Influence of Scum Layer on Methane Emission from Open Anaerobic Pond of Palm Oil Mill

Kannikar Kongwarin

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Engineering in Environmental Engineering

Prince of Songkla University

2012

Thesis Title	Influence of Scum Layer on Methane Emission from Open Anaerobic Pond of Palm Oil Mill
Author	Ms.Kannikar Kongwarin
Major Program	Environmental Engineering

Major Advisor :

……

(Asst. Prof. Dr. Charongpun Musikavong)

Co-advisor:

…….....……..…..……

(Asst. Prof. Dr. Chaisri Suksaroj)

…….....

(Asst. Prof. Dr. Thunwadee Suksaroj)

Examining Committee :

……..Chairperson

(Assoc. Prof. Dr. Aran H-Kittikun)

……...……..

(Assoc. Prof. Dr. Suraphong Wattanachira)

(Dr. Watsa Khongnakorn)

……...…..…

(Asst. Prof. Dr. Charongpun Musikavong)

..……...……..…

(Asst. Prof. Dr. Thunwadee Suksaroj)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirement for the Master of Engineering Degree in Environmental Engineering.

……..……...…..……

(Prof. Dr. Amornrat Phongdara)

Dean of Graduate School

ชื่อวิทยานิพนธ์ ผลของชั้นฝ้าไขต่อการปลดปล่อยก๊าซมีเทนจากบ่อหมักไร้อากาศแบบเปิดของโรงงานสกัดน้ำมันปาล์ม

ผู้เขียน นางสาวกรรณิการ์ คงวาริน

สาขาวิชา วิศวกรรมสิ่งแวดล้อม

ปีการศึกษา 2554

บทคัดย่อ

งานวิจัยนี้มุ่งศึกษาผลของชั้นฝ้าไขต่อการปลดปล่อยก๊าซมีเทนจากบ่อหมักไร้อากาศแบบเปิดของโรงงานสกัดน้ำมันปาล์มดิบ โดยประเมินการปล่อยก๊าซมีเทนด้วยแบบจำลองคณิตศาสตร์ (Methane Recovery in Wastewater Treatment AMS III-H version 16) ซึ่งพัฒนาโดย United Nations Framework Convention on Climate Change ผลการศึกษาพบว่า บ่อหมักไร้อากาศแบบเปิดบ่อที่หนึ่ง และบ่อที่สองเป็นแหล่งปล่อยก๊าซมีเทนมากกว่าบ่ออื่นๆ ถึง 30 เปอร์เซ็นต์ ถึงแม้ว่าจะมีการติดตั้งระบบผลิตก๊าซชีวภาพเพิ่มเติม ซึ่งจากการพิจารณาบ่อหมักดังกล่าว พบว่า มีชั้นฝ้าไขปกคลุมทั่วพื้นผิว เมื่อทำการตรวจวัดการปลดปล่อยก๊าซมีเทนด้วย close flux chamber ในพื้นที่จริง พบว่า บริเวณที่ไม่มีชั้นฝ้าไข ปลดปล่อยก๊าซมีเทนสูงกว่าบริเวณที่มีชั้นฝ้าไขปกคลุม อีกทั้งบริเวณที่มีชั้นฝ้าไขสามารถลดการปลดปล่อยก๊าซมีเทนได้มากถึง 60 เปอร์เซ็นต์ที่ความหนาของชั้นฝ้าไขเฉลี่ย 2 – 3 เซนติเมตร จากการศึกษาปริมาณจุลชีพในชั้นฝ้าไข พบว่า ในชั้นฝ้าไขของบ่อหมักไร้อากาศแบบเปิดบ่อที่สองมีปริมาณจุลินทรีย์ที่เติบโตบนอาหาร AMS agar ที่ไม่มีเมทานอลในสภาวะที่มีเทน 50 เปอร์เซ็นต์ มากกว่าในชั้นฝ้าไขจากบ่อหมักไร้อากาศแบบเปิดบ่อที่หนึ่ง โดยมีค่า 28 และ 0.12 CFUs/g ตามลำดับ โคโลนีที่พบมี 2 ลักษณะ ได้แก่ โคโลนีสีขาว และสีชมพู ขอบมน กลม นูน ซึ่งมีลักษณะทางสัณฐานวิทยาเป็นแกรมลบ รูปแท่ง และกลม ตามลำดับ การศึกษาการลดก๊าซมีเทนโดยจุลินทรีย์ทั้งสอง พบว่า จุลินทรีย์มีการใช้ก๊าซมีเทนในการเจริญเติบโต ส่งผลให้ก๊าซมีเทนลดลงแบบปฏิกิริยาลำดับที่ 1 โดยโคโลนีสีขาว และสีชมพูมีค่าคงที่ของอัตราการลดลงของก๊าซมีเทนเฉลี่ย 0.20 และ 0.34 ต่อวัน ตามลำดับ จึงทำการบ่งชี้เอกลักษณ์ทางชีวภาพด้วยเทคนิค Polymerase Chain Reaction โดย 16S rRNA gene พบว่า โคโลนีสีขาวมีความคล้ายคลึงร้อยละ 99.19 กับ *Rahnella aquatilis* และ โคโลนีสีชมพูมีความคล้ายคลึงร้อยละ 98.73 กับ *Pseudomonas psychrophila*

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Academic Year	2011

ABSTRACT

The aim of this work was to study an influence of scum layer on methane emission from open anaerobic pond of palm oil mills. The evaluation of methane emission using the mathematical model (Methane Recovery in Wastewater Treatment AMS III-H version 16) developed by United Nations Framework Convention on Climate Change showed that the first and the second anaerobic ponds emitted methane more than the other ponds 30 percent. Even in the mill that had the biogas recovery system for wastewater treatment. The first and the second anaerobic ponds were covered by the scum on the surface. When the evaluation of methane emission by actual measurement using close flux chamber was conducted, it was found that the area without scum covered layer emitted methane higher than the area with scum covered layer. This scum layer with the range of scum thickness 2 – 3 cm can reduce the methane emission by 60 percent. The study of the microbial community in the scum was done by growing on the AMS agar without methanol under 50 percent methane in the headspace. The number of microorganisms which grew in the scum from the second anaerobic pond (28 CFUs/g) was more than that of the first anaerobic pond (0.12 CFUs/g). Two dominant colonies found included the white and pink colonies, round, with even edges, smooth surface. Their morphological characteristics were rod and cocci shape, gram-negative and they consumed methane in the headspace. The reaction order for methane reduction was the first order reaction with rate coefficient of 0.20 and 0.34 day^{-1} for the white and pink colonies respectively. The 16S ribosome RNA gene sequening was used to identify the white and pink colonies. The white colony had 99.19 percent similar with *Rahnella aquatilis* strain and the pink colony had 98.73 percent similar, Its with *Pseudomonas psychrophila*.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my thesis advisor, Assistant Professor Dr. Charongpun Musikavong, for his kindness, valuable suggestions guidance and strong encouragement during the thesis work. Special respect and thanks are also extended to Assistant Professor Dr. Thunwadee Suksaroj and Assistant Professor Dr. Chaisri Suksaroj, for the valuable suggestion and guidance as co-advisor and Dr. Aramas Suttinun for the valuable suggestion.

I would like to thank my committee members: Associate Professor Dr. Suraphong Wattanachira Associate Professor Dr. Aran H-Kittikun, and Dr. Watsa Khongnakorn.

Special thanks also go to all of the staff members of the Department of Civil Engineering, Faculty of Engineering and the Faculty of Environmental Management, Prince of Songkla University, Songkhal Province, Thailand for the supporting of the laboratory facilities and providing me with valuable information. I also wish to thank all friends at the Miner of Environmental Engineering, Prince of Songkla University for their warm support and helps over the entire period of this research.

I would like to sincerely thank the National Research Center for Environmental and Hazardous Waste Management (NCE-EHWM), Prince of Songkla University for their financial support and Palm oil mill Industry in Krabi Province, Thailand for in assistant with sample collection and providing all information.

Finally, I wish to express my gratitude thanks my parents for their wholehearted understanding, unconditional love, supports and never giving up on me for inspiration throughout my thesis.

Kannikar Kongwarin

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LIST OF ABBREVIATIONS AND SYMBOLS

GHGs	=	Green house gases
CH_4	=	Methane gas
CO_2	=	Carbon dioxide
UNFCCC	=	United National Framework Convention on Climate Change
MOB	=	Methane oxidizing bacteria
SRB	=	Sulfate reducing bacteria
COD	=	Chemical Oxygen Demand
BOD	=	Biological Oxygen Demand
cm	=	Centimeter
GC-TCD	=	Gas - chromatography with a thermal conductivity detector
PCR	=	Polymerase chains reaction
16S rRNA	=	16S ribosome ribonucleic acid
POME	=	Palm oil mill effluent
CSTR	=	Completely Stirred Tank reactor
FFB	=	Fresh fruit bunch
CPO	=	Crude palm oil
GWP	=	Global warming potential
PK	=	Palm kernel
BE	=	Baseline emission
PE	=	Project emission
g/L	=	Gram per litre
mg/L	=	Milligrams per litre
CFUs/g	=	Colony-forming units per gram

CHAPTER 1

INTRODUCTION

1.1 Motivation

Currently, global climate has been changed because of the increasing of greenhouse gases (GHG). GHG is a major cause of global warming that build up many aspects such as seasonal change, some animal species extinction, or mutation for survival and outbreaks of diseases, etc. Some important GHG such as methane (CH_4), carbondioxide (CO_2), nitrousoxide (N_2O), chlorofluorocarbon (CFCs) and tropospheric ozone (O_3) are accumulating in the atmosphere radiation (Webb *et al.,* 1997 and Chomsuri, 1997). The methane gas is classified as the one of important GHG because of its ability to cause global warming by up to 25 times higher than the carbondioxide (Bracmor *et al.,* 2009 and McKeown and Gardner, 2009) and its concentration in atmosphere has increased by 1 – 2 percents per year (Webb *et al.,* 1997). Methane can be emitted to the atmosphere by both natural and human sources. The natural sources of methane emission include wetlands, lagoon, swampland wildfire, while, the human source is municipal solid waste landfills and wastewater treatment system, etc. (U.S. Environmental Protection Agency, 2010).

Methane is a part of biogas produced by the biological breakdown of organic matter in the absent of oxygen. Biogas that is produced by anaerobic digestion or fementation, is carbondioxide, methane (Yacob *et al.,* 2005) and other gases. The generation of methane from anaerobic process is shown in equation (1.1).

$$\text{Biodegradable waste} \xrightarrow[\text{(Anaerobic process)}]{} CO_2 + CH_4 + H_2O + \text{organic acids} + \text{degradable waste} \quad (1.1)$$

In Thailand, palm oil industry is one of the major agro-industry especially in the south of Thailand. The palm oil mills normally use wet extraction process to convert fresh fruit bunch (FFB) to crude palm oil (CPO). In the wet extraction process in the palm oil production system, high quantities of water are utilized during the process of crude palm oil (CPO) extraction. Chavalparit *et al.* (2006) reported that water consumption in this production process was in the range of 1.0 – 1.3 m^3/ton FFB. About 50 – 79 percents of the water used results in palm oil mill effluents (POME). This wastewater contains high organic content which required well management in order to reduce the environmental impact. The stabilization pond system including anaerobic, facultative, and detention ponds is the conventional system widely used in the mills to treat wastewater. The anaerobic ponds, hence, could be the one of methane emission sources into the atmosphere.

Recently, the biogas system is applied in wastewater treatment process of palm oil mill. The mills use the anaerobic pond as the equalization system for reducing the temperature. Since, wastewater from the mill has high concentration of biochemical oxygen demand (BOD) and chemical oxygen demand (COD) which are higher than 50,000 and 100,000 mg/L, respectively (Praseatsun *et al.,* 1990 and Ahmad *et al.,* 2006). The COD of wastewater in palm oil mill 1.0 kilogram can produce methane 0.1 kilogram (Yacob *et al.,* 2005). After anaerobic pond, the wastewater was flowed to biogas recovery system. Biogases from the biogas recovery system of palm oil mill had been used to produce electricity. After biogas recovery system, the wastewater is flowed to the stabilization ponds which consisted of anaerobic, facultative and detention ponds. On this basis, even though the biogas treatment is used, anaerobic pond that could emit methane is still utilized in the system. Therefore, it is important to evaluate the amount of methane emission and to find out the solution for optimizing methane emission. There are two methods that could be used to determine the methane emission from the wastewater 1) Mathematical model by United Nations Framework Convention on Climate Change (UNFCCC) and 2) Actual measurement by flux chamber method.

Siriluksopon (2009) studied the methane emission from anaerobic pond of palm oil mill; it was found that the solid layer called “scum” covered on surface area of anaerobic pond affected on the methane emission from these ponds. The measurements of methane emission by close flux chamber between with and without scum layer were 397.6 and 640.2 tonCO_2/year, respectively. The reduction of methane emission by scum cover may due to 1) It block methane emission into atmosphere and 2) Biological characteristics such as methane oxidation bacteria (MOB) and sulfate reducing bacteria (SRB) which used methane as sole carbon and energy source for biomass production (Anthony, 1982).

1.2 Objectives

1.2.1 To study the methane emission from the wastewater treatment system of palm oil mill with/without biogas recovery system by mathematical model.

1.2.2. To compare the methane emission from with and without scum layer cover on surface area of the anaerobic ponds.

1.2.3 To study the effect of scum layer on surface of anaerobic pond on methane emission.

1.3 Limitations of the study

1.3.1 Evaluate methane emission from the wastewater treatment system of palm oil mill with/without biogas recovery system by mathematical model.

1.3.2 Compare the methane emission from area which covered by scum and non-scum of the first and second anaerobic ponds of the palm oil industry.

1.3.3 Study the physical, chemical, and biological characteristics of wastewater and scum in the first and second anaerobic ponds of the palm oil mill.

1.3.4 Study the type of microorganism by using selective media (AMS without methanol) and Polymerase Chains Reaction (PCR) technique with 16S ribosome RNA (16S rRNA) (Figure 1.1).

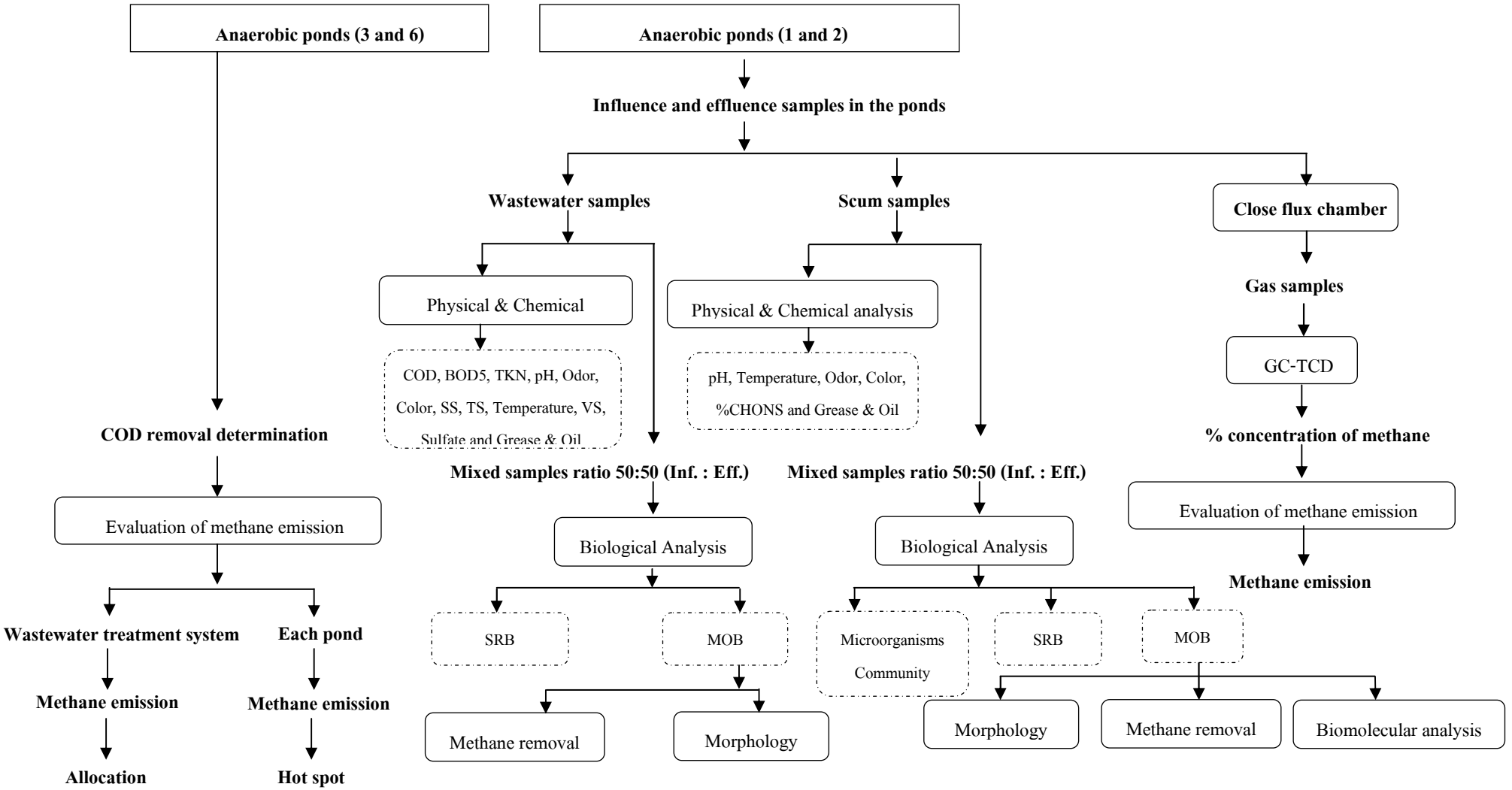


Figure 1.1 Experimental procedure diagram.

1.4 Significance of the study

1.4.1 To know the quantity of methane emission from the wastewater treatment system of palm oil mill with/without biogas recovery system.

1.4.2 To know the physical, chemical and biological properties of wastewater and scum in the first and second anaerobic ponds of the wastewater treatment system in the palm oil mill industry.

1.4.3 To know the effect of scum covered to methane emission from anaerobic pond into atmosphere.

1.4.4 To know a type of microorganisms which lives in the scum and effect to methane emission into atmosphere.

CHAPTER 2

LITERATURE REVIEWS

2.1 Palm oil mill

The oil palms (*Elaeis*) comprise two species of the Arecaceae, or palm family. They are used in commercial agriculture in the production of palm oil. Mature trees are single-stemmed, and grow to 20 m. tall. The leaves are pinnate, and reach between 3-5 m. long. A young tree produces about 30 leaves a year. Established trees over 10 years produce about 20 leaves a year. The flowers are produced in dense clusters; each individual flower is small, with three sepals and three petals.

The palm fruit takes five to six months to mature from pollination to maturity. The palm fruit is reddish, about the size of a large plum and grows in large bunches. Each fruit is made up of an oily, fleshy outer layer (the pericarp), with a single seed (the palm kernel), also rich in oil. When ripe, each bunch of fruit weighs 40-50 kilogrammes.

Oil is extracted from both the pulp of the fruit (palm oil, edible oil) and the kernel (palm kernel oil, used in foods and for soap manufacture). For every 100 kilograms of fruit bunches, typically 22 kilograms of palm oil and 1.6 kilograms of palm kernel oil can be extracted (Hamid, 2008).

The high oil yield of oil palm trees (as high as 7,250 liters per hectare per year) has made it a common cooking ingredient in Southeast Asia and the tropical belt of Africa. Its increasing use in the commercial food industry in other parts of the world is buoyed by its cheaper pricing (United Stated Department of Agriculture, 2006), the high oxidative stability of

the refined product (Che Man *et al.,* 1999 and Bertrand, 2007) and high levels of natural antioxidants (Sundram *et al.,* 2003).

2.1.1 Oil palm in Thailand

Oil palm is now increasingly noticed when the Thai government has set its policy on producing palm oil-based biodiesel as a renewable energy. Therefore since 2006, a variety of discourse on oil palm has emerged to promote its plantation as are newable source of energy, a country savior, a reforestation scheme, a wind-protection zone, and a transformation of deserted rice fields into palm fields (Yangdee B., 2006).

Malaysia has established its oil palm industry since 1917 while Thailand's industry has begun in 1969, or 50 years behind that of Malaysia. During 2005 until 2009, Thailand has oil palm plantations areas increase averages ratio 11.65 percents and 15.12 percents per year respectively. In 2009, Thailand has 3.20 million hectares of oil palm plantation areas and total production of 8.61 million-tones FFB, 2,694 kilograms oil/hectare shown in Table 2-1. Figure 2.1 shows the location of palm oil mill in Thailand, most the mill 88 percent located in the south of Thailand including Krabi, Suratthani, Chaumphon (Palm Oil Products and Technology Research Center : POPTEC, 2010).

Table 2-1 The data of oil palm in Thailand in 2005 – 2010**.**

Year	plant areas (million hectares)	Oil palm plant areas (million hectares)	Fresh fruit bunch (million tons)	Products per areas (kilograms/hectares)
2005	2.75	2.03	5.00	2,469
2006	2.95	2.37	6.72	2,828
2007	3.20	2.66	6.39	2,399
2008	3.63	2.87	9.26	3,225
2009	3.95	3.20	8.61	2,694
Rate increase (%)	9.76	11.65	15.12	3.11
2010*	**4.33**	**3.53**	**10.49**	**2,974**

Source : Office of Agricultural Economics (2009).

Remake: * Expectations.

