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สำนักงาน : สถาบันวิจัยและพัฒนาแห่งมหาวิทยาลัยเกษตรศาสตร์
มหาวิทยาลัยเกษตรศาสตร์ จตุจักร กรุงเทพฯ 10900

วิทยาสารเกษตรศาสตร์เป็นวารสารของมหาวิทยาลัยเกษตรศาสตร์ ซึ่งพิมพ์เผยแพร่ผลงานทางวิชาการแขนงต่างๆ ทางด้านวิทยาศาสตร์และสังคมศาสตร์ ทั้งจากภายในและภายนอกมหาวิทยาลัย โดย**วิทยาสารเกษตรศาสตร์ สาขาวิทยาศาสตร์** มีกำหนดออกปีละ 4 ฉบับ คือ เดือนมีนาคม มิถุนายน กันยายน และธันวาคม และ**วิทยาสารเกษตรศาสตร์ สาขาสังคมศาสตร์** มีกำหนดออกปีละ 2 ฉบับ คือ เดือนมิถุนายน และธันวาคม

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Effect of Hydropriming and Redrying on the Germination of Triploid Watermelon Seeds

Rukui Huang[1], Sutevee Sukprakarn[1], Thammasak Thongket[1] and Sunanta Juntakool [2]

ABSTRACT

Two triploid watermelon cultivars 'Gold Prince' and 'Guangxi 5' were subjected to hydropriming by soaking in deionized water for 2 hrs with aeration following 24 and 48 hrs incubated at saturated relative humidity. Hydropriming had a promotive effect on germination performance in both cultivars but the overall seed germination percentages did not increase in 'Gold Prince'. Furthermore, hydrated seeds were redried under different conditions to low down the seed moisture content to 6-7 % and the two cultivars responded differently. The highest germination percentages and lowest mean germination time were obtained from medium drying (40%RH, 20°C) for 'Gold Prince' and quick drying (20%RH, 20°C) for 'Guangxi 5'.

Key words: triploid watermelon seed, hydropriming, seed germination

INTRODUCTION

Triploid watermelons [*Citrullus lanatus* (Thunb.) Matsum & Nakai] were first developed in the early 1950's at Kyoto University in Japan (Kihara, 1951). Besides the well known character of being seedless, triploid watermelon has its superior attributes, e.g. field resistance to Fusarium wilt (Kihara, 1951), watermelon fruit blotch (*Acidivorax avenae* subsp. *citruli*) (Garret *et al.*, 1995), long shelf life and high total soluble solid content (Rhodes and Zhang, 1999). Although triploid watermelons gained great market potential, the production remains low. Poor germination, slow growth at seedling stage, and high seed cost are main factors limiting the production.

Mechanical weakening of the seed coat structure such as scarification, seedcoat nicking, and seed coat lateral splitting has been reported to successfully enhanced germination performance of triploid watermelon seed (Duval and NeSmith, 1998; Grange and Leskovar, 2000). However, some drawback effects such as embryo injury and time involved are the negative effects usually associated with these treatments.Therefore, researchs are needed to detect a practical and easily handling method to improve the germination performance of triploid seeds aiming to promote triploid watermelon production.

Seed priming is a treatment that partially hydrates seeds so that germination process begins, but radicle emergence does not occur. Priming has been widely reported to enhance seed germination performance of various species of field crops, vegetables, and other plants (Welbaum *et al*., 1998). Short time hydration treatments, e.g. hydropriming, humidification (incubating seed at high relative humidity) have been widely used to increase seed vigour and extend seed longevity in many plant species (Burgass and Powell, 1984; Powell *et al*.,

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2000).

Redrying after priming is a critical step for maintaining seed quality.Rapid drying following priming can damage seeds resulting in the loss of advancement obtained during priming (Parera and Cantliffe,1992). Mechanical embryo damages have been reported in soybean, pine, and larch as a result of drying after osmotic priming (Armstrong and McDonald, 1992; Huang and Zou, 1989). On the contrary, primed pansy seeds increased longevity after fast drying (20°C, 40% RH) (Bruggink *et al*., 1999).

In cucurbits, priming has been proven successful to increase either germination rate or percentages in muskmelon (Bradford *et al.*, 1988; Nerson and Govers, 1986), diploid watermelon (Demir and Van de Venter, 1999; Sachs, 1977), and bitter gourd (Lin and Sung, 2000), etc. Unfortunately, few reports were documented on priming treatments of triploid watermelon seeds. In this work ,we attempt to assess the influence of hydropriming and redrying conditions on germination performance of triploid watermelon seeds.

MATERIALS AND METHODS

Seed materials: Seeds of two triploid watermelons, 'Gold Prince' and 'Guangxi 5', from the Vegetable & Flower Institute of Guangxi Academy of Agriculture Science, P.R. China, were employed in the experiment. The initial seed moisture contents of 'Gold Prince' and 'Guangxi 5' were 5.3% and 6.1%, respectively while the initial seed germination percentages were 83% and 67%, respectively.

Water uptake pattern: To determine the imbibition curve, seeds of 'Gold Prince' and 'Guangxi 5' were treated by seedcoat laterally split or not split (25 seeds per treatment), then soaked in deionized water for a period of 8 hours. Seeds were taken out from the water hourly and surface dried by blotter paper, weighed, and immediately put back to the water. The water uptake pattern was determined by measuring the water absorbed at every hour intervals related to the fresh weight (Le Deunff *et al.*,1989).

Hydration: The seeds of triploid watermelon were placed in glass columns (30 cm in height and 7 cm in diameter) containing 400 ml of deionized water and kept at 25°C under natural light for 2 hours. The duration of soaking was determined based on the water uptake pattern. Aeration was provided at 15 min/hr, 30 min/hr, and 45 min/hr, continuous, and non-aeration. After soaking, seeds were taken out from the water and put in a suspension of 0.2% Mancoseb for 10 minutes, in controlling fungus infection. Seeds were then rinsed off in running tap water for 10 minutes followed by surface drying with blotter paper and incubated in sealed plastic boxes under saturated humidity at 20±1°C for 24hrs or 48 hrs.

Germination tests were carried out immediately after incubation with 4 replicates of 25 seeds each in moist sand (1 liter deionized water per 10 kg sand). Seeds were carefully placed horizontally at 1 cm depth to prevent any orientation advantages (Maynard, 1989) in plastic boxes (7.5 × 11.5 cm). The boxes were covered with plastic lids to control evaporation and placed in germination chamber at 25°C. Seedlings were evaluated at 4^{th} and 14^{th} day after seeding and the mean normal seedlings, abnormal seedlings and dead seeds were calculated (ISTA, 1999), untreated seeds(original seeds) served as control. Mean germination time (MGT) was also obtained based on daily counting of normal seedlings using the following formula proposed by Alvarado and Hewitt (1987):

$$MGT = \sum Ti.Ni / \sum Ni$$

where Ni = number of newly germinated seeds at time Ti

Redrying: After the incubation process, the seeds were redried to lower seed moisture content to 6-7 % in a humidity controllable electronic dry cabinets (DRY-60, WEIFO) at different drying conditions: slow drying (60% RH, 20°C), medium

drying (40% RH, 20°C), and quick drying (20% RH, 20°C). Germination test was carried out immediately after the drying process as described above, compared to surface dried seeds and control. The seed moisture content was determined by hot air oven method at 103±1°C for 17 hours (ISTA, 1999). Two replications of 1 gram each were used for the test.

Statistical analysis: Data analyses were performed using the SAS statistical software (Version 6.12). Completely randomized design was used in this experiment. Mean separations were performed by Duncan's multiple range test (DMRT) at 5% level. The values in percentage were arcsine transformed before analysis.

RESULTS AND DISCUSSIONS

Hydration: The water uptake of triploid and diploid watermelon seeds showed a triphasic pattern, which is similar to the pattern of most of the seed kinds (Bewly and Black, 1994). However, there were no significant differences in water uptake between seed coat lateral splitting and non-splitting treatment especially in 'Guangxi' triploid watermelon (Figure 1). Therefore, water impermeability may not be a limiting factor to inhibit germination in both 'Gold Prince' and 'Guangxi 5'. The water uptake was attained rapidly during the first two hours, and continued to slowly increase with a steady rate in all treatments.

From the water uptake pattern, 2 hours duration was then chosen for soaking triploid watermelon seeds and found no significant differences on germination percentages among 2 hours soaking treatments in relation to aerating time (Figure 2). However, the highest germination percentages were obtained from primed seeds soaking in water for 2 hours with continuous aeration for 'Guangxi 5', and 45 min/hour aeration for 'Gold Prince'.

After 2 hours soaking with aeration (Figure

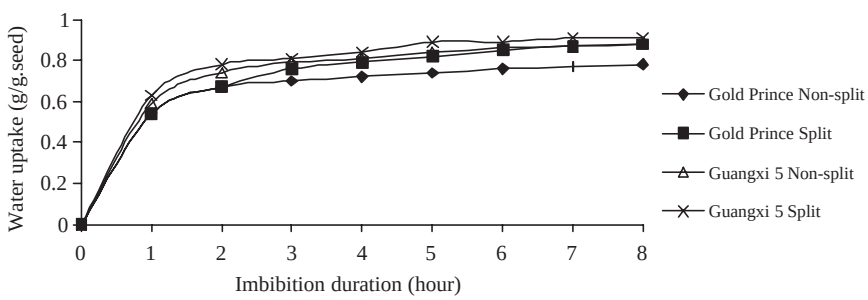

Figure 1 Water uptake pattern in watermelon seeds under 8 hours imbibition period.

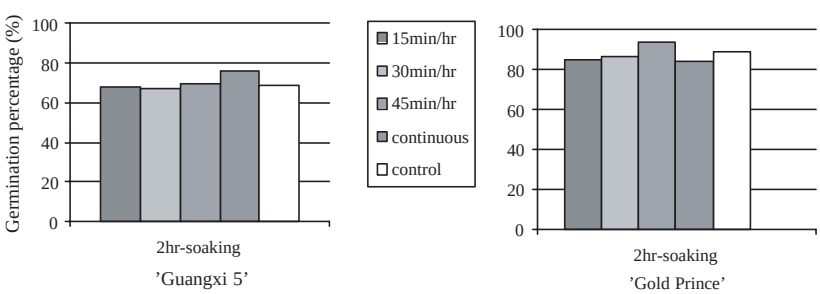

Figure 2 Germination of 'Guangxi 5' and 'Gold Prince' after hydropriming treatments at different aeration times.

2), primed seeds were further incubated for a period of 24 hrs or 48 hrs. The germination percentages of 'Gold Prince' were not significantly improved by both 24 or 48 hours incubation, but primed seeds from 48 hrs incubation dramatically increased the germination percentages of 'Guangxi 5'. However, MGT was significantly reduced by incubation time in both cultivars (Table 1) which indicate that the incubation treatment could enhance the speed of germination (Alvarado and Hewitt, 1987).

Redrying: When hydrated seeds (2 hrs soaking + 48 hrs incubation) were redried, all the seeds lost their moisture rapidly from approx.30% to 10% within one day (Figure 3). Under quick drying condition (20% RH, 20°C), both cultivars reached their equilibrium moisture content (5.09 % for 'Gold Prince' and 4.6 % for 'Guangxi 5') within two days. While medium drying (40% RH, 20°C) brought seeds to an equilibrium moisture content within 4 days of redrying (6.35% for 'Gold Prince' and 6.95% for 'Guangxi 5'). On the contrary, even after 4 days of drying under slow drying condition (60% RH, 20°C), the moisture contents were still high (9.63% for 'Gold Prince' and 8.42% for 'Guangxi 5'). The seeds of both cultivars in slow drying treatment were then transferred to medium drying condition and dried back to approx. 6~7% moisture content at the 5th day.

After redrying, the highest germination percentages and MGT were obtained from medium drying for 'Gold Prince' and quick drying for 'Guangxi 5' (Table 2). Since redrying after priming

Table 1 Germination percentages and mean germination time(MGT) of triploid watermelon seeds after soaking in water for 2 hours following 24 or 48 hours incubation.

Treatments	Gold Prince		Guangxi 5	
	Germination(%)	MGT(day)	Germination(%)	MGT(day)
48 hrs incubation	84.0a	4.11b	80.0a	4.08b
24 hrs incubation	80.5a	4.13b	67.0b	4.16b
Control	83.0a	4.38a	67.0b	4.63a
F-test	ns	**	*	**
C.V.(%)	5.92	2.33	8.49	3.76

Means within each column followed by the same letter are not significantly different at P<0.05 level by Duncan's multiple range test.

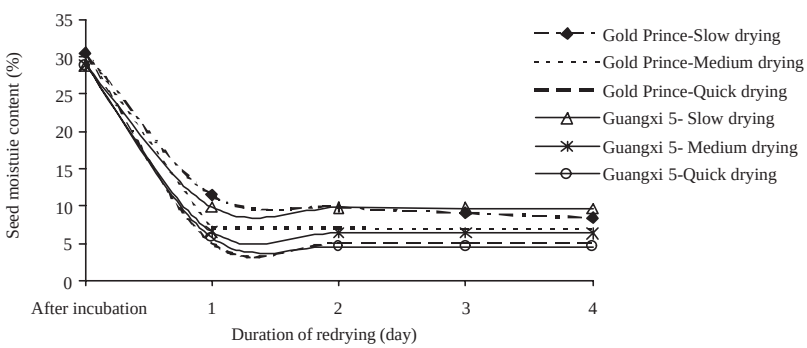

Figure 3 Changes in seed moisture content under different drying conditions of hydroprimed triploid watermelon seeds.

Table 2 Germination percentage and mean germination time (MGT) of hydroprimed triploid watermelon seeds after redrying.

Redrying conditions	Gold Prince		Guangxi 5	
	Germination (%)	MGT (days)	Germination (%)	MGT (days)
Surface drying	85 b	4.10b	76 bc	4.11 bc
Slow drying	80 b	4.20 ab	80 ab	4.52 a
Medium drying	92 a	4.06 b	82 ab	4.17 bc
Quick drying	84 b	4.29 ab	89 a	4.07 c
Control	83 b	4.36 a	67 c	4.41 a
F-test	*	*	**	*
C.V. %	4.48	2.12	6.20	3.64

Means within each column followed by the same letter are not significantly different at P<0.05 level by Duncan's multiple range test.

is critical step for maintaining seed quality, it could be explained that rapid drying might have altered the soluble carbohydrates content which in turn reduce desiccation tolerance and speed of germination (Bruggink *et al*., 1999). Further study is needed to investigate the relationship between redrying and seed longevity of primed seed.

CONCLUSSION

Hydropriming treatment can be successfully applied on triploid watermelon seeds to improve germination performance. A treatment combination of 2 hrs soaking in water following 48 hrs incubation at saturated relative humidity increased germination percentages and reduced mean germination time of both triploid watermelon cultivars 'Gold Prince' and 'Guangxi 5'. Hydrated seeds can be redried without the lost of physiological advancement obtained from hydration phase. However, the efficacy of priming treatments is also cultivar dependent.

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Varietal Evaluation and 30 kDa Protein Studies in Local Bitter Gourd (*Momordica charantia* L.)

Charan Ditchaiwong[1], Prachya Kongtawelert[2], Surapol Natakankitkul[3], Manoch Tongjiem[4] and Maneechat Nikornpun[5]

ABSTRACT

Twelve accessions of local bitter gourds were tested in comparison with a cultivated bitter gourd at Phichit Horticultural Research Center in summer 2000. Botanical and horticultural characteristics of leaf, pistillate flower, staminate flower, fruit and seeds were recorded. Ripe fruit yield of the accessions ranged from 3,227 to 6,912 kg/ha. Yield was not significantly different among the accessions but showed high variation in protein levels in extraction from endosperm. The partial protein powder ranged from 104.33 to 208.67 mg/5 g of endopsperm while the specific activity protein at 30 kDa evaluated by the polyacrylamide gel electrophoresis (PAGE) method ranged from 104.86 to 265.42 μg/5g of endosperm. One specific accession (No.16) gave the highest level of the specific activity protein at 30 kDa, however, it gave high level of the partial protein powder, 168.33 μg/5g. Contradictory, the other accession (No.11) which had more or less the same level of total protein as the mentioned accession, had rather low level of the specific activity protein at 30 kDa, 149.96 μg/5g of endosperm.

Key words: bitter gourd, 30 kDa protein, partial protein, specific activity protein

INTRODUCTION

Rural Thai people have used several indigenous vegetables and spices as medicine since the ancient times. The local medicine is called herbal medicine. Nowadays, there are various kinds of modern medicine, therefore the interest towards herbal medicine is quite limited. Some researchers are searching for new compounds to use for these disease such as HIV and cancer. It was found that bitter gourd reduced oxidation of methmyoglobin by hydrogenperoxide in laboratory. The reduction as such may be able to reduce the action of the virus.

Bitter gourd has long been used as local medicine in Asia. The plant has been investigated for the active ingredients and their effects. Ng *et al*.(1992 and 1997) at the Chinese Medical Material Research Centre of the Chinese University of Hong Kong found a series of protein in bitter gourd which has anti-HIV activity. Lee-Huang *et al*. (1994-1995a and 1995b) isolated MAP30 (*Momordica* anti-viral protein of 30 kDa) that has anti-HIV activity. There are several proteins involved in HIV replication, such as reverse transcriptase which converts the viral RNA genome to DNA, protease which modifies the protein products of the viral genome for new viral particles and integrase which inserts and removes the DNA from the host genome.

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The protein can block the infection of T lymphocytes and monocytes by HIV and inhibits the replication of HIV in already infected cells *in vitro*. (Lee-Huang *et al*., 1990) Although, MAP30 shows the inhibitory effect, but it appears to have limited toxicity to uninfected cells. MAP30 has a direct action against integrase activity and may inhibit the virus in other ways as well (Lee-Huang *et al*.,1995b). It has been quite sometimes that *M. charantia* protein was shown to have an action on HIV, but the protein has not been developed further for HIV treatment.

There are two types of bitter gourd in Thailand; local and cultivated types, the former scattered in Thailand as weeds. Variations of size and shape of fruits, plant type and leaf type are always observed. Because of its low economical values, little attention has been paid to the crop in terms of research. Collection of germplasm of local and cultivated bitter gourd and evaluation of horticultural characterization are the primary step for the breeding program. Subsequently, identification and separation of MAP30-like substance of the collected accessions would be operated. Then, breeding and development of varieties for high MAP30 variety should be the important step for the production of MAP30 in large scale.

MATERIALS AND METHODS

Twelve accessions of local bitter gourds were collected from various locations in Thailand in 1999. They were grown in the field at the horticultural research center, Department of Agricultural Extension in Phichit province, Thailand in 2000. Randomized complete block design with 4 replications was used. One cultivated bitter gourd called Deak bin brand or No.20 was used as a control variety. The experimental period was about 4 months. Fruits were harvested at ripe stage, they were weighted and seeds were taken out for seed weight.

General horticultural and botanical characteristics such as leaf shape and size, male and female flowers, fruit and seeds were recorded. They were grown for botanical and horticultural characterization.

Partial protein extraction, total protein analysis and specific activity protein at 30 kDa analysis were obtained by using frozen endosperm of bitter gourd seeds. The endosperm was ground at 4°C with normal saline solution at pH 3.6-4.0. The solution was filtered with cloth before it was centrifuged at 12,000 rpm, 4°C for 30 minutes for 3 times. The supernatant was shaked with 50%$(NH_4)_2SO_4$ and 50 mM Na_2PO_4 buffer, pH 6.3 before it was centrifuged again. The residue was dialysed with 20 mM Na_2PO_4 buffer, pH 6.3. Then active protein fraction was dried with a lyophilizer for 24 hrs for partial protein powder.

Total protein was analysed from the partial protein powder, using Bio-Rad protein assay reagent (Bradford) (Bio-Rad Laboratories, U.S.A.Cat.No. 500-0006). Five milligrams of the partial protein was mixed with 1 ml dei. H_2O and shaken by vortex. Ten µl of the solution was dropped into microliter plate wells. Various concentrations of Bovine Serum Albumin (BSA), 0 to 1000 µg/ml were dropped into adjacent wells. Then 200 µl/well of dyereagent was mixed in each well. They were measured at 620 nm absorbance. A regression line of standard protein (BSA) was calculated. Solution of each accession was compared with standard prtoein (BSA), to calculate total protein of each accession.

Specific activity of protein at 30 kDa was analysed from the partial protein powder. Deionized water was added into the powder. Buffer was added into the solution before it was heated at 95°C for 5 min. The sample was used for electrophoresis. Gels were stained with coomassie blue before washing and drying. Then specific activity of protein at 30 kDa was measured by an imaging densitometer, then calculated by comparison both amount of protein.

RESULTS

Characterization of botanical and horticultural traits of 12 local bitter gourds (Table 1-8) revealed that many botanical characteristics of leaf of all accessions were similar to each other. They had simple type of leaf, pubescent on surface covering and alternate arrangement, with palmate venation. Leaf shape of all accessions were the same, which showed circular shape of leaf, open shape of upper leaf sinus, concave shape of teeth, 7 lobes, acute apex of leaf, cordate base of leaf and parted margin of leaf. They were different on leaf size and color (Table 1). Leaves of all accessions were green in color but different in degree of greenness as measured by Hunter L, a, b color.

Pistillate flowers of all accessions were the same on many characteristics. (Figure 1). The flowers were regular (actinomorphic), solitary type of inflorescence, rotate corolla shape, polypetalous of corolla with 5 petals, yellow corolla, inferior ovary, epigynous of flower part, calyx adnates to ovary, 5 calyx lobe, 3 parietal placenta and reniform shape of sessile bract. Size of petal, peduncle length and size of sessile bract were different among the accessions (Table 2).

Staminate flowers of all accessions were the same on many characteristics (Figure 2). They had regular staminate flower (actinomorphic), axillary inflorescence, rotate corolla shape, polypetalous corolla with 5 petals, yellow corolla, 5 calyx lobe and reniform shape of sessile bract. Size of petal, peduncle length, size of sessile bract and distance of sessile bracts to peduncle base of the accessions were different (Table 3).

Fruit of bitter gourd was pepo type with 7 lobes. Size and weight of the fruits in various accessions were different (Table 4). The accessions showed differences in number of seeds per fruit and seed size (Table 5). Ripe fruit yield among the accessions was not significantly different (Table 6). The yield ranged from 3,227 to 6,912 kg/ha. Accession No.13 gave the highest fruit yield. Other high yield accessions were No.21, 11, 7 and 16. Fruits and seeds of accession 11, 13 and 16 were shown in Figures 3, 4 and 5. Fresh seed yield of the accessions was significantly different (Table 7). Seed yield ranged from 312.5 to 842.5 kg/ha. Accession No.13 gave the highest seed yield. The accession also gave the highest number of ripe fruit, 680,313 fruit/ha (Table 7). However, high fruit yielding accession was not always gave high seed yield.

Accession No.13 was among the accessions that had the longest harvesting life, 117-118 days after germination (Table 8). The last harvesting of ripe fruit after germination for Accession No.13 was 117 days. Number of times to harvest ripe fruits of this accessions was the highest, 50 times (Table 8).

Figure 1 Pistillate (female) flower of bitter gourd.

Figure 2 Staminate (male) flower of bitter gourd.

Table 1 Botanical characteristics of leaf of bitter gourd accessions.

Acc. No.	Size of leaf (cm)[1]		
	Width	Length	Petiole length
1	11.9	8.0	6.7
3	10.0	7.1	4.7
5	11.4	7.2	5.8
6	14.8	11.2	7.5
7	13.4	9.2	8.8
8	15.4	9.9	7.3
10	13.5	8.3	9.3
11	10.7	7.5	5.4
12	10.8	7.9	5.6
13	10.0	7.7	5.8
16	10.8	7.1	6.9
20	14.3	10.2	7.6
21	10.5	7.7	5.1

[1] Average of 10 mature leaves

Table 1 (continue) Botanical characteristics of leaf of bitter gourd accessions.

Acc. No.	General shape of petiole sinus	Colour of leaf[1]		
		L[2]	a[3]	b[4]
1	Wide open	35.19	-5.22	10.33
3	Open	53.05	-4.97	9.18
5	Wide open	87.19	-1.23	1.79
6	Wide open	87.44	-1.3	1.66
7	Wide open	78.85	0.94	10.05
8	Wide open	51.34	-4.89	7.67
10	Open	38.01	-6.83	14.83
11	Open	51.72	-4.89	9.67
12	Wide open	34.96	-6.35	13.03
13	Wide open	65.64	-2.16	7.52
16	Open	65.64	-2.16	7.52
20	Open	51.37	-4.8	8.41
21	Wide open	50.36	-4.03	6.74

[1] Color QUEST Hunter Lab
Evaluated date: 5 April 2000

[2] L - lightness

[3] a – redness or greenness, - a green, + a red

[4] b – yellowness or blueness, - b blue, + b yellow

Table 2 Botanical characteristics of pistillate flower of bitter gourd accessions.

Acc. No.	Pistillate flower			Sessile bract of pistillate flower[1]		
	Petal (mm)		Peduncle length	Width	Length	Distance to peduncle base
	Width	Length	(mm)	(mm)	(mm)	(mm)
1	8.4	10.9	29.0	7.7	5.5	3.5
3	11.2	14.5	47.8	8.4	6.2	9.3
5	10.3	13.5	29.4	8.9	5.8	7.3
6	9.3	11.6	37.4	7.4	5.1	2.9
7	8.4	11.1	33.8	10.6	8.3	3.9
8	7.5	11.3	52.7	11.0	6.7	5.2
10	9.8	12.6	41.7	6.7	4.6	2.8
11	6.7	10.0	44.6	9.5	5.9	8.5
12	11.3	18.9	87.7	8.9	5.6	8.8
13	10.1	16.1	63.7	7.2	4.7	5.0
16	7.1	9.5	45.3	6.3	4.6	3.9
20	8.1	10.7	55.1	3.7	3.2	5.8
21	6.8	10.2	51.1	7.4	4.9	6.6

[1] Average of 10 pistillate flowers

Table 3 Botanical characteristics of staminate flower of bitter gourd accessions.

Acc. No.	Staminate flower			Sessile bract of staminate flower[1]		
	Petal (mm)		Peduncle length	Width	Length	Distance to peduncle base
	Width	Length	(mm)	(mm)	(mm)	(mm)
1	9.4	15.1	66.2	8.5	6.9	10.1
3	13.6	20.3	69.6	7.6	5.4	20.4
5	10.7	15.8	50.9	8.8	6.7	15.2
6	9.5	13.9	52.2	10.0	7.4	8.6
7	10.4	15.1	29.9	6.6	4.3	4.6
8	8.4	16.3	61.8	10.5	7.5	10.5
10	10.1	16.5	48.6	6.0	4.2	5.2
11	10.1	14.8	51.8	10.3	6.6	14.1
12	12.9	21.3	68.3	11.2	6.2	12.6
13	11.3	17.1	75.5	6.2	4.5	7.6
16	9.0	12.3	54.7	5.7	3.5	5.6
20	10.9	14.8	77.2	7.7	5.7	28.0
21	7.8	12.7	45.2	7.8	5.6	12.4

[1] Average of 10 staminate flowers

Table 4 Size and shape of bitter gourd fruit in 13 accessions.

Acc. No.	Length[1] (cm)	Diameter[1] (cm)	Fruit base to sessile bract[1] (cm)	Weight[1] (g)
1	4.02	3.00	2.96	12.03
3	4.72	2.11	3.82	5.31
5	4.81	2.18	2.78	5.87
6	6.70	3.26	4.39	20.30
7	10.29	3.59	2.92	42.89
8	11.44	3.72	4.38	51.21
10	8.52	4.23	5.70	45.75
11	6.88	3.02	4.42	16.12
12	4.70	2.08	6.48	4.69
13	4.69	2.20	7.04	4.94
16	6.08	3.25	4.50	19.89
20	6.10	3.18	5.78	17.91
21	5.20	2.93	5.37	13.73

[1] Average of 10 fruits.

Table 5 Number of seed per fruit and size of bitter gourd accessions.

Acc. No.	Size (mm)[1]			Number of seeds per fruit[2]
	Width	Length	Thickness	
1	7.25	12.40	3.91	8
3	4.78	9.65	3.22	14
5	5.75	9.43	3.25	9
6	7.37	12.36	4.05	8
7	6.63	12.76	3.85	31
8	7.80	14.93	4.07	28
10	7.00	12.51	3.89	26
11	6.95	13.64	3.48	15
12	5.23	10.66	3.23	9
13	5.53	11.16	3.22	13
16	7.64	12.91	3.49	17
20	7.43	12.34	4.09	11
21	7.32	12.18	4.06	8

[1] Average of 10 seeds

[2] Average of 10 mature fruits

Table 6 Days to 50% female flowering, first harvest of ripe fruit and yield of ripe fruit of bitter gourd accessions.

Acc. No.	Days to 50% female flowering[2/]	Days to first harvest (days)[2/]	Weight of ripe fruit[1] (kg/ ha)[2/]
1	57 abcd	68 c	3,227
3	59 bcd	70 cd	3,853
5	55 ab	68 bc	4,403
6	61 cd	71 cd	4,956
7	53 e	59 a	5,188
8	62 de	69 c	4,516
10	56 abc	62 ab	4,878
11	66 a[1]	75 d	5,376
12	57 abcd	66 bc	4,827
13	55 ab	66 bc	6,912
16	56 abc	68 bc	5,168
20	57 abcd	70 c	4,640
21	55 ab	67 bc	5,701
F-test	**	**	NS
CV (%)	5.96	5.36	27.11

1 Harvested ripe fruits per area.

2 Same letters indicate no differences in means (DMRT).

Table 7 Number of ripe fruit and seed yield of bitter gourd accessions.

Acc. No.	No. of ripe fruit (fruit/ha)	Fresh seed yield (kg/ha)
1	170,938 cde	360 cd
3	448,750 b	390 cd
5	575,625 a[1]	533.75 bc
6	176,563 cde	420 bcd
7	89,063 de	382.5 cd
8	63,125 e	380 cd
10	72,813 e	312.5 d
11	190,625 cd	485 bcd
12	655,938 a	610 b
13	680,313 a	842.5 a
16	150,000 cde	477.5 bcd
20	138,438 de	428.75 bcd
21	255,313 c	501.25 bcd
F-test	**	**
CV (%)	25.08	25.33

1 Same letters indicate no differences in means (DMRT)

Table 8 Days to last harvest and number of time to harvest ripe fruit of bitter gourd accessions.

Acc. No.	Last harvesting of ripe fruit after germination (days)	No. of times to harvest ripe fruits
1	107 cd	33 de
3	117 a[1]	45 ab
5	117 a	48 a
6	118 a	39 bcd
7	98 f	23 f
8	109 bc	24 f
10	101 e	24 f
11	117 a	36 cde
12	117 a	49 a
13	117 a	50 a
16	106 d	34 de
20	107 cd	32 e
21	110 b	41 bc
P	< 0.01	< 0.01
CV (%)	1.81	11.56

[1] Same letters indicate no differences in means (DMRT)

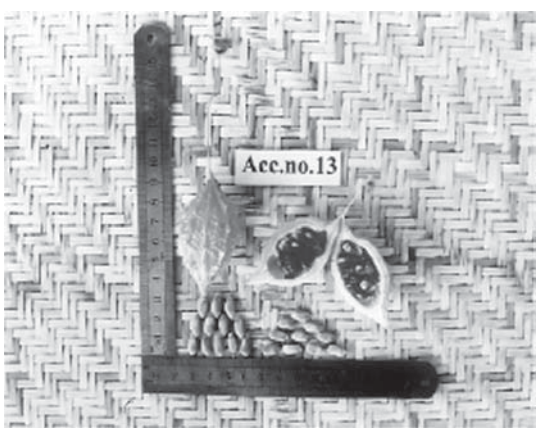

Figure 3 Fruit and seeds of bitter gourd, accession No. 13.

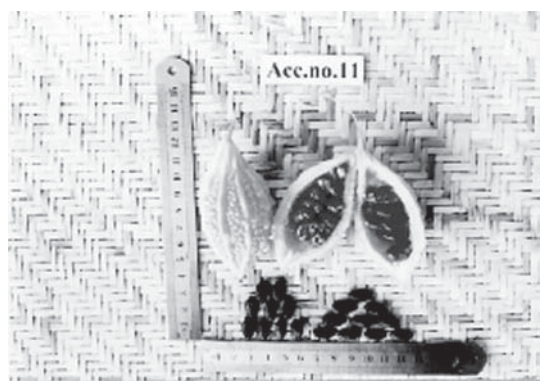

Figure 4 Fruit and seeds of bitter gourd, accession No. 11.

Total protein, and specific activity protein at 30 kDa in 5 grams of endosperm are showed in Table 9. Levels of these proteins showed variation among accessions of bitter gourd. The levels of total protein did not have any correlation correlated with the levels of the specific activity protein at 30 kDa. The levels showed the range of 104.86 to 265.42 μg/5g of endosperm.

When total protein and specific activity at 30 kDa were extracted from the endosperm of each accessions, it was found that high fruit and seed yielding accession such as No.13 gave low level of total protein 106.67 mg/ml H_2O and low level partial protein and specific activity protein. It gave

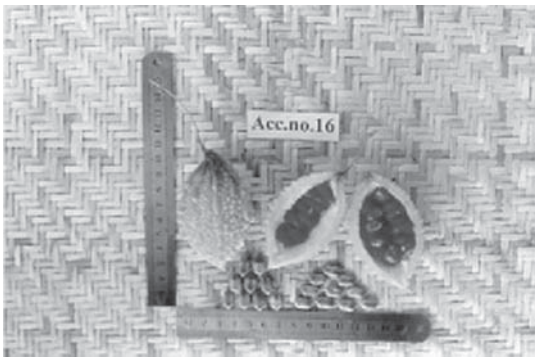

Figure 5 Fruit and seeds of bitter gourd, accession No. 16.

104.33 μg/5 g of endosperm of partial protein powder while the highest level was 208.67 mg/5g and gave 104.86 mg/5g of endosperm of specific activity protein at 30 kDa while the highest level was 265.42 μg/5g of endosperm. Accession No.16 gave the highest level of specific activity protein at 30 kDa. The accession gave lower yield of ripe fruit than accession No.13. However, the difference was not statistically significant.

DISCUSSION

Accession No.13 gave the highest fruit yield. It gave also the highest number of harvesting times and number of fruits. Number of female flowers and number of fruit per plant were found to be yield components of bitter gourd (Ramachandran and Gopalakrishnan, 1979, Paranjape and Rajput, 1995 and Rajput *et al*., 1995). Fruit yield was not as important as seed yield because only endosperm was used for extraction for specific protein at 30 kDa. Accession No.13, again, gave the highest seed weight.

When specific protein at 30 kDa or MAP30 was extracted from the bitter gourd accessions, wide range of protein levels was observed. Lee Huang *et al*. (1995a) reported the identification and purification of MAP30 in bitter melon *Momordica charantia* were different cultivar from our local bitter gourd.

High yielding variety such as accession No.13 did not give high level of MAP30. It gave the lowest level of MAP30, 104.80 μg/5g endosperm.

Table 9 Total protein , partial protein and specific activity protein at 30 kDa in 5g of endosperm of bitter gourd accessions.

Acc. No.	Total protein (μg/ml dei.H_2O)	Partial protein (mg)	Specific activity protein at 30 kDa (μg)[1]
1	148.17	155	158.40
3	142.5	163.33	206.39
5	126	115	121.96
6	93.17	142	162.30
7	162	142	197.51
8	172	179	179.86
10	138.3	208.67	215.91
11	160.8	207.33	149.96
12	87	110	120.22
13	106.67	104.33	104.86
16	136.50	168.33	265.42
20	150.67	117.67	139.28
21	175.33	162.67	147.81

[1] Specific activity protein calculated from imaging densitometer (model Bio-Rad GS-700).

While accession No.16 gave lower fruit yield than accession No.13 but it gave the highest level of MAP30, 265.42 μg/5g of endosperm. Since the difference in fruit yield was not statistically significant, therefore accession No.16 would better than accession No.13 for MAP30 extraction, eventhough seed yield of this accession was about half of accession No.13.

CONCLUSION

General botanical characteristics of local bitter gourd and cultivated bitter gourd were the same on type of leaf, pistillate flower, staminate flower fruit and seed. The differences were found only size fruit yield and seed yield. When total protein, and specific activity protein at 30 kDa were extracted from endosperm of the seeds. It was found that high yielding accessions did not always give high level of protein content in the seed. Some low yielding accessions gave high specific protein at 30 kDa levels.

Variation of specific activity protein at 30 kDa of bitter gourd accessions as shown in Table 9 illustrated differences in genetic control of MAP30 biosynthesis in the plants. It is very interesting that the range of production is wide, from 104.86 to 265.42 μg/5 g of endosperm. The substance which was extracted from bitter gourd fruit so called MAP 30 kDa is under investigation for its characteristics such as amino acid sequences. Plant breeding may play an important role on improvement for high production of the protein. Many methods of breeding such as pedigree method, mass selection, recurrent selection and F_1 hybridization can be used for varietal improvement.

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Spatial Dispersion and Optimum Sample Size for Cotton Bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) Larvae on Cotton

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ABSTRACT

Field experiments were conducted in the irrigated and the rain-fed crops of 2000 and 2001 at Suwan Farm, Pak Chong, Nakon Ratchasima, Northeastern Thailand to determine the spatial dispersion of cotton bollworm, *Helicoverpa armigera* (Hübner) larvae on four cotton varieties/lines, namely, AP1 and AP2, the mutant lines, SR60 (Sri Samrong 60) and SD1 (Sarid1), the recommended varieties. RCB was used with four replicates for both crops. The spatial dispersion was analyzed using the variance-to-mean ratio ($s^2/\bar{x}$), Morisita's Index (I_δ) and the negative binomial parameter (k). Although cotton bollworm larvae exhibited mainly clumped distribution, sometimes it was found to be randomly disperse during the sampling periods of both crops. In general, *H. armigera* larvae were at high aggregation (k=0.10) on the irrigated crop and low aggregation (k=3.92) on the rain-fed crop. The dispersion information was used to select an optimum sample size at the 0.05 probability. The optimum sample size of 15 and 30 plants per 140 m^2 were needed to monitor low and high aggregation levels of *H. armigera* larvae, respectively.

