45.85 ± 1.49 $^{b, C}$	38.3 ± 2.4 $^{b, C}$
SDCOB6	70.75 ± 4.31 $^{b, A}$	69.55 ± 3.75 $^{ab, A}$	51.1 ± 7.50 $^{bc, B}$	51.0 ± 6.93 $^{bc, B}$	42.35 ± 2.19 $^{d, BC}$	40.4 ± 1.56 $^{bc, BC}$	31.15 ± 0.300 $^{b, C}$
SDCOB7	83.0 ± 2.55 $^{ab, A}$	62.0 ± 1.84 $^{b, B}$	60.3 ± 0.71 $^{b, B}$	57.1 ± 3.96 $^{ab, B}$	41.3 ± 0.57 $^{d, C}$	41.5 ± 1.84 $^{bc, C}$	30.95 ± 0.35 $^{b, D}$
SDCOB8	94.95 ± 6.86 $^{a, A}$	62.9 ± 8.49 $^{b, B}$	46.4 ± 1.84 $^{bc, C}$	44.05 ± 2.9 $^{c, C}$	34.6 ± 6.51 $^{b, CD}$	33.65 ± 2.33 $^{c, CD}$	25.95 ± 1.06 $^{e, D}$

a) Mean value followed by different small letters in the same row are significant different at p < 0.05
b) Mean values followed by different capital letters in the same column are significant different at p <0.05

The water activity changes of sugar free dark chocolate with functional oils during storage

The water activity (A_w) is a numerical value for measuring the amount of free water that microorganisms can use for their growth, and it is known that microorganisms capable of proliferation vary depending on that value. The A_w of chocolate is generally low ranging from 0.1 to 0.2 because it contain cocoa butter and sugar (sucrose) which bound the water presence in the formulation with the moisture content as low as 1% and none of microorganisms could grow in this condition. Sucrose is a low hygroscopic sugar which had low capability of absorbing moisture from atmosphere.

Figure 2 shows that all samples have the same pattern of A_w changes during storage where there were slightly high values at 0 month ranging from 0.52 to 0.66 and suddenly dropped at the 1st month within 0.45 to 0.51 and slowly increased at the 2nd month onward until the end of the 6th month of storage duration. The value of Aw range at the end of storage study was from 0.74 to 0.79. The values were high enough to stimulate the particular mold to grow. Both samples of SDCOB2 and SDCOB3 which contain minimum and medium amount fish oil besides minimum amount of pumpkin and flax seed oil were significantly high on A_w value especially at stages 3 to 4 months of storage compared to other samples.

The hygroscopicity of the chocolates will absorb high moisture during storage and will affect the water activity in the products. Proper storage with right packing material at correct storage temperature and relative humidity could minimize the increment of A_w and shelf life of the products.

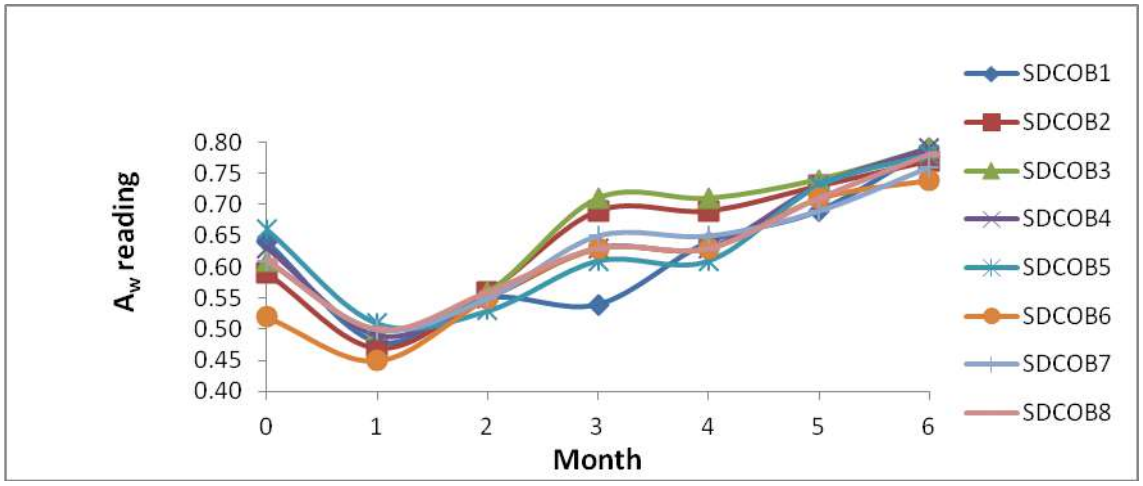

Figure 2. Water activity (A_w) reading of sugar free dark chocolates with different portion of functional oils within 6 months storage

Table 3. Statistical analysis on water Activity of sugar free dark chocolate with functional oils blend during 6 months storage.

	Month						
Sample	0	1	2	3	4	5	6
SDCOB1	0.64 ± 0.01 $^{a, BC}$	0.48 ± 0.03 $^{ab, E}$	0.55 ± 0.01 $^{a, D}$	0.54 ± 0.01 $^{c, D}$	0.64 ± 0.01 ^{b,C}	0.69 ± 0.02 $^{b, B}$	0.79 ± 0.01 ^{a,A}
SDCOB2	0.59 ± 0.00 ^{ab,D}	0.47 ± 0.01 ^{ab,F}	0.56 ± 0.00 $^{a, E}$	0.69 ± 0.00 ^{ab,C}	0.69 ±0.00 ^{ab,C}	0.73 ± 0.01 ^{ab,B}	0.77 ± 0.01 $^{ab, A}$
SDCOB3	0.61 ± 0.01 ^{ab,C}	0.50 ± 0.01 $^{a, E}$	0.56 ± 0.02 $^{a, D}$	0.71 ± 0.00 $^{a, B}$	0.71 ± 0.00 $^{a, B}$	0.74 ± 0.00 $^{a, B}$	0.79 ± 0.01 $^{a, A}$
SDCOB4	0.63 ± 0.09 $^{ab, B}$	0.49 ± 0.00 ^{ab,C}	0.55 ± 0.02 $^{a, BC}$	0.63 ± 0.01 ^{b,B}	0.63 ±0.01 ^{b,B}	0.73 ± 0.00 $^{ab, AB}$	0.79 ± 0.03 $^{a, A}$
SDCOB5	0.66 ± 0.02 $^{a, B}$	0.51 ± 0.01 $^{a, C}$	0.53 ± 0.01 $^{a, C}$	0.61 ± 0.04 ^{b,B}	0.61 ± 0.04 $^{b, B}$	0.73 ± 0.01 $^{ab, AB}$	0.78 ± 0.01 $^{ab, A}$
SDCOB6	0.52 ± 0.01 ^{b,C}	0.45 ± 0.02 $^{b, D}$	0.55 ± 0.01 $^{a, C}$	0.63 ± 0.00 ^{b,B}	0.63 ± 0.00 ^{b,B}	0.71 ± 0.01 $^{ab, A}$	0.74 ± 0.00 $^{b, A}$
SDCOB7	0.61 ± 0.02 ^{ab,C}	0.5 ± 0.01 $^{a, D}$	0.55 ± 0.01 $^{a, D}$	0.65 ± 0.01 $^{b, BC}$	0.65 ± 0.01 $^{b, BC}$	0.69 ± 0.02 $^{b, B}$	0.76 ± 0.02 $^{ab, A}$
SDCOB8	0.61 ± 0.01 $^{ab, CD}$	0.5 ± 0.01 $^{a, E}$	0.56 ± 0.00 $^{a, D}$	0.63 ± 0.03 ^{b,C}	0.63 ± 0.03 $^{b, C}$	0.71 ± 0.03 $^{ab, B}$	0.78 ± 0.01 $^{ab, A}$

a) Mean value followed by different small letters in the same row different at p < 0.05

b) Mean values followed by different capital letters in the same column are sig. different at p <0.05

Peroxide value of sugar free dark chocolate with functional oils during storage

The peroxide value is the number that expresses, in milliequivalent (mEq) of active oxygen, the quantity of peroxide contained in 1000 g of the substance. A low peroxide value meant the chocolate are more stable towards oxidation.

The result shows that there is a correlation between peroxide value and amount of each of functional fat in the chocolate where the value was increased by increasing amount of functional oils in the formulations *(Figure 3,* Table 4). Control sample of sugar free dark chocolate which contain only cocoa butter (SDCOB1) was significantly lowest in peroxide value while SDCOB2 and SDCOB3, both contain minimum pumpkin and flax seed oil followed by minimum and medium amount of fish oil respectively were significantly found to be the highest level of peroxide value compared to others ($p<0.05$). All samples containing flax seed oil tend to give high level of peroxide value while samples which contain blended cocoa butter with only fish oil in any RDA level (SDCOB4, SDCOB5 and SDCOB6) gave relatively low peroxide value. However, the level of the peroxide value for all chocolate samples was acceptable since below than 10 mEq/kg (The Codex Alimentarius, 2001).

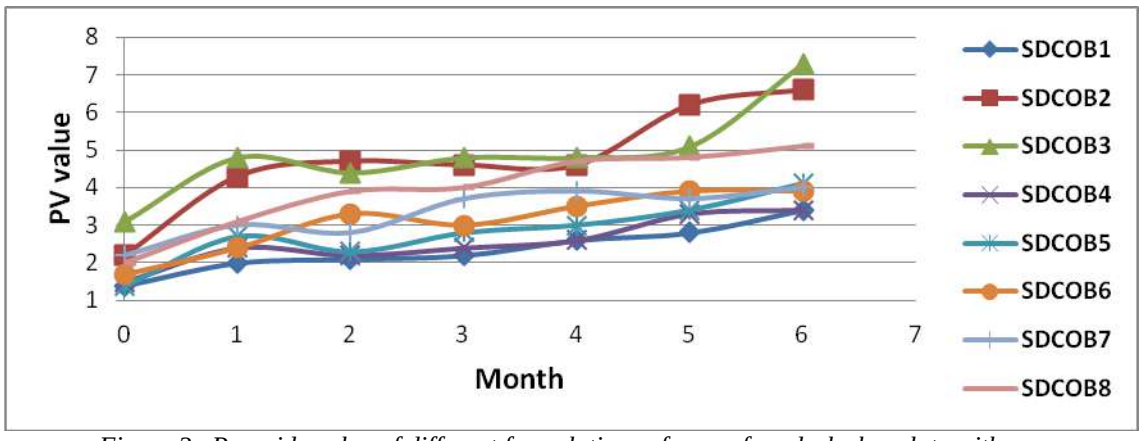

Figure 3. Peroxide value of different formulations of sugar free dark chocolate with functional oils within 6 month of storage

Table 4. Statistical analysis on peroxide value of sugar free dark chocolate with functional oils during 6 months of storage

	Month						
Sample	0	1	2	3	4	5	6
SDCOB1	1.4 ± 0.28 $^{b, B}$	2.0 ± 0.00 ^{b,B}	2.1 ± 0.42 $^{b, B}$	2.2 ± 0.28 ^{d,B}	2.6 ± 0.28 $^{b, AB}$	2.8 ± 0.00 ^{c,AB}	3.4 ± 0.57 $^{b, A}$
SDCOB2	2.2 ± 0.00 ^{ab,C}	4.3 ± 1.14 $^{ab, B}$	4.7 ± 0.14 $^{a, B}$	4.6 ± 0.28 $^{ab, B}$	4.6 ± 0.28 ^{ab,B}	6.2 ± 0.28 ^{a,A}	6.6 ± 0.85 $^{ab, A}$
SDCOB3	3.1 ± 0.14 ^{a,C}	4.8 ± 0.28 $^{a, B}$	4.4 ± 0.57 ^{ab,B}	4.8 ± 0.28 $^{a, B}$	4.8 ± 0.28 ^{a,B}	5.1 ± 0.14 ^{ab,B}	7.3 ± 0.14 $^{a, A}$
SDCOB4	1.5 ± 0.42 $^{b, B}$	2.4 ± 0.57 $^{b, AB}$	2.2 ± 0.28 $^{b, AB}$	2.4 ± 0.00 ^{cd,AB}	2.6 ± 0.28 ^{b,AB}	3.3 ± 0.42 ^{c,A}	3.4 ± 0.57 ^{b,A}
SDCOB5	1.4 ± 0.28 $^{b, B}$	2.7 ± 0.99 $^{b, AB}$	2.3 ± 0.42 $^{b, B}$	2.8 ± 0.00 $^{cd, AB}$	3.0 ± 0.57 ^{b,AB}	3.4 ± 0.57 ^{c,AB}	4.1 ± 0.42 ^{b,A}
SDCOB6	1.7 ± 0.42 $^{b, B}$	2.4 ± 0.57 $^{b, B}$	3.3 ± 0.70 ^{b,AB}	3.0 ± 0.00 ^{c,AB}	3.5 ± 0.14 ^{b,AB}	3.9 ± 0.14 ^{bc,A}	3.9 ± 0.71 ^{b,A}
SDCOB7	2.2 ± 0.00 $^{ab, B}$	3.0 ± 0.00 $^{b, AB}$	2.8 ± 0.28 ^{b,B}	3.7 ± 0.42 $^{bc, AB}$	3.9 ± 0.42 $^{ab, AB}$	3.7 ± 0.42 $^{bc, AB}$	4.0 ± 0.57 $^{b, A}$
SDCOB8	2.0 ± 0.57 $^{b, B}$	3.1 ± 0.14 $^{b, B}$	3.9 ± 0.42 $^{ab, AB}$	4.0 ± 0.00 ^{b,AB}	4.7 ± 0.42 ^{a,AB}	4.8 ± 0.85 $^{b, AB}$	5.1 ± 0.71 ^{b,A}

a) Mean value followed by different small letters in the same row different at $p < 0.05$

b) Mean values followed by different capital letters in the same column are sig. different at $p < 0.05$

CONCLUSIONS

All samples show the same increasing pattern in physicochemical changes of peroxide value, water activity and glossiness during storage. Control sample which contain cocoa butter without functional oils gave significantly lower value of peroxide value, A_w and higher gloss value compared to other samples containing functional oils. Proper packaging materials is necessary to properly pack the sugar free chocolate and to ensure that it is tight enough to protect from moisture absorption from outside the packaging.

ACKNOWLEDGEMENTS

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A PRELIMINARY INVESTIGATION OF A NOVEL PROCESS OF WET FERMENTED BEANS MICRONIZING AND ITS EFFECT ON BEANS FINAL QUALITY AND POLYPHENOLS CONTENTS

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ABSTRACT - *The aim of this study was to introduce the micronizing step earlier before drying process in order to explore the potential of consolidating the cocoa bean processing steps. Four levels of wet bean moisture content (33%, 28% and 23%, 16%) were micronized at three different levels of infra-red temperatures (220°C, 200°C and 180°C). The micronizing time was set constant at six minutes. The control experiment was conducted using dried beans with 7.5% moisture content and 165°C infra-red temperature for three minutes. The effectiveness of the separation process, measured by the present amounts of the non-retrieved beans (U%) was assessed. On top of that, the final nibs moisture content (Mcf) and the polyphenols contents was analyzed and determined. The highest percentage of non-retrieved beans (U%) was detected in the 28 and 33% wet beans moisture and 180°C temperature. The non-retrieved beans was not detected in the 23% and 16% moisture for all three temperatures. The lowest nibs final moisture content (Mcf) was 3.26% in the 16% wet beans moisture and 220°C temperature. This value was found to be not significantly different ($p<0.05$) from the control. This suggests that the micronizing process could be successfully conducted on wet fermented cocoa beans. The polyphenols quantification analysis generally results in a reduction of the total polyphenols, catechin and epicatechin contents. Results from this study shows potential of early micronizing with wet beans, which could path the way for consolidating cocoa processing steps. Early micronizing process, especially the one that can be conducted during the drying stage, will not only reduce the drying time, but also creates the prospect of trading processed nibs as a semi-finished product.*

***Keywords**:* Cocoa bean processing, wet beans, micronizing, polyphenols

INTRODUCTION

Micronizing is a heating procedure conducted on dried cocoa beans in the processing factory. The purpose of micronizing is to give the cocoa beans a short high temperature thermal shock in order to expand the beans and loosen the shell from the nibs (Beckett, 2009). The beans will normally appear puffy after the micronizing treatment. The infrared burner is widely used in the cocoa bean processing factory for this purpose. The standard procedure of micronizing is by heating the dry beans to 165°C for two to three minutes, although the temperature and the duration might vary depending on the beans condition (Mulato, 2012). Immediately after micronizing, the beans are subjected to the breaking and winnowing process. The broken beans known as 'nibs' will be retrieved and used in further processing steps. On the other hand, the shells are collected and market as cocoa by products (Kealey *et al.*, 2000; DeZann, 1999). If for some reasons the micronizing process does not being conducted properly, then the separation process will not be effective. When this happens, the nibs will be contaminated with higher amounts of shells and vice versa. Additionally, the beans will be poorly broken and the amounts of unbroken beans will be higher. Higher percentage of unbroken beans will affect the processing quality as well as delaying the processing time. On the other hand, the cocoa bean moisture content which stood at 7.5% at the beginning of the micronizing process will be reduced due to the heating and drying effect. Typically, the micronized bean moisture content is reduced to $3.9\pm0.5\%$ (Anshary, 2015) . Factors such as micronizing temperature, heating time and the moisture condition of the beans would affect the separation performance and the final moisture contents of the nibs. Similarly, the other valuable anti-oxidants compounds such as total polyphenols and other phenols which is heat

sensitive will be affected (Andújar, Recio, Giner, & Ríos, 2012). However, up to this date, there has been no reported study on the effect of infrared micronizing conducted on wet beans. We envisage that if wet beans can be micronized, then cocoa processing at the primary processing stage can be consolidate and merged with the secondary processing stage. A few advantages can be derived from this process merging. Firstly, the whole cocoa process can be intensified and more option for the drying method can be introduced. One particular drying method which draws our interest is the use of electromagnetic fields in the form of microwave to speed up the drying process in the primary processing. Similarly, the use of electromagnetic can be applied in the micronizing process. Secondly, the nibs having been separated from its shell will have no protective layer. This layer acts as a barrier for the hot air to penetrate the kernel. The present of the layer in the beans will slow down the removal of gaseous vapor to the surroundings and this translates as longer drying time. Other advantages include less shipment weight, less energy and ready material for factory used.

Therefore, the aim of this study was to introduce the micronizing step earlier before drying process in order to explore the potential of consolidating the cocoa bean processing steps. For this purpose, different levels of wet beans moisture were subjected to selected infrared temperature and its effect on separation efficiency, final nibs moisture content and polyphenols contents was determined.

MATERIALS AND METHODS

Sample preparation

Fresh fermented beans were obtained from Stesen Penyelidikan dan Pembangunan Koko Jengka Pahang. The samples were stored in a chiller (-30ºC) before used. Initial moisture content of the sample was determined immediately using AOAC (2000) method. Four different moisture conditions were prepared by drying the sample in the convection laboratory oven (Memmert) from initial moisture to the desired moisture content by weight change method.

Micronizer apparatus

An electrical heated infrared drum micronizer (G.W. Barth GmbH) was used to conduct the micronizing procedure. The unit has a temperature setting range from 90 to 250ºC and its rotation speed can be set from 5 to 15 rpm. Normally, the micronizer is set at 165 º C for 3 minutes as a standard procedure to micronized dry beans. Based on our experiences, three temperature setting was selected to conduct wet beans micronizing. The selected temperature were 180, 200 and 220 ºC. The drum rotation speed was selected to rotate at 10 rpm and the micronizing times were maintained for a duration of 6 minutes. For each test, 200gm of beans were used as sample and the experiments were conducted in triplicates and the average value was reported. The control experiments were conducted using dried beans with 7.5% moisture and 165 ºC infrared temperatures for three minutes.

Experimental procedure

This study investigates the potential of wet fermented cocoa beans micronizing, its physical quality and its polyphenols content. Four different levels of beans moisture content were identified in this experiment, i.e.; 33 %, 27%, 23% and 16%. These four different moisture levels were subjected to three different infrared temperatures. The selected infrared temperature were 180, 200 and 220ºC. A total of 200 gm of wet fermented beans was used in each treatment and the treatment was replicated three times. After the beans has been micronized, it went to the breaking process using a mechanical breaker (Gordon, UK). A proper micronized beans will appear plum and puffy and can be easily broken. The broken beans contain a mixture of broken nibs and fragments of shells. This mixture then goes through a winnowing process using a winnower machine (Gordon, UK) to separate the broken nibs and the shell fragments. The effectiveness of the separation process in terms of the percentage of unbroken beans, denotes as non-retrieved beans (U%) was identified as one of the determining factors in this study. Besides the separation efficiency, the nibs final moisture contents (MCf) and its total polyphenols and other phenol derivatives, namely epicatechin and catechin contents were also measured and analyzed.

Breaking

The breaking of the micronized beans was conducted immediately after the micronizing process. A laboratory size electric motor driven impact breaker (John Gordon & Co. UK) was used to break the micronized beans. The breaker has rotating disc which turn clockwise and the beans were compressed against a static disc. The gap between the static and the rotating disc can be adjusted by turning the control knob.

Total Polyphenol determination

The amount of total polyphenols was determined using a method described by Markham and Bloor (1998). The total polyphenol content was determined by the spectrophotometric method of Folin-Ciocalteu. Quantitative HPLC preparative method was used for the determination. The sample was grounded in a high speed laboratory mill until its particle size was reduced to approx 90µm. The mill cocoa samples (1 g) will be extracted with 100 mL of acetone:water (70:30; v:v) under reflux at 60ºC for 2 h. Acetone was removed under vacuum at 30ºC. The analysis was performed using UV-Visible Spectrometry Shimadzu. The blank was used as a reference cell, the slid width 0.2mm with 765nm of wavelength. The corvette was used as a sample container which was transparent to the electromagnetic radiation. The standard was prepared by diluting gallic acid. The analysis was carried out using defatted sample. The result was expressed as gallic acid equivalent on a dry weight basis.

Preparation of extracts for phenolics content determination

Defatted cocoa beans samples were extracted with 80% aqueous acetone (80 ml) for 30 min at 50°C using a sonicater. The mixture was then filtered through a filter paper (Whatman No. 4) and the residue of glassware was washed with 80 % aqueous acetone and total volume of filtrate was made up to 100 ml in a volumetric flask. 10 ml of the extract was dried on a rotary evaporator at 45°C. The extraction obtained was suspended with 5ml deionised water. For following filtration, SPE cartridge C18 was preconditioned using 5 ml MeOh and continuously preconditioned by 5 ml deionised water. Then inject the pooled extract through the SPE cartridge, the resulting residue was discarded. The epicatechin, which was retained by the SPE cartridge was eluted with about 10 ml of 40 % aqueous MeOH. The final volume elute was made up to 10 ml in a volumetric flask. 1.5 ml of final extraction was filtered through a nylon filter into a vial 10 microliter of this final solution was injected during the HPLC analysis.

RESULTS AND DISCUSSIONS

Separation efficiency of the micronized beans

The effectiveness of the separation process was considered crucial in this study. The effectiveness of the separation process was assessed by determining the presents of non retrieved beans after the micronizing process. *Figure 1* shows the result of wet beans micronizing conducted at 180ºC. *Figure 2* shows the result for wet micronizing at 200ºC, whereas *Figure 3* shows the result conducted at 220ºC. Generally, the effectiveness of the separation improved with lower amount of non retrieved beans present at low beans moisture and low micronized temperature. At 23% and 16% moisture content, there is no present of non retrieved beans except for 220ºC temperature treatment. This could be due to the conditions of the beans, where at 23% mc, the beans had achieved its skin dry condition (Faborode, Favier, & Ajayi, 1995), and further heating will be concentrated at the kernel (the nibs). Heating the kernel rapidly at this level will quickly convert the water to steam and this will eventually lead to the bean expansion. The expanded bean caused the detachment of the kernel from the shell and this condition is believed to facilitate the breaking and separation process.

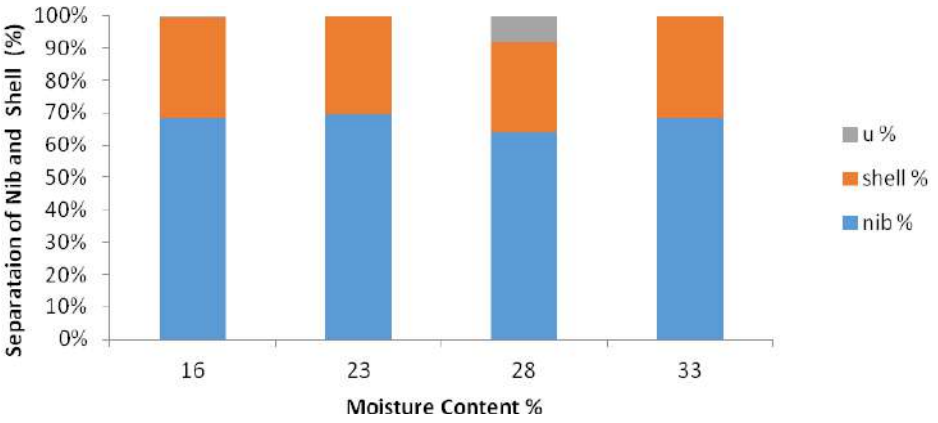

Figure 1. Separation efficiency in the 180°C temperature

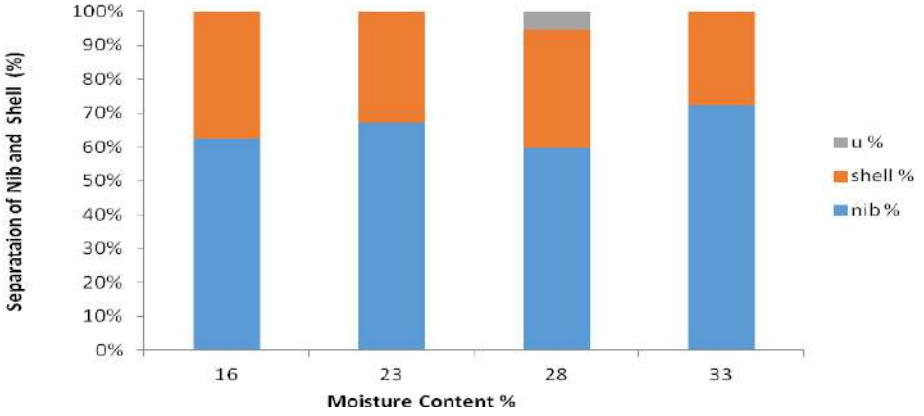

Figure 2. Separation efficiency in the 200°C temperature

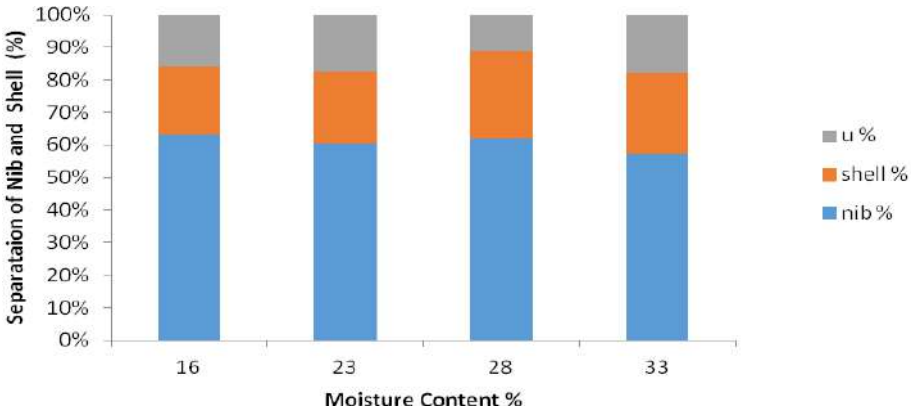

Figure 3. Separation efficiency in the 220°C temperature

The results of one way ANOVA on the unbroken beans percentage (U%) are as shown in Table 1. The present of the non retrieved beans was detected during sieve shaker operations and mostly was retained in the 4 mm mesh sieve size. The percentage of U% ranges from 12.43 to 5.37%. The non retrieved beans were detected at 33 and 28% wet beans moisture for all three temperatures. High percentage of U% was observed in 180ºC infrared temperature and 33% wet beans moisture. The high percentage of U% detected could be due to the physical condition of the beans, which was elastic and therefore failed to break when passed through the impact breaker. The elasticity of the beans was mainly detected in the high wet beans moisture. This could be due to the transfer of moisture from the internal bean to skin layer when the skin becomes drier due to the infrared heating. The other reason could be a slower rate of moisture migration from the internal bean since the mechanism of infrared heating is effective to dry the outer layer (Li *et al.*, 2014). The slower moisture rate will result in dense and non porous nibs which contribute to the elasticity characteristic (Nganhou, 2004). In one of the infrared micronizing study using high moisture lentils for pre-treatment, the end product of lentils also resulted in a softer texture (Arntfield, S.D. 1997). In the low wet beans moisture (23% and 16%), less residual moisture was available for the moisture migration to happen, therefore the beans become crisper and expanded. In addition, in the 23% wet beans moisture, the beans had achieved a dry skin condition and any further heating will heat the nibs (de Brito *et al.*, 2001). Heating the nibs at this level will rapidly convert the water to steam and this will eventually lead to the bean expansion. The crisped and expanded beans could be easily broken. This was observed in the 23 and 16% wet beans moisture and thus explained the absence of non retrieved beans in these two samples. Table 1 shows the mean value of the non retrieved beans for each wet beans moisture and infrared temperatures. The non retrieved beans (U%) were not detected in 23% and 16% wet beans moisture, which was similar to the control experiment.