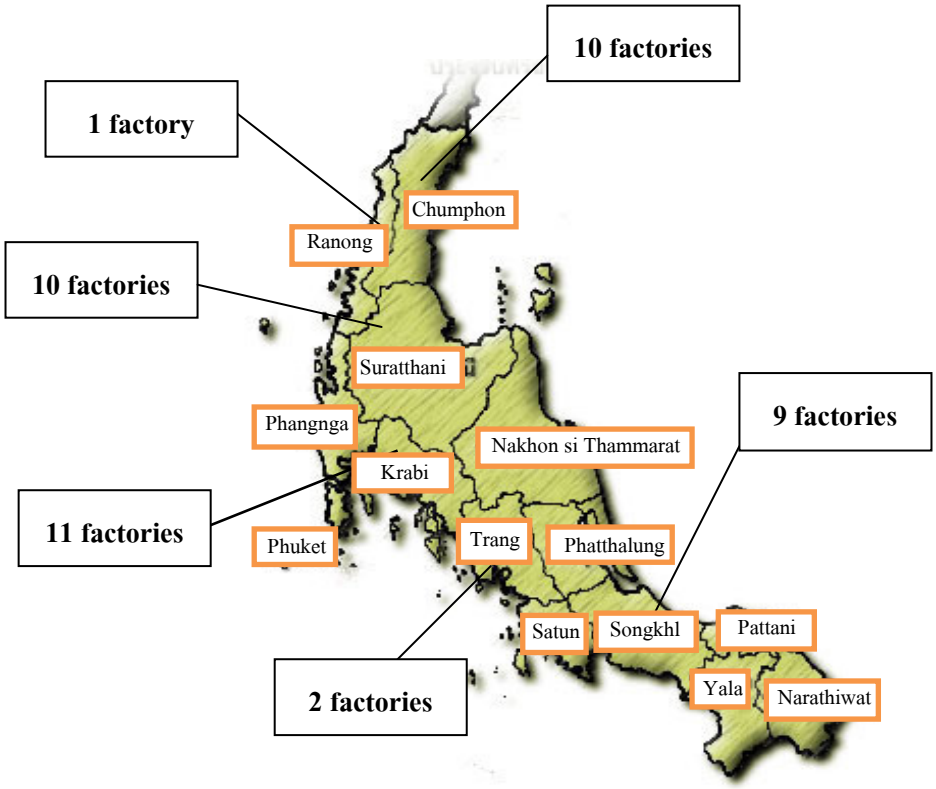


Figure 2.1 Palm oil mill in the South of Thailand.

Source : Adapted from Palm oil Products and Technology Research Center (POPTEC) (2010).

2.1.2 Palm oil mill process

2.1.2.1 Types of process of palm oil mill

1) Dry mill process is process of purchasing only of palm fruit. Dry mill process doesn't separate oil from fiber and kernel. Consequently, quality of crude palm oil is lower than standard wet mill process.

2) Standard wet mill process is wet process of palm oil milling consists of bunch sterilization, fruit stripping, digestion, screw pressing for liquid extraction and centrifugation for oil separation. FFB is sterilized by stem. The fruits are stripped off by a rotary thresher and subsequently mashed in a digester. A twin screw press in used to separate liquid, which is a mixture of water, oil and solid. A vibrating screen removes large size solid particles. Mixing water is added to the digester, the screw press and the screening unit to improve extraction efficiency and flow ability of the processing stream. The extracted product, called palm

oil mixture, is the mixture of palm oil, water and fine solid particles. Consequently, a large amount of water used in process, a larger amount of palm oil mill effluent (POME) is generated with high organic load (Prasertsan *et al.,* 2005)**.**

2.1.2.2 Extraction of crude palm oil mill

There exist several processing stages in the extraction of CPO from fresh fruit bunched. The schematic flow diagram of the standard wet process is shown in Figure 2.2. The detail of palm oil mill processes that consume water and generate pollution is described in the following.

1) Arrival and storage of fresh fruit bunches (FFB) at factory

Soon after harvesting, the FFB must be brought to the mill. The FFB are unloaded on a ramp and put into containers of 2.5 to 3 tones transport capacity. The time from harvesting until sterilizing of the FFB should be as short as possible as and no longer than 72 hours. This is to avoid excessive production of free fatty acids (FFA) by the natural enzyme present in the mesocarp. Palm oil of fresh fruits contains about 1 percent FFA but increase by ageing of the fruits.

2) Sterilization

Sterilization of the FFB is done batch wise in autoclaves of 20 to 30 ton FFB capacity. Depending on that capacity 7 to 9 containers of FFB can be put into the “sterilizer”. The FFB is sterilized in order to inactivate the natural enzymes which stops splitting of fat into FFA and the subsequent loss of oil.

3) Bunch stripping

The containers with the sterilized bunches are emptied into a rotary drum threshers where the fruits are separated from the bunch stalk. The empty fruit bunches (EFB) are at present often separately stored for incineration to reduce the mass of residues and for simultaneous production of ash which has plant fertilizing value.

In addition, the sterilizing loosens the fruit of the FFB, and softens the mesocarp, resulting in easier extraction of oil.

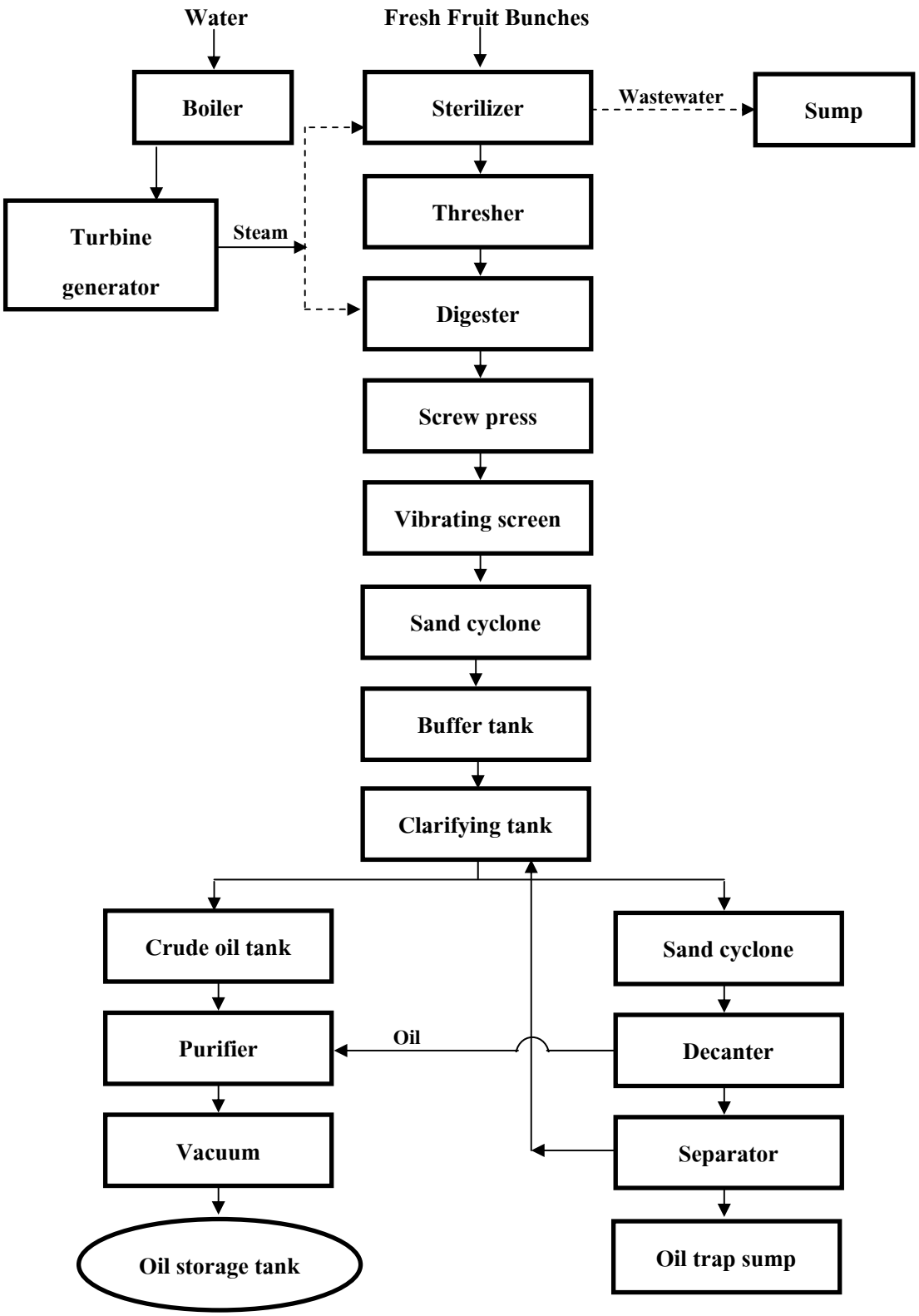


Figure 2.1 A block flow diagram of the palm oil mill process.

Source : Chavalparit *et al.* (2006)

4) Digestion

The separated fruits are discharged into vertical steam-jacketed drums (digesters). Here the fruits are treated mechanically by to convert them into a homogeneous oily mash. Hot water is added to the digester to facility homogenization. This mash is subsequently fed into the oil extraction press.

5) Oil extraction and handing of solid residues

Extraction of palm oil is done by means of a continuous screw press system. The extracted oil phase is collected and is discharged to the purification section. The remaining press cake is transported to a separation system consisting of air classifiers and cyclones (depericarper or fiber separation) for recovery of the nuts and fibers. The nuts and fiber are dried during this separation process by hot air, which is indirectly heated by steam to a temperature of 135ºC. Kernels are recovered from nuts in crackers and are normally sold to kernel oil mills. Fibers and shells are sent to the boilers hose and used as fuel. The screw press produces raw crude oil which contains a high concentration of suspended matter, resulting in difficulties in oil water separation and a high organic loading in the wastewater discharged from the palm oil mill.

6) Oil purification: Clarification and drying

The production process described in oil purification and treatment of setting tank underflow take place, it called “oil room”.

Screening of raw crude oil

For improvement of the following separation steps of oil clarification, hot water is added to the raw oil and then passed through a vibrating screen (Johnson – screen or Sweco – screen) to separate larger size solids as dirt, fibers and fragments of the pericarps from the liquid phase. The oil, after sieving, still contains small size solids and water. The large surfaces of these types of sieves result in intensive contact of oil with air which has a negative effect on oil quality due to oxidation of oil.

Separation of suspended solids from oil

The conventional procedure for separation of oil from water and suspended solids is the “setting tank” method. The raw oil is heated either by the introduction of live steam

or with close steam heating coils which facilitates gravity separation. Depending on the applied setting tank surface loading rate and retention time, this procedure, however, has a low separation efficiency of about 50 percents only. As a result, either the separated oil still contains a high concentration of suspended solids or the settled residue (setting tank bottom sludge) contains a high content of oil. Long retention times combined with high temperature also reduce the oil quality. To improve overall oil yield of the process, some mills switched from the setting tank procedure to a more efficient oil clarification system using a 3-phase centrifuge (decanter). This equipment, however, is not part of the original standard wet process. The separated oil floating on top of the setting tank is collected by a funnel system and sent to the oil purification system. The setting tank underflow is collected in the sludge tank and subsequently treated recovery of oil.

Separation and removal of fine suspended solids from the oil

The raw crude oil from the setting tank (top oil) is combined with recovered oil from the treatment of the setting tank underflow. These results in a total crude-oil production of about 163 kg/ton FFB processed. Centrifuges carry out this final oil purification (solids removal) step. For easy operation, these centrifuges are equipped with an automatic cake discharge and cleaning system Because of the low suspended solids content in the raw crude oil this process step dose not generate large volumes of solid residue and hence, has a very low impact on the environment.

Oil drying and cooling

The purified crude oil, after the centrifugation step, still contains water, which is removed by a vacuum evaporation system. Subsequently, the dried crude oil is kept in storage tanks and sold to an oil refinery. This process-step of crude oil drying has very little environmental impact.

7) Treatment of setting tank underflow (bottom sludge)

The bottom sludge from the “setting tank” is characterized by high oil content (around 14 percent), high concentration of organic substances (both in the dissolved form and as suspended solids) and water soluble substances. In addition, the water phase contains fine fibers and sand. In order to recover oil and to decrease the organic load of the liquid residue, the setting tank bottom sludge is further treated as described below.

8) Straining and desanding

In order to protect the equipment in the subsequent process steps (in particular centrifuges) against clogging, the bottom sludge is pre-cleaned by means of microstrainers/hydrocyclones. These "desanders" are frequently cleaned by discharging the accumulated solids to the drain, followed by the injection of fresh water. Desander washwater consumption is normally around 5 litres/ton FFB

9) Centrifuging

The pre-cleaned setting tank bottom sludge is collected in a buffer tank ("sludge tank") and then pumped to two-phase centrifuges (separators) for oil recovery. To improve oil separation it is common practice to add water to the bottom sludge to improve the oil separation efficiency, which is normally about 92 percent. Water consumption is about 200 kg/ton FFB processed.

Improved oil recovery efficiency can be achieved by using the decanter process in place of the setting tank – separator process. This process, using a three – phase centrifuge (decanter) in place of the setting tank system.

2.1.3 Palm oil mill waste

The palm oil mills generate many by-products and wastes beside the liquid wastes that have been mentioned. They may have a significant impact on the environment if they are not properly dealt with. Palm oil waste products from processing including:

2.1.3.1 Palm oil mill effluent (POME)

Wastewater is generated from moisture in raw material and water is added in the process. Oil room wastewater, sterilizer condensate and turbine cooling water are collective called palm oil mill effluent (POME). The POME is treated anaerobically in a series of wastewater treatment ponds, which required a large land area. There is a concern that the wastewater might pollute underground water. Since the final effluent is of brownish color, it cannot be discharged into the natural water ways (Praseartsan *et al.,* 2005). Effluent water is defined as water

discharged from industries, which contains soluble materials that are injurious to the environment. Such soluble materials may be gases such as CH_4, SO_2, NH_3, halogen or soluble liquids or solids which contain ions of either organic or inorganic origin and with their concentration above the threshold value (Pfaffin-Zirgler, 1980).

Standard/wet process showed that fresh fruit bunch one ton can be generating wastewater about one cubic meter or in the range from 0.98 to 1.05 cubic meters. Point is an important source of wastewater and high concentration of contaminants in wastewater including wastewater from the steam of palm and separator/decanter which the rate of generated wastewater about 0.15 and 0.48 to 0.74 cubic meters per ton FFB, respectively (Phongpeta, 2000).

Characteristics of wastewater from palm oil mill

Wastewater from palm oil mill has different characteristics. It depended on the processes that generated wastewater (Prasertsan, 2000). Wastewater from the palm oil mill process is generated from 5 sources.

1) Wastewater from steam palm has characteristic is oil but is low the suspended solid (SS) and doesn't emulsion. General, the oven 25 tons of FFB can generated wastewater from steaming about 2.0 to 3.0 cubic meters.

2) Wastewater from separated water and decanter cake out-to oil (more than) high suspended solid.

3) Wastewater from clean tools such as separation gravel separate water and slugged and separation high centrifuge.

4) Wastewater from cooling steam tank and evaluation from wastewater has very low the SS and clean.

5) Wastewater from centrifuge generated about 0.03 - 0.15 m^3/ton FFB

Specifically, palm oil mill effluent (POME) is a general phase referring to the effluent from the final stages of palm oil production in the mill. It includes various liquids, dirties, residual oil and suspended solids. POME in its untreated form is a very high strength waste, depending on the operation of the process, that is; informal, semi-formal and formal processes,

the BOD of these wastes ranges from 25,000 to 35,000 mg/L. It contains about 94 percent water. POME actually is the sum total of liquid waste which cannot be easily or immediately reprocessed for extraction of useful products.

The compositions of effluent from these various sources are mainly water, oil solids (suspended and dissolved) sand. Adapalm (1992) reported the flowing composition as percentage of total sludge:

Water	93 – 95 %
Solid	3 – 4 %
Oil	0.5 – 2 %

Characteristic of wastewater and sludge is shown in Table 2-2.

2.1.3.2 Others

Biomass products consist of fibers, shells and EFB. They can be used as biomass fuel for producing energy in process. Decanter cake was considered as waste.

2.1.4 Palm oil mill effluence management

Effluent management involves the typical handing of liquid waste. The physical technique often involves sedimentation, filtration and decolonization of effluent. Physical technique normally at the first stage of purification process to remove suspended solid particles. This is called primary treatment. The commonly used devices include sieve, sedimentation bed and filter. Physicochemical technique involves coagulation of finely dispersed and suspended solid particles, adsorption of the dissolves impurities such as heavy metals (Gang and Weixing, 1998., Gardea *et al.*, 1998., Igwe and Abia, 2003., and Abia *et al.,* 2003.) and selective crystallization, reverse osmosis and ion-exchange processes (Chow *et al.,* 1981). Reverse osmosis is most often used at the final stage of effluent treatment. Secondary treatment is biological process following primary treatment. The forms of secondary biological process include tricking filters, contact stabilization, etc. There are widely known methods of effluent treatment in palm oil mill industries (Chow *et al.,* 1981).

Table 2-2 Properties of wastewater and sludge from palm oil mill industry.

Properties	Source of wastewater				
	steam palm	**Separator**	**Centrifuge**	**Sludge**	**Collected treatment**
Color	Brown	Brown	Dark brown	Brown	Dark brown
pH	5.12	4.61	4.89	4.84	4.05-4.62
BOD	31,620	21,000	45,375	66,550	50,000-60,000
COD	65,969	38,246	67,567	105,955	80,000-150,000
Volatile acid (acetic acid)	3,150	1,638	2,273	5,355	3,100-5,800
Alkalinity	1,576	480	86	200	68-200
Oil & Grease	20	-	4	1,130	15-2,500
TS	54,546	25,634	47,242	448,570	49,000-88,500
Volatile solids	44,354	23,056	39,617	108,590	4,200-82,000
SS	2,600	2,900	20,300	40,000	18,500-52,000
NH_3- TKN	43	23	22	61	23-61
Organic	22	-	518	1,352	550-1,400

Remake : Alkalinity unit mg/L as $CaCO_3$ and other parameter unit mg/L (except for color and pH).

Source : Prasertsun *et al.* (2000).

2.1.4.5 Anaerobic and facultative ponds

Usually, palm oil mill have used anaerobic ponds that this system consists of a series of pond connected in series for different purpose. The effluent after oil trapping is retained in an acidification buffering pond for about two or three days, the resulting effluent is then treated in an anaerobic pond with a hydraulic retention time of thirty to eighty days depending on the mills. This digested liquid is further treated in a series of facultative ponds before it is discharged. In some cases, part of the digested liquid is recycled to the acidification and buffering pond. The total hydraulic retention time of the system ranges from 75 to 120 days (Donne, 1981).

Anaerobic ponds are deep treatment ponds with commonly 2-5 m deep and receive such a high organic loading (usually > 100 g $BOD/m^3/d$ equivalent to > 3000 kg/ha/d for a depth of 3 m.). They contain an organic loading that is very high relative to the amount of oxygen entering the pond, which maintains anaerobic conditions to the pond surface that exclude

oxygen and encourage the growth of bacteria, which break down the effluent. The anaerobic pond acts like an uncovered septic tank. Anaerobic bacteria break down the organic matter in the effluent, releasing methane and carbondioxide. Sludge is deposited on the bottom and a crust forms on the surface as shown in Figure 2.2 (Srisukpan, 1994). Anaerobic ponds don't contain algae, although occasionally a thin film of mainly *Chlamydomonas* can be seen at the surface. They work extremely well in warm climate (can attain 60-85 percents BOD removal) and have relatively short retention time (for BOD of up to 300 mg/l, one day is sufficient at temperature > 20^{o}C)

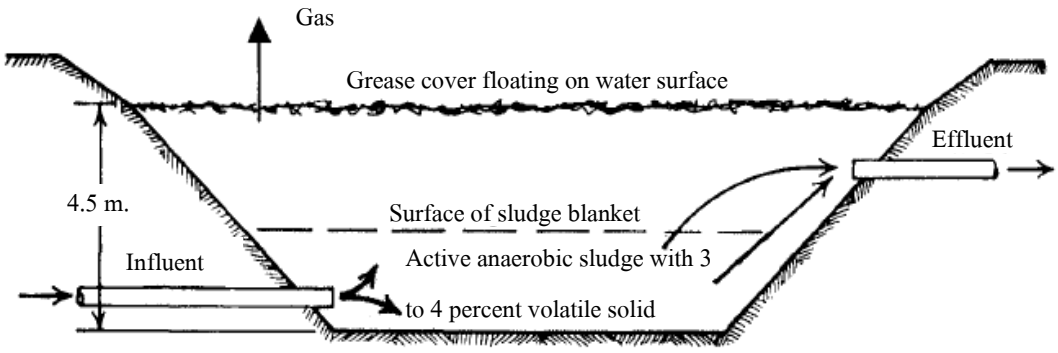


Figure 2.2 Anaerobic pond.

Source: Srisukpan (1994).

2.2 Methane

Biogas typically refers to a gas produced by the biological breakdown of organic matter in the absence of oxygen. Biogas originates from biogenic material and is a type of biofuel. Biogas is produced by anaerobic digestion or fermentation of biodegradable materials such as biomass, manure, sewage, municipal waste, green waste, plant material and energy crops. (National Non-Food Crops Centre, 2003). This type of biogas comprises primarily methane and carbon dioxide. Other types of gas generated by use of biomass are wood gas, which is created by gasification of wood or other biomass. These types of gas consist primarily of nitrogen, hydrogen, and carbon monoxide, with trace amounts of methane.

The composition of biogas varies depending upon the origin of the anaerobic digestion process. Raw biogas produced from digestion is roughly 60 percent CH_4 and 29 percents $CO_2$and gases such as H_2S and N_2 (2 percent) (Richards *et al*., 1991). Table 2-3 demonstrates the composition of biogas.

2.2.1 Chemical stages of anaerobic process

In anaerobic digestion process biodegradable material is broken down by the microorganisms in the absence of oxygen. The process of anaerobic digestion is mainly used to manage waste in domestic and industrial procedures. Through this process huge amount of organic matter is reduced, which results in the reduction of matter to be poured into natural water resources. Although there are number of different microorganisms that are used in the process of anaerobic digestion, the important once is acetic acid-forming bacteria (acetogens) and methane-forming archaea (methanogens). These microorganisms first convert the initial biodegradable material into sugar, acetic acid and hydrogen and then finally results in the production of biogas. Microorganisms that can work within the temperature 35-40ºC are known as mesophiles or mesophilic bacteria. The one that works in very high temperature is called thermophiles or thermophilic bacteria.

Organic compound is protein carbohydrate and lipid component which is solid and solute. Process of decay organic in wastewater is anaerobe and generated biogas. It has 4 stages and each stage has bacterial for the degradation as shows in Figure 2.6. The detail information is descanted as follows:

The first stage : hydrolysis or solubilisation is first stage in anaerobic in which large chains of polymers are broken down into smaller constituents or monomers like sugar, fatty acids and amino acids. These products are then easily available to the microorganisms for their energy and survival (Water Treatment Plant, 2010).

- Hydrolytic and Fermentative bacterial : this group includes both obligate and facultative anaerobes, and may occur upto 108-109 cells/ml of sewage sludge digesters. They remove the small amounts of O_2 present and create anaerobic conditions. These bacteria hydrolyze and ferment the organic materials, e.g., cellulose, starch, proteins, sugars,

lipids, etc., and produce organic acids, CO_2 and H_2. But usually only 50% of the polysaccharides present in the waste may be digested (molecular-plant-biotechnology, 2009).