Key words : *Helicoverpa armigera*, dispersion, cotton, sample size

INTRODUCTION

The cotton bollworm, *Helicoverpa armigera* (Hübner), is described as the most destructive and persistant key pest of cotton (*Gossypium* spp.) in Thailand. The larval stage of *H. armigera* causes the greatest amount of damage to cotton with each destroying up to 15 cotton bolls during its development. Seriously damaged cotton fruiting bodies (buds, open flowers and bolls) can be considered as total crop loss, however larvae will eat leaves and young stems when nothing else is available. Consequently, chemical control still remains the backbone of management tactics for *H. armigera*. Before implementing a spray program, pest scouting and economic threshold levels should be determined. In addition, the spatial dispersion of *H. armigera* and optimum sample size should be investigated before sampling decisions are made.

The two most commonly tested mathematical distributions, the Poisson distribution and the negative binomial distribution, are useful for describing random and aggregated distributions, respectively. Dispersion of population can be classified by calculating the indices such as the variance-to-mean ratio ($s^2/\bar{x}$) and Morisita's Index (I_δ). Variance-to-mean ratio is the simplest and most fundamental index to access an agreement of the data set to the Poisson distribution in which the variance equals the mean (Davis, 1994; Wilson,

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1994), while Morisita's Index has the advantages of being relatively independent type of distribution, the number of samples, and the size of mean (Morisita, 1962; Pieters and Sterling, 1974). Moreover, Morisita (1962) noticed its relationship to the binomial distributions and relative parameter for appropriate samples and stratified random sampling. In general, the values of both indices can be used to estimate whether a populaiotn's spatial pattern is uniform, random, or aggregated (Pieters and Sterling, 1974; Taylor, 1984; Davis, 1994).

Southwood (1978), Taylor (1984), Perry (1997), and Southwood and Henderson (2000) advocated that analysis of spatial dispersion of insects provides the estimation of pest densities and minimal costs which were the basis for making decision in pest management. Nachapong (1980) and Mabbett and Nachapong (1979, 1983), stated that the egg distribution of cotton bollworm was adequately described by the negative binomial and the spatial distribution remained contagious over the period of sampling. It was also revealed that the taller and healthier plants were more frequented targets for egg laying moths and the eggs were deposited on these plants in higher numbers (Mabbett *et al*., 1978, 1979). Reliable sampling plans are essential for monitoring pest population densities where timely pest management decision is necessary (Kuno, 1991). Buntin (1994) noticed that stratified random sampling technique could improve sampling efficiency by reducing sample variation that samples were collected from all areas of the habitat. Southwood (1978) and Southwood and Henderson (2000) stated that the number of samples could be varied with the distribution of the insect. Although several studies have been conducted on the dispersion of *H. armigera* eggs and larvae in Thailand, dispersion of insect may vary with densities and environmental conditions. The objectives of this study were to describe the spatial dispersion of *H. armigera* larvae and to develop the optimum sample size needed to be taken for desired precision of estimates of an effective sampling program.

MATERIALS AND METHODS

Studies were investigated at Suwan Farm (356 m above sea level and 101.25° N, 14.42° E), Pakchong, Nakon Rachasima Province for two growing crops of 2000 and 2001. A field of 0.30 ha was planted with four cotton varieties/lines, namely, Sri Samrong 60 (SR60), Sarid1 (SD1) and new mutant lines AP1 and AP2 of moderate resistance to bollworm, obtained from gamma-irradiated SD1 with a spacing of 1.0×1.0 m. The experiments were arranged in randomized complete block design with four replicates. An individual plot consisted of 7 rows, each 20 m long (140 m^2). All varieties/lines were planted on 1 October 2000 for the irrigated crop and 21 July 2001 for the rain-fed crop, respectively. Plots were managed in the same way as commercial cotton planting in this region. Sprinkler irrigation system was applied as required. The field was treated with 38 kg/ha Furadan 3% G and 0.06 kg/ha Imidacloprid 70% WS at planting time of the irrigated and the rain-fed crops, respectively. Carbosulfan 20% EC 50 ml per 20 litres of water and Omethoate 50% SL 40 ml/20 litres of water were alternately applied 5, 6, 7, 8 weeks after sowing (WAS) for the irrigated crop and Azodrin 60% WSC 40 ml per 20 litres of water applied once 4 WAS for the rain-fed crop, to control severe attack by leafhopper.

For the purpose of stratified random sampling, individual plot divided into four strata and 10 plants chosen at random per each plot, totally 160 plants, were visually inspected. *H. armigera* larvae were counted weekly from 10 to 14 WAS in the irrigated crop and from 7 to 13 WAS in the rain-fed crop, respectively. To analyze the spatial dispersion, means ($\bar{x}$) and variances (s^2) for counts of larvae per plant were calculated for each sampling date.

Although the variance mean ratio is commonly considered to be a useful index to describe

the spatial dispersion of a given insect, a single index could not serve well both as a measure of departure from randomness or as a measure of aggregation (Kuno, 1991). The two statistical models; Morisita's Index (I_δ) with F_0 value and the negative binomial parameter *k* were examined to confirm the values of $s^2/\bar{x}$ ratio (Morisita, 1962; Southwood, 1978; Davis, 1994). The parameter *k* of the negative binomial distribution is one measure of aggregation that can be used for insect species having clumped or aggregated spatial pattern. As *k* becomes smaller, the degree of clumping increases; and as the value of *k* increases, the negative binomial distribution approaches the random Poisson.

The optimum sample size (n) was computed to give a reliable estimate. Calculation of optimum sample size depends on the statistical dispersion of the target population and how precision is defined (Southwood, 1978; Southwood and Henderson, 2000). The adjusted sample size (n′) is also required to be calculated (Taylor, 1984). The calculations were based on the 4 varieties/lines altogether with 5 sampling dates (10 WAS–14 WAS) for the irrigated and 7 sampling dates (7 WAS–13 WAS) for the rain-fed crops, respectively. The mean temperatures during the irrigated and the rain-fed crops were 25.15°C and 26.02°C, respectively. The total rain-days for the irrigated and the rain-fed crops were 33 days and 75 days, respectively.

RESULTS AND DISCUSSION

The dispersion indices ($s^2/\bar{x}$ and I_δ) of *H. armigera* larvae on four cotton varieties/lines during the 2 years are presented in Tables 1 and 2. All values of both indices were greater than 1 and were confirmed by t-value and F_o value (p=0.01), showing that the dispersion of *H. armigera* larvae among cotton plants was clumped. It was found that data of both crops for each cotton variety/line was in consistent with the report that the distribution of cotton bollworm eggs and larvae fitted the negative binomial (Nachapong, 1980; Mabbett and Nachapong, 1983; Wilson and Room, 1983). Overdispersion could be apparent when the sample variance was significantly higher than the expected Poisson theory as judged by the index of dispersion (Kuehl and Fye,1972). The values of negative binomial parameter *k* were also described ranging from 0.10 to 0.21 in the irrigated and from 2.89 to 9.00 in the rain-fed crops, respectively (Tables 1 and 2). According to Southwood (1978), increasing in the value of *k* was associated with decreasing tendency for aggregation, whereas the decrease in *k* value indicated high aggregation at that time. Among the cotton varieties/lines, the highest aggregation was found in SR60 for both crops (Tables 1 and 2). This might be due to the different plant types and moderate resistance to the cotton

Table 1 Evaluation of spatial dispersion of *Helicoverpa armigera* (Hübner) larvae for four cotton varieties/lines at weekly intervals (10 WAS- 14 WAS) during the irrigated crop.

Variety /line	Variance mean ratio ($S^2/\bar{x}$)	t-value [1/]	Distribution pattern	Morisita's Index (I_δ)	F_o [1/]	Distribution pattern	k-value	Distribution pattern
AP1	1.17	17.00	clumped	6.20	1.17	clumped	0.21	clumped
AP2	1.27	18.62	clumped	7.18	1.27	clumped	0.18	clumped
SD1	1.37	28.03	clumped	12.44	1.37	clumped	0.10	clumped
SR60	1.64	46.7	clumped	23.33	1.64	clumped	0.05	clumped

[1/] P=0.01

Table 2 Evaluation of spatial dispersion for *Helicoverpa armigera* (Hübner) larvae for four cotton varieties/lines at weekly intervals (7 WAS-13 WAS) during the rain-fed crop.

Variety /line	Variance mean ratio ($S^2/\bar{x}$)	t-value [1/]	Distribution pattern	Morisita's Index (I_δ)	F_o [1/]	Distribution pattern	k-value	Distribution pattern
AP1	1.04	1.00	clumped	1.20	1.04	clumped	4.00	clumped
AP2	1.02	0.67	clumped	1.11	1.02	clumped	9.00	clumped
SD1	1.07	1.75	clumped	1.40	1.07	clumped	3.00	clumped
SR60	1.06	2.25	clumped	1.40	1.06	clumped	2.89	clumped

[1/] P = 0.01

bollworm of the other varieties/lines.

Table 3 expresses an overall dispersion of *H. armigera* for four cotton varieties/lines. The highly aggregated level (k=0.10) in the irrigated crop was observed, while low aggregation (k=3.92) fell in the rain-fed crop (Table 3). The low value of I_δ (1.27) in the rain-fed crop showed that even at a very low density, individuals tended to aggregate. High causes of aggregation (I_δ=10.40) was recorded in the irrigated crop (Table 3). The reason might be due to the heterogeneity of the environment such as microclimate, preferred parts of the plant and occurrence of natural enemies.

For the irrigated crop, fractional values of k were obtained for each sampling date in each cotton variety/line ranging from 0.001 to 0.25 (Figure 1). The peak (0.25) was observed 10 WAS in AP1, then sharply declined until 14 WAS and never exceeded even at a value of 0.50 (Figure 1), showing the bollworm populations to be highly aggregated in this crop. In the rain-fed crop, the individual k values for each sampling date differed considerably, ranging from 0.001 to 4.5 (Figure 2). The peak (4.5) was recorded 7WAS in AP2 and rapidly decreased in later sampling dates (8 WAS to 13 WAS) (Figure 2). The aggregation peaks of both crops might be coincided with the early larval stage of bollworm. The results confirmed the previous findings in revealing that as most population aged they became progressively less clumped (Pieters and Sterling, 1974; Wilson and Room, 1983). Southwood (1978) mentioned that k value could be influenced by predation, size of sampling units and weather. It was reported that the actions of predators and other

Table 3 Summary of spatial dispersion for *Helicoverpa armigera* (Hübner) larvae during the irrigated and the rain-fed crops of 2000 and 2001.

Cropping seasons	Variance mean ratio ($S^2/\bar{x}$)	t-value [1/]	Distribution pattern	Morisita's Index (I_δ)	F_o [1/]	Distribution pattern	k-value	Distribution pattern
Irrigated	1.34	34.00	clumped	10.40	1.34	clumped	0.10	clumped
Rain-fed	1.05	2.50	clumped	1.27	1.05	clumped	3.92	clumped

[1/] P = 0.01

mortality factors could be implicated in the progressive reduction in aggregation of bollworm life stages (Pieters and Sterling, 1974).

The overall dispersion of *H. armigera* at each sampling date in the irrigated crop revealed mostly an aggregated dispersion (Figure 3). However, in a few cases the expected frequencies of the negative binomial dispersion did not fit the observed dispersion. On those occasions, $s^2/\bar{x}$ did not depart significantly from 1. It was observed that larvae were randomly disperse at two sampling dates (10 WAS and13 WAS) in the irrigated crop (Figure 3). The peak of $s^2/\bar{x}$ (3.4) was recorded 11 WAS (Figure 3). The result was similar to those reported by Kuehl and Fye (1972) and Wilson and Room (1983) that the bollworm larvae tended to be randomly distributed when population density was low.

A similar trend was recorded for the rain-fed crop that samples collected from 7 WAS to 9 WAS (early stages of the sampling period) were found randomly disperse (Figure 3). Thereafter, *H. armigera* larvae exhibited clumped dispersion alternated with random dispersion. The distinct peak of $s^2/\bar{x}$ (2.2) was found 10 WAS (Figure 3). The finding was in line with the report that spatial dispersion of *H. armigera* larvae was random during the early development period of the cotton plants after which they exhibited either random or clumped distribution (Buranapanichpan, 1989). Morisita (1962) and Taylor (1984) stated that change in distribution from clumped to random resulted from the alteration of the size of the area occupied by the insects related to that of the sample or decreased population density. In both crops, there was a significant number of most samples ($s^2/\bar{x}$ >1)

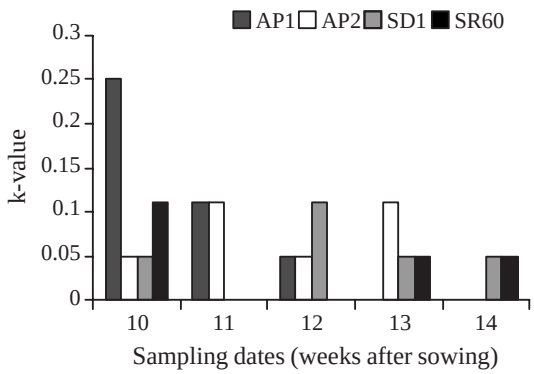

Figure 1 Negative binomial parameter *k* of *H. armigera* larvae calculated from each sampling date of four cotton varieties/ lines of the irrigated crop.

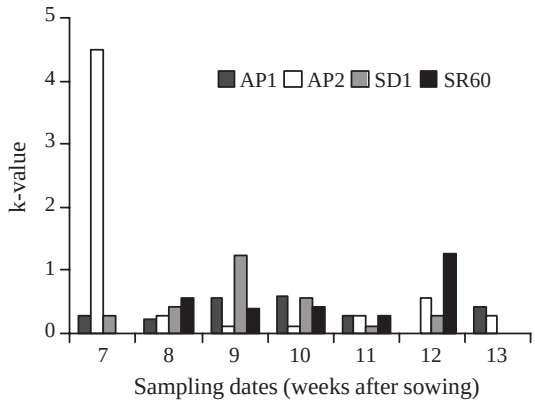

Figure 2 Negative binomial parameter *k* of *H. armigera* larvae calculated from each sampling date of four cotton varieties/ lines of the rain-fed crop.

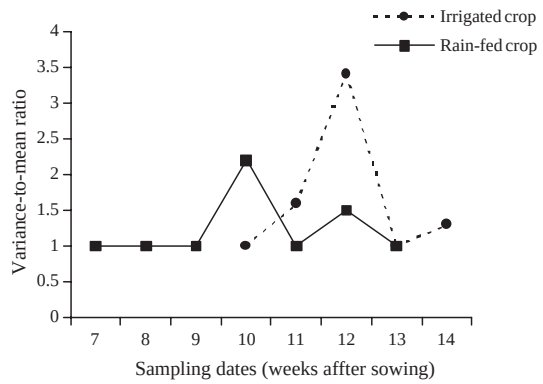

Figure 3 Index of dispersion, variance mean ratio ($s^2/\bar{x}$) of *H. armigera* larvae observed on four cotton varieties/lines during 5 sampling dates (10 WAS–14 WAS) in the irrigated crop and 7 sampling dates (7 WAS–13 WAS) in the rain-fed crop.

expressing agreement with negative binomial dispersion probabilities. Southwood (1978) revealed that when the population of an area became sparse, the chances of an individual occurring in any sample unit was low and continuous, the dispersion was effectively random. He also stated that dispersion might be changed because of the movement of medium and large larvae in some situations. In addition, Pieters and Sterling (1974) reported that cannabalistic behavior of *H. armigera* would help reducing aggregation. As different larval instars were present at some sampling dates and so different behaviors exhibited leading to different times of adult emergence, which could result in clumped together with random distribution during the investigation period. Moreover, the cotton field sprayed with some insecticides might affect the level of oviposition and dispersion pattern of *H. armigera* larvae. Nachapong (1980) previously stated that the distribution of cotton bollworm was dependent on both egg laying habits of the female moths and variation in growth among the cotton plants.

The information of spatial dispersion could be used to prepare sequential sampling plan of *H. armigera*. It was found that stratified random sampling and whole plant visual sampling method could be applied for bollworm scouting program. Minimum 15 cotton plants could be monitored at low aggregation level (k >1) for 0.05 probability of being within 10% of the mean number of bollworm larvae per plant. This result agreed with Wilson (1994) who reported that a smaller sample size was required at lower population aggregation for a given level of reliability. However, when population was highly aggregated (k <1), sample size should be increased up to 30 plants (p=0.05) per 140 m^2.

CONCLUSION

The dispersion pattern of *H. armigera* larvae among cotton plants changed over the season, showing first an aggregation and subsequently, a random dispersion. The overall pattern of *H. armigera* dispersion remained essentially the same whatever the cotton growing crop as well as the cotton variety/line. Random dispersion might be observed at the low population densities expressing consistency with Poisson distribution. Samples obtained from relatively dense insect population were the negative binomial dispersion. The sample size was influenced by the statistical distribution of the samples. It was possible to assume that optimum sample size of 15 plants and a considerably larger sample size of 30 plants per 140 m^2 were required at low and high aggregations of *H. armigera* larvae, respectively. Although the population density should be monitored throughout the season, it is possible that the sampling times and labor costs could be reduced.

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Effect of Soil Amendment with Urea and Calcium Oxide on Survival of *Ralstonia solanacearum*, the Causal Agent of Bacterial Wilt or Rhizome Rot of Ginger

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ABSTRACT

The effect of soil amendment with urea and calcium oxide on survival of *Ralstonia solanacearum*, the causal agent of bacterial wilt or rhizome rot of ginger, was studied by mixing urea and calcium oxide at the rate of 80 : 800 kg/rai in the artificial infested soil with 2.83×10^7 cfu/ml of bacteria. The treated soil was left one week before transplanting the two months old gingers. The soil was sampled during week 0 - 4 to evaluate the population of *R. solanacearum* and to compare with the control by serial dilution method and spread plate on SM-1 medium. The experiment showed that the population of *R. solanacearum* in the soil amendment with urea and calcium oxide decreased from 0.88×10^7 cfu/ml to 0.15×10^5 cfu/ml in week one, 0.1×10^4 cfu/ml in week two and 0 cfu/ml in week three. The control treatment still contained high population level of 0.26×10^7 cfu/ml in week one, 0.13×10^6 cfu/ml in week two and three and 0.11×10^6 cfu/ml in week four. This population level could cause typical wilt of the tested gingers. The tested gingers transplanted in the treated soil showed no symptom. The decrease of bacteria in the treated soil was due to the toxicity of ammonium, ammonia and nitrate degraded from urea in high pH soil condition (average 7.0-7.2). Therefore the soil amendment with urea and calcium oxide at the rate of 80 : 800 kg/rai is recommended to decrease population of *R. solanacearum* in the infested fields for bacterial wilt control. The treated soil should be left at least 3 weeks before planting a new crop to avoid toxicating to ginger seedlings.

Key words: ginger, bacterial wilt, rhizome rot, *Ralstonia solanacearum*, *Pseudomonas solanacearum*, soil amendment, urea and calcium oxide

INTRODUCTION

Bacterial wilt or rhizome rot of ginger caused by the soilborne bacteria *Ralstonia (Pseudomonas) solanacearum* is the serious problem in ginger growing area in both tropical and sub tropical regions. This disease usually occurs in late rainy season. The diseased plant showed inward curling, yellowing and browning of the entire shoot and almost dead. The basal portion of the yellow stem (shoot) is water-soaked and easily broken off from the underground rhizome and there is milky bacterial ooze exuding from cut stem or rhizome. According to the examination of the causal agent founded in Thailand by Koch's postulate and identification indicated that the causal agent was *Pseudomonas solanacearum*, ginger strain, belonging to biotype 3 and 4. It caused severe disease to tomato, potato, egg plant, phuttaraksa, edible ginger, creater-galangal, kachai, phai, and tumeric. All inoculated

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plants died 100 % in two weeks. Ginger strain could not infect sesame, peanut, bird chille, cowpea, yard long bean, triploid banana and bird of paradise. The relationship between bacterial causal agent and other organisms such as *Fusarium oxysporum, Sclerotium rolfsii, Pythium sp., Bacillus megatherium, Bacillus subtilis* etc are as follow : the symptom and diseased development are not different when inoculated with *P. solanacearum* alone and *P. solanacearum* mixed with the other organism. There were no other organisms in the experiment that could inhibit the growth of *P. solanacearum* or retard wilt symptom. On testing of 9 chemicals by petridish zonal inhibition and tube dilution technique, it was found that aureomycin at 50 ppm, streptomycin at 100 ppm and captan at 500 ppm could inhibit the growth of this organism. But in dipping ginger-seed into these chemicals before planting at concentration 2000 ppm for aureomycin and streptomycin, and 5000 ppm for captan, the chemicals could not prevent ginger-seed from disease (Chantaraotan, 1982). The infectivity tritration of *P. solanacearum* indicated that the concentration of inoculum at 10^5 - 10^8 cfu/ml could cause bacterial wilt symptom (Vudhivanich, 1997). Yabuuchi *et al*. (1992) reported some physical and biochemical characteristics and nucleotide sequences of *P. solanacearum* are different from *P. aeruginosa* which was the type strain of bacteria in genus Pseudomonas. Therefore, it was transfer to new genus, *Burholderia solanacearum* ,and finally changed to *Ralstonia solanacearum* in 1995. The disease can widespread by water, rain and by seed (rhizome) transmittion (Chantaraotan *et al*., 1986). A wide host range have been described for this pathogen (Atabug and San Juan, 1981 ; Vudhivanich and Soontarasing, 1994). It could survive in debris in soil for many years (Smith, 1944 ; Kelman, 1953). Chemical control of the disease has been attemptd with little success, beacause the pathogen resisted to many chemicals and antibiotics (Garner *et al*., 1917 ; Well and Roldan, 1922 ; Miller and Harvey , 1932 ; Chantaraotan, 1982). This pathogen has high genetic variation, new race or strain often occurred (AVRDC, 1974).

For many last years, the farmers have avoided the disease by shifting the ginger growing area to new location. It caused the increase of deforestation and widespread of the disease to the new cultivated area. One approach to help the farmer growing ginger in their own infested fields is soil amendment with some materials to decrease the population of the soilborne pathogen. There are many reports of soil amenment attempting to control the disease. Elphinstone and Aley (1993) reported the integrated control for bacterial wilt of potato race 1 biovar1 by crop rotation with maize and using 0.5 kg/ha metriburin , soil amendment with 5 ton/ha calcium oxide and 200 kg/ha urea could reduce the population of *R. solanacearum* and nematode in soil. It was also found that the population of *Pseudomonas cepacia*, the antagonistic bacteria increased. Michel (1997) reported that intercropping tomato with cowpea and the application 200 : 5000 kg/ha of urea and calcium oxide in soil amendment could reduce the population of *P. solanacearum* and bacterial wilt of tomato in Taiwan. However the experiment result of each location was not consistent. Patcharin (1997) reported soil amendment with urea and calcium oxide at the rate of 68.5 : 800 kg/rai could reduce the bacterial wilt of tomato by 60 % at greenhouse condition and 81 % at field condition. There were no significant differences among the types of calcium oxide such as calcium hydroxide, marl or dolomite. Furthermore the increase of calcium oxide have greater effect on the disease control than the increase of urea.

The objective of this experiment was to study the effect of soil amendment with urea and calcium oxide on survival of *R. solanacearum*, the causal agent of bacterial wilt or rhizome rot of ginger. The relationship between the population level and the bacterial wilt of ginger was also studied.

MATERIALS AND METHODS

Inoculum preparation

Ralstonia solanacearum was isolated from diseased ginger by cross streak on Tetrazolium medium (TZC) [Kelman, 1954] for 48 hour at room temperature (30-32°C). Virulent colony of small irregularly round, fluidal, white with pink in center were picked up and maintained in sterile distilled water and kept in a cooler at 13°C. The inoculum was prepared from virulent colony and increased on TTC medium for 30 hours and then suspended in sterile distilled water. The optical density (O.D.) was measured by spectrophotometer to reach 0.5 at wavelength 590 nanometer. Some of the inoculum was inoculated into disease -free ginger shoot by scalpel leaf clip method for pathogenicity test.

Artificially infested soil preparation

Artificially infested soil was prepared by mixed inoculum 1 liter per 16.2 kilograms of steriled soil. A half of infested soil was filled into 12 clay pots as the control and the rest used as the treatment for soil amendment with urea and calcium oxide.

Survival of *R. solanacearum* in infested soil (control)

Two months old disease - free gingers were transplanted in artificial infested soil in 12 clay pots. Each clay pots had 4-5 shoots. Wilt symptom was checked everyday. The infested soil was sampled every week from week 0 (initial state) to week 4. The evaluation of population of *R. solanacearum* in infested soil was made by serial dilution method and spread plate on SM-1 medium (Granada *et al*., 1983).

Survival of *R. solanacearum* in infested soil amending with urea and calcium oxide

The infested soil was amended with urea and calcium oxide at the rate of 80 : 800 kg/rai (50 g urea mixing with 500 g calcium oxide per 16.2 kg of infestd soil). The soil was waterred and wrapped with plastic sheet and left for 1 week. Afterward, the treated soil was divided into 12 clay pots. Two months old disease - free gingers were transplanted into those 12 clay pots. Wilt symptom was checked everyday. The treated soil was sampled every week from week 1 to week 4. The evaluation of population of the pathogen in treated soil was made by the same method as in the control. Soil pH was also recorded.

RESULTS AND DISCUSSION

The disease-free gingers which inoculated with bacterial suspension at O.D. of 0.5 by Scalpel leaf clip method for pathogenicity test showed typical wilt in 3 days. The tested gingers transplanted in the artificial infested soil (control pots) showed wilt symptom in 4 days and died within one week. It confirmed that the inoculum used in this experiment was virulent and high pathogenicity. The diseased plants showed inward curling, yellow and wilt of the lower leaves, followed by complete yellowing and browning of the entire shoot. The rhizome of the infected gingers showing water soak area in the succulent part and having milky bacterial ooze exuding from the cut rhizome. The virulent colony which was small irregularly round, fluidal, white with pink in center could isolate from the inoculated plant (Figure 1). The bacterial population in the control pots slowly decreased from 0.88×10^7 cfu/ml in the initial stage to 0.26×10^7 cfu/ml in week one, 0.13×10^6 cfu/ml in week two and three and 0.11×10^6 cfu/ml in week four . All of the tested gingers showed typical wilt symptom in 4-5 days after transplanted into the control pots and died in a few days. The bacterial population in the infested soil amending with urea and calcium oxide at the rate of 80 : 800 kg/rai decreased from 0.88×10^7 cfu/ml to 0.15×10^5 cfu/ml in week one, 0.1×10^4 cfu/ml in week two and was not founded in week three. The tested gingers transplanted into treated pots showed no symptom. The population dynamic of the pathogen during week 0 - 5 showed in Table

Figure 1 Bacterial wilt or rhizome rot of ginger, symptom and colony of *R. solanacearum.*

a. Tested ginger transplanted in the infested soil showing inward curling and complete yellowing in 4 days.

b. Typical wilt symptom in the ginger field occurred during rainy-season.

c. Rhizome of the infected ginger showing water soak area in the succulent part.

d. Milky bacterial ooze exuding from the cut rhizome.

e. Virulent colony of *R. solanacearum* on TZC medium formed an irregularly round, fluidal, white with pink in center.

f. Virulent colony of *R. solanacearum* on SM1 medium formed round, pulvinate, fluidal and tan in color.

1 and Figure 2.

Soil amending with urea and calcuim oxide at the rate of 80 : 800 kg/rai could decrease the population of *R. solanacearum* in the infested soil due to the toxicity of ammonium, ammonia and nitrate degraded from urea in high pH soil condition (average 7.0 - 7.2). The experimental result was confirmed the conclusion of Elphinstone and Aley (1993) ; Michel (1997) and Patcharin (1997) that soil amendment with urea and calcium oxide could reduce the population of *R. solanacearum* and bacterial wilt of tomato . However the rate of urea and calcuim oxide was different depending on soil type ,soil pH , soil moisture and soil microorganisms. Thus the soil amendment with urea and calcium oxide at the rate of 80 : 800 kg/rai is recommended to reduce the bacterial wilt of ginger in the severe infested area. The treated soil should be left 3 weeks before planting to avoid toxicating to ginger seedlings.

CONCLUSION

The soil amendment with urea and calcium oxide at the rate of 80 : 800 kg/rai could decrease population of *R. solanacearum* in the infested soil

Table 1 Population of *R. solanacearum* in the control and treated soil during week 0 – 4.

Week	Control (cfu/ml)	Treated soil (cfu/ml)
0	0.88×10^7	-
1	0.26×10^7	0.15×10^5
2	0.13×10^6	0.1×10^4
3	0.13×10^6	0
4	0.11×10^6	0

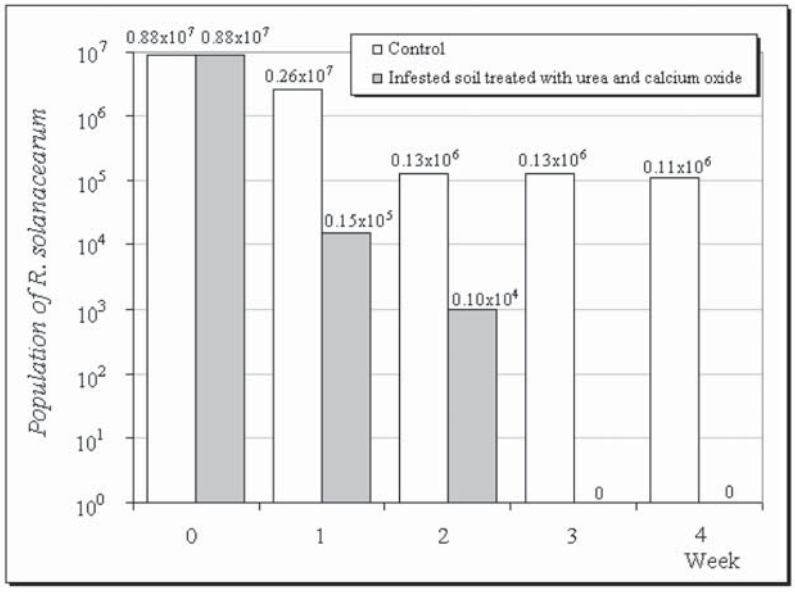

Figure 2 Population of *R. solanacearum* in the control and treated soil with urea and calcium oxide at the rate of 80:800 kg/rai.

in greenhouse condition. The decrease of bacteria was due to the toxicity of ammonium, ammonia and nitrate degraded from urea. This method can be recommended to decrease bacteria in the infested area for bacterial wilt control. The treated soil should be left at least 3 weeks before planting a new crop to avoid toxicating to plant.

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Genetic Diversity of Four Pig Breeds in Thailand Based on Microsatellite Analysis

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ABSTRACT

Fourteen microsatellite loci were analyzed in 79 random individuals to characterize the genetic variability of Thai native pig breed (TN), Large White (LW), Spotted Large White (SLW), and Pietrain (PT). Mean numbers of allele per locus, mean numbers of shared allele per locus and average gene diversity per locus were 8.86, 1.86 and 0.7875, respectively. Polymorphism information content (PIC), observed and expected heterozygosity of TN (0.7555, 0.5335 and 0.7925) were higher than the values in LW (0.5468, 0.5571 and 0.6707), SLW (0.5300, 0.5800 and 0.6492) and PT (0.4874, 0.4545 and 0.5986). Base on Nei' standard genetic distance and phylogenetic tree constructed using Nei' standard genetic distance matrix, there were a great genetic differentiation between TN and the other three breeds.

Key words: genetic diversity, pig, microsatellite

INTRODUCTION

Knowledge of genetic variation within and among different breeds is very important for understanding and developing endogenous economic genetic traits of breeds. Microsatellites or simple tandem repeatedly genetic markers have been used to define genetic structures and genetic relationships among different breeds. Microsatellites are highly polymorphic, they provide extremely useful markers for comparative studies of genetic variation, parentage assessment and studies of gene flow and hybridization. However, most of the studies using microsatellites have concentrated on cattle, sheep, and pig (Buchanan *et al*., 1994; MacHugh *et al*., 1998; Martinez *et al*., 2000; Hanslik *et al*., 2000), while not much information is available about the genetic diversity of native pigs. Some studies reported variation between Chinese indigenous Meishan and western breeds (Paszek *et al.*, 1998), and four indigenous pig breeds and three introduced breeds in China (Li *et al*., 2000), but no information is available on the population structure and genetic diversity of Thai native pigs. In this study, the genetic variability and population structures of four pig breeds in Thailand were conducted using 14 microstellite loci. Gene differentiation and the genetic relationship within and between these four pig breeds are estimated.

MATERIALS AND METHODS

Sample collection and DNA extraction

Blood samples stabilized with EDTA were collected from a total of 79 individuals belonging to four breeds: Thai native pig (n=49), Spotted Large White (n=10), Large White (n=10) and Pietrain (n=10). Genomic DNA was extracted by an organic solvent method. The white cells were washed in phosphate-buffered saline (PBS) centrifugation and

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resuspension, then lysed with SDS detergent in the present of 10 mM Tris buffer (pH 8.0), 25 mM EDTA and 100 mg/ml proteinase K. The samples were incubated at 55°C for 60 min, and overnight at 37°C. Then, the mixture was filled with 500 µl phenol:choloform:isoamyl alcohol (25:24:1), centrifuged and resuspended after that it was extracted with 500 µl choloform:isoamyl alcohol (24:1), centrifuged and resuspended two times. The aqueous phase of this extraction was combined with 1 µl 95% ethanol to precipitate the DNA, which was washed in 70% ethanol and, finally, resuspended in 10 mM Tris/25 mM EDTA buffer.

Microsatellite analysis

Fourteen microstellite markers (ATP2, IGF1, PgHAS, S0010, S0086, S0107, S0151, S0227, S0352, SS13N17R, SS13R44R, SW957, SW1066 and TNFB) were used for the analysis of the pig breeds (Table 1). Samples were adjusted to a concentration of 5 ng/µl before PCR amplification. The PCR amplification was performed on individual animal DNA. The PCR reaction was accomplished in a total volume of 5 µl using 5 ng of genomic DNA, 1 mM dNTP, 5 µM of each primer, 1 unit of *Tag* DNA polymerase and 10X PCR buffer. The 10X PCR buffer consisted of 100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl and pH 8.3. The PCR reaction cycle was accomplished by an initial denaturation at 94°C for 3 min, second denaturation at 94°C for 30 sec, primer annealing for 30 sec at the desired temperature, extension at 72°C for 1 min and final extension at 72°C for 5 min. The PCR were analyzed on 4.5% polyacrylamide denaturing sequencing gel, which was then dyed in $AgNO_3$ solution.

Data analysis

Allele frequency, the mean number of alleles per locus, average gene diversity (Nei, 1973), polymorphism information content (Botstein *et al.*, 1980), observed and expected heterozygosity and Nei' standard genetic distance (Nei, 1978) were computed. UPGMA dendrogram was constructed using PHYLIP software package (Felsenstein, 1993).

Table 1 Microsatellite marker names, primer oligonucleotide sequences and PCR conditions.

Marker name	Forward primer	Reverse primer	PCR temp.
ATP2	GCTGCATAGGGAGCTGTAGG	TAATGATGGTGGTGGTTAGTGC	65
IGF1	GCTTGGATGGACCATGTTG	CACTTGAGGGGCAAATGATT	60
PgHAS	GTCACAGTGGATGGCATTTG	ACATCCCTAAGGTCGTGGC	60
S0010	TTAACATGGCTGTCTGGACC	GTCCCTGTCCAACCATAAGA	60
S0086	GCACAGTCTATTGATACTGGCGTC	CTGAGAACTTCCATATGCTCCTGG	65
S0107	CAAGGATGCCTGTAACTGGTGCAG	TCCTTAAGGCCTCGTAGGATCTG	65
S0151	CACCACACAACCACAGACTCCA	ACTTGAAGCCATAGCCACACCT	65
S0227	GATCCATTTATAATTTTAGCACAAAGT	GCATGGTGTGATGCTATGTCAAGC	60
S0352	CGTTAAGCCACTTTCTGTGG	AAGGGGGAGGGCGAGGGA	60
SS13N17R	ATGTGCCGCAGTGCGTGCTAG	GCCAGTGTCCCCACAAGGG	60
SS13R44R	CCTCCTGGTGACCTGACAGG	GTGTGATATGGAAAAGGACC	60
SW957	AGGAAGTGAGCTCAGAAAGTGC	ATGGACAAGCTTGGTTTTCC	65
SW1066	GCAGGATGAACCACCCTG	CTCTTGAGGCAACCTGCTG	65
TNFB	CTGGTCAGCCACCAAGATTT	GGAAATGAGAAGTGTGGAGACC	65

RESULTS AND DISCUSSION

The number of alleles observed at each locus in Thai native pig (TN), Large White (LW), Spotted Large White (SLW) and Pietrain (PT) and the numbers shared among the four breeds, were given in Table 2. A total of 124 alleles were detected across the 14 loci analyzed. The number of alleles per locus varied form three (SS13N17R) to 15 (PgHAS) with a mean value of 8.86 (the mean of TN, LW, SLW and PT were 8.64, 4.00, 3.29 and 3.36). The mean number of alleles per locus was similar in all breeds. But there were marked differences for some loci, e.g. S0086 with 10 alleles in TN and only four, one, and one in LW, SLW and PT, respectively; PgHAS with 15 alleles in TN and five, two and two in LW, SLW and PT, respectively. The mean number of shared alleles was 1.86. Average gene diversity over all loci was 0.7875 (Table 2) while, for individual loci, average gene diversities ranged from 0.5635 (SS13N17R) to 0.9068 (S0107). Across loci, average gene diversity increased with increasing number of alleles (regression coefficient = 0.0282 ± 0.004, $P < 0.001$).