Table 1. The mean value ±std deviation of non retrieved beans (U%) using one way ANOVA

Wet beans moisture	Infrared Temperature ºC			
	180ºC	200ºC	220ºC	Control (165ºC and 7.5% beans moisture)
33	$12.34 \pm 1.00^{b,A}$	$8.60 \pm 0.70^{a,A}$	$6.76 \pm 0.25^{a,A}$	Not detected (nd)
28	$8.46 \pm 0.40^{b,B}$	$6.76 \pm 0.42^{a,B}$	$5.36 \pm 1.01^{a,B}$	
23	nd	nd	nd	
16	nd	nd	nd	

*Means (value ± std.dev.) with the same small letter in a same column and capital letter in a same row are not significantly different at p>0.05. Means (value ± std.dev.) followed by * are not significantly different at p>0.05.*

Final moisture content

Another determining factor in this study was the nibs final moisture content (Mcf). This factor determined the effect of the infrared temperature towards the reduction of bean moisture content. A reduction in bean moisture contents is an indicator for the dryness level of the nibs. It is believed that dry nibs will make the separation process easier. Results show that from three temperatures, only 200ºC temperature showed a linear correlation towards the moisture contents reduction as shown in *Figure 4.* The correlation can be represented by the linear equation y=0.670x-5.014 with 0.99 R-squared value. The other two temperatures did not result in a positive correlation, and therefore was not shown in the graph. However, in the 16% beans moisture and 200ºC temperature, the nibs moisture had significantly reduced to 4.5%, a condition that is close to the desired nibs moisture after micronizing process (3.9%). This result was considered as one of the major findings in this study, particularly in term of the potential in conducting micronizing process using wet beans.

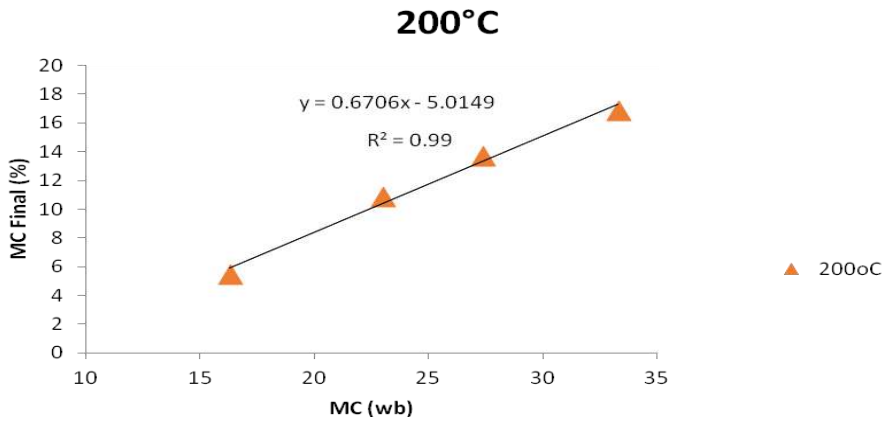

Figure 4. Moisture content reduction of the beans at 200°C

Analysis of Total polyphenols, catechin and epicatechin content

The samples from each treatment were further analyzed and quantified for its total polyphenols and phenolic contents. All the samples were freeze dried prior to the sample preparation steps. Chemical analysis was conducted in triplicates and the average value is reported here. *Figure 5* shows the results of total polyphenols analysis of the dried nibs in three different moisture content and three different temperatures. In the 180°C temperature, the total polyphenols contents reduced progressively from 98.44 to 87.96 mg/g GAE equivalent for 33% and 23% moisture content respectively. However, in the 200°C temperature, the total polyphenols content showed only a marginal reduction, from 92.17 to 91.74 mg/g GAE equivalent in the 33% and 23% moisture content. In the 220°C temperature, the total polyphenols showed a contradiction pattern. The total polyphenol content in the 33% moisture content was 94.92 mg/g GAE equivalent. However, the amount increased to 96.31 mg/g GAE equivalent in the 23% moisture content. The reasons for the increase was uncertain. It could probably due to the error in the analysis since total polyphenols is a highly sensitive compounds. Other study related to the total polyphenols determination also reported a degradation in the amounts when treated with higher temperature and relative humidity (Daud, Talib, & Kyi, 2007).

Similar reducing trend was observed in the catechin content as shown in *Figure 6*. The amount of catechin contents was 0.46 mg/g in the 33% moisture and 0.21 mg/g in the 23% moisture at 180°C temperature. In the 200°C temperature, the catechin contents were in the range of 0.2 mg/g for both 23 and 28% beans moisture content. The highest catechin contents was detected in the 180°C temperature and 33% beans moisture content. Between the three temperatures, only 180°C temperature showed a progressive retention of catechin. This means that the amount of moisture available probably assists in retaining the catechin amount. A study on the impact of cocoa processing on the phenols content reported a reduction of up to 80% in the catechin amount when subject to 'Dutch processing' which involves high temperature (Payne, Hurst, Miller, Rank, & Stuart, 2010). It also suggests that the increased on the amounts of catechin could be due to epimerization of (-)-epicatechin.

On the other hand, the amount of epicatechins was found to be generally higher compared to catechins as shown in Figure 6. The results show the epicatechin contents was in the range of between 2.04 mg/g to 6.94 mg/g. These values were found to be in confirmation with other researchers finding (Albertini *et al.*, 2015, Hurst *et al.*, 2011, Kothe, Zimmermann, & Galensa, 2013). The highest amount of epicatechin content was 6.96 mg/g, in the 180°C temperature and 28% moisture. The retention amounts of epicatechin did not follow a similar pattern as in the catechin. However, in the 220°C temperature, the epicatechin content was found to be reduced, which could well be the effect of high temperatures. In the 23% moisture, the epicatechin amounts were 4.36 mg/g but in 33% moisture the epicatechin was found to reduce to almost half of the amount (2.04 mg/g). Finally,

the epicatechin contents showed a marginal variation amongst the wet beans at 200ºC temperature. Similar reduction pattern of epicatechin has been reported in cocoa drying study, where approximately 75% of epicatechin concentration was reduced at the end of fermentation and drying (Albertini *et al.*, 2015).

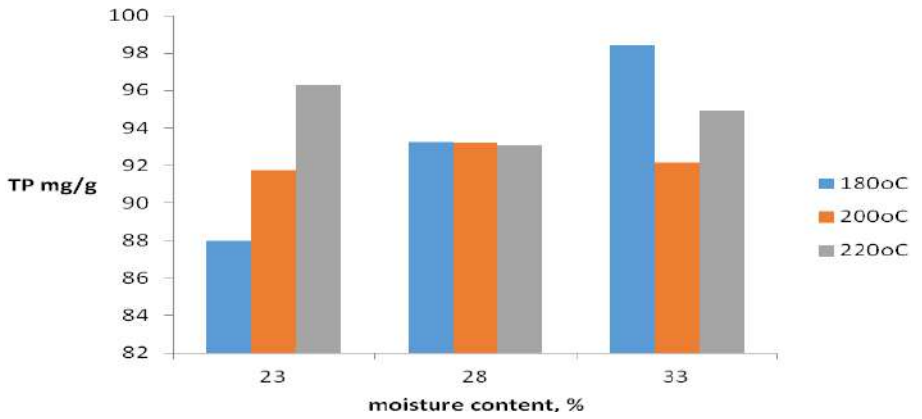

Figure 5. Total polyphenols content at three temperature treatment and three wet beans moisture

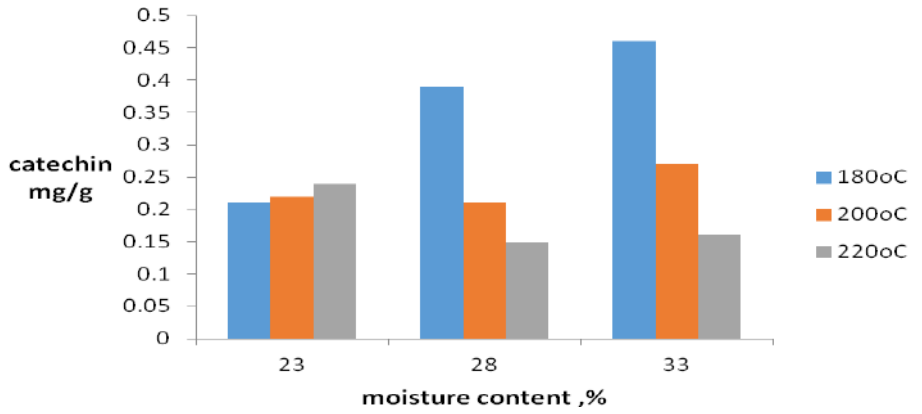

Figure 6. Catechin contents at three temperature treatment and three wet beans moisture

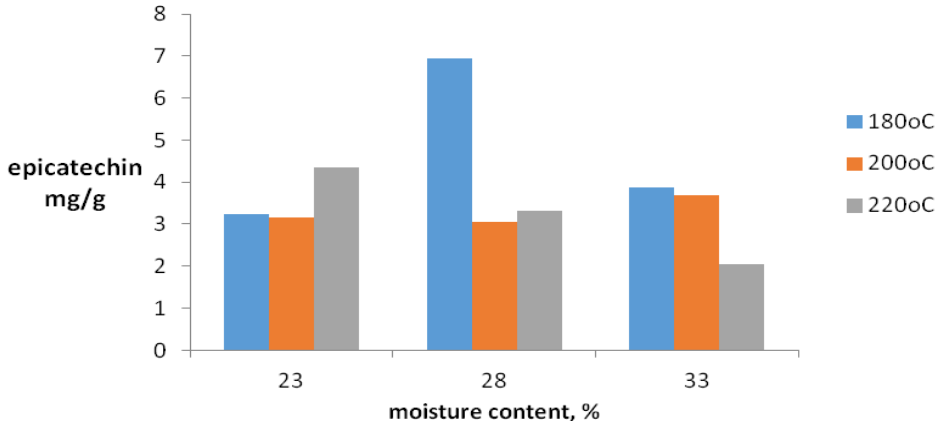

Figure 7. Epicatechin contents at three temperature treatments and three wet beans moisture

CONCLUSION

This paper highlight some of the results on the potential of wet beans micronizing using fermented wet cocoa beans. The results provide some avenues to explore the works of the intensification process of the cocoa beans processing techniques. Particularly, the results which showed that the wet fermented beans can be micronized at moisture content higher than 7.5%, with an acceptable level of separation efficiency. This means that the wet beans could be partially dried in the primary processing and then continues to the micronizing steps, and this will make the separation process easier. If the cocoa beans can be dry without the shells, the drying process are expected to be more efficient. The polyphenols quantification analysis indicates a certain level of preservation could be achieved with gentle heat treatment combined with higher moisture contents. Unfortunately, the polyphenols analytical data for 16% beans moisture was not available, and therefore the phenolic compound analysis was not presented. The next step in this project is to conduct trials on nibs drying using a domestic microwave oven in order to find a better and more efficient method of drying with the emphasis to preserve the physicochemicals contents of the beans.

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SENSORY PROPERTIES OF COCOA LIQUOR FROM DIFFERENT PODS STORAGE AND FERMENTATION DURATION USING SHALLOW BOX

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ABSTRACT - *Malaysian dried cocoa beans are reported as low in flavour quality and sold at discounted prices. In order to improve the flavour quality of Malaysian cocoa beans, the shallow box fermentation and pods storage up to ten days are suggested. However, the flavour quality of Malaysian cocoa beans is still inconsistent and often faced over-fermented problem. Therefore, the effect of pods storage (0, 2, 4 and 6 days) and fermentation at 0, 24, 48, 72, 96 and 120 hours using a shallow box on sensory properties of cocoa liquor were investigated in order to determine time to end up fermentation. Four main attributes of flavour namely cocoa, bitterness, astringent and sourness were scored by seven trained panellists. The study showed that as duration of pods storage extended, the cocoa attribute was increased and decreased in between the fermentation duration. PS6 and PS4 exhibited strongest cocoa flavour in liquor from 24 hours of dried fermented cocoa beans, while PS2 and PS0 have their strongest cocoa flavour in liquor from 72 hours of dried fermented cocoa beans. The bitterness and astringent attributes were decreased but in between 48 to 120 hours of fermentation duration, the intensities reached plateau regardless the duration of pods storage. Similar to cocoa, the sourness attribute was increased and decreased in between the fermentation duration. However, the sourness attribute was decreased in dried unfermented cocoa beans as pods storage extended to six days. Two-way ANOVA exhibited that the duration of pods storage and fermentation have significant interaction effect on the intensity of cocoa flavour, bitterness, astringent and sourness. These findings suggested that duration of fermentation should be terminated early and depending on duration of pods storage.*

Keywords: Cocoa beans, fermentation, pod storage, sensory, duration

INTRODUCTION

The primary raw materials of the chocolate and cocoa-based industries are derived from seeds of the cocoa tree (*Theobroma cacao* L.). The seeds lay inside the fruits, which better known as pod and have unpleasant taste with excessively bitter and astringent. The seeds need to be processed by fermentation, followed by drying, and roasting to obtain the full spectrum of chocolate flavour (Vázquez-Ovando, Chacón-Martínez, Betancur-Ancona, Escalona-Buendía, and Salvador-Figueroa *et al.*, 2015; Aculey, Snitkjaer, Owusu, Bassompiere, Takrama, Nørgaard, Petersen, and Nielsen *et al.*, 2010). Among that process, fermentation is the most important process as any imperfections during this stage especially in flavour is irreversible and will affect the taste in the next final products. In order to produce dried cocoa beans with high quality of flavours, the fermentation process need to be carried out perfectly and terminated through drying at the correct duration (Khairul Bariah, 2014; Sukha, Butler, Umaharan and Boult *et al.*, 2008).

Termination of fermentation too early will prevent the flavour precursors from fully developed hence produced the under-fermented beans. The under-fermented beans is characterised by slaty and purple appearance with low cocoa aroma intensity (Jinap, Ikrawan, Bakar, Saari, and Lioe *et al.*, 2008). Whereas if termination of fermentation is too late, the developed flavour precursors is destroyed and resulted in over-fermented beans (Aprotosoaie, Luca, and Miron *et al.*, 2016; Khairul Bariah, 2014). The over-fermented dried cocoa beans is presented by dull dark or blackish colour and has unpleasant smells of ammonia or putrid. Powder

or chocolate produced from the over-fermented beans will have a typical “hammy” off-flavour due to short-chain fatty acids produced by *Bacillus* spp. and filamentous fungi. Most importantly, the products is lack of cocoa/chocolate taste because of excessive proteolysis of protein (Amoa-Awua, 2014; Lima, and Rob Nout, 2014; Yusep, Jinap, Jamilah and Nazamid *et al.,* 2002).

In general, the world market has distinguished cocoa into two broad categories of cocoa beans which are "fine" or "flavour" and "bulk" or "ordinary". The "fine" or "flavour" cocoa beans such as Trinitario beans from Trinidad is sought after by chocolate makers for a distinctive flavour and have more pronounced organoleptic characteristics. Meanwhile, the beans "bulk" or "ordinary" is abundantly available on the market such as Amazonian Forastero from Ghana. The Ghana cocoa liquor is characterised as having strong intensity of cocoa and nutty flavours, moderate in bitterness and astringency with slightly acidic/sour, fruity, floral and raw/beany/green flavours (Kadow, Bohlmann, Phillips, and Lieberei *et al.,* 2013; Sukha *et al.,* 2008). Malaysian cocoa beans are also categorized as "bulk" or "ordinary" beans but the quality are far behind to match with Ghana. The Malaysian dried fermented cocoa beans is well known as having low quality of flavour with a weak cocoa aroma, high acidity, as well as strong astringent and bitter taste and often sold at discounted prices. Although the shallow box fermentation and pods storage up to ten days are suggested to improve the flavour quality of Malaysian cocoa beans, the result is still inconsistent and often faced with over-fermented problem. Therefore, this study is trying to investigate how the pods storage and fermentation using a shallow box will affect sensory properties of cocoa liquor. So subsequently, the optimum time to end up the fermentation process can be determined.

MATERIAL AND METHOD

Sample Preparation

Fermentation: Ripe and healthy cocoa pods from mixed clone were harvested from Cocoa Research and Development Centre (CRDC) Perak, Malaysia. Pods storage was carried out as described by Khairul Bariah, Yang and Nur Hamizah *et al.* (2016). The harvested pods were stored in a basket and left for 2, 4 and 6 days under dry and well aerated conditions of roof. Prior to fermentation, the pods were opened, extracted and sorted manually for healthy fresh cocoa seeds. Subsequently, four fermentations with capacity of 150 kg were carried out concurrently in shallow box measuring 90 X 60 X 31 cm^3. During fermentation, the fermenting mass was covered with gunny sacks and turned at 72 hours by transferring from one box to another. About 15 kg of wet cocoa beans were taken out randomly from the top, middle and the bottom layer of mass at 0, 24, 48, 72, 96 and 120 hours of fermentation, respectively.

Drying: The respective wet cocoa beans were subsequently dried using natural sun drying under transparent roof. The beans were spread out on the drying platform in one layer of cocoa beans thickness. During daylight, the beans were turned using stainless steel rake at every three hours to ensure the beans were uniformly dried. While at night, the platforms were covered with gunny to avoid dew drop. The practise was carried out until the moisture content of the cocoa beans reduced to about 7.5 percent. Upon dried, sampling were performed using quartering tools until each quarter contain about 250 gram. The samples were placed in vacuum sealed container, labelled and stored accordingly until further analysis.

Sensory Analysis (Quantitive Desciptive Analysis-QDA)

Cocoa liquor: Cocoa liquor of each samples were prepared according to Federation of Cocoa Commerce (FCC, 2012) with slight modification. The dried beans were roasted in a forced airflow-drying oven for 15 min at 150^0C. Roasted beans were left to cool to approximately 50^0C before shells removed and broken into nibs. Subsequently, the roasted nibs (50g) were grinded in IKA Mill (IKA, Germany) until a smooth cocoa paste obtained.

Sensory: Sensory analysis was carried out at Cocoa Innovative and Technology Centre (CITC), Nilai by seven Malaysian Cocoa Board (MCB) trained panellist. A quantitative descriptive analysis with the scale of “0” to “10” was used. The scale of “0” indicates the absence

or minimum intensity and "10" indicates the maximum intensity. Samples were labelled randomly with selected 3-digits numerical code and commercial Ghana cocoa liquor was used as reference. Four flavour attributes which were cocoa, bitter, astringent and sour/acidic were evaluated.

Statistic analysis

All the statistical analyses were carried out using Minitab version 16.1.0. Analysis of variance (ANOVA) was performed in order to evaluate the effect of pods storage and fermentation duration on sensory properties of cocoa liquor. Comparison of treatment means and significant differences ($P < 0.05$) between treatments means separated were assessed using Tukey Method and reported as means ± standard error of the mean.

RESULT AND DISCUSSION

Cocoa

Scores for cocoa attribute of all liquors from dried cocoa beans at different durations of pods storage and fermentation are presented in *Figure 1*. The highest intensity of cocoa attribute (2.9 ± 1.8) was in liquor from dried unfermented cocoa beans which were stored for six days (PS6). Followed by decreasing order were liquor from dried unfermented cocoa beans which were stored for four (PS4), two (PS2) and 0 (PS0) days with the intensity of 2.1 ± 1.0, 1.5 ± 0.8 and 1.3 ± 0.5, respectively. A one-way analysis of variance (ANOVA) revealed that the effect of pods storage duration on the cocoa flavour was statistically significant ($F_{3, 52} = 4.27$, $p = 0.009$). Pairwise comparison by tukey method supports the finding where the PS6 have stronger cocoa flavour compared to others. The PS4 and PS2 were indistinguishable from each other and finally the PS0 had the weakest cocoa flavour intensity.

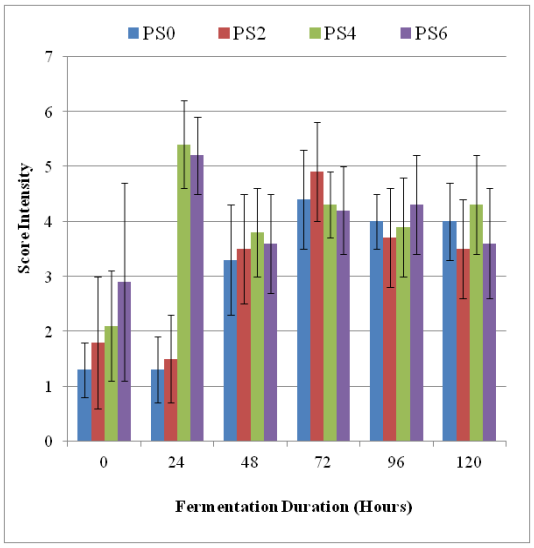

Figure 1. The cocoa attribute of all cocoa liquors from the dried cocoa beans at different durations of pods storage and fermentation.

Regardless of the duration of pods storage, the score of cocoa attribute among liquors from dried cocoa beans at different durations of fermentation was fluctuated. Apparently, the duration of pods storage and fermentation have significant interaction effects, ($F_{15, 315} = 14.52$, $p = 0.000$) on the intensity of cocoa flavour. Whereby, PS6 and PS4 exhibited strongest cocoa flavour in liquor from 24 hours of dried fermented cocoa beans. While, PS2 and PS0 have their strongest cocoa flavour in liquor from 72 hours of dried fermented cocoa beans and afterwards the flavour weakened.

The cocoa attribute is formed as a result of reaction between amino acids and peptides with reducing sugar in maillard reaction. The amino acids, petides as well as reducing sugar are derived from catalytic reaction of protease and invertase during fermentation (Voigt and Lieberei, 2014). The fluctuation of score of cocoa attribute among liquors from dried cocoa beans at different fermentation duration might be reflected to the finding by Buyukpamukcu, Goodall, Hansen, Keely, Kochhar and Wille, et al., (2001). Their works revealed that two oligopeptide namely nonapeptide (APLSPGDVF) and hexapeptide (SPGDVF) are derived from vicilin 7S class globulin and suggested to be responsible for cocoa flavour. The nonapeptide is formed early during fermentation and degraded after day three. While, the hexapeptide which is

also formed at initial stage of fermentation but lag phase in its formation and has a second increase from day four onward. In addition, Marseglia, Sforza, Faccini, Bencivenni, Palla, and Caligiani *et al.,* (2014) suggested that effect of different fermentation processes caused of variable in peptide patterns of cocoa beans.

Bitterness

The bitterness attribute is reported as a result of polyphenolic compounds which endogenously available in cocoa and considered as a negative attribute in food (Harwood, Ziegler, and Hayes *et al.*, 2013). Hence, the lower the intensity of bitterness would be the better for the quality of cocoa liquor. The scores for bitterness attribute of all liquors in this study are presented in *Figure 2*. Overall, the bitterness attribute varied and reduced at different durations of pods storage and fermentation. In dried unfermented cocoa beans, the bitterness attribute was very with average scores of over 6.0 in most samples except PS6. PS6 had the lowest score for bitterness attribute followed by PS4, PS0 and PS2 as duration of pods storage extended to 6 days. However, the effect of pods storage durations were not statistically significant, ($F3, 52 = 1.12$, $p = 0.350$) to the bitterness attribute.

The study also revealed that the intensity of bitterness was reduced despite the fluctuated scores in between the fermentation duration. The intensity of bitterness was still strongly presented with the score above 5.0 in liquor from 24 hours of PSO and PS2 dried fermented cocoa beans. While in PS4 and PS6, the bitterness was beyond reasonable intensity with the score of 4.1 ± 0.7 and 4.3 ± 1.0, respectively. Reduction of bitterness is suggested to be due to gradually decrease of the content of methylxanthines after the first 72 h of fermentation (Aprotosoaie *et al.,* 2016). Additionally, this study also revealed that the bitterness attribute reached a plateau in between 48 to 120 hours of fermentation duration. Two way ANOVA exhibited that there was a significant interaction effects between the duration of pods storage and fermentation on intensity of bitterness flavour, $F (15, 315) = 2.11$, $p = 0.009$. Thus indicated that the fluctuation of the bitterness during fermentation depends on the pods storage.

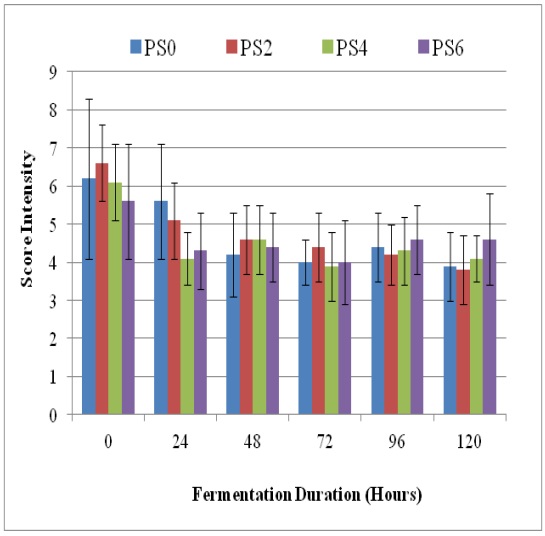

Figure 2. The bitterness attributes of all the 24 dried cocoa beans from 0 (PS0), 2 (PS2), 4 (PS4) and 6 (PS6) days of pods storage at different duration of fermentation

Astringent

Similar to bitterness, the polyphenols and methylxantines are reported to be responsible for astringency in cocoa beans. Conversion of the compounds during fermentation is suggested to reduce bitterness and astringent attributes in cocoa beans (Aprotosoaie *et al.,* 2016; Cruz, Leite, Soares and Bispo *et al.*, 2015). The scores for astringent attribute of all liquors from dried cocoa beans at different durations of pods storage and fermentation are presented in *Figure 3*. Similar with bitterness, all cocoa liquor prepared from dried unfermented cocoa beans has very strong intensity of the astringent attribute with average scores over 6.0. This finding is in well agreement with previous report that dried unfermented cocoa beans has an unpleasant taste with high astringency and bitterness (Aprotosoaie *et al.*, 2016).