Table 2-3 Typical composition of biogas.

Compound	Chemical	%
Methane	CH_4	50–75
Carbon dioxide	CO_2	25–50
Nitrogen	N_2	0–10
Hydrogen	H_2	0–1
Hydrogen sulfide	H_2S	0–3
Oxygen	O_0	0–0

Source: Basic Information on biogas (2007).

The second stages : acidogenes is the large chains of fatty acids are broken down by acidogenic (fermentative) bacteria. Acetate and hydrogen that are produced during the first stage are directly consumed by methanogens. But the volatile fatty acids (VFA's) whose chain length is greater than acetate can be digested directly hence these are first to be catabolised into smaller compounds. So during acidogenesis these are broken down (Water Treatment Plant, 2010).

- This stage using many bacterial groups. The main group is *Clostridiumspp.* and *Bacteriodesspp.* are obligate anaerobic (anaerobic condition only and inhibited by oxygen)

The third stages: acetogenesis is product from methane because methane forming bacteria want high specific substrate. Methane forming bacteria use acetic acid, formic acid, hydrogen, methanol and methylamine but can't use volatile fatty acids at carbon atom more than 2 atoms (Water Treatment Plant, 2010). Bacterial group, that can degrade volatile fatty acids that has carbon atom more than 2 atoms to acetic acid such as acetoclastic group bacterial or acetogens group bacterial. Hydrolysis, acedogenesis and acetogenesis is continual reaction. So, these called acidification process and called bacterial is acid formers bacterial.

- Acetogenic Bacteria these bacteria oxidise H_2 by reducing CO_2 to acetic acid, which is then used up by methanogens to generate methane, CO_2 and H_2. Thus acetogenic bacteria also remove H_2 and enable the obligate H_2 producing bacteria to continue their function (molecular-plant-biotechnology, 2009).

The four stages : methanogenes phase is susceptible to the environment. Since, changes in environmental conditions like temperature would affect its required performance. Thus, it is best carried out in a tank with so many advantages which are found using a tank digester and this includes organic acid (small molecule), carbon dioxide (CO_2) and hydrogen (H_2) generated from acetogenesis process to change to methane and carbon dioxide by methane forming bacteria is methaogenic bacteria.

- Methanogenic bacteria : This group of bacteria converts acetate and CO_2 and H_2 into CH_4. Thus methanogens remove the hydrogen produced by obligate H_2 prosing bacteria, thereby lowering the H_2 partial pressure and enabling the latter to continue producing H_2. Methanogenic bacteria are the strictest possible anaerobic known (molecular-plant-biotechnology, 2009).

When considering a group of bacterial that live together in anaerobic pond, a group of methane formers bacteria, a major bacterial groups to control the speed of reaction in the system was formed. Since bacteria have the slowest growth rate and limited environment of bacteria than other groups.

2.2.2 Factors which affecting the methane generation rate (Kothri *et al.,* 2008)

1) Oxygen: The oxygen must be absence in anaerobic pond because it effects to methanogens or methane formers stop grown.

2) Temperature optimum: Temperature affects bacterial activity; methane formation is optimum in the temperature range 35 – 38ºC. In cold regions a solar canopy is built over the biogas plants to maintain the desired temperature.

In hot regions, another micro-organism called 'thermophilic' is utilized for anaerobic fermentation in the temperature rage 55 – 60ºC. Gas production rises with the increase in average ambient air temperature. As the temperature increase, the total retention period decrease and vice-versa. However, the total gas production remains practically the same.

3) Solids Content: For high efficiency concentration of solid in pond should be add organic between 5 – 10 percents and should be has value about 25 percen for batch operation.

4) pH value: Measure of pH value indicates the concentration of hydrogen ions. Micro-organism is sensitive to pH of the digested slurry. For optimum biogas production, pH can be varied between 6.8 and 7.8. At pH of 6.2, acid conditions prevail which restrain the growth of methanogenic bacteria. Control on pH should be exercised by adding alkali when it drops below 6.6.

5) Seeding: Contain both acid forming bacteria and methane forming bacteria. Acid forming bacteria multiply fast, while the methane forming bacteria grow slowly. To start and accelerate fermentation, seeding of methane forming bacteria is required. Accordingly, a small quantity of digested slurry rich in methane-forming bacteria is added to freshly charged digester.

6) Solid-to-water ratio. Cattle dung (gobar) contains about 18 percent solid matter and the remaining 82 percent is water. Anaerobic fermentation proceeds at a faster rate if the surry contains about 9 percent solid matter. Digester feed is prepared by mixing water and solid in the ratio 1:1 by weight to reduce the solid content. To increase the solid matter, crop residues and weed plants may be mixed with the feed stock.

7) Volumetric loading rate: It is expressed as the quantity of organic waste fed into the digester per day per unit volume. In general, the municipal sewage treatment plants operate at a loading rate of 1.0 to 1.5 kg/m^3/day. Overloading and underloading reduce the biogas production with a fixed retention time.

8) Retention Time: The period for which the biomass slurry is retained inside the digester is called 'retention time'. It refers to the volume of digester divided by the volume of slurry assed per day. Thus, a 120 liters digester which is fed at 5 liters per day would have a

retention time of 24 days. It is optimized to achieve 80% complete digestion considering ambient temperature.

9) C/N Ratio: Methanogenic bacteria need carbon and nitrogen for its survival. Carbon is required for energy while nitrogen for building cell protein. The consumption of carbon is 30 to 35 times faster than that of nitrogen. A favorable ratio of C:N can be taken as 30:1. Any deviation from this ratio lowers the biogas production. A proper balance of C:N ratio is maintained either by adding saw dust having a high C:N ratio or by poultry waste having a low C:N ratio.

10) Bacteria affect to methane gas. It has 3 types, which consist of methane production bacteria (MPB), sulfate reduction bacteria (SRB) and Methane Oxidation Bacteria (MOB) (Sangnil and Treampanit, 2007) in section 2.3.

2.2.3 Benefit of methane

By using biogas, many advantages arise. In North America, utilization of biogas would generate enough electricity to meet up to three percent of the continent's electricity expenditure. In addition, biogas could potentially help reduce global climate change. Normally, manure that is left to decompose releases two main gases that cause global climate change: nitrous dioxide and methane. Nitrous dioxide warms the atmosphere 310 times more than carbon dioxide and methane 21 times more than carbon dioxide. By converting cow manure into methane biogas via anaerobic digestion, the millions of cows in the United States would be able to produce one hundred billion kilowatt hours of electricity, enough to power millions of homes across the United States. In fact, one cow can produce enough manure in one day to generate three kilowatt hours of electricity; only 2.4 kilowatt hours of electricity are needed to power a single one hundred watt light bulb for one day. (State Energy Conservation Office (Texas), 2009). Furthermore, by converting cow manure into methane biogas instead of letting it decompose, we would be able to reduce global warming gases by ninety-nine million metric tons or four percent . (Webber *et al.*, 2008).

The 30 million rural households in China that have biogas digesters enjoy 12 benefits: saving fossil fuels, saving time collecting firewood, protecting forests, using crop residues for animal fodder instead of fuel, saving money, saving cooking time, improving hygienic conditions, producing high-quality fertilizer, enabling local mechanization and electricity production, improving the rural standard of living, and reducing air and water pollution.

2.2.4 Impact of methane on the Earth

Methane is the main component of the biogas which causes the main global warming. Methane in the Earth's atmosphere is an important greenhouse gas with a global warming potential of 25 compared to CO_2 over a 100-year period (Shindell *et al.,* 2009). This means that a methane emission will have 25 times the impact on temperature of a carbon dioxide emission of the same mass over the following 100 years. Methane has a large effect for a brief period (a net lifetime of 8.4 years in the atmosphere), whereas carbon dioxide has a small effect for a long period (over 100 years). Because of this difference in effect and time period, the global warming potential of methane over a 20 year time period is 72. The Earth's methane concentration has increased by about 150 percent since 1750, and it accounts for 20 percent of the total radiative forcing from all of the long-lived and globally mixed greenhouse gases as shown in

Figure **2.3** (United Nations Environment Program, 2010). Usually, excess methane from landfills and other natural producers of methane are burned so $CO_2$is released into the atmosphere instead of methane, because methane is such a more effective greenhouse gas. Recently, methane emitted from coal mines has been successfully utilized to generate electricity.

2.3 Bacteria affect the biogas methane

2.3.1 Methanogens or methane formers

Methanogens or methane formers create methane which doesn't use oxygen thus, has a little amount of oxygen. They are in a group of kemo-hecterotropth bacteria and degraded organic as sole and energy source for growth and composed of 3 groups (Sanning, 2005).

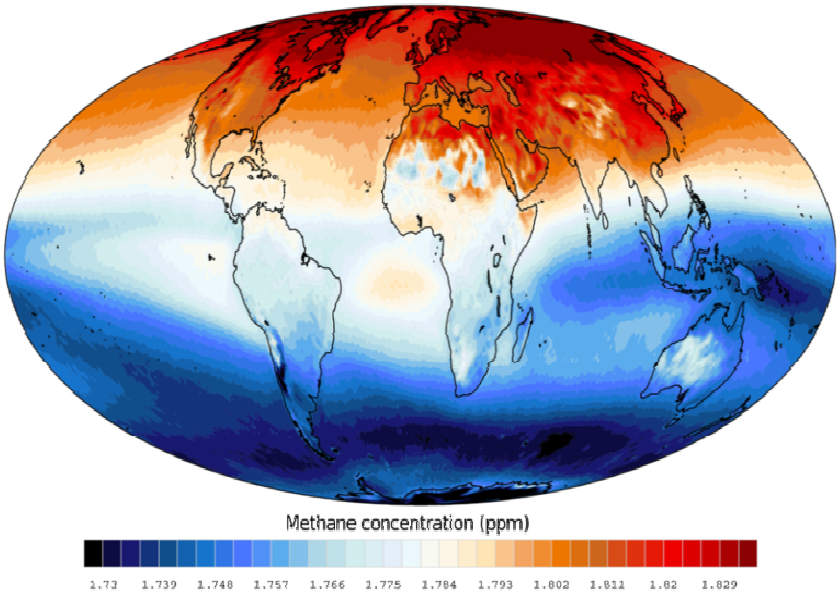


Figure 2.3 Methane concentration in the upper troposphere. (In 2006-2009).

Source : United Nations Environment Program (2010).

1) Acetate Utilizing Bacteria or Acetotrophic Methanogens or Acetoclastic Methanogens which oxidized acetate to methane and changed acetic acid (about 70%) to methane and carbondioxide (Sa-ngonkripong, 2008). This bacterial group has 2 types consists of:

a. *Methanosaeta spp.* (*Methanotrix spp.*) grows in low concentrated acetate condition.

b. *Methanosarcina spp.* grows in high concentration acetate condition.

2) Hydrogen Utilizing Bacteria or Hydrogenotrophic Methanogens are bacteria change hydrogen and carbondioxide to methane and water. These group bacteria grow faster.

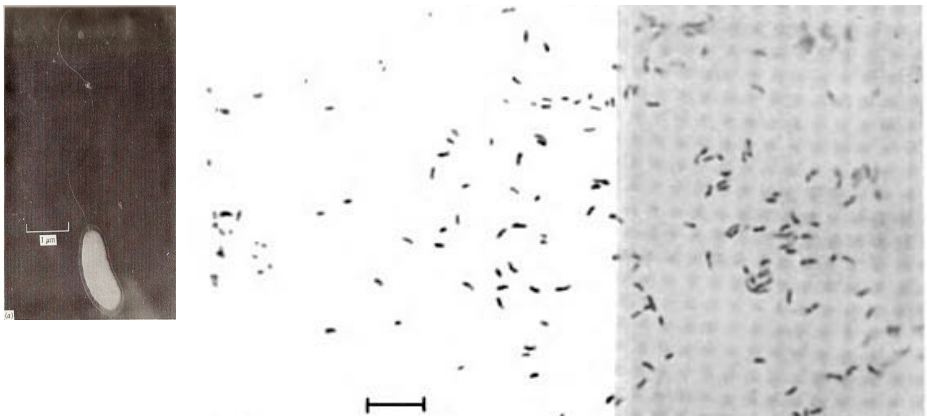
3) Methyllotrophic Methanogens the groups bacteria use Methyl group (CH_3^-) as a substrate for the products of CH_4 such as methanol and methylamine [$(CH_3)_3$-N] (Sanning, 2005)

2.3.2 Sulfate reduction bacteria (SRB)

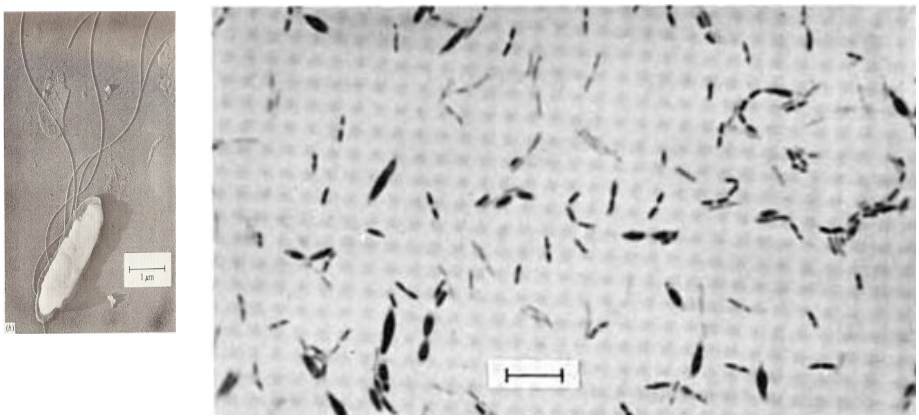
Sulfate Reducing Bacteria (SRB) the extract oxygen from sulfate compounds and change the sulfite in the form of sulfate is hydrogensulfite (H_2S). Sulfate Reducing Bacteria (SRB) group compete with the Methanogens in the degradation of organic compounds using sulfate compounds as the electron which it called "Sulfate Reduction" (Visser, 1994). The two well-established genera of sulfate reducing bacteria, *Desulfotomaculum* and *Desulfovidrio* (Lindsey and Creaser, 1975):

1) *Desulfotomaculum* is bacterial, that may be mesophilic or thermophilic range of 35 - 60°C but naturally occurring halophilic strains' are not know. It is slowly grown bacterial at temperature lower than 35°C. Bacterial in natural has life in rage mesophilic (Nazina and Rozanova, 1978). The thermophilics species, *Desulfotomaculum nigrificans*, was earlier known as *Clostridiuum nigrificans.* All members of the genus form spore and genera are Gram-negative (Postgate and Raymond, 1979). Morphologically most desulfomacula are straight, but departures from the rule exist as shown in Figure 2.4 a (Postgate, 1979).

2) *Desulfovidrio* is the best know, largely because its members are usually somewhat easier to isolate and purify, they are usually mesophilic (45°C - 48°C) and can be halophilic. They do not form spores. Earlier synonyms for this genus were *Spirillum, Microspira, Vibrio* and *Sporovibrio* (Pochon and Barjac, 1954) that were typed species is *Desulfovibrio desulfuricans* (Postgate and Raymond, 1979). Morphologically most dusulfovidrios are curved (Figure 2.5 b) and tends to form spirilloid forms. *Desulfovibrio gigas* is exceptional because of its large size, spirilloid appearance and instracellular granules as shown in Figure 2.4 b (Postgate, 1979).



a) *Desulfotomaculum*



b) *Desulfovidrio*

Figure 2.4 Characteristic of sulfate reduction bacteria group.

Source : Postgate (1979).

Sulfate reducing bacteria (SRB) decompose organic matters by use SO_4^{2-} as electron acceptor and obtain sulfide as product. SRB plays an important role in anaerobic digestion of complex substrates: as shown in Figure 2.6.

1) It generates sulfides that cause product inhibition of SRB. It also competes with MB for substrate, acetate and/or hydrogen, lead to decreasing of methane production.
2) It causes alkalinity resulting from conversion of sulfate to sulfide.
3) It accelerates the oxidation of organics, such as lactate, which are commonly degraded at a lower rate by incomplete oxidation of non-SRB.

Sulfate reduction results in accumulation of sulfide. Sulfide is most toxic in its undissociated form because the neutral molecule can permeate the cell membrane. Sulfide production cause several disadvantages as follows:

1) It's toxic to methanogenic bacteria (MB), acetogenic bacteria (AB), and SRB.
2) Fraction of organic compounds is used by SRB rather than MB, results in decreasing of methane production
3) Quality of biogas is reduced since a part of produced sulfide ends up as H_2S in the biogas. So removal of H_2S from the biogas is required.
4) H_2S gas is toxic, malodorous and corrosive.
5) It accelerates the oxidation of intermediate organic acids, such as lactate, which are normally degraded at a lower rate by incomplete oxidizing non-SRB. Acetate and sulfide are products from oxidation of the organic acids.

2.3.3 Methanotrophs

Methanotrophs or methanotrophic bacteria are a subset of physiological group of bacteria known as methylotrophs which are a group of gram-negative bacteria and widespread in the environment, that have characterized over 100 new methane-utilizing bacteria. Methanotrophic bacteria are unique in their ability to utilize C_1 compounds, including methane, methanol, and methylamine as sole source of carbon and energy (Anthony, 1982., Bowman *et al.,* 1990., Hou, 1984. and Whittenbury *et al.,* 1970). They are unable to utilize as sole energy source any compounds with carbon-carbon bonds (Bowman *et al.,* 1991). The organisms are ubiquitous, have growth optimum in the mesophilic zone, but also grow under thermophilic conditions (Jewell *et al.,* 1992). Biological methane oxidation consists of anaerobic, facultative and aerobic. (Andrew *et al.,* 1999).

2.3.3.1. Anaerobic methanotrophs bacteria

Anaerobic oxidation of methane (AOM) is a microbially mediated phenomenon and represents a significant component of carbon cycling in marine environments (Barnes and Goldberg, 1976) thus are a specialized group of archaea. AOM is believed to be mediated by a

consortium of archaea and sulfate reducing bacteria (Amann *et al.,* 1995, Boetius *et al.,* 2000, and De Long *et al*., 1989).

AOM is performed by two paraphyletic groups of methanogenic archaea. One of these (ANaerobic MEthanotroph: ANME-2) is distantly related to the acetoclastic methanogen *Methanosarcina*. The other (ANaerobic MEthanotroph: ANME-1) forms a separate cluster branching off between the acetoclastic and nonacetoclastic methanogens thus these are normally found in association with sulfate-reducing bacteria (Valentine, 2002) in Figure 2.6. The two groups have approximately 81% sequence identity in the 16S rRNA genes (Hallam *et al.*, 2003 and Orphan *et al.,* 2003). This indicates that methanogenic archaea involved in AOM might have different phenotypes and that at least twice the capability for reversed methanogenesis has volved from methanogenesis (or vice versa). There is some preliminary evidence that the biochemical mechanism of AMO by these phylogenetic groups is at least somewhat different (Hallam *et al.,* 2003 and Kruger *et al.,* 2003). As mentioned above, the syntrophic sulfate-reducing partners are often related to the *Desulfosarcinales*.

Geochemical evidence indicates that the net consumption of methane (CH_4) in these anoxic environments is linked to the consumption of sulfate (SO_4^{2-}) (Barnes and Goldberg, 1976., Devol and Ahme,1981., Hoehler *et al.,* 1994., Iversen and Jorgensen,1985 and Reeburgh, 1976). The net reaction was described as in equation (2-1):

$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + 2H_2O \qquad (2\text{-}1)$$

Global Nutrient Cycling (1980) reported that in marine sediments methane concentrations decreased from the sediments toward the water column. At the same time, sulfate concentrations decreased from the water column into the sediments, indicating that sulfate might be the electron acceptor for anaerobic methane oxidation (AMO). Anaerobic methane oxidation has two properties in common: slow growth and mutualism. AMO is mediated by syntrophic reversed methanogenic archaea and sulfate-reducing bacteria. The two are always found in close proximity to one another, although there is some recent evidence for the occurrence of

methanogenic archaea involved in AMO without a sulfate reducer nearby (Orphan *et al.,* 2003). It is still unknown if these archaea are simply inactive, are capable of AMO without a sulfate-reducing partner, or are doing something completely different.

2.3.3.2 Aerobic methanotrophs bacteria

Aerobe methanotrophs bacteria are a unique group of gram-negative bacteria capable of utilizing methane as sole carbon and energy source (Haber *et al.,* 1983 and Patt *et al*., 1974).The most methylotrophic bacteria are those aerobic bacteria that utilize one-carbon compounds more reduced than formic acid as sources of carbon and energy and assimilate formaldehyde as a major source of cellular carbon (Dijkhuizen *et al.,* 1992., Lidstrom, 1991., and Whittenbury and Dalton, 1981)**.** Methylotrophic bacteria utilize a variety of different one-carbon compounds including methane, methanol, methylated amines, halomethanes, and methylated compounds containing sulfur (Dijkhuizen *et al.,* 1992., Hanson *et al.,* 1990., and Lidstrom, 1991). Some cleave methyl groups from organic compounds including choline or the pesticide carbofuran (Topp *et al.,* 1993.) and utilize them as sole sources of carbon and energy (Patt *et al*., 1974). Bacteria that utilize formate, cyanide, and carbon monoxide have different modes of metabolism including pathways for the assimilation of one-carbon units. In addition, Whittenbury *et al.* (1970) and Anthony (1982) also reported that methane-oxidizing bacteria more than 100 types used oxygen and gram-negative by used methane and ethanol as sole carbon and energy source to emission H_2 and CO_2 to the atmosphere.

Methane oxidation is bacteria. It grows in the area that oxygen can diffuse passes down. In addition, Kjeldsen *et al.* (1997) reported oxygen demand in methane oxidation reaction, that has the value in rage 3.6-4.0 g O_2/g CH_4but high moisture, it was less diffusion passes down caused of increase methane oxidation therefore, factor in methane oxidation include temperature moisture and oxygen (Kightley *et al.,* 1995., Whalen *et al.,* 1990., Boeckx and Cleemput, 1996 and Park *et al.,* 2002).