Eleven loci were polymorphic in all breeds, while three were monomorphic in one to two breeds (namely S0227 in SLW, S0086 and SS13R44R in SLW and PT). Measures of genetic variation for each population (polymorphism information content, PIC; observed, H_O; and expected, H_E heterozygosity) were given in Table 3. The highest PIC was found in TN (0.7555), while the lowest PIC was found in PT (0.4874). Observed heterozygosity of TN, LW, SLW and PT (0.5335, 0.5571, 0.5800 and 0.4545) was less than expected heterozygosity (0.7925, 0.6707, 0.6492 and 0.5986) in all breeds. The lowest heterozygosity was showed in PT, while the highest heterozygosity was showed

Table 2 Number of microstellite alleles at each locus in Thai native pig (TN), Large White (LW), Spotted Large White (SLW) and Pietrain (PT), number shared among the four breeds and average gene diversity within populations.

Locus	Numbers of alleles						Average gene diversity
	Total	TN	LW	SLW	PT	Shared	
ATP2	5	5	2	2	2	2	0.7010
IGF1	10	10	3	3	4	2	0.8592
PgHAS	15	15	5	2	2	1	0.8815
S0010	12	12	5	2	3	2	0.8399
S0086	10	10	4	1	1	1	0.7978
S0107	12	12	7	7	6	4	0.9068
S0151	9	7	4	6	5	2	0.8514
S0227	5	5	2	1	2	1	0.7006
S0352	7	7	5	3	3	2	0.7406
SS13N17R	3	3	2	2	2	2	0.5635
SS13R44R	6	6	2	1	1	1	0.6484
SW957	8	8	3	3	2	1	0.7661
SW1066	11	11	6	5	8	1	0.8800
TNFB	11	10	6	5	6	4	0.8876
Mean/locus	8.86	8.64	4.00	3.29	3.36	1.86	0.7875
(SE)	0.30	0.30	0.23	0.29	0.31	0.20	

Table 3 Sample size, polymorphism information content (PIC) and heterozygosity (standard errors in parenthesis) averaged over 14 microstellite loci.

Breed	Sample size	PIC	Mean heterozygosity	
			Observed	Expected
TN	49	0.7555 (0.02)	0.5335 (0.03)	0.7925 (0.01)
LW	10	0.5468 (0.06)	0.5571 (0.10)	0.6707 (0.07)
SLW	10	0.5300 (0.06)	0.5800 (0.09)	0.6492 (0.07)
PT	10	0.4874 (0.07)	0.4545 (0.09)	0.5986 (0.08)
Average		0.5799	0.5313	0.6778

in TN. The genetic diversity of LW(H_O= 0.5571) in this study was higher than that of Yorkshire in China (H_O=0.526), while genetic variations of TN (H_O= 0.5335) was lower than that of indigenous pig breeds in China (H_O= 0.561-0.719) reported by Li *et al.* (2000). The difference of genetic diversity value obtained could be due to the choice of microsatellite loci as well as the choice of population. All of the results indicated that diversity of TN was higher than that of exotic breeds. It may be long-time selection that resulted in the low variation in the exotic breeds.

Nei' standard genetic distance based on allele frequencies between TN and LW; TN and SLW; TN and PT; LW and SLW; LW and PT; and SLW and PT; were 0.6141, 0.5299, 0.5697, 0.0849, 0.2426 and 0.1297, respectively (Table 4). The value showed a high degree of genetic divergence between TN and the other three pig breeds and the closest was found to be LW and SLW.

Nei' standard genetic distance matrix was used in order to build phylogenetic tree with the UPGMA method (Figure 1). In this tree, the TN population were clearly differentiated from the remaining breeds.

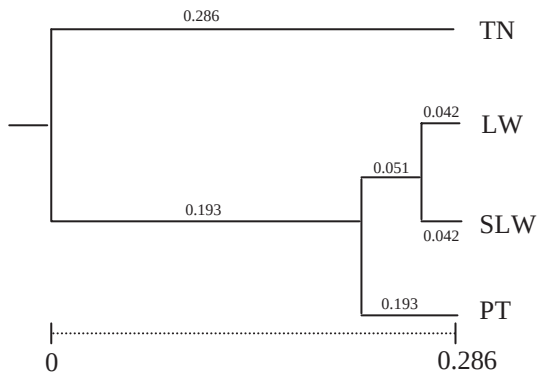

Figure 1 Dendrogram showing the genetic relationship among four pig breeds in Thailand. Phylogenetic tree was constructed from Nei' standard genetic distance by the UPGMA method, which was based on 14 microsatellite loci. Numbers were the branch lengths.

Table 4 Matrix of Nei' standard genetic distance among Thai native pig (TN), Large White (LW), Spotted Large White (SLW), and Pietrain (PT).

Breed	TN	LW	SLW	PT
Thai native pig (TN)	-			
Large White (LW)	0.6141	-		
Spotted Large White (SLW)	0.5299	0.0849	-	
Pietrain (PT)	0.5697	0.2426	0.1297	-

CONCLUSION

Fourteen microsatellite loci were analyzed in 79 random individuals to characterize the genetic variability of TN, LW, SLW and PT. Mean numbers of allele per locus, mean numbers of shared allele per locus and average gene diversity per locus were 8.86, 1.86 and 0.7875. The highest genetic diversity was found in TN (PIC, H_O and H_E of TN) , while the lowest value was found in PT (PIC, H_O and H_E of PT). However, the genetic variations of TN were lower than that of native pigs in China. Base on Nei' standard genetic distance, there was a great genetic differentiation between TN and the other three breeds. Similarly, phylogenetic tree was constructed using Nei' standard genetic distance matrix showed that TN was clearly distinct from the other three breeds.

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Preliminary Studies on Recycling Spent Brine in Green Mango Fermentation

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ABSTRACT

A preliminary study for alleviating pollution from mango processing brine was conducted by recycling spent brines. Spent brines were reclaimed by a sand filtration and an acitvated carbon system. The comparative physico-chemical characteristics of influent and effluent spent brines were investigated. It was found that the values of pH and acidity of effluent brine changed significantly while there was no change in NaCl content. The effluent brine became a visual clear corresponding to a settleable solid, and there were no substantial changes in the total solid without an objectionable odor. Mango brining experiment from defrozen, reclaimed brine and fresh brine were conducted. Pickled mango from reclaimed brine was as good as the pickled mango from fresh brine. The quality of reclaimed brine should be studied during extensive storage at room temperature. A comparison of 4 samples of sweet and sour pickled mango were done and evaluated by a preference test. The overall conclusion from the tasting panel on all samples of sweet and sour pickled mango was that there was not a significant difference in color, odor, flavor, texture or acceptability. Furthermore, the capacity of the reclamation system was also estimated. Results showed that a substantial changes in the chemical composition were significantly different for 15 liters of effluent brine, but for 12 liters of effluent brine, there was only some changes in total solid and dissolved solid. Therefore, further experiment should be performed to large - scale operations for potential recycling brine evaluation.

Key words: pickled mango, reclaimed brine, brine disposal, activated carbon

INTRODUCTION

Mango (*Mangifera Indica* Linn) played an important role on the country's economic development. It was found that the most popular types of preserved mangoes for consumers are sweet and sour pickle mangoes, by intermediate raw mango obtained from green mango fermentation in salted stock brine. The pickle solution is the spent brine from these operations. It was noticed that the spent brine constituted a considerable volume of strong waste with an objectionable odor. The brine disposal problem was compounded by the close proximity of many fruit and vegetable processors to residential areas and by the lack of adequate municipal sewage treatment facilities. Spent brine is a strong pollutant and creates serious disposal problems, because of the large quantities of organic matter and salt contained therein. The organic matter can be removed by biological treatment, but NaCl and other mineral contents are not biodegradable and could pollute the water or soil during land application. Recycling of food processing brine, therefore, could reduce the amount of salt and

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minerals to be discharged as well as conserving water. Several brine recycling systems have been studied. Geisman and Henne (1973), Palnitkar and McFeeters (1975) and McFeeters *et al*. (1977) reported on the recycling of brine in cucumber fermentations. Mercer *et al*. (1970) described a brine recycling and reconditioning system using activated carbon for olive processing. Soderquist (1971) described an activated carbon renovation of spent cherry brine. A filter and activated charcoal system for recycling spent bisulfite brines used in sweet cherry processing was conducted by Panasiuk *et al.*(1977). Whereas Romero *et al*.(2001) described the treatment and recycling of spent brines and osmotic solutions from pickling of olives, cabbage, cucumbers, prunes and cherries. They found that the reclaimed brine may contain polygalacturonase which softens cherries, but thermal inactivation of polygalacturonase could prevent softening. Athanasopoulos (1976) studied the kinetics of thermal inactivation of polygalacturonase in spent brine. However, the recycling of pickling mango's spent brine could not be found in any literature.

This preliminary experiment was confined to the treatment and reuse of brine generated during the production of green mango fermentation. The information developed in this study should be useful for solving potential water pollution problems of salted stock brine factories. It might be the way to alleviate the critical pollution potential of saline liquid wastes discharged from the fermentation process. However, the experiment should be applied to a large scale operation to further evaluate its reuse potential.

MATERIALS AND METHODS

Mango

Fresh green mangoes (Kaew Dhum cultivar) were purchased from the wholesale market. They were washed, and selected for sound and mature fruit prior to pickling.

Spent brine

The spent brine used in the experiment was obtained from a fruit pickling processor in Angthong province from its stock of 12 month old in salted mango brine. It was sampled in 4 brine plastic pails. Each of these pails contained the salted stock brine from the 4 fermentation pails after they were filtered with muslin cloth to remove suspended solids. They were then kept at 10°C in a chill room prior to conducting the experiment.

Brine reclamation system

A laboratory scale brine reclamation system (Panasiuk *et al*., 1977) was assembled for the quantitative measurement of the effluent (schematic diagram on Figure 1). The system consisted of a glass column (36×400 mm), containing 0.45 — 0.60 mm water filtration grade sand, in series with three glass columns, each 33×600 mm, containing activated carbon (CGC 12×40 C, C. Gigaetic Carbon Co. LTD). The total weight of the activated carbon was 456 g. The columns were plugged with glass wool at both ends and were connected by glass tubing attached to rubber stopper joints. Brine was pumped through the system (from the bottom of each column to the top of the succeeding column) with an oil rotary vacuum pump (RP—S50H) at a flow rate of approximately 20 ml / min, controlled by a stopcock at the bottom of the last column.

Each batch of sampled spent brine (influent) was passed through the sand/activated carbon system. The cumulative volume of 15 liters of effluent brine was collected and the samples were taken to be analyzed for the physico-chemical quality, after the completion of each day's operation, The influent brine was also analyzed in the same manner as the effluent brine. All effluent brines were stored at –18°C in a freezing room in order to maintain their stability until green mango could be purchased for pickling.

The green mango brining process

Green mangoes were immersed in reclaimed

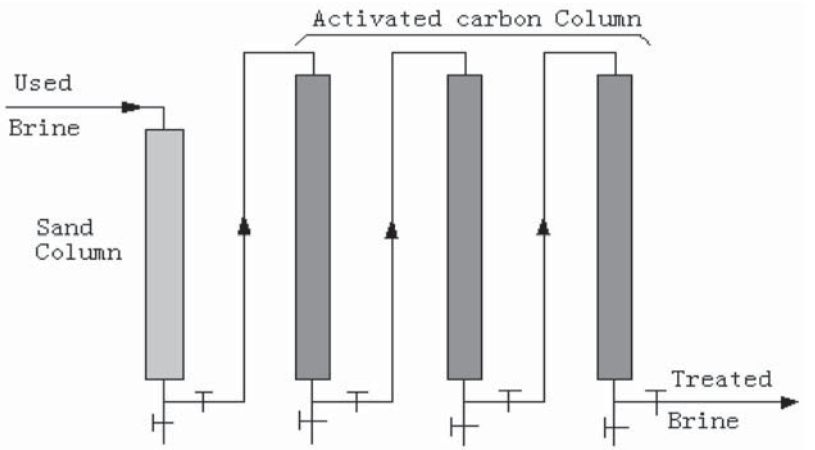

Figure 1 Spent brine treatment system.

brine as a control formulated brine using approximately 1 kg of mango per one liter of brine. The 3 kilograms of fruit was held under the brine with a perforated stainless steel plate fitted to the pail. Three pails of brine were prepared as a control sample that consisted of 10% salt, 0.5% $CaCl_2$ and 0.1% $K_2S_2O_5$. Each pail of reclaimed brine was formulated with the same composition as the control sample by adding NaCl, $CaCl_2$ and $K_2S_2O_5$. During the fermentation period , brine samples were periodically withdrawn for acidity, pH, NaCl determination and microbial analysis. After 40 days, the salted pickled mango stock was considered fully cured and ready for further processing. The remaining brine was taken from each pail for chemical analysis, while the salted pickled mango stock was prepared for sweet and sour pickled mango.

Test methods

Total acidity in the brine was determined by titration with NaOH and expressed as acetic acid. The pH measurement was determined by Orion Model 410A pH meter. Salt content, settleable solids, filterable solids and dissolved solids were determined using a standard method for the examination of water and wastewater(Andrew *et al*., 1995). Calcium was determined using a standard APHA (1998); 3111. Sulfur dioxide was determined using the Optimized Monier-William Method (AOAC, 1995).

Pickled mango process

The following process was used to prepare sweet and sour pickled mango. (1) Wash, peel, pit and cut each fruit into 6 pieces; (2) Refreshen the salted pickled mango stock by soaking it in water for half an hour, in the final washing, 0.5% $CaCl_2$ solution is added to firm the tissue for 1 hour before it is made into sweet and sour pickled mangos; (3) Prepare the syrup by combining 42% sugar, 12% vinegar, 2% salt and 0.15% citric acid; (4) Soak the refreshed pickled mangoes in the syrup for 24 hours at room temperature; (5) Draw off the syrup and reheat; (6) Quickly dip the mango pieces into boiled syrup; (7) Fill in PE bag (flesh: syrup = 1:1) seal and keep in the refrigerator.

Sensory evaluation

Sweet and sour pickled mango products from the reclaimed brine and fresh brine were carried out for organoleptic testing by using the nine point hedonic scale for the subjective evaluation of the product. The panelists were researchers of IFRPD and were asked to evaluate the differences on a nine point scale (1 = extremely dislike, 9 =

extremely like) of flavor, taste, color, texture and acceptability. The experimental design was a Randomized Complete Block Design (RBD) and the data was statistically analyzed using the Analysis of Variance (ANOVA) and Duncan's Multiple Range test with a 95% confidence level.

Investigation of reclamation system capacity

The capacity of the three activated carbon column system was investigated by treating a fixed volume influent. Fifteen liters of effluent was collected and the changes in composition and performance were measured. The experiment was conducted in 5 cycles from the same salted sample stock.

RESULTS AND DISCUSSION

Brine reclamation system

Three influent brine and three effluent brine samples were each analyzed for physico-chemical composition as shown in Table1. The storage spent brine used in the experiment was not significantly different in pH, acidity and NaCl content. A comparison between each batch of the influent brine and effluent brine showed that they contained significant differences in the composition mentioned above with no change in NaCl content. It indicated that NaCl could not be removed by the system. The effluent brine contained less sulfurdioxide and calcium than in the influent brine. The treated brine

Table 1 Physico-chemical characteristic of spent brine from salted mango stock.

Particular	Influent brine			Effluent brine		
	1	2	3	1'	2'	3'
pH ± SD	2.66^{a}±0.06	2.64^{a}±0.01	2.65^{a}±0.03	3.11^{c}±0.01	2.95^{b}±0.01	2.97^{b}±0.01
Ac. ± SD (%)	0.91^{c}±0.01	0.90^{c}±0.03	0.92^{c}±0.01	0.48^{a}±0.01	0.54^{b}±0.01	0.51^{b}±0.00
NaCl±SD (%)	4.76^{d}±0.02	4.68^{ab}±0.04	4.73^{bc}±0.04	4.74^{bc}±0.03	4.61^{a}±0.05	4.70^{bc}±0.05
SO_2 ± SD (ppm)	123.39±1.4	112.2±1.15	125.57±1.5	99.35±3.69	85.51±0.04	91.05±1.24
Ca (mg/l)	997	985	1003	955	970	978
SS ± SD (ml/l)	1.7^{d}±0	1.45^{b}±0.05	1.5^{c}±0	0^{a}±0	0^{a}±0	0^{a}±0
FS ± SD (g/l)	0.66^{b}±0.04	0.83^{b}±0.13	0.94^{c}±0.06	0.08^{a}±0.02	0.22^{a}±0.14	0.15^{a}±0.19
DS ± SD (g/l)	63.95^{a}±0.59	70.23^{c}±0.76	75.81^{d}±0.99	66.07^{ab}±2.84	69.34^{bc}±4.02	70.62^{c}±2.75
TS ± SD (g/l)	75.41^{c}±0.40	79.93^{d}±0.36	80.66^{d}±3.15	68.84^{a}±1.82	70.20^{ab}±0.59	72.22^{b}±0.88

Note: Ac. = acidity as acetic acid; SS = settleable solid; FS = filterable solid; DS = dissolved solid; TS = total solid; SD = standard deviation

The figure on the same row with the same letter are not significantly different ($p>0.05$)

was clear because the total solids were reduced significantly, while the settleable solids became free.

Microorganism examination of the influent brine and the effluent brine revealed the presence of a significant number of microorganisms (Table2). Nevertheless, the effluent brine provided no objectionable odor. All effluent brine samples were kept at –18°C in a freezer in order to retain stability prior to further processing. While Panasiuk *et al*. (1977) reported that the untreated and reclaimed brine were similar in stability during 24 weeks of storage at room temperature.

Green mango brining experiment

Formulated reclaimed brine having the same formular as the fresh brine produced in laboratory was used in the fermentation process. Figure 2 and Figure3 indicated that the initial pH in reclaimed brine was lower but higher in titratable acidity compared to the control brine due to the acid present in the spent brine. Acidity in brine could prevent fruit deterioration from putrefactive and pectolytic organisms at the beginning of fermentation (Desorosier, 1963). The pattern of pH decline and titratable acidity increase occurred during fermentation. Each sample of reclaimed brine and fresh brine showed substantial changes in NaCl (Figure 4).

Table 2 Gross floral in spent brine from green mango fermentation.

Gross floral	Influent brine			Effluent brine		
	1	2	3	1’	2’	3’
TVC (log cfu /ml)	6.04	5.85	6.28	5.48	5.48	6.15
Yeast (log cfu /ml)	6.08	6.18	6.15	5.20	5.08	5.34
Mold (log cfu/ml)	0	0	0	0	0	0
Coliform (MPN/ml)	< 3	< 3	< 3	< 3	< 3	< 3

Note : TVC = Total viable count

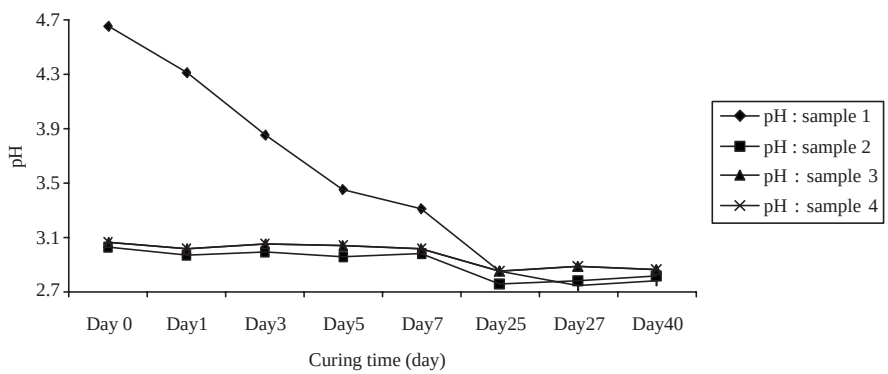

Figure 2 Changes in pH in brine during green mango fermentation.

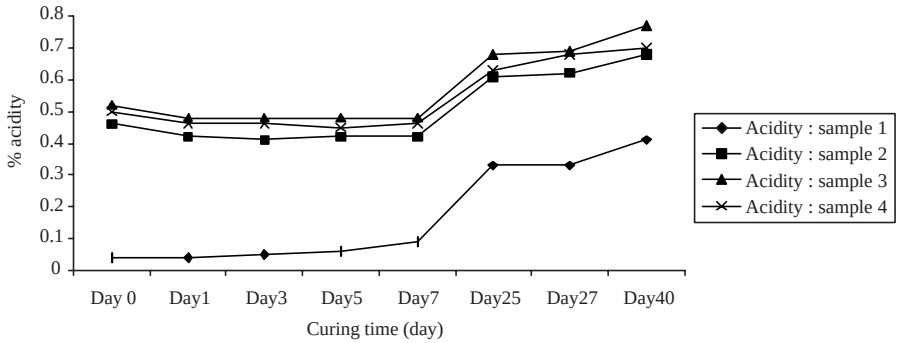

Figure 3 Changes in acidity in brine during green mango fermentation.

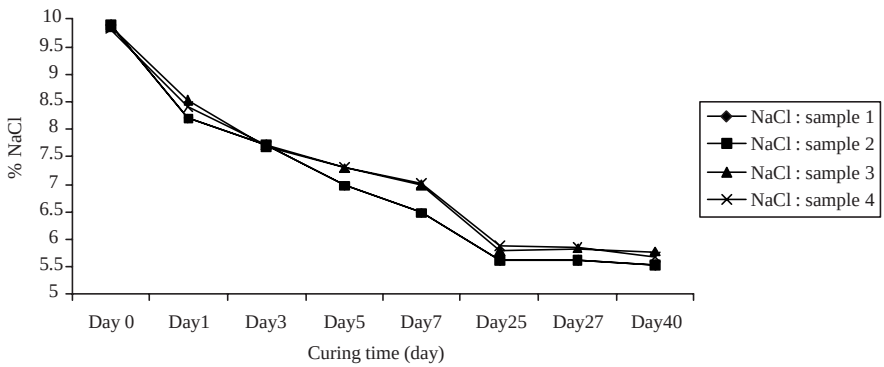

Figure 4 Changes in NaCl content in brine during green mango fermentation.

Pickled mango process and sensory evaluation

All salted mangoes stock appeared to be undergoing a normal fermentation and storage behavior. Salted pickled mango stock is not considered a consumer commodity due to containing high NaCl content (Table 3). Refreshening pickled mango was accomplished by leaching salt from salted mango stock. Sulferdioxide concentration was also minimized during the process with only 24 ppm SO_2 remaining in the final products. Whereas the optimum content of NaCl and total acidity as acetic acid in the products was 1.7% and 0.55%, respectively. It corresponded to the acceptability factor of the panelists (Table 4).

The subjective evaluation data (Table 4) showed no discernible significant differences in color, odor, flavor, texture or acceptability among sweet and sour pickled mangoes from the reclaimed brine and the control. This result showed that the quality of the pickled mango from the individual storage pails was not different.

Investigation of reclamation system capacity

A comparison was made on the investigation of effluent brine for 5 cycles of influent brine from the same sample of stored brine. Substantial changes in the pH value, total acidity and NaCl were significantly different ($p<0.05$). Decreases in the pH of effluent brine and increases in the total acidity were found as influent volume increased in contact time with the activated carbon system. The retention of total acidity in the effluent brine from carbon treatment is desirable since lactic acid is known to be a preservative in the brine of stored olives (Water Pollution Control Research Series, 12060 EHU 03 / 71). However, the activated carbon

became saturated after 4 cycles of influent were treated, because, showed no substantial changes in total solid of effluent brine (Table 5). After 5 cycles treatment, settleable solids (SS) were still not found in the effluent brine due to the sparadic discharge of fine carbon and retained settleable solids.

CONCLUSION

The reclaimed brine from the spent mango processing brine had an average salt content of 4.68%, pH 3.01 and a total acidity as acetic acid 0.51%. It could reduce the waste disposal

Table 3 Composition of salted mango stock and pickled mango products.

Sample number	Salted pickled mango stock		Sweet and sour pickled mango		
	Acid ±SD (%)	NaCl±SD (%)	Acid (%)±SD	NaCl ±SD (%)	SO_2 (ppm)
1	0.68 ± 0.04	4.64 ± 0.005	0.51 ± 0.002	1.73 ± 0.02	24.35
2	0.85 ± 0.09	4.87 ± 0.04	0.55 ± 0.007	1.67 ± 0	24.78
3	0.92 ± 0.005	4.77 ± 0.085	0.55 ± 0.004	1.57 ± 0	24.49
4	0.84 ± 0.045	4.75 ± 0.17	0.53 ± 0.003	1.73 ± 0	24.44

Note: Acid = acidity as acetic acid; solid; SD = standard deviatio

Table 4 Organoleptic evaluation of sweet and sour pickled mango products.

Sample No.	Color	Flavor	Odor	Texture	Acceptability
1	6.89^{a} ± 0.74	7.26^{a} ± 0.81	6.87^{a} ± 0.97	7.30^{a} ± 0.72	6.89^{a} ± 1.23
2	6.89^{a} ± 0.60	7.41^{a} ± 0.77	7.22^{a} ± 0.95	7.24^{a} ± 0.80	7.28^{a} ± 0.91
3	6.98^{a} ± 0.91	7.30^{a} ± 1.02	7.00^{a} ± 1.13	7.11^{a} ± 0.90	6.83^{a} ± 0.98
4	7.33^{a} ± 0.58	7.41^{a} ± 0.65	7.43^{a} ± 0.79	7.48^{a} ± 0.59	7.37^{a} ± 0.77

The figure on the same column with the same letter are not significantly different ($p>0.05$)
1 = Control; 2 — 4 = Treated Sample; SD = Standard Deviation from 23 panelist.

Table 5 Quality of recycling spent brine from capacity evaluation of activated carbon system.

Sample No.	pH ± SD	Ac.(%) ± SD	NaCl(%) ± SD	TS (g/l) ± SD	DS (g/l) ± SD	SS (ml/l) ± SD
Pre-treatment	2.65^{f} ± 0.01	0.96^{f} ± 0.02	5.01^{e} ± 0.00	78.10^{b} ± 0.95	74.10^{d} ± 0.25	1.60 ± .00
1	3.94^{e} ± 0.01	0.15^{a} ± 0.01	4.55^{a} ± 0.01	68.79^{a} ± 0.05	62.71^{a} ± 1.99	0
2	2.90^{d} ± 0.01	0.53^{b} ± 0.00	4.69^{b} ± 0.00	69.13^{a} ± 0.02	68.96^{b} ± 0.14	0
3	2.87^{c} ± 0.01	0.59^{c} ± 0.01	4.78^{c} ± 0.01	70.79^{a} ± 1.67	69.62^{bc} ± 0.52	0
4	2.77^{b} ± 0.01	0.71^{d} ± 0.00	4.80^{c} ± 0.01	75.13^{b} ± 4.24	72.07^{cd} ± 2.56	0
5	2.75^{a} ± 0.01	0.76^{e} ± 0.01	4.92^{d} ± 0.02	77.39^{b} ± 0.32	75.45^{e} ± 1.80	0

The figure on the same column with the same letter are not significantly different ($p > 0.05$).
Ac. = acidity as acetic acid; SS = settleable solid; DS = dissolved solid; TS = total solid;
SD = standard deviation

requirement without adversely affecting product quality. The pickled mango in the reclaimed brine was as good as the pickled mango from the fresh brine. The reclaimed brine should be studied for further processing after long term storage at normal temperature. For the acitivated carbon system capacity in laboratory scale was not still clearly defined in the conclusion. The experiment should be applied to large scale operations and evaluated for reuse potential. Cost analysis is required to establish the economic feasibility of brine reclamation. These analyses must consider the logistics of brining, finishing, disposal cost, storage losses and the unit operations cost for the brine treatment .

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Biotreatment of High Fat and Oil Wastewater by Lipase Producing Microorganisms

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ABTRACT

Studies on biotreatments of high fat and oil wastewater by selected lipase producing bacteria were carried out in two experiments with two groups of bacteria, single culture and mixed culture.

In the first experiment, wastewater of bakery industry was treated with 4 bacteria isolates. Results showed that grease in the form of fat and oil and COD decreased remarkably with treatments. With single culture, the removal of fat and oil and COD were 73%-88% and 81%-99% during 7 days treatment. Isolates KUL8 and KUL39 showed better activities. However, better treatments were found in experiment treated with single culture than using mixed culture.

In the second experiment, wastewater of palm oil and bakery industries were treated with 6 isolates within 48 hours. All 6 isolates showed better degradation in palm oil wastes than bakery wastes. However, KUL8 and KUL39 still showed better degradation activities. Both isolates can removed fat and oil by 87.7% and 80.6% in palm oil wastewater and 70% and 64% in bakery wastes respectively. The decreasing of COD was found to be 90%-96%. When mixed culture of KUL8, KUL39 and KLB1 were applied for both kinds of wastewater. It was found that better results were observed in palm oil treated with single culture KUL8, KUL39, and KUB1 while mixed culture KUL8 mixed with KLB1 was suitable for use in bakery waste. The three isolates were identified as *Acinetobacter* sp. (KUL8), *Bacillus* sp. (KUL39), and *Pseudomonas* sp. (KLB1). They were all mesophilic strains. Besides, it was found that all three strains also produced amylase and protease which stimulate better waste treatments.

Key words: lipase producing bacteria, fat and oil wastewater, wastewater treatment

INTRODUCTION

Fat and oil (i.e. “grease”, defined as plant oil/animal fats) are among the stable organic compounds in wastewater. Fat and oil have been discharged from food industries, restaurants, slaughterhouse, household (Becker *et al.*, 1999; Stoll and Gupta, 1997). High concentration of these compounds in wastewater often causes major problem in biological wastewater treatment processes. Because of their nature are pounds will form layer on water surface and decrease oxygen transfer rate into the aerobic process (Becker et *al.*, 1999). Therefore, these is a need of which operate them separately. Currently, preparation of fat and oil is usually done by physical and chemical pretreatment processes such as using a dissolved air floatation and skimming (Chigusa *et al.*, 1996). However, floating fat and oil waste must be operated by safety process in order to safe cost of the operation process.

The application of lipase producing

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microorganisms into wastewater treatment system to degrade fat and oil and eliminate pretreatment process is an interesting strategy. The isolation of lipase producing microorganisms capable of degrading fat and oil in wastewater are interested in order to utilize fat and oil and eliminate the pretreatment processes The lipase producing microorganisms from variety sources have been isolated and studied on the degradable efficiency as both single culture and mixed culture formula. Chigusa *et al*. (1996) obtained 9 yeast isolates from food and oil wastewater. These were mixed, then applied into soybean oil producing wastewater. The results showed that fat amount was decreased by 94%.

In the same case, Wakelin and Forster (1997) found a single culture and mixed culture which were isolated from grease trap degraded over 90% of fat content in activated sludge system.

Microbial lipases have been produced by yeasts, fungi, and bacteria as extracellular, intracellular, and cell-bound enzyme. The extracellular lipases from yeast and bacteria were interesting because of easier application. Yeasts produced extracellular lipase are *Candida deformans* (Muderwa and Ratamahenina, 1985), *C. rugosa* (Rao *et al*., 1993) while bacteria are *Pseudomonas aeruginosa* EF2 (Gilbert *et al*., 1991), *P. fragi* CRDA 323 (Pabai *et al*., 1995) and *Alcaligenes* sp. Strain No. 679 (Kokusho *et al*., 1982)

In fact, the lipase producing microorganisms is found in fat and oil contaminate sources. Thus, they were isolated from bakery and palm oil industry wastewater. The present study was aimed to isolate lipase producing microorganisms inducing their degradation capacities of various levels of fat and high fat and oil waste water in laboratory level.

MATERIALS AND METHODS

Isolation of lipase producing microorganisms

Microorganisms were isolated from soil and wastewater samples which rich in fat and oil. Enrichment culture were carried out on medium containing olive oil as a sole carbon source. When grown cultures could be observed, transfer 0.1 ml of them to double agar (Suwansuthichai, 1989). The single colony capable of lipase production was screened by the appearance of clear zone around its colony according to tributyrin hydrolysis (Chappe *et al*., 1994). Then, selection of strains with widely clear gone were selected and confirmed for their lipase production and assay lipase activity (Horani, 1994).

Some selected isolates with better lipase activities were identified by The Department of Medical Science, Ministry of Public Health.

Media and growth conditions

Enrichment media contained : 5.0 g/l $(NH_4)_2SO_4$, 0.5 g/l K_2HPO_4, 0.3 g/l $MgSO_4.7H_2O$ and 20 ml/l olive oil. Double layer agar media is the same as the enrichment media but supplied with 2% (w/v) agar. The bottom layer has no olive oil while on top layer was supplement with olive oil. Tributyrin agar contained : 3 g/l yeast extract, 5 g/l peptone, 12 g/l agar and 10 ml tributyrin. The lipase production medium was contained with 1.3 g/l $(NH_4)_2SO_4$, 0.9 g/l K_2HPO_4, 0.6 g/l KH_2PO_4, 0.2 g/l $MgSO_4.7H_2O$, 0.1 g/l yeast extract, 0.5 g/l $CaCO_3$ and 10 ml/l olive oil. All cultures were incubated at 30°C. Liquid medium was carried out on rotary shaker with 250 rpm. The supernatant of selected isolates was assayed as lipase activity by following method described by Horani (1994). One lipase unit (LU) is the amount of enzyme which will liberate one μm of fatty acid in one hour under the condition assay (30°C)

Study on fat and oil degradation with isolated microorganisms

Two experiments of isolated microorganisms for fat and oil degradation ability studying were investigated. The first, isolated bacteria strains including KUL8, KUL12, KUL17 and KUL39 were examined. The second was carried out with

two bacteria strains (KLB1,and KLB2) and two yeast strains (KLY1,and KLY2). The high fat and oil degrading activity bacteria (KUL8 and KUL39) included, also. All studies were carried out with single culture and mixed culture.

The first experiment, single culture and mixed culture of KUL8, KUL12, KUL17 and KUL39 were grown in wastewater from bakery industry. pH of wastewater was adjusted to 7.0, then 5% inoculum of each isolated or total mixed culture was added and shaken at 250 rpm, 30°C. Samplings were carried out every 24 hours for 7 days . COD and fat and oil contents were analyzed with standard method (APHA, 1989) and presented as percent degradation.

The second experiment, four bacterial strains, KUL8, KUL39, KLB1, KLB2 and two yeast strains, KLY1, KLY2 were used. The 5% of inoculum was added to each media single culture and mixed culture which grew in wastewater from bakery and palm oil industry. An experiment was carried out as described in the first experiment.

Wastewater characterization

Wastewater generated from bakery industry was composed of 12,000 -14,000 mg/l, BOD ; 15,000 – 25,000 mg/l, COD ; 10 – 15 g/l, fat and oil and pH range 5.6 – 6.1. Wastewater generated from palm oil industry was composed of 12,000 – 14,000 mg/l, BOD ; 15,000 – 25,000 mg/l, COD ; 10 – 15 g/l, fat and oil and pH range 5.6 – 6.1. (Bhumibhamon and Funtong, 2001)

RESULTS AND DISCUSSION

In this study, 200 isolates microorganisms were isolated in media 25 isolates with large clear zone were selected contained olive oil by double layer technique. Then cultures turn on tributyrin agar and further part inoculation on tributyrin agar hydrolysis media. The result showed 8 isolates with sharp clear zone KUL8, KUL12, KUL17, KUL39, KLB1, KLB2< KLY1, KLY2. All 8 selected isolates were grown in liquid media for 7 days. The supernatant were contained isolate lipase activity (3 – 7 units) with pH range 5 – 8 and optimum temperature at 30 – 40°C. For basic test, it was found that KUL8, KUL12, KUL17, KUL39, KLB1 and KLB2 were gram negative bacteria and KLY1, KLY2 were yeast strains.

Degradation of fat and oil in the first experiment

The bakery wastewater were treated with individual isolates KUL8,KUL12, KUL17, and KUL39. Results were shown on Figures 1 – 4. When treating with single strain, it was found that the degradation of organic (in formed COD), fat and oil were higher than control (no added strains). The treatment apply with isolated strains could be reduced fat and oil content by 73% to 88% (Figure 1), and COD conteny by 81% to 99% (Figure 2) within 7 days. The KUL8 and KUL39 strains were shown higher efficiency activities than KUL12 and KUL17. The degradation of fat and oil and COD of KUL8 were 88.8% and 98% and of KUL39 were 81.6%, and 99%, respectively. Since, KUL8 and KUL39 have more ability to producing amylase and protease enzymes than the others and could be promoted better COD degradation, the mixed culture strains was performed and compared with the result of single strains. When mix-isolated strains in different ratio was treated, results were not better than that treated with single strain. That might be dued to on interactions between differently strains. The fat and oil and COD degradation when treated with mixed culture were 32% - 74% (Figure 3) and 56% - 95% (Figure 4), respectively.

Degradation of fat and oil in second experiment

The experiment of high fat and oil wastewater from bakery and palm oil manufacturing were treated with single culture and mixed cultures. The results were shown on Figures 5-6.