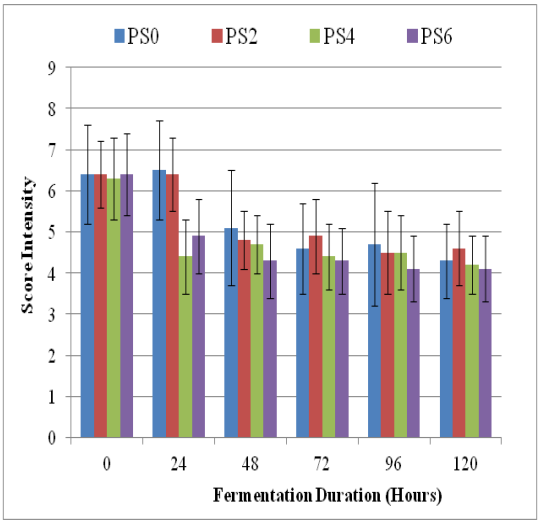

Figure 3. The astringent attributes of all the 24 dried cocoa beans from 0 (PS0), 2 (PS2), 4 (PS4) and 6 (PS6) days of pods storage at different duration of fermentation

Drying of fermented cocoa beans will reduce the astringent. In this study, the astringent attribute were still strong in cocoa liquor prepared from cocoa beans for batch PS0 and PS2 which were dried at 24 hours of fermentation duration. Whereas, cocoa liquor prepared from dried cocoa beans for batch PS4 and PS6 were beyond reasonable intensity. Similar to bitterness attribute, the astringent scores for all the liquor except from cocoa beans for batch PS0 which were dried at 48 hours of fermentation and onward were also beyond reasonable intensity. One way ANOVA demonstrated that the astringent attribute of cocoa liquor from dried unfermented cocoa liquor was not significantly ($F_{3, 52} = 0.04$, $p = 0.990$) affected by duration of pods storage. However, two ways ANOVA showed significant interaction between duration of pods storage and fermentation ($F_{15, 315} = 2.35$, $p = 0.003$).

Sourness

The acid or sourness attribute for all cocoa liquors are presented in *Figure 4*. As expected, very slightly acid or sourness attributes presence in liquor prepared from unfermented dried cocoa beans with the scores less than 2. The score of acid or sourness attribute for unfermented dried cocoa beans followed in increasing order of PS6 < PS4 < PS2 < PS0 as duration of pods storage extended to 6 days. One way ANOVA demonstrated that the acid or sourness attribute of cocoa liquor from dried unfermented cocoa liquor was not significantly ($F_{3, 52} = 0.45$, $p = 0.716$) affected by duration of pods storage. As the duration of fermentation extended, the scores of acid or sourness attribute were increased but decreased after certain duration. In addition, the scores of acid or sourness attribute for liquor from PS4 and PS2 dried fermented cocoa beans were slightly dropped and increased in between 96 and 120 hours of fermentation duration. Two ways ANOVA showed significant interaction between duration of pods storage and fermentation ($F_{15, 315} = 3.25$, $p = 0.000$).

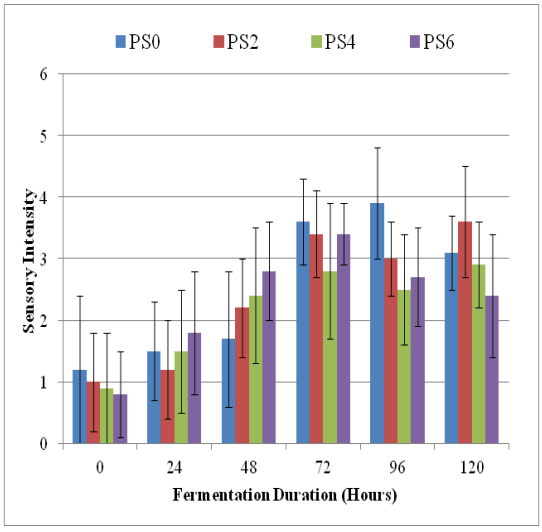

Figure 4. The acid/sour attributes of all the 24 dried cocoa beans from 0 (PS0), 2 (PS2), 4 (PS4) and 6 (PS6) days of pods storage at different duration of fermentation

CONCLUSION

Comparison of sensory properties among all cocoa liquors demonstrated that cocoa liquour from PS6 and PS4 which were fermented for 24 hours have significantly strongest cocoa attribute, moderately bitter and astringent and slightly low in acidic or sourness attribute. Therefore, suggesting Malaysian cocoa beans should be fermented accordingly in order to obtain cocoa liquor that is comparable to Ghana.

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QUALITY ASSURANCE ASSESSMENT OF COCOA-BASED LIQUID FOUNDATION COSMETIC

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ABSTRACT – *This article presents part of analyses subjected to quality assurance of cocoa-based liquid foundation formulation prior to commercialization. Total viable count for aerobic mesophilic microorganisms, i.e. bacteria (aerobic plant count), yeast and moulds (yeast and mould count) were less than 10 colony forming unit (cfu)/g after 6 months assessment. Sensorial attributes in terms of color, odor, texture, smoothness, spreadibility, greasiness, product adhesion to skin, occurrence of skin irritation as well as overall acceptability, have been well conducted on 10 selected panels. The sensory evaluation comparative assessment data provides a documentation of perceived characteristics with overall acceptability for the tested sample and commercial product at 7.90±0.74 and 8.20±1.03, respectively with no significant difference (p>0.05). The Ocular and Dermal Irritection Assay results exhibited that the product was classified as very minimal irritant and non-irritant with highest Irritection Draize Equivalent (IDE) and Human Irritancy Equivalent (HIE) scores of 0.12 and 0.13, respectively. Meanwhile, the in vitro SPF value of cocoa-based liquid foundation was 7.62 whereas emulsion-based of commercial brands, tagged as Comm1, Comm2 and Comm3 were 6.48, 8.96 and 6.79, respectively. Diversification of cocoa products range through development of cocoa-based liquid foundation cosmetic is one of Malaysian Cocoa Board attempts towards sustainability of cocoa industry in Malaysia.*

***Keywords**:* Quality assurance; microbial property; sensory evaluation; *in vitro* irritection assay; *in vitro* SPF

INTRODUCTION

Generally, cosmetic products are suitable media for microorganisms to grow due to the materials in their compositions. Contamination of cosmetics products such as adverse effects regarding the product smell, changes of color and viscosity, could cause abrasion, irritation as well as allergies on the skin as a result of endotoxins and metabolites produced by microorganisms (Smart and Spooner, 1972). Given the wide range of cosmetic products, it can be seen that microorganism contamination may pose a significant risk in term of consumer health. Another essential element that needs to be considered in a new product development is sensorial analysis (Isaac *et al.,* 2012). It is a discipline that interprets, assesses and measures characteristics of a product, after stimulating people in relation to their vital senses, i.e. vision, touch, smell and taste (Stone and Sidel, 1993). Nowadays, all activities are developed around preference of consumers. In this context, sensory evaluation has been widely practiced in many sectors such as food, cosmetic, textile, medical, chemical as well as packaging (Piggot and Hunter, 1999; Stone and Sidel, 1993). In the present study, the cocoa-based liquid foundation has yet to perceive a symbolical value or positive emotion through integration of sensorial attributes that would stand a better chance for the product to be successful in the market.

Non-animal testing methods are needed for the skin irritation potential of consumer product formulations in order to support safety assessment efforts (Laquieze *et al.,* 2015). The *in vitro* Dermal and Ocular Irritection assay are based on the principle that chemicals known to induce alterations in the structure of keratin, collagen and other dermal proteins, will promote measurable changes in target biomolecules and macromolecular structures. Previous studies have clearly demonstrated that the processes of protein denaturation and disaggregation that are induced in this *in vitro* assay mimic the effects produced

when these types of irritants are applied to the skin. The test may be employed to predict the *in vivo* toxic effects of chemicals and formulations. The quantitative Irritection *in vitro* assay has been found to be highly reproducible. Of even greater relevance, this method can be readily employed to evaluate multiple samples at varying volumes or concentrations. Thus, the test serves as an extremely useful screening tool that facilitates all stages of raw material selection, formulation development and final product selection (Zafarizal *et al.,* 2005). Constant exposure to ultraviolet (UV) irradiation may cause skin to age through a complex process. UV generates reactive oxygen species (ROS) which has always been associated with collagen deficiency and subsequent skin wrinkling (Fischer *et al.*, 1997, Varani *et al.*, 2000). Currently, emulsions containing natural bioactives have emerged as promising vehicle for the delivery of such component to the human skin due to ease of application (Foldvari, 2000). Incorporation of antioxidants in topical application preparation has been widely investigated especially for the decreasing of skin aging (Bisset *et al.,* 1990). Therefore, this study was also to evaluate the photoprotective potential of cocoa-based liquid foundation cosmetic in comparison to commercial brands with synthetic UV filters. Overall, the main objective of this study was to formulate and evaluate the cocoa-based liquid foundation cosmetic for the requirement of quality assurance. Artificial perfumes, dyes and harsh chemicals which are all known to irritate skin are prohibited and only skin-friendly ingredients that are "kind" to the skin, permitted in the development of cocoa-based liquid foundation formulation.

MATERIALS AND METHODS

Microbiological analysis

The contamination of cosmetic products by microorganisms can be detected by determining the presence of bacteria via the aerobic plate count (APC) and the presence of other microorganisms via yeast and mould count (YMC). The total microbial count was calculated from the total count of APC and YMC. Samples were prepared accordingly and serially diluted in modified leetheen broth. Each dilution (0.1 mL) was aseptically pipetted and spread onto the surface of modified leetheen agar for APC and potato dextrose agar for YMC. Plates were incubated for 48 hours (for APC) and 7 days (for YMC) at 30^{o}C (Madden & Dallas, 1998). The colony forming units for each diluted sample was then counted and recorded. The analysis was conducted at 3 months interval for 6 months assessment.

Skin feel sensory analysis

The panelists between 25 to 40 years of age rated their appreciation on the formulations based on these attributes; (i) color, (ii) odor, (iii) texture, (iv) smoothness, (v) spreadibility, (vi) greasiness, (vii) product adhesion to skin, (viii) any occurrence of irritation, and (ix) overall acceptability. Initially, they were briefed on how they should perform the evaluations. A ten-level hedonic scale, with word anchors ranging from "dislike very much" to "like very much" was used. Scores were attributed to each level, ranging from 1 to 10. For each skin feel attribute, the procedure, definition and scale were demonstrated with reference. Standard materials were selected for each attribute, based on the ASTM E1490 Standard and bibliographic references (Civille & Dus, 1991), and the panelists were trained to remember their intensities as reference.

In vitro dermal and ocular irritation assay

The Ocular and Dermal Irritection assays (In Vitro International, Irvine, CA) are quantitative *in vitro* test methods that mimic biochemical phenomena is an alternative method to animal irritancy studies (Draize Test). The *in vitro* irritation assays have been reported to correlate well with the *in vivo* irritancy tests (Sina *et al.,* 1995).

The ocular irritection assay (In Vitro International, Irvine, CA) requires two essential components: a membrane disc that permits controlled delivery of the test material to a reagent solution, and a proprietary reagent solution of proteins, glycoproteins, lipids and low molecular weight components that self-associate to form a complex macromolecular matrix. Controlled mixing of the test material and reagent solution during the assay incubation period promotes protein denaturation and disaggregation of the

macromolecular matrix. The dermal irritection assay (In Vitro International, Irvine, CA) also requires two components: a membrane substrate modified by covalently cross linking a mixture of keratin, collagen and an indicator dye, and a reagent solution consisting of an organized globulin/protein matrix. The changes in protein structure induced by the test material were readily quantified by the changes in turbidity at 405 nm (OD_{405}) of the reagent solution for ocular irritation, while in the dermal irritation assay, the extent of dye release and protein denaturation was quantified by measuring the changes in optical density of the reagent solution at 450 nm (OD_{450}). The ocular irritancy potential of the cocoa-based liquid foundation was expressed as an Irritation Draize Equivalent (IDE) whereas the dermal irritancy potential as a Human Irritancy Equivalent (HIE). The IDE and HIE have been reported to correlate well with *in vivo* investigations by the Draize method and human test, respectively. The predicted *in vivo* classifications, based on these scoring systems, are shown in Tables 1 and 2.

Table 1. Classification of irritation draize equivalent score to *in vivo* irritancy classification (Draize test)

Irritection Draize Equivalent (IDE) Score	Predicted Ocular Irritancy Classification
0.0 – 12.5	No or Minimal Irritant
12.5 – 30.0	Mild Irritant
30.0 – 51.0	Moderate Irritant
> 51.0	Severe Irritant

Table 2. Classification of human equivalent score to *in vivo* irritancy classification

Human Irritancy Equivalent (HIE) Score	Predicted Dermal Irritancy Classification
0.00 – 0.90	Non-Irritant
0.90 – 1.20	Non-Irritant/Irritant
1.20 – 5.00	Irritant

In vitro determination of SPF

Hundred mg (100 mg) of the tested sample was spread on the 56 cm^2 area to obtain an even film thickness of 2 $\mu L/cm^2$ on Transpore Tape as suggested in the operation manual of the UV-2000S Ultraviolet Transmittance Analyzer for the sample preparation and application technique (Curtis, 1994). The prepared sample was exposed to Xenon flash lamp for determining the Sun Protection Factor as follows:

$$SPF = \frac{\int_{290}^{400} E\lambda \cdot S\lambda \cdot d\lambda}{\int_{290}^{400} E\lambda \cdot S\lambda \cdot T\lambda \cdot d\lambda} \quad \text{Eq. 1}$$

Where, Eλ is the erythema action spectrum, Sλ is the solar spectral irradiance, Tλ is the spectral transmittance of the sample with the integral is calculate across the 290-400 nm wavelength limits. The equation shows that the higher the amount of transmittance, the lower the SPF value.

Statistical analysis

All statistical analyses were performed using MINITAB package ver. 14 and significance levels were taken to be 0.05 (unless stated otherwise).

RESULTS AND DISCUSSION

Microbial enumeration of bacteria, yeasts and moulds count

The tested sample was comply to Category 1 cosmetic products (A guidance document on microbiological control of cosmetic products - total viable count for aerobic mesophilic microorganisms, i.e. bacteria, yeast and moulds

must not more than 10^2 cfu/g or mL in 0.5 g or 0.5 mL of the product) (Detmer *et al.*, 2007). The formulation showed growth (bacterial count) after storage except for yeasts and moulds (not detected) of only 2.0±0.0 and 6.0±1.0 cfu/g at 3rd and 6th month of assessment, respectively (Table 3). The result was also subjected to microbiologically quality management (MQM) system of the Regulation (EC) No 1223/2009 (EU Cosmetics Regulation) which stated that in products with unremarkable microbiological findings, the result is generally < 10 cfu/g or microbe(s) absent in 1 g of product. The compliance to the recommended microbial limit in the finished product showed that it was adequately preserved. In addition to the microbial limit specified, no product shall have a microbial content recognized as harmful to the consumer. Implementation of Good Manufacturing Practices (GMP) has been the foundation for improving industrial quality control analyses. Other recommended tests include the identification of *Pseudomonas aeruginosa*, *Candida albicans* and *Staphylococcus aureus*, however, many manufacturers do not carry out identification on these organisms as their normal quality control procedure.

Table 3. Microbiological analysis of cocoa-based liquid foundation

Parameter	[cfu/g]			
	Month 0	Month 3rd	Month 6th	Acceptability according to Category I products (<10^2cfu/g)
Bacterial count#	n.d.	2.0	6.0	√
	n.d.	2.0	7.0	√
	n.d.	2.0	5.0	√
Mean ± SD	-	2.0 ± 0.0	6.0 ± 1.0	
Yeasts &	n.d.	n.d.	n.d.	√
Moulds count+	n.d.	n.d.	n.d.	√
	n.d.	n.d.	n.d.	√
Mean ± SD	-	-	-	

#Test method: IOCCC 118:1990 / ISO 4833 "General guidance for enumeration of microorganisms. Colony count at 30ºC"; +Test method: IOCCC 118:1990 / ISO 7954 "General guidance for enumeration of yeasts and moulds. Colony count at 25ºC"

Cosmetics that have held an important place in human life since the beginning of time are products that are not required to be sterile microbiologically but have to be of proper quality in terms of consumer health (Steinberg, 2006). Although it may have not demand sterile products, the products may neither contain excessive quantities of microorganisms nor specific microorganisms which have the potential to compromise the quality of the product (ISO 17516). It has been determined that these products could be contaminated with microorganisms found in the production environment or in raw materials, especially water, and that the contamination could occur after production due to unhealthy storage conditions or during consumer use (Underwood, 1998). Compliance with GMP standards strictly during the production as well as incorporation of preservatives as determined by regulations, are vital procedures for the sustainability and longevity of cosmetic product particularly for cocoa-based liquid foundation.

Skin feel sensory analysis

Sensory analysis has become an essential methodology in research and development departments of large cosmetic companies. Applied to the skin which is a widely sensitive material, the perceptions are felt at the application and upon application giving to the skin measuring sensations, i.e. visual (appearance), olfactory (fragrance/smell), tactile (texture/feel) and

somesthetic. In this study, ten panelists between 25 to 40 years of age were selected among MCB's staffs and the public. The panelists were asked to apply the samples and assessed their degree of liking subjected to ten-point hedonic scale according to color, odor, texture, softness, spreadibility, greasiness, product adhesion to skin, any occurrence of irritation and overall acceptability (1 [dislike very much] until 10 [like very much] in relation to the consumer's preferences). ANOVA statistical analysis by Fisher's comparison was used to determine whether the differences between measurements were significant. According to Table 4, there were no significant differences regarding the color, odor, smoothness, spreadibility, greasiness, product adhesion to skin as well as overall acceptability. However, significant difference could be seen for its odor/aroma. In this case, the aroma of commercial brand was being preferable compared to the cocoa-based liquid foundation might be due to the cocoa aroma which are rarely found in make-up products.

Table 4. Score obtained from sensorial attributes performed on commercial brand and cocoa-based liquid foundation

No.	Sensorial attributes	Sample	
		Standard (Commercial brand)	Cocoa-based liquid foundation
1	Color	$6.90^{a} \pm 2.56$	$5.20^{a} \pm 2.25$
2	Odor	$7.70^{a} \pm 0.95$	$5.90^{b} \pm 1.73$
3	Texture	$7.50^{a} \pm 1.35$	$6.30^{ab} \pm 1.06$
4	Smoothness	$7.80^{a} \pm 1.14$	$7.10^{a} \pm 1.29$
5	Spreadibility	$7.90^{a} \pm 1.10$	$7.50^{a} \pm 1.18$
6	Greasiness	$7.80^{a} \pm 1.03$	$7.70^{a} \pm 0.82$
7	Product adhesion to skin	$7.50^{a} \pm 1.18$	$8.50^{ab} \pm 0.85$
8	Any occurrence of irritation?	NIL	NIL
9	Overall acceptability	$8.20^{a} \pm 1.03$	$7.90^{a} \pm 0.74$

**Values are expressed as mean ± SD. Means with the different letters in each column are significantly different ($p<0.05$), by ANOVA ($n = 10$)*

Despite of the unfavorable aroma by consumers, cocoa-based liquid foundation however has indicated slightly higher preference in term of its adhesion to skin property, hence giving a lightweight longer wear formulation *(Figure 1)*. The sensorial features of a formulation are mainly related to the raw materials and package (Dooley *et al.*, 2009). The raw materials influence comprehensively in what the consumer feels when applies the cosmetic. The emollients in emulsion-based, for example, are raw materials of marked influence in the tactile (sense of touch) attribute (Parente *et al.*, 2008; Gorcea and Laura, 2010). In this study, the main representatives of cocoa-based liquid foundation are Cocoate BG (emollient) and Plurol diisostearique (emulsifier). Furthermore, the MCB is now focused on further exploring inspiring textures and sensorial attributes that innovative ingredients can bring to cosmetic products. Therefore, an addition of cocoa product (17.54%, w/w) into the emulsion-based cocoa liquid foundation may enhances skin to remain healthy especially with the existence of polyphenol compound, an antioxidant that helps in anti-collagenase and anti-elastase activities (Wahab *et al.*, 2014).

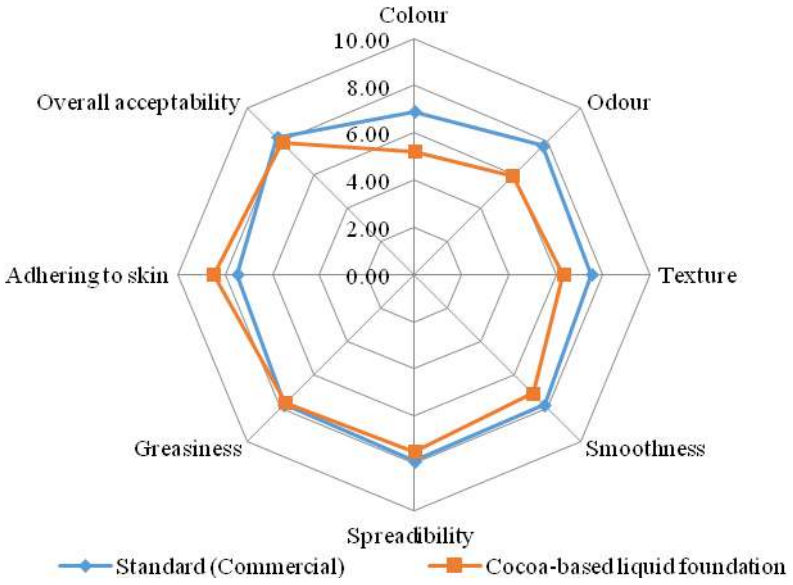

Figure 1. Spider web diagram of sensory evaluation means of cocoa-based liquid foundation with comparison to a commercial product

***In vitro* ocular and dermal irritation assay**

The quantitative Ocular and Dermal Irritection *in vitro* assays have been found to be highly reproducible. Of even greater relevance, the Irritection assay methods can be readily employed to evaluate multiple samples at varying volumes or concentrations. Thus, these tests serve as extremely useful screening tools that facilitate all stages of raw material selection, formulation development and final product selection. Therefore, the cocoa-based liquid foundation was evaluated with the Irritection Assay System in order to predict their potential to cause dermal irritation. To achieve the objective, standard volume-dependent dose-response studies based on cosmetic protocol was performed with the Ocular and Dermal Irritection Assay test methods and the results for both assays are illustrated in the Tables 5 and 6, respectively. The Ocular and Dermal Irritection Assay results indicated that cocoa-based liquid foundation has been classified as very minimal irritant with the highest Irritection Draize Equivalent (IDE) score of 0.12 whereas non-irritant for prediction of dermal irritancy showed by the highest Human Irritancy Equivalent (HIE) score of 0.13 as shown in Tables 5 and 6, respectively.

Table 5. Irritection Draize Equivalent (IDE) score of cocoa-based liquid foundation for Ocular Irritection Assay

Dose (μL)	IDE Score**	Predicted Ocular Irritancy Classification
50	0.12*	Minimum
75	0.10	Minimum
100	0.09	Minimum
125	0.08	Minimum

**maximum qualified score; **IDE scores in ranges of 0.00-12.5, 12.5-30.0, 30.0-51.0 and >51.0 are considered as minimal irritant, mild irritant, moderate irritant and severe irritant, respectively*

Table 6. Human Irritancy Equivalent (HIE) score of cocoa-based liquid foundation for Dermal Irritection Assay

Dose (μL)	HIE Score**	Predicted Dermal Irritancy Classification
50	0.04	Non-Irritant
75	0.06	Non-Irritant
100	0.13*	Non-Irritant
125	0.12	Non-Irritant

**maximum qualified score; **HIE scores in ranges of 0.00-0.90, 0.90-1.20 and .20-5.00 are considered as non-irritant, non-irritant/irritant and irritant, respectively*

All these readings comply with the MCB's specification and also meet the requirements of quality control, safety and the regulation. The skin irritation analysis is a quantitative *in vitro* test that utilizes changes of relevant macromolecules to predict the acute skin irritancy of cosmetic formulations and ingredients. This assay is based on the principles that chemical compounds that cause skin irritation are known to induce alterations in the structure of keratin, collagen and other dermal proteins. The processes of conformational change that are induced in this *in vitro* assay mimic the effects that are produced when these types of irritants are applied to the skin. One aspect of safety that concerns consumers can be described as mildness which is the product ability to perform its intended function without irritating the skin or eyes. Products that harsh could cause irritant contact dermatitits (ICD), the symptoms include erythema (redness), burning, itching and flaking. Skin irritation is one of the most common adverse effects of cosmetic products in humans depended in many factors, including the concentration, duration and frequency of exposure, exposed skin site, rate of penetration and intrinsic toxic potential of the substance in the product (Nawanopparatsakul *et al.*, 2005). In order to make sure that the product is safe and does not cause any irritation to the users, skin and eye irritation analysis is carried out. Only the product that comes in contact with the eyes is subjected to eye irritation analysis. Consequently, the *in vitro* skin irritation test is a reasonable strategy to determine the potential skin irritancy and to predict the *in vitro* toxic effect of chemicals, cosmetic ingredients, formulations and new cosmetic products (Genno *et al.*, 1998). The test serves as an extremely useful screening tool that facilitates all stages raw materials selection, formulation development and final product selection.

In vitro determination of photoprotective efficacy of cocoa-based liquid foundation

The results obtained for photoprotective efficacy of the cocoa-based liquid foundation against ultraviolet radiation in comparison to other commercial brands are exhibited in *Figure 2*. The *in vitro* sun protection factor (SPF) value of cocoa-based liquid foundation was 7.62 whereas other commercial brands, tagged as Comm1, Comm2 and Comm3 were 6.48, 8.96 and 6.79, respectively. According to the recommendations of the European Union (2006/647/EC) concerning the effectiveness of sunscreen products (European Commission Recommendation, 2006), the tested sample meet the current standards for protection against UVA. The value of UVA protection factor (PF-UVA) *in vitro* for the tested sample (7.62) was much greater than the minimal recommended value, which is at least 2.00. Ranging from 290 to 400, the cocoa-based formulation was in compliance with the minimum requirements of at least 370 nm of the critical wavelength (European Commission Recommendation, 2006). In comparison with the commercial formulations, the formulation of cocoa-based formulation provided higher protection against UVA (PF-UVA), in particular, they had much higher values of critical wavelength (λc) and PF-UVA factor. As have been proved before (Hojerová, 2011; Velasco *et al.*, 2008), the critical wavelength may well describe the width of the spectrum of photoprotection, but is not suitable for measuring the intensity of protection.