The reactions oxidize methane to carbon-dioxide and water by methanotroph in soil in aerobe condition (Croft *et al.,* 1989 and Visser, 1994) in equation (2-2).

$$CH_4 + 2O_2 \text{ ----------> } CO_2 + H_2O + \text{Heat} + \text{New Cell} \quad (2\text{-}2)$$

Pathways for methane oxidation (Hanson and Hanson, 1996) through the enzyme methane monooxygenase (MMO) for oxidize methane to methanol (CH_3OH). These MMOs can cometabolizer or transform non-growth by either growing or resting cell (Brigmon, 2001). Therefore, dehydrogenation reaction was formaldehyde (HCHO), formate (HCOOH) and carbon-dioxide (CO_2), respectively by using the enzyme methanol dehydrogenase (MDH) formaldehyde dehydrogenase (FADH) and formate-dehydrogenase (FDH) relatively (Figure 2.6). Therefore, methanotroph utilizing methanol and formaldehyde as sole carbon and energy source (Humer *et al*., 2001).

Methanotrophs are also of special interest to environmental microbiologists because of their capability to degrade various environmental contaminants, their potential for single cell protein production, and other novel aspects of their biochemistry (Hanson and Hanson, 1996). The methanotrophs bacteria were separated into three assemblages consists of:

Type I : Methanotrophs are γ- proteobacteria that have stacked membranes harboring methane monooxygenase (pMMO) the emzyme for primary methane oxidation, and that use ribulose monophosphate (RuMP) cycle, which converts formaldehyde into muticarbon compounds, for building cell biomass (Lidstrom, 2001). They are including the genera *Methylomonas* and *Methylobacter* which included three broadly homologous clusters of species and proposed that the family *Methylococcaceae* should contain the genera *Methylococcus, Metahylomicrobium, Methaylobacter* and *Methylomonas*as redefined in their publications (Bowman *et al.,* 1993., and Bowman *et al*, 1995).

Type II : Methanotrophs belong to the α- proteobacteria, have rings of pMMO-harboring membranes at the periphery of the cells, and use the serine cycle, an alternative pathway for converting formaldehyde into biomass; these bacteria also often contain a soluble (s) MMO in addition to pMMO (Lidstrom,2001). They are including the genera *Methylosinus* and *Methylocystis* (Hanson *et al.,* 1991., and Whittenbury and Krieg, 1984)

Remark: All type I cells use the hexulose monophosphate pathway for formaldehyde incorporation

All type II cells use the serine pathway for formaldehyde incorporation. (Lawrence *et al.,* 1970.)

Type X : Methanotrophs was a new group, belong to the genus *Methylococcuscapsulatus* (γ - proteobacteria) and combine features characteristic of the other two types: they have stacked membranes and the RuMP cycle have elements of the serine cycle and sMMO (Lidstrom,2001).

Remark : Type X methanotrophs were distinguished from type I methanotrophs because they also possessed low level of enzymes of the serine pathway ribalose-bisphosphate carboxy lase and enzyme present in Cavin Benson cycle (Whittenbury and Dalton,1981. and Whittenbury and Krieg, 1984).

2.3.3.3 Facultative methanotrophs bacteria

Facultative methylotrophs can use compounds containing no carbon-carbon bonds as well as complex organic compounds with carbon-carbon bonds as sole sources of carbon and energy (Colby and Zatman, 1972).

Green (2005) reported that the genus *Methylobacterium* is composed of a variety of pink-pigmented facultatively methylotrophic (PPFM), which can grow on one-carbon compounds such as formate, formaldehyde and methanol as sole source of multicarbon growth substrates. Most, but not all, strains can grow on nutrient agar and some can grow on methylated amines. Only one strain has been reported to utilize methane as sole carbon source consists of *M. rhodesianum, M. zatmanii* and *M. fuiisawaense.*

In addition, Prior to 1960, The taxonomy of many of the isolates now assigned to *Methylobacterium* was still uncertain. Although regarded as Gram – negative, these organisms often stained Gram-variable. This Gram – variability, coupled with their morphological properties (mainly rods, which are occasionally branched are exhibit polar growth), has contributed to much of the confusion surrounding their checkered taxonomic history and in 1974, two facultative methanotrophs have been reported, both of which contained peripheral (type II) internal membrane systems and utilized the serine pathway for formaldehy defixation (Patel *et al.,* 1978., and Patel *et al.,* 1979). The isolation of these organisms has opened the way for detailed study of C-1 assimilation processes in methanotrophs (O'Connor *et al.,* 1975 and O'Connor *et al.,* 1978). However, neither facultative methanotroph has proven suitable for genetic studies of methane oxidation, nitrogen fixation, and related functions.

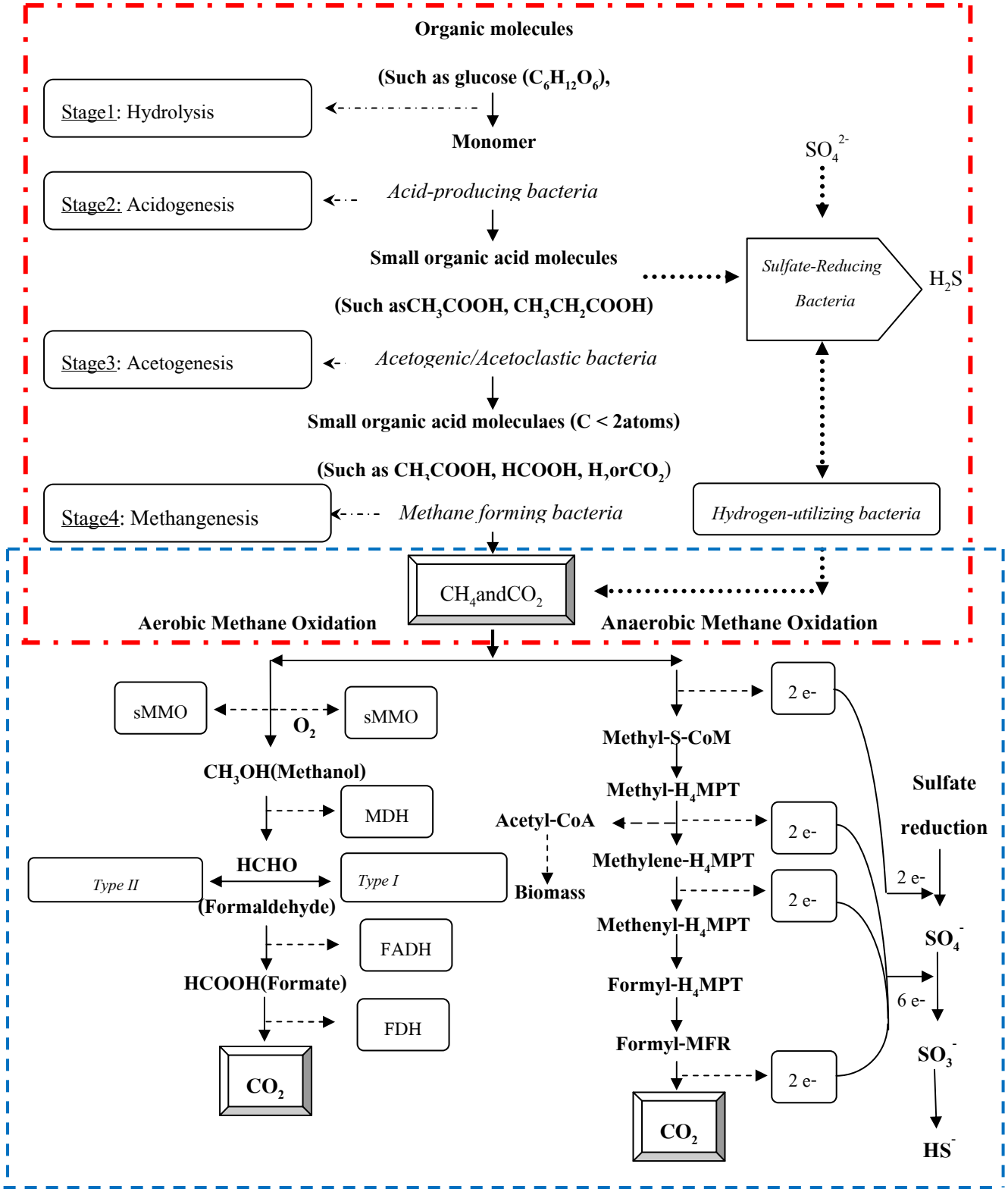


Figure 2.6 Stage of generate methane and methane oxidation.

Adapted from: Sanning (2005), Humer and Lechner (2001), and Hallam *et al.*(2004).

Green and Bousfield (1981) reported that non-pink pigmented facultative methylotrophs claimed to be able to utilize methane as sole source of carbon and energy and belong to the genus *Methylobacterium* consists of *M.ethanolicum* and *M.hydrolimneticum* (Lynch *et al.,* 1980).

2.4 The evaluation of methane emission

The Kyoto Protocol is an international agreement linked to the United Nations Framework Convention on Climate Change (UNFCCC). The major feature of the Kyoto Protocol is that it sets binding targets for 37 industrialized countries and the European community for reducing greenhouse gas (GHG) emissions. GHGs consist of 6 gases, which have different Global Warming Potential (GWP). (McKeown and Gardner, 2009, and Bracmort *et al.,* 2009).

Methane is one of a gas that causes global warming such as carbon dioxide, methane, nitrous-oxide (N_2O), hydrofluoro-carbon est. Carbon dioxide is cause of global warming more than other gas because it release more than other gases is 68 percent of GHG, The second is methane 27 percent.

2.4.1 Mathematical model

The evaluation of methane emission with mathematical model (Methane Recovery in Wastewater Treatment AMS III.H version 16), that developed by UNFCCC for methane recovery in wastewater treatment. Figure 2.7 shows the CDM concept. Characteristic of CDM project activity categories can be classified according to the amount of gas and size of the project. It consist of three types including bundle of small scale CDM, small scale CDM and large scale CDM.

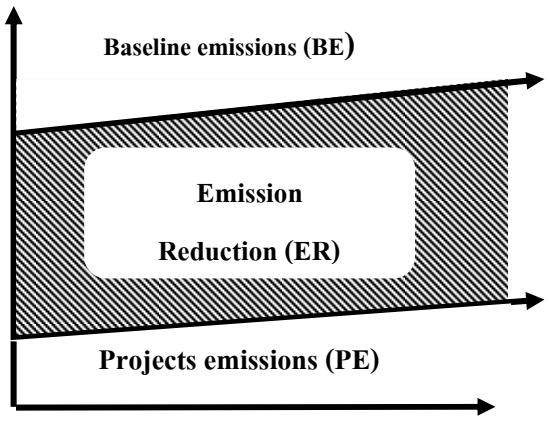


Figure 2.7 Baseline and project emissions and emissions reductions

Adapted from : UNFCCC (2009)

Figure 2.7 shows the reduction of GHG emission, it can calculated in equation (2-3)

$$ER_{y,ex\ ante} = BE_{y,ex\ ante} - \left(PE_{y,ex\ ante} + LE_{y,ex\ ante}\right) \qquad (2\text{-}3)$$

Where:

ER_y = Emission reduction in year *y* (tCO_2e)

BE_y = Baseline emission in year *y* calculated as equation 2.2 (tCO_2e).

PE_y = Project emission in year *y* calculated as equation 2.4 (tCO_2e).

LE_y = Leakage emission in year *y* (tCO_2e)

Baseline Emission

Equation (2-4) is equation for the evaluation of methane emission of baseline emissions (tCO_2e) that emit the maximum quality of gas. In part of the project emission, that it benefit the gas emission from the baseline emission. Therefore, the gas emission has the value less than baseline emission. (Methane Recovery in Wastewater Treatment AMS III.H version 16, UNFCCC, 2009)

$$BE_y = \{BE_{power,y} + BE_{ww,treatmen,y} + BE_{s,teratment,y} + BE_{ww,disch\,arg\,e,y} + BE_{s,final,y}\} \quad (2.4)$$

Where:

BE_y = Baseline emissions in year y (tCO_2e)

$BE_{power,y}$ = Baseline emission from electricity or fuel consumption in year y (tCO_2e)

$BE_{ww,treatment,y}$ = Baseline emission of the wastewater treatment systems affected by the project activity in year y (tCO_2e)

$BE_{s,treatment,y}$ = Baseline emission of the sludge treatment systems affected by the project activity in year y (tCO_2e)

$BE_{ww,disch\,arg\,e,y}$ = Baseline methane emission from degradable organic carbon in treated wastewater discharged into sea/river/lake in year y (tCO_2e).

$BE_{s,final,y}$ = Baseline methane emission from anaerobic decay of the final sludge produced in year y (tCO_2e).

The evaluation of GHG emissions from wastewater treatment system with mathematical model (Methane Recovery in Wastewater Treatment AMS III.H version 16, 2009) developed by UNFCCC. In palm oil mills, there was no sludge treatment. The $BE_{s,treatment,y}$ and $BE_{s,final,y}$ were not counted. In addition, the treated wastewater was utilized for oil palm plantation or retention. $BE_{ww,disch\,arg\,e,y}$ was considered to be zero. The palm oil mill used the electricity produced from biomass or biogas, $BE_{power,y}$ was considered to be zero. The equation 2-5 was used to calculated baseline emission for the wastewater treatment of palm oil mill.

$$BE_{ww,treatment,y} = \sum_i Q_{ww,y} * COD_{\inf low,y} * MCF_{ww,teratment,BL} * B_{o,ww} * UF_{BL} * GWP_{CH4} \quad (2\text{-}5)$$

Where:

$Q_{ww,y}$ = Volume of wastewater treated in baseline wastewater treatment system in year *y* (m^3).

$COD_{\inf low,y}$ = Chemical oxygen demand of the wastewater inflow to the baseline treatment system in year *y* (t/m^3).

$MCF_{ww,teratment,BL,i}$ = Methane correction factor for baseline wastewater treatment system (MCF values as per Table 2-4).

$B_{o,ww}$ = Methane producing capacity of the wastewater (IPCCC lower value of 0.25 $kgCH_4/kgCOD$).

UF_{BL} = Model correction factor to account for model uncertainties (0.89).

GWP_{CH4} = Global Warming Potential for methane (value of 21).

Project Emission

The equation (2-6) is equation for evaluation project emissions (tCO_2e)

$$PE_y = \left\{ \begin{matrix} PE_{power,y} + PE_{ww,treatment,y} + PE_{s,treatment,y} + PE_{ww,discharge,y} + \\ PE_{s,final,y} + PE_{fugitive,y} + PE_{biomass,y} + PE_{flaring,y} \end{matrix} \right\} \quad (2\text{-}6)$$

Where:

PE_y = Project activity emission in the year y (tCO_2e)

$PE_{power,y}$ = Emission from electricity or fuel used by the project facilities in the year y (tCO_2e).

$PE_{ww,treatment,y}$ = Methane emission from wastewater treatment systems affected by the project activity, and not equipped with biogas recovery in the project scenario, in year y (tCO_2e).

$PE_{s,treatment,y}$ = Methane emission from sludge treatment systems affected by the project activity, and not equipped with biogas recovery in the project situation, in year y (tCO_2e).

$PE_{ww,discharge,y}$ = Methane emission on account of inefficiency of the project activity wastewater treatment systems and degradable organic carbon in treated wastewater in year y (tCO_2e).

$PE_{s,final,y}$ = Methane emission from anaerobic the decay of the final sludge generated by the project activity treatment systems in year y (tCO_2)

$PE_{fugitive,y}$ = Methane fugitive emission due to inefficiencies in capture systems in year y (tCO_2e).

$PE_{flaring,y}$ = Methane emission due to incomplete flaring in year y (tCO_2e).

$PE_{biomass,y}$ = Methane emission from biomass stored under anaerobic conditions which would not have occurred in the baseline situation (tCO_2e)

Methane emission form the baseline wastewater treatment systems affected by the project ($PE_{ww,treatment,y}$) are determined using the methane generation potential of the wastewater treatment systems shall be calculated as per equation (2.7) and $PE_{power,y}$, $PE_{s,treatment,y}$, $PE_{y,ww,discharge}$, $PE_{s,final,y}$, $PE_{fugitive,y}$ $PE_{flaring,y}$ and $PE_{biomass,y}$ have the values of this terms are Zero.

$$PE_{ww,treatment,y} = \sum_{i} Q_{ww,y} * COD_{\inf low,y} * MCF_{ww,teratment,PJ,k} * B_{o,ww} * UF_{PJ} * GWP_{CH4} \quad (2.7)$$

Where:

$Q_{ww,y}$	=	Volume of wastewater treated in baseline wastewater treatment system in year y (m^3).
$COD_{removed,PJ,k,y}$	=	Chemical oxygen demand removed by project wastewater treatment system k in year y (tonnes/m^3), measured as the difference between inflow COD and the outflow COD in system k.
$MCF_{ww,teratment,PJ,k}$	=	Methane correction factor for project wastewater treatment system k (MCF values as per Table 2.4).
$B_{o,ww}$	=	Methane producing capacity of the wastewater (IPCCC lower value of 0.21 $kgCH_4$/kgCOD).
UF_{PJ}	=	Model correction factor to account for model uncertainties (1.12).
GWP_{CH4}	=	Global Warming Potential for methane (value of 21).

Table 2-4 IPCC default values for methane correction factor (MCF).

Type of wastewater treatment and discharge pathway or system	MCF value
Discharge of wastewater to sea, river or lake	0.1
Aerobic treatment, well managed	0.0
Aerobic treatment, poorly managed or overloaded	0.3
Anaerobic digester for sludge without methane recovery	0.8
Anaerobic reactor without methane recovery	0.8
Anaerobic shallow lagoon (depth less than 2 metres)	0.2
Anaerobic deep lagoon (depth more than 2 metres)	0.8
Septic system	0.5

Source : Methane Recovery in Wastewater Treatment AMS III.H version 16 (2009)**.**

2.4.2 Actual measurement

Chamber method is on the popular measurement because it can be used for large area and its process in not complicated. The chambers can be classified in two typed. Those with forced flow through air circulation indicated as “open chamber”, and those with close-loop air circulation, are “close chamber” (Hettiaratchi and Hansen, 1996 and Cheema, 1997). Table 2-5 presents the comparison of close and open flux chamber for gas measurement.

2.4.2.1 Open chamber method

Open chamber method, cleaned air must be forced to the chamber. The pressure of outside air should exceed a pressure of wastewater gas and velocity of outside air sir must be controlled. Pressure deficits in chamber caused by induced air flow can cause artificial high flux. The close chamber method is less complicated. It is simple to construct, do not disturb a site, easy to install or remove, can be used in a place with no electrical supply. Gas flux from wastewater can be calculated from the change in concentration with time. Figure 2.8 shown Open flux chamber method.

Table 2-5 Comparison of close and open flux chamber for gas measurement**.**

Close Chamber	Open Chamber
Characteristics	
Simple cylindrical chamber for homogenous mixing of gas.	Coupled to the atmosphere by air inlet thorough which outside air is continuously drawn, forced dry sweep air flow over surface at signicantly exceeding the gaseous release rate.
Gas flux Determination	
The change in concentration with time (periodically collecting gas sampling).	The concentration difference between incoming and outgoing air.
Advantages	
Small flux can be measured. No electrical demand. Sample to construct. No disturbance to the site. Easy to install and remove.	Maintain environmental conditions close to those of the uncovered field. More applicable for continuous long term monitoring of gas flux.
Problems	
1. Inhibition of normal gas distribution by the increasing of gas concentration in enclosure head space. *Solves by short collection period and correction equation.* 2. Pressure change in soil by inserting chamber into the soil. *Solved by installing collar to the soil and sealing the cover to the collar.* 3. The change of temperature in the soil and atmosphere under the chamber. *Solved by insulating the chamber and reflective material covering and short gas collection period.* 4. Disturbance of soil air boundary layer.	1. Sensitive to pressure deficit inside the chamber caused by the induced air flow. 2. Artificially high flux. *Solved by larger inlet gas orifices than size of outlet.* 3. Requirement of electricity for clean air pressurization. 4. Requirement of laboratory tests; mixing efficiency, bias testing, sweep air flow rate test, etc. 5. For high purity, sweep air is proposed to be nitrogen or oxygen – difficult for on – site installation.

Source: Chomsurin (1997)**.**

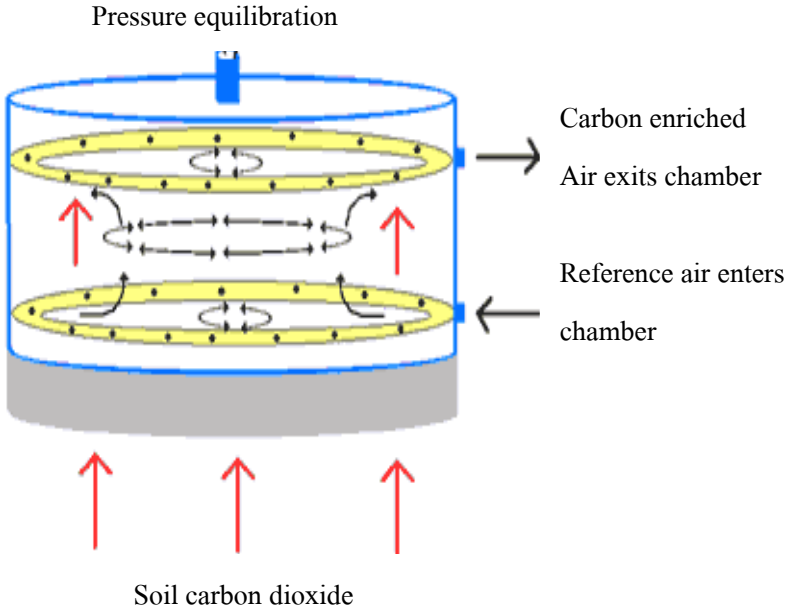


Figure 2.8 Open flux chamber method.

Source: United States Department of Agriculture (2006).

2.4.2.2 Close flux chamber method

Close flux chamber operates by allowing the wastewater gas to accumulate in the chamber and by withdrawing samples at timed intervals. The samples are later analyzed for the change of gas concentration, and the flux is found according to the following equation (2-8) (Hettiaratchi and Hansen, 1996) and relationship between concentration of gas and time (min) as shown in Figure 2.9.

$$F = \frac{\rho V \Delta C}{A \Delta t} \qquad (2\text{-}8)$$

Where:

F	=	Flux of gas, $g/m^2/s$
ρ	=	Density of the gas, kg/m^3 (Table 2-6)
V	=	Volume of the chamber, m^3
A	=	Surface that are enclose by the chamber, m^2
ΔC	=	Change in concentration of the gas, ppm converted to g/g or kg/kg.
Δt	=	Time interval over which the samples are taken.