Single culture of KUL8 showed the highest fat and oil degradation in both bakery and palm oil wastewater were 87.72%, and 70.27%, respectively

(Figures 5A-5B). Degradation of fat and oil were gradually decreased to 64.34%, 57.34%, 52.01%, 45.64%, 44.56% in bakery industry wastewater and 80.12%, 67.25%, 50.88%, 49.70%, 45.03% in palm oil industry wastewater treated with KUL39, KLB1, KLB2, KLY2 and KLY1, respectively. Moreover, the degradation of organic compounds (COD) were higher than control (without any adding selected isolates). The degradation efficiency of COD in wastewater from bakery and palm oil industry were found to be 94%-96% and 90%-92% (Figures 5B), respectively. The three best isolated (KUL8, KUL39,

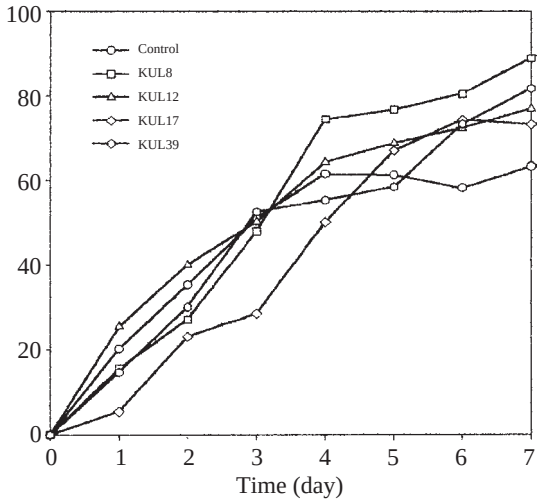

Figure 1 Biodegradation of fat and oil in bakery' s wastewater by single culture of isolated bacteria.

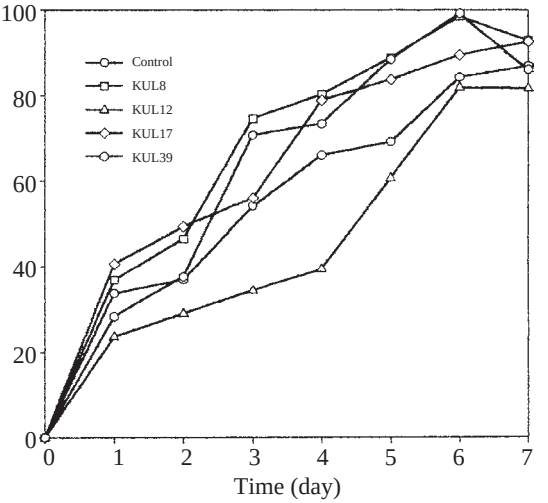

Figure 2 Biodegradation of organic compound in akery's wastewater by single culture of isolated bacteria.

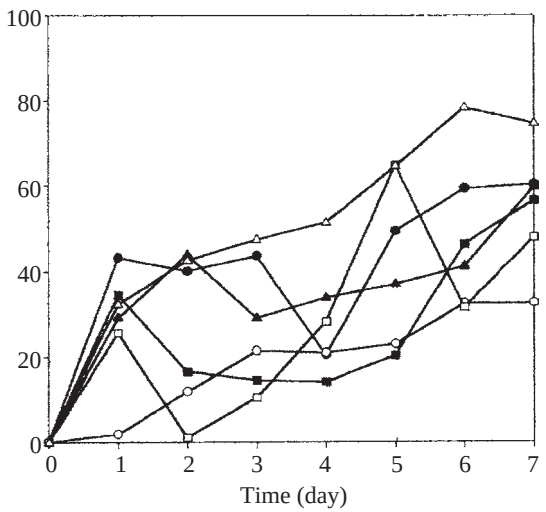

Figure 3 Biodegradation of fat and oil in bakery' s wastewater by mixed culture of isolated.

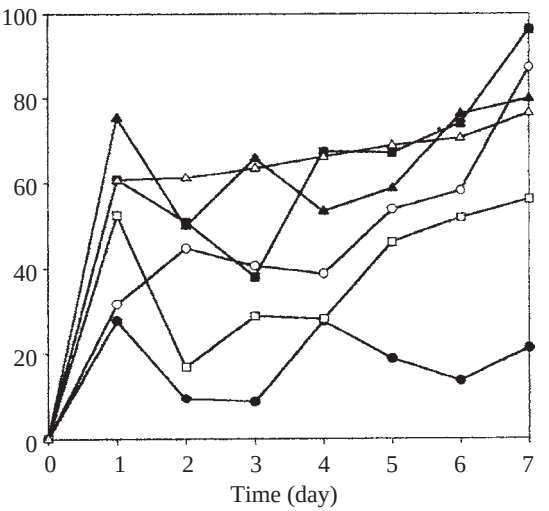

Figure 4 Biodegradation of organic compound in bakery's wastewater by mixed culture of isolated.

KLB1) were selected for further experiment

Another experiment of wastewater from palm oil and bakery treating with mixed culture were examined. Four mixed culture of KUL8, KUL39 and KLB1 were set in 2 and 3 mixed-isolates as MC1, MC2, MC3 and MC4.

Mixed culture of three isolates (MC4) degraded palm oil wastewater better than the two-isolates mixed culture (Figure 6A) whereas two isolates mixed culture (MC2) was suitable for bakery

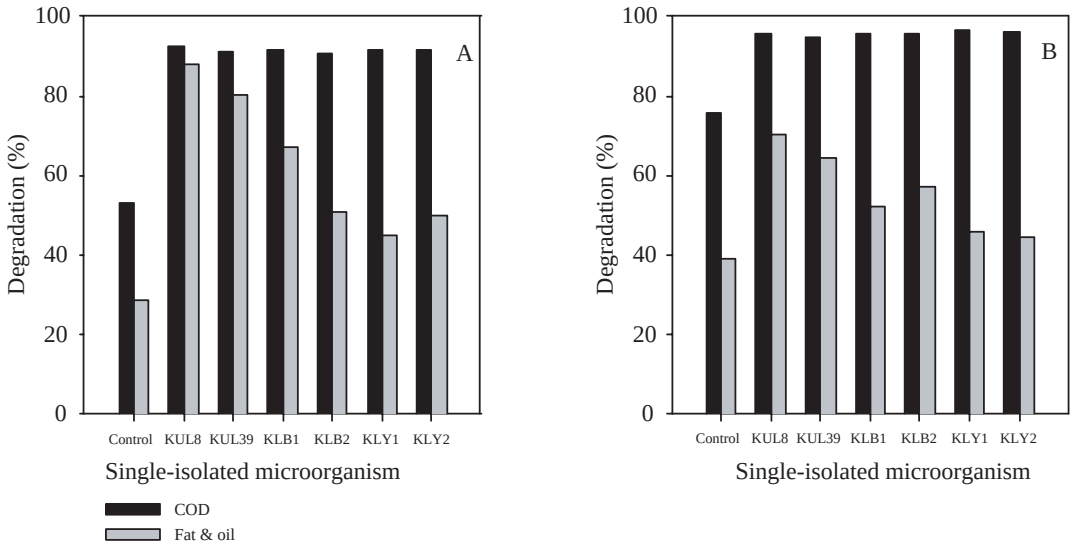

Figure 5 Biodegradation of fat & oil industrial and bakery's wastewater by each single-isolated microorganism A: Plam oil industrial wasterater B: Bakery's wastewater

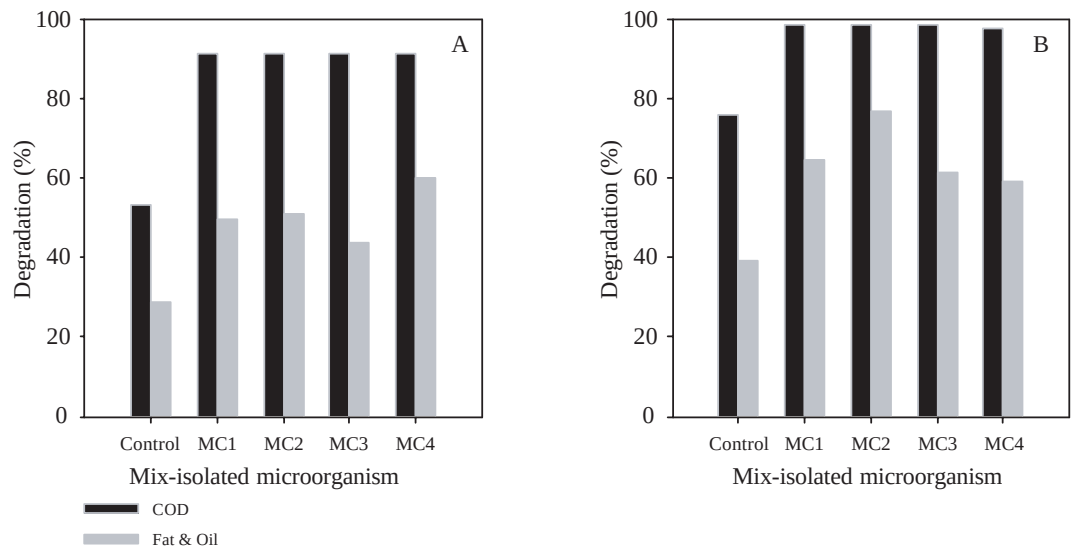

Figure 6 Biodegradation of fat & oil industrial and bakery's wastewater by mix-isolated microorganism. A: Plam oil industrial wasterater B: Bakery's wastewater II

wastewater (Figure 6B). However, the efficiency of selected microorganism for fat and oil degradation depended much on the component of wastewater. The single isolate of KUL8, KUL39 were shown promising for palm oil wastewater treatment than mixed culture. Single isolate of KUL8 was suggested to be used in bakery wastewater treatment.

The results of degradation of fat and oil between mixed cultures and single culture in two experiments indicated that the very different degradation efficiency might be dued to the different reaction system of lipase from each culture. Lipase present not only catalyzed hydrolysis reaction but also catalyzed interesterification reaction, depending on the source of lipase and reaction condition (Macrae, 1983). Lipase produced by different organisms might have different reaction such as *Pseudomonas cepacia* (Dunhaupt *et al*., 1992). *Acinetobacter radioresistant* CMC1 (Hong and Chang, 1998) were dominated hydrolytic reaction of lipase. On the other hand *P. fragi* CRDA323, *P. fluorescens, Pantoea aggomerans* (Pabai *et al.*, 1995) dominated on interesterification reaction. Therefore, in the mixed culture system of the present study hydrolysis reaction occurred but interesterification reaction had not determined either some reaction or occurred on both reactions. Moreover, the isolates also contained protease which able to decrease lipase activity and lower the degradation activities. However, fat and oil and organic compound degradation with organisms still activities of better than without using organisms.

The supernatant of isolates KUL8, LUL39 and KLB1 were also found particularly ∝-amylase and protease. Therefore, bacteria not only degraded fat and oil, but also degraded organic compounds. According to the result studies the isolated lipase producing bacteria were suggested to be used in wastewater treatment which will increased wastewater treatment efficiency.

The selected isolates with better degraded fat and oil were KUL8, KUL39 and KLB1, which were identified as *Acinetobacter* sp., *Bacillus* sp. and *Pseudomonas* sp., respectively by The Department of Medical Science, Ministry of Public Health.

CONCLUSION

In the isolation of lipase producing microorganisms for high fat and oil wastewater treatment, the result showed that bacteria is the most appropriate isolate. Single culture of isolate KUL8, KUL39 and KUB1 were suitable for palm oil wastewater. Mixed culture of KUL8 and KUB1 was better used in bakery wastewater. Department of Medical Science, Ministry of Public Health, identified the three isolates studied as *Acinetobacter* sp. (KUL8), *Bacillecs* sp. (KUL39) and *Pseudomonas* sp. (KLB1). Thailand, which has authority in controlling the safety microorganisms for use in waste treatment.

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Influence of Physical Factors and Various Complex Media on Growth and Bacteriocin Production of Two-synergistic Peptide with Heat Stable Bacteriocin Producer, *Enterococcus faecium* NKR-5-3, Isolated from Thai Fermented Fish

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ABSTRACT

Enterococcus faecium NKR-5-3, isolated from Thai fermented fish (Pla-ra), produced an interested two-synergistic peptide bacteriocin with heat stable and broad spectrum activity. The influence of complex media and culture condition on growth as well as bacteriocin production of *E. faecium* NKR-5-3 has been studied. From 8 indicator strains used, *Enterococcus faecalis* ATCC 19433 was found to be the most sensitive strain. The bacteriocin to be diluted with distilled water containing 0.1% (v/v) Tween 80 increased 2-fold to 4-fold of its activity against indicator strains when compared with distilled water. In M-MRS broth, bacteriocin production reached maximum level at 30°C and decreased with the increasing of culture temperature. Among different 6 complex media for lactic acid bacteria cultured at 30°C, M 17 broth with the initial pH of 7.5 yielded the maximum growth and bacteriocin production of *E. faecium* NKR-5-3. Growth and bacteriocin production decreased when it was cultured in M 17 broth containing more than 1% NaCl and they were completely inhibited in M 17 broth containing more than 9% NaCl. Bacteriocin activity reached a detectable level at the early exponential phase and increased due to the cell growth to the maximum level at the end of exponential phase in 22 hr of incubation, which displayed a primary metabolite production.

Key words: two-synergistic peptide bacteriocin, growth and bacteriocin production, *Enterococcus faecium*, Thai fermented fish, Pla-ra

INTRODUCTION

Bacteriocins are proteinaceous antimicrobial compounds with a bactericidal mode of action against bacteria closely related to the producer strain (Tagg *et al*., 1976). Some bacteriocins produced by lactic acid bacteria (LAB), such as nisin, inhibit not only closely related species but are also effective against food-borne pathogens such as *Listeria monocytogenes, Clostridium perfringens, Bacillus cereus, Staphylococcus aureus* and many other gram-positive spoilage bacteria (Cleveland *et al*., 2001; O'Sullivan *et al*., 2002). Recently, the use of either bacteriocin-producing LAB starter cultures or their bacteriocins for food preservation has received much interest (Ennahar *et al*., 1999; Franz *et al*., 1999).

Among LAB, *Enterococcus* is widely

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distributed and associated with food substrate. Enterococci are also used in food fermentation and some strains are routinely employed as starter culture in the manufacture of fermented food (Ennahar *et al*., 1999; Franz *et al*., 1999). Several strains of enterococci are known to produce bacteriocin, enterocin, and most of them belong to class II bacteriocin with heat stability and anti-*Listeria* activity (Franz *et al*., 1999).

Bacteriocin production in LAB usually occurs throughout the growth phase and ceases at the end of the exponential phase (Parente *et al*., 1994; De Vuyst *et al*., 1996). However, many factors affect on bacteriocin production in LAB such as medium component and culture condition (Parente and Hill, 1992). All of these factors are the most important factors for large-scale production of bacteriocin in food industry.

In recent years, there have been numerous reports on bacteriocin production by *E. faecium* isolated from various sources (Franz *et al*., 1996; Ennahar *et al*., 1998). However, only one report on bacteriocin producing *E. faecium* isolated from Thai fermented fishes (Pla-ra) of Thailand was described (Wilaipun *et al*., 2002). In summary, 11 bacteriocin producing LAB were isolated from 80 Pla-ra samples and one potent strain with high bacteriocin activity was selected. According to the result of API 20 Strep system and 16S rDNA sequences it was identified to be *E. faecium* and named *E. faecium* NKR-5-3. Beside its high bacteriocin activity production, *E. faecium* NKR-5-3 produced two-synergistic peptide bacteriocin with heat stable and a broad spectrum activity against some food pathogenic bacteria and spoilage LAB. The purpose of this study was to characterize the growth and bacteriocin production of *E. faecium* NKR-5-3.

MATERIALS AND METHODS

Bacterial strains and media

The two-synergistic peptide bacteriocin producer, *E. faecium* NKR-5-3, has been previously isolated from Thai fermented fish (Pla-ra) (Wilaipun *et al*., 2002). The culture was maintained as frozen stocks and held at -80°C in TSBYE (tryptic soy broth, Difco Laboratory, Detroit, MI, USA, supplemented with 6 g l^{-1} yeast extract, Difco Laboratory, Detroit, MI, USA) containing 15% (v/v) of glycerol. Throughout the experiments, this strain was subcultured every 2 weeks on TSAYE (TSBYE plus 12 g l^{-1} agar) slant and kept at 4°C. The cultures were propagated twice in TSBYE (pH 6.5) at 30°C for 18-24 hr before used. The following selected indicator strains were grown in the indicated media and temperature: *Bacillus cereus* JCM 2152 and *Bacillus coagulans* JCM 2257 in brain heart infusion broth (Difco Laboratory, Detroit, MI, USA) at 30°C and 37°C, respectively, *Lactobacillus sakei* subsp. *sakei* JCM 1157, *Lactobacillus plantarum* ATCC 14917, *Lactococcus lactis* subsp. *cremoris* TUA 1344L, *Leuconostoc mesenteroides* subsp. *mesenteroides* JCM 6124 in de Man, Rogosa and Sharpe (MRS) broth (Oxoid, Hampshire, England) at 30°C, *Enterococcus faecalis* ATCC 19433 and *Listeria innocua* ATCC 33090 in TSBYE at 37°C for determining bacteriocin activity.

For bacteriocin production, *E. faecium* NKR-5-3 was cultured in a production medium and incubated at each indicated temperature with no aeration and pH control. Unless otherwise noted *E. faecalis* ATCC 19433 was used as an indicator strain for bacetriocin activity determination.

Bacteriocin activity assay

The cell-free neutralized supernatant (CFNS) of *E. faecium* NKR-5-3 grown in each medium at 30°C for 18-24 hr was obtained by centrifugation at 10,000x*g* for 15 min at 4°C, neutralization with 1 M NaOH to pH 6.5 and subsequent sterilization by heating at 100°C in water bath for 5 min. The bacteriocin activity of CFNS was determined against indicator strains using critical dilution method (Mayr-Harting *et al*., 1972). The CFNS were twofold serially diluted with sterile diluent in microtiter

plate and aliquots (10 µl) of each dilution were spotted onto TSAYE plate overlaid with 5 ml of TSAYE soft agar media (1% agar, w/v) seeded with 10^7 CFU ml^{-1} of overnight (18 hr) cultured indicator strain. The arbitrary activity unit was defined as the reciprocal of the highest dilution producing a distinct inhibition of the indicator lawn and expressed in terms of arbitrary units per milliliter (AU ml^{-1}).

Selection of the most sensitive strain and influence of diluents on bacteriocin activity

Modified MRS broth described by Tichaczek *et al*. (1992) with 2% glucose (M-MRS+2% Glu broth) at pH 6.5 was inoculated with 1% (v/v) 18 hr culture broth of *E. faecium* NKR-5-3 and incubated at 30°C for 24 hr. The bacteriocin activity of CFNS was determined comparing with 8 different indicator strains (Table 1) using two different diluents of sterile distilled water and sterile distilled water containing 0.1% Tween 80.

Production of bacteriocin at different temperatures

M-MRS+2% Glu broth was inoculated with 1% (v/v) 18 hr culture broth of *E. faecium* NKR-5-3 and incubated at different temperatures (25, 30, 35, 40 and 45°C). After 24 hr of incubation, the pH and OD_{600} of culture broth as well as bacteriocin activity in CFNS were determined.

Production of bacteriocin in various media conditions

For complex media study, APT broth (Difco; pH 7.7), Elliker broth (Difco; pH 6.8), M 17 broth (Merck; pH 7.2), MRS broth (Oxoid; pH 6.2), M-MRS broth (pH 6.5) and M-MRS+2%Glu broth (pH 6.5) were inoculated with 1% (v/v) 18 hr culture broth of *E. faecium* NKR-5-3 and incubated at 30°C. At the selected time intervals, the pH and OD_{600} of culture broth as well as bacteriocin activity in CFNS were determined.

For the influence of pH and sodium chloride, M 17 broth (Merck) adjusted to pH 4.0, 5.0, 6.0, 7.0, 7.5, 8.0, 9.0 and 9.5 were inoculated with 1% (v/v) of bacteriocin producing culture grown for 18 hr at 30°C. To determine the effect of sodium chloride concentration in the medium, sodium chloride was added to M 17 broth (pH 7.5) to a final concentration of 1, 3, 5, 7, 9, 11, 13 and 15% (w/v). The pH and OD_{600} of culture broth as well as bacteriocin activity in CFNS were determined after incubation at 30°C for 24 hr.

Kinetics of bacteriocin production

M 17 broth (1,000 ml, pH 7.5) was inoculated with 1% (v/v) 18 hr culture broth of *E. faecium*

Table 1 Bacteriocin activity of *E. faecium* NKR-5-3 CFNS against various indicator strains and the enhancement of Tween-80 on bacteriocin activity.

Indicator strains	Bacteriocin activity (AU ml^{-1})	
	Distilled water	0.1% Tween 80
Bacillus cereus JCM 2152	800	1,600
Bacillus coagulans JCM 2257	1,600	6,400
Enterococcus faecalis ATCC 19433	6,400	12,800
Lactobacillus plantarum ATCC 14917	1,600	6,400
Lactobacillus sakei subsp. *sakei* JCM 1157	1,600	6,400
Lactococccus lactis subsp. *cremoris* TUA 1344L	800	3,200
Leuconostoc mesenteroides subsp. *mesenteroides* JCM 6124	400	1,600
Listeria innocua ATCC 33090	800	3,200

NKR-5-3 and incubated at 30°C. At the selected time intervals, the pH and OD_{600} of culture broth and bacteriocin activity in CFNS were determined.

RESULTS

Selecting for the most sensitive strain and influence of diluent on bacteriocin activity

Bacteriocin activity in CFNS of *E. faecium* NKR-5-3 was determined comparing to eight indicator strains using two different diluents. According to the highest antibacterial activity of bacteriocin produced by *E. faecium* NKR-5-3 to *E. faecalis* ATCC 19433 (Table 1), it suggested that *E. faecalis* ATCC 19433 was the most sensitive strain among eight indicator strains used. Comparing of the diluted solution between sterile distilled water containing 0.1% Tween 80 and distilled water (Table 1), the higher bacteriocin activity of 2-4 folds was obtained when Tween 80 was added. According to these results, *E. faecalis* ATCC 19433 was selected as an indicator strain and sterile distilled water containing 0.1% Tween 80 was chosen as a diluent for bacteriocin activity determination in further experiments.

Production of bacteriocin at different temperatures

E. faecium NKR-5-3 exhibited the maximum cell density when grew in M-MRS+2%Glu at 25°C for 24 hr. However, the maximum bacteriocin activity (12,800 AU ml^{-1}) was obtained when it was grown at 30°C and yielded lower cell density (Table 2). On the other hand, at the high growing temperatures of 40 and 45°C bacteriocin activity in CFNS were found to be as low as 200 and 0 AU ml^{-1}, respectively.

Production of bacteriocin in different complex media

E. faecium NKR-5-3 was cultured in six different complex media at 30°C. After 12 hr of incubation, bacteriocin activity could be detected from most of the six complex media and after 24 to 36 hr *E. faecium* NKR-5-3 exhibited both of the maximum cell density (OD_{600} = 3.10) and highest bacteriocin activity (51,200 AU ml^{-1}) when grown in M 17 broth (Table 3). Therefore, M 17 broth was selected for further experiment. Meanwhile, in M-MRS+0.2%Glu it gave the lowest growth and lowest bacteriocin activity.

Influence of initial pH medium on bacteriocin production

E. faecium NKR-5-3 grew to the maximum cell density (OD_{600} = 3.3) in M 17 broth with the initial pH = 7.5 and 8.0, meanwhile, bacteriocin production was maximum (51,200 AU ml^{-1}) at initial pH = 7.5. In M 17 broth adjusted initial pH below 7.5 and above pH 8.0 the growth was reduced

Table 2 Growth and bacteriocin production of *E. faecium* NKR-5-3 in M-MRS+2% glucose at different temperatures.

Temperature (°C)	pH	OD_{600}	Bacteriocin activity[a] (AU ml^{-1})
25	4.65	3.47	6,400
30	4.55	3.18	12,800
35	4.45	2.96	6,400
40	4.35	2.63	200
45	5.55	1.50	0

[a] *E. faecalis* ATCC 19433 was used as an indicator strain

Table 3 Growth and bacteriocin activity on different complex medium of *E. faecium* NKR-5-3 at 30°C.

	APT broth	Elliker broth	M 17 broth	MRS broth	M-MRS broth (0.2% glucose)	M-MRS broth (2% glucose)
12 hours						
pH	4.85	5.15	6.38	4.96	6.05	5.15
OD_{600}	2.44	1.63	2.34	2.48	1.64	2.52
Activity[a] (AU ml^{-1})	3,200	1,600	12,800	3,200	1,600	3,200
18 hours						
pH	4.68	5.00	6.02	4.52	6.05	4.64
OD_{600}	2.63	1.67	2.98	2.67	1.58	2.70
Activity[a] (AU ml^{-1})	6,400	3,200	25,600	6,400	1,600	6,400
24 hours						
pH	4.61	5.00	5.85	4.52	6.05	4.53
OD_{600}	2.68	1.68	3.10	2.65	1.56	2.72
Activity[a] (AU ml^{-1})	6,400	3,200	51,200	6,400	1,600	12,800
36 hours						
pH	4.56	4.95	5.80	4.48	5.98	4.49
OD_{600}	2.46	1.70	2.96	2.61	1.48	2.64
Activity[a] (AU ml^{-1})	6,400	3,200	51,200	6,400	1,600	12,800

[a] *E. faecalis* ATCC 19433 was used as an indicator strain

and bacteriocin production was lower than at pH 7.5. *E. faecium* NKR-5-3 was not capable of growth and bacteriocin production at pH 4.0. Although the growth of *E. faecium* NKR-5-3 was detected at pH 5.0 and 9.5, but it gave very low bacteriocin activity of 100 AU ml^{-1} and no activity were obtained at all in these two pH (Table 4).

Influence of sodium chloride on bacteriocin production

E. faecium NKR-5-3 exhibited the maximum growth (OD_{600} = 3.3) and bacteriocin production (51,200 AU ml^{-1}) when it was grown in M 17 broth containing 0 to 1% NaCl. However, having NaCl concentration of 3 to 7%, drastically decreased both of growth and bacteriocin production. No growth or bacteriocin could be detected when higher NaCl concentration of 9% was used (Table 5).

The kinetics of bacteriocin production

Optical density (OD_{600}), pH of culture medium and bacteriocin production during the growth of *E. faecium* NKR-5-3 in M 17 broth at 30°C are shown in Fig. 1. OD_{600} increased from an initial ca. 0.03 to 3.0 during the first 24 hr of incubation. The OD_{600} was then stabilized at ca. 3.0 and remained at this level up to 36 hr of incubation. OD_{600} was found to decrease and reached the level of 2.5 at the end of the 72 hr incubation period. The initial pH of culture medium at 7.4 was decreased to 5.7 at the end of incubation period. Bacteriocin production was initially detected at 4 hr after inoculation and increased to a maximum of 51,200 AU ml^{-1} after 22–24 hr. Furthermore, bacteriocin activity remained stable at this level till the end of incubation period.

Table 4 Influence of initial pH of M 17 broth on growth and bacteriocin production of *E. faecium* NKR-5-3.

Initial pH	pH of culture broth after 24 hr	OD_{600}	Bacteriocin activity[a] (AU ml^{-1})
4.0	3.98	0.03	0
5.0	4.83	0.67	100
6.0	4.99	2.18	6,400
7.0	5.65	2.70	12,800
7.5	5.84	3.28	51,200
8.0	6.07	3.30	25,600
9.0	6.52	3.00	6,400
9.5	8.06	1.44	0

[a] *E. faecalis* ATCC 19433 was used as an indicator strain

Table 5 Influence of sodium chloride concentration in M 17 broth on growth and bacteriocin production of *E. faecium* NKR-5-3.

NaCl concentration (%)	pH of culture broth after 24 hr	OD_{600}	Bacteriocin activity[a] (AU ml^{-1})
0	5.90	3.33	51,200
1	5.84	3.26	51,200
3	6.45	1.56	1,600
5	6.59	0.95	400
7	6.89	0.37	200
9	7.27	0.03	0
11	7.26	0.03	0
13	7.23	0.03	0
15	7.20	0.03	0

[a] *E. faecalis* ATCC 19433 was used as an indicator strain

DISCUSSION

According to the highest antibacterial activity (6,400 AU ml^{-1}) of bacteriocin produced by *E. faecium* NKR-5-3 to *E. faecalis* ATCC 19433. It suggested that *E. faecalis* ATCC 19433, which is the closely related strain to *E. faecium*, was the most sensitive strain among 8 indicator strains used. This result comply with bacteriocins produced by another LAB, those are usually exhibit the highest antibacterial activity against the target strains which are closely related to the producing strain (De Vuyst and Vandamme, 1994; Tagg *et al.*, 1976). On the comparison of two diluents on bacteriocin activity, sterile distilled water containing 0.1% Tween 80 increased 2-4 folds of bacteriocin activity against indicator strains when compared to distilled water. The enhancement of Tween 80 on bacteriocin

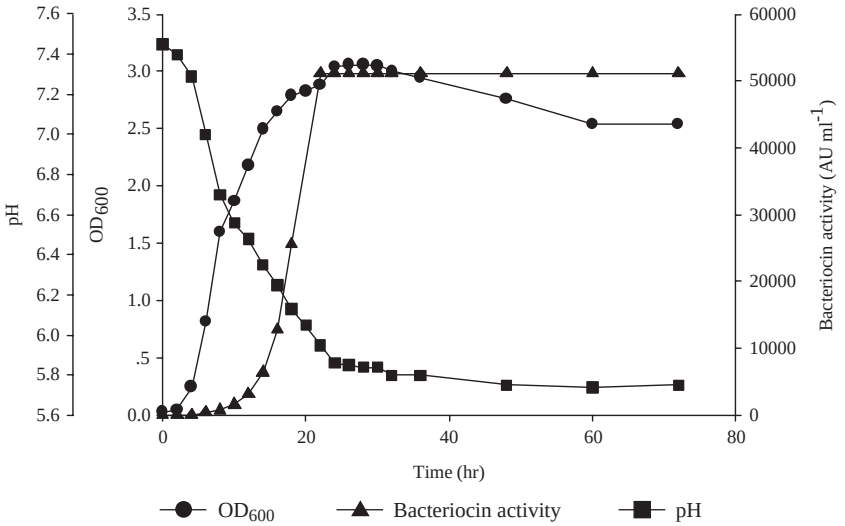

Figure 1 The growth (OD_{600}), bacteriocin production and pH of culture broth of *E. faecium* NKR-5-3 during incubation in M 17 broth at 30°C.

activity was due to a reduction of the binding of bacteriocin to the surface of plastic microtiter wells since most bacteriocins are proteinaceous with hydrophobic compound which tend to aggregate to form large complexes and to adhere to hydrophobic surfaces (Nissen-Meyer *et al*., 1992). Nisin and enterocin 4 were previously reported to be rapidly adsorbed to polypropylene and glassware. The addition of 0.1% Tween 80 to the diluent buffer could reduce these adsorption from 45-75% to less than 5% (Joosten and Nunez, 1995). In addition, the adsorption of nisin to hydrophilic surfaces by electrostatic interactions and desorption by Tween has also been studied by Daeschel *et al*. (1992). Furthermore, Nissen-Meyer *et al*. (1992) suggested that Tween 80 may involve in the stabilization of a favorable configuration of the bacteriocin molecules and/or the sensitization of the target cell, perhaps through destabilization of its membrane.

In general, bacteriocin production is growth-associated (Parente *et al*., 1994; De Vuyst *et al*., 1996). However, in some bacteriocin producing strains such as *Lactobacillus acidophilus* and *Lactococcus lactis* the maximum bacteriocin levels or production rates did not correlate directly with cell mass or growth rate (Kim *et al*., 1997; Bogovic-Matijasic and Rogelj, 1998). As it also was found in *E. faecium* NKR-5-3 grown at 25 and 30°C. Moreover, the lower growth rate or some unfavorable growth conditions was also reported to enhance bacteriocin production in some bacteriocin producing strains (De Vuyst *et al*., 1996). At 40 and 45°C, *E. faecium* NKR-5-3 showed the low level and lost of bacteriocin production, respectively. The similar result was also found in *Enterococcus faecium* DPC 1146 showing the low level of enterocin production at 42-45°C (Parente and Hill, 1992). However, enterocin P and enterocin Q production of *E. faecium* L50 were reported to reach the maximum level at 47°C and 37-47°C of incubation temperature, respectively (Cintas *et al*., 2000).

The growth and bacteriocin production of *E. faecium* NKR-5-3 was studied in six different complex media. After 24 hr of incubation, growth of *E. faecium* NKR-5-3 in M 17 broth gave the maximum cell density and maximum bacteriocin production. This result resembles enterocin 1146 production in which *E. faecium* DPC 1146 grow best with the highest antimicrobial compound in M 17 broth supplemented with 0.5% glucose (Parente and Hill, 1992).

In general, LAB are fastidious microorganisms with respect to nutrient requirement so that a rich medium with yeast extract and protein hydrolysates is required for good growth and bacteriocin production (Parente and Hill, 1992; De Vuyst *et al*., 1996). The growth and bacteriocin production of LAB is often limited by organic nitrogen sources rather than by the carbon sources (Parente and Ricciardi, 1999). Furthermore, M 17 broth contains more various organic nitrogen sources, as in digested form of beef, casein and soybean, than those of another 5 media used. Moreover, M 17 broth contains large amount of sodium beta-glycerophosphate, which increases the buffering capacity of medium and also promotes the growth of lactic streptococci (Merck, 2000). Consequently, M 17 broth is suitable for the growth and bacteriocin production of *E. faecium* NKR-5-3. Beside the previously optimum properties, M 17 medium does not contain Tween 80 as seen in most complex media for bacteriocin production, which will be the advantage in a bacteriocin purification step since Tween 80 was reported to interfere with the bacteriocin purification procedure (Muriana and Klaenhammer, 1991).

The growth and bacteriocin production of *E. faecium* NKR-5-3 occurred in the neutral or slightly alkaline medium condition. This finding is different from what is known for the optimum pH for bacteriocin production in previous reports which were usually at pH 5.5-6.0 and were often lower than the optimum pH for growth (Parente *et al*., 1994; Matsusaki *et al*., 1996). On the other hand, it complies with the optimum pH range of enterocin 900 production by *E. faecium* BFE 900 (Franz *et al*., 1996). However, the optimum pH for bacteriocin production may also be affected by the culture medium (Parente and Ricciardi, 1999). According to this study, the large scale production of bacteriocin by *E. faecium* NKR-5-3 in batch culture should be done in M 17 broth with initial pH value of 7.5. Moreover, the application of this strain as a protective culture in situ, should therefore be used in food systems with pH more than 6.0.

Although *E. faecium* NKR-5-3 was isolated from Thai fermented fish (Pla-ra) containing large amount of NaCl it exhibited a poor growth and bacteriocin production in the medium containing more than 1% NaCl. In addition, the growth and bacteriocin production were completely inhibited in the medium containing more than 9% NaCl. Hence, *E. faecium* NKR-5-3 could not be classified as a halophilic bacteria that played an important role in fermented fish process but rather a halotolerant bacteria that could survive during fermented process. In a previous report, the nukacin ISK-1 production by *Staphylococcus warneri* ISK-1 was shown to increase with increasing NaCl and the highest level of production was reached in the medium having 1.4 M NaCl. Meanwhile, cell growth, glucose consumption and lactate production were inhibited by the increase in NaCl concentration (Sashihara *et al*., 2001). In our previous study, bacteriocin from *E. faecium* NKR-5-3 cultured in M 17 broth without sodium chloride could stabilize under different sodium chloride concentration (0-24%) environment at 4°C for more than 30 days (data not shown). By this finding, the application of *E. faecium* NKR-5-3 in high salt containing food system should be done by using its bacteriocin as a biopreservative compound rather than using it as a starter or biopreservative culture.

The growth and bacteriocin production of *E. faecium* NKR-5-3 was studied at selected time intervals throughout the incubation period. From our finding, bacteriocin activity could be detected at early exponential phase and increased with the increasing of cell growth until reached the maximum production in early stationary growth phase. It suggested that bacteriocin production of *E. faecium* NKR-5-3 was the primary metabolite production. This result complied with almost of bacteriocin production by LAB those were reported to be the primary metabolite production (De Vuyst and Vandamme, 1994). The maximum production of bacteriocin occurred at high cell density in early

stationary phase, as was reported also for bacteriocin produced by *Enterococcus faecium* BFE 900 (Franz *et al*., 1996), *Enterococcus faecium* RZS C5 (Leroy and De Vuyst, 2002). However, some types of bacteriocin were reported to be a secondary metabolite production such as bacteriocin produced by *Lactobacillus lactis* subsp. *lactis* (Rattanachaikunsopon and Phumkhachorn, 2000), pediocin AcH and Mesenteroicin 5 which were produced by *Pediococcus acidilactici* and *Leuconostoc mesenteroides* strain UL5 (Biswas *et al*., 1991; Lewus *et al*., 1991).

CONCLUSION

It was shown that *E. faecalis* ATCC 19433 was the most sensitive strain to the bacteriocin of *E. faecium* NKR-5-3 and Tween 80 could increase bacteriocin activity against the selected indicator strains. Bacteriocin production of *E. faecium* NKR-5-3 is a growth-associated process and it was influenced by some controllable environmental factors such as temperature, nutrient component and initial pH of culture media.