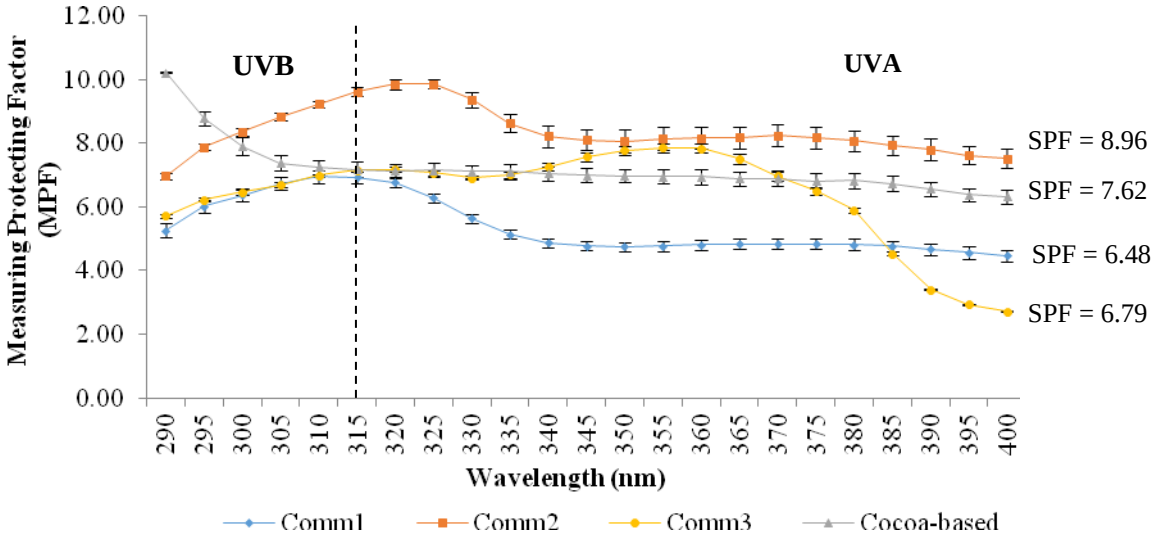

Figure 2. Photoprotective efficacy of the cocoa-based liquid foundation against ultraviolet radiation in comparison to commercial brands, tagged as Comm1, Comm2 and Comm3

The cocoa-based liquid foundation product, like the formulations containing individual polyphenolic fractions, also met current standards of the European Union (2006/647/EC) for protection against UV radiation, classifying them as the products of medium photoprotection $SPF_{labeled}$ = 15. Analysis of the result leads to the conclusion that the cocoa-based emulsion, regardless of the intensive UV irradiation, exhibited excellent photostability, especially in the UVA region. For the majority of them the values of $\%PF\text{-}UVA_{in\ vitro}$ exceeded 90%. Similar results were obtained by Choquenet *et al.* (2008) who measured the photostability of emulsion systems containing individual polyphenolic compounds, namely quercetin and rutin. They found high photostability (more than 90%) and compatibility with other commercially available sunscreen filters. Moreover, as was described in previous publications, flavonoids such as quercetin significantly reduce the photodegradation of the chemical UV filters without any changes in the effectiveness of the sunscreen formulations (Scalia and Mezzena, 2010; European Commission Recommendation, 2006; Badea *et al.*, 2008). The multiple effects of polyphenolic compounds, as antioxidants, photostabilizers and UV filters, incorporated into cosmeceutical formulations represent a promising strategy for the development of broad-spectrum cosmeceutical products with enhanced efficacy and safety.

CONCLUSION

The cocoa-based liquid foundation has been classified as an exclusive and innovative product development due to the involvement of intense cocoa product, whereby duplication of marketplace liquid foundation was initially observed for customization activity. In essence, the Cocoa Cosmetic & OTC Products Pilot Plant (CCOPP) of Malaysian Cocoa Board intends to continue pursuing work focused on cocoa-based liquid foundation and development process based on natural cocoa production through sustainable process technology. A key area of focus will remain on the utmost quality approach throughout utilization of cocoa products in a production of high quality liquid foundation cosmetic. In addition, as the health/nutritional cosmetic industry is expected to continue at a high rate, more healthcare cosmetic products and development process will be soon established. Mass-production of cocoa-based liquid foundation is expected to be conducted at the CCOPP to further activity of pre-commercialization.

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SENSORY EVALUATION AND PHYSICAL CHARACTERISATION OF WHITE CHOCOLATE WITH FREEZE-DRIED COCOA POLYPHENOL NANOEMULSION

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ABSTRACT - *In this study, physical and sensory properties of white chocolate added with (1%, 5% and 10%) freeze-dried cocoa polyphenol nanoemulsion with xanthan gum or maltodextrin were evaluated. The addition of 1% freeze-dried cocoa polyphenol nanoemulsion did not significantly affect the instrumental hardness and all sensory attributes evaluated except smoothness of the chocolate compared to the control white chocolate. Higher addition of freeze-dried cocoa polyphenol nanoemulsion significantly reduced the chocolate hardness, increase the moisture content and particle size.*

Keywords: White chocolate, texture analysis, sensory analysis

INTRODUCTION

White chocolate contains cocoa butter, milk powder and sugar. It does not contain any non-fat cocoa solid either from cocoa liquor/cocoa mass or cocoa powder. Therefore white chocolate does not contain any cocoa polyphenol unlike milk chocolate and dark chocolate. White chocolate is often used as a control in human intervention studies. However some authors argue that white chocolate is not suitable to be used as a placebo or control since it does not only lacks polyphenol but also other compounds such as methylxanthines (theobromine and caffeine). White chocolate also have different fatty acid and carbohydrate composition compared to dark and milk chocolate (Rimbach, Egert, de Pascual-Terese, 2011 and Rostami *et al,* 2015).

Rostami *et al*., (2015) studied daily consumption of 25g of either 83% cocoa solid chocolate and iso-caloric white chocolate for 8 weeks by patient with hypertension and type-2 diabetes. The results showed improved cardiovascular risk indices by decreasing systolic and diastolic blood pressure without detrimental effect on triglyceride, weight, insulin resistant and BMI.

Cocoa beans have high polyphenol content which consist of (-)-epicatechin, (+)-catechin, procyanidin B2, procyanidin C1, cinnamtannin A2 and other oligomeric procyanidin which have been shown to have antioxidant activity in vitro. Cocoa polyphenol has been reported to reduce the risk of cardiovascular diseases including atherosclerosis, hypertension, and platelet activity. Intake of cocoa powder rich in proanthocyanidins reduces susceptibility of low-density lipoprotein to oxidation. Since bioavailability of polyphenol in the intestinal tract is low, nanoemulsion techniques was applied to cocoa polyphenol to increase the bioavailability.

This study aims to determine the effect on the texture and sensory of white chocolate after the addition of freeze-dried cocoa polyphenol nanoemulsion.

MATERIALS AND METHOD

White chocolate formulation

Based on previous study on freeze-drying of nanoemulsion containing cocoa polyphenol, two type of additive were selected as the best i.e. xanthan gum and maltodextrin.

Recipe for the white chocolate is 48% icing sugar, 29.5% whole milk powder, 22% cocoa butter and 0.5% lecithin. Icing sugar, milk powder and cocoa butter were mixed into paste and pass through a three roll mill (Capco, UK) for three times to obtain the required particle size. The

refined mixture was conch for six hours in an end runner mill (Pascal, UK). During the conching process the remaining 50g cocoa butter and half of the required lecithin was added. The rest of the lecithin was added 2 hour before the end of conching.

1%, 5% and 10% of freeze-dried cocoa nanoemulsion either with xanthan gum or maltodextrin were mixed with cocoa butter before added into the white chocolate. The coding for the samples are as follows; CNEX5(1%) for chocolate with 1% freeze-dried cocoa nanoemulsion with xanthan gum' CNEX5(5%) for chocolate with 5% freeze-dried cocoa nanoemulsion with xanthan gum, CNEX5(10%) for chocolate containing 10% freeze-dried cocoa polyphenol nanoemulsion with xanthan gum, CNEM15(1%) for chocolate containing 1% freeze-dried cocoa polyphenol nanoemulsion with maltodextrin, CNEM15(5%) for chocolate containing 5% freeze-dried cocoa polyphenol nanoemulsion with maltodextrin and lastly CNEM15(10%) for chocolate with 10% freeze-dried cocoa polyphenol nanoemulsion with maltodextrin. The white chocolate was then tempered and molded into chocolate with size of 37cm (L) x 14cm(W) x13cm(H). The chocolates were stored at 20^0C with 65% humidity before further analyses.

Texture

The texture analysis of white chocolate comprise of two test i.e penetration test and breaking test. The equipment used for both these testing is Texture Analyser from Stable Microsystem (Surrey, UK). For the penetration testing, a needle probe, P/2N was used. The trigger force used for penetration was 10g while the distance of the probe was 6mm (half the thickness of the test chocolate). A 5kg load cell was used. The samples were penetrated at a speed of 2.0mm/s. The samples were taken out of the chiller immediately analysed at room temperature. Two pieces of chocolates were use for this test. Each chocolate was penetrated twice towards the end of the piece. Four data were obtained for each chocolate formulation.

A mini 3 point bend rig (HDP/3PB) was used for snap test. This rig is placed on the heavy duty platform and principally measures the fracture characteristics or brittleness of a product. The same chocolates were used for snap test however only 2 data were obtained since each piece of chocolate was snap in the middle.

Moisture content

Moisture content was carried out using a Karl-Fischer auto-titrator apparatus (Mettler-Toledo Model DL-38). Solvent followed by Karl-Fischer was added into the reaction vessel until the electrode was immersed. Pre-titration was performed followed by drift analysis. The drift value should be between 5-50 µg/min. The water equivalent of the reagent was performed by conc analysis using known mass of water. 5g of sample was added and the mixture was stirred for 30s to disperse the sample into the solvent. Titration with Karl-Fischer reagent was carried out until the end point was reached. Each sample was tested twice (IOCCC, 1988).

Colour

The color of white chocolate was determined using Colorimeter (Konica Minolta, Model CM-5, Japan). The color was described using the CIE system developed by Lab System in 1976. The CIE system uses three axes i.e L* (z axis) which range from 0 (black) to 100 (white), a* (x axis) denotes red when on the positive direction and green when on the negative direction while b* (y axis) denotes yellow when on the positive direction and blue on the negative direction.

Particle size

Particle size of chocolate was determined using laser diffraction technique with Chocosizer (Malvern Instruments, UK). White chocolate samples were melted in an oven at 50^0C. About 100mg of sample were deposited using a plastic syringe into a beaker containing 20ml sunflower oil. The sample and sunflower oil was sonicated using the build-in sonicator for 2 minutes to ensure the entire chocolate particle are well dispersed and no agglomeration of chocolate particles. When the instrument background is stable, and the obscuration reading is 0.0%, the sample was added into the small volume sample dispersion unit containing sunflower oil. The sample was added using a plastic dropper until the obscuration

reading reached 20%. The instrument will measure the particle size and provide a distribution of the sample particle size (Malvern, UK).

Sensory evaluation on chocolate incorporating freeze-dried cocoa polyphenol nanoemulsion

Sensory evalution sessions were conducted at the Cocoa Innovation and Technology Centre by experience panelist. The panelists consist of three female and three male aged 39 to 51 years old. Chocolate in the form of small bars were presented to panelist and evaluated for several attributes i.e glossiness, odour, hardness, melting time, melting characteristics, smoothness, color, off-flavour and acceptability.

Statistical evaluation

All data were analyzed using Minitab 14. All analysis of variance were conducted at significance value of $P<0.05$.

RESULTS AND DISCUSSION

The physical characteristics of white chocolates are tabulated in Table 1. The instrumental hardness of white chocolate was reduced significantly when more than 5% of freeze-dried cocoa polyphenol nanoemulsion was added. Belscak-Cvitnovic *et al.* (2012) reported that addition of 1% freeze-dried extract of red raspberry leaf significantly reduced the instrumental hardness of milk chocolate. Sensory data also indicate that panelist observed that chocolate with 10% freeze-dried cocoa polyphenol nanoemulsion to be less hard.

Snap force showed higher value compared to penetration force. This could be due to the type of instrument/probe, penetration was done with a needle with sharp point and easily penetrate the chocolate while snap test used an knife-like attachment with smooth edge. A lot of information can be gained from the three-point bend analysis. This analysis is also known as three-point break or three-point snap test. 1. Breaking strength (force per unit width) or breaking stress (force per unit area) of a sample is taken as the maximum strength or stress value of the curve. 2. The distance to break gives an indication of the brittleness of the sample as it shows how far a sample can be deformed before fracture. 3. The gradient of the slope indicates sample toughness; the higher the gradient, the tougher the sample. But Gaines et al (1992) suggest that from three-point break test, the hardness is obtained from peak force while brittleness is obtained from the slope. This slope is also referred to as Young Modulus.

All chocolates with addition of freeze-dried cocoa polyphenol nanoemulsion have significantly higher particle size compared to the control white chocolate. The control chocolate has high particle size. The freeze-dried cocoa polyphenol nanoemulsion with xanthan gum have more apparent affect on the chocolate particle size compared to freeze-dried cocoa polyphenol nanoemulsion with maltodextrin. This results have relation with the smoothness sensory result where CNEX5(10%) and CNEX5(5%) have the least and second least smooth results *(Figure1)*.

Similarly the moisture content of white chocolates with added freeze-dried cocoa polyphenol nanoemulsion have significantly higher moisture content compared to control white chocolate. Belscak-Cvitnovic *et al.*(2012) reported that the plastic viscosity of semisweet chocolate enriched with 3% concentrated red raspberry leaf extract increased significantly and proposed this may be due to presence of moisture. Water on the surface of sugar particles causes the sugar to stick together and impede flow. For every 0.3% of moisture in chocolate (above the level of 1%) a further 1% fat must be added to compensate and restore the viscosity. However in this study we did not measure the viscosity, but the increase in moisture may contribute to the changes in white chocolate texture for example smoothness and hardness.

Initial white chocolate showed that the control white chocolate to be lighter in color. Addition of 10% freeze-dried nanoemulsion with 5% xanthan gum has the lowest L* and b* values but highest a* value. However panelist scored the control white chocolate and CNEX5(1%) to be slightly darker compared to the other formulation.

Table 1. Physical characteristics of white chocolate

Sample	Penetration force (g)	Snap force (g)	Particle size µm, D(v, 0.9)	Moisture content (%)	L*	a*	b*
White chocolate	608.84±137.73^{a}	10,908.52±0.00^{a}	52.78±0.38^{e}	0.93±0.06^{c}	83.01±0.18^{a}	-0.27±0.06^{b}	27.19±0.07^{a}
CNEX5 (1%)	655.00±42.13^{a}	10,864.18±25^{a}	56.26±1.13^{d}	1.22±0.01^{ab}	79.59±0.16^{c}	-0.01±0.01^{ab}	27.19±0.04^{a}
CNEC5(5%)	535.12±53.48^{ab}	9,711.99±64^{b}	73.32±1.39^{b}	1.15±0.07^{b}	77.62±0.16^{f}	0.00±0.48^{ab}	24.48±0.32^{cd}
CNEX5(10%)	449.93±26.83^{b}	7,648.28±191^{bc}	85.96±2.08^{a}	1.12±0.06^{b}	75.66±0.11^{g}	0.48±0.04^{a}	22.82±0.13^{e}
CNEM15(1%)	636.39±29.79^{a}	8,445.34±75^{b}	56.92±0.64^{cd}	1.13±0.04^{b}	79.92±0.01^{bc}	-0.08±0.17^{ab}	26.76±0.80^{ab}
CNEM15(5%)	597.75±67.20^{a}	7,132.61±220^{d}	64.18±0.54^{bc}	1.27±0.09^{ab}	79.18±0.18^{d}	0.06±0.11^{ab}	25.20±0.31^{c}
CNEM15(10%)	423.86±11.66^{b}	6,852.94±426^{d}	60.77±0.31^{c}	1.44±0.01^{a}	78.72±0.08^{e}	0.28±0.06^{ab}	24.20±0.45^{cd}

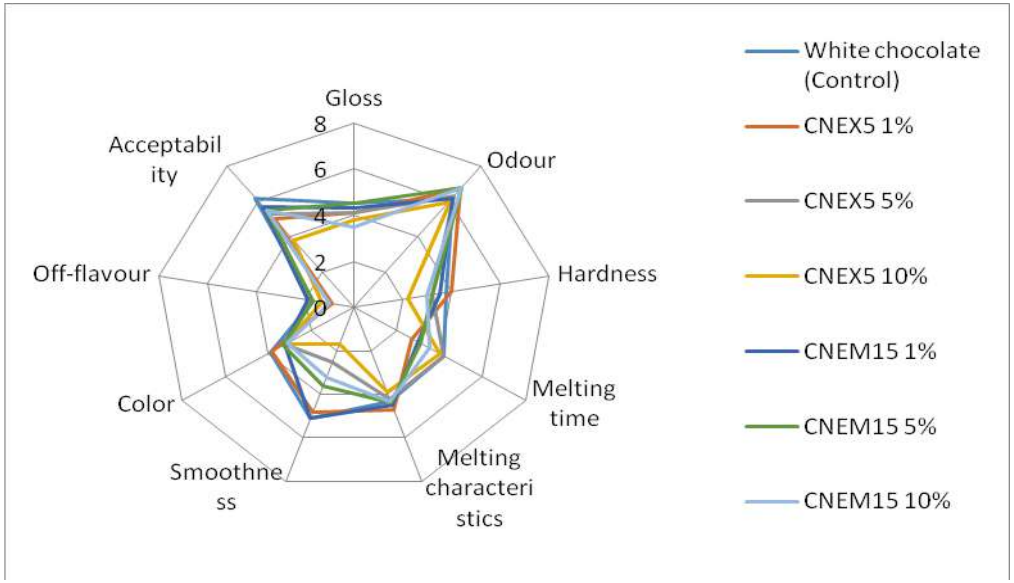

Figure 1. Sensory attributes of control white chocolate and white chocolate with freeze-dried cocoa polyphenol nanoemulsion.

Figure 1 show the results obtained from the sensory evaluation. Statistical analysis did not show any significant difference among the chocolates except for smoothness. Smoothness refers to the sensation on the tongue and roof of mouth while the product is melting. The anchor used is 0 for very sandy and 10 for very smooth. Mean comparison for smoothness using Dunnet's test showed that CNEX5 10% to be the least smooth among the white chocolate. Panelist did not detect significant difference in CNEX5 1% and CNEM15 1% smoothness from the control white chocolate.

Erdem *et al.* (2014) reported that the addition of maltodextrin and lemon fiber into dark chocolate significantly impact the sweetness, firmness and adherence features of the dark chocolates compare to control sample. Firmness refers to force required to compress samples between tongue and palate while adherence refers to level of stickiness to molar teeth.

Panelist also rated chocolate with the addition of 10% freeze-dried cocoa polyphenol nanoemulsion with xanthan gum to be the least acceptable and also with the least hardness although both attributes are not significantly different.

CONCLUSION

Data showed that addition of 10% freeze-dried cocoa polyphenol nanoemulsion affect the texture, color and moisture content of white chocolate. Sensory data showed that only the smoothness showed significant difference. Only chocolate incorporated with 10% freeze-dried cocoa polyphenol nanoemulsion with xanthan gum was significantly less smooth compared to the control white chocolate.

ACKNOWLEDGEMENT

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HARDNESS AND SURFACE GLOSSINESS OF MILK CHOCOLATE CONTAINING 1-3% MOISTURE ON STORAGE

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ABSTRACT - *A storage study was conducted for milk chocolates containing 5 levels of moisture (1-3%) incubated at 16°C and 30°C for duration of 6 months. The effect on hardness and surface glossiness of the chocolate were determined. Results showed that no significant effect ($p>0.05$) of moisture (1-3%) in chocolate to its hardness measured both as penetration and breaking force. After 6 months of storage at 30℃ however, the hardness was significantly reduced ($p<0.05$) with the increase in moisture content of chocolate. Storage of chocolate at 16℃ showed no significant changes of hardness throughout the 6 month study. At 30℃ however, the force needed to penetrate the chocolate increased with storage time for chocolate containing less than 2.5% moisture. The force needed to break the chocolate also increased with storage time, but stabilised after 3 months. Chocolate containing more than 2.5% moisture was seen to decrease in hardness after 3 months storage at 30℃.The glossiness of the surface was not significantly ($p>0.05$) affected by the moisture content in chocolate but was mainly influenced by its storage temperature and time. The glossiness value was significantly reduced when the chocolate was stored at 30°C after a period of one month. The finding demonstrated that the incubation temperature significantly ($p<0.05$) affected textural characteristics and surface glossiness of milk chocolate during storage.*

Keywords: Chocolate, moisture content, hardness, glossiness.

INTRODUCTION

The quality of chocolate depends on its physical and organoleptic properties. It should be solid at room temperature with a good snap and shiny appearance along with easy melting in the mouth, giving a pleasant mouthfeel sensation (Afoakwa, 2016). Chocolate products can be classified as "medium" or "long life" products which, when packaged, should have a storage life of 12 months or longer (Man & Jones, 2000). Chocolate should be held at 60% relative humidity and temperature within the range 16-18℃ (Lees & Jackson, 1985). It is a very stable product when stored under correct conditions of temperature and humidity mainly due to its low moisture content (1.0-1.9%). Nevertheless, degradation in quality may occur at earlier stages whereby its physical and/or organoleptic properties no longer correspond to the desired parameters. Factors causing deterioration of chocolate may be those which are inherent in the product itself such as its moisture level, and those which are dependent on the environment such as its storage condition. The moisture content in chocolate may vary due to differing compositions and ingredients used in the formulations, and the storage conditions may depend on the location and climates.

This paper will look into the effect of different moisture contents (1-3%) in chocolate and storage temperatures of 16℃ and 30℃ on the hardness and surface glossiness of milk chocolate stored for duration of 6 months. The temperature 16℃ correspond to the chill condition normally used to store products prior to marketing and 30℃ would represent a slightly warmer condition during handling, transportation and storage of products normally experienced in tropical climate.

MATERIALS AND METHODS

Materials

Milk chocolate was made using commercially sourced ingredients. Cocoa liquor (natural

Malaysian cocoa liquor) and cocoa butter (deodorized, pure prime pressed of Malaysian origin) were purchased from Barry Callebaut Malaysia Sdn. Bhd., Klang, Selangor. Whole milk powder (New Zealand) was purchased from Promac Enterprises (Malaysia) Sdn. Bhd., Klang, Selangor. Lecithin (L-phosphatidylcholine from soya bean) was purchased from Akashi Biosystems (Malaysia) Sdn. Bhd. Shah Alam. Sugar and vanillin powder were purchased from local supermarket.

Sample preparation

Milk chocolate was formulated to contain a total fat content of 33.5% inclusive of cocoa butter and milk fat, 6% fat free cocoa solid, 17% fat free milk solid and 43% sugar. Mixing of ingredients with small doses of cocoa butter fraction was carried out using a laboratory scale mortar and pestle mill (Pascal, UK) at 45°C. Subsequently the mixture was refined using a three roll refiner (Pascal, UK) to attain chocolate particle of less than 40µm in size. The size was measured using portable digital micro-screw meter (Mitutoyo, Japan). The refined chocolate mass was then transferred back into mortar and pestle mill and the remaining fraction of cocoa butter was added to the mixture and conched for 6 hour at 55ºC. Lecithin was added and the conching process was continued for another two hours. Finished liquid chocolate was tempered manually according to method described by Talbot (1994). Approximately two-third of melted chocolate from a bowl was poured onto a cold marble slab in room temperature of 24-26ºC. Using a stainless steel scrapper, the chocolate mass was spread out on the cold marble slab surface and then folded back. The process was repeated until the chocolate mass reached a temperature of 27 – 28ºC measured by a portable digital thermometer (Thermamite 1, U.K). The chocolate was added back to the rest of the warm chocolate in the bowl and mixed thoroughly. The temperature of the final chocolate mass should be around 29-30ºC.

The moisture content in the chocolates were varied to contain 5 levels of moisture by adding solid particulate cocoa butter emulsion (1:1 of cocoa butter:water) at 0.0, 1.0, 2.0, 3.0 and 4.0% into the tempered liquid milk chocolate. The chocolate was then poured into chocolate mould, vibrated to remove air bubbles, and cooled in a chiller at 16ºC for 30 min. The chocolates were then removed from mould and packed in airtight plastic container. Three replications were made. Moisture content of samples was determined using HR73 Halogen moisture analyzer (Metler Toledo, Malaysia).

Storage condition

Chocolates samples with 5 levels of moisture content were placed in airtight container and stored in incubators (IFE 550, Memmert, Germany) at temperatures 16℃ and 30℃ (± 1°C) for 6 months. Data for hardness, glossiness and moisture content were collected monthly for duration of six months.

Hardness analyses

Hardness of chocolate was determined at 24°C (ambient temperature) with an SMS Texture Analyzer (TA.XT plus, UK). The force needed to break the chocolate (breaking force) was measured by means of the peak force (expressed in gram) required to break a piece of chocolate (16 mm width x 35 mm length x 15 mm height), using a three-point break test with HDP/3PB probe attached to a 30 kg load cell, moving at a pre-test speed of 2.0 mm/s, test speed of 2 mm/s, post-test speed of 10.0 mm/s and the target mode was set at 5 mm distance. The force needed to penetrate the chocolate (penetration force) was measured by means of peak force needed to penetrate the chocolate at 5 mm depth using a 2 mm stainless steel needle probe, with other setting parameters similar to that of breaking test. Measurement was determined in triplicate and the mean value was used.

Surface gloss analyses

Glossiness of chocolates was measured by Tri-microgloss (Sheen, England) where a beam of light was projected on to the flat surface of chocolate and the strength of light reflected within a narrow angle was measured. Projection angle of 20°, 60° and 85° were used. The numerical scale used was a reference to 100 for a polished black glass plate, which was calibrated at each time of measurement. Measurement was made at 2 positions for each chocolate samples and 3

replication was made. The mean value from a total of six readings was calculated.

Moisture content analyses

The moisture content of cocoa butter emulsion and chocolate samples was determined using HR73 Halogen moisture analyzer (Metler Toledo, Malaysia). Chocolate samples were separately cut into small pieces and then each milled using a dry kitchen blender into fine solid particles. 5 mg of the respective sample was placed on the pan and heated to 105ºC until constant weight was achieved. Measurement was determined in triplicate and the mean value calculated.

Statistical analyses

Data were analyzed statistically using Minitab-14 software for analysis of variance (one-way ANOVA) and Tukey multiple comparison test at a significance level of 0.05.

RESULTS AND DISCUSSION

Moisture content in chocolate samples

The actual moisture content of chocolates added with 5 levels of cocoa butter emulsions (1:1 of cocoa butter:water) were measured and result is shown in Table 1. The moisture level was found to range between 1.02 to 2.95% where control sample (sample 1) of 1.02% was within the typical moisture content of commercial chocolate (Afoakwa *et al*., 2007). Samples with added cocoa butter emulsion contain slightly less moisture than expected. The reducing value may be resulted from moisture loss and uneven distribution of water-in-oil particles during the tempering procedure.