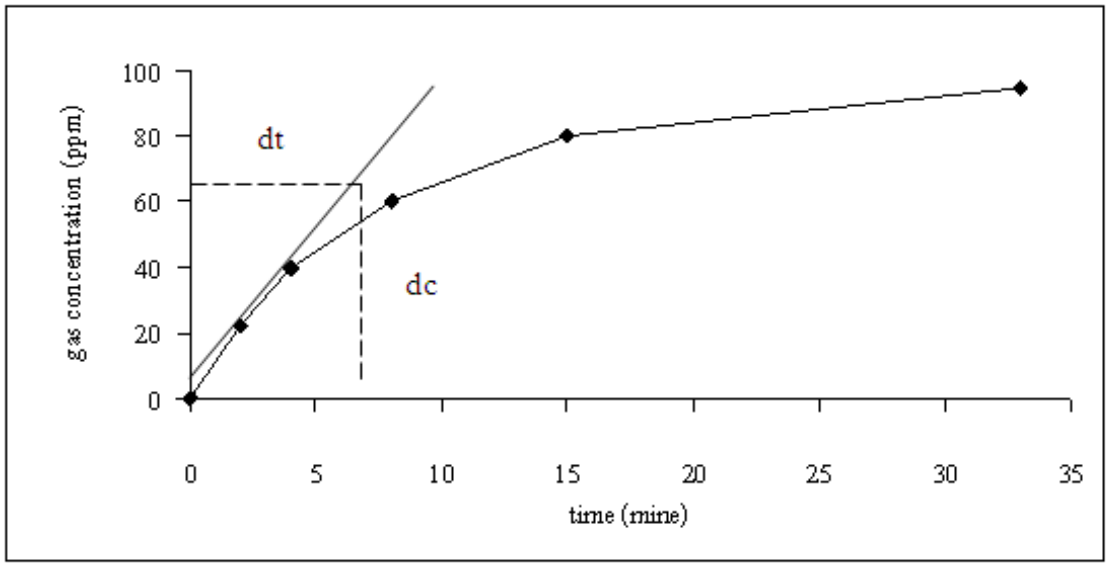


Figure 2.9 Concentration of gas in chamber.

Source : Maurine and Lagerkvist (1998).

Table 2-6 Density of temperature.

Temperature (°C)	Density (kg/m^3)	Temperature (°C)	Density (kg/m^3)	Temperature (°C)	Density (kg/m^3)
0	1.2923	29	1.1684	40	1.1127
10	1.2467	30	1.1644	41	1.1107
20	1.2042	31	1.1592	42	1.1087
21	1.2002	32	1.1541	43	1.1066
22	1.1962	33	1.1489	44	1.1046
23	1.1923	34	1.1437	45	1.1026
24	1.1883	35	1.1386	46	1.1005
25	1.1843	36	1.1334	47	1.0985
26	1.1832	37	1.1282	48	1.0965
27	1.1763	38	1.1231	49	1.0944
28	1.1724	39	1.1179	50	1.0924

Source : http://www.courseware.rmutl.ac.th/courses/55/unit213.html.

CHAPTER 3

THE METHANE EMISSION FROM THE WASTEWATER TREATMENT SYSTEM OF PALM OIL MILL

3.1 Introduction and objective

A palm oil processing is carried out in palm oil mills where crude palm oil (CPO) is extracted from fresh fruit bunch (FFB). Large quantity of water is used during the extraction process and about 50 percent of water supply was converted to wastewater (Ahmad *et al.,* 2006). This wastewater contains high organic matter content and it may have a significant impact on the water environment if it is not managed properly.

In the past, a series of anaerobic ponds was used to treat wastewater as general practice. Currently, the technology for treating wastewater from palm oil mills (POME) has been developed and applied a biogas recovery system (Quah *el at.*, 1982). In the biogas system, the wastewater flows into the stabilization ponds for reducing temperature and stabilizing of wastewater (The water treatments, 2008). After the anaerobic ponds, the wastewater flows to a biogas recovery system. The treated wastewater from biogas plant is fed to stabilization pond which consists of anaerobic, facultative and detention ponds for treating the wastewater. The anaerobic ponds before and after the biogas recovery system could emit greenhouse gases (GHGs) to the atmosphere in term of carbondioxide (CO_2) and methane (CH_4). The methane is classified as one of important GHGs that emitted from anaerobic pond and has the global warming potential (GWP) of 25 times of CO_2 (Climate Change, 1995) and compost of 50-60 percents of biogas (UNFCCC, 1998). On this basis, the methane emission from wastewater treatment system must be managed properly. In order to gain the better understanding on the methane emission, it is important to determine the amount of the methane emission from each

treatment unit of the wastewater treatment system. This information could be used to find out the hot spot of methane emission source. Presently, the mathematical model is commonly used to determine the methane emission from many sources.

Therefore, the major objectives of this chapter are; 1) to determine the methane emission from the wastewater treatment system with the mathematic model, and 2) to determine the hot spot of methane emission source from wastewater treatment system of palm oil mill.

3.2 Materials and methods

3.2.1 Study site

The wastewater treatment plant of the selected palm oil mill in Krabi Province, Thailand is the studied site. This mill has production capacity about 45 – 60 tons of fresh fruit bunch (FFB)/hour. It produces crude palm oil (CPO) by using wet extraction process. The time frame of study was two year in 2009 and 2010. The wastewater treatment process in 2009 utilized a series of anaerobic ponds whereas in 2010 the biogas recovery (Continuous Stirred Tank Reactor; CSTR) and a series of anaerobic ponds were employed to treat the wastewater. In 2009, the mill produced CPO, palm kernel (PK) and shell by 31,205, 12,458 and 14,084 tons (metric ton), respectively whereas, in 2010, it produced CPO, PK and shell by 29,456, 12,371 and 10,559 tons, respectively.

3.2.2 Samples collection and experimental procedure

The evaluation of methane emission from the wastewater treatment system was conducted using the mathematical model (Methane Recovery in Wastewater Treatment AMS III.H version 16) developed by United Nations Framework Convention on Climate Change (UNFCCC, 2009). The methane emission from anaerobic pond system in 2009 was used to represent the baseline emission (BE). The methane emissions from anaerobic pond with the CSTR (biogas recovery system) and stabilization pond in 2010 were used to represent the project emission (PE). The value of COD obtained from the palm oil mill for one year period and the laboratory analysis in this study were used in calculation. Wastewater samples were collected on

November 28, 2009 (for baseline emission), February 9, 2010 and April 9, 2010 (for project emission). The experimental procedure diagram is presented in Figure 3.1.

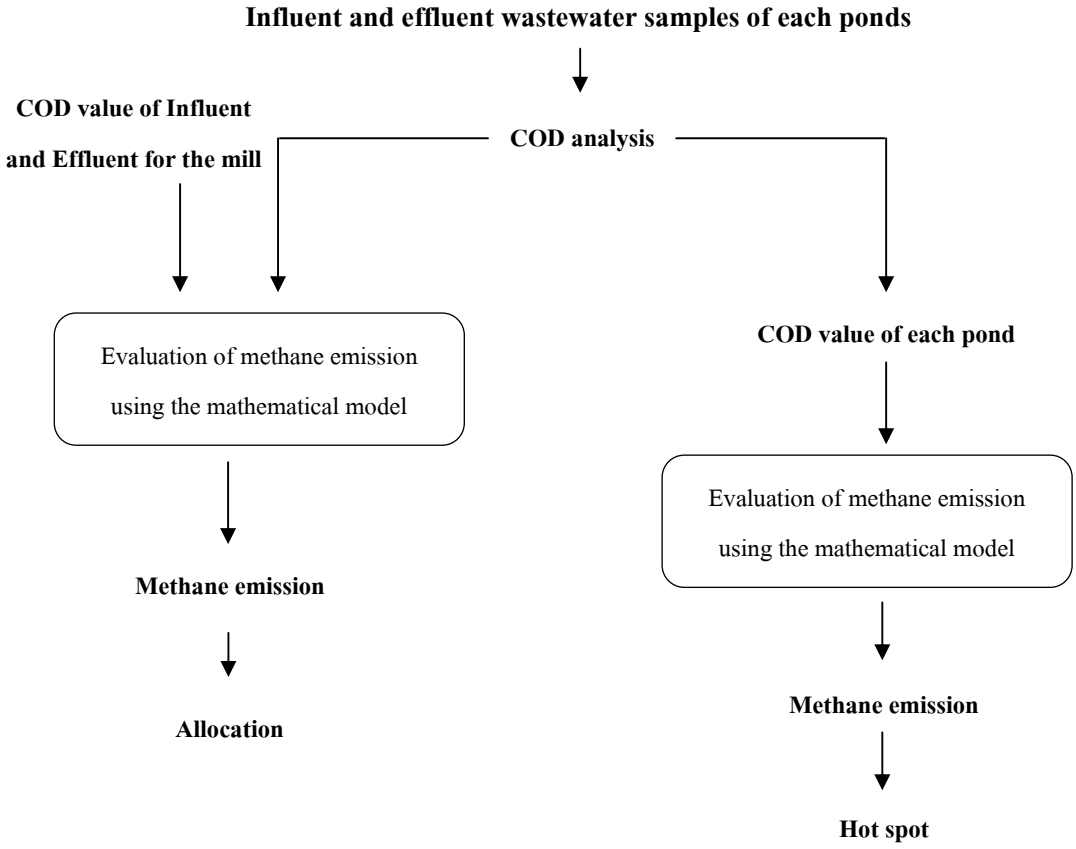


Figure 3.1 Experimental procedure diagrams

3.2.3 Mathematical model

The mathematical model that is commonly used for the evaluation of methane emission from the wastewater treatment system is Methane Recovery in Wastewater Treatment AMS III.H version 16 (UNFCCC, 2010). The structure of model composes of baseline, project and reduction emission estimated as follow:

3.2.3.1 The methane emissions reduction

Emission reduction shall be estimated with the equations 3.1, that it consists of the baseline, project and leakage emissions sections:

$$ER_y = BE_{wastewater,y} - \left(PE_{wastewater,y} - LE_{wastewater,y}\right) \quad (3.1)$$

Where;

ER_y = Emission reduction in year (tCO_2e)

$BE_{wastewater,y}$ = Baseline emission of the wastewater treatment system in year (tCO_2e)

$PE_{wastewatr,y}$ = Project emission of the wastewater treatment system with biogas recovery system in year (tCO_2e)

$LE_{wastewater,y}$ = Leakage emission in year (tCO_2e)

Remark : The leakage emissions are not included in this work because the mill does not have the leakage emission from the biogas plant.

3.2.3.2 The anaerobic ponds (baseline emission; BE)

In 2009, the general wastewater treatment of the mill was through six cascading anaerobic pond system without biogas recovery. Wastewater samples were collected from the sampling points as shown in Figure 3.2 on November 28, 2009 (Appendix A). The depth of these ponds is approximately 3.5 m. The BE can be calculated using equation 3.2. The required data for the evaluation of baseline emission is presented in Table 3-1.

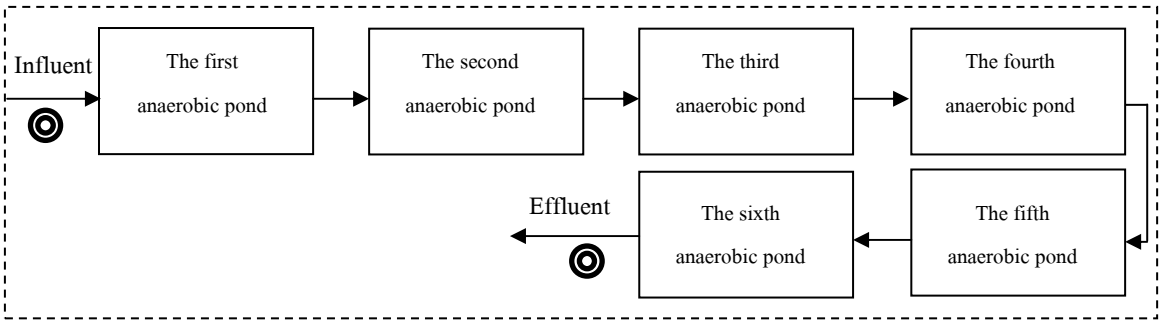


Figure 3.2 The wastewater treatment system of studied mill without biogas recovery system in 2009.

◎ The sampling point

$$BE_{wastewater,y} = BE_{power,y} + BE_{ww,treatment,y} + BE_{s,treatment,y} + BE_{ww,discharge,y} + BE_{s,final,y} \quad (3.2)$$

Where;

$BE_{wastewater,y}$ = Baseline emission of wastewater in year (tCO_2e)

$BE_{power,y}$ = Baseline emission from electricity fuel consumption in year (tCO_2e)

$BE_{ww,treatment,y}$ = Baseline emission of the wastewater treatment system affected by the project activity in year (tCO_2e)

$BE_{s,treatment,y}$ = Baseline emission of the sludge treatment systems affected by the stabilization pond activity in year (tCO_2e)

$BE_{ww,discharge,y}$ = Baseline emission from degradable organic carbon in treated wastewater discharge into sea/river/lake in year (tCO_2e)

$BE_{s,final,y}$ = Baseline emission from stabilization decay of the final sludge produced in year (tCO_2e)

Table 3-1 The required data for the evaluation of methane emission from anaerobic pond system without biogas recovery system.

Environmental index	**Unit index**
Volume of wastewater treated in wastewater treatment system	m^3/year
COD of wastewater from production process (inflow COD)(1)	g/m^3
COD of treated wastewater in final anaerobic pond (outflow COD)(1)	g/m^3
COD of wastewater from production process (inflow COD(2)	g/m^3
COD of treated wastewater in final anaerobic pond (outflow COD)(2)	g/m^3
Electricity or fuel consumption for treated wastewater	tCO_2e/year
Discharge of treated wastewater	tCO_2e/year
Sludge treatment system	tCO_2e/year
Sludge final treatment system from anaerobic decay	tCO_2e/year

Remark: (1) Average COD, this value was averaged from monthly COD value in 2009.

(2) COD of the wastewater samples that were collected on November 28, 2009.

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The mill used the electricity produced by using biomass fuel. Therefore, there was no GHG emission from electricity. The wastewater treatment operation did not discharge treated wastewater and sludge, $BE_{ww,discharge,y}$ and $BE_{s,treatment,y}$ were considered to be zero. Since, there was no sludge treatment in the system. The $BE_{s,final,y}$ was considered to be zero. Therefore, the equation 3.3 was used to calculated GHG emission of wastewater treatment system in the year baseline.

Quantity of fresh fruit bunch (FFB) and quantity of crude palm oil (CPO) were used to evaluate BE (in equation 3.3) in 2009. They were 200,000 ton FFB and 31,205 ton CPO. The wastewater quantity of the mill in 2009 was calculated by using the value of 0.55 m^3 wastewater per ton FFB. This mentioned data was obtained from the mill. The values of COD at the sampling points were obtained from the mill for one year period and also sampling analysis one times**.** The average value of the mill data and the analysis was averaged again and used for calculation of BE.

$$BE_{ww,treatmenty} = \sum_i Q_{ww,i,y} * COD_{inflow,y} * MCF_{ww,teratment} * B_{o,ww} * UF_{BL} * GWP_{CH_4} \quad (3.3)$$

Where;

$Q_{ww,y}$ = Volume of wastewater treated in the anaerobic pond system in year (m^3).

$COD_{inflow,y}$ = Chemical oxygen demand of the wastewater inflow to the anaerobic pond system in year (t/m^3).

$MCF_{ww,treatment}$ = Methane correction factor for anaerobic pond system (baseline treatment) that the wastewater treatment was anaerobic deep lagoon (depth more than 2 meters) value of 0.8 as per Table 3-2.

$B_{o,ww}$ = Methane producing capacity of the wastewater, IPCC lower value of 0.25 $kgCH_4/kgCOD$.

UF_{BL} = Model correction factor to account for model uncertainty (0.89)

GWP_{CH_4} = Global Warming Potential for methane (value of 25)

Table 3-2 Methane correction factor (MCF) for methane emission evaluation by IPCC.

Type of wastewater treatment and discharge pathway or system	MCF value
Discharge of wastewater to sea, river or lake	0.1
Aerobic treatment, well managed	0.0
Aerobic treatment, poorly managed or overloaded	0.3
Anaerobic digester for sludge without methane recovery	0.8
Anaerobic reactor without methane recovery	0.8
Anaerobic shallow lagoon (depth less than 2 meters)	0.2
Anaerobic deep lagoon (depth more than 2 meters)	0.8
Septic system	0.5

Source: Methane Recovery in Wastewater Treatment AMS III.H version 16 (UNFCCC, 2010).

3.2.3.3 Biogas recovery system (project emission; PE)

In 2010, most palm oil mills used anaerobic ponds and the biogas recovery system to treat wastewater. Wastewater samples were collected from the sampling points as shown in Figure 3.3 on February 9, 2010 and April 9, 2010. Subsequent to the treatment in the CSTR, the treated wastewater flows to the existed anaerobic pond system which serves as post treatment and water reservoir. The CSTR system is enable for capturing and utilizing captured methane to generate the electricity by gas engine.

The methane emission from wastewater treatment with biogas recovery system consists of (1) the first and the second anaerobic ponds (2) the biogas recovery system (CSTR system) and (3) the final anaerobic ponds (the third to the sixth anaerobic ponds). Methane emission was evaluated by using the equation 3.4 and 3.5, the requirement data for the evaluation of project GHG emission is shown in Table 3-3.

As mentioned earlier, the palm oil mill utilized the electricity produced by biomass fuel. Therefore, there was no GHG emission for electricity. The wastewater treatment operation did not discharge treated wastewater and sludge. $PE_{ww,discharge,y}$ and $PE_{s,treatment,y}$ was considered to be zero since there was no sludge treatments in the system. The $PE_{s,final,y}$ was

considered to be zero. For $PE_{fugitive,y}$ of the biogas recovery system, the capture efficiency value of 0.9 shall be used, therefore the fugitive emission was 10% of biogas recovery system. But no flaring of gases containing methane, $PE_{flaring,y}$ was considered to be zero. Therefore, the equation 3.5 was used to calculate GHG emission of wastewater treatment system with biogas recovery system.

Table 3-3 The required data for the evaluation of methane emission from wastewater treatment system with biogas recovery system.

Environmental index	**Unit index**
Volume of wastewater treatment in wastewater treatment system	m^3/year
COD of wastewater from production process (inflow COD)(1)	g/m^3
COD of inlet to CSTR system(1)	g/m^3
COD of outlet from CSTR system(1)	g/m^3
COD of treated wastewater in final anaerobic pond (outflow COD)(1)	g/m^3
COD of wastewater from production process (inflow COD)(2)	g/m^3
COD of inlet to CSTR system(2)	g/m^3
COD of outlet from CSTR system(2)	g/m^3
COD of treated wastewater in final anaerobic pond (outflow COD)(2)	g/m^3
Power from electricity or fuel consumption of CSTR system	tCO_2e/year
Sludge treatment system from CSTR system	tCO_2e/year
Biomass treatment system	tCO_2e/year
Fugitive from biogas release	tCO_2e/year
Flare from biogas capture system	tCO_2e/year
Discharge of treated wastewater	tCO_2e/year
Sludge final treatment system from wastewater treatment system	tCO_2e/year

Remark: (1) Average COD, this value was average from monthly COD value in 2010.

(2) Average COD analysis of the wastewater samples that were collected on February 9, 2010 and April 9, 2010.

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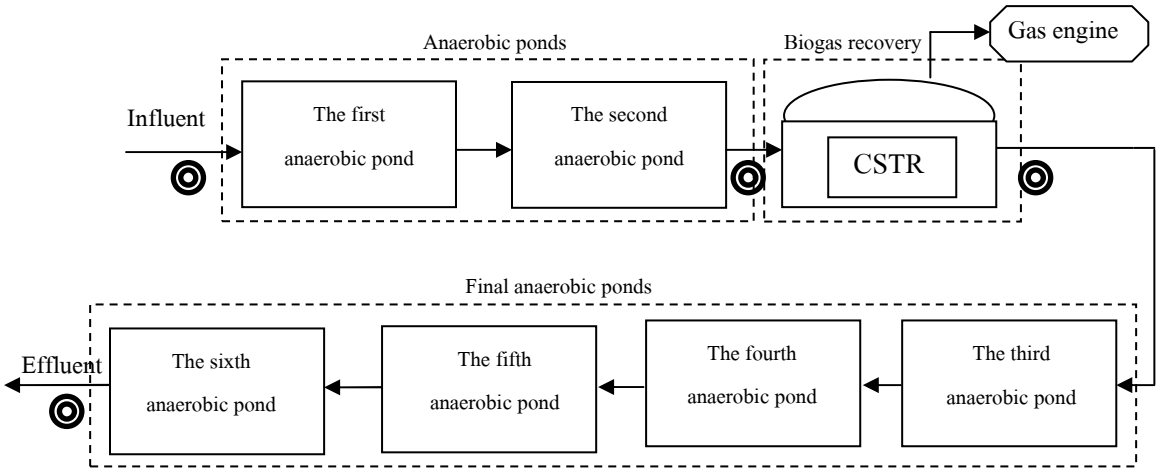


Figure 3.3 The wastewater treatment system with biogas recovery system in 2010.

◎ The sampling point

$$PE_{wasteawter,y} = PE_{power,y} + PE_{ww,treatment,y} + PE_{s,teratment,y} + PE_{ww,dich\arg e,y} + PE_{s,final,y} + PE_{fugitive,y} + PE_{biomass,y} + PE_{flaring,y} \quad (3.4)$$

Where:

$PE_{wasteawter,y}$ = Project activity emissions of wastewater treatment system in year (tCO_2e)

$PE_{power,y}$ = Emissions from electricity or fuel consumption in year (tCO_2e)

$PE_{ww,treatment,y}$ = Methane emissions from CSTR system in year (tCO_2e)

$PE_{s,treatment,y}$ = Methane emissions from sludge treatment system affected by the CSTR system in year (tCO_2e)

$PE_{ww,dich\arg e,y}$ = Methane emission from degradable organic carbon in treated wastewater in year (tCO_2e)

$PE_{s,final,y}$ = Methane emissions from anaerobic decay of final sludge produced in year (tCO_2e)

$PE_{fugitive,y}$ = Methane emissions from biogas release in capture system in year (tCO_2e)

$PE_{biomass,y}$ = Methane emissions from biomass stored under anaerobic conditions in year (tCO_2e)

$PE_{flaring,y}$ = Methane emissions due to incomplete flaring in year (tCO_2e)

$$PE_{ww,treatment,y} = \sum_i Q_{ww,i,y} * COD_{removed,PJ,y} * MCF_{ww,treatment} * B_{o,ww} * UF_{PJ} * GWP_{CH4} \quad (3.5)$$

Where:

$Q_{ww,y}$ = Volume of wastewater treated in wastewater treatment system in year (m^3)

$COD_{removed,PJ,y}$ = Chemical oxygen demand removed by project wastewater treatment system *k* in year (tonnes/m^3), measured as the difference between inflow COD and the outflow COD in biogas recovery system

$MCF_{ww,treatment,}$ = Methane correction factor for project wastewater treatment system CSTR that the wastewater treatment was anaerobic digester for sludge without methane recovery value of 0.8 as per Table 3-2.