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Salinity Levels on Survival Rate and Development of Mud Crab *(Scylla olivacea)* from Zoea to Megalopa and from Megalopa to Crab Stage

Pattanee Jantrarotai[1], Krailerk Taweechuer[1]and Suparp Pripanapong[2]

ABSTRACT

Effect of salinity on survival rate and development of mud crab (*Scylla olivacea*) were conducted into two phases: phase I from zoea 1 to megalopa and phase II from megalopa to the crab stage. In phase I, four different levels of salinity, 28, 30, 32 and 34 parts per thousand (ppt) were used. The results showed that zoea reared at 28, 30, 32 and 34 ppt developed to megalopa stage with survival rates of 13.16, 22.19, 8.25 and 7.08 percent respectively. At 30 ppt, zoea developed to megalopa stage with better survival rate than at the other salinity levels ($P<0.05$). In all salinity levels, high mortality rate of zoea occured during the development of zoea 1 to zoea 3 and was higher than that of zoea developed from stage 3 to megalopa. Duration for development of zoea 1 to megalopa at all salinity levels were 23.64-24.22 days which were not significantly different ($P>0.05$) among treatments.

In phase II, the effect of salinity on survival rate and developmental period of megalopa to the fifth crab stage (C5) at salinity levels of 12, 16, 20, 32, 36 and 40 ppt were studied. The results showed that survival rates of the crab developed from megalopa at 16, 20, 32 ppt were 80.95, 76.19, and 80.95 percent respectively. These figures were significantly higher ($P<0.05$) than those at 12, 36, and 40 ppt. Time used for megalopa to develop into the fifth crab stage (C5) was influenced by salinity. The developmental period to the fifth crab stage from megalopa were 29.14, 28.25, 30.10, 31.75, 32.55, and 43.50 days, respectively. Aging also had influenced on time for crab development. The more advanced crab stage, the longer time used for the development of crab to the next stage.

Key words: *Scylla olivacea*, salinity, zoea, megalopa, crab

INTRODUCTION

The portunid crab *scylla* known as mangrove or mud crab, is the largest species of edible crab in the Indo-Pacific region. Its habitat is in brackish waters between the lower intertidal zone and offshore such as mangrove estuaries and creeks. (Hyland *et al*. 1984; Macintosh, 1988). Mud crab is important to socio-economic of Australia, Japan, Taiwan, Indonesia, Philippines and Thailand (Keenan, 1999). The export value for mud crab of Thailand is approximately 180 million baths with the total volume of 3,867 tons. Mud crabs produced in Thailand are mostly wild catches. However, due to the degradation of natural habitats, increasing of water pollution, and overexploitation of crab, the wild population of this shellfish decreased significantly.

Development of mud crab aquaculture has started for some times but not much success has been acheived due to mass mortality of seed crab especially during the zoea and megalopa stage.

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Therefore, it is necessary to identify the certain factors affecting survival and development of mud crab seed. Salinity is one of the factors related to the rearing conditions needed to be determined. This study was conducted to define optimal salinity levels for survival and development of mud crab *scylla olivaceae* in two developmental periods. Experiment 1: study from zoea 1 to the megalopa stage and experiment 2: study from megalopa to the crab stage.

MATERIALS AND METHODS

Experiment 1

An ovigerous female crab was raised in 50-L plastic tanks at the salinity 30 parts per thousand (ppt) and fed daily with chopped fishes and squids until spawning. After the eggs hatched into zoea1, the zoea were randomly stocked in 12, 31.0×43.5×29.5 cm styrofoarm boxes at density of 400 zoea/box. Each box was allocated to each salinity level, 28, 30, 32 and 34 ppt at three replicates/ treatment. Each box contained 15 liter of water at its respective salinity. At the stocking, the zoea were gradually acclimated to the increasing salinity until reaching the required salinity. Zoea were fed with rotifer 60 ind/mL and artemia 10 ind/mL. Dead zoea were removed and saline water was totally changed daily prior to feeding the zoea. Alive zoea were picking up in a wide-bore pipette and recorded daily. The survival rate and the development of zoea were examined by counting the number and identifying the stage of the larvae (zoea 2, 3, 4, 5 and megalopa). Time (days) for the development of zoea 1 to megalopa stage in each salinity level was recorded and used as indicator for zoea development.

Experiment 2

The effect of salinity on the survival and development of megalopa to crab stage was investigated. Each of eighteen styrofoarm boxes divided into seven compartments using seven punched plastic cups as the cell to prevent cannibalism. The cup had a diameter of 8 cm. Each of three replicates of divided box was filled with 15 liters of water at salinity levels of 12, 16, 20, 32, 36 and 40 ppt. A megalopa was randomized into each cup and daily fed with newly hatch artemia 10 ind/ mL and chopped fishes soon after daily change of new water . Survival rate (%) and time (days) of the larvae molted to the next stage were determined.

Analysis of variance

Survival rate (%) and time (days) used for the development of mud crab larvae at various salinity levels in both experiments were subjected to analysis of variance. If the differences were significant ($P<0.05$), then Duncan 'new multiple range test (DMRT) was conducted to find out which treatments were different.

RESULTS AND DISCUSSION

Experiment 1

The survival of zoea 1 that successfully developed to megalopa stage at salinity level of 28, 30, 32 and 34 ppt were 13.16, 22.91, 8.25 and 7.08 percent, respectively (Table 1, and Figure1). The zoea reared at 30 ppt developed to megalopa stage with significantly higher survival rate ($P<0.05$) than those at the other levels. At this salinity level, zoea 1 survived into zoea 2, 3, 4, 5 and megalopa at percentages of 51.75, 35.58, 31.75, 30.08 and 22.91 respectively. There were no significant differences ($P>0.05$) in survival rates of zoea at various stages of development when reared at 28, 32 and 34 ppt. High mortality of zoea was observed at 34 ppt. The result agreed with Heasman and Fielder (1983) and Hoang (1999) who found that optimum salinity level for survival of *Scylla serrata* could be varied from 30-34 ppt. They also reported that survival and megalopa production depended on the ability of the zoea to maintain the body fluid in the isotonic environment. Zoea raised in the hypo or hypertonic solution would lose some energy for adjusting body fluid, the energy left for molting might not be

enough and cause mortality in zoea. This study showed that zoea raised at 30 ppt had the highest survival rate, this probably due to the tested zoea was in isotonic of 30 ppt water which was right for the zoea to hatch.

At all salinity levels, the accumulate mortality occurring during zoea 1 developing to zoea 3 were significantly higher ($P<0.057$) than those of the other developmental stages. Worner (1997) reported that in the wild, mass mortality of the early stage larvae always occured in marine animals that produced abundance of larvae. Furthermore, the early stage larvae, zoea 1 in this study had not yet developed the eyestalk which was the organ producing water balance regulating hormone. When crab larvae developed to zoea 3, 4, 5, the rate of mortality was found to decrease and survival rates were not significantly differences ($P>0.05$). The reason was that zoea at these stages were acclimated and adjusted to the salinity so that the efficiency in controlling fluid-balancing was better as appeared in *Macrobthalmus setosus* (Nongnut, 1999).

Time for the developing of zoea 1 to

Table 1 Mean percent survival of zoea 1 (Z1) to megalopa (M) reared under four different salinity ; 28, 30, 32 and 34 parts per thousand (ppt).

Treatment	Z1	% survival				
		Z1-Z2	Z1-Z3	Z1-Z4	Z1-Z5	Z1-M
1. (28 ppt)	$100\pm0.00^{a,A}$	$37.58\pm7.60^{b,B}$	$18.50\pm5.71^{b,C}$	$15.75\pm6.66^{b,C}$	$14.50\pm6.08^{b,C}$	$13.16\pm6.14^{b,C}$
2. (30 ppt)	$100\pm0.00^{a,A}$	$51.75\pm1.08^{a,B}$	$31.75\pm3.07^{a,C}$	$35.58\pm3.68^{a,CD}$	$30.08\pm3.39^{a,D}$	$22.91\pm2.40^{a,E}$
3. (32 ppt)	$100\pm0.00^{a,A}$	$30.08\pm5.39^{b,B}$	$14.16\pm5.13^{b,C}$	$11.00\pm3.76^{b,C}$	$9.75\pm4.54^{b,C}$	$8.25\pm3.47^{b,C}$
4. (34 ppt)	$100\pm0.00^{a,A}$	$31.66\pm2.02^{b,B}$	$13.33\pm3.14^{b,C}$	$9.58\pm1.87^{b,D}$	$8.91\pm1.50^{b,D}$	$7.08\pm0.62^{b,D}$

Note : Data are expressed as mean ± SD
Mean in the same rows (capital letter) or column (lower case) with the same letter are not significantly different ($P>0.05$)

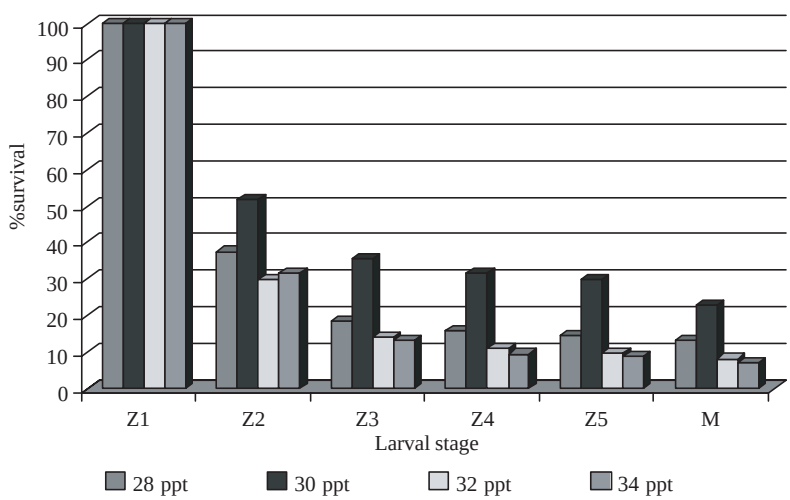

Figure 1 Percentage of zoea surviving reared under four different levels of salinity; 28, 30, 32 and 34 parts per thousand (ppt).

megalopa at the salinity levels of 28, 30, 32 and 34 ppt were 23.64, 24.22, 23.76 and 23.95 days, respectively (Table 2). There were no significant differences (P>0.05) of the development period among treatments. This was probably because zoea was the stage of crab whose life cycle developed in marine environment, therefore, salinity levels might not have much effect on its development (Nongnut, 1999).

Experiment 2

The survival rates of the fifth crab stage developed from megalopa at the salinity levels of 12, 16, 20, 32, 36 and 40 ppt were 42.85, 80.95, 76.91, 80.95, 52.38 and 9.52 percent, respectively (Table 3 and Figure 2). Megalopa in different treatments were found to tolerate salinity and developed to crab stage differently. Survival rate of crab at all stages developed from megalopa were not significantly different among salinity levels from16-32 ppt (P>0.05) and were significantly higher (P<0.05) than those at 12 and 40 ppt. High mortality of megalopa when developed to crab 1 were recorded at 40 ppt. It showed that crab stage tended to have greater tolerance to wide ranges of salinity particularly between 16-32 ppt. This result agreed with the study in *Scylla serrata* by Trans *et al*. (1998). However, too low or too high salinity as evidented in 12 and 40 ppt, will affect survival rate of crab. This was because crab had to spend more energy to regulate body fluid at extreme salinity. Furthermore, crab might have mineral deficiency if reared at too low salinity levels (Holliday, 1969).

At salinity levels of 12, 36 and 40 ppt , the mortalities of crab 1 developing from megalopa were high similar to that occured in Zoea 2

Table 2 The average developmental period (days) from zoea 1 to megalopa reared under four different salinity.

Treatment	Developmental period (days)
1 (28 ppt)	23.64 ± 0.78^{a}
2 (30 ppt)	24.22 ± 0.71^{a}
3 (32 ppt)	23.76 ± 0.30^{a}
4 (34 ppt)	23.95 ± 0.48^{a}

Note : Mean in the same column with the same letter are not significantly different (P>0.05)

Table 3 Mean percent survival of megalopa (M) to crab 1, 2, 3, 4 and 5 (C1-C5) reared under six different salinity ; 12, 16, 20, 32, 36 and 40 parts per thousand (ppt).

Treatment	M	% survival				
		M-C1	M-C2	M-C3	M-C4	M-C5
1. (12 ppt)	$100\pm0.00^{a,A}$	$57.14\pm50.70^{b,B}$	$47.61\pm51.17^{b,BC}$	$42.85\pm50.70^{b,BC}$	$42.85\pm50.70^{b,BC}$	$42.85\pm50.70^{b,BC}$
2. (16 ppt)	$100\pm0.00^{a,A}$	$95.23\pm21.82^{a,A}$	$90.47\pm30.07^{a,A}$	$85.71\pm35.85^{a,A}$	$85.71\pm35.85^{a,A}$	$80.95\pm40.23^{a,A}$
3. (20 ppt)	$100\pm0.00^{a,A}$	$95.23\pm21.82^{a,AB}$	$95.23\pm21.82^{a,AB}$	$95.23\pm21.82^{a,AB}$	$90.47\pm30.07^{a,AB}$	$79.19\pm43.64^{a,B}$
4. (32 ppt)	$100\pm0.00^{a,A}$	$95.23\pm21.82^{a,A}$	$95.23\pm21.82^{a,A}$	$95.23\pm21.82^{a,A}$	$95.23\pm21.82^{a,A}$	$80.95\pm40.23^{a,A}$
5. (36 ppt)	$100\pm0.00^{a,A}$	$52.38\pm51.17^{b,B}$	$52.38\pm51.17^{b,B}$	$52.38\pm51.17^{b,B}$	$52.38\pm51.17^{b,B}$	$52.38\pm51.17^{ab,B}$
6. (40 ppt)	$100\pm0.00^{a,A}$	$9.52\pm30.07^{c,B}$	$9.52\pm30.07^{c,B}$	$9.52\pm30.07^{c,B}$	$9.52\pm30.07^{c,B}$	$9.52\pm30.07^{c,B}$

Note : Data are expressed as mean ± SD

Mean in the same rows (capital letter) or column (lower case) with the same letter are not significantly different (P>0.05)

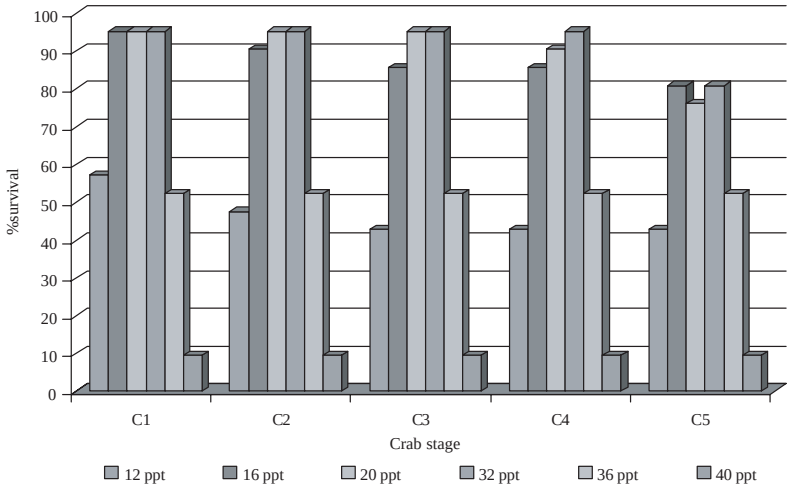

Figure 2 Percentage of megalopa surviving reared under six different levels of salinity; 12, 16, 20, 32, 36 and 40 parts per thousand (ppt).

developing from Zoea l. After developing to crab 1 stage, mortality of further crab stages were low. Therefore, there were no significant differenes ($P>0.05$) in the survival rates of crab at each development stage. The study also showed that salinity levels had effect on time for the development of megalopa to crab 5. The developmental period to crab 5 from megalopa raised at salinity level of 16, 20, 32, 36 and 40 ppt were 28.25, 30.10, 31.75, 32.55 and 43.50 days, respectively (Table 4 and Figure 3). At higher salinity, the trend for longer time was needed for the development of megalopa to crab stage. Crab age also influenced time for development to the next stages. The more advanced crab stage, the longer times spent for the development. This was because the setting period during the molting process in the older crab was longer than that of the younger one (Marichamy and Rajapackiam,1991).

Table 4 The average developmental period (days) from megalopa (M) to crab 1, 2, 3, 4 and 5 (C1-C5) reared under six different salinity ; 12, 16, 20, 32, 36 and 40 parts per thousand (ppt).

Treatment	Developmental period (day)				
	M-C1	C1-C2	C2-C3	C3-C4	C4-C5
1. (12 ppt)	$9.83\pm1.46^{a,C}$	$4.10\pm0.31^{a,A}$	$4.22\pm0.66^{a,A}$	$4.55\pm0.88^{ab,A}$	$6.44\pm1.13^{ab,B}$
2. (16 ppt)	$9.70\pm1.41^{a,C}$	$3.73\pm0.87^{a,A}$	$4.27\pm0.82^{a,A}$	$4.44\pm0.78^{a,A}$	$7.11\pm1.36^{ab,B}$
3. (20 ppt)	$10.30\pm1.52^{a,C}$	$4.45\pm0.75^{a,A}$	$4.45\pm1.09^{a,A}$	$4.57\pm1.10^{ab,A}$	$6.37\pm1.14^{a,B}$
4. (32 ppt)	$11.30\pm1.45^{a,C}$	$4.35\pm0.81^{a,A}$	$3.90\pm0.91^{a,A}$	$4.85\pm0.67^{ab,A}$	$7.05\pm1.29^{ab,B}$
5. (36 ppt)	$13.54\pm1.50^{a,C}$	$4.00\pm0.63^{a,A}$	$4.27\pm0.64^{a,AB}$	$4.81\pm0.87^{ab,AB}$	$6.81\pm1.47^{ab,B}$
6. (40 ppt)	$20.00\pm2.82^{a,C}$	$4.50\pm0.70^{a,A}$	$5.00\pm0.70^{b,A}$	$5.50\pm0.70^{b,A}$	$8.00\pm1.41^{a,AB}$

Noet : Data are expressed as mean ± SD

Mean in the same rows (capital letter) or column (lower case) with the same letter are not significantly different ($P>0.05$)

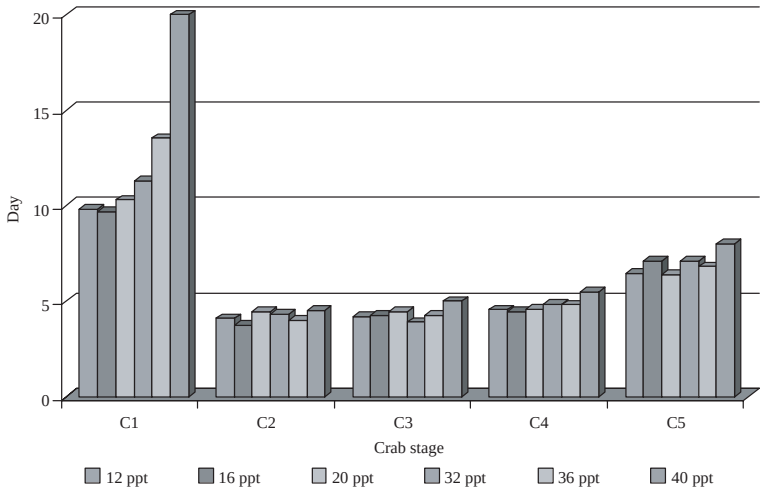

Figure 3 The average developmental period (days) from megalopa (M) to crab 1, 2, 3, 4 and 5 (C1-C5) reared under six different salinity.

CONCLUSION

Zoea reared at 30 ppt developed to megalopa stage with better survival rate (22.19 percent) than those at the other salinity levels of 28, 32 and 34 ppt. In all salinity levels, high mortality rate of zoea occured during the development of zoea 1 to zoea 3. Duration for development of zoea 1 to megalopa at all salinity levels were 23.64-24.22 days. For survival rates of crab developed from megalopa, the survival rates of crab were high among salinity levels from16-32 ppt (76.91-80.95 percent) and were significantly higher ($P<0.05$) than those at 12 and 40 ppt (42.85 and 9.52 percent, respectively). The developmental period to the fifth crab stage from megalopa was influenced by salinity. At higher salinity, the trend for longer time was needed.

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Ultrastructure of Connective Tissue Cells of Giant African Snails *Achatina fulica* (Bowdich)

Viyada Seehabutr

ABSTRACT

The connective tissue sheath of cerebral ganglion of giant African snails *Achatina fulica* (Bowdich) contain many types of cells such as pore cells, granular cells, vesicular connective tissue cells, amoebocytes, fibroblasts and muscle cells. Pore cells are characterized by numerous invagination of the cell membrane. Granular cells contain numerous granules. The vesicular connective tissue cells contain round nuclei, located in the centre of the cells. Amoebocytes have pseudopodia. Fibroblasts are spindle-shaped or elongate cells. They contain ovoid or elongate nuclei. Muscle cells have ovoid nuclei located in the centre of the cells.

Key words: connective tissue cells, pore cell, vesicular connective tissue cell, amoebocyte, fibroblast, muscle cell

INTRODUCTION

Achatina fulica (Bowdich) belongs to Phylum Mollusca, Class Gastropoda, Subclass Pulmonata, Order Stylommatophora, Family Achatinidae. The nervous system of this snail consists of 11 ganglia. Each ganglion is covered with connective tissue. There are two layers, a thick outer layer and a thin inner layer.The connective tissue of *Helix aspersa* the pulmonate snail, has a large number of globular cells that contain glycogen, muscle cells and fibroblasts (Kerkut and Walker, 1975).

In *Archachatina marginata* (Pulmonata) all ganglia are enclosed in a thick and loose connective tissue which contain many cell types and muscle fibers (Nisbet, 1961). In *Lymnaea stagnalis* (Pulmonata), the main cellular constituents of connective tissue surrounded the ganglia are fibrocytes, pigment cells, amoebocytes, granular cells and smooth muscle fibers (Wendelaar Bonga, 1970). Sminia (1972) reported that there are 8 different cell types in the connective tissue of *Lymnaea stagnalis.* These are the pore cells, the granular cells, the vesicular connective tissue cells, the amoebocytes, the fibroblasts, the undifferentiated cells, the pigment cells and the muscle cells. At the ultrastructural levels, Wendelaar Bonga (1970) found that granular cells of *Lymnaea stagnalis* contain large granules of variable sizes (0.5-4 μm) whereas the muscle cells contain thick filaments (ϕ300-600 A°). In addition, Sminia (1972) reported that the pore cells of this snail are the most conspicuous cell types in the connective tissue. These cells have many invaginations which are bridged by cytoplasmic tounges (Sminia *et al.*, 1972; Sminia and Boer, 1973). Sminia and Boer(1973) suggested that the pore cells in the connective tissue of *Lymnaea stagnalis* are protein producing cells, as they contain very extensive granular endoplasmic reticulum. Fernandez and Fernandez (1972) found that the ganglionic sheath

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of *Helix aspersa* contains pigment cells which have melanosomes (0.5-1 μm in length). They concluded that these cells are involved in melanin synthesis. In *Lymnaea stagnalis*, the amoebocytes contain round or oval nuclei, many vacuoles and a few granules in the cytoplasm. In addition, the cells possess many long pseudopodia (Sminia, 1972). Sminia (1972) suggested that these cells are involved in endocytosis. Moreover,Sminia(1972) found the vesicular connective tissue cells,the fibroblasts and the muscle cells in *L. stagnalis*. The vesicular connective tissue cells are very large (up to 60 μm). The cytoplasm of this cell is present as a thin rim against the cell membrane. The space in the cell is the storage for reserve material (Sminia, 1972). The fibroblasts are spindle-shaped or elongate and have several branching processes. Moreover, there are collagen fibrils in the cytoplasm (Sminia, 1972). The muscle cells contain the thin (ϕ 50-100 A°) actin and the thick (ϕ 300-600 A°) paramyosin filament in the cytoplasm (Sminia, 1972).

MATERIALS AND METHODS

Preparation of the specimen

The mature snails, *Achatina fulica* were collected from the wild during the rainy season. The snails with the shell length of 6.7 cm or the approximate weight of 25-30 g were used in the experiment. After the snails were anesthetized with 1 % nembutal for 30 min, they were sacrificed and the cerebral ganglia together with surrounding connective tissue sheath were dissected out.

Preparation for transmission electron microscopy

The specimens were fixed in a mixture of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 at 4°c for 1.5 hrs and washed three times with cacodylate buffer. They were post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 hrs and washed three times with distilled water. After tertiary fixation in 1% uranyl acetate for 0.5 hr, the specimens were washed in distilled water and dehydrated in graded series of ethanol (50% to 100%) and propylene oxide. Then the specimens were embedded in araldite. Semi-thin and ultra-thin sections were cut with MT-2 ultramicrotome using glass knives.The semi-thin sections were stained with 1% methylene blue in 1% borax and examined with light microscope. In addition, the ultra-thin sections were stained with saturated uranyl acetate in 70% alcohol (Watson, 1985) and lead citrate (Reynold, 1963), for 7-12 min each. Sections were then examined under a Hitachi h-300 TEM operating at 75 Kv.

RESULTS AND DISCUSSION

There are 6 different cell types found in the connective tissue sheath of cerebral ganglia (Figure 1). These are pore cells, granular cells, vesicular connective tissue cells, amoebocytes, fibroblasts,and

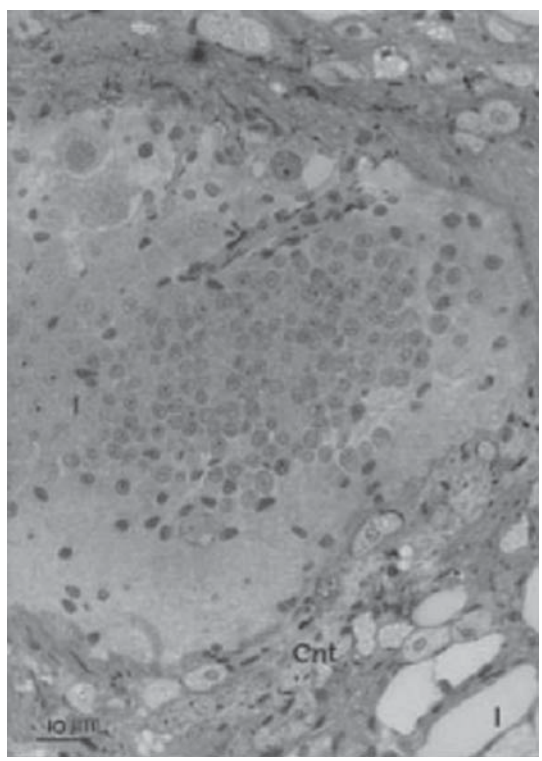


Figure 1 Photomicrograph of cerebral ganglion. Cnt = connective tissue

muscle cells.

Pore cell. There are many tubular and vesicular invaginations of the cell membrane in the pore cell. Moreover, there is many pores at the periphery of the cell, oval nucleus and many granules in the cell (Figure 2). These cells are similar to those found in *Lymnaea stagnalis* (Sminia, 1972).

Granular cell. It can be indicated that the Golgi apparatus is involved in the formation of granules in the granular cell(Figure 3) because the Golgi apparatus is rather extensive. The stacked lamellae are swollen. There are numerous round heterogeneous granules (∅2-3 μm) in the cell. The rough endoplasmic reticulums are well developed. They are located between the granules. The granular cells are commonly found in the connective tissue of gastropod (Sminia, 1972).

Vesicular connective tissue cells. These cells are called vesicular cells because they have spaces in the cell after preparing with routine fixation and staining techniques. There are round nuclei, mitochondria, small vesicles and tubules in the cell (Figure 4). These cells are abundant in the digestive gland and the gonad of gastropods (Sminia, 1972).

Amoebocyte. They are the round in shape and possess many pseudopodia (Figure 5-6).In addition,they contain nuclei, mitochondria, small vesicles and many vacuoles. These cells are also found in the blood of gastropoda (Sminia, 1972).

Fibroblasts.These cells contain elongate nuclei, rough endoplasmic reticulum, electron dense elementary granules, mitochondria, lysosome-like structures and connective tissue fibrils (Figure 7). This type of cell can be found in connective tissue of *Lymnaea stagnalis* (Sminia, 1972).

Muscle cell. They have ovoid nuclei located in the centre of the cells. The cytoplasm is occupied by filaments. Moreover, there are many pinocytic and small vesicles in the peripheral cytoplasm of the cells (Figure 8). These cells are similar to those of muscle cells of *Lymnaea stagnalis* (Sminia, 1972).

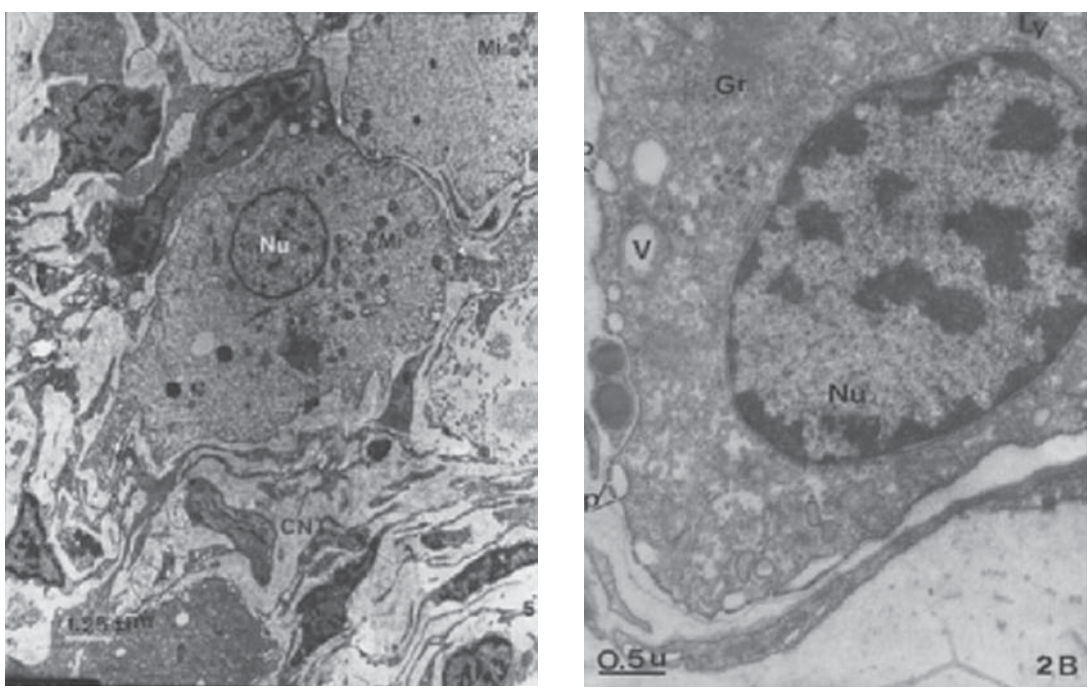


Figure 2 (A-B) Transmission electron micrographs of pore cells. (Lower left in Figure 2A) Nu = nucleus, P = pore, V = vesicular, Gr = granule

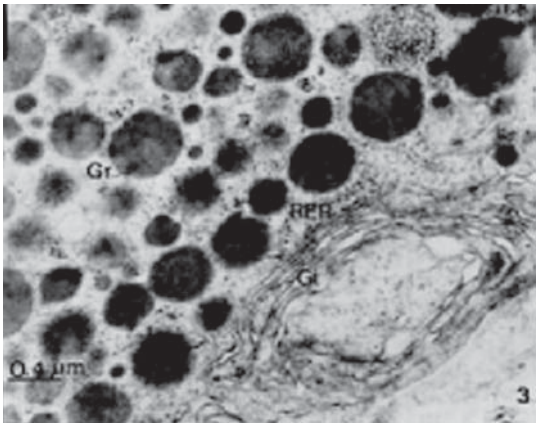


Figure 3 Transmission electron micrographs of granular cells.
Gr = granule, RER = rough endoplasmic reticulum
Gi = Golgi apparatus

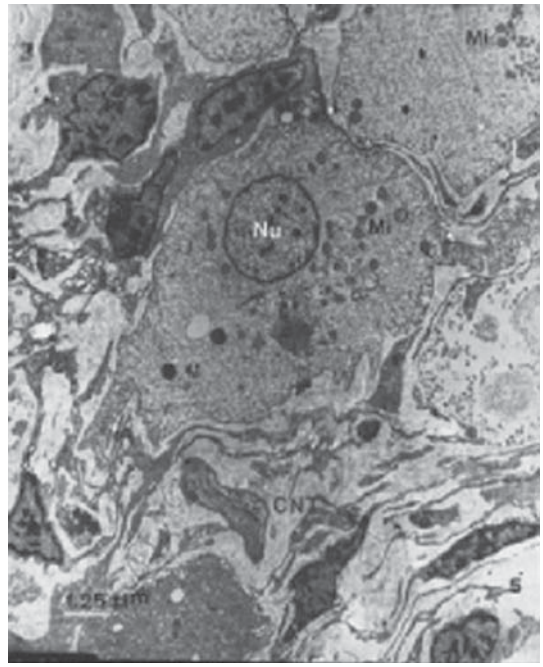
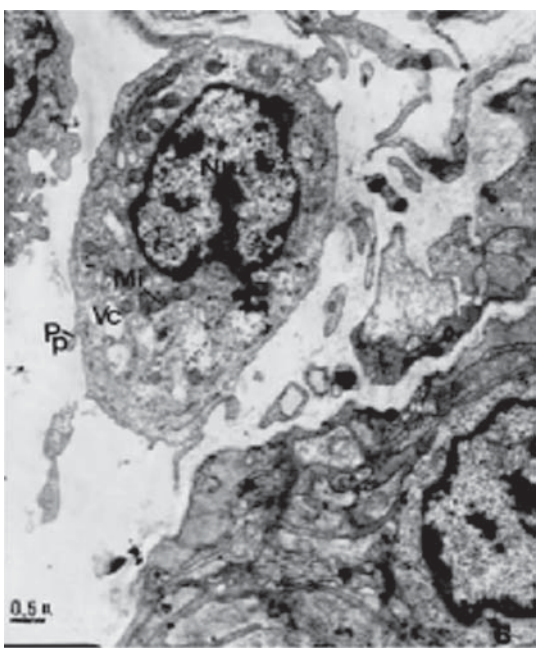


Figure 4 Transmission electron micrographs of vesicular connective tissue cell.
Nu = nucleus, Mi = mitochondria



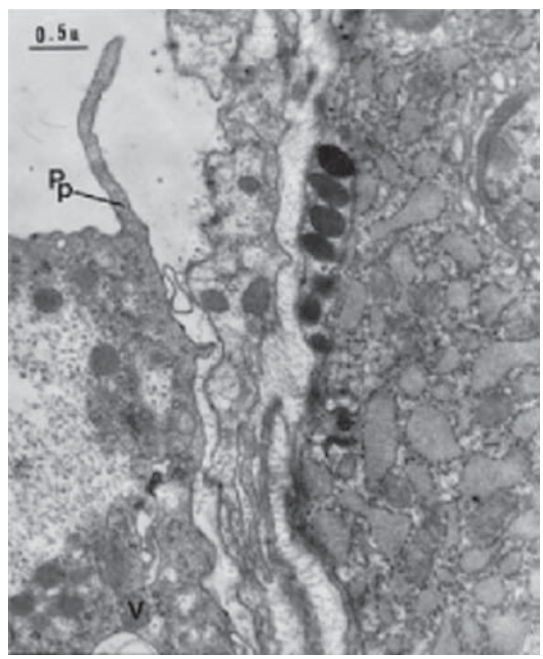


Figure 5-6 Transmission electron micrographs of amoebocytes.
Nu = nucleus, Vc = vacuole, Pp = pseudopodium, Mi = mitochondria
V = small vesicle

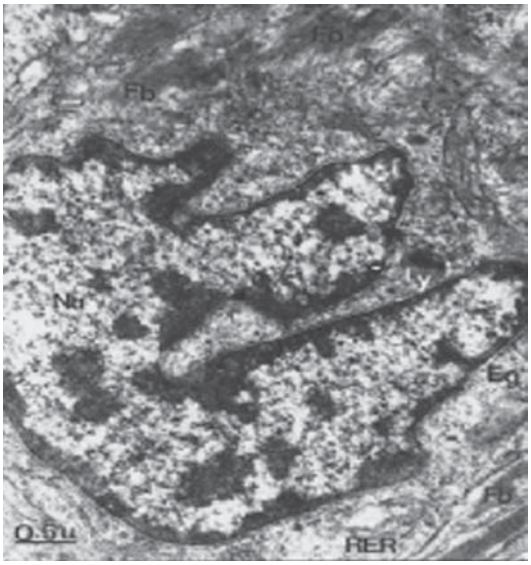


Figure 7 Transmission electron micrographs of fibroblast, Nu = nucleus, Ly = lysosome like structure, RER = rough endoplasmic reticulum, Eg = elementary granule, Fb = fibril

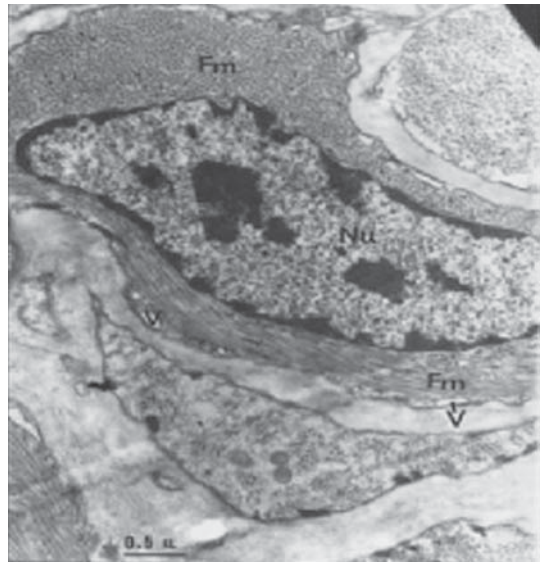


Figure 8 Transmission electron micrographs of muscle cell, Nu = nucleus, Fm = myofilaments, V = vesicle

CONCLUSION

The connective tissue sheath of the cerebral ganglion of *Achatina fulica* is composed of several cell types: pore cells, granular cells, vesicular connective tissue cells, amoebocytes,fibroblasts,and muscle cells.