Table 1. Moisture content of milk chocolate samples

Sample code	1	2	3	4	5
Added cocoa butter emulsion (%)	0.0	1.0	2.0	3.0	4.0
Moisture content (%)	1.02±0.17	1.44±0.26	2.01±0.09	2.54±0.07	2.95±0.13

Mean moisture content ± standard deviation, n=3.

Effect of moisture content and storage temperature on hardness of chocolate

In this study, hardness was measured as the force needed to penetrate and break a piece of chocolate which could represent the hardness felt on the first bite of chocolate. The effect of moisture level and storage temperature on force needed to break and penetrate a chocolate at ambient temperature is shown in *Figure 1 and 2*. The moisture level (1-3%) in chocolate did not significantly affect ($p>0.05$) its hardness measured both as penetration and breaking force. Chocolate stored at 30°C showed a significantly lower ($p<0.05$) breaking and penetration force than chocolate stored at 16°C. This was probably due to the weakening of fat crystals network as its solid fat content was reduced due to the increase of storage temperature. After 6 months of storage at 30℃ however, the hardness was seen to be significantly increased, probably due to the stabilisation of fat crystals packing over time. On the other hand, the increase in moisture content of chocolate stored at 30℃ after 6 month resulted in a decrease in chocolate's hardness. Moisture presence in the chocolate could lead to aggregation of sugar particles that reduces the relative strength of particle to particle interaction (Afoakwa, 2008) thus reduces its hardness and this effect increases with storage time.

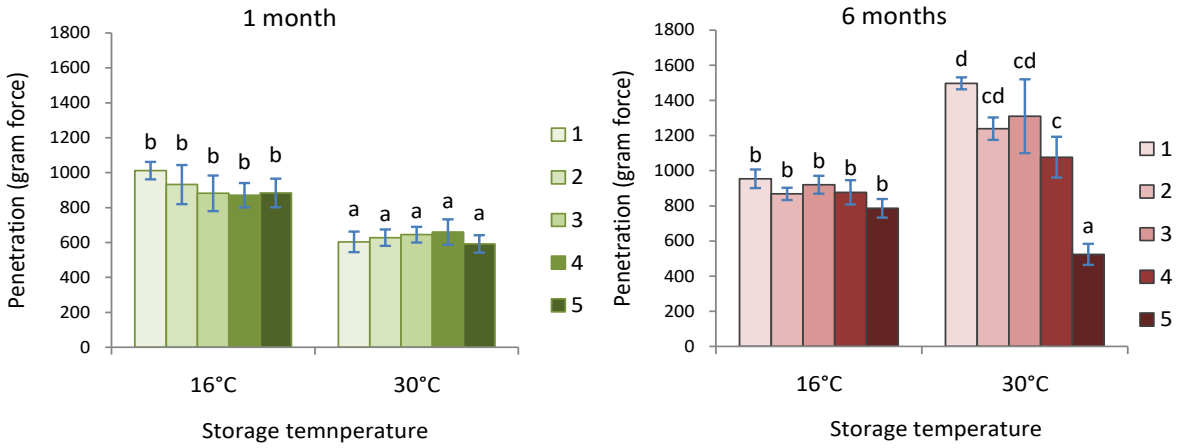

Figure 1. Penetration force for chocolate samples (1-5) containing different moisture contents stored at 16 and 30°C after 1 and 6 months storage.
Error bars are 95% confidence intervals for mean based on standard deviation, n=3. Different letters indicate significant different at p<0.05.

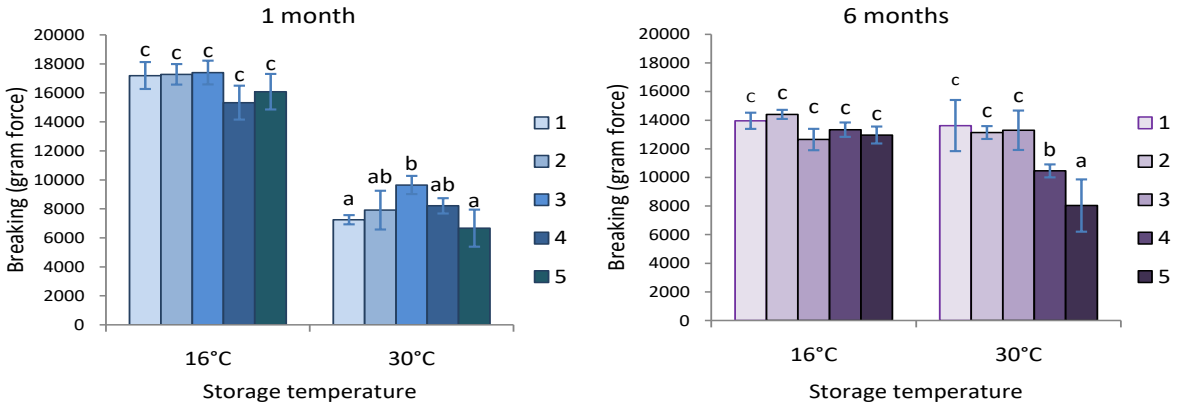

Figure 2. Breaking force for chocolate samples (1-5) containing different moisture contents stored at 16 and 30°C after 1 and 6 months storage.
Error bars are 95% confidence intervals for mean based on standard deviation, n=3. Different letters indicate significant different at p<0.05.

Overall, chocolates stored at 16°C showed no significant changes of hardness throughout the 6 month study *(Figure 3 and 4)*. At 30°C however, the force needed to penetrate the chocolate increased with storage time for chocolate containing less than 2.5% moisture. The force needed to break the chocolate also increased with storage time, but stabilised after 3 months. Chocolate containing more than 2.5% moisture was seen to decrease in hardness after 3 months storage at 30°C.

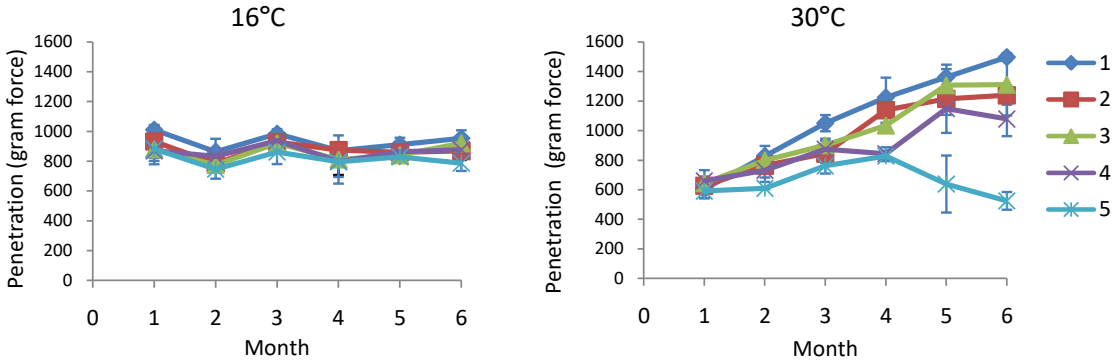

Figure 3. Penetration force of chocolate samples (1-5) with different moisture content stored for 1-6 months at 16°C and 30°C. Error bars are 95% confidence intervals for mean based on standard deviation, n=3

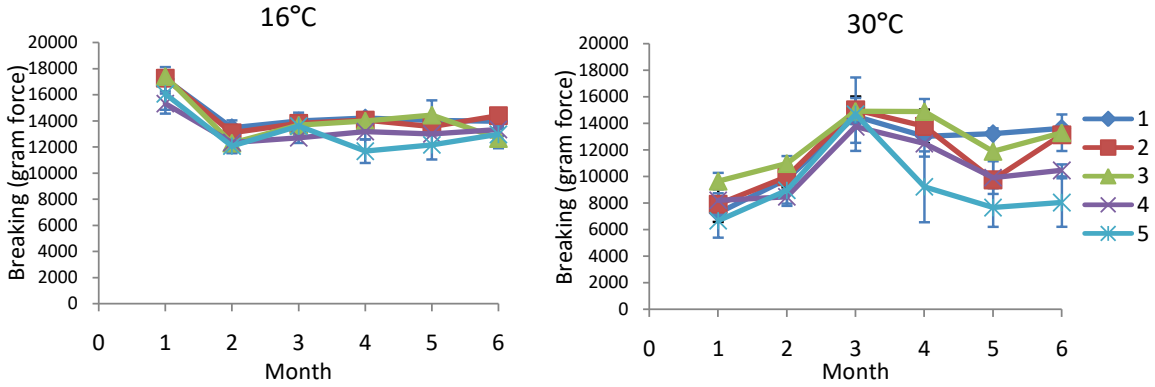

Figure 4. Breaking force of chocolate samples (1-5) with different moisture content stored for 1-6 months at 16°C and 30°C. Error bars are 95% confidence intervals for mean based on standard deviation, n=3

Effect of moisture content and storage temperature on surface glossiness of chocolate

The measurement of gloss in chocolate correlates with visual observation of surface shininess and represent the capacity of a surface to reflect directed light (ASTM, 1995). The glossiness of chocolate surface is dependent upon the polymorphic form of cocoa butter crystals which is determined by the tempering temperature of chocolate (Afoakwa, 2008; Beckett, 2000). The surface topography of chocolate was also reported to influence its glossiness (Briones, 2006). In this study, all chocolate samples were tempered manually using the same procedure and moulded into polycarbonate mould of the same shape and size. This would eliminate variations in tempering

parameters and surface topography. The gloss reading at incidence light angle of 85° was selected since the readings obtained at 60° and 20° angle were below 10 gloss unit which were considered as low gloss surface (Briones, 2006; Mendoza *et al.*, 2010).

Figure 5 shows the effect of moisture and storage temperature on the glossiness of the chocolates after one and 6 month storage. Chocolates stored at 30°C were significantly less glossy than chocolates stored at 16°C. The effect of moisture content in each storage temperature was seen to fluctuate but did not differ significantly ($p>0.05$).

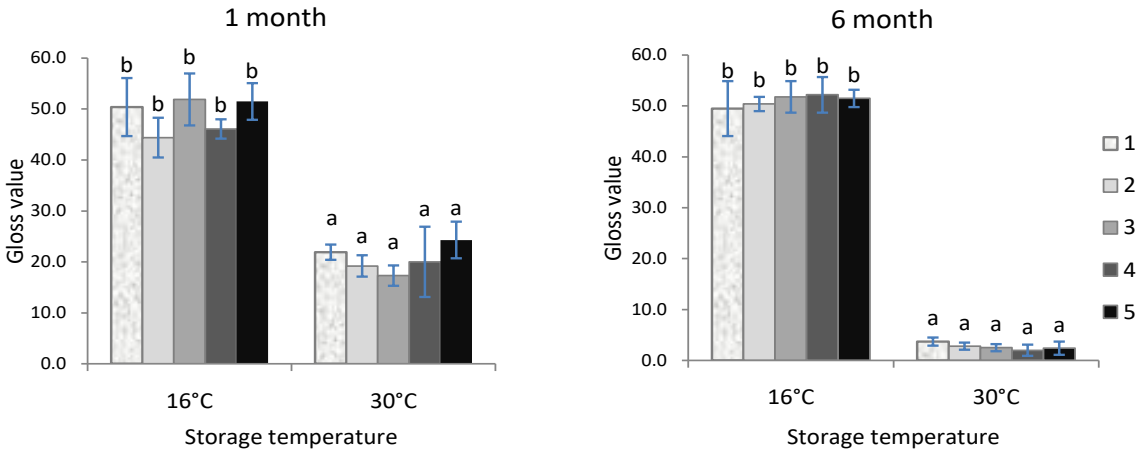

Figure 5. Surface gloss for chocolate samples (1-5) containing different moisture contents stored at 16°C and 30°C after 1 and 6 months storage measured at 85° angle.
Error bars are 95% confidence intervals for mean based on standard deviation, n=6. Different letters indicate significant different at p<0.05.

The effect to surface gloss of chocolate can be explained in terms of changes of cocoa butter crystal forms as temperature increases. When chocolate was tempered (at 28-29°C) and subsequently cooled (16°C), a closed-packed shiny stable beta crystal was formed (Beckett, 2000). The decrease in glossiness of chocolate with storage time and temperature *(Figure 6)* could be related to the whitening formation on the surface of chocolate or also known as bloom formation. It could either result from the crystallization of sugar (sugar bloom) or fat (fat bloom) molecules on the surface of chocolate. Fat bloom was caused by the fat migration to the surface of chocolate which could occur at temperature above 18°C and is accelerated with the increase of storage temperature (Lonchampt & Hartel, 2004; Ali *et al.*, *2001*). This explains the reduction of gloss value when chocolate was stored at 30°C. Sugar bloom on the other hand occurs in the presence of moisture that would dissolve sugar particles on the surface of chocolate and recrystallizes it to give a whitish surface appearance (Lees & Jackson, 1985). From the experiment it seems that the increase in moisture content in chocolate did not significantly affect their gloss value at individual storage temperatures. Since the chocolates were stored in airtight container, the atmospheric humidity may not contribute to the formation of sugar bloom. Therefore the glossiness of chocolate was mainly affected by storage temperature and storage time. In this study, the gloss values of chocolates were markedly reduced (less than 10 gloss value) when chocolates were stored at 30°C after 3 months storage. Storage at 16°C for 6 months did not significantly affect chocolate's glossiness.

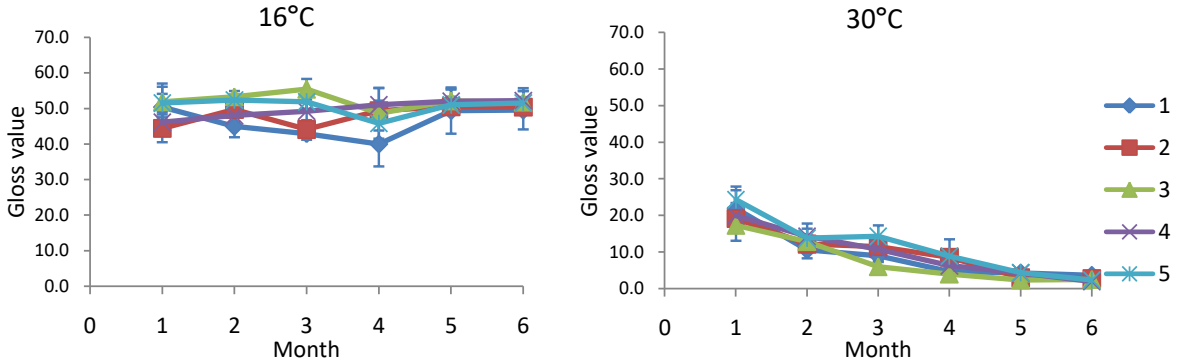

Figure 6. Surface gloss of chocolate samples(1-5) with different moisture content stored at 16 and 30ºC for 1-6 months. Error bars are 95% confidence intervals for mean based on standard deviation, n=6.

CONCLUSION

The incubation temperature significantly ($p<0.05$) affected textural characteristics and surface glossiness of milk chocolate during storage. The moisture content of 1-3% in milk chocolate however did not significantly ($p>0.05$) affect their physical properties during storage.

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INDUCTION AND PROPAGATION OF SOMATIC EMBRYOS FROM CELL SUSPENSION CULTURES OF *Theobroma cacao* L.

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ABSTRACT - *Two different formulations of liquid medium culture were investigated and compared to overcome poor somatic embryo development and mass propagation in selected genotypes of* Theobroma cacao *L.. Embryogenic cells induced from staminodes and petals, cultured on modified Nestle induction medium, was used as inoculum for liquid cultures. Approximately 0.2 g (fresh weight) of somatic embryos were used as starting culture. After 3 months of culture, the frequency of somatic embryo formation differed between genotype and the medium used. Embryos produced were transferred to agar medium for further development and conversion into plantlets.*

Key words*:* Liquid medium, embryogenic cells, somatic embryos, *Theobroma cacao* L.

INTRODUCTION

In plants, embryo-like structures can be generated from cells other than zygote. The origin of these asexual embryos is diverse: apomictic embryos derive from unfertilized eggs or even from surrounding maternal tissue, androgenetic embryos develop from *in vitro* cultured immature pollen grains (microspores) and somatic embryos originate from somatic cells grown *in vitro* (Mordhorst *et al.,* 1998). Somatic embryogenesis itself is defined as a process which involves the development of nonzygotic plant cells, also known as somatic cells to differentiate into embryos which later regenerate into whole plants.

Somatic embryo (SE) may develop directly from somatic cells or indirectly from calli aggregates during the culture of a plant cell, tissue or organ (Sadeq *et al.,* 2014). It offers numerous advantages which include the production of synthetically coated seeds and unlimited production of clones with elite traits (Aslam *et al.,* 2011). The pattern of the developmental response of cultured tissue is epigenetically determined. The process of somatic embryogenesis is mainly regulated by several factors, including plant growth regulator, transcription factor and other related substances during each stage of development.

However, the efficiency of primary SEs to produce a large number of plants is relatively low and often develop into abnormal plants. Therefore, secondary SEs, which are initiated from primary SEs were established.

Based on the previous study, secondary somatic embryogenesis has the potential to increase embryo production up to 30-fold and help to reduce its cost (Maximova *et al.,* 2002). The secondary embryos produced are more uniform, well-developed and offers the advantages of a high multiplication rate, independence from explants source effects, repeatability and can be maintained for a prolonged period by repeated cycles of secondary embryogenesis (Yang *et al.,* 2013).

Regardless of medium types or plant species, the multiplication of embryogenic cells is a key step to evolve from a small research scale (petri dishes) to large volume, therefore increasing the number of plantlets. However, this technique, also called mass propagation is considered as labour intensive when semi-solid medium is used. To overcome this problem, a liquid system in which SEs are freely suspended and grown in a liquid medium could be an alternative.

The use of liquid medium enables the embryogenic tissue proliferation and mass production of SEs in Erlenmeyer flasks or bioreactor of more than 20 species (Etienne-Barry *et al.,* 1999). Liquid culture systems also provide various advantages for mass propagation such as culture condition are much more uniform and medium can be changed easily (Martre *et al.,*

2001). This liquid technique has been the subject of many studies over many years (Jalal and Collin, 1979; Leathers and Scragg, 1989; Tsai and Kinsella, 1981, 1982; Gurney *et al.,* 1992; Rojas *et al*., 2008)

This short study focussed on the selection of the most suitable medium to mass propagate *Theobroma cacao* secondary SEs via liquid culture by using selected cocoa genotypes from class I, II, III and IV, recommended by Malaysian Cocoa Board as well as other genotypes that bear excellent traits and quality.

MATERIALS AND METHODS

Plant materials

Unopened two-to-three-week old (4 to 6 mm in length) cocoa flower bud from eight cocoa genotypes (PBC123, PBC154, KKM19, QH441, QH1176, QH1287, MCBC2 and MCBC7) were used as a source of explants in this study.

The selection was based on the SEs performance on a solid medium study carried out separately (data not shown). Samples were collected from field-grown clonal plants in the Center of Cocoa Biotechnology Research, Malaysian Cocoa Board.

Embryogenic calli and somatic embryos induction

Unopened flower buds were washed with tap water and surface sterilized as described in Norasekin *et al.* (2013).

For induction of embryogenic calli, both staminodes and petals were extracted and cultured in 90 mm diameter plastic petri dishes containing 30 ml of modified Nestle induction medium supplemented with plant growth regulators. Dishes were incubated for 2 weeks in the dark at 25 ± 2^{O}C. Then, the cultures were transferred to a modified secondary calli growth medium (NIM2) for another 2 weeks.

For induction of SEs, the cultures were transferred to a differentiation medium (NIM3). On this medium, the cultures were maintained with periodic subculturing at an interval of three weeks until primary SEs were observed.

Somatic embryogenesis from cell suspension culture

Two formulations of basal culture medium were used in this study: NIM3liq and CC21liq. To establish cell suspension culture, primary SEs established from NIM3 were used.

Approximately 0.2 g (fresh weight) of SEs were inoculated in an Erlenmeyer flask containing 50 ml liquid medium. Cultures were placed on a rotary shaker with continuous shaking at 120 rpm in the dark at 25 ± 2^{O}C. The cultures were maintained in continuous repetitive cell cycles by subculturing the embryogenic cells every 2 - 3 weeks (depending on the degree of responsiveness of the cell sample to the medium used) onto fresh liquid medium. The number of SEs produced was recorded after minimum 3 cycles or 8 weeks.

The experiment was carried out in five replicates per genotype per medium. Cultures with normal and good proliferation of SEs were agitated continuously until adequate culture establishment was reached before SEs undergo maturation, germination and conversion to plantlets.

RESULTS AND DISCUSSIONS

Embryogenic calli and somatic embryos induction

Suitable combination of PGR used in the induction medium results in dedifferentiation of explants' cells to form calli within 2-4 weeks. A visible morphological variation was observed in calli formation which are fluffy, compact white and/or yellow calli and friable calli with an occasional mixture of both. The difference in calli morphology was also reported in some other plants (Junaid *et al.*, 2007a).

According to a method described by Siti Norhana *et al*. (2016), primary SEs from selected genotypes were consistently produced at a higher mean percentage in between four to eight weeks after culture initiation on modified Nestle induction medium. These embryos were formed on the surface of the calli and appeared globular in structure.

After several weeks of embryos development, embryogenic structures of primary

SEs observed were classified into three morphological categories; (a) normal embryos, which are usually in a globular, heart and torpedo shape; (b) fasciated embryos, which consist of two or more fused embryos with multiple cotyledons and (c) abnormal embryo-like structure which is formed in groups, missing apical meristem development and lateral symmetry.

Figure 1 represents SEs derived from the cocoa staminode undergoing calli formation and primary SEs at different stages of embryogenesis.

However, as in many other species, the number of *Theobroma cacao*'s normal SEs formed was very low. Therefore, the embryogenic calli and primary SEs *(Figure 1b)* were selected and introduced to suspension culture for secondary SEs induction and proliferation.

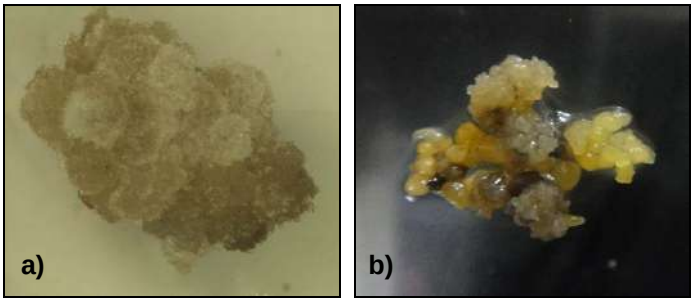

Figure 1. Somatic embryogenesis of cocoa. (a) Calli tissues from cocoa staminode; (b) Primary somatic embryos at different morphological categories and developmental stages.

Somatic embryos formation from cell suspension culture

After approximately 12 weeks in liquid culture, embryos were harvested and counted. All genotypes used in this study produced secondary SEs from the primary SEs after 2-3 weeks inoculation in NIM3liq medium, but only six genotypes produced secondary SEs after inoculation in CC21liq medium (Table 1). The multiplication efficiency was categorized in four stages: no proliferation of SEs; low proliferation of SEs (<2-fold); medium proliferation of SEs (>2- fold); and high proliferation of SEs (>5-fold).

Table 1 showed the observation of culture after three cycles in both medium. No proliferation of the SEs was recorded for all five replicates of genotypes PBC154 *(Figure 2a)* and PBC123 when the SEs were inoculated in CC21liq (PGR-free differentiation medium) after three cycles or minimum nine weeks of subculture.

On the contrary, for genotype MCB7, the suspension culture did not grow robustly, although the multiplication efficiency of the SEs was moderate at an average of 5-fold.

Table 1 also showed that the rest of the genotypes (KKM19, QH1176, MCBC2, QH441 and QH1287) produced a sub par proliferation rate. Also, few SEs only started to develop and propagate at the third cycles. The average of the low proliferation rate was below than 2-fold.

Contrary to the result mentioned above, SEs from several genotypes inoculated in NIM3liq showed better proliferation performance. Culture from genotypes PBC123, MCBC7 and MCBC2 responded well even though the proliferated embryos were obtained from non-synchronized embryogenesis culture, which consisted of a mixture of embryos from all developmental stages i.e. globular, heart and torpedo stage *(Figure 2b and 2c).* SEs from genotype PBC123 showed more uniform development if compared to genotypes MCBC7 and MCBC2 (figure not shown). However, cultures from genotypes KKM19, QH1176, PBC154, QH441 and QH1287 responded laboriously to the same medium.

However, the number of normal SEs only counted once the culture reached adequate culture establishment (data not shown) before SEs undergo maturation, germination and conversion to plantlets.

These results showed differences in embryogenesis that might be attributed to the genotypes used, physiological status of the donor plants and culture time (Quainoo and Dwomo, 2012).

Table 1. Observation of somatic embryos proliferated in CC21liq and NIM3liq medium.

Genotype	Class	Medium	Observation after 3 cycles of subculture
PBC123	I	CC21liq	No proliferation of SE
		NIM3liq	Moderate proliferation of SE
KKM19	II	CC21liq	Low proliferation of SE
		NIM3liq	Low proliferation of SE
MCBC7	III	CC21liq	Moderate proliferation of SE
		NIM3liq	Moderate proliferation of SE
QH1176	III	CC21liq	Low proliferation of SE
		NIM3liq	Low proliferation of SE
MCBC2	III	CC21liq	Low proliferation of SE
		NIM3liq	Moderate proliferation of SE
QH441	IV	CC21liq	Low proliferation of SE
		NIM3liq	Low proliferation of SE
QH1287	IV	CC21liq	Low proliferation of SE
		NIM3liq	Low proliferation of SE
PBC154	?	CC21liq	No proliferation of SE
		NIM3liq	Low proliferation of SE

Figure 2. Somatic embryos performance after inoculation in a liquid expression medium. (a) Culture from PBC154 in CC21liq medium after 3 cycles of inoculation; (b) and (c) Mix of secondary SEs formed-normal and abnormal SEs.

From this study, both medium are capable to develop and produce secondary SEs from several cocoa genotypes. The recorded multiplication efficiency is between low to medium. However, not only well developed SEs was produced, but undeveloped embryos with numerous globule shaped also formed.

Decrease or increase of embryogenic capacity among the explants of the same genotype between replicates could be attributed to the differentiation process and is related to the reduction in the number of cells competent to undergo somatic embryogenesis (Vooková and Kormuťák, 2005).

Growing embryogenic cultures in suspension allow a better synchronisation of the cultures (Von Arnold, 2008). However, prolonged culturing in a liquid medium generally induces degeneration of cultures and loss of embryogenic capacity or increase the risk of the appearance of somaclonal variation (Etienne and Bertrand, 2003).

One of the main problems encounters in cocoa culture is a high browning phenomenon of the cultured cells. Generally, it is regarding the naturally high polyphenolic compound in cocoa-tree. Therefore, it is necessary to avoid browning by subculture the cells frequently (every 2-3 weeks), replenish with a new medium to prevent the cell suspension from becoming brownish, which is will decrease the growth rate and finally declined.

Other than that, a major issue related to the use of liquid medium is hyperhydricity of the cocoa cultured embryo. It is a physiological disorder that results in morphological and physiological alteration of plants, often giving them a 'glassy' appearance, due to apoplastic water accumulation (Watt, 2012). The cause of the hyperhydricity is the constant immersion of the explants which will lead to the abnormal growth and necrosis. Because of this, application of temporary immersion bioreactor after culturing explant in a certain period in Erlenmeyer flasks is an alternative. However, adequate culture establishment is recommended before temporary immersion system can be initiated.