$B_{o,ww}$ = Methane producing capacity of the wastewater that IPCCC lower value of 0.21 $kgCH_4/kgCOD$

UF_{PJ} = Model correction factor to account for model uncertainties (1.12)

GWP_{CH4} = Global Warming Potential for methane (value of 25)

In 2010, the quantity of FFB and the quantity of CPO were 194,325 ton FFB and 29,456 ton CPO. They were used in the evaluation of project GHG emission in 2010 (the equation 3.5). The wastewater quantity of industry in 2010 was calculated by using the value of 0.55 m^3 wastewater per ton FFB. This mentioned data was obtained from the mill. The values of COD at the sampling points were obtained from the mill for one year period and also sampling analysis 2 times**.** The average value of the mill data and the analysis were averaged again and used for calculation of PE.

3.2.3.4 The evaluation of methane emission from each pond from the wastewater treatment system

The previous section was determined the methane emission from the overall system of anaerobic pond and biogas recovery system. In this section, the evaluation of methane emission from each treatment unit of the wastewater treatment system (Figure 3.2 and 3.3) was conducted and it was used to find out the hot spot of methane emission source. The wastewater samples were collected in each treatment unit of the wastewater treatment system in 2009 and 2010 at influent and effluent. The COD of wastewater samples were analyzed in laboratory in order to evaluate the methane emission with the mathematical model.

3.2.4 Allocation method

According to the output from process of the mill, CPO is counted as main product whereas palm kernel (PK) and shell are counted as by-products of wet extraction process. The GHG must be allocated to all products and by-products. In general, the allocation process could be done by mass, price and energy. Considering the allocation by mass, the results of allocation by mass normally presented in term of percent allocation which is not suitable for this study. The allocation by energy can be used and more suitable in this study. The following equations 3.6 and 3.7 were used for the calculation.

The equation for the calculation the emission of product was;

$$Emission_{product,allocated} = Total\ GHG\ emission(kgCO_2e) \times (\%Energy\ distribution_{product}) \quad (3.6)$$

And,

$$\%Energy\ distribution_{product} = \frac{\left[LHV_{product}(MJ/ton) \times Yield_{product}(ton)\right]}{\left[\sum\begin{pmatrix} LHV_{product}(MJ/ton) \times Yield_{product}(ton) \\ +\sum\left(LHV_{Byproduct}(MJ/ton) \times Yield_{Byproduct}\right)\end{pmatrix}\right]}$$

The equation for the calculation the emission of by-products was;

$$Emission_{Byproduct,allocated} = Total\ GHG\ emission(kgCO_2e) \times (\%Energy\ distribution_{Byproduct}) \quad (3.7)$$

And,

$$\%Energy\ distribution_{product} = \frac{\left[LHV_{product}(MJ/ton) \times Yield_{product}(ton)\right]}{\left[\sum\begin{pmatrix} LHV_{product}(MJ/ton) \times Yield_{product}(ton) \\ +\sum\left(LHV_{Byproduct}(MJ/ton) \times Yield_{Byproduct}\right)\end{pmatrix}\right]}$$

Where:

$LHV_{product}$ = Lower heating value of product (MJ/ton product)

$LHV_{Byproduct}$ = Lower heating of byproduct (MJ/ton byproduct)

3.3 Results and discussion

3.3.1 The evaluation of methane emission by the mathematical model

3.3.1.1 The anaerobic pond system (BE)

The wastewater treatment system used for treated wastewater in 2009 consisted of six anaerobic ponds. The baseline methane emission from the anaerobic pond system was estimated by using the mathematical model and the data for evaluation is presented in Table 3-4.

Table 3-4 The data for the evaluation of methane emission from anaerobic pond system (baseline emission: BE).

Environmental index	The value	Unit index
Volume of wastewater treated in wastewater treatment system	110,000	m^3/year
COD wastewater from production process (inflow COD)(1)	88,121	g/m^3
COD treated wastewater in final anaerobic pond (outflow COD)(1)	819	g/m^3
COD wastewater from production process (inflow COD)(2)	96,000	g/m^3
COD treated wastewater from production process (outflow COD)(2)	1,280	g/m^3

Remark: (1) Average COD, this value was averaged from monthly COD value in 2009.

(2) COD of the wastewater samples that were collected on November 28, 2009 (Appendix A).

Calculation result:

1)Anaerobic ponds system

$$BE_{ww,treatment,y} = \sum_i Q_{ww,i,y} * COD_{\inf low,i,y} * MCF_{ww,teratment,BL,i} * CH_4 * B_{o,ww} * UF_{BL} * GWP$$

$$= (110{,}000\ m^3/year \times (92{,}061 - 1{,}050)\ g/m^3 \times 0.8 \times 0.94 \times 25 \times 21\ kgCH_4/kgCOD$$

$$= 39{,}524\ tonCO_2e/year$$

Thus,

$$Emission_{ww,treatment} = 39{,}524\ tonCO_2e/year$$

Therefore, emission of wastewater treatment from production process part:

$Emission_{wastewater}$ = 39,524 $tonCO_2e$/year

Finally,

Total emission = 39,542 $tonCO_2e$/year

The result of this model (BE) shows that the conversion of 200,000 ton FFB per year to 31,205 ton CPO per year emitted methane 39,542 $tonCO_2e$/year (Figure 3.4). This observation is corresponded with the study of Musikavong and Suksaroj (2009). They reported that the anaerobic wastewater treatment process of palm oil mills with a production capacity from 10 to 90 ton FFB/hour emitted methane of 10,920 - 49,140 $tonCO_2e$/year. The GHG emission could be converted to the GHG emission per FFB and CPO without allocation to by-products. The GHG emission per one ton of FFB and CPO were 198 $kgCO_2e$/ton FFB and 1,267 $kgCO_2e$/ton CPO, respectively.

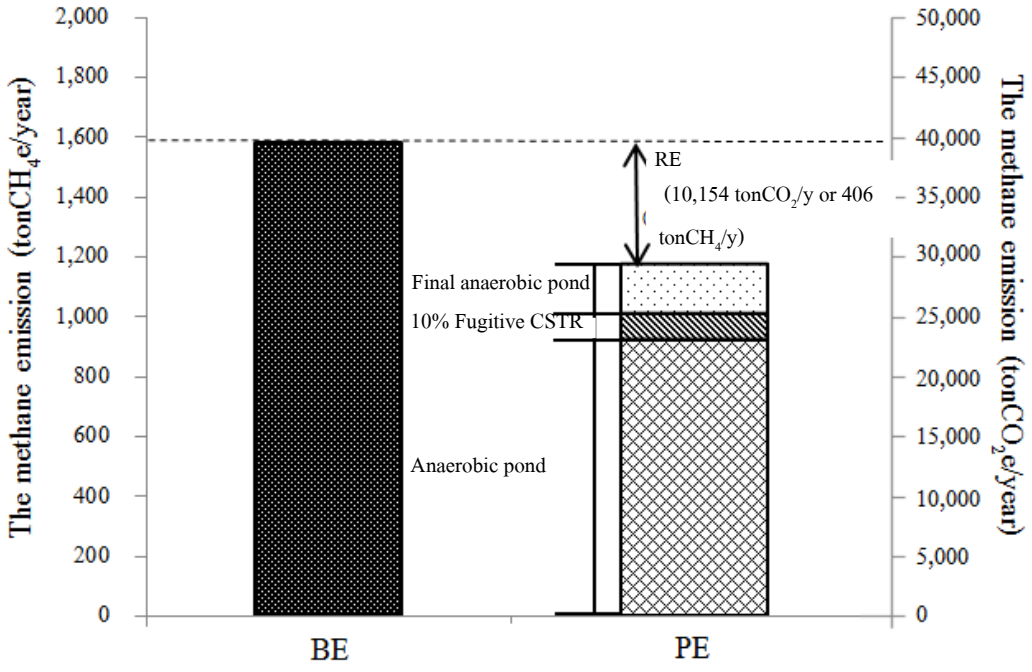


Figure 3.4 Methane emission of wastewater treatment system with/without biogas recovery system.

PE : Project Emission BE : Baseline Emission RE : Reduction Emission

3.3.1.2 Biogas recovery system (PE)

Currently, the wastewater treatment system of the mill is the anaerobic pond followed by CSTR and anaerobic pond. The project methane emission from the biogas recovery system with CSTR reactor was estimated using the mathematical model by UNFCCC and the data for evaluation of GHG emission is presented in Table 3–5.

Table 3-5 The data for the evaluation of methane emission from CSTR system (project emission: PE).

Environmental index	values	Unit index
Volume of wastewater treatment in wastewater treatment system	106,879	m^3/year
COD wastewater from production process (inflow COD)(1)	112,840	g/m^3
COD inlet to CSTR system(1)	68,000	g/m^3
COD outlet from CSTR system(1)	7,000	g/m^3
COD treated wastewater in final anaerobic pond (outflow COD)(1)	2,195	g/m^3
COD wastewater from production process (inflow COD)(2)	88,800	g/m^3
COD inlet to CSTR system(2)	42,000	g/m^3
COD outlet from CSTR system(2)	14,760	g/m^3
COD treated wastewater in final anaerobic pond (outflow COD)(2)	3,120	g/m^3
Fugitive from biogas release	10% of the biogas capture	

Remark: (1) Average COD, this value was average from monthly COD value in 2010.

(2) Average COD analysis of the wastewater samples that were collected on February 9, 2010 and April 9, 2010 (Appendix A).

Calculation result:

1) Wastewater treatment system

(1) Anaerobic ponds

$$PE_{ww,treatment,y} = \sum_i Q_{ww,i,y} * COD_{inflow,i,y} * MCF_{ww,teratment,BL,i} * B_{o,ww} * UF_{BL} * GWP_{CH}$$

$$= 106{,}879\ m^3/year \times (100{,}820 - 55{,}000)\ g/m^3 \times 0.8 \times 0.21\ kgCH_4/kgCOD \times 1.12 \times 25$$

= 23,036 $tonCO_2e$/year

(2) CSTR reactor of biogas recovery system (This value must be used in the calculation of fugitive emission)

$PE_{ww,treatment,CSTR}$ = 106,879 m^3/year x (55,000 – 10,880) g/m^3 x 0.8 x 0.21 $kgCH_4$/kgCOD x 1.12 x 25

= 22,182 $tonCO_2e$/year

(3) Final ponds

$PE_{ww,treatment,finalpond}$ = 106,879 m^3/year x (10,880 – 2,658) g/m^3 x 0.8 x 0.21 $kgCH_4$/kgCOD x 1.12 x 25

= 4,134 $tonCO_2e$/year

Thus,

$Emission_{ww,treatment}$ = (23,036 + 4,134 $tonCO_2e$/year

= 27,170 $tonCO_2e$/year

2) $Emission_{PE,fugitive}$

According to UNFCCC 2010, the fugitive emission is 10 percent of total emission of wastewater treatment system using biogas recovery system.

$PE_{fugitive\ CSTR}$ = (0.1 x 22,182) $tonCO_2e$/year

= 2,218 $tonCO_2e$/year

Therefore, emission of GHG from production process wastewater:

$Emission_{wastewater}$ = (27,170 + 2,218) $tonCO_2e$/year

= 29,388 $tonCO_2e$/year

Finally,

$Total\ emission$ = 29,388 $tonCO_2e$/year

The result of PE shows that the biogas recovery system (CSTR system) could capture methane 22,182 tonCO_2e/year. It was used to generate the electricity that could be used in the mill and sold to province electricity authority (PEA). However, the methane 2,218 tonCO_2e/year still was emitted into atmosphere from the fugitive emission of biogas recovery system. In addition, the methane from the anaerobic pond before and after the biogas recovery system were also emitted into atmosphere 27,170 tonCO_2e/year. The total GHG emission from the wastewater treatment system with biogas recovery system was 29,388 tonCO_2e/year as shown in Figure 3.4. The GHG emission without allocation to other by-products per ton of FFB and CPO were 151 kgCO_2e/ton FFB and 998 kgCO_2e/ton CPO, respectively (Figure 3.5).

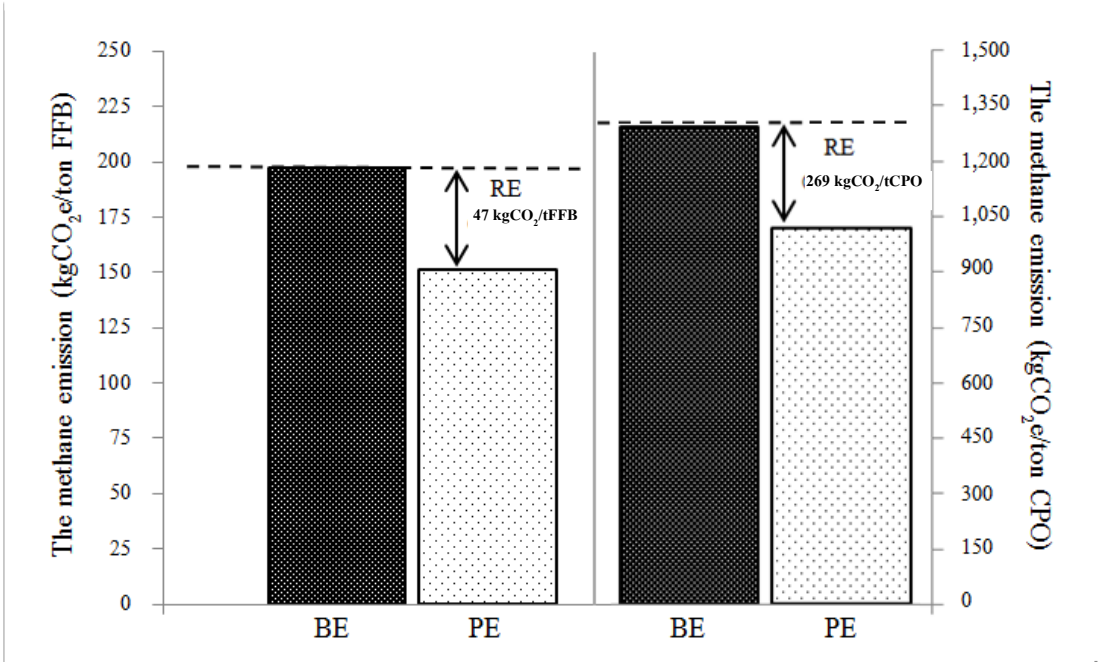


Figure 3.5 Methane emissions from wastewater treatment system per one ton of FFB and CPO.

PE : Project Emission BE : Baseline Emission RE : Reduction Emission

3.3.1.3 Total emission reductions

From the results, the methane emission of BE and PE (with CSTR system) was 39,542 and 29,388 tonCO_2e/year, respectively. The total emission reductions can be calculated as below:

$$ER_{wastewater\ treatment} = BE_{ww,treatment} - (PE_{ww,treatment,(CSTR\ system)} - LE_{ww,treatment,(CSTR\ system)}$$

$$= (39{,}542 - 29{,}388)\ \text{ton}CO_2\text{e/year}$$

$$= 10{,}154\ \text{ton}CO_2\text{e/year}$$

The biogas recovery system (CSTR reactor) can reduce GHG 10,154 $tonCO_2e$/year as shown Figure 3.4. The GHG emission reduction per ton of FFB and CPO without allocation to other by-products was 47 $kgCO_2e$/ton FFB and 269 $kgCO_2e$/ton CPO, respectively as shown in Figure 3.5.

3.3.2 GHG emission of product and by-products

Methane emission was allocated to the product and by-products. CPO is product, shell and PK are by-products. The lower heating value of product and by-products were used in allocation. LHV_{CPO}, LHV_{shell}, and LHV_{PK} are 39,212 MJ/ton CPO, 24,207 MJ/ton shell and 18,915 MJ/ton PK, respectively (Keawmai *et al.*, 2011). The calculation examples of allocation procedure for baseline methane emission and project methane emission are shown in Appendix B.

The baseline emission in 2009 was 39,542,000 $kgCO_2e$ and project emission in 2010 was 29,388,000 $kgCO_2e$. The GHG emissions from the wastewater treatment system without biogas recovery system per one ton of CPO, shell, and PK were 861 $kgCO_2$/ton CPO, 532 $kgCO_2$/ton shell and 416 $kgCO_2$/ton PK, respectively. Whereas the GHG emission from the wastewater treatment system with biogas recovery system per one ton of CPO, shell, and PK were 700 $kgCO_2$/ton CPO, 431 $kgCO_2$/ton shell and 338 $kgCO_2$/ton PK, respectively. The installation of the biogas recovery system into the wastewater treatment system could reduce methane emission by 161 $kgCO_2e$/ton CPO, 101 $kgCO_2e$/ton shell and 78 $kgCO_2e$/ton PK (Figure 3.6).

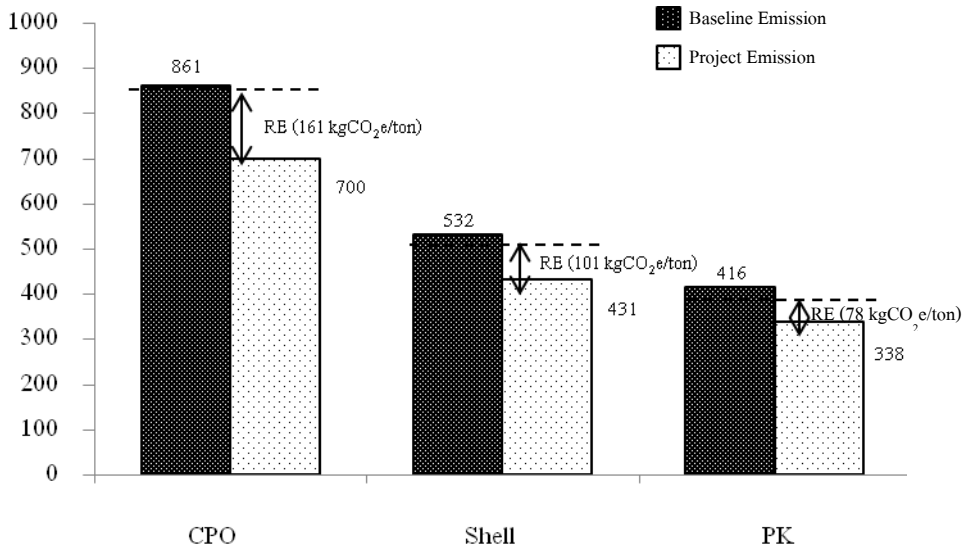


Figure 3.6 Methane emissions per ton of product and by products allocated by LHV.

PE : Project Emission BE : Baseline Emission RE : Reduction Emission

Considering the palm oil industry that installed the CSTR reactor in Thailand (Table 3-8), the baseline emission was 148 – 221 $kgCO_2e$/ton FFB or 872 – 1,300 $kgCO_2e$/ton CPO. By using weighting average for calculation, baseline emission was 160 $kgCO_2e$/ton FFB or 942 $kgCO_2e$/ton CPO. The project emission ranged between 61 – 98 $kgCO_2e$/ton FFB or 358 – 578 $kgCO_2e$/ton CPO (74 $kgCO_2e$/ton FFB or 437 $kgCO_2e$/ton CPO for weighting average values). The biogas system could reduce GHG in the range of 82 – 125 $kgCO_2e$/ton FFB or 485 – 731 $kgCO_2e$/ton CPO (95 $kgCO_2e$/ton FFB or 556 $kgCO_2e$/ton CPO for the weighting average value). The methane emission in BE case fall into the range of BE of palm oil industry in Thailand. The PE in this study was considerably high, this lead to the low RE value. It must be notes that during the sampling period, the wastewater treatment plant operated in the initial state of the biogas system with CSTR reactor.

Table 3-6 The methane emission from wastewater treatment plant with CSTR reactor of palm oil mill evaluated by mathematical model of UNFCCC from literature data and this study.

Palm oil mill	Baseline emission (BE)			Project emission (PE)			Reduction emission (RE)			FFB	CPO	Biogas	Reference
Faculty	tCO_2e/y	$kgCO_2e/tFFB$	$kgCO_2e/tCPO$	tCO_2e/y	$kgCO_2e/tFFB$	$kgCO_2e/tCPO$	tCO_2e/y	$kgCO_2e/tFFB$	$kgCO_2e/tCPO$	(t/y)	(t/y)	system	(version AMS.III.H)
In Suratthani, Thailand	23,551	160	942	11,417	78	457	12,134	82	485	147,103	25,008	CSTR	AMS-III.H. ver. 5, 2006
In Krabi, Thailand	34,302	158	931	13,192	61	358	21,110	97	573	216,720	36,842	CSTR	AMS-III.H. ver. 5, 2006
In Suratthani, Thailand	33,602	168	988	15,243	76	448	18,359	92	540	200,000	34,000	CSTR	AMS-III.H. ver. 9, 2008
At Sikao in Trang, Thailand.	27,129	148	872	11,697	64	376	15,432	84	496	183,000	31,110	CSTR	AMS-III.H. ver. 9, 2008
At Saikhueng in Surattani, Thailand	33,945	171	1,008	15,205	77	452	18,739	95	557	198,000	33,660	CSTR	AMS-III.H. ver. 9, 2008
At Bangsawan in Suratthani, Thailand	33,139	221	1,300	14,743	98	578	18,396	123	721	150,000	25,500	CSTR	AMS-III.H. ver. 9, 2008
At Sinpun in Suratthani, Thailand	32,848	164	966	14,693	74	432	18,155	91	534	200,000	34,000	CSTR	AMS-III.H. ver. 9, 2008

Table 3-6 The methane emission from wastewater treatment plant with CSTR reactor of palm oil mill evaluated by mathematical model of UNFCCC from literature data and this study (Cont.).