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Comparative Hematology, Morphology and Ultrastructure of Blood Cells in Monocellate Cobra (*Naja kaouthia*), Siamese Spitting Cobra (*Naja siamensis*) and Golden Spitting Cobra (*Naja sumatrana*)

Chaleow Salakij[1], Jarernsak Salakij[2] and Lawan Chanhome[3]

ABSTRACT

Blood samples of 17 monocellate cobras (*Naja kaouthia*), 12 Siamese spitting cobras (*Naja siamensis*) and 6 golden spitting cobras (*Naja sumatrana*) in the Queen Saovabha Memorial Institute were collected from ventral caudal vein for basic hematology, light microscopic, scanning and transmission electron microscopic features of blood cells. There was no hematozoa detected in all monocellate cobras. Five Siamese spitting cobras (42%) and four golden spitting cobras (74%) were positive for *Hepatozoon* sp. Lymphocytes were the most commonly observed leukocytes in all cobras and average 6-8 μm in diameter. Azurophils were the second most commonly observed leukocytes, averaging 10-17 μm in diameter. Azurophils in golden spitting cobra contained more number of azurophilic granules and ribosomes than the other cobras. Heterophils were the largest leukocytes, averaging 16-19 μm in diameter. Eosinophils in monocellate cobras revealed large bulging granules and usually were medium-sized cells, averaging 10-14 μm in diameter. Eosinophils in Siamese spitting cobra were larger than those in monocellate cobra but contained smaller granules. Eosinophils in golden spitting cobra showed bulging granules but smaller than those in monocellate cobra. Basophils in all kind of cobras were similar and as small as lymphocyte but revealed many small granules polarity. Scanning electron microscopic examination revealed the surface of thrombocytes, erythrocytes, lymphocyte, azurophil and heterophil in monocellate cobra. Transmission electron microscopic examination showed organelles within azurophil and thrombocyte of Siamese spitting cobra and azurophis and gamonts of *Hepatozoon* sp. in erythrocytes of golden spitting cobra.

Key words: cobra, hematology, *Hepatozoon*, *Naja*, morphology, ultrastructure

INTRODUCTION

The cobras are in the genus *Naja*, subfamily Bungarinae which includes king cobra (*Ophiophagus*) and krait (*Bungarus*). The cobras in Thailand compose of monocellate or monocled cobras (*Naja kaouthia*), Siamese or Indo-Chinese spitting cobras (*Naja siamensis*) and golden or equatorial spitting cobras (*Naja sumatrana*), (Cox *et al*., 1998). The Queen Saovabha Memorial Institute (QSMI) has initiated a captive-breeding program since 1994 to supply healthy snakes for venom and antivenom production. These venomous snakes prey on mice and occasionally on puff-faced watersnakes. Most venomous snakes were highly infected with *Hepatozoon* sp. (Salakij *et al*., 2001).

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Morphologic characteristics of reptile blood cells are heterogeneous. Variations in cell characteristics and cell populations existed between species within the order Squamata (Alleman *et al*., 1999). Hematologic examination would be useful for evaluating the health of the snakes, however, basic hematologic values and morphology have not been described in these species. The purpose of this study was to obtain the hematologic data and morphologic characterization of blood cells in three species of cobras.

MATERIALS AND METHODS

Blood samples of 17 monocellate cobras, 12 Siamese spitting cobras and 6 golden spitting cobras were collected from ventral caudal vein during December 2000 to June 2001. The monocellate cobra was captive-breed in QSMI while the Siamese spitting cobras and the golden spitting cobras were wild-caught and reared in QSMI for 3 years. Blood smears were prepared immediately, then air-dried and stained by Wright's (W) and Wright's-Giemsa (WG) stains. The whole blood samples were kept in EDTA, stored at 4°C and processed within 2 hours. The complete hematology was performed as previous described by Salakij *et al*., 2002a.

Blood smears were fixed in methanol and stained by WG stain (Benjamin, 1978) for determination of differential leukocyte count, identification of hematozoa infection and morphological evaluation of all blood cells. *Hepatozoon* sp. infection was graded by quantifying the number of infected erythrocytes (RBCs), as described by Salakij *et al*. (2001). At least 200 WBCs were counted for differential WBC determination. For comparison of pattern of staining, blood smear from 2 samples of each cobras were stained with 1-step Wright's staining method that did not require methanol fixation prior to staining (Benjamin, 1978).

For each parameter obtained, data from each group of cobra were calculated for means, variances and standard error (SE) using SPSS for window™ (Norusis, 1993). Significant difference among means were determined using an independent sample T-test model.

For scanning electron microscopy (SEM), a drop of blood from two monocellate cobra were fixed using 1.5% glutaraldehyde (GA) in 0.1 M phosphate buffer pH 7.3 (PB) at 4°C overnight. Specimens were dehydrated through a graded acetone series, coated with gold and viewed under Jeol JSM-5600 LV scanning electron microscopy. For transmission electron microscopy (TEM), buffy coats from 2 Siamese spitting cobra and 2 golden spitting cobras were fixed in 2.5% GA and PB for 24 hours, postfixed with 1% osmium tetroxide and embedded in Spurr's epoxy resin. Ultrathin sections stained with uranyl acetate and lead citrate, were examined using Jeol 1200Ex TEM. Identification of blood cells by TEM was based on the relative number, size, shape and distribution of granules and on nuclear appearance.

RESULTS

There was no hematozoa detected in all monocellate cobras. Five Siamese spitting cobras (42%) and four golden spitting cobras (74%) were positive for *Hepatozoon* sp. (Table 1). There were no significant differences of all hematologic values among the cobras except the MCHC, absolute number of heterophils, eosinophils, plasma protein, fibrinogen and reticulocyte count (Table 2).

The *Hepaozoon* sp. gamonts found in Siamese spitting cobras were small (Figure 1a) while in golden spitting cobras the gamonts were very large (Figure 1b). The gamonts were in the cytoplasm of enlarged RBCs (Figure 1a, 1b). Some gamonts were free from RBCs but still within their parasitophorous vacuole membrane (PVM, Figure 1c). Ultrastructurally, intra-RBC *Hepaozoon* gamont was in PVM and displaced the nucleus (Figure 7c and d). Hook-like formation of PVM was seldom detected (Figure 7c). There was an

Table 1 Numbers and percentages of *Hepatozoon*-negative and positive in the cobras subgrouped according to sex.

Groups	*Hepatozoon* negative		*Hepatozoon* positive		Total
	Male	Female	Male	Female	
Monocellate cobras	15	2	0	0	17
Siamese spitting cobras	5	2	5	0	12
Golden spitting cobras	1	1	3	1	6
Total	21	5	8	1	35
%	60	14	23	3	100

Table 2 Comparative hematology (mean ± SE) among the cobras.

	Monocellate cobras	Siamese spitting cobras	Golden spitting cobras
Number	17	12	6
PCV (%)	21.2 ± 1.2	21.3 ± 1.8	18.8 ± 2.4
Hb (g/dl)	6.5 ± 0.4	6.9 ± 0.6	4.8 ± 0.7
RBC ($x10^6/\mu l$)	0.616 ± 0.052	0.576 ± 0.042	0.657 ± 0.086
MCV (fl)	362.7 ± 18.9	371.6 ± 24.9	289.3 ± 9.6
MCH (pg)	110.1 ± 5.90	120.3 ± 9.62	71.2 ± 3.1
MCHC (g/dl)	30.5 ± 0.7^a	32.3 ± 1.5^b	24.8 ± 1.7^c
WBC ($x10^3/\mu l$)	14.316 ± 1.265	12.025 ± 0.880	9.816 ± 1.046
Azurophils ($x10^3/\mu l$)	3.993 ± 0.785	3.089 ± 0.512	2.632 ± 0.623
Heterophils ($x10^3/\mu l$)	0.664 ± 0.170^a	0.226 ± 0.069^b	0.473 ± 0.146^{ab}
Eosinophils ($x10^3/\mu l$)	0.197 ± 0.061^a	0.011 ± 0.011^b	0 ± 0^{bc}
Basophils ($x10^3/\mu l$)	0 ± 0	0 ± 0	0.015 ± 0.015
Lymphocytes ($x10^3/\mu l$)	9.259 ± 0.903	8.562 ± 0.709	6.695 ± 0.590
Monocytes ($x10^3/\mu l$)	0 ± 0	0 ± 0	0.03 ± 0.2
Azurophils (%)	26.1 ± 3.7	25.2 ± 3.5	26.0 ± 4.5
Heterophils (%)	4.4 ± 1.0	1.9 ± 0.5	4.7 ± 1.3
Eosinophils (%)	1.1 ± 0.08	1.4 ± 0.3	0 ± 0
Basophils (%)	0 ± 0	0 ± 0	0.2 ± 0.2
Lymphocytes (%)	66.9 ± 4.4	71.8 ± 3.5	69.2 ± 4.1
Monocytes (%)	1.2 ± 0.4	1.0 ± 0.5	0 ± 0
PP (g/dl)	5.56 ± 0.43^a	6.84 ± 0.63^b	9.6 ± 0.4^c
Fibrinogen (mg/dl)	28.6 ± 28.6^a	0 ± 0^a	200.0 ± 44.7^c
Agg. Reticulocytes (%)	1.6 ± 0.6^{ac}	4.2 ± 0.8^b	1.5 ± 0.3^c
Punct. Reticulocytes (%)	6.8 ± 1.0^{ac}	32.7 ± 6.9^b	13.8 ± 4.5^c

The figures on the same row with the same letter are not significantly different ($p > 0.05$).

electron-lucent space between the *Hepaozoon* gamont PVM and RBC cytoplasm (Figure 7b). The gamonts possessed a nucleus, many micronemes (Figure 7b and 7c), dense granules (Figure 7c) and vacuoles.

Erythrocytes

Erythrocytes (RBCs) from all cobras were homogeneous in color but moderately anisocytosis (Figure 1). Cytoplasmic vacuoles were found in less than 1% of RBCs (Figure 1d). The young RBCs were seldom shown (Figure 1d). *Hepatozoon*–infected RBCs were larger than those non-infected ones (Figure 1a and b) and hypochromic (Figure 1a). The mitotic figure of RBCs in golden spitting cobra rarely appeared (Figure 1e). By SEM, RBCs were ellipsoid, lacking a dome appearance at the site of the nucleus (Figure 5a and b). The width of RBCs in monocellate cobra were significantly less than in Siamese spitting cobra and golden spitting cobra whist the length of RBCs in Siamese spitting cobra were significantly less than in golden spitting cobra (Table 3).

Thrombocytes

Thrombocytes were elongate and approximately half the size of mature RBCs (Figure 1f). Their aggregation turned into round cells (Figure 1f and 5a). However, they could be differentiated from lymphocytes by the characteristic perinuclear and cytoplasmic vacuolation (Figure 1f). Ultrastructurally, thrombocytes contained a nucleus, mitochondria, vacuoles and dense core granules (Figure 7a).

Leukocytes

Leukocytes (WBCs) of the cobra were categorized into 6 groups; azurophil, heterophil, eosinophil, basophil, lymphocyte and monocyte. For comparison, the blood smears stained with one step Wright's stain provided staining quality for identification of all blood cell type but in Wright's stain the RBCs stained more basophilic (Figure 1g and h).

Lymphocytes in cobras were the most prevalent circulating cells (Table 2). They were small, well differentiated averaging 6-8 μm in diameter (Figure 2e, 3e, 4e, Table 3). There were no morphologic difference among lymphocytes in three kinds of cobras. Some vacuoles were present in the cytoplasm of lymphocytes in monocellate cobras (Figure 2e) and Siamese spitting cobras (Figure 3e).

Azurophils were the second most commonly

Table 3 Comparative blood cell diameters in micrometer (mean ± SE) among the cobras.

	Monocellate cobras	Siamese spitting cobras	Golden spitting cobras
Number of cells	40	40	40
RBC (width)	9.45 ± 0.11^{a}	10.10 ± 0.12^{b}	9.90 ± 0.09^{b}
RBC (length)	16.65 ± 0.12^{a}	16.25 ± 0.18^{ab}	16.70 ± 0.16^{ac}
Azurophils	12.95 ± 0.22^{a}	11.38 ± 0.30^{b}	13.23 ± 0.27^{a}
Heterophils	16.05 ± 0.19^{a}	15.13 ± 0.25^{b}	14.95 ± 0.26^{b}
Eosinophils*	13.23 ± 0.39^{a}	15.65 ± 0.34^{b}	14.70 ± 0.50^{b}
Basophils*	8.87 ± 0.17^{a}	8.90 ± 0.19^{a}	10.05 ± 0.34^{b}
Lymphocytes	7.48 ± 0.23^{ab}	6.63 ± 0.19^{b}	7.53 ± 0.18^{a}
Monocytes*	15.87 ± 0.37^{a}	15.40 ± 0.53^{ab}	15.00 ± 0.30^{b}

The figures on the same row with the same letter are not significantly different ($p > 0.05$).
*Only 20 cells from each group were measured.

observed leukocytes, which contained fine indistinct azurophilic granules. They were round and 11-14 μm in diameter (Table 3). The nuclei were round to irregular with clump chromatin locating centrally to eccentric (Figure 2a, 3a, 4a). The cytoplasm of azurophils in golden spitting cobra were stained more azuroplilic than those of the other two cobras. Ultrastucturally, they contained numerous membrane-bound granules, some mitochondria, rough endoplasmic reticulum and many ribosomes (Figure 6a, 6b). The organelles in azurophils of Siamese spitting cobra were less than those of golden spitting cobra (Figure 6c, 6d). Monocytes were not frequently observed and their characters were similar to mammalian monocytes (Figure 2f, 3f, 4f).

Heterophils were the largest of the WBCs averaging 14-18 μm in diameter (Table 3). They

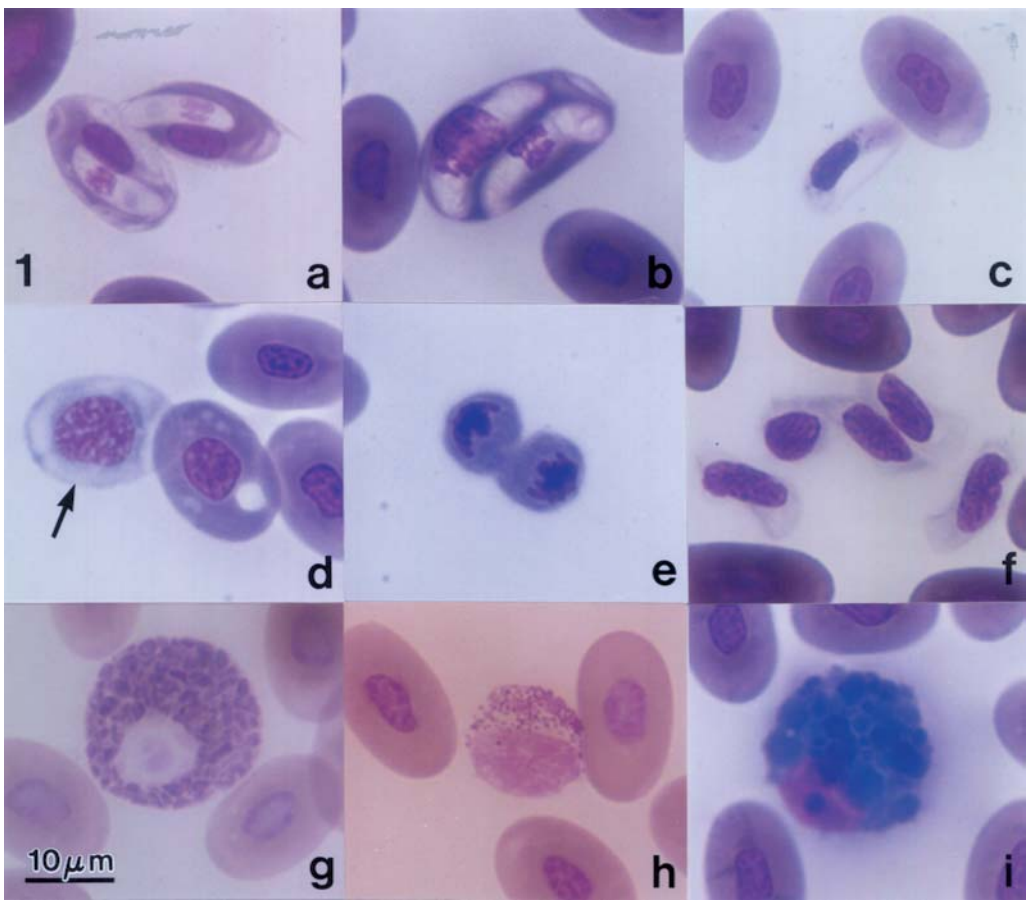

Figure 1 (a) Two gamonts of *Hepatozoon* sp. in two erythrocytes (RBCs) of Siamese spitting cobra. Note the hypochromic RBCs. (b) Two large gamonts of *Hepatozoon* sp. in one RBC of golden spitting cobra. Note the larger size than RBCs in (a). (c) Free *Hepatozoon* sp. in golden spitting cobra. (d) Young erythrocyte (arrow) and vacuole in RBC of golden spitting cobra. (e) Two daughter cells of RBCs in golden spitting cobra. (f) Five thrombocytes in monocellate cobra. Wright's-Giemsa stain (WG). (g) Hetrophil in golden spitting cobra. Wright's stain (W). (h) Basophil in golden spitting cobra. W stain. (i) The large eosinophil with 18 μm in diameter in golden spitting cobra. Note the variation in size of granules and eccentric nucleus. WG stain.

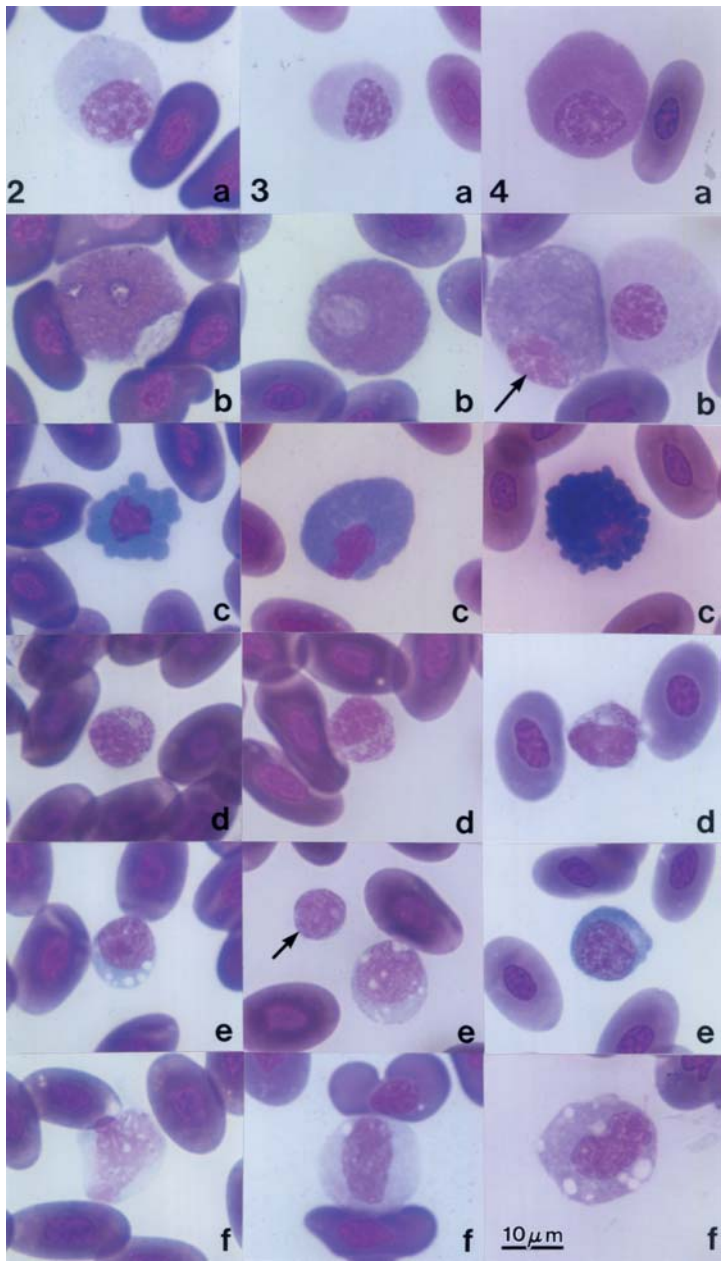

Figure 2 WBCs in the monocellate cobra. (a) An 16 μm azurophil. (b) A 19 μm heterophil. (c) An eosinophil with bulging large granules. (d) A 9 μm basophil. (e) A 10 μm lymphocyte with vacuolated cytoplasm. (f) A 17 μm monocyte. WG stain.

Figure 3 WBCs in the Siamese spitting cobra. (a) A 13 μm azurophil. (b) A 18 μm heterophil. (c) An eosinophil with many large granules packed in the cytoplasm. (d) A 10 μm basophil. (e) A 8 μm (arrow) and a vacuolated 10 μm lymphocytes. (f) A 14 μm monocyte. WG stain.

Figure 4 WBCs in the golden spitting cobra. (a) An 16 μm azurophil with dark margenta cytoplasm. (b) A 19 μm heterophil (arrow) and an azurophil. (c) An eosinophil. (d) A 10 μm basophil. (e) A 11 μm lymphocyte. (f) A 16 μm monocyte. WG stain.

contained large numbers of irregular shape, dull eosinophilic granules (Figure 2b, 3b, 4b). By Wright's stain, the heterophil granules were easily seen by reddish-orange bright color (Figure 1g). There were no morphologic difference among heterophils in three kinds of cobras. By SEM, their granule contour was bulging showing the custard apple-liked appearance (Figure 5d).

Eosinophils in monocellate cobras revealed very large bulging granules (Figure 2c) and usually were medium-sized cells, averaging 11-15 μm in diameter (Table 3). Eosinophils in Siamese spitting cobra were larger than those in monocellate cobra but contained smaller granules (Figure 3c). Eosinophils in golden spitting cobra revealed bulging granules but smaller than those in monocellate cobra (Figure 4c) and in some occasion very large cells were also detected (Figure 1i). By Wright's stain, eosinophil granules were stained dark blue the same as by WG stain. The eosinophil numbers were very low in all kinds of cobra (Table 2).

Basophils were very small, averaging 9-11 μm in diameter (Table 3) and slightly smaller than eosinophils (Figure 2d, 2c, 3d). They contained small dark purple staining metachromatic granules polarized opposite to the round eccentric nucleus. By Wright's stain, basophil granules stained darker than those in WG stain (Figure 1h).

DISCUSSION

The incidence of hematozoa infection in the Siamese spitting cobras and golden spitting cobras

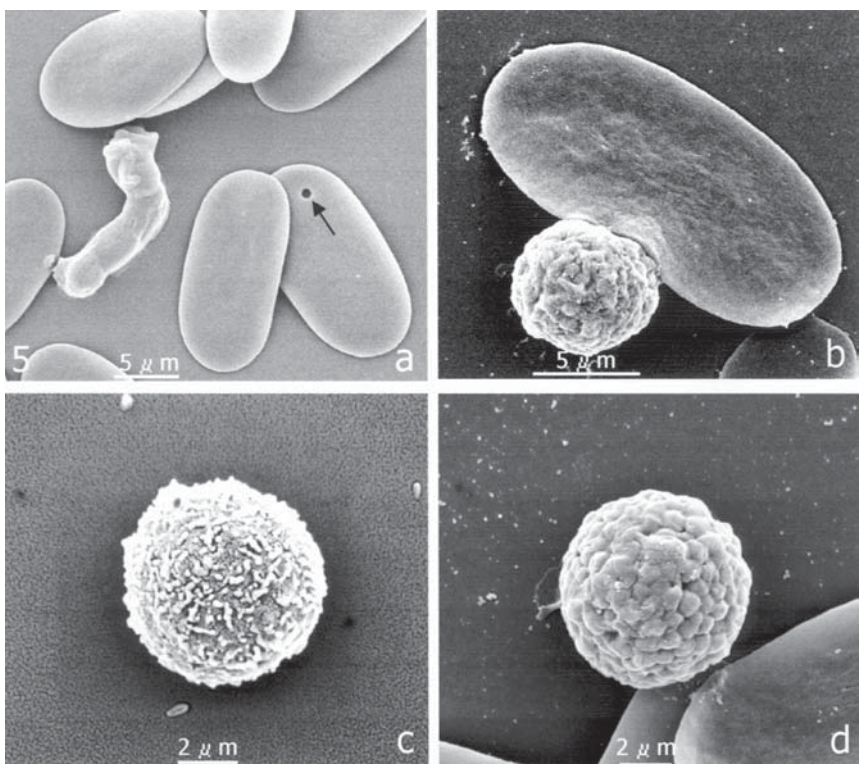

Figure 5 Scanning electron micrographs of blood cells in monocellate cobra. (a) Four aggregated thrombocytes (left) and RBC with small cytoplasmic vacuole (arrow). (b) Lymphocyte with irregular surface membrane. (c) Azurophil, showing villi-like surface. (d) Heterophil with custard apple-like appearance of the granule contour and some micropores.

were as high as the other snakes in Queen Saovabha Memorial Institute (Salakij *et al*., 2001). Some hematological values were different from the normal hematologic parameters for reptile (Mader, 2000) such as the PCV was lower whist the total WBC and the plasma protein was higher than the reference (Mader, 2000). This study also revealed that hematozoa parasitism of Siamese spitting cobras and golden spitting cobras RBCs had no effect on anemia since there were no significant differences of all RBC parameters except the MCHC. These results supported the finding of no clinical disease demonstrated in infected snakes (Campbell, 1986).

Lymphocytes in the cobras were the most prevalent circulating cells like those in King cobra (Salakij *et al*., 2002a), puff-faced watersnakes (Salakij *et al*., 2002b) and the other snakes (Mader, 2000). Some researchers characterize azurophils as monocytes with azurophilic granules (Campbell, 1986).

It was quite difficult to differentiate eosinophils from basophils in WG stained smears

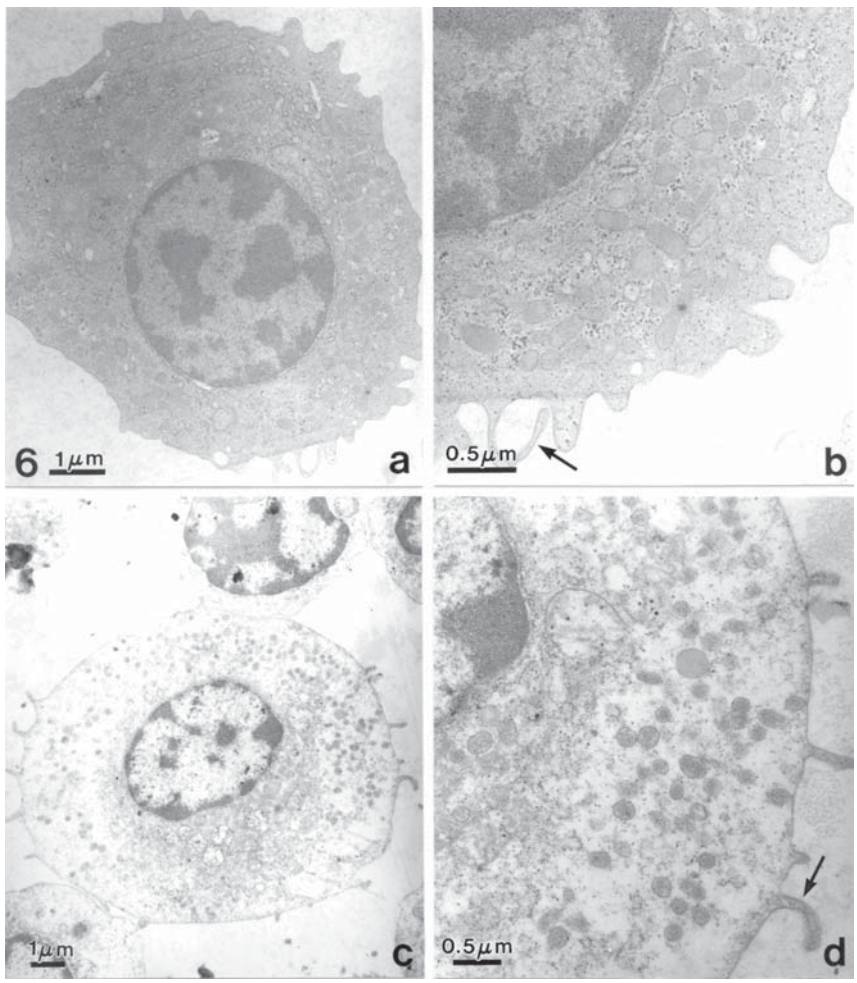

Figure 6 Transmission electron photomicrographs of (a) Azurophil from golden spitting cobra. (b) Higher magnification of the azurophil in (a) showing many granules, ribosomes and villi-like surface (arrow). (c) Azurophil from Siamese spitting cobra. (d) Higher magnification of the azurophil in (c). Note the cytoplasmic process (arrow).

because of the bluish coloration of their granules. They were identifed more easily on Wright's stained preparation. The eosinophil granule characteristic in the cobras was similar to those of iguanas and psittacines (Hawkey and Dannett, 1989) which contained dark purple staining metachromatic granules obscuring the unlobed nucleus. The large-sized eosinophils found in golden spitting cobra should be the characteristic of eosinophil in snakes which were larger than those of the other reptiles (Mader, 2000). The large bulging granules of eosinophils in monocellate cobra and golden spitting cobra were similar to some eosinophils in king cobra (Salakij *et al*., 2002a). But the granules of eosinophil in Siamese spitting cobra were not bulged out the surface but seemed to be packed in the

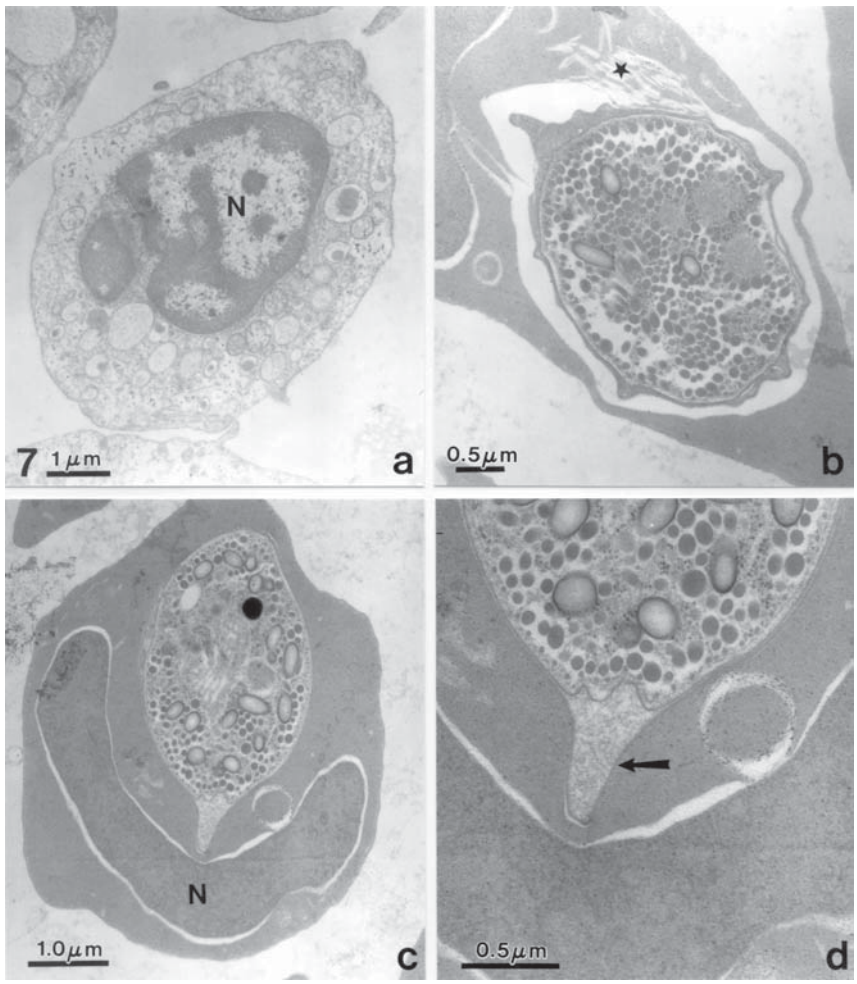

Figure 7 Transmission electron photomicrographs of (a) Thrombocyte of Siamese spitting cobra containing nucleus (N), mitochondria, vacuoles and dense core granules. (b) Cross-section of *Hepatozoon* sp. in RBC of golden spitting cobra. The hemoglobin around the protozoa was denatured (*). (c) Cross section of RBC in golden spitting cobra containing *Hepatozoon*. Note the displacement of the host nucleus (N). The *Hepatozoon* gamont contains Golgi apparatus, one dense granule and may micronemes. (d) Higher magnification of a *Hepatozoon* in (c). Note hook-appearance of the parasitophorous vacuole membrane (arrow).

cytoplasm.

The low number of eosinophils in the cobras (Table 2) and their bluish granules might make it misidentified as basophils. The finding of eosinophils in all kinds of cobras supported the existence of these leukocytes in snakes eventhough they were not identified in eastern diamondback rattlesnakes (Alleman *et al*., 1999).

The small gamonts of *Hepatozoon* sp. found in Siamese spitting cobras were similar to those found in the banded krait (*Bangarus fasciatus*) of the QSMI (Salakij *et al*., 2001). The large gamonts found in golden spitting cobras were referred as the large gamonts found in mangrove snakes and mangrove pit vipers of the QSMI (Salakij *et al*., 2001). The other hematozoas; *Haemogregarina* sp. and trypanosomes found in puff-faced watersnakes of the QSMI (Salakij *et al*., 2002b) were not detected in the cobras.

These results provided comparative hematologic data and a guide for identification of blood cells in nonocled, Siamese spitting and golden spitting cobras. *Hepatozoon* infection was relatively common in wild-caught Siamese spitting and golden spitting cobras but not in captive-bred monocellate cobras. The study may be beneficial for further study and related research.

ACKNOWLEDGMENTS

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Synchronous Fluorescence Spectroscopic Technique: The Tool for Rapid Identification of Polycyclic Aromatic Hydrocarbons (PAHs) at Sub-ppm Level in Liquid Samples

Apisit Songsasen, Sornnarin Bangkedphol and Pornpun Pornsinlapatip

ABSTRACT

Various polycyclic aromatic hydrocarbons (PAHs) at sub-ppm level were identified qualitatively by synchronous scanning fluorescence technique at various wavelength intervals ($\Delta\lambda$). Due to the difference in chemical structure, each PAHs gives specific characteristic spectrum for each wavelength intervals ($\Delta\lambda$). This work demonstrated that the synchronous scanning fluorescence method can be used as a tool for the rapid identification of PAHs in ethanolic sample which contain three or six types of PAHs such as fluorene, truxene, benzo(k) fluoranthene, carbazole, chrysene, anthracene, acenaphthene and indeno(1,2,3,cd)pyrene in mixture.

Key words: polycyclic aromatic hydrocarbons, synchronous scanning, spectrofluorometry

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants in environment, consisting of two or more fused benzene rings in linear, angular or cluster arrangement. Substitution of carbon in benzene ring with nitrogen, sulfur, oxygen or other elements gives heterocyclic compounds which are also classified as PAHs. Crystalline solid of PAHs has high melting points and low vapor pressures. Unlike most hydrocarbons, PAHs are usually colored. PAHs are produced naturally by combustion processes, e.g. forest fires, volcanic activity, etc., and anthropogenically via industrial processes, particularly the combustion of fossil fuels for heating, power and transport. There was also strong evidence indicating that PAHs may be produced by bacteria and plants (Gibson, 1995).

PAHs in the atmosphere can be polluted in many kinds of environmental samples such as soil, rain, river, underground water, plants, etc., which many are known to be carcinogenic agents. Figure 1 shows the chemical structure of the sixteen unsubstituted PAHs identified by The Environmental Protection Agency (EPA) as potential carcinogens (Boonyatumanond, 1999).

The relation between PAHs and human cancer is strongly suggested by their occurrence in environment and their carcinogenic pathways. For example, lung cancer mortality that related to PAHs has been increasing in many countries. It is not only necessary to distinguish between acute and chronic toxicity thresholds, but also important to know the toxic chemical species (Lakowicz, 1999).

The toxicity of the PAHs in environment depends on the types and quantity of each PAHs. Environmental samples, usually consist of more than one PAHs. Therefore, a rapid and simple qualitative analysis for preliminary identification of PAHs in samples is important especially when a

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large number of samples need to be analyzed. The structures of polycyclic aromatic hydrocarbons (PAHs) are have aromatic system that electron can delocalize from $\pi \rightarrow \pi^*$ which cause fluorescence phenomena (Skoog, 1985). Therefore, the spectrofluorometric method was considered to be simple and time saving method. Thus, the development of the spectrofluorometric method to make it more suitable for the determination of PAHs in sample is necessary (Schulman, 1999).