Results from this short study revealed that selected cocoa genotypes suspended in CC21liq medium showed much slower multiplication rate compared to when suspended in NIM3liq medium. This result was similar to their sub par performance observed in the solid state of the same medium line (data not shown).

Because of the low proliferation rate, temporary immersion system was not initiated. All normal SEs with bipolar torpedo shape and starchy SEs with at least 2cm in length with developed cotyledons or with just an apical dome but developed radicle end were allowed to undergo maturation. Once matured, they were transferred to rooting medium.

Cocoa somatic embryogenesis from calli or cell suspension culture is the most common alternative and efficient method to get high frequency of SE formation in order to increase plant regeneration.

In many cases, *in vitro* developed SEs are liable to form an embryogenic calli or secondary embryos (Choi *et al.,* 1998). Therefore, in this study, SEs of *Theobroma cacao* were used to induce secondary somatic embryogenesis. However, there is a limitation of this protocol on cocoa mass propagation, which is the secondary SEs efficiency was genotype dependent as in primary SEs (Masseret *et al.*, 2009). It is also possible to have numerous genotypes that react well to the medium to produce primary SEs yet respond laboriously to propagate to secondary SEs.

The finding of genotype dependent of cocoa SEs is supported by the previous research done by Malaysian Cocoa Board, Nestle Research Centre and other cocoa research institution. Hence, it is a challenge to devise the best-suited medium for each genotype recommended by MCB to ensure the highest number of SEs produced.

CONCLUSIONS

A system for the establishment of cocoa SEs or cocoa cell suspension is described in this study and evidently not too far different compared to other crops. It is a fast and efficient method that potentially gives high frequency of plant regeneration. However, further improvement on the formulation of the most suitable liquid medium for the selected cocoa genotypes is vital to increase the propagation of elite trees as recommended by MCB.

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IDENTIFICATION OF GENETIC MARKERS FOR BLACK POD, CPB, YIELD, BUTTER CONTENT, SELF-COMPATIBILITY, VSD & POD HARDNESS USING GENOMIC SELECTION

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ABSTRACT- *"Genomic selection" comprises methods that use genotypic data across the whole genome to predict any trait with an accuracy sufficient to allow selection on that prediction alone. The work begins with chipping a seed or leaves, extract its DNA, and discard it or select it as a parent of the next generation. The development of high density SNP information of fourteen local cocoa germplasm has enabled genomic selection in selective breeding programs in association with the mapping of the genetic basis of complex traits. However, to genotypes all cocoa clones available routinely for high density SNP may be prohibitively expensive. One means to reduce genotyping cost is the use of genotype imputation, were selected cocoa clones (fourteen cocoa clones) are genotyped at high density, and the majority of individuals (for selection of candidates) as in this study, three hundred of them were genotyped at much lower density, followed by imputing to the high density SNPs. The performance of genomic prediction using imputed genotype data was comparable to using true genotype data, and both were superior to pedigree-based prediction as evidenced in research in animal and crop improvement using genomic selection worldwide. Statistical analysis of the training sets simultaneously estimates allele effects at all loci. Other selection candidates, are genotyped allowing their genomic estimated breeding values (GEBVs) to be calculated. Selection for breeding with desirable traits will proceed on the basis of these GEBVs. Ultimately a four-year breeding cycle, including three years of field testing, can be reduced to only the four months required to grow and cross a plant. Thousands of selection candidates can be evaluated without ever taking them out to the field. Ongoing field trials are still very much a part of a breeding program using genomic selection (GS), but the purpose, and therefore the practice, shifts: phenotypes are no longer used to select but to train a prediction model.*

***Keyword**:* Genomic selection, cocoa, phenotype, GEBV, breeding

INTRODUCTION

Plant breeding can be broadly defined as alterations caused in plants as a result of their use by humans (Lateef, 2015; Jannink *et al.*, 2010), ranging from unintentional changes resulting from the advent of agriculture to the application of molecular tools for precision breeding (Bevan, 2007; Sakiyama *et al.*, 2014; Jiang, 2013). The vast diversity of breeding methods can be simplified into three categories: i) plant breeding based on the selection of observed variants, disregarding their origin; (ii) generation and selection of expanded variation by controlled mating; and (iii) monitoring the inheritance of within-genome variation and selection of specific recombinants.

The ultimate output for plant breeding is the selection of better types among variants, in terms of yield and quality of edible parts; ease of cultivation, harvest, and processing; tolerance to

environmental stresses; and resistance against pests (Bhat *et al.,* 2016; Jarquin *et al.,* 2014). Manipulating a single trait, disregarding all others, is relatively straightforward; however, this is unlikely to result in a useful variety. The challenge of plant breeding resides in improving all of the traits of interest simultaneously, a task made more difficult by the genetic correlations between different traits, which may be due to genes with pleiotropic effects, to physical linkage between genes in the chromosomes, or to population genetic structure. Selecting for one trait will change correlated traits, sometimes in the desired direction, other times in an unfavorable way (Grant-Downton *et al.,* 2005; Endo *et al.,* 2016).

Genomic selection is a form of marker-assisted selection in which genetic markers covering the whole genome are used so that all quantitative trait loci (QTL) are in linkage disequilibrium with at least one marker (Goddard *et al.,* 2007; Heffner *et al.,* 2009; Lorenz *et al.,* 2011). This approach has become feasible thanks to the large number of single nucleotide polymorphisms (SNP) discovered by genome sequencing and new methods to efficiently genotype large number of SNP (Calus, 2010; Meuwissen *et al.,* 2001; Varshney *et al.,* 2014). The objective in genomic prediction is to estimate effects of individual SNP alleles on a trait of interest (Mammadov *et al.,* 2012; Liu *et al.,* 2016).

By linking the genotypic and phenotypic information together, estimates for each of the SNPs are obtained using genotype imputation. Genotype imputation involved high density genotyping of certain key individuals, while the majority of individuals are screened with a lower density SNP panel (Rafalski, 2010; Voss-Fels *et al.,* 2016). These genotype data are then used to impute the non-genotyped markers for the individuals genotyped at low density (Tsai *et al.,* 2017; Brøndum *et al.,* 2014; Dimauro *et al.,* 2013). Imputation approaches have been successfully and widely applied in breeding programs for several livestock and crop species (Poland *et al.,* 2012; Tsai *et al.,* 2017). The last step in the process involves genotyping of young selection candidates, whose GEBVs are obtained by summing up all the relevant SNP effects (Heffner *et al.,* 2010; Jan *et al.,* 2016; Rabier *et al.,* 2016).

MATERIAL AND METHOD

Plant leaves and phenotypes

The genotype and trait data used in the study were collected from our local 300 cocoa clones from Bagan Datuk, Jengka, Madai and Tawau. The samples comprised of seven phenotypes such as black pod, Cocoa Pod Borer, yield, butter content, self-compatibility, Vascular Streak Dieback and pod hardness. These traits are suspected to be heritable, but with a predominantly polygenic genetic architecture.

Low coverage re-sequencing

The whole genome re-sequencing of 300 DNA libraries was done on the illumina NGS platform to generate at least 360 Gb of raw data in order to produce around three to four times genome coverage per sample based on an estimated genome size of 400Mb. Image analysis was performed in real time by Illumina HiSeq Control Software (HCS) v2.2.68 and Real Time Analysis (RTA) v1.18.66.3. The Illumina bcl2fastq 2.17.1.14 pipeline was then used to generate the sequenced data.

RESULTS AND DISCUSSION

Pre-processing of 300 genomes data

Low quality reads were filtered and potential sequencing errors were removed by k-mer frequency-based error correction. The following types of reads were removed:

- Reads having at least one 'N' base.
- Reads with an average Phred quality ≤30.
- Reads shorter than 80bp.

Reads were trimmed according to a quality score, from the 5'-end to the 3'-end, with a threshold mean score of 30. As shown in Table 1 below, 91% of reads from the 300 cocoa genomes, with a total size of approximately 500Gb, were considered as high quality preprocessed reads and were utilized as input for the SNP analysis.

Table 1. Statistical summary of sequencing reads before and after preprocessing for 300 *cocoa genomes*. (shown here is only the forst fourteen samples) A total of 91% of reads retrieved were of high quality

		Sequencing reads		Filtered reads			
No.	300 cocoa Sample ID	Number of reads	Sequence size (Gbp)	Number of reads	Sequence size (Gbp)	Number of reads (%)	Estimated times coverage (based on 400Mb genome size)
1	GSA00243	14,013,572	1.75	12,755,476	1.58	91.02%	3.96
2	GSA00244	14,507,334	1.81	13,245,714	1.64	91.30%	4.11
3	GSA00245	11,488,072	1.44	10,434,662	1.30	90.83%	3.24
4	GSA00246	12,716,028	1.59	11,448,798	1.42	90.03%	3.55
5	GSA00247	13,550,206	1.69	12,265,086	1.52	90.52%	3.81
6	GSA00248	14,292,690	1.79	13,074,020	1.62	91.47%	4.06
7	GSA00249	14,047,204	1.76	12,726,472	1.58	90.60%	3.95
8	GSA00250	14,341,934	1.79	12,940,298	1.61	90.23%	4.02
9	GSA00251	13,018,142	1.63	11,856,564	1.47	91.08%	3.68
10	GSA00252	13,457,068	1.68	12,215,572	1.52	90.77%	3.79
11	GSA00253	11,244,764	1.41	10,163,618	1.26	90.39%	3.15
12	GSA00254	12,738,924	1.59	11,365,162	1.41	89.22%	3.53
13	GSA00255	12,265,044	1.53	11,051,152	1.37	90.10%	3.43
14	GSA00256	12,411,992	1.55	11,350,868	1.41	91.45%	3.52

Reference Genome Assembly Database

A database was built for the repeat masked *Theobroma cacao* reference genome using MGRC synabase in house construction tool. Table 2 below summarizes the genome statistical parameters for 24,386 scaffolds of the *Theobroma cacao* reference genome version tc1.1.

Table 2. Assembly statistics summary of *Theobroma cacao* genome with a minimum contig size of 1kb. N90, N50 and N10 represent the length of the smallest contig in the set that contains contigs with combined lengths representing at least 90%, 50%, and 10% of the assembly respectively.

Maximum	Total scaffolds	Total size	Average	N90	N50	N10	%GC
23,032,738	24,386	396,242,104	16,248	5,140	7,557,474	22,025,072	34.53

SNP Calling

i) SNP statistics

Processed sequencing reads of the 300 cacao samples were mapped against the *Theobroma cacao* draft genome to identify SNPs.

Table 3. Number of raw SNPs and the average supporting reads depth distribution for each sample (shown the first 30). The average depth per SNP is around 4 supporting reads across 300 cocoa samples.

No.	Sample ID	Number of SNPs	Total Depth (DP)	Average DP per SNP
1	GSA00243	265,548	1,040,108	3.9
2	GSA00244	269,050	1,048,177	3.9
3	GSA00245	189,315	627,805	3.3
4	GSA00246	243,114	861,731	3.5
5	GSA00247	224,561	872,217	3.9
6	GSA00248	256,513	991,792	3.9
7	GSA00249	215,209	791,178	3.7
8	GSA00250	281,122	1,150,217	4.1
9	GSA00251	244,085	896,553	3.7
10	GSA00252	273,967	994,977	3.6
11	GSA00253	263,562	851,174	3.2
12	GSA00254	211,936	778,399	3.7
13	GSA00255	261,272	910,332	3.5
14	GSA00256	233,403	838,162	3.6
15	GSA00257	247,403	921,049	3.7
16	GSA00258	243,542	913,609	3.8
17	GSA00259	206,542	732,409	3.5
18	GSA00260	215,193	819,750	3.8
19	GSA00261	208,884	646,734	3.1
20	GSA00262	263,648	844,681	3.2
21	GSA00263	222,175	774,220	3.5
22	GSA00264	226,363	759,717	3.4
23	GSA00265	271,439	956,075	3.5
24	GSA00267	237,951	904,521	3.8
25	GSA00268	261,257	983,231	3.8
26	GSA00269	222,453	740,103	3.3
27	GSA00270	183,271	630,622	3.4
28	GSA00271	251,739	860,561	3.4
29	GSA00272	220,843	825,620	3.7
30	GSA00273	251,014	966,673	3.9

In order to obtain optimum high quality SNPs, all 300 samples were combined and treated as a single sample to do initial SNP calling. Based on the SNP Depth coverage distribution plot asshown on *Figure 1* below, SNPs with supporting reads between 200 to 1300 depth were considered for downstream analysis.

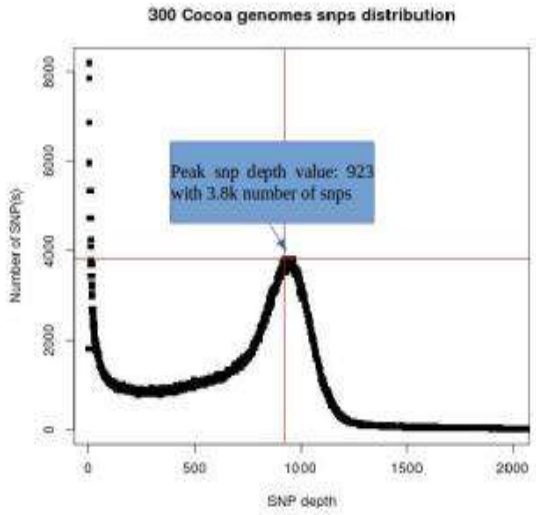


Figure 1. SNPs distribution by number of supporting reads from SNPs calling results of merged 300 cocoa genomes samples. Around 1.6 Million SNPs were considered for downstream analysis

ii) SNP classifications

Based on the filtered SNPs results, a total of 1.6 million SNPs with read support ranging from 200 to 1300 read depth were further classified. The statistical summary of the SNP Classification are provided in Table 4 and *Figure 2* below:

Table 4. SNP classification statistics by snpEff based on the genome annotation.

CLASSIFICATION OF SNPs	
DOWNSTREAM	536,096
INTERGENIC	780,851
INTRAGENIC	73
INTRON	174,854
NON_SYNONYMOUS_CODING	43,325
NON_SYNONYMOUS_START	11
START_GAINED	1,745
START_LOST	91
STOP_GAINED	1,628
STOP_LOST	152
SYNONYMOUS_CODING	27,013
SYNONYMOUS_START	1
SYNONYMOUS_STOP	50
UPSTREAM	15,967
UTR_3_PRIME	11,854
UTR_5_PRIME	6,232
Total SNPs	1,599,943

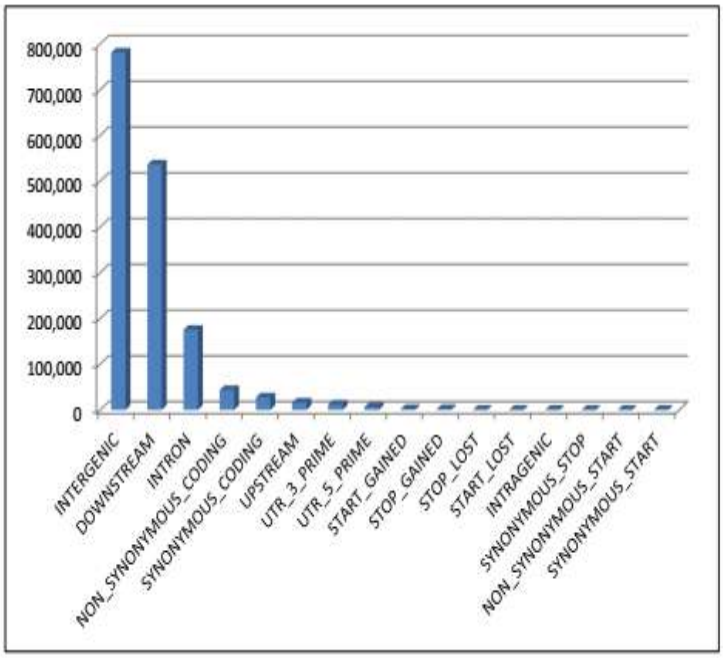


Figure 2. Histogram of SNP classifications statistics by snpEff and based on the genome annotation

iii) SNP density

SNPs from all scaffolds that mapped against public reference genome of *Theobroma cacao* cultivar of Matina 1-6, were used for SNPs density construction. SNP densities across the chromosomal regions of the genome were obtained using a 200 kb bin size for each chromosome. 10 SNP density graphs, one for each chromosome, were generated as shown below *(Figure 3)*.

Genome annotation

Gene annotation statistics

Transcript and protein alignments were performed on the repeat-masked genome by WindowMasker. Augustus was run across the scaffold assembly with *Arabidopsis thaliana* parameters for comparability as a plant model organism for gene identification. Besides the *Theobroma cacao's* transcripts sequences, we also used other cocoa species as a source of evidence (hints) for prediction and annotation of the *Theobroma cacao* draft genome (Table 5).

Table 5. Predicted gene statistics by utilization of the reference cocoa transcripts, *Arabidopsis thaliana* and other public cocoa species peptides as a source of evidence (hints)

Feature	Count	Minimum length (bp)	Maximum length (bp)	Average length (bp)
Genes	47,426	110	73,989	3,288

Genomic selection

Breeding Value (BV) predicted in GS is known as the genomic estimated breeding value (GEBV). In this project, 7 traits from 300 cocoa trees were sequenced to identify the SNP markers that mainly contribute on the effect of trait phenotypes differences. The list of traits measured is as follows (Table 6):

Table 6. List of the 7 traits from 300 cocoa samples that were sequenced to identify genomic selection markers.

No.	Trait	Phenotype Classification Indicator	
1	Black Pod (B)	Moderate Tolerence (MT)	Moderate Susceptible (MS)
2	CPB (C)	0-2 Resistance (R),2-3 Moderate (MR), 3-4 Susceptible (S)	
3	Yield (H)	>= 3000KG/HA/YEAR (HIGH)	<= 2000KG/HA/YEAR (LOW)
4	Butter Content (L)	>= 2880KG/HA/YEAR (HIGH)	<= 2310KG/HA/YEAR (LOW)
5	SCSI (S)	Self Compatibility (SC)	Self In Compatibility (SI)
6	VSD (V)	Moderate Tolerence (MT)	Moderate Susceptible (MS)
7	Pod Hardness (P)	> 110 kgcm-2 Hardest (HH), 95 - 110 kgcm-2 Hard (H), 65 - 80 kgcm-2 Moderate Soft (SM), < 65 kgcm-2 Soft (S)	

Figure 3 (a) Chromosome 1

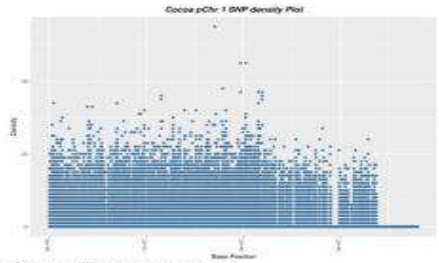


Figure 3 (b) Chromosome 2

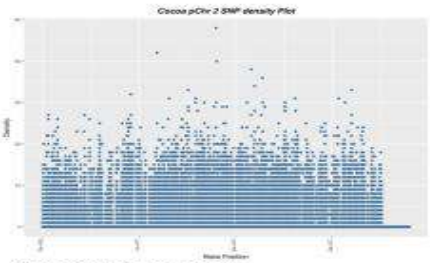


Figure 3 (c) Chromosome 3

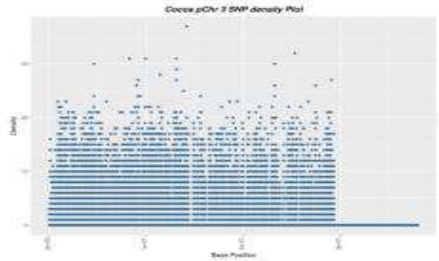


Figure 3 (d) Chromosome 4

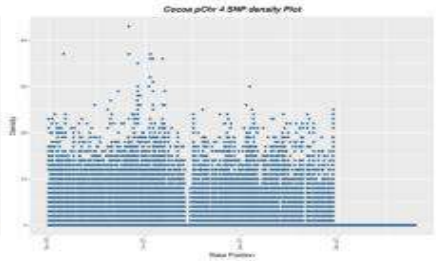


Figure 3 (e) Chromosome 5

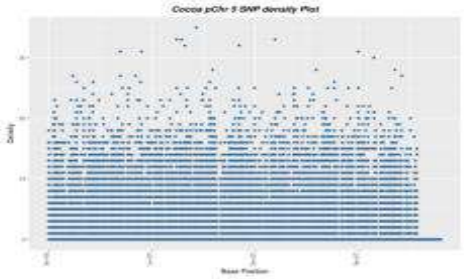


Figure 3 (f) Chromosome 6

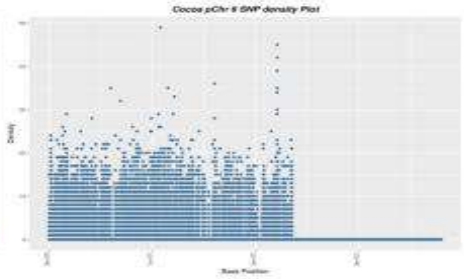


Figure 3 (g) Chromosome 7

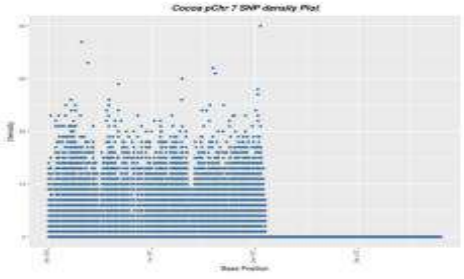


Figure 3 (h) Chromosome 8

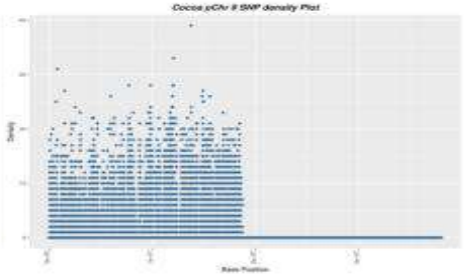


Figure 3 (i) Chromosome 9

Figure 3 (j) Chromosome 10

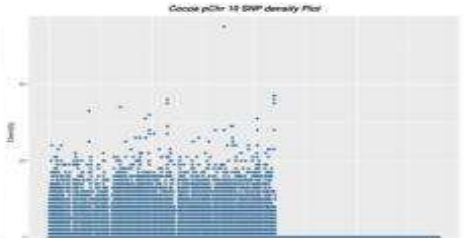


Figure 3. 10 SNP density graphs, one for each chromosome, were generated as shown

Table 7. Phenotypes value information for each sample of 300 cocoa genome samples (shown here as the first fifty samples) across 7 traits provided by MCB. (-) symbol represents the phenotype value that is not available or missing.

No.	MCB_code	Factor location (S=sabah ;P=perak)	Black Pod	CPB ADSI	Yield	Content Butter	SCSI	VSD	Pod Hardness
1	B01_s_TM	S	MT	-	5740	-	-	MS	-
2	B02_s_TM	S	MT	2.28	3930	-	SI	MT	-
3	B04_s_TM	S	MT	-	3760	-	-	MT	-
4	B06_s_TM	S	MT	-	-	-	SI	MS	-
5	B07_s_TM	S	MT	-	-	-	-	MS	-
6	B08_s_TM	S	MT	-	-	3460	-	MS	-
7	B12_s_TM	S	MT	-	-	3140	-	S	-
8	B15_s_TM	S	MT	-	-	-	-	S	-
9	B17_s_TM	S	MT	-	-	2770	-	S	-
10	B18_s_TM	S	MT	-	-	2690	SC	MT	-
11	B19_s_TM	S	MT	-	-	2600	-	MT	-
12	B24_s_TM	S	MT	-	-	-	-	-	-
13	B29_s_TM	S	MT	1.12	-	2030	SI	MT	-
14	B33_s_TM	S	MT	-	1590	-	-	MT	-
15	B34_s_TM	S	MT	1.88	-	1560	-	MS	-
16	B35_s_TM	S	MT	1.59	1530	-	-	MT	-
17	B36_s_TM	S	MT	2.2	-	3560	-	MT	-
18	B37_s_TM	S	MT	1.11	-	3510	-	MT	-
19	B40_s_SM	S	MS	2.55	-	2020	SI	-	-
20	B42_s_SM	S	MS	-	-	1590	-	MT	-
21	B44_s_SM	S	MS	-	-	2390	-	MT	-
22	B45_s_SM	S	MS	1.03	-	2040	SI	MT	-
23	B47_s_SM	S	MS	2.43	-	1738	-	MT	-
24	C02_s_R	S	-	0.75	-	-	SI	-	-
25	C04_s_R	S	MS	1.03	-	2040	SI	MT	-
26	C05_s_R	S	-	1.09	-	-	-	-	-
27	C06_s_R	S	MT	1.11	-	3510	-	MT	-
28	C07_s_R	S	MT	1.12	-	2030	SI	MT	-
29	C09_s_R	S	-	1.56	-	2550	-	MT	-
30	C11_s_R	S	MT	1.59	1530	-	-	MT	-
31	C15_s_R	S	MT	1.88	-	1560	-	MS	-
32	C22_s_GSA00275	S	MT	2.28	3930	-	SI	MT	-
33	C31_s_SMR	S	-	2.78	-	-	-	-	-
34	C48_p_R	P	-	1.26	-	3880	-	-	-
35	C49_p_R	P	-	1.45	-	-	SI	-	-
36	C50_p_R	P	-	1.89	-	-	-	-	-
37	C51_p_R	P	MT	1.94	-	3510	-	MT	-
38	C54_p_GSA00282	P	MT	2.2	-	3560	-	MT	-
39	C55_p_GSA00283	P	-	2.22	-	-	-	-	-
40	C56_p_GSA00284	P	-	2.23	-	-	-	-	-
41	C57_p_GSA00285	P	-	2.26	-	-	-	MT	-
42	C58_p_GSA00286	P	-	2.28	-	-	-	-	102.5
43	C60_p_GSA00287	P	-	2.32	-	3150	-	-	-
44	C62_p_GSA00288	P	MS	2.43	1738	-	-	MT	-
45	C63_p_GSA00289	P	-	2.44	-	-	-	-	-
46	C64_p_GSA00290	P	-	2.48	3925	-	-	-	-
47	C66_p_GSA00291	P	MS	2.55	-	2020	SI	-	-
48	C68_p_GSA00292	P	-	2.56	-	-	-	-	-
49	C69_p_GSA00293	P	-	2.57	-	2550	SI	-	-
50	C70_p_GSA00294	P	-	2.63	-	-	-	-	-

Methodology

For each trait, In order to estimate the SNPs effect probabilities based on phenotypes value, for statistical significance, Fisher's exact test was used. In other words, the test is used to examine the significance of the association (contingency) between the phenotype values. In order to identify genotype-phenotype correlations, the Fisher exact test was applied only to those samples with known genotypes and phenotypes information as shown in Table 7 (above). Table 8 below shows the statistical summary of a number of samples used with the statistically significant test for each trait.

Table 8. Statistical summary of samples used for Fisher's exact test calculation by trait. Overall, 205 different cocoa genome samples are involved.