Palm oil mill Faculty	Baseline emission (BE)			Project emission (PE)			Reduction emission (RE)			FFB (t/y)	CPO (t/y)	Biogas system	Reference (version AMS.III.H)
	tCO_2e/y	$kgCO_2e/tFFB$	$kgCO_2e/tCPO$	tCO_2e/y	$kgCO_2e/tFFB$	$kgCO_2e/tCPO$	tCO_2e/y	$kgCO_2e/tFFB$	$kgCO_2e/tCPO$				
Summary	**218,516**	**(148 – 221)**	**(872– 1.300)**	**96,190**	**(61 – 98)**	**(358 – 578)**	**122,325**	**(82 – 125)**	**(485 – 731)**	-	-	-	-
Weighting average ($tonCO_2e$/year)	-	**160**	**942**	-	**74**	**437**	-	**95**	**556**		-	-	-
This study in Krabi, Thailand	39,542	198	1,267	29,388	151	998	10,154	47	269	*197,163	*30,331	CSTR	AMS-III.H. ver. 16, 2010

Remark * Average of FFB and CPO in 2009 and 2010 of the mill

Oil yield 17 percents (Thamsiriroj and Murhy *et. al.*, 2009).

( ) unit $tonCO_2$/year

3.3.3 The evaluation of the methane emission from each pond of the wastewater treatment system

3.3.3.1 The wastewater treatment system without biogas recovery system

The previous results presented the methane emission from the anaerobic pond system. This information did not show the detail of methane emission from each pond and the hot spot of methane emission among these ponds. Therefore, the evaluation of methane emission from each pond (the first to the sixth pond) in 2009 was conducted by the mathematical model on BE. The wastewater samples were collected on November 28, 2009 from each pond for measuring COD in laboratory for calculation.

Table 3-7 The chemical oxygen demand and the methane emission of each pond.

Pond	COD (mg/L)		COD removal	The methane emission		% methane
	Influent	Effluent	(mg/L)	tonCH_4e/year	tonCO_2e/year	emission
1	96,000	64,000	32,000	606	15,160	34
2	64,000	33,600	30,400	576	14,402	32
3	33,600	20,800	12,800	243	6,064	14
4	20,800	9,600	11,200	212	5,306	12
5	9,600	4,800	4,800	91	2,274	5
6	4,800	1,280	3,520	67	1,668	4
Total				**1,795**	**44,874**	**100**

Remake: Quantity of palm fruit bunch (FFB) was 200,000 tons in 2009.

Quantity of wastewater was 0.55 m^3 per ton FFB in 2009.

The COD value of wastewater samples that was collected on November 28, 2009

Table 3-7 shows the methane emission from each anaerobic pond in order to determine the hot spot by the mathematic model. The first and the second ponds were the hot spot of methane emission from wastewater treatment system because they emitted methane 34 and 32 percents of total methane emission, respectively while the summation of methane emission from the third to the sixth pond was 35 percent of total methane emission.

3.3.3.2 The wastewater treatment system with biogas recovery system

The result of methane emission evaluated from overall biogas recovery system could not show the hot spot of methane emissions because it did not show the breakdown methane emission from each treatment unit. The hot spot of methane emission from biogas recovery system therefore, could not be determined. The evaluation of methane emission from each treatment unit of biogas recovery system in 2010 was conducted using the mathematical model. The wastewater samples were collected on February 9 and April 9, 2009 from each pond of the wastewater treatment system for measuring COD for the evaluation of methane emission.

Table 3-8 shows the methane emission from each pond of the wastewater treatment with the biogas recovery system in 2010. It was found that the first and the second anaerobic ponds emitted methane by 45 and 29 percents of the total methane emission, respectively. The biogas recovery system (CSTR reactor) emitted methane 7 percent. Consider the summation of methane emission from the third to the sixth anaerobic ponds, it emitted methane by 18 percent. The first and the second ponds were the hot spot of methane emission source. It can be stated that even though the biogas recovery system was employed to treat the wastewater, the methane emission still continuously emitted due to the open pond systems, especially the anaerobic pond before biogas system. Therefore, the first and the second anaerobic ponds must be managed well in order to reduce the methane emission into an atmosphere.

The analysis of COD from the wastewater of the first and the second anaerobic ponds in Table 3-7 and 3-8 showed that the degradation of COD in the first and the second anaerobic ponds yielded average methane production by 0.19 and 0.16 m^3CH_4 per kg of COD removed (CODR), respectively (Table 3-9). The theoretical yield of methane from an anaerobic pond is ranged between 0.29 and 0.36 m^3 per kg CODR (Rajeshwan *et al*., 2000 and Meat technology update, 2010), The Australian Meat Industry Council presented the biogas production from anaerobic ponds of 0.5 m^3 per kgCODR at 60 percent CH_4 which was equivalent to 0.3 m^3CH_4 per kgCODR (Meat technology update, 2010).

Table 3-8 The average of chemical oxygen demand and the methane emission of each pond (project emission: PE).

Pond	Average of COD (mg/L)		COD removal	The methane emission		% methane
	Influent	Effluent	(mg/L)	tonCH_4e/year	tonCO_2e/year	emission
1	88,800	60,600	28,200	537	13,418	45
2	60,600	42,000	18,600	354	8,850	29
Biogas	42,000	14,760	27,240	*89	*2,218	*7
3	14,760	10,560	4,200	80	1,999	7
4	10,560	7,920	2,640	50	1,256	4
5	7,920	5,295	2,625	50	1,249	4
6	5,295	3,120	2,175	41	1,035	3
Total				**1,201**	**30,025**	**100**

Remark: Quantity of palm fruit bunch was 194,325 ton FFB in 2010.

Quantity of wastewater was 0.55 m^3 per ton FFB in 2010.

* The methane emission of biogas system (CSTR reactor), due to the fugitive emission, is equal to 10 percent of methane production from biogas recovery system (UNFCCC, 2010).

The average of COD values of wastewater samples collected on February 9, 2010 and April 9, 2010 (Appendix A).

Table 3-9 The methane yield from chemical oxidation demand degradation in the first and the second anaerobic ponds

Anaerobic pond	COD removal (kg/m^3)	Average of methane emission (m^3CH_4/m^3)	COD to yield of methane (m^3CH_4/kgCOD)
The first pond			
In 2009	32.00	5.73	0.18
In 2010	28.20	5.23	0.19
Average			**0.19**
The second pond			
In 2009	30.40	4.71	0.16
In 2010	18.60	2.98	0.16
Average			**0.16**

3.4 Concluding remarks

The mathematic model (Methane Recovery in Wastewater treatment AMS III.H version 16) developed by UNFCCC was used to evaluate the methane emission from the wastewater treatment system of the palm oil mill. In 2009; the mill used the series of anaerobic ponds for treating wastewater. It emitted methane by 39,542 tonCO_2e. Until, the mill installed the CSTR system in 2010 can capture the methane by 22,182 tonCO_2e for producing electricity. Currently, the biogas recovery system of the mill could reduce the methane emission by 10,154 tonCO_2e/year (47 kgCO_2e/ton FFB or 269 kgCO_2e/ton CPO). By allocation of GHG to product and by-products, 861 kgCO_2e/ton CPO, 532 kgCO_2e/ton shell and 416 kgCO_2e/ton PK were found in 2009. In 2010, the production process emitted methane by 700 kgCO_2e/ton CPO, and 431 kgCO_2e/ton shell and 338 kgCO_2e/ton PK, respectively. The biogas recovery system installation could reduce the methane emission by 161 kgCO_2e/ton CPO or 101 kgCO_2e/ton shell or 78 kgCO_2e/ton PK, respectively. The first and the second ponds emitted methane higher than other ponds. Their emissions were more than 65 percent of total methane emission. The average COD degradation rate for the production of methane from the first and the second ponds were 0.19 and 0.16 m^3CH_4 per kg of CODR, respectively. The first and the second ponds were identified as the hot spot of methane emission into the atmosphere. Although, the mill added the biogas recovery system but the first and the second ponds still were identified as methane emission hot spots. Therefore, the mill should be install the other treatment process such as cooling tower and heat exchanger system for reducing temperature before sent during to the biogas recovery system.

CHAPTER 4

INFLUENCE OF MICROORGANISMS IN SCUM LAYER ON METHANE EMISSION FROM ANAEROBIC POND

4.1 Introduction and objective

Methane is one of the important greenhouse gases (GHGs) that cause global warming. Its concentration in the atmospheric has been increasing for many decades (United Nations Environment Program, 2010). Currently, a wastewater treatment system of palm oil mills in Thailand consists of two systems 1) stabilization pond system, and 2) biogas recovery system. The stabilization pond consists of series of anaerobic ponds. The biogas recovery system composes of anaerobic pond, biogas recovery system and stabilization pond. As presented in the previous chapter, the anaerobic pond in stabilization pond system was one of the major methane emission sources, while the anaerobic pond before and after biogas recovery system was the major methane emission source from the biogas recovery system.

The major source of methane emission from the wastewater treatment system of the palm oil mill was the first and second anaerobic ponds that emitted methane higher than other pond in wastewater treatment system. Therefore, these ponds must be managed well in order to reduce the GHG emissions. By consideration in detail, the surfaces of the first and second anaerobic ponds were covered by scum layer. The previous research reported that the anaerobic pond without scum layer emitted the methane higher than that of with scum layer (Siriruksoporn, 2008). It is expected that there are physical or biological characteristics in scum layer affected on the methane emission.

Therefore, the major hypothesis of this study is that some groups of microorganisms in scum layer of the anaerobic pond could degrade methane and decrease the methane emission into the atmosphere. In order to prove the reduction of methane by scum layer, there are two research approaches. Firstly, the amount of methane emission from the anaerobic pond with and without scum layer covered on the surface should be determined. Secondly, the specific types of the methanotroph group in the scum layer should be confirmed. These two aspects were the main focus of this chapter. Finally, the results obtained could be used to further determine the method for reducing the methane emission from the anaerobic pond of the palm oil mill.

4.2 Materials and methods

4.2.1 Study site

The first and second anaerobic ponds were the major sources of methane emission from palm oil mill wastewater by either treatment with stabilization pond system or biogas recovery system. By observation, the first and second anaerobic ponds were covered by scum layer on surface as shown in Figure 4.1 and 4.2. These ponds were used as the sampling sites.

Figure 4.1 The first anaerobic pond.

Figure 4.2 The second anaerobic pond.

4.2.2 Experimental procedure

The experimental procedure consisted of three parts. The first part was the evaluation of methane emission using the actual measurement technique by close flux chamber for collection gas (as shown in Appendix C). The second part was the analysis of physical and chemical characteristics of wastewater and scum samples in the first and second anaerobic ponds. The third part was study of biological characteristics of wastewater and scum samples in the first and second anaerobic ponds, which it consists of bacteria, yeast, fungi, sulfate reducing bacteria (SRB) and methane oxidation bacteria (MOB) as shown in Figure 4.3.

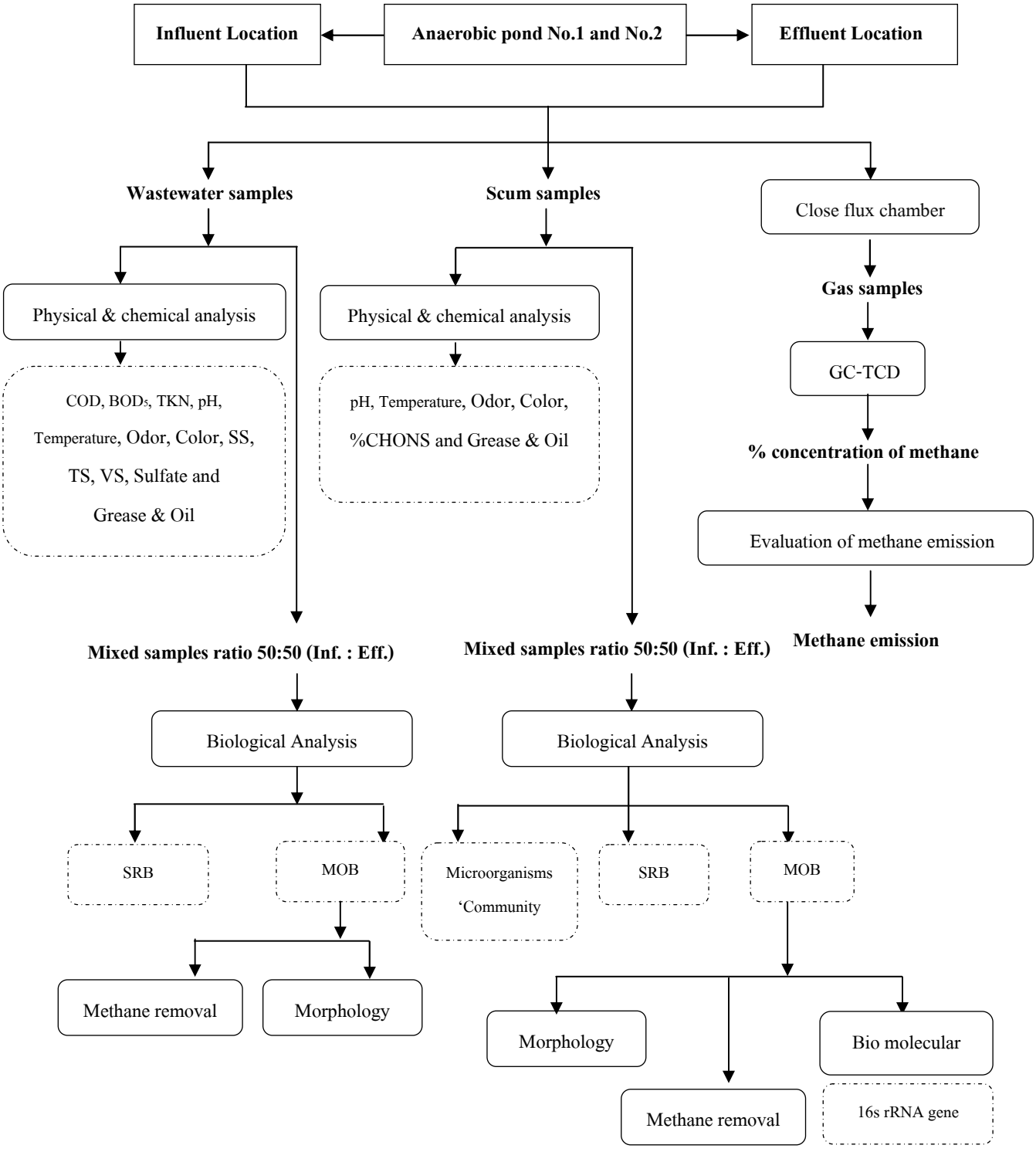


Figure 4.3 Experimental procedure diagram for the evaluation of methane emission by actual measurement.

4.2.3 The evaluation of methane emission by actual measurement technique

The evaluation of methane emission by the actual measurement can be conducted by using close flux chamber. The close flux chambers were put on the surface area with/without scum layer at influence and effluent of the anaerobic pond in order to collect the gas samples with gas tight syringe and store in vacuum tube. The gas samples were collected two times on February 9, 2010 and April 9, 2010.

The time for the collection of gas samples in the chamber was during 0 to 180 minutes. The gas samples were collected 10 times at 0, 10, 20, 30, 50, 80, 100, 120, 150 and 180 minutes, respectively, by gas tight syringe size 10 milliliters (Figure 4.4 and 4.5). To analyze the components of gas samples, the samples were injected into the Gas-Chromatography with a Thermal Conductivity Detector (GC-TCD) series GC 7890A, with capillary column (Shincarbon ST packed column), helium was used as the carrier gas at 60 ml./min. The previous research reported that the percent of gas concentration in close flux chamber increased during the time 0 to 60 minutes and stable at the time 60 to100 minutes (Siriruksoporn, 2008). The time for the samples collection in this study was designed in order to cover this range.

The gas samples were analyzed the concentration of methane with GC-TCD. These data were used to plot the graph for determining methane concentration by times ($\Delta C / \Delta t$). The equation 4.1 (Hettiaratchi and Hansen, 1996) was used to calculate the flux of gas. The global warming potential for methane (GWP_{CH4}) of 25 was used in the calculation.

$$F = \frac{\rho v \Delta C}{A \Delta t} \tag{4.1}$$

Where:

F	=	Flux of gas ($g/m^2/s$)
ρ	=	Density of the gas (kg/m^3) (Appendix D)
ΔC	=	Change in concentration of the gas (ppm converted to g/g or kg/kg)
V	=	Volume of the chamber, m^3
Δt	=	Time interval over which the samples are taken.
A	=	Surface that are enclosed by the chamber (m^2)

Figure 4.4 Close flux chambers put on surface area with/without scum layer.

Figure 4.5 The gas samples were collected by gas tight syringe in vacuum tube.

4.2.4 The physical and chemical characteristics of the wastewater and scum samples

The wastewater and scum samples from the first and the second anaerobic ponds were collected to study the physical and chemical characteristics. They were collected at influent and effluent points of the first and the second anaerobic ponds three times on February 9, and April 9 and 28, 2010. Figure 4.6 shows the location for collection of wastewater and scum samples. The wastewater and scum samples were separately kept in the bottles at 4°C until analysis. Their physical and chemical characteristics were presented in the Table 4.1 and 4.2, respectively.

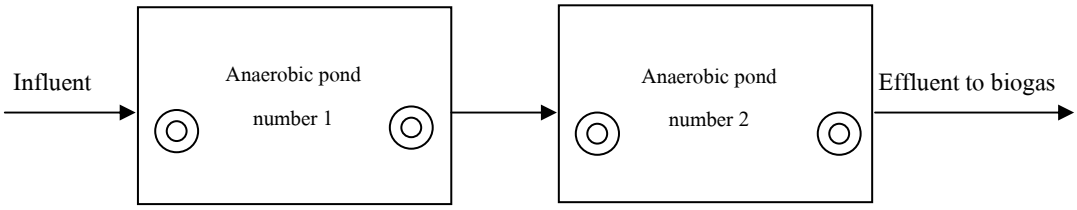


Figure 4.6 The location for collection of the wastewater and scum samples.

◎ Sampling points.

Table 4-1 Physical and chemical parameters used for determining wastewater characteristics.

Parameter	Analysis Methods/Instruments*
Color	Observation
Odor	Sensation
pH	pH meter
Temperature (ºC)	Thermometer
Chemical Oxygen Demand : COD (mg/L)	Open flux chamber
Biological Oxygen Demand : BOD_5 (mg/L)	Azide modifica
Total Solid : TS (mg/L)	Evaporation
Suspended Solids : SS(mg/L)	FilterGF/C and muffle oven
Volatile Solid : VS (mg/L)	Oven
Total Kjeldahl Nitrogen : TKN (mg/L)	Kjeldahl nitrogen method
Grease and Oil (mg/L)	Soxhlet extraction method
Sulfate (mg/L)	Turbidimetric method

***Source**: Standard Method for the Examination of Water and Wastewater 21th Edition (APHA, AWWA and WEF, 2005).

Table 4-2 Physical and chemical parameters used for scum characteristic determining.

Parameters	Analysis	References
Color	Observation	Standard Methods for the Examination of Water and Wastewater 21th Edition (APHA, AWWA and WEF, 2005)
Odor	Sensation	
pH	pH meter	
Temperature	Thermometer	
Grease and Oil (%)	Partition-Gravimetric Method	
%C	Dynamic Flash Combustion	WI-RES-CHINS-O-001
%H	Dynamic Flash Combustion	WI-RES-CHINS-O-001
%O	Dynamic Flash Combustion	WI-RES-CHINS-O-001
%N	Dynamic Flash Combustion	WI-RES-CHINS-O-001
%S	Dynamic Flash Combustion	WI-RES-CHINS-O-001

4.2.5 The biological characteristics of wastewater and scum samples

The previous research found that the surface area of anaerobic ponds of the palm oil mill covered by scum layer emitted methane lower than that of without scum layer (Sirirulsoporn, 2008). Therefore, this work was focused on microbes groups that used methane as carbon sources for biomass that grown in scum.

The wastewater samples were collected at depth of 15-20 cm and the scum samples were collected from the surface area of these ponds at influent and effluent point three times on February 9, and April 9 and 28, 2010 (Figure 4.6). They were separately kept in sterilized polypropylene bottles at 4˚C for microorganisms' community study which consisted of bacteria, yeast and fungi. The specific medias consisted of (E2) and AMS agar without methanol were used for SEB and MOB as shown in Table 4-3.

Table 4-3 Media and conditions for study of biological characteristics of the wastewater and scum samples in the first and the second anaerobic ponds.

Biological Characteristics	Media	Condition	Samples		Reference
			Wastewater	Scum	
Microorganisms' community					
- Bacteria	NA	At 35°C in aerobe condition	✗	✓	Sridang, 2005
- Yeast	YMA	At 35°C in aerobe condition	✗	✓	
- Fungi	PDA	At 27°C in aerobe condition	✗	✓	
Specific media					
- SRB	E2	At 25°C in anaerobe condition	✓	✓	Bass *et al.* (1996)
- MOB	AMS	At 25°C under 50% methane	✓	✓	Whittenbury *et al*. (1970)

Remark : ✓ = The experiment was conducted. ✗ = The experiment was not conducted.

NA = Nutrient Agar YMA = Yeast Malt Agar

PDA = Potato Dextrose Agar

AMS = Ammonium Mineral Salts (AMS) without methanol agar

4.2.5.1 Samples preparation of wastewater and scum

Each sample of the wastewater and scum were mixed together between influent and effluent point collected samples of each pond. Each influent and effluent wastewater samples of 10 ml were mixed together at the ratio of 50:50 while the scum samples 3 g of the influent and effluent samples were mixed together and diluted with 85 percent NaCl.

4.2.5.2 Study microorganisms' community in scum

Numeration : The scum samples were prepared as shown in 4.2.5.1 and were diluted by serial dilution technique (until 10^{-5}) before numeration by spread plate technique. The studied microorganisms' community consisted of the total bacteria, yeast and fungi in aerobic condition. The NA was used for bacteria count, whereas YMA and PDA were used for yeast and fungi, respectively. The bacteria and yeast cultures were incubated at 35°C for 7 days whereas fungi cultures were incubated at 27 °C for 7 days.

Morphological study : The characteristics of colonies grown on each medium (NA, PDA and YMA) were observed by visual aspect. The colonies were purified using streak-plate technique on new medium and incubated in order to study the morphology. The morphology of the colonies grown on YMA and PDA were observed by microscope whereas the pure colonies grown on NA were stained by the method of Gram strains before the microscope observation (X100) (Olympus BX50).