Vo-Dinh (1978) used synchronous excitation technique to improve the selectivity of luminescence spectrometry. This approach offered several advantages, including narrowing of spectral bands,

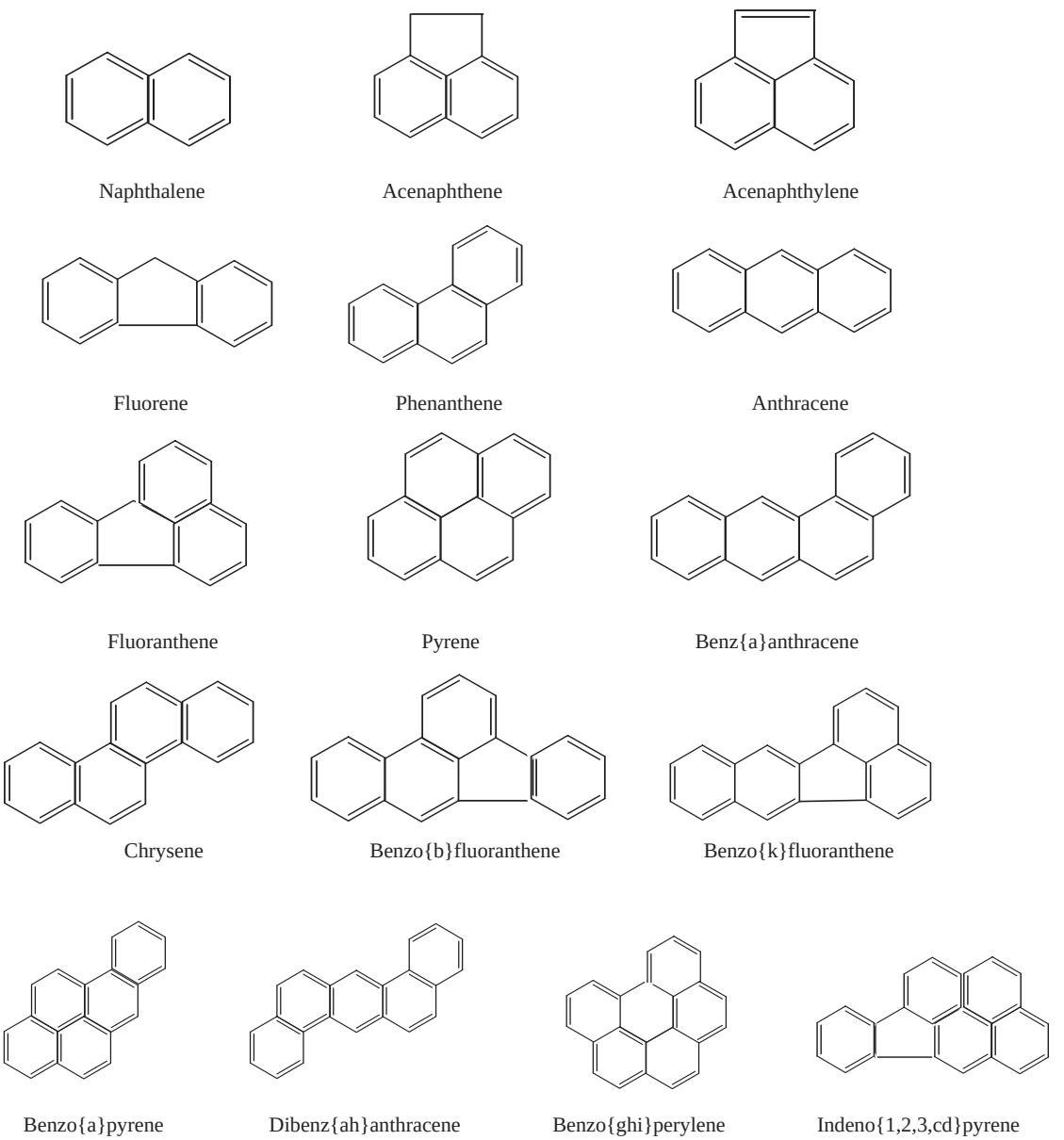

Figure 1 Chemical structure of the sixteen PAHs which were identified by The Environmental Protection Agency (EPA) as priority pollutants.

an enhancement in selectivity by spectral simplification, and a decrease of measurement time in multicomponent analysis. Vo-Dinh *et al*. (1980) used fluorometric method to identify polycyclic aromatic compounds in a synthetic fuel. Several trace and major components including benzo[a]pyrene, chrysene, fluorene, fluoranthene, phenanthrene and pyrene has been identified and determined at a concentration range of 10 - 1,000 ppm. It was found that the selectivity of the fluorometric analysis is greatly improved by synchronous excitation scanning. In this work, synchronous scanning technique was used for qualitative analysis of PAHs mixture in liquid samples at a sub-ppm level.

MATERIALS AND METHODS

Reagents

All reagents used were analytical reagent grade. Truxene, fluorene and carbazole were purchased from Chem Service (West Chester, USA). Benzo(k)fluoranthene, anthracene and chrysene were purchased from Fluka (Steinheim, Switzerland). Acenaphthene and indeno(1,2,3,cd)pyrene were purchased from BDH (Poole, England). Ethanol was purchased from Merck (Darmstadt, Germany). Hydrochloric acid was purchased from Lab-Scan (Bangkok, Thailand).

Experimental procedures

- Preparation of standard solution

Stock standard solution (10 ppm)

Five hundred micrograms of each PAHs standard was accurately weighed. Each PAHs standard was dissolved with ethanol and then made up to the volume of 50 ml in a volumetric flask. The stock solution was kept at the temperature close to 0°C and absence of light.

Intermediate standard solution

Each of the intermediate standard solution of PAHs was diluted from stock standard solution (10 ppm) with ethanol to 1 ppm.

Working standard solution

Working standards were prepared by diluting the intermediate standard solution (1 ppm) to sub-ppm levels (0.005-0.04 ppm). The dilution of each PAHs standard depended on the sensitivity of the synchronous signal obtained from the spectrofluorometer in the presence of ethanol as solvent. Each of the working standard solution and pure ethanol were measured by spectrofluorometer using synchronous scanning technique at various wavelength interval ($\Delta\lambda$) from 1-300 nm.

- Preparation of synthetic samples

A mixture consisting of benzo(k)fluoranthene, fluorene and truxene standard, a mixture consisting of chrysene, acenaphthene and indeno(1,2,3,cd)pyrene standard and a mixture consisting of benzo(k)fluoranthene, carbazole, chrysene, anthracene acenaphthene and indeno(1,2,3,cd)pyrene standard

Each mixture was prepared by diluting each of the intermediate standard solution (1 ppm in ethanol) to 0.01 ppm in the presence of ethanol as solvent. Each mixture was analyzed by spectrofluorometer using synchronous scanning technique at various wavelength interval ($\Delta\lambda$) from 1-300 nm.

Apparatus

Spectrofluorometer

The samples were analyzed by using a Varian Cary Eclipse spectrofluorometer. The spectrum was recorded at the wavelength interval ($\Delta\lambda$) of 1-300 nm, excitation wavelength of 200-500 nm for each standard PAHs and synthetic samples.

Electrical balance

An analytical balance (PERKIN ELMER AD-4 Autobalance, 0.05 mg-200 mg, USA.) was used for preparation of standard solution and synthetic samples.

RESULTS AND DISCUSSION

Table 1 shows the wavelength interval (Δλ) which give characteristic synchronous scanning fluorescence spectrum for each PAHs in ethanol. Some of these spectra are shown in this article. The results suggested that synchronous fluorescence spectroscopic method can be used as a tool for the qualitative analysis for the PAHs in mixtures. Each PAHs gave characteristic spectrum at each Δλ due to the difference in chemical structure of each PAHs. The characteristic peaks of fluorene, truxene, benzo(k)fluoranthene, carbazole and anthracene begin to appear when Δλ is less than 10 nm. For acenaphthene and chrysene, the characteristic peaks appear at Δλ equal to 10 nm and higher than 30 nm, respectively. The characteristic peaks of indeno (1,2,3,cd)pyrene begin to appear at Δλ equal to 110 nm. These suggested that six PAHs including benzo(k)fluoranthene, carbazole, chrysene, anthracene, acenaphthene and indeno(1,2,3,cd) pyrene can be separated qualitatively by synchronous scanning fluorescence.

Synthetic sample between benzo(k) fluoranthene, fluorene and truxene

To identify each PAHs in the synthetic mixture of benzo(k)fluoranthene, fluorene and truxene, the synchronous fluorescence spectrum at Δλ 20 nm of synthetic sample at the concentration of 0.01 ppm for each PAHs (Figure 2) was compared with the synchronous fluorescence spectra of each standard PAHs at the same Δλ (Figure 2 to Figure6).

From Figure 2, there were three groups of peak in the spectrum which indicated the presence of benzo(k)fluoranthene, fluorene and truxene. The triplet peaks at the excitation wavelength of 277.96, 289.06 and 299.07 nm indicated the presence of fluorene. The peak at the excitation wavelength of 336.00 nm indicated the presence of truxene. The doublet peaks at the excitation wavelength of 381.07 and 401.07 nm indicated the presence of benzo(k)fluoranthene. Thus, this showed that synchronous scanning fluorescence method at only Δλ of 20 nm can be used to identifiy the species of each PAHs in the mixture consisting of 0.01 ppm of benzo(k)fluoranthene, fluorene and truxene.

Synthetic mixture of acenaphthene, chrysene and indeno(1,2,3,cd)pyrene

To identify each PAHs in synthetic mixture of acenaphthene, chrysene and indeno(1,2,3,cd) pyrene by synchronous scanning fluorescence technique, Δλ at 95 and 110 nm have to be considered. Acenaphthene and chrysene in the sample can be identified by comparing the synchronous fluorescence spectra at the Δλ of 95 nm of synthetic sample at the concentration of 0.01

Table 1 Wavelength interval (Δλ) which give characteristic synchronous scanning fluorescence spectrum for each PAHs when ethanol was used as solvent.

Type of PAHs	Wavelength interval (Δλ)
Truxene	5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65
Fluorene	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35 40, 45, 50, 55, 60
Benzo(k)fluoranthene	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70,75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200
Carbazole	2, 3, 4, 5, 6, 7, 8, 9, 10, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110
Anthracene	2, 3, 4, 5, 6, 7, 8, 9, 10, 120, 130, 140, 170
Acenaphthene	10, 15, 20, 25, 30, 35, 45, 90, 95
Chrysene	50, 90, 95, 100, 110, 120
Indeno(1,2,3,cd)pyrene	110, 120, 180,190

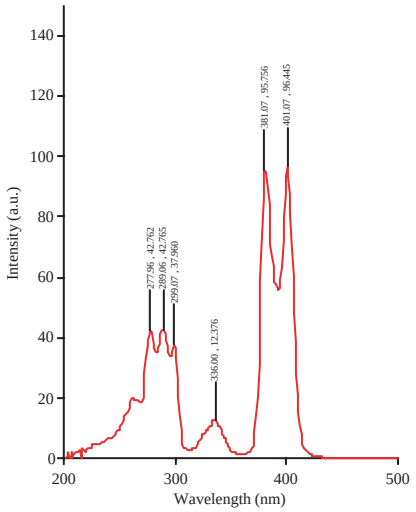

Figure 2 The synchronous spectrum of benzo(k) fluoranthene, fluorene and truxenein ethanol at Δλ = 20 nm.

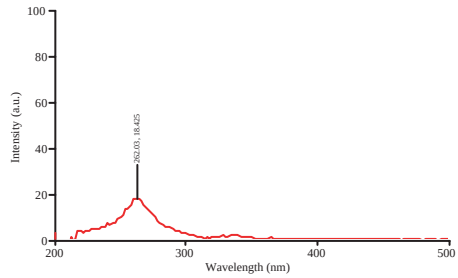

Figure 3 The synchronous spectrum of solvent (ethanol) at Δλ = 20nm.

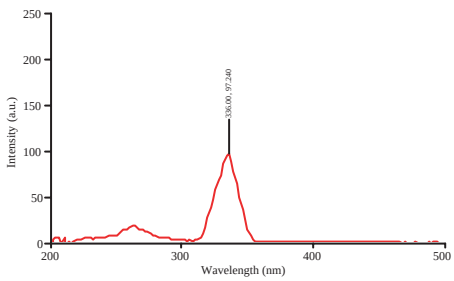

Figure 4 The synchronous spectrum of truxene at Δλ = 20nm.

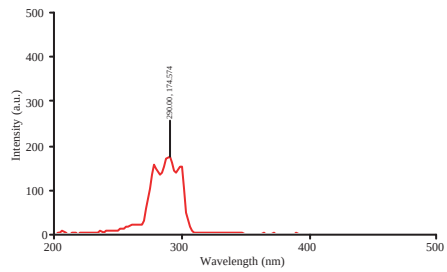

Figure 5 The synchronous spectrum of fluorene at Δλ = 20nm.

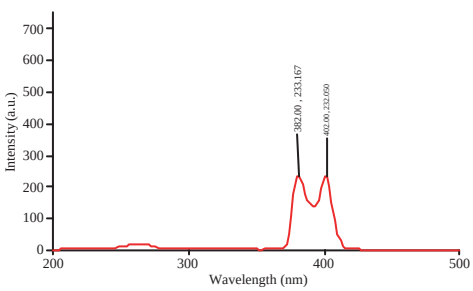

Figure 6 The synchronous spectrum of benzo(k) fluoranthene at Δλ = 20 nm. 2220 nm.

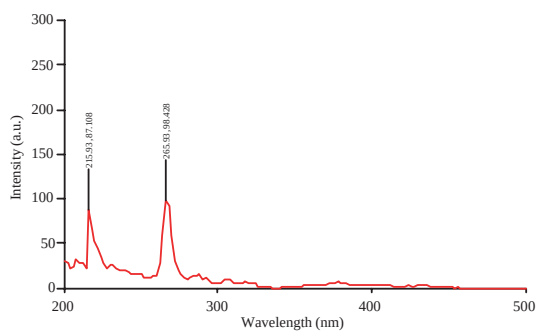

Figure 7 The synchronous spectrum of chrysene, acenaphthene and indeno(1,2,3,cd) pyrene.

ppm for each PAHs (Figure 7) with the synchronous fluorescence spectra of ethanol, acenaphthene and chrysene at the same Δλ (Figure 8 to Figure 10).

The peak at the excitation wavelength of 215.93 and 265.93 nm indicated the presence of acenaphthene and chrysene in the sample respectively. However, the peak of acenaphthene was shifted by 10 nm from the standard acenaphthene (excitation wavelength of 225.93 nm). Nevertheless, this still confirms that there was acenaphthene in the sample because acenaphthene is the only PAHs that can give the peak at the excitation wavelength around 225.93 nm. The presence of indeno(1,2,3,cd)pyrene in the sample was identified by considering the synchronous spectra of synthetic mixture, ethanol, indeno (1,2,3,cd)pyrene and the other two PAHs in the mixture at the Δλ of 110 nm (Figure 11 to Figure 15). At the Δλ of 110 nm, only indeno(1,2,3,cd) pyrene give characteristic peak at the excitation wavelength higher than that of 350 nm. This showed that the synchronous scanning fluorescence technique can be used to identify each PAHs from the synthetic mixture of acenaphthene, chrysene and indeno(1,2,3,cd)pyrene.

Synthetic mixture of benzo(k) fluoranthene, carbazole, chrysene, anthracene acenaphthene and indeno(1,2,3,cd)pyrene at the concentration of 0.01 ppm for each PAHs

To identify each PAHs in the synthetic mixture of benzo(k)fluoranthene, carbazole, chrysene, anthracene acenaphthene and indeno (1,2,3,cd)pyrene by synchronous scanning fluorescence technique, the Δλ at 6 nm, 20 nm, 95 nm and 110 nm have to be considered. Carbazole, benzo (k) fluoranthene and anthracene in the sample can be identified by comparing the synchronous fluorescence spectra at the Δλ of 6 nm of synthetic

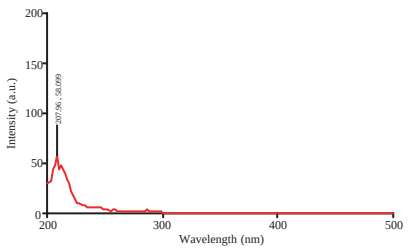

Figure 8 The synchronous spectrum of solvent (ethanol) at Δλ = 95nm.

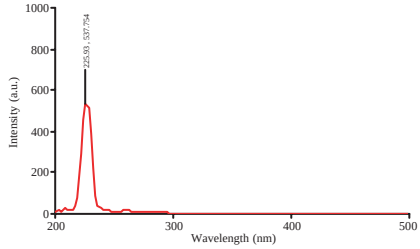

Figure 9 The synchronous spectrum of acenaphthene at Δλ = 95nm.

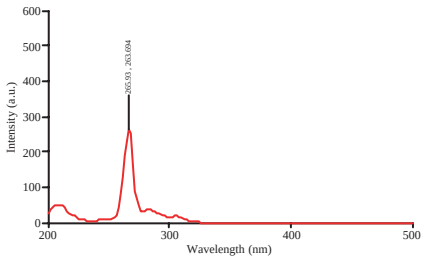

Figure 10 The synchronous spectrum of chrysene at Δλ = 95 nm.

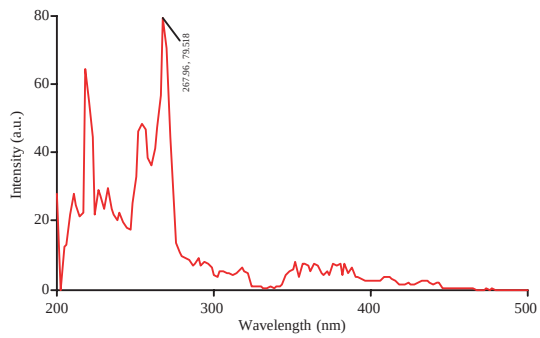

Figure 11 The synchronous spectrum of chrysens, acenaphthene and indeno(1,2,3,cd) pyrene in ethanol at Δλ = 110 nm.

sample at the concentration of 0.01 ppm for each PAHs (Figure 16) with the synchronous fluorescence spectra of ethanol, carbazole, benzo(k)fluoranthene and anthracene at the $\Delta\lambda$ of 6 nm (Figure 17 to Figure 20).

The peaks at the excitation wavelength of 336.00, 374.00 and 400.00 nm indicated the presence of carbazole, anthracene and benzo(k)fluoranthene

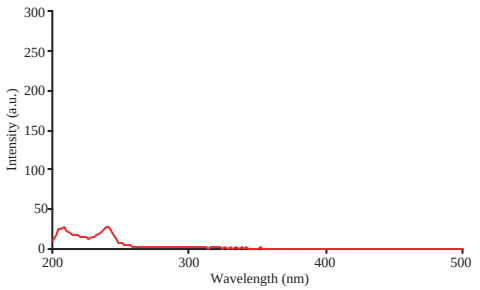

Figure 12 The synchronous spectrum of solvent (ethanol) at $\Delta\lambda$ = 110 nm.

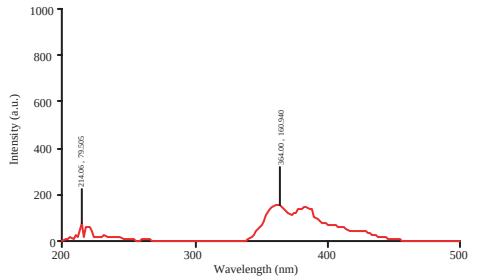

Figure 13 The synchronous spectrum of indeno (1,2,3,cd)pyrene at $\Delta\lambda$ = 110 nm.

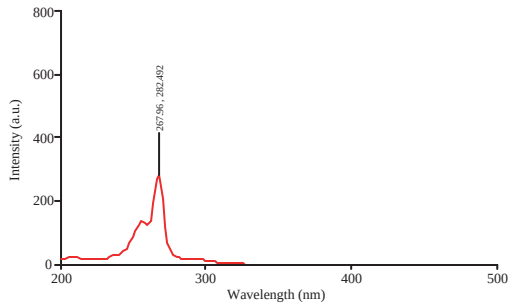

Figure 14 The synchronous spectrum of chrysene at $\Delta\lambda$ = 110 nm.

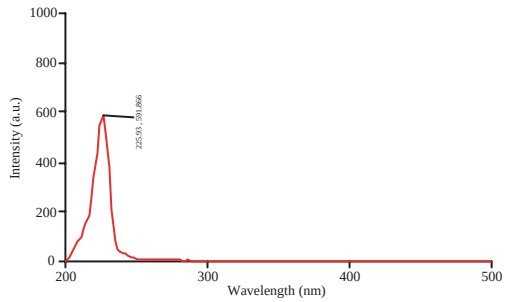

Figure 15 The synchronous spectrum of acenaphthene at $\Delta\lambda$ = 110 nm.

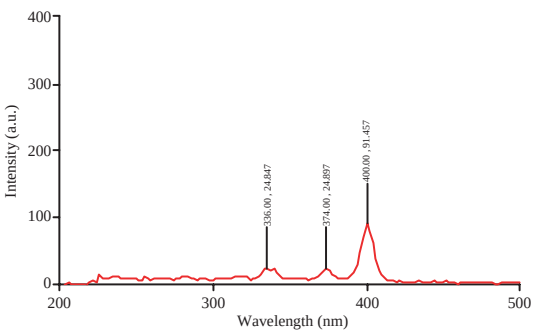

Figure 16 The synchronous spectrum of benzo(k) fluoranthene, carbazole, chrysene, anthracene, acenaphthene and indeno (1,2,3,cd)pyrene in ethanol at $\Delta\lambda$ = 6 nm.

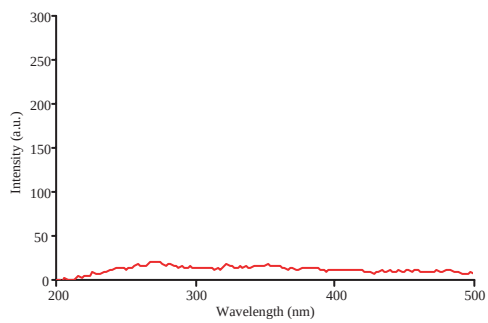

Figure 17 The synchronous spectrum of solvent (ethanol) at $\Delta\lambda$ = 6 nm.

in the sample respectively. For the identification of acenaphthene, the synchronous fluorescence spectra at the $\Delta\lambda$ of 20 nm have to be considered (Figure 21 to Figure 23).

The presence of acenaphthene in the sample was suggested by a peak at the excitation wavelength of 302.00 nm. However, the peak of acenaphthene was shifted by 2 nm from the standard acenaphthene

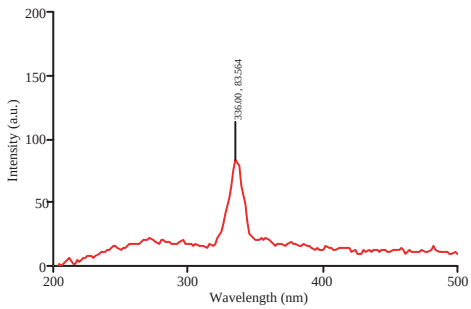

Figure 18 The synchronous spectrum of carbazole at $\Delta\lambda$ = 6 nm.

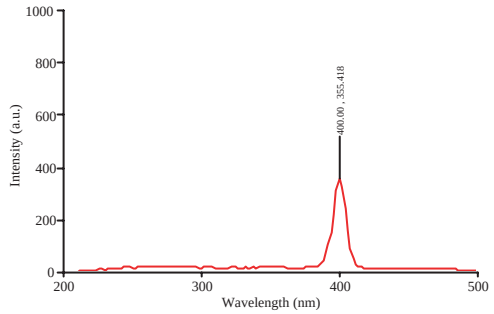

Figure 19 The synchronous spectrum of benzo(k) fluoranthene at $\Delta\lambda$ = 6 nm.

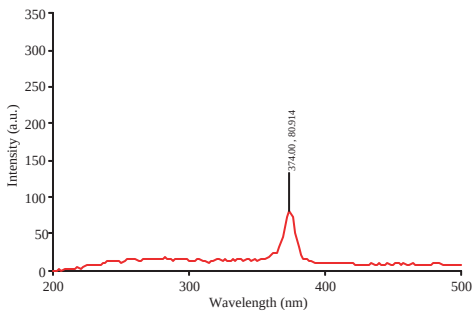

Figure 20 The synchronous spectrum of anthracene at $\Delta\lambda$ = 6 nm.

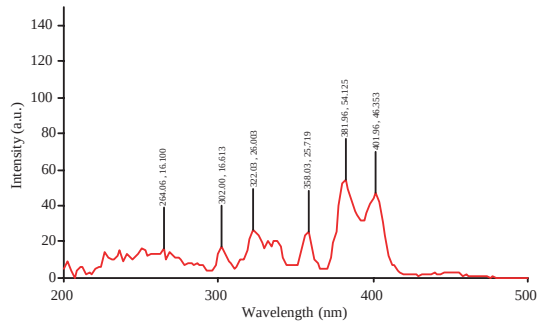

Figure 21 The synchronous spectrum of benzo(k) fluoranthene, carbazole, chrysene, anthracene, acenaphthene and indeno (1,2,3,cd)pyrene in ethanol at $\Delta\lambda$ = 20 nm.

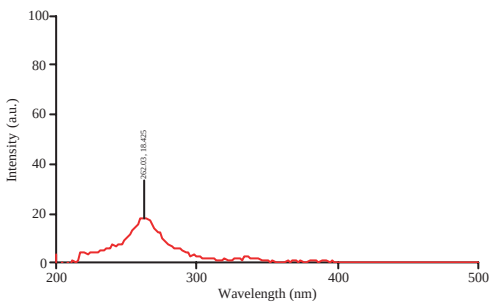

Figure 22 The synchronous spectrum of solvent (ethanol) at $\Delta\lambda$ = 20 nm.

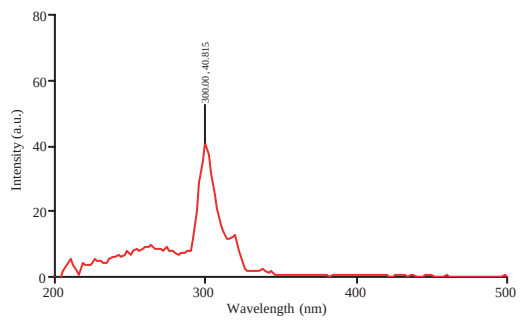

Figure 23 The synchronous spectrum of acenaphthene at $\Delta\lambda$ = 20 nm.

(excitation wavelength of 300.00 nm). Nevertheless, this still confirms that there was acenaphthene in the sample because acenaphthene is the only PAHs that can give the peak at the excitation wavelength around 300.00 nm. The presence of chrysene in the sample was proved by comparing the chrysene of synthetic mixture, ethanol and chrysene at the Δλ of 95 nm (Figure 24 to Figure 26).

The peak at the excitation wavelength of 265.93 nm indicated that there was chrysene in the sample. The presence of indeno(1,2,3,cd)pyrene in the sample was identified by considering the synchronous spectra of synthetic mixture, ethanol, indeno(1,2,3,cd)pyrene and other PAHs in the mixture at the Δλ of 110 nm (Figure 27 to Figure 34). At the Δλ of 110 nm, only indeno(1,2,3,cd) pyrene gives the characteristic peak at the excitation wavelength higher than that of 350 nm. These results suggested that the synchronous scanning fluorescence technique can be used to identify each PAHs from synthetic mixture of benzo(k) fluoranthene, carbazole, chrysene, anthracene acenaphthene and indeno(1,2,3,cd)pyrene.

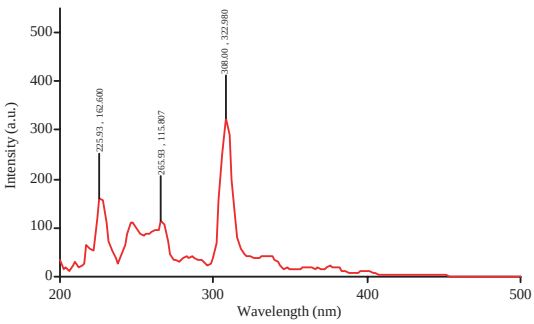

Figure 24 The synchronous spectrum of benzo(k) fluoranthene, carbazole, chrysene, anthracene, acenaphthene and indeno (1,2,3,cd)pyrene in ethanol at Δλ = 95 nm.

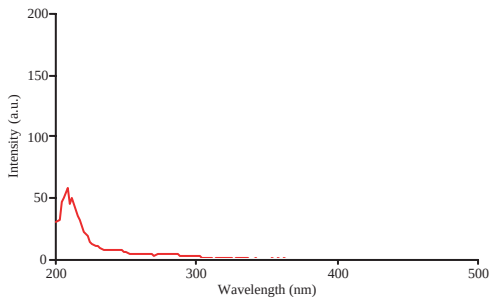

Figure 25 The synchronous spectrum of solvent (ethanol) at Δλ = 95 nm.

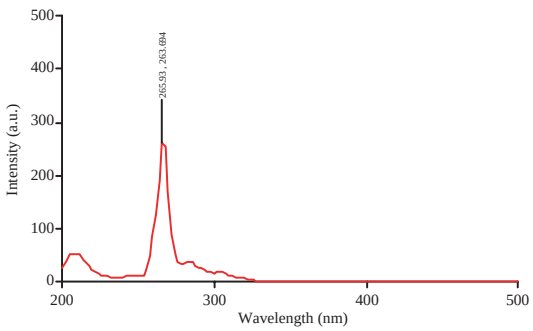

Figure 26 The synchronous spectrum of chrysene at Δλ = 95 nm.

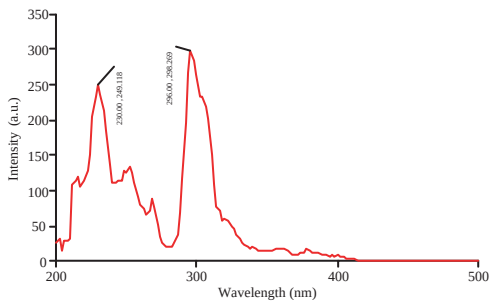

Figure 27 The synchronous spectrum of benzo(k) fluoranthene, carbazole, chrysene, anthracene, acenaphthene and indeno (1,2,3,cd)pyrene in ethanol at Δλ = 110 nm.

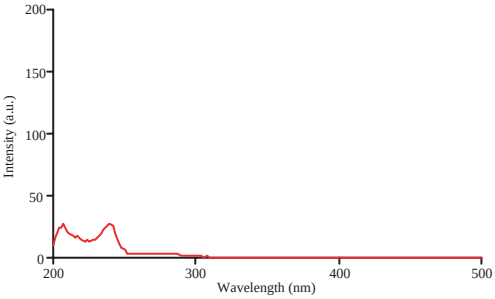

Figure 28 The synchronous spectrum of solvent (ethanol) at Δλ = 110 nm.

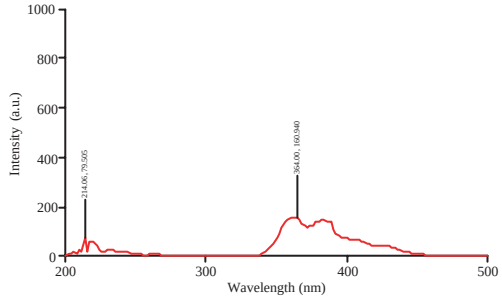

Figure 29 The synchronous spectrum of indeno (1,2,3,cd)pyrene at Δλ = 110 nm.

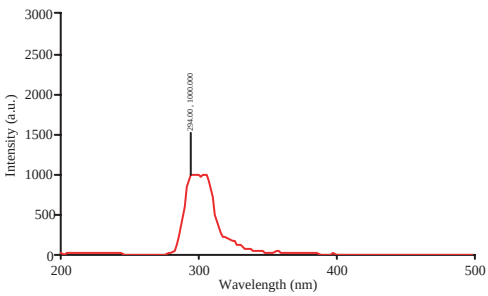

Figure 30 The synchronous spectrum of benzo(k) fluoranthene at Δλ = 110 nm.

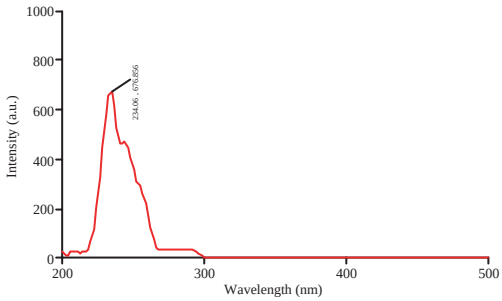

Figure 31 The synchronous spectrum of carbazole at Δλ = 110 nm.

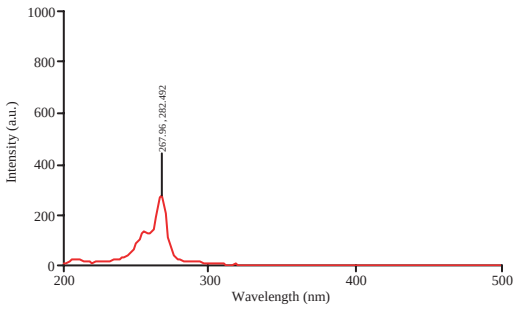

Figure 32 The synchronous spectrum of chrysene at Δλ = 110 nm.

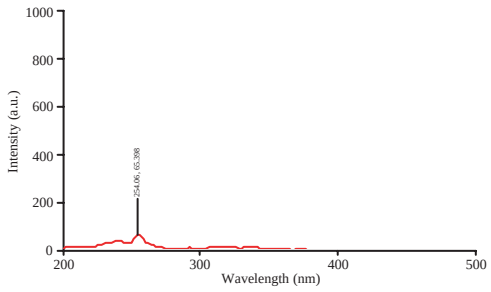

Figure 33 The synchronous spectrum of anthracene at Δλ = 110 nm.

CONCLUSION

The results of the three synthetic mixtures from the previous section suggested that the synchronous scanning fluorescence method can be used to identify each PAHs in synthetic mixtures. The number of wavelength interval (Δλ) to be considered depending on the species and numbers of PAHs presence in the analytical mixture. Comparing with other methods, such as gas chromatography and high-performance liquid chromatography, the synchronous scanning fluorescence method is more convenient than other methods because of less time consuming and the simplicity in preparation of samples. This works shown that the synchronous scanning fluorescence

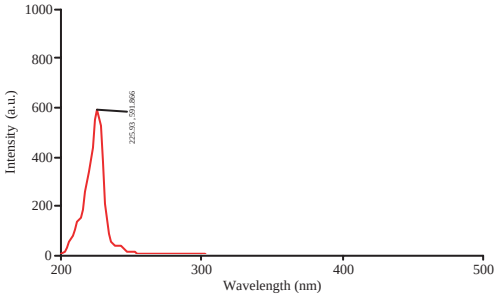

Figure 34 The synchronous spectrum of acenaphthene at $\Delta\lambda$ = 110 nm.

technique can be applied for the qualitative analysis of PAHs from the environmental samples which were extracted by ethanol. However, the use of synchronous scanning fluorescence technique for the qualitative analysis of PAHs in others organic solvents is also possible and would be more useful for the identification of PAHs in the environmental samples.

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The Study of Water Allocation in Klong Yai Basin

Thanet Somboom and Kobkiat Pongput

ABSTRACT

Klong Yai Basin is sub-basin of east coast basin, and watershed area is 1,830 sq. kilometers in Rayong and Chonburi Province. The existing Dokrai reservoir and Nongpralai reservoir are main reservoirs and Klong Yai reservoir was suggested to support more water usage.

A mathematical model, Acres Irrigation Support Package (AISP) was selected to simulate water allocation in Klong Yai basin at normally case (in 2000) and forecasting case (in 2009). AISP will calculate results from 1970 to 2000 to decide the operating rule curve and evaluation water delivery from external indicator was selected for water allocation in Klong Yai Basin.

It was found that, the effective rainfall in dry seasons is more than wet season, at the same time the irrigation efficiency in dry season is 60 % and wet season is 55 %. Normally case in 2000, inflow rule and reservoir rule curve of Dokrai reservoir and Nongpralai reservoir will be used to operate the reservoirs in each year. In forecasting case in 2009, Reservoir rule curve of Klong Yai reservoir will be used to forecast water allocation in Klong Yai basin. The evaluation of water delivery found that, irrigation supply is 0.449 from industry supply, at the same time industry supply is 0.459 from reservoir capacity and irrigation supply is 1.697 from net irrigation demand which irrigation supply in dry season more than wet season.

Key word: water allocation, Klong Yai Basin

INTRODUCTION

As the result of agricultural, industrial and consumption expansion, the demand of water in Thailand is increasing rapidly. It is, however, difficult to use any constructions such as dams or opening new irrigating area dealing with water management because of shortage of capital and being fought against by disadvantageous groups. In stead of constructing, water allocation and water delivery system are likely to be the efficiently measures.

To fulfil water allocation from cost water source, the relevant offices must completely share their responsibility. The basin consisting of many cost water sources and varieties of water use activities needs good water management, suitably for its activities and avoiding water shortage. Accordingly, to the National Economic and Social Development Plan, the development was designed to utilize for economy, society and the people's life folk.

The reason for choosing Klong Yai Basin as the demonstrated area is that there are varieties of water use in this area- agricultural, industrial and consumption as well as water delivery between existing water supply and cross-cost water source. It is essential to allocate water in order to avoid shortage of water in this area. Water allocation in Klong Yai Basin has been model by applying the

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Acers Irrigation Support Package (AISP) as a measure in water allocation and water use from existing cost water source.

The purposes of the study are to:

1. investigate the activities of usage water in Klong Yai Basin

2. model the water allocation condition in Klong Yai Basin using the Acres Irrigation Support Package (AISP) Approach and adjust the model suitably for water usage in study area.

3. suggest how to allocate water suitably with existing cost water source and water supply system for efficient water allocation

4. suggest the future water allocation after the construction of Klong Yai Reservoir.

Klong Yai Basin is a sub-basin in the eastern coast of Thailand. Its basin code is 18.03, establishing at 13^0 15' – 12^0 45' N. and 101^0 00' – 101^0 30' E. Within 1,830 sq. km., in area, 1,454.43 sq. km. is in Rayong Province and 349.59 sq. km. in Chonburi Province. Rivers and canals in the basin flow into Rayong River and consequently to the gulf of Thailand. (RID 2000, illustrated in Fig. 1)

At present, water supply in this area consists of 2 reservoir – Dokrai reservoir and Nongpralai reservoir is the main reservoir to supplying water for varieties of activities. Water release of the main reservoir was drained to Dokrai Canal and Nongpralai Canal, and flowing to Rayong River. In the middle of Rayong River, the water level was risen by Ban Khai weir and the water from Ban Khai weir will be released to the both sides of the river for agricultural demand for 30,000 Rai of Ban Khai Irrigation area and water supply in Rayong Province.