No.	Trait	Number of Genome Samples	Phenotype Classification
1	Black Pod (B)	18 vs 5	Tolerance vs Susceptible
2	CPB (C)	15 vs 13	Resistance vs Susceptible
3	Yield (H)	10 vs 7	High vs Low
4	Butter Content (L)	12 vs 12	High vs Low
5	SCSI (S)	19 vs 18	Self Compatibility vs Self In Compatibility
6	VSD (V)	17 vs 8	Tolerance vs Susceptible
7	Pod Hardness (P)	18 vs 33	Soft vs Hard

The P-value for each SNP obtained from the Fisher exact test analysis was sorted in ascending order for the top 10k most significant p-value SNPs for each trait. The 70,000 combined SNPs from all 7 traits were then used for downstream analysis. For each of the 70,000 SNPs across all cocoa genome samples discovered as significant SNPs effect p-value, the same SNPs coordinates were each given a score based on the SNP genotypes for each trait. Table 9 below shows the score substitution example for the Butter Content trait (The same method was also applied to the other 6 traits respectively). After scoring across 10k total number of SNPs being made for each trait, summation of SNPs scores by a cocoa sample was calculated. Table 10 below shows an example of the simplified calculation of SNPs scores assuming only 4 SNPs were used.

Table 9. Fisher's exact test P value score substitution by genotype classification (1/1, 0/1, 0/0). Only genotype 1/1 being score with the related p-value of the SNP. Other genotypes become 0 as a score.

Cocoa sample		SNP1	SNP2	SNP3	SNP4
L01_p_H	Genotype	1/1	0/1	0/1	0/1
	Value	9.61483E-06	0	0	0
L01_s_H	Genotype	1/1	1/1	0/0	0/1
	Value	9.61483E-06	9.61480E-06	0	0
L03_p_L	Genotype	1/1	1/1	1/1	0/0
	Value	9.61483E-06	9.61480E-06	6.73040E-05	0
L03_s_L	Genotype	1/1	1/1	1/1	0/0
	Value	9.61483E-06	9.61480E-06	6.73040E-05	0

Table 10. Example of a simplified calculation of the Fisher score summation with 4 single nucleotide polymorphisms (SNP) for 4 cocoa samples.

Cocoa sample	SNP1	SNP2	SNP3	SNP4	Score Summation (GEBV)
L01_p_H	9.614830E-06	0	0	0	9.614800E-06
L01_s_H	9.614830E-06	9.614830E-06	0	0	1.923000E-05
L03_p_L	9.614830E-06	9.614830E-06	6.730400E-05	0	8.653300E-05
L03_s_L	9.614830E-06	9.614830E-06	6.730400E-05	0	8.653300E-05

Genomic Estimation Breeding Value (GEBV)
Genomic estimated breeding value of SNP score summation (Fisher's exact test value) across 300 cocoa samples; the results are displayed in Table 11 below:

Table 11. GEBV of Fisher score summation across 300 samples (shown for the first ten samples) for 7 traits

No.	MCB_code	Black Pod	CPB	Yield	Butter Content	SCSI	VSD	Hardness Pod
1	B01_s_TM	40.352938	9.38766	62.419529	38.239445	18.361485	15.626376	6.358914
2	B02_s_TM	42.752207	12.438234	70.691176	44.809785	22.683935	15.415667	9.344171
3	B04_s_TM	40.571815	11.639961	65.729021	43.262798	19.159002	14.238582	8.146424
4	B06_s_TM	45.156052	11.036401	67.606283	49.595747	23.178542	18.149049	9.583566
5	B07_s_TM	40.610211	10.338601	64.361117	47.349448	20.066399	16.548907	8.870509
6	B08_s_TM	41.858569	10.37136	66.602684	42.960779	19.751396	18.531198	7.937615
7	B12_s_TM	44.107314	12.212266	77.580677	51.789114	24.662117	19.159977	9.8554
8	B15_s_TM	38.954798	8.962207	60.34893	37.865082	17.432909	15.547262	6.116128
9	B17_s_TM	39.940177	9.618203	61.933309	39.723107	17.691771	15.332731	6.469774
10	B18_s_TM	43.004844	13.37399	69.428784	46.212146	21.834927	14.367132	7.686006

After imputation, a total Fisher exact test score was made for each trait across 300 samples, value indicator range by traits were summarized (based on Table 12) as shown in *Figure 4 and 5* below:

Table 12. Normalization values across the first 10 samples for 7 traits

No.	MCB_code	BlackPod	CPB	Yield	Butter content	SCSI	VSD	Hardness Pod
1	B01_s_TM	0.714393	0.902461	0.7358	0.933766	0.797478	0.722935	0.081105
2	B02_s_TM	0.6429	0.727956	0.543077	0.68783	0.482813	0.735861	0.410079
3	B04_s_TM	0.707871	0.77362	0.658691	0.745735	0.73942	0.808071	0.278088
4	B06_s_TM	0.57127	0.808146	0.614952	0.508685	0.446807	0.56818	0.43646
5	B07_s_TM	0.706727	0.848063	0.690562	0.592767	0.673364	0.666342	0.357881
6	B08_s_TM	0.669528	0.846189	0.638335	0.75704	0.696296	0.544736	0.255077
7	B12_s_TM	0.602521	0.740882	0.382557	0.426584	0.338806	0.506163	0.466416
8	B15_s_TM	0.756055	0.926799	0.784043	0.947779	0.865076	0.727789	0.05435
9	B17_s_TM	0.726693	0.889273	0.747128	0.878231	0.846232	0.740949	0.093322
10	B18_s_TM	0.635372	0.674427	0.57249	0.635337	0.544619	0.800185	0.22735

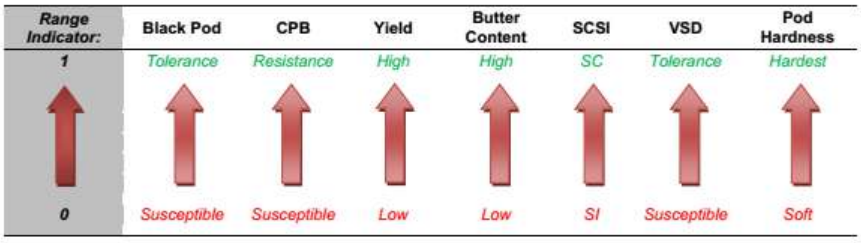


Figure 4. Seven traits Normalization values range from 0 to 1. A larger value means the cocoa sample has more desirable phenotype properties.

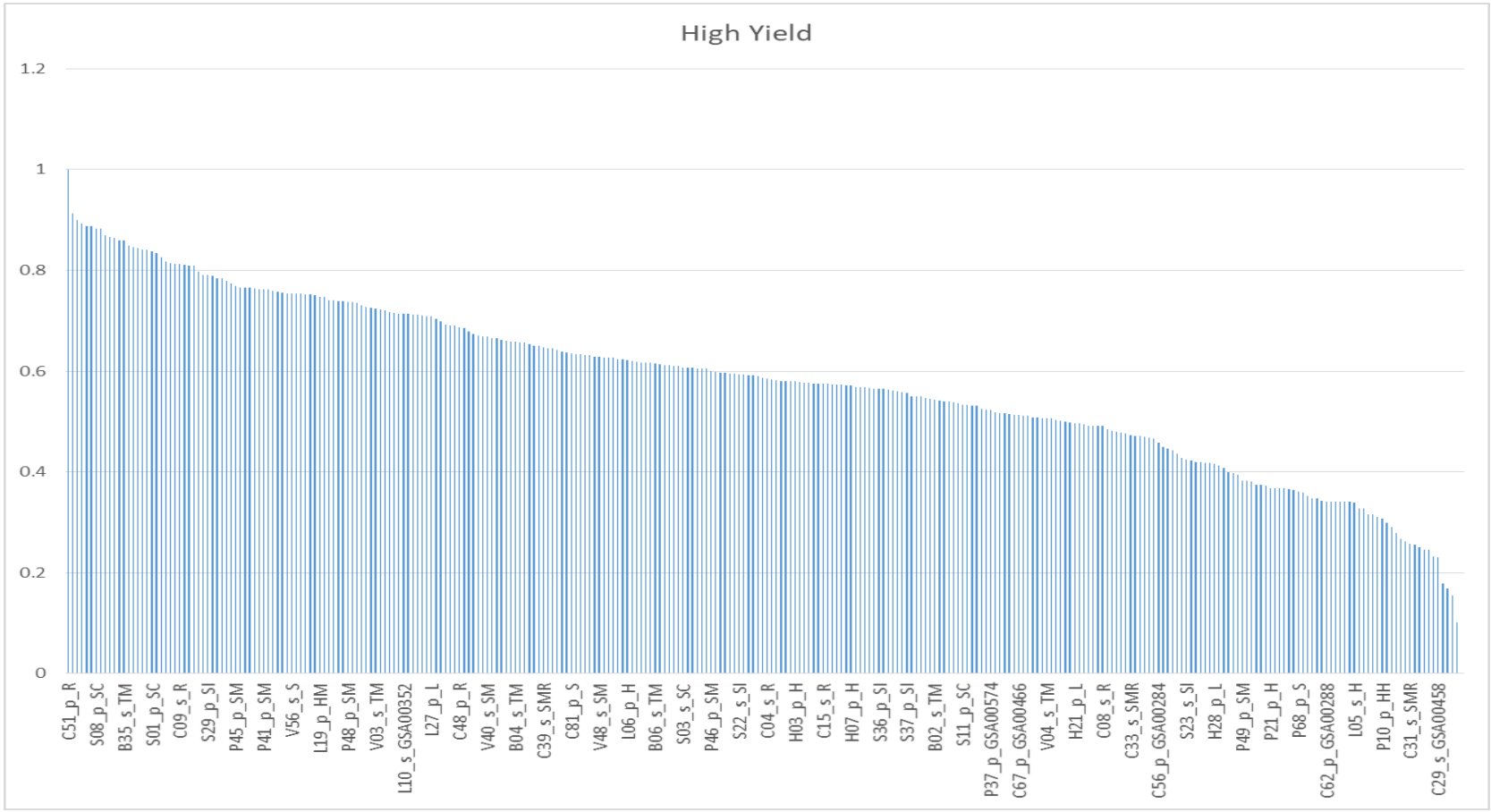


Figure 5. High yield trait for Normalization values plot. A larger value means that the cocoa samples have more desired phenotype figure

GEBV value for different traits combination

Table 13 and *Figure 6* showing a top 20 combination for high yield and high cocoa butter traits.

Table 13. Top 20 combinations for high yield & high cocoa butter traits

MCB_code	High Yield * High Butter	Top 10 which clone represented
C51_p_R	0.873558961	QH22, HP
L20_p_H	0.81338384	QH 326, HP
P56_p_SM	0.808810167	EET 381, HP
L25_s_GSA00374	0.795722488	PBC123, M
P25_p_GSA00562	0.792858461	EET236, HP
S29_p_SI	0.790546863	CCM10, HP
L13_p_GSA00356	0.78402155	MCB C4, HP
P53_p_SM	0.772330136	ICS40, HP
L25_p_GSA00373	0.768931682	PBC123,HP
C21_s_GSA00443	0.761744092	NA33
P31_p_GSA00568	0.752580753	CCN 51, HP
S34_p_SI	0.750763098	EET 308, HP
S19_p_SC	0.747891305	EET 236, HP
B15_s_TM	0.743099164	MCBC 2, M
P60_p_SM	0.728565592	GS 36, HP
V57_s_S	0.724098631	MCBC4, M
V56_s_S	0.724029499	MCBC2, M
P51_p_SM	0.72103109	R 21, HP
P42_p_SM	0.718329875	R 8, HP
P45_p_SM	0.711569156	R 15 , HP
L13_s_GSA00357	0.704393945	MCB C4, M

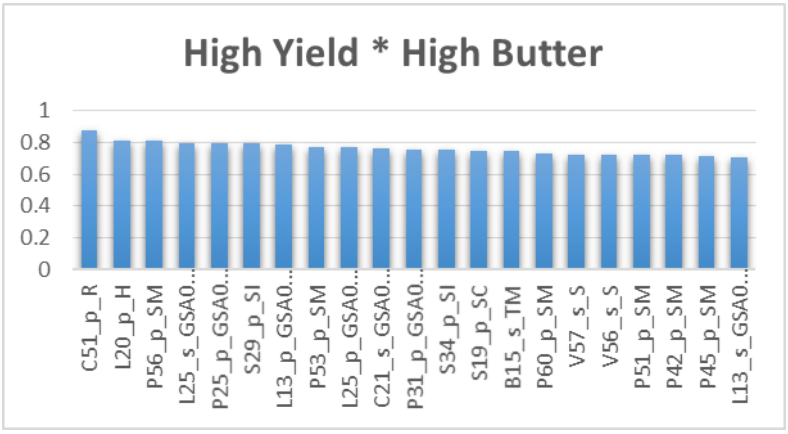
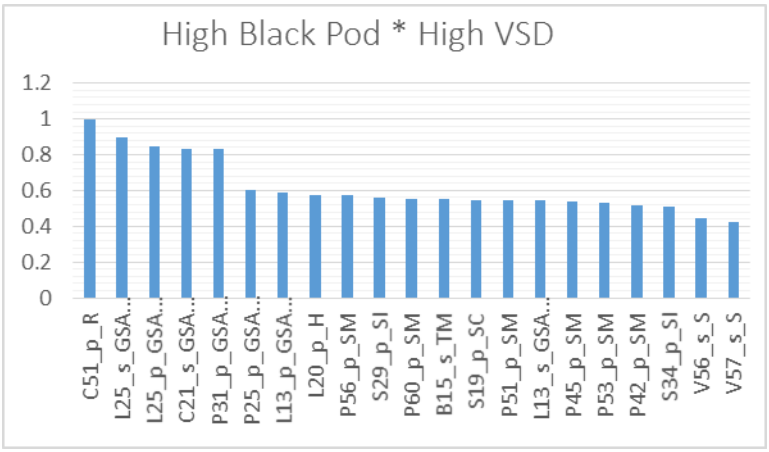


Table 14 and *Figure 7* showing a top 20 combination for high tolerance to black pod and VSD.

Table 14. Top 20 combinations for high tolerance to black pod and VSD

MCB_code	High Black Pod * High VSD	Top 10 which clone represented
C51_p_R	1	QH22, HP
L25_s_GSA00374	0.90017743	PBC 123, M
L25_p_GSA00373	0.849126322	PBC 123, HP
C21_s_GSA00443	0.830670916	NA33 , M
P31_p_GSA00568	0.830293142	CCN 51, HP
P25_p_GSA00562	0.60373479	EET 236, HP
L13_p_GSA00356	0.591050911	MCB C4, HP
L20_p_H	0.577022464	QH 326, HP
P56_p_SM	0.575005642	EET 381, HP
S29_p_SI	0.561135923	CCM 10, HP
P60_p_SM	0.551375592	GS 36, HP
B15_s_TM	0.550248059	MCB C2, M
S19_p_SC	0.548348046	EET 236, HP
P51_p_SM	0.546512512	R 21, HP
L13_s_GSA00357	0.5421244	MCB C4, M
P45_p_SM	0.536636961	R 15, HP
P53_p_SM	0.533855305	ICS 40, HP
P42_p_SM	0.520237059	R 8, HP
S34_p_SI	0.507761716	EET 308, HP
V56_s_S	0.448030823	MCB C2, M
V57_s_S	0.425858778	MCB C4, M



CONCLUSION

With the availability of GEBV value for each clones, this will served as a background for breeding selection for the next generation. As evidence in Table 13 and Table 14 with their respective *Figure 6 and 7*, cross breeding between tables will compromise at least four important traits and their progeny need to go another round of validation. Selection for breeding with desirable traits will proceed on the basis of these GEBVs.

Ultimately a four-year breeding cycle, including three years of field testing, can be reduced to only the four months required to grow and cross a plant. Thousands of selection candidates can be evaluated without ever taking them out to the field. Ongoing field trials are still very much a part of a breeding program using genomic selection (GS), but the purpose, and therefore the practice, shifts: phenotypes are no longer used to select but to train a prediction model.

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MICROANALYSIS OF COCOA BEANS FOR DETERMINATION OF TANNIN CONTENT CONTRIBUTED TO COCOA FLAVOR

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ABSTRACT - Cocoa beans classified as valuable food that contributed all over around the world. One of the important things that mostly researcher focus are concentrated on flavor analysis and investigated their chemical compound that useful to human being such as in pharmaceutical industries and food industries. Cocoa flavor turn to be low when tannin content was high and cocoa flavor was high when tannin level was low (Clapperton et al., 1994). This is common situations when cocoa beans have been fermented and roasted for several days. Chemical changes were shown at the pre-fermentation and after fermentation process. Microanalysis is a one of powerful techniques that can be used as new tools for cocoa compound analysis.

Key words: Microanalysis, tannin, cocoa flavor.

INTRODUCTION

Chocolate flavor development involves two complexes processes: fermentation and roasting. During fermentation storage protein degrade into amino acids and short oligopeptides. They can reach with reducing sugar to produce a complex mixture of compounds during roasting. These compounds are the major contributors to chocolate flavor.

Besides components that contributed to chocolate flavor, seeds also contain ones that detract from chocolate flavor, noably condensed tannins and methylxanthines (theobromine and caffeine). Tannins reduce perceives chocolate flavor and cause astringency, and methyxanthines are bitter. Malaysian beans are discounted due to their low chocolate flavor. Producing is also currently low in Malaysia. Producing beans with novel and exceptional properties can revive and revolutionize the Malaysian cocoa industry. The objectives of this research were to determination of tannin level for different beans and development of tannin analysis by using microanalysis techniques

MATERIALS AND METHODS

Sample preparation and tannin analysis

Cocoa sample was taken from Tenom and stored at one day after harvest from the trees. 20 cocoa seeds were used along the experiment. Every single bean was peels from the pod (separated from the pod). After that, every single bean was moved from their mucilage by using wooden waste and leaves it for a while to make sure it dry. Then, 10 mg from the single beans was weighed and put into methanol. There are 3 replications been used for every sample. After that, 10mg of the sample was crushed by small mortar and mix it with glass powder. After that, sample was centrifuge 5000rpm at 5min. Supernatant of the sample was take out and fill-up in a new tubes and been analyses.

Vanillin-HCL Assay (Yamiko et al., 1998)

Proanthocyanidins in the sample solutions of 5 GSEs, 4 health foods, and 2 grape seed oils were determined by the vanillin-HCL assay described by Sun *et al.* (1998). To 1 ml of CT (catechin) solution (0-300ug/ml in methanol) or test solution (150-250ug/ml polyphenol in methanol) in a test tube, 2.5ml in methanol (control) or 1% vanillin solution in methanol (sample) and 2.5ml of 9

mol/LHCL in methanol was added. The reaction mixture was incubated for 20 min at 30^0C and the absorbance at 500nm was measured by using UV Spectrometer (UV Lambda 35, Perkin Elmer).

The following A_0, A_b, A_c, A_s was measured for each standard and sample.

A_0 = Absorbance at 500nm of the control of o mg CT (1ml methanol + 2.5ml methanol + 2.5ml 9 mol/L HCL.

A_b = Sample of 0 mg CT (1ml methanol + 2.5ml 1% vanillin solution + 2.5ml 9 mol/L HCL.

A_c = control (1 ml CT (20-300ug/ml) or test solution + 2.5ml methanol + 2.5ml 9 mol/L HCL.

A_s = sample (1 ml CT (20-300ug/ml) or test solution + 2.5 ml 1% vanillin 2.5ml 9 mol/L HCL.

A was calculated as follows for each standard and sample solution:
$A = (A_s - A_b) - (A_c - A_0)$

A calibration curve was prepared using A for the CT solution using the above calculation. Total flavan-3-ol in each test was calculated from the calibration curve.

The Vanillin-HCL assay was performed 3-5 times for each sample.

RESULTS AND DISCUSSIONS

Result obtained from *Figure 1,* it was shown total 20 samples listed from samples 1 to 20. The entire sample was calculated by conversion to 10mg because all the samples were targeted to be uniform 10mg of every singles beans. There are three sample which was shown highest levels of the tannin content, with a high catechin content with absorbance below than 0.5 (500nm). The high tannin samples are sample no. 8, 14, and 16. And the rest of the samples had shown lower levels of the tannin content. Sample no 1-7, 9-13, 15, 17- 20 shown lower tannin levels.

It was shown that every single cocoa bean has tannin and can be separated into low and high levels of the tannin. This is due to different types of clones and their genetic variations between the clones. In additional, information of the color in the cocoa beans also contributed for levels of tannins in every single beans.

The result of this analysis shows the absorbance of the samples that were analyzed in a day. This result shows that there are varibilities of the flavan-3-ol level in each bean that were analyzed. The variability of flavon-3-ol concentrations level in each samples tell us that each bean in the same pod not necessarily have the same level of flavon-3-ol concentration in each sample. There are reasons that lead to the variability of flavon-3-ol concentration in each sample. During the preparation of the beans, we observed that the size of each beans are different from each other, thus the weight of each beans are different too. We are also observed that the intensity of the colour in each bean also different. These factors may affect the level of flavon-3-ol concentration in each bean. A darker bean may have high level of flavon-3-ol than a less dark.

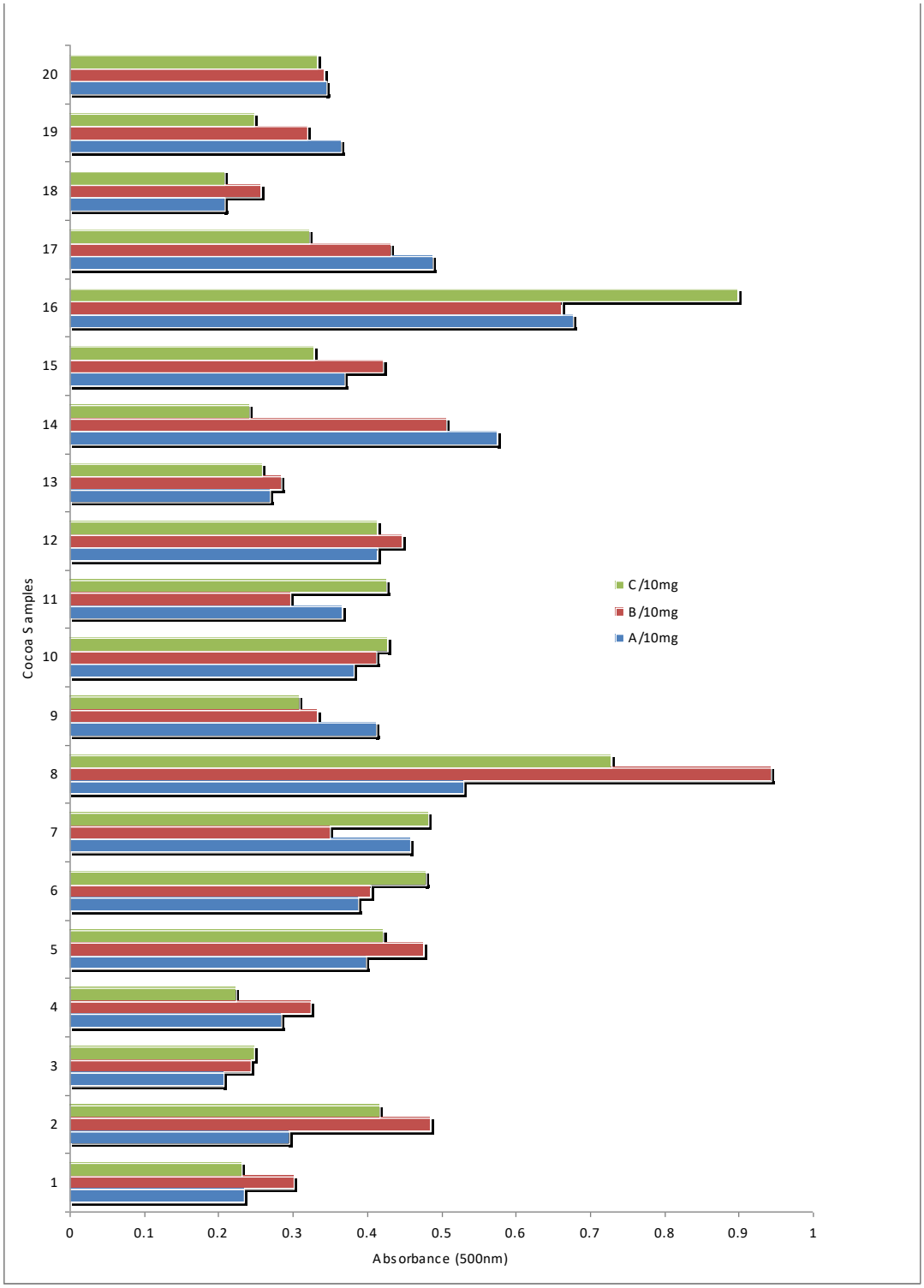

Figure 1. Tannin levels for 20 cocoa beans samples (3 replications for each samples)

Table 2. The absorbance and concentration of the samples.