4.2.5.3 Microorganism identification in wastewater and scum

Sulfate Reducing Bacteria (SRB) : The wastewater and scum samples were prepared as shown in 4.2.5.1. They were cultivated in anaerobic selective medium (E2) for SRB that consisted of KH_2PO_4 (0.4 g/L), NH_4Cl (1.0 g/L), $CaCl_2$ (0.1 g/L), $MgSO_4 \cdot 7H_2O$ (2.0 g/L), $FeSO_4 \cdot 7H_2O$ (0.5 g/L), yeast extract (1.0 g/L), $Na_2SO_4 \cdot 2H_2O$ (1.0 g/L), $NaHCO_3$ (2.4 g/L) and sodium lactate (7.0 g/L) and added to distilled water. Enrichment bottles (size 50 ml) were incubated at 25°C in anaerobic condition with anaerobic jar for 5 weeks. Indications of sulfate reducing activity included the production of hydrogen sulphide gas and blackening of the culture in bottles caused by ferrous sulfide precipitation (Bass *et al.,* 1996).

Methane Oxidizing Bacteria (MOB) : The wastewater and scum sample were prepared as shown in 4.2.5.1. They were diluted by the serial dilution technique (until 10^{-5}) before numeration with spread plate technique on AMS agar without methanol that consisted of $MgSO_4 \cdot 7H_2O$ (1.0 g/L), K_2HPO_4 (0.7 g/L), KH_2PO_4 (0.54 g/L), NH_4Cl (0.5 g/L), $CaCl_2 \cdot 2H_2O$ (0.2 g/L), $FeSO_4 \cdot 7H_2O$ (4.0 g/L), H_3BO_4 (0.3 mg/L), $CoCl_2 \cdot 6H_2O$ (0.2 mg/L), $ZnSO_4 \cdot 7H_2O$ (0.1 mg/L), $Na_2MoO_4 \cdot 2H_2O$ (0.06 mg/L), $MnCl_2 \cdot 4H_2O$ (0.03 mg/L), $NiCl_2 \cdot 6H_2O$ (0.02 mg/L), $CuCl_2 \cdot 2H_2O$ (0.01 mg/L), and agar (15.0 g/L) at pH 6.8 ± 0.2 and added to distilled water. The MOB was cultured on AMS agar without methanol and methane 50% in headspace and incubated in the polythene container. The headspace gas of the polythene container was contained mixed gas that composed of 60 percent of methane, 20 percent of oxygen and 20 percent of other gas at 25 °C for one week. Everyday, the gas samples in headspace were collected by using gas tight syringe and storage in vacuum tube. Then, the gas samples were analyzed with GC-TCD, series GC 7890A coupled with capillary column (Shincarbon ST packed column). The carrier gas was helium flowed at 60 ml./min.

Morphological study of MOB: The colonies of MOB were observed by visual aspect and numerated. Each colony of MOB was purified by streak-plate technique and incubated for one week. The subculture was done more than 3 times to obtain pure culture. Then, they were studied by Gram stain before the morphology observation under the microscope (1000X) (Olympus BX50).

Polymerase Chains Reaction (PCR) technique for 16S ribosome RNA (16S rRNA gene) : Each colony grown on AMS agar without methanol was pured and was sent to analyze the bimolecular characteristics using Polymerase Chains Reaction (PCR) technique 16S ribosome RNA (16S rRNA gene) at Department of Microbiology, Kasetsart University, Thailand, in order to identify microorganism of the colonies.

The study of methane oxidation in headspace by microorganisms

The pure colonies grown on AMS without methanol agar were used to study the methane consumption in the polythene container. Headspace gas of the polythene container contained mixed gas that composed of 60 percent of methane, 20 percent of oxygen and 20 percent of other gas at 25 °C for one week. Everyday, the gas samples in headspace were collected by using gas tight syringe and storage in vacuum tube. Then, the gas samples were analyzed with GC-TCD, series GC 7890A coupled with capillary column (Shincarbon ST packed column). The carrier gas was Helium flowed at 60 ml./min..

4.3 Results and discussion

4.3.1 The evaluation of methane emission by actual measurement

The first and the second anaerobic ponds were used to treat the wastewater and to reduce the wastewater temperature. Their surface areas were covered by scum. This research was to evaluate the methane emission from the first and the second anaerobic ponds by close flux chamber. The sampling points were at the surface area of anaerobic pond 1) covered with scum layer, and 2) without scum layer.

4.3.1.1 The methane emission rate of the first and second anaerobic ponds

In the experiment, the gas samples in close flux chamber were collected at 0, 10, 20, 30, 50, 80, 100, 120, 150 and180 minutes, respectively. The percent of concentration of gas increased by time (ΔC /Δt) as can be seen in Figure 4.7 to 4.10 and in Appendix C. Linear function (Y = mx±C) was used to determine the rate of methane emission as shown in Table 4.4.

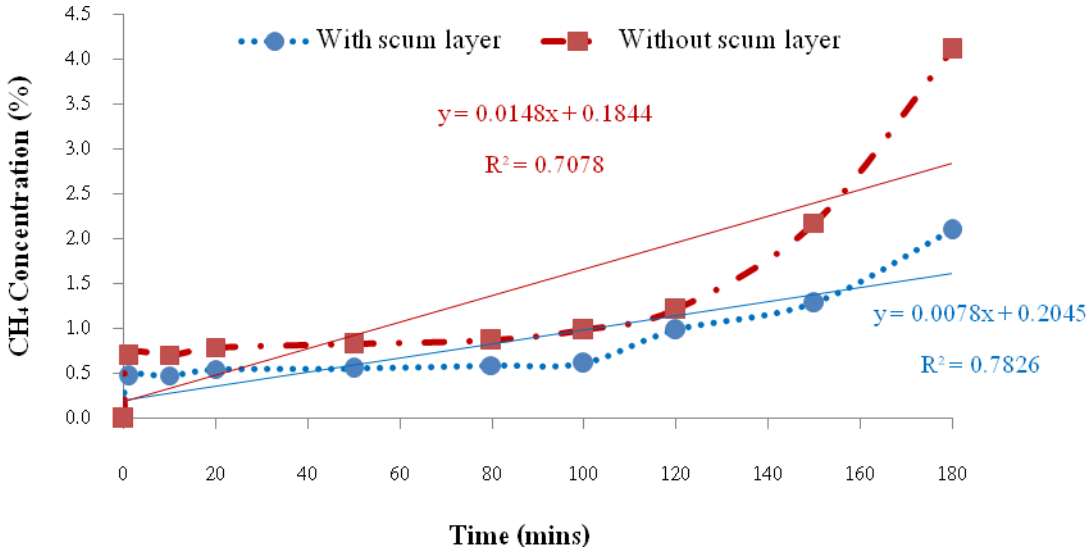


Figure 4.7 Methane emission in close flux chamber by time from the first anaerobic pond at influent point (first sampling).

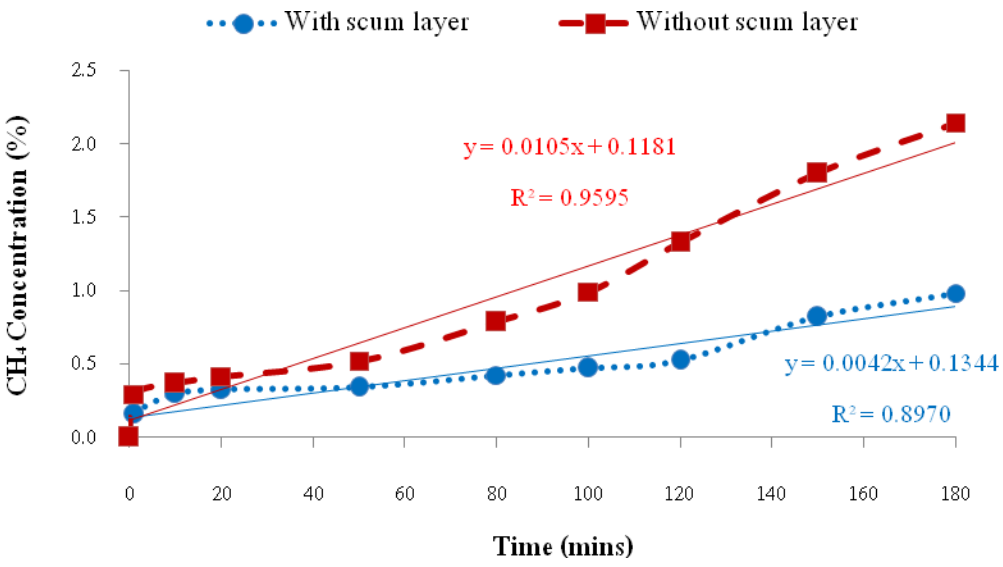


Figure 4.8 Methane emission in close flux chamber by time from the first anaerobic pond at effluent point (first sampling).

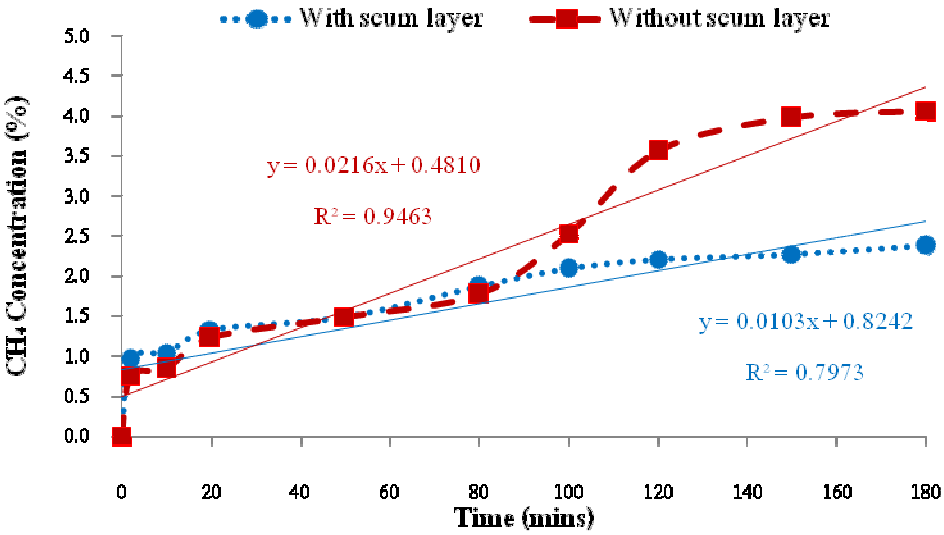


Figure 4.9 Methane emission in close flux chamber by time from the second anaerobic pond at influent point (first sampling).

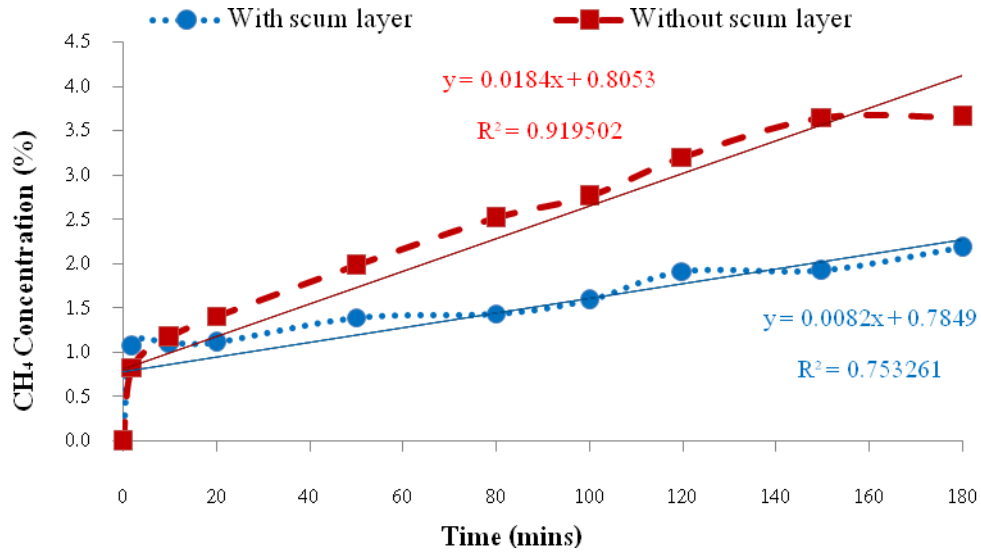


Figure 4.10 Methane emission in close flux chamber by time from the second anaerobic pond at effluent point (first sampling).

Table 4-4 The methane emission rate of the first and the second anaerobic ponds ($\Delta C / \Delta t$).

Locations		$\Delta C / \Delta t$ of the first anaerobic pond			$\Delta C / \Delta t$ of the second anaerobic pond		
		First time	Second time	Average	First time	Second time	Average
Influent	**With scum**	0.0078	0.0085	0.0082	0.0103	0.0173	0.0138
	Without scum	0.0148	0.0118	0.0133	0.0216	0.0244	0.0230
Effluent	**With scum**	0.0042	0.0079	0.0061	0.0082	0.0186	0.0134
	Without scum	0.0105	0.0148	0.0127	0.0184	0.0237	0.0211

Remark : The first time was conducted on February. 9, 2010 and the second time was conducted on April 9, 2010. The previous research showed $\Delta C/\Delta t$ of the second anaerobic pond at influent point with and without scum layer of 0.03158 and 0.04105, respectively (Siriluksopon, 2008).

The slope ($\Delta C / \Delta t$) from linear function was used to calculate methane emissions from the first and the second anaerobic ponds by equation 4.1. To consider the methane emission rate between the ponds, it was found that methane emission rate of the second anaerobic pond was higher than that of the first anaerobic pond. It may due to the operation condition. The second anaerobic pond was operated under stabilized temperature than that of the first pond. Average temperature in the first and the second anaerobic ponds were 59 ºC and 40 ºC, respectively.

4.3.1.2 Total methane emission from the pond area with/without scum layer

For the influent location, the first anaerobic pond emitted average methane into atmosphere 376 and 611 tonCO_2e/year from the surface area with/without scum covered, respectively. At the effluent location, the average methane emission of 280 and 586 tonCO_2e/year from the surface area with/without scum covered surface was detected, respectively, as shown in Appendix C. The average methane emission of the first anaerobic pond was 328 and 598 tonCO_2e/year at the surface area with/without scum covered layer, respectively. The thickness of the scum layer was in the range of 2.5 – 3 cm. For the influent location of the second pond, it emitted average methane from the surface area with/without scum layer covered 400 and 665 tonCO_2e/year, respectively. At the effluent location of the second pond average methane emissions of 413 and 667 tonCO_2e/year was found, respectively. The average methane emission of the second anaerobic pond was 406 and 666 tonCO_2e/year at the surface area with/without scum covered layer, respectively. The thickness of the scum layer in this pond was in the range of 2.5 – 3 cm. It can be stated that the area without scum covered layer of the first and the second anaerobic ponds emitted methane higher than the area covered by scum layer. This was correspondence to the research of Siriruksopon (2008) which reported that the anaerobic ponds without scum layer emitted the methane higher than that of with scum layer covered area. The examples of the calculation for total methane emission from the area with and without scum layer covered were showed in Appendix C.

The results showed that the scum covered layer effected the methane emission from the anaerobic ponds. The scum layer of the first anaerobic pond at influent and effluent points could decrease the methane emission by 39 and 52 percents, respectively, when compared with that of area without scum covered (Figure 4.11). While the second anaerobic pond at influent and effluent points, scum layer could decrease 60 and 62 percents of methane emission, respectively (Figure 4.12). The methane emission from the first and the second anaerobic ponds of palm oil mill could affect by scum layer. The next step was to study the physical, chemical, and biological characteristics of the wastewater and scum samples in order to investigate the possibility of the methane emission reduction at scum layer covered on surface of the anaerobic ponds.

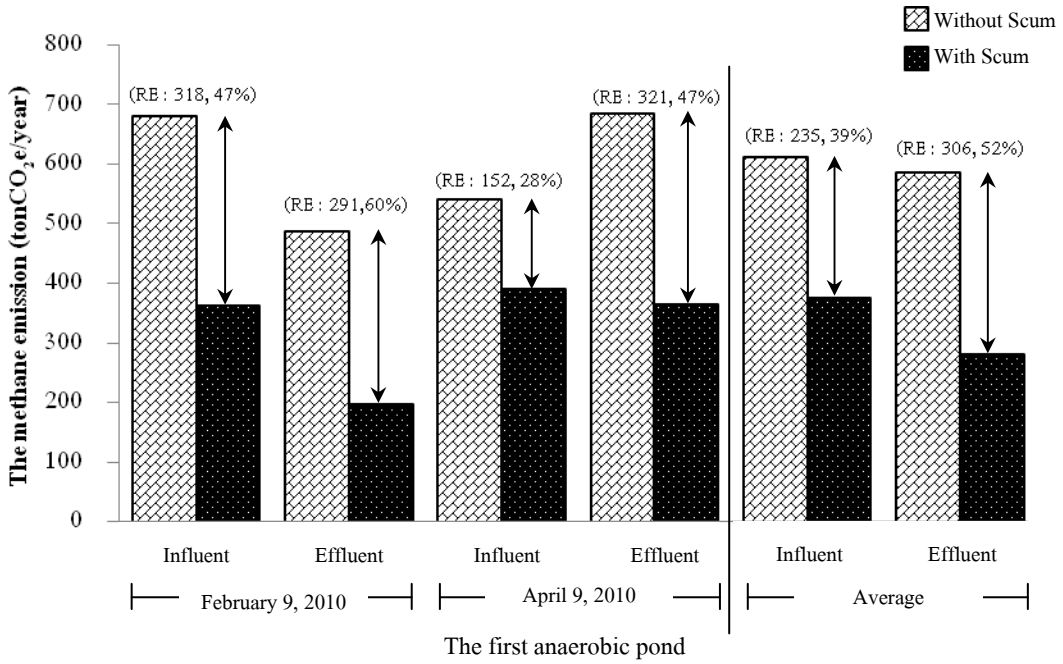


Figure 4.11 The methane emission of the first anaerobic pond at with/without scum layer (at influent and effluent points of the pond).

RE = Reduction Emission

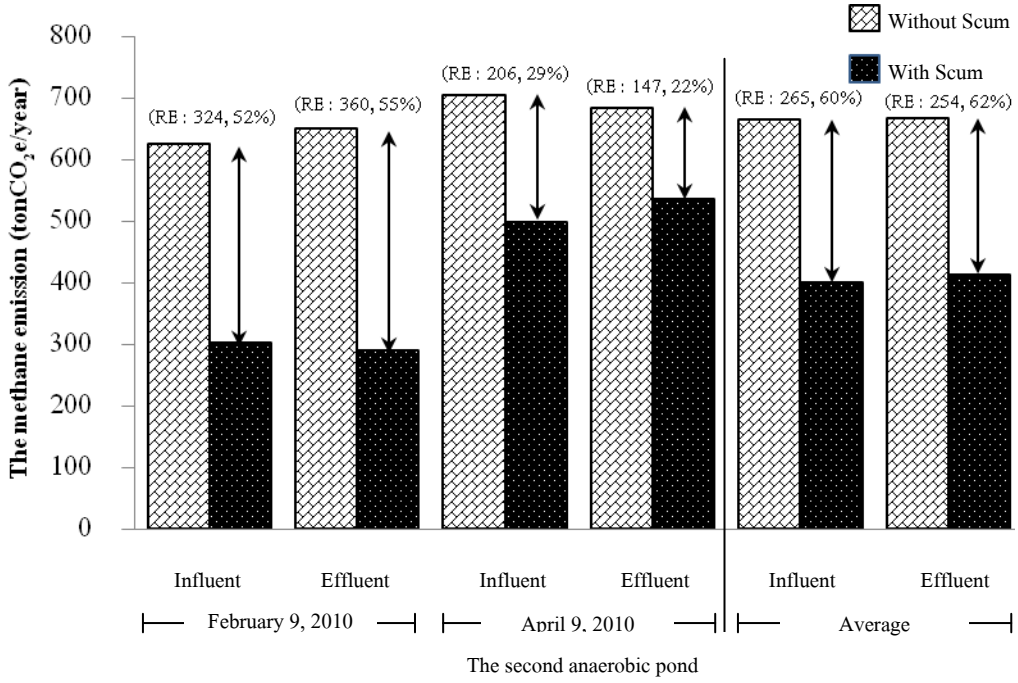


Figure 4.12 The methane emission of the second anaerobic pond at with/without scum layer (at influent and effluent points of the pond).

RE = Reduction Emission

4.3.2 Physical and chemical characteristics of the wastewater and scum samples

4.3.2.1 The physical and chemical characteristics of palm oil mill wastewater

Large quantities of water were used during the extraction of CPO from the FFB, and about 50 percent of the water results in POME (Ahmad *et al.,* 2003). POME from the first and the second anaerobic ponds was a dark black liquid that contains high amounts of total solids (13,804 - 49,080 mg/L), oil and grease (4,710 - 29,228 mg/L), COD (39,200 - 93,850 mg/L) and BOD (21,000 - 70,520 mg/L), as presented in Table 4-5. This was in accordance with the results from the research of Prasertsan *et al.* (2000) and Muksikavong (2009). They reported that the concentration of COD in palm oil mill wastewater was in range from 80,000 to 150,000 mg/L. Ahmad *et al.* (2003) reported that the POME contained high concentration of organic matter, high amounts of total solids (40,500 mg/L), oil and grease (4,000 mg/L), COD (50,000 mg/L) and BOD (25,000 mg/L). Grease and oil have been discharged from the process into the pond that high concentration of these compounds in wastewater often causes major problem in biological wastewater treatment process. They could form layer on water surface and decrease oxygen transfer rate into the aerobic process (Becker *et al.,* 1999).

4.3.2.2 The physical and chemical characteristics of scum

Scum is one of the solid by-products generated during wastewater treatment. In general, it may be defined as a layer of floating material that develops on the surface of the pond. According to Metcalf and Eddy (1991), scum may consist of fats, oils, waxes, soaps, food leftover, fruit and vegetable skins, hair etc. A brief review from literature suggested that scum constitution depended essentially on the characteristic of the raw sewage. According to Laubscher *et al.* (2001), the thickness of the scum layer would depend more on the characteristics of the influent wastewater than on change of biomass and reactor operational parameters. The accumulation of scum occurred in two distinct compartments: on the surface of the settler compartment and inside the three-phase separator. According to Lettinga and Hulshoff Pol (1991), an accumulation of scum inside the three-phase separator may block the natural passage of gas, hence impairing its collection.

Table 4-5 The physical and chemical characteristics of wastewater in the first and the second anaerobic ponds of palm oil mill.

Parameter	The first anaerobic pond			The second anaerobic pond		
	Inf.	**Eff.**	**Average (Inf. – Eff.)**	**Inf.**	**Eff.**	**Average (Inf. – Eff.)**
Color	Dark Black	