MATERIALS AND METHODS

Acres Irrigation Support Package is designed to perform planning and operations for large irrigation systems. Systems with multiple reservoirs, irrigation projects and channels, and conflicting water supply demands can be analyzed. To provide this functionality, the package includes a set of computer programs that are designed to give the user several degrees of freedom to perform a system analysis. The user can set up different configurations of an irrigation system to perform studies of different development plans. As well, the analysis programs can work with monthly or weekly time steps. The package also provides access to the tools required for management of the input and output data of the computer programs. (ACRES,1979, 1999)

AISP was the mathematical model to provides several modules which are as follows:

- Main Control program (MC)
- Database Module for Time Series Data (DBM)
- Reference Evapotranspiration Module (ETM)(developed by International Institute for Land Reclamation and Improvement (ILRI))
- Irrigation Demand Module (IDM)
- Water Balance Module - Acres Reservoir Simulation Package (ARSP)
- Editing Modules for ARSP, IDM, and ETM
- Backrouting Module (BRM)
- Tools (Utilities) Module which provides access to PKZIP for file backup and to Microsoft Office for statistical analysis and data reporting.

The process of study is to

1) collect the data on meterology, hydrography and physical feature of the Basin and other data of water use for agriculture, industry and consumption, compared with the mode, accordingly to the factual conditions,

2) calculate the rain supply in the Basin,

3) analyze water balance module in the Basin used for other water use activities (Illustrated in picture No.1)

Setting measures and analysis of the result

1. The calculation of average annual water supply and multi-saved water statistically to set up the measure of water supply- dry, wet and normal level, flowing into the Dokrai, Nongpralai and Klang Yai Reservoir. The data were drawn from the

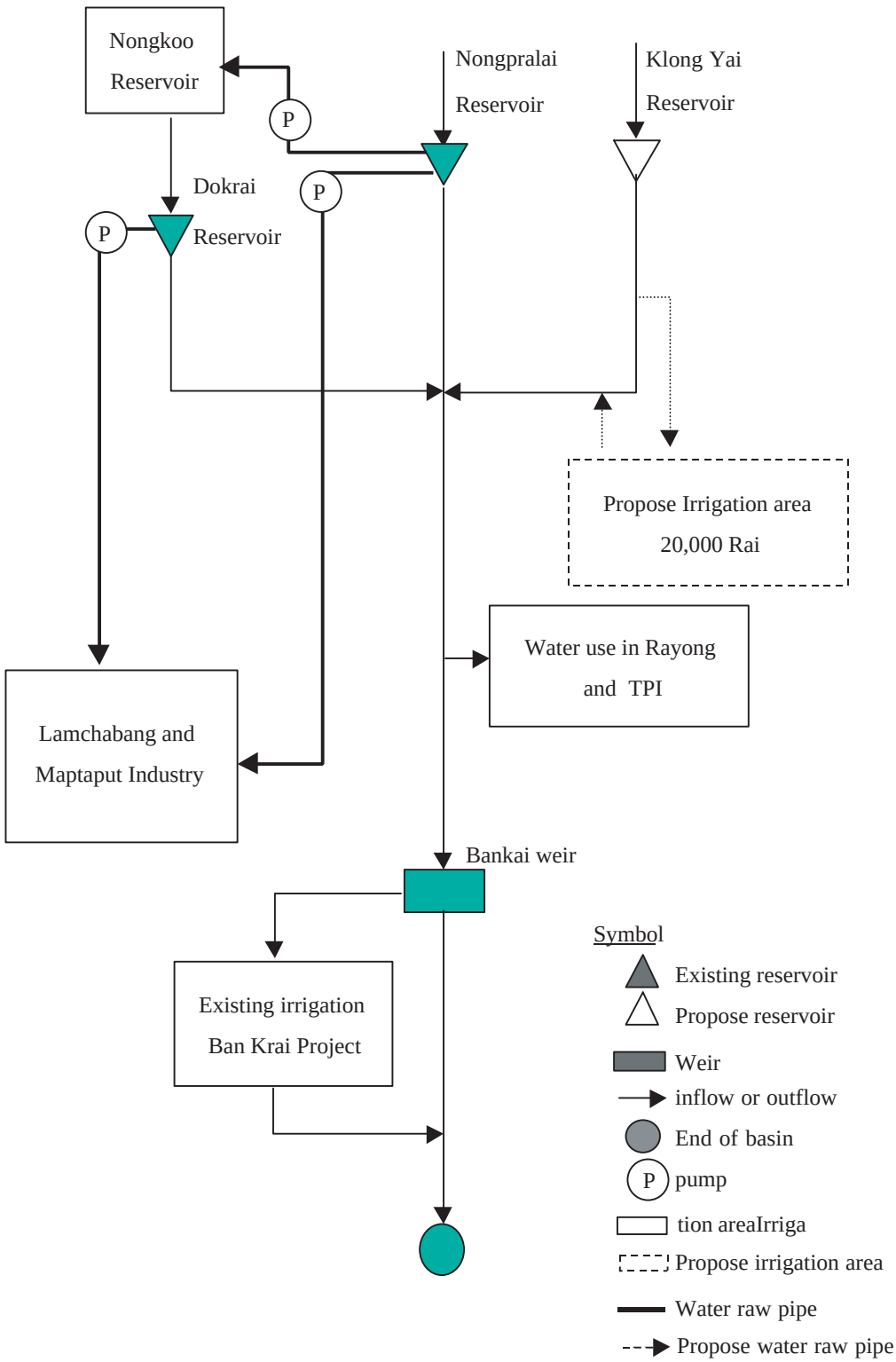

Figure 1 Schematic diagram water allocation in Klong Yai Basin.

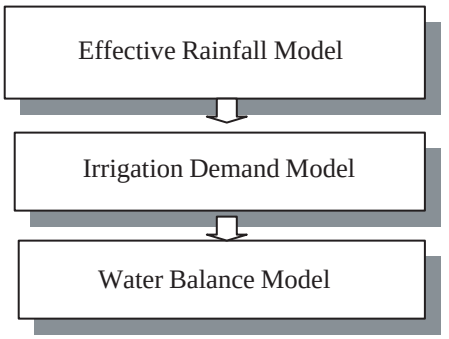

Figure 2 Schematic diagram to calculation for AISP Model.

current flowing into the reservoirs starting in January yearly per multi-saved water at the end of each month yearly, ranked respectively from high to low then calculate for percentage at 10, 30, 50 70 and 90% of the time.

2. Evapotranspiration (ETO) was calculated from weathers data of the Rayong Weather Station, Department of Meteorology, (Station Code No. 48478) by Penman-Monteith Method.

3. Analyze the quantities of effective rainfall within Bankai Irrigation Project and Klong Yai Irrigation Project (suggestion). The data were collected in daily rainfall format by the Station No. 48022 established in Bankai District, Rayong Province as representative for rainfall in both Irrigation Projects. The evaluation of effective rainfall was calculated by Effective rainfall Module of AISP mathematical model. The specification of capacity in the paddy field can be identify in 3 types capacity, accordingly to their water height in the paddy field – high (120 mm), medium (90 mm) and lower (45 mm) in two periods- dry season (January to June) and wet season (July to December).

4. The calculate of water demand in Irrigation Area

4.1 Demonstrated area was divided into 2 blocks – existing area in Bankai Irrigation Project and the propose area in 20,000 Rai of Klong Yai Irrigation Project. Case study was drawn from the surveying results, collected by Rayong Irrigation Project. The suitable conditions of Klong Yai Reservoir were reported by the Resource engineering and Macro-Consultant Engineering Ltd (1996).

4.2 In the calculation of the Evapotraspiration Module, Coefficient of plants (Kc) from Cropwat mathematical model (version 7.0) applied to plants being grown in the irrigation area which were classified into 5 groups- grains, crops, vegetables, long-life trees and fishing pond.

4.3 The evaluation of Irrigation Demand Module, the water provided for paddy plant is 250 mm. and the average soaking water ratio per day is 1.5 mm.

4.4 1.5 mm of Investigation of Klong Yai Basin

Three main reservoirs - Dokrai, Nongpralai and Klong Yai Reservoir (currently constructed) are utilized in supplying for consumption, agricultural and industrial demand within the Basin. Water Balance Module in AISP was evaluated into 2 study cases:

1. The evaluation in the present case, it consist of existing water supply water and water demand conditions in 2000 – Dokrai reservoir and Nongpralai reservoir, water demand within planting area of Bankai Irrigation Project and non-agricultural demand in 2000.

2. The evaluation in the future case, it consist of existing water supply and water demand conditions in 2009- Dokrai reservoir, Nongpralai reservoir, Klong Yai reservoir is the main reservoirs. The water demand in Klong Yai Basin consist of water demand within planting area of Bankai Irrigation Project and Klong Yai irrigation project (currently constructed by the 2004-2006) and water usage for non-agricultural demands in 2010.

RESULTS AND DISCUSSION

Measure of the backrouting module

The Backrouting Module to Dokrai and Nongpralai Reservoir could be divided into 2 periods- dry season and normal season. (Figure 3)

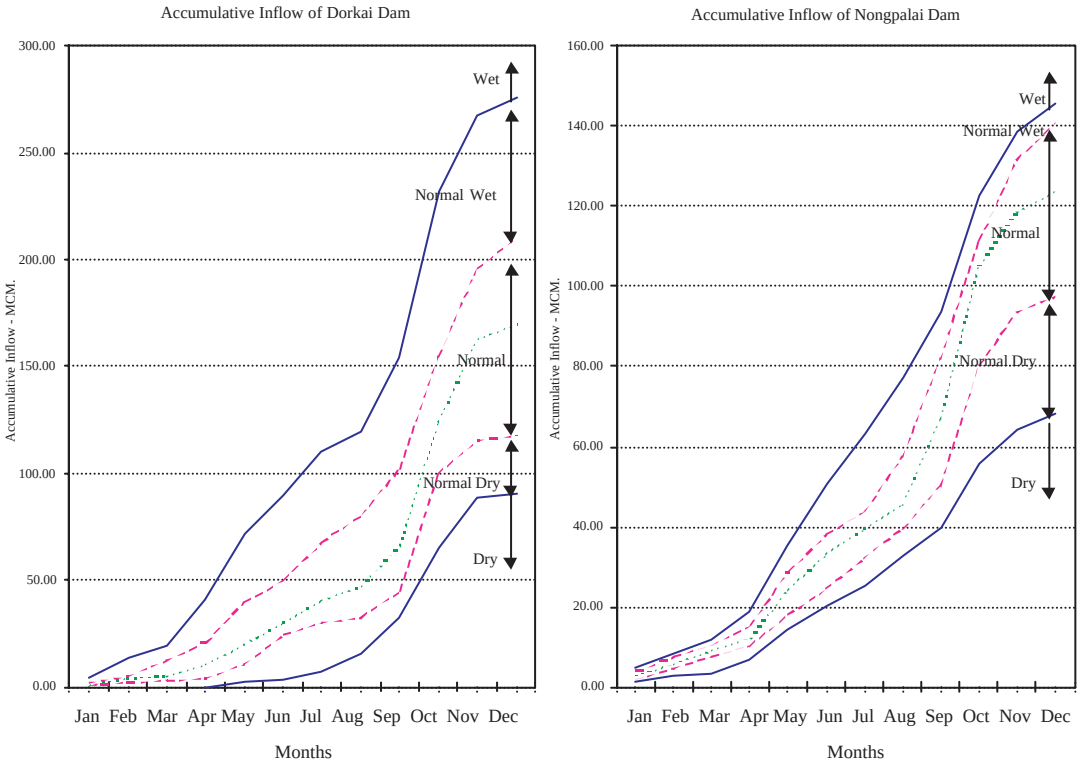

Figure 3 Inflow rule of Dokrai and Nongpralai Reservoir.

The study of water system in Klong Yai Basin

The study of water system in Klong Yai Basin by AISP Approach measuring the relationship within the Basin for water allocation from the existing water supply. The result of existing case in 2000, it can be set Operating rule curve of Dokrai reservoir and Nongpralai reservoir from early of the year (January 1), illustrated in Table 1.

The case study for measuring water use from Reservoirs and delivery system in Irrigation area in 2009 could be estimated for the suitable condition in Klong Yai Reservoir Project using AISP Approach. (Table 2)

Water supplied evaluation

Water Supply Indicators was used as an approach to evaluate the consumption and industrial demand within the Basin. (Table 3)

CONCLUSION

The study of water allocation in Kong Yai Basin was found that irrigation demand ratio in 2000 was about 50 % of industrial and consumption demand. When comparing to industrial water demand per reservoir capacity in Dokrai and Nongpralai Reservoir, industrial water demand was as much as an half of reservoir capacity. It was shown that water allocation in the Basin, principally, depends on industry and consumption.

Net irrigation demand in Bankai Irrigation Project in the dry season is not different from the wet season. However, net irrigation supply in dry season was shown less than in wet season. When comparing to water delivery capacity, It was formed that irrigation demand is higher than any other demand (both sides of Bankai Irrigation Project). In 2000, Water delivery capacity can be delivered at

Table 1 Operating rule curve in Dorkrai and Nongpralai reservoir.

Unit: Million Cubic Meters (MCM)

Month	Volume	
	Dokrai reservoir	Nongpralai reservoir
January	73.0	164.0
February	60.0	144.0
March	50.0	133.0
April	49.0	122.0
May	47.0	116.0
June	49.0	110.0
July	53.0	105.0
August	54.0	103.0
September	59.0	122.0
October	68.0	159.0
November	72.0	164.0
December	73.0	165.0

Table 2 Operating rule curve in Klongyai reservoir.

Unit: Million Cubic Meters (MCM)

Month	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Volume	40.0	30.0	28.0	25.0	24.0	22.0	23.0	27.0	32.0	37.0	39.0	40.0

Table 3 Water supplied evaluation in Bankai irrigation project.

Index	Variable	Volume of variable (MCM)	Result of eveuation
1. Water delivery capacity (%)	Canal capacity to delivery	122.52	3.389
	Peak irrigation demand	36.15	
2. Dry season RIS.	Irrigation supply	28.34	1.665
	Irrigation demand	17.02	
3. Wet season RIS.	Irrigation supply	19.61	1.731
	Irrigation demand	11.33	
4. Annual RIS.	Irrigation supply	47.95	1.691
	Irrigation demand	28.35	
5. Industrial water delivery	Industrial supply	109.16	0.459
	Reservoir capacity	238.00	
6. Ratio to water use	Irrigation supply	48.97	0.449
	Industrial supply	109.16	

Remark: The Result of evaluation was calculated from water usage conditions in 2000
RIS.= Relative Irrigation Supply

3.398 time of irrigation demand. (The data in this study were yearly total amount with Bankai Irrigation Project. It is essential to consider water delivery during the crisis time as well as efficient value in a period of time.)

The amount of water flowing into the Reservoirs each year during the wet season is one of the most important factors to be considered (dry, wet or normal level) for water delivery and water draining. To prevent water shortage and flooding in the wet season, agricultural and consumption demand is also a factor to evaluate amount of water in the reservoirs and operating rule curve. In present, if we change the habitual way of water use and cost water supply within the irrigation area, for example, the construction of a new reservoir changing insteaded of good water allocation (kinds of growing plants, planting area, duration of planting, and an amount of water use in Industrial demand ,etc.), we will reduce the cost for planting development and social problems.

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Parallel Logic Synthesis Optimization for Digital Sequential Circuit

Aswit Pungsema and Pradondet Nilagupta

ABSTRACT

High-level synthesis tools are very important for designing electronic circuits. A lower level logic gates are synthesized by optimization of the circuit's combination part, which is then realized by mapping on programmable devices such as FPGAs. This synthesis process is a computation intensive task. In this paper, we propose an alternative method to synthesis a sequential logic circuit which reduces time consuming in synthesis process. First using a parallel partitioning algorithm partition the whole circuit into sub-circuits and then using parallel sub-circuit synthesis in order to reduce computation. The LGSynth'91 benchmark suite used for experiment is in net-list format. Our result shows that the number of partition is increasing whereas the synthesis time is reduced as the number of processor is increased.

Key words: parallel logic optimization, graph partitioning, logic synthesis, sequential circuit

INTRODUCTION

With general purpose parallel processing machines becoming more commonplace, parallel processing is being used extensively to solve a variety of VLSI CAD algorithms. In recent years, there has been a tremendous amount of research on algorithms for optimization problems. Many engineering design problems which are classified as optimization problems have benefited from the results of this research. In particular, design problems can be modeled mathematically and solved by mathematical programming techniques. There remain many logic synthesis optimization design problems which are considered too complex to be solved in a reasonable amount of time using single-processor computers.

Logic synthesis forms an important part of many VLSI CAD applications. In this paper we concentrate on circuits partitioning for parallel logic synthesis in which we exploit parallelism by partitioning the circuit being synthesis. The logic synthesis problem is started as follows. Given a state-transition-graph (STG), generate a logic function from STG, and then optimize the logic (logic synthesis) and finally device mapping. Logic synthesis, given a logical function $f(x_1,\ldots,x_s)$, composed of an on-set $F(x_1,\ldots,x_s)$ and a don't-care-set $D(x_1,\ldots,x_s)$, finds a sum-of-products expression (a cover of f), $G = g_1+\ldots+g_t$ with minimum t, such that $F \subseteq G \subseteq F+D$.

Many algorithms have been published for various stages of sequential synthesis. For synchronous circuits, these include methods for state assignment (Lin and Newton, 1989 and Villa and Vincentell., 1990), state minimization (Hachtel *et al*., 1991), testing (Ghosh, 1991), retiming (Leiserson *et al*., 1983.), technology mapping (Moon *et al*., 1989), verification (Bryant. 1986 and Coudert *et al*., 1989), timing analysis, and optimization across register boundaries (DeMicheli, 1991 and Malik *et al*., 1991). For asynchronous circuits, these include methods for hazard-free synthesis (Lavagno *et al*., 1991). However, no comprehensive

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evaluation of the algorithms and no complete synthesis system in which all of these algorithms are employed, has been reported to date. A complete sequential circuit synthesis system is needed, both as a framework for implementing and evaluating new algorithms, and as a tool for automatic synthesis and optimization of sequential circuits.

MATERIALS AND METHODS

At present the device density is very high, enabling the circuit designer to design a larger circuit. However, processing problems arise when the netlist is too large and consumes all of the available memory. In this paper, we propose an alternative method to synthesis a sequential logic circuit which reduces time consuming in synthesis process. First using a parallel partitioning algorithm partition the whole circuit into sub-circuits and then using parallel sub-circuit synthesis in order to reduce computation as shown in Figure 1 and Figure 2.

There are potential problems with this approach. The first problem is that the large circuit must be partitioned into N sub net-lists (define 'N' before the partition) which balance the size of each sub net-list with respect to performance of the next process. The second problem is that the connections between nodes during partitioning process must be minimal, because the network speeds is slower than CPU. The last problem is the global minimizations of synthesis results which consumes computation time.

This paper focused on circuit partitioning which is modeled as a graph. Since the next step, a parallel synthesis uses a partition circuit as an input. The graph partition algorithms we choose for circuit

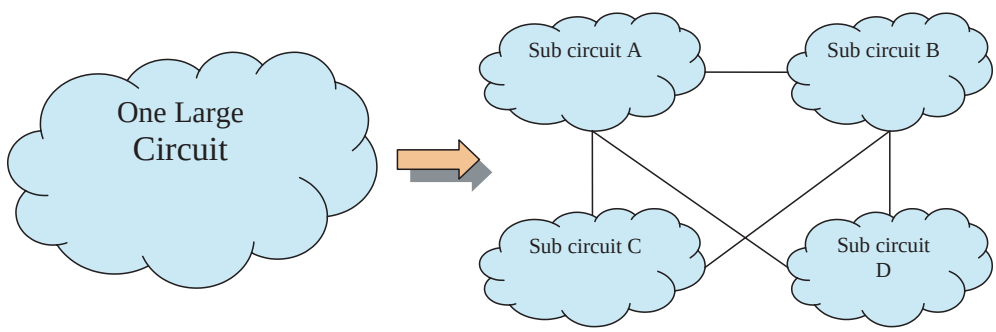

Figure 1 The first step of partitioning a circuit.

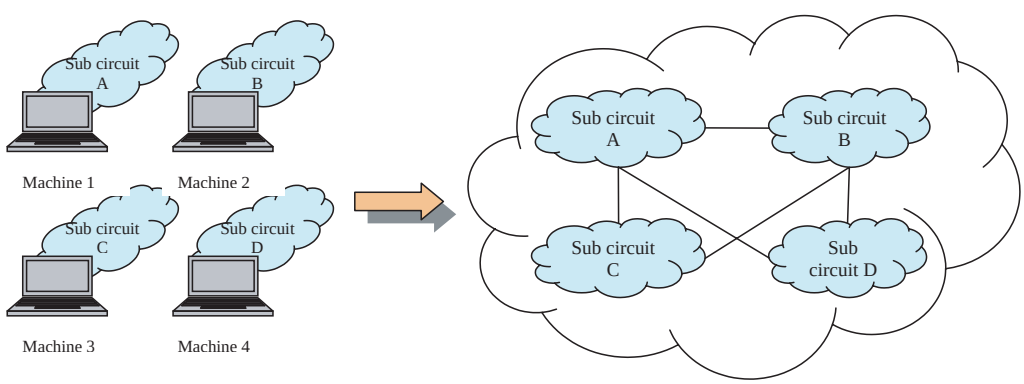

Figure 2 Thesecond step is to distribute the logic synthesis. The third step is to join the sub-circuits back into one circuit.

partitioning must be possible to use in parallel for increase speed and reduce share memory. For instance, the solutions for a linear equation ($Ax = b$) applied using iterative methods on a parallel computer gives rise to a graph partitioning problem. A key step is a multiplication of a sparse matrix and a dense vector. Graph partitioning corresponded to matrix A, is used to significantly reduce the amount of communication time. If direct parallel methods are used to solve a sparse system of equations, then a graph partitioning algorithm can be used to compute a fill reducing order that leads to a high degree of concurrency in the factorization phase. The multiple minimum degrees ordering used almost exclusively in serial direct methods, is not suitable for direct parallel methods, as it provides limited concurrency in the parallel factorization phase.

The graph partitioning problem is NP-complete. However, many algorithms have been developed that result in a reasonably good partition. Recently, a new class of multilevel graph partitioning techniques were introduced by Hendrickson and Leland (1993), and further studied by Karypis *et al.*, (1998). This multilevel algorithm provides an excellent graph partitioning and a moderate computational complexity. Even though these multilevel algorithms are quite fast compared with spectral methods, parallel formulations of multilevel partitioning algorithms are needed for the following reason. The amount of memory on serial computers is insufficient to allow the partitioning of graphs corresponding to large problems that can now be solved on massively parallel computers and workstation clusters. Subsequently, a parallel graph partitioning algorithm can take advantage of the significantly higher amount of memory available in parallel computers.

Unstructured graph partitioning algorithm

The problem is to partition the vertices of a graph into *p* roughly equal parts, such that the number of edges connecting vertices in different parts is minimized. The ***p-way*** graph partitioning problem is defined as followed : Given a graph G = (V, E) with |V| = n, partition V into p subset, V_1, V_2,…, V_p such that $V_i \cap V_j = \varnothing$ for $i \neq j$, $|V_j| = n/p$, and $\cup_j V_j = V$, consequently the number of edges of E whose incident vertices belong to different subsets is minimized. A p-way partitioning of V is commonly represented by a partitioning vector P of length n, so that for every vertex ($v \in V_1$,P[v]) is an integer between 1 and p. This indicated the partition to which the vertex (v) belongs. Given a partitioning (P), the number of degrees whose incident vertices belong to different partitions is called the *edge-cut* of the partition.

The various phases of the multilevel graph bisection are shown in Figure 3. There are three phases in multilevel graph bisection, *coarsening phase*, *initial partitioning phase*, and *uncoarsening and refinement phase*. During the coarsening phase, the size of the graph is successively decreased. During the initial partition phase, a bisection of a smaller graph is computed, and then during the uncoarsening phase, the bisection is successively refined as it is protected to a larger graph. During

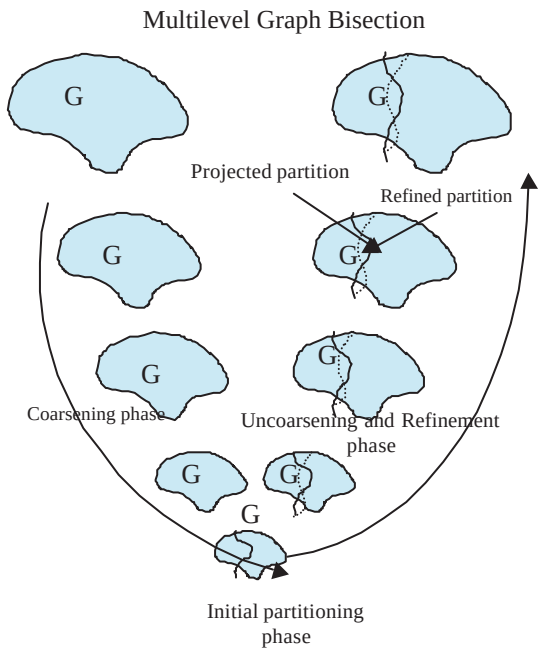

Figure 3 The multilevel graph bisection.

the uncoarsening phase a dotted lines indicate projected partition, and a solid line indicate partitions that are produced after refinement.

Parallel multilevel graph partitioning algorithms

The basic concept of parallel multilevel graph partitioning algorithms is as follows. There are two parallel methods that can be exploited in the p-way graph partitioning algorithm based on the multilevel bisection, as described in previous section. The first parallel method is due to the recursive nature of the algorithm (Karypis, 1996). Initially a single processor finds a bisection of the original graph, and then two processors find bisections of those two newly created sub-graphs and so on. However, this scheme can only use up to log p processors, and only reduces the overall run time of the algorithm by a factor of O (log p). We will refer to this parallel method as the procedure associated with the recursive step.

The second parallel method can be exploited during the bisection step. In this case, instead of performing the bisection of the graph on a single processor, we perform it in parallel. We will refer to this parallel method as the procedure associated with the bisection step. Note that if the bisection step is done in parallel, then the increase in speed obtained by the parallel graph partitioning algorithm can be higher than O(log p).

The parallel graph partitioning algorithm we have been describing in this section exploits both of these parallel methods. Initially all the processors cooperate to bisect the original graph G, into G^0 and G^1. Subsequently half of the processors bisect G^0, while the other half of the processors bisects G^1. This step creates four subgraphs G^{00}, G^{01}, G^{10}, and G^{11}. Then each quarter of the processors bisect one of these subgraphs and so on. After a log p steps, the graph G has been partitioned into p parts.

The ParMETIS library (Karypis *et al*., 1998) provides a variety of algorithms. ParMETIS is an MPI-based parallel library that implements a variety of algorithms for partitioning unstructured graphs, meshes, and for computing fill-reducing orderings of sparse matrices. A brief overview of the functionality of PARMETIS is shown in Figure 4. ParMETIS extends the functionality provided by METIS and includes routines that are especially suited for parallel AMR computations and large scale numerical simulations. We investigated some partitioning algorithm matching our problem. We

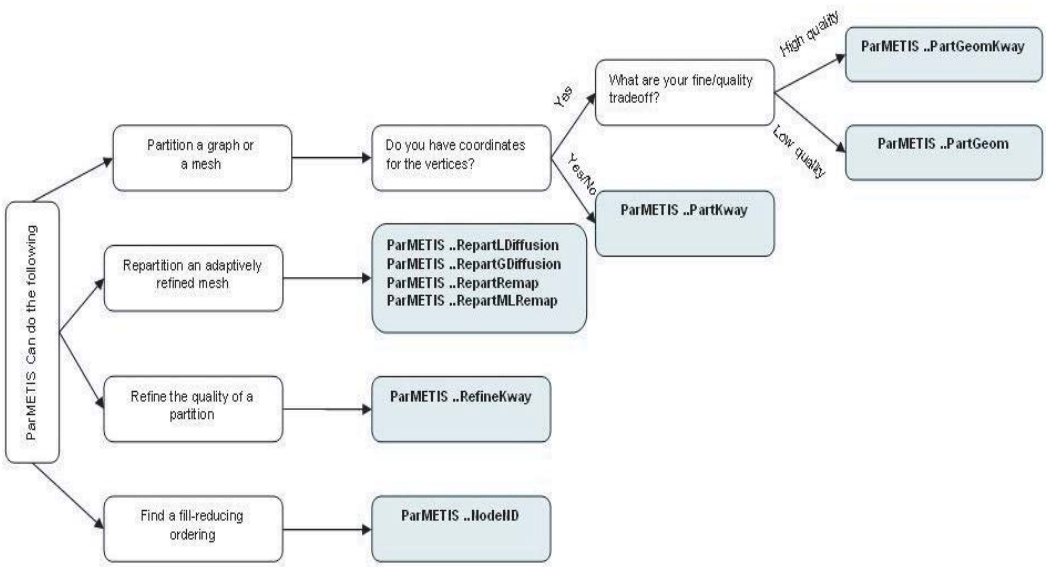

Figure 4 Abrief overview of the functionality provided by partMETIS.

started verifying those algorithms in order to find the algorithm that gives the best result respect to our scope. PARMETIS_PARTKway is based on the serial multilevel k-way partitioning algorithm describe in previous section and used the second part of the parallel method. This k-way partitioning algorithm shows that it produces a quick partition and high quality partition. The multilevel k-way partitioning algorithm works as follow. First, a graph is gradually coarsened down so it contains a few hundred vertices. Second, a k-way partition of this smaller graph is computed, and then this partitioning graph is projected back to the original graph (finer graph) by periodically refining thee partition. Since the finer graph has more degrees of freedom, such refinements improve the quality of the partition.

Implementation

We started our implementation by porting SIS version 1.2 (Sentovich *et al*., 1992) to the Linux platform in order to parse the netlist file format. After that we built the module to map the netlist in to the graph structure for processing and testing by ParMETIS for partitioning and finally we built the module to merge the sub-circuits and verify the circuit correction. The experiments on the LGSynth'91 benchmark suite (Saeyang, 1991) were performed in PIRUN cluster (Uthayopas *et al*., 2000) using 16 nodes of the Beowulf PC Cluster, Pentium III 500 MHz processors, with 128MB memory and 100Mbps Ethernet connections.

RESULTS AND DISCUSSION

The first phase of mapping netlist to graph is to analyses the characteristic of sub circuit results. This paper will present results using time and the quality of circuit output in order to select the best parameter for the next step. We focused on the boolean part of RTL circuits. The set of equations describing the circuit is represented by a boolean network. This network is a directed acyclic graph (DAG). Each node of DAG is associated to each boolean equation, and there is an arc between two nodes N_i and N_j if the output of N_i is an input to N_j.

Figure 5-7 show the partitioning results for each circuit into 2, 4, 6, 8, 10, 12, 14 and 16 partitions running on 2, 4, 8, and 16 processors (workstations). The results show time consumption will be affected by the number of processors. The network I/O is leaking the CPU time. From the partitioning algorithm, the graph image will be distributed to each process and then synchronized at a random node to perform the bisection algorithm. This process consumes a lot of network I/O of communicating to each other. The time consume for partition on each circuits trend to increase since the complexity of each circuits are increase. The time consuming for partition circuit into bigger partition is decrease i.e. partition circuit into 16 partitions is better than partition into other partition size when the number of processor is increased. However, there are some partition size is better than partition into 16 partition because of the complexity of circuit. When the complexity of a circuit is increased, the more partition divides, the time consume is better. The s38417 is more complicate than s15850 and s1488.

Table 1 and 2 show the synthesis runtime and edge-cut of sub-circuit VS. number of processors. Edge-cut is total number of links to each of the other sub-circuits. From Tables 1-2 we choose the optimum number of sub-circuits by the number of processors. The synthesis runtime is decreased when we use a processor up to 16 processors. The speed up of synthesis runtime is increased as the circuit is more complex. The speed up of circuits1488, s15850, and s38417 are 0.6, 8.2 and 7.7 respectively. The number of edge-cut is increased as the number of processor is increased. The results, as compared to sequential execution, are obtained through a complex circuit taking a long time at one processor. Parallel runtime is calculated from logic synthesis time and merge time (sequential). Since parallel runtime is calculated by

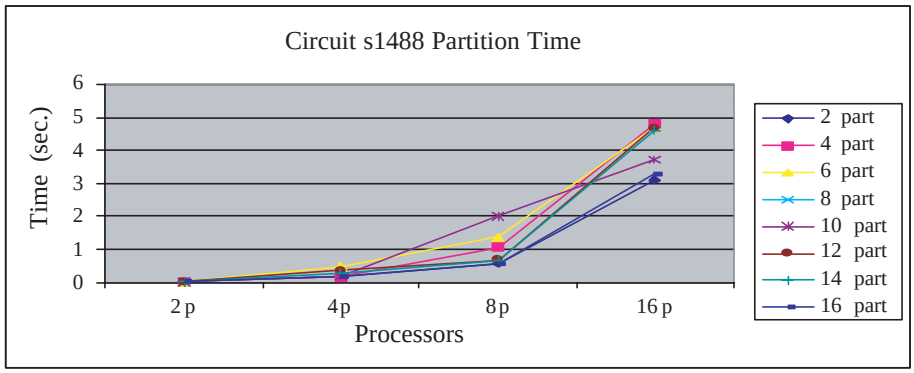

Figure 5 The time consume for partitioning circuit s1488 into 2-16 partitions VS. the number of processors.

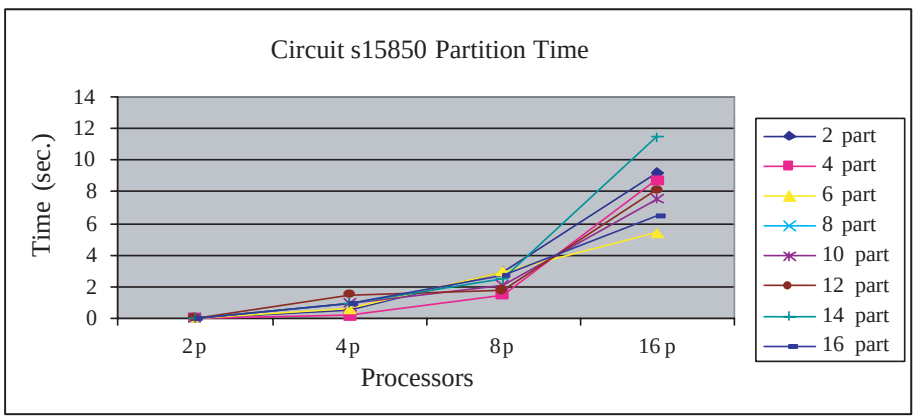

Figure 6 The time consume for partitioning circuit s15850 into 2-16 partitions VS. the number of processors.

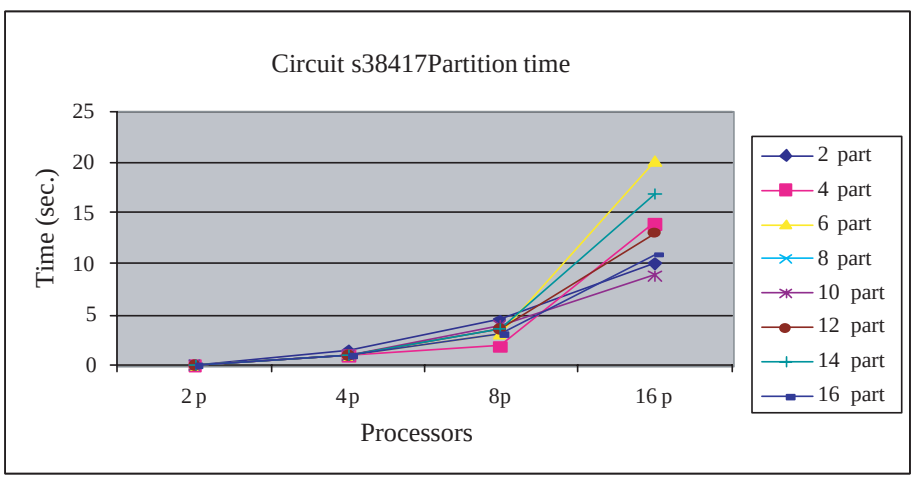

Figure 7 The time consume for partitioning circuit s38417 into 2-16 partitions VS. the number of processors.

Table 1 Synthesis runtime VS. number of processors (sec.).

Circuit	1P	2P	4P	8P	16P
S1488	52	38	42	56	76
S15850	793	435	138	111	96
S38417	953	492	314	167	125
Total	1789	965	494	334	297

Table 2 An edge-cut VS. number of processors.

Circuit	2P	4P	8P	16P
S1488	1	185	274	345
S15850	35	56	103	217
S38417	40	106	166	274

the longest processing time, the more processors we have the longer delay time the Network-File-System takes to load the sub netlist into memory. In addition, merge time is dependent on the number of circuits to merge.

CONCLUSION

We have proposed an alternative method to synthesis a sequential logic circuit which reduces time consuming in synthesis process. The LGSynth'91 benchmark suite used for experiment is in net-list format. Our result shows that the number of partition is increasing where as the synthesis time is reduced as the number of processor is increased up to 16 processors. The number of edge-cut is increased as the number of processor is increased. The speed up on each circuit is 0.6, 8.2 and 7.7 respectively. Future work, we will try on other parallel optimization which may be improved performance and also we will try to test more examples.

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