Number of beans	Beans ID	Absorbance at 500nm	Concentration
1	4617	0.4744	94.88
2	4618	0.3842	76.84
3	4619	0.5208	104.16
4	4701	0.7593	151.86
5	4702	0.9515	190.30
6	4703	0.8335	166.70
7	4704	0.5381	107.62
8	4705	0.6978	139.56
9	4707	0.6489	129.78
10	4708	0.4269	85.38
11	4710	0.5760	115.20
12	4711	0.6630	132.60
13	4713	0.4513	90.26
14	4714	0.6450	129.00
15	4715	0.6814	136.28
16	4716	0.6310	126.20
17	4717	0.5993	119.86
18	4719	0.5785	115.70
19	4720	0.5695	113.90
20	4721	0.4813	96.26
21	4724	0.6333	126.66
22	4726	0.2777	55.54
23	4727	0.4467	89.34

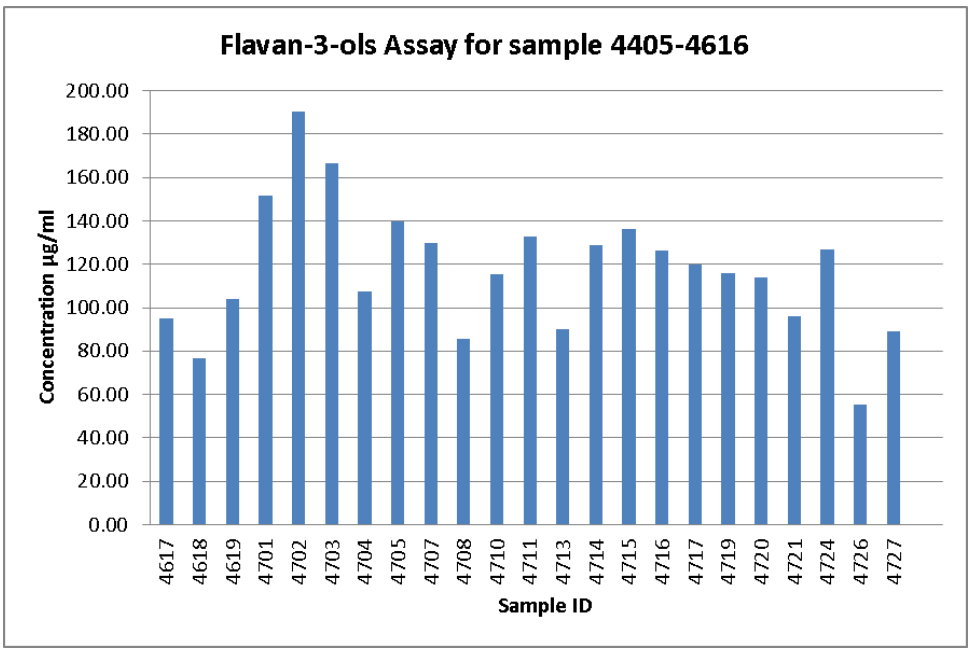

Figure 2. Concentration of samples at 500 nm

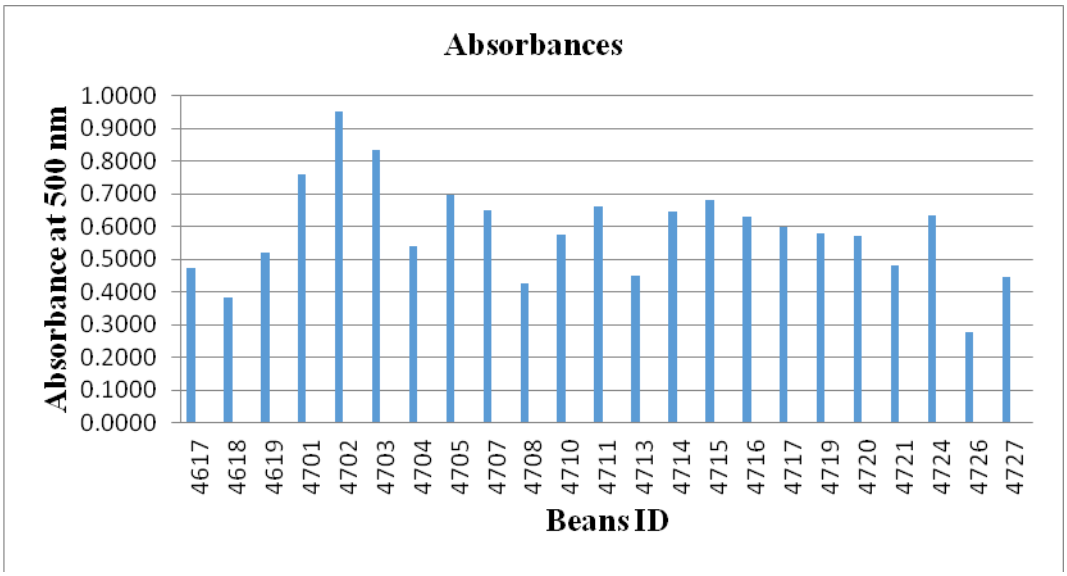

Figure 3. Absorbance of samples at 500nm

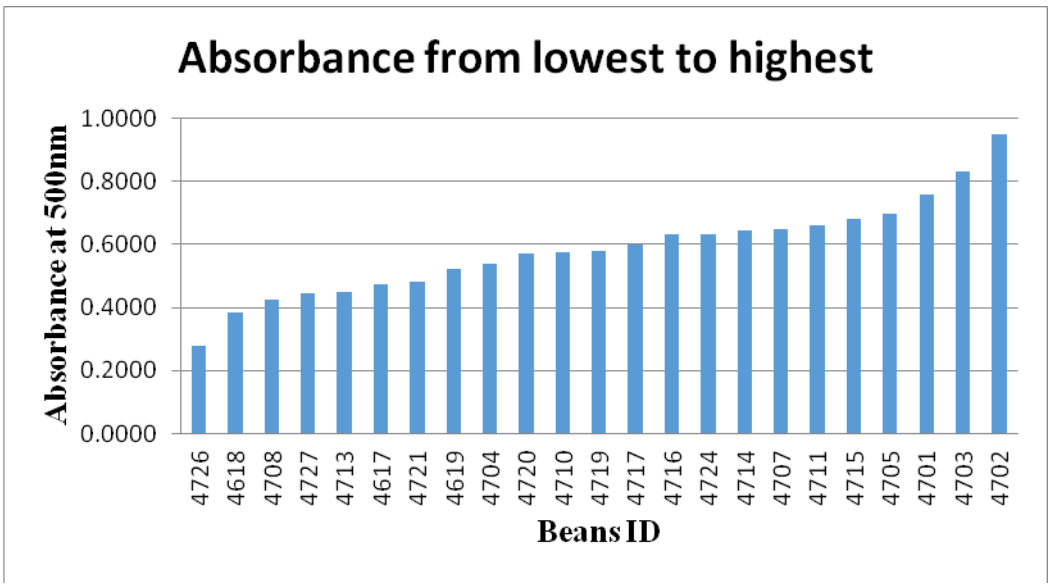

Figure 4. The absorbance from lowest to highest at 500nm

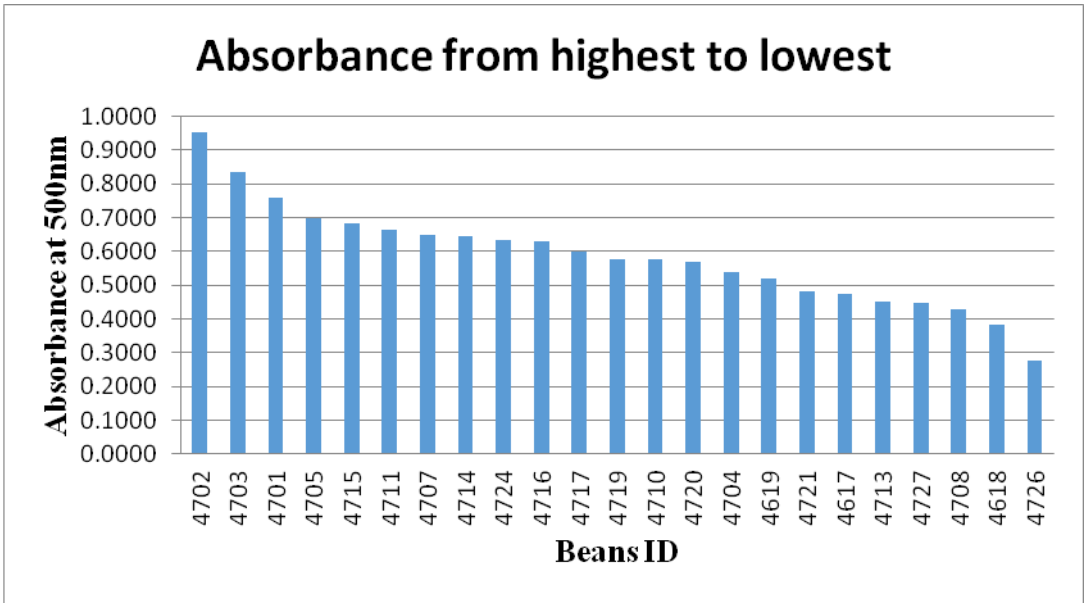

Figure 5. The absorbance from highest to lowest at 500nm

The purpose of this assay was to analyze the level of flavon-3-ol in each bean in every cocoa pod. The type of cocoa pod may be selected from different clone that had been successfully pollinated. As the analysis were done, the result will be assorted and a number beans will be selected with the highest flavon-3-ol concentration which considered as the health promoting cocoa beans. A number of beans also will be selected from the lowest flavon-3-ol concentration which considered as high flavor cocoa beans. This process will continue from year to year to find the higher and the lowest level of flavon-3-ol concentration in cocoa beans. This analysis allows us to control the production of cocoa beans with high flavon-3-ol content to promote good health as we consume the cocoa product. This is also allows us to control the flavor of cocoa product in the market as we produce cocoa beans with low flavon-3-ol content.

Figure 6 shows the plotted catechin standard calibration curve was assorted from lowest to highest. We had determined the concentration of flavan-3-ols in each sample. From the curve, it shows the result of flavan-3-ol content which is the bean ID 4702 has the highest content of flavan-3-ol while the bean ID 4726 has the lowest. The highest content flavon-3-ol is considered as the health promoting cocoa beans while the lowest is considered as the high flavor cocoa beans.

Table 2. Concentration and absorbance of catechin standard in Vanillin/H2SO4 at 500 nm

µg /ml	Absorbance at 500nm
7.8125	0.0261
15.625	0.0551
31.25	0.1364
62.5	0.3392
125	0.6505
250	1.2496
500	2.2476
1000	3.4216

In this analysis, the reagent used in the reaction of flavon-3-ol with the vanillin had been charged with H_2SO_4 are used instead of HCl. This is because H_2SO_4 are more stable than HCl. The stability of H_2SO_4 and HCl was determined from the previous analysis. In the previous analysis, the reaction of 35% HCl was compared to the 99% H_2SO_4. The result shows a better stability when H_2SO_4 was used in the analysis.

The main reason of the stability of the reaction was the concentration of the reagent used. A less concentration reagent have more water compared to more concentration reagent. At the same normality, H_2SO_4 is a better catalyst than HCl. These differences was distributed due to the different water content of the two acids. At the same normality, concentrated HCl contain more water than H_2SO_4.

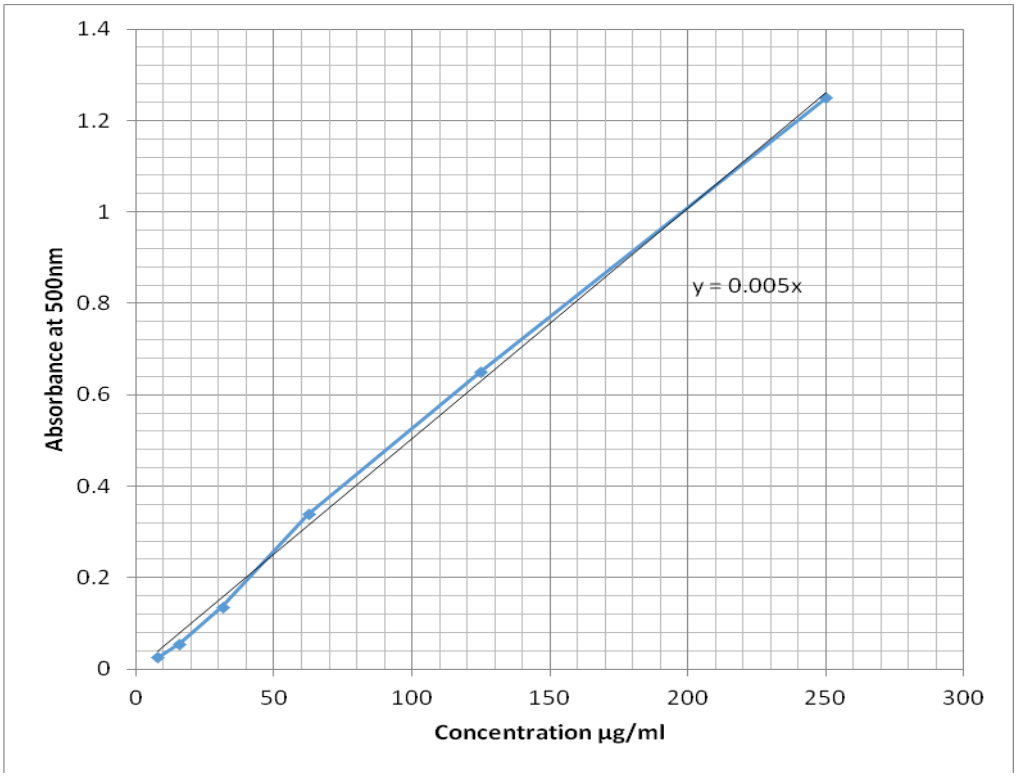

Figure 6. Catechin standard curve in Vanillin/H2SO4 at 500 nm.

CONCLUSION

It was shown that analysis of tannin levels on cocoa beans by observation of the color intensity. This experiment was successfully found that with light color have a high level of tannin and blackish color of the beans shown that low tannin levels. This experiment will be applied in a thousand cocoa beans analysis for the improvement of the fast, cheap and accurate techniques for microanalysis of individual's beans.

As a conclusion, the analysis shows that each cocoa bean have different flavon-3-ol concentration. Within this analysis, we can produce the grade A of cocoa bean that been chosen by level of flavan-3-ol concentration, because level of flavon-3-ol are the main factor that influence the flavor of cocoa bean.

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DRAFT GENOME SEQUENCE OF *Conopomorpha cramerella,* A DEVASTATING INSECT PEST OF COCOA IN SOUTHEAST ASIA

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ABSTRACT - Conopomorpha cramerella *is a moth of the Gracillariidae family and was recognized as the major threatening pest in Southeast Asia's cocoa industry. Here, we report the draft sequence of approximately 498 Mbp genome of this species. The genome contains 35,750 CDSs and have an overall C + G content of 38.15%. To the best of our knowledge, this is the first genome sequence of* Conopomorpha *species reported. The genome sequence may provide fundamental molecular information on elucidating the metabolic pathway of pest-host interaction.*

Keywords: Cocoa pod borer, *Conopomorpha cramerella*, lepidoptera, draft genome, *de novo* assembly

GENOME ANNOUNCEMENT

Conopomorpha cramerella which commonly known as cocoa pod borer is a devastating pest of cacao in Southeast Asia, particularly in Malaysia, Indonesia and the Philippines (Shapiro *et al*., 2008). These borers were extremely difficult to control because of their small size and larval development takes place inside the pod and they are responsible for yield losses of up to 80% (Day, 1989). The cocoa pod borer is believed to be originated from rambutan (*Nephelium lappaceum* L.), pulasan (*Nephelium* mutabile Blume) and nam-nam (*Cynometra cauliflora* L.) pest before shifted to cocoa and became endemic to Southeast Asia (Malaysian Plant Protection Society, 1987). The infestation of this pod borer was noticeable for decades to growers in Indonesia especially in Sulawesi where it caused a rapid decline of the cocoa industry in the mid-1800s (Shapiro *et al*., 2008). Whereas, in Malaysia, *C. cramerella* nearly caused a collapse of the cocoa industry in early 1990s (Shapiro and Rosenquist, 2004) and the declining trend of the cocoa production are still prominent till now. The economic impact of this pest on cacao-dependent economies is enormous and became the primary limitation to growing cacao in Indonesia, Malaysia, and the Philippines (Shapiro *et al*., 2008). Many initiatives were implemented to address this issue and collaborative approaches were initiated between ASEAN communities.

Here we report the draft genome of the *C. cramerella*, a lepidoteran that became a major threat and concern towards cocoa industry in Southeast Asia by using hybrid assembly approach which was the combination of second-generation Illumina HiSeq sequencing technology of high accuracy with third-generation Pacific Biosciences (PacBio) single-molecule real-time (SMRT) sequencing technologies allows for the bridging of complicated genomic regions, such as internal repeats.

To access the insect genome sequence, total DNA was extracted using GeneAll Exgene^{TM} Tissue SV kit (GeneAll Biotechnology, Korea). The extracted DNA was subjected to assessment to check for its quality, quantity and integrity. The DNA quality and quantity were then measured using a NanoDrop (Thermo Fisher Scientific, USA) and Qubit^{TM} dsDNA BR Assay kit. A total of 8 SMRTcells of data was delivered in this project with a total throughput of 9.38 Gbp. The average throughput per SMRTcell is around 1,177 Mbp. A post-filtered polymerase read- length N50 of 15,033 nt and a filtered Subread N50 of 9,151 nt was achieved. This is expected to give a longer horizontal "reach" to the assembly process. Quality-filtered reads from the Illumina platform and PacBio RS II platforms were used as input for the hybrid genome assembly to overcome challenges posed by the highly heterozygous *C. cramerella* genome. The assembled draft *C. cramerella* genome was 498.43 Mb, containing 69,085 contigs with an N50 size of 12.98 kb. The longest contig was 380.85 kb (Table 1).

Table 1. Summary of *C. cramerella* genome assembly statistics

Assembly Statistics	
Genome assembly size (Mbp)	498.43
Number of contigs	69,085
Sum of contigs size (bp)	498,435,22
Longest contigs (bp)	380,851
Smallest contigs (bp)	1,000
N50 length (bp)	12,988
Mean length (bp)	7,215
%GC	38.15

Augustus (Stanke and Morgenstern, 2005) was run across the scaffold assembly using splicing parameters tuned for use on *Drosophila melanogaster* (fruit fly). A hints file was included for gene prediction to improve exon detection. The *C. cramerella* transcriptome sequenced previously was mapped to the genome assembly to furnish one set of hints. Finally, Blat peptide matches to *D. melanogaster* and *Bombyx mori* genes predicted peptides was used as another hint source. A total of 35,750 predicted protein coding genes were identified with 38,543 predicted transcripts and predicted peptides, with an average gene length of 5861.69 bp *(Figure 1)*.

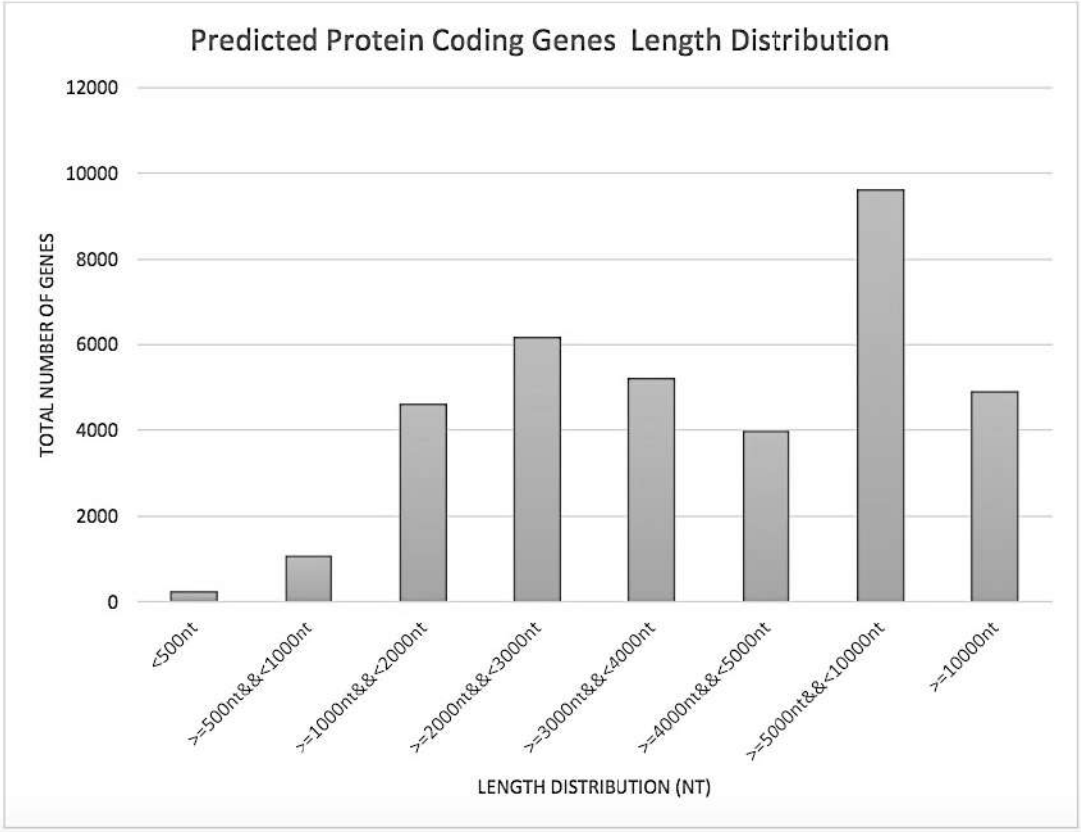

Figure 1. Distribution of predicted protein coding gene lengths. D. melanogaster and Bombyx mori were used as the hint for gene prediction.

To estimate the quality of the genome annotation and contiguity of the genome assembly, all predicted peptides were mapped to *B. mori* (a well annotated closely related genome) using blastP. The number of *C. cramerella* peptides matching at 90% or more to the best matching *B. mori* peptide gives a rough estimate of the completeness of the predicted peptides and how many peptides are split across scaffolds. The results show that around 12.52% are full length genes. This is due to the high heterozygosity of the genome assembly of *C. cramerella*.

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THE COMPLETE GENOME SEQUENCE OF *Oncobasidium theobromae,* AN EPIDEMIC PATHOGEN ISOLATED FROM VASCULAR STREAK DIEBACK DISEASE INFECTED COCOA TREES

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ABSTRACT – Oncobasidium theobromae *is an infectious basidiomycete fungus pathogen that cause vascular streak dieback disease in cocoa with higher severity and incident reported in Southeast Asia. Here, we report the complete genome sequence of approximately 49 Mbp genome of this species isolated from infected cocoa trees. The genome contains 13,830 CDSs and have an overall C + G content of 52.48%. These genomic data generate insights about the pathogen-host interaction as well elucidating the biological mode of infection at genomics level.*

Keywords: Vascular streak dieback disease, *Oncobasidium theobromae,* complete genome, *de novo* assembly

GENOME ANNOUNCEMENT

Among other fungal diseases of Cocoa (*Theobroma cacao*) are the Vascular Streak Dieback (VSD) which is caused by *Oncobasidium theobromae*, a basidiomycete fungus as reported by Prior (1980) in Southeast Asia and Melanesia. Infected trees exhibit characteristic symptoms like elevated lenticels, darkened vascular traces at the leaf scars, green-spotted chlorosis and falling leaves that usually began on the second or third flush behind the stem apex and infected xylem. Total defoliation would occur eventually and death is unavoidable once the fungus spreads to the trunk (Keane and Prior, 1991). Recognized as a highly specialized near-obligate parasite of cocoa, *O. theobromae* is a leaf-penetrating, vascular pathogen that is windborne and might have originated from evolved endophyte of yet to be identified indigenous host (Guest and Keane, 2006). Since basidiocarp development necessitates fresh leaf scars during wet weather and the basidiospores' viability last only for mere hours when shed in the night (Keane, 1981) the disease spread on cocoa is not prodigious. Although systemic triazole fungicides with sufficient efficacy have been identified, as a vascular pathogen an effective *O. theobromae* commercial scale VSD control remains at an impasse.

VSD incident was first reported in Papua New Guinea (PNG) in the early 1960s (Bridgland *et al*., 1966a) and has been a major problem in the large commercial plantations in West Malaysia and Sabah and influenced growers to replace cacao with oil palm. The disease is widespread in Indonesia, including in the fine flavor cocoa plantations in East and West Java, and in the large areas of newer cocoa plantings in Sulawesi (Guest and Keane, 2006). In highly susceptible material, the losses incurred averaged from 25 to 50%, with the possibility of total loss (Dennis, 1991).

To better understand the genetic basis for VSD infection by *O. theobromae*, we sequenced the whole genome of *O. theobromae* isolated from infected cocoa trees by using second-generation Illumina HiSeq sequencing technology. The fungal genome was accessed by extracting the DNA using DNeasy Plant Kit (Qiagen, USA). The extracted DNA was subjected to assessment to check the quality, quantity and integrity. The DNA quality and quantity were measured using Nanodrop and Qubit dsDNA BR assay. DNA was fragmented using Covaris S220 (Covaris Inc, USA) to a targeted size of 300– 400bp (for short- insert size) and >600bp (for long-insert size). The fragmented DNA was end-repaired, ligated to adapters and PCR-enriched using NEBNext Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) according to manufacturer's protocol. The final library was quantified using Qubit DNA HS assay. Library size was determined using Bioanalyzer High Sensitivity DNA chip. The resulting library was sequenced

on Illumina flow cell, consisting of 200 cycles on Illumina HiSeq 2000 (Illumina, USA). The sequencing run generated a total of 16.8 Gb of raw data and 14.78 Gb for the pre-processed data. All of the filtered sequences were *de novo* assembled using MGRC Assembler pipeline (version 1.4) and gap closing were performed after contigs had been generated from overlapping reads. All ~ 15 Gb of clean reads obtained from the Illumina Hiseq 2000 platforms were subjected to the 23- mer frequency distribution analysis with Synamer (MGRC K-mer occurrences counter program) for the estimation of the genome size. The assembled *O. theobromae* genome was 49.18 Mb, containing 4,135 contigs with an N50 size of 38.5 kb. The longest contig was 495.4 kb (Table 1).

Table 1. Summary of *O. theobromae* genome assembly statistics

Assembly Statistics	
Genome assembly size (Mbp)	49.18
Number of contigs	4,135
Average of contigs size (kb)	10.2
Longest contigs (kb)	495.4
N90 length (kb)	3.3
N50 length (kb)	38.5
%GC	52.48

Augustus (Stanke and Morgenstern, 2005) was run across the scaffold assembly using splicing parameters tuned for *Coprinus cinereus*. Blat peptide matches to *Rhizoctonia solani* AG-1 IA and *Rhizoctonia solani* AG-3 Rhs1AP were used as hint files for Augustus. A total of 13,830 predicted protein coding genes were identified with 13,934 predicted transcripts and predicted peptides, with an average gene length of 1,784 bp. BlastP was used to map predicted peptides against the UniProt database with a cutoff e-value of 1e-5.

To estimate the quality of the genome annotation and contiguity of the genome assembly, all predicted peptides were mapped to *C. cinerea* okayama7#130 using blastP. The number of *O. theobromae* peptides matching over 90% of the length of the best *C. cinerea* peptide gives a rough estimate of the completeness of the predicted peptides and how many peptides are split across scaffolds. The result show that 100% are full length genes in the genome assembly of *O. theobromae*.

Small subunit ribosomal RNA (SSU rRNA) genes are the standard reference sequences for taxonomic classification of organisms. 18S rRNA has played a dominant role in the estimation of relationships among eukaryotes. We have predicted 18S rRNA in the *O. theobromae* genome sequences based on hidden Markov models (HMMs) of structural alignments. Using the predicted 18S sequence and the SSU Ref NR 99 128 dataset downloaded from SILVA, a phylogenetic tree was generated based on the Ceratobasidiaceae family 18S rRNA from SILVA Database together with the *O. theobromae* predicted 18S rRNA *(Figure 1).*

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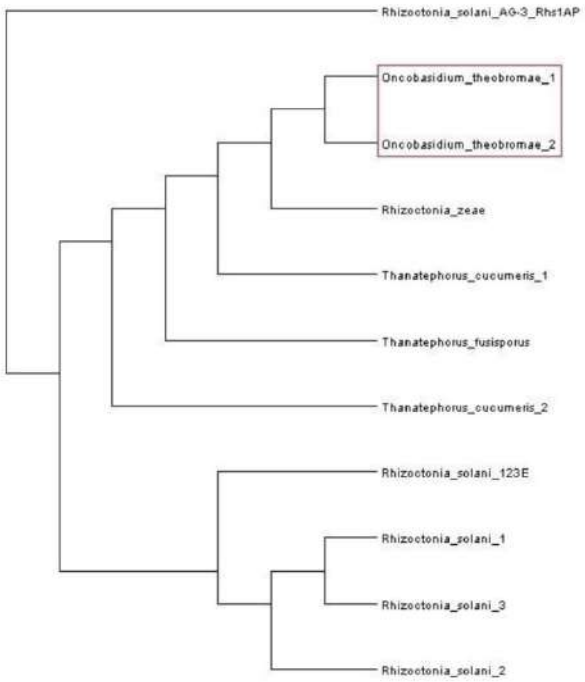

Figure 1. Phylogeny of Ceratobasidiaceae family 18S rRNA data.
This cladogram was constructed using a neighbour-joining (NJ) method.

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Aims & Scope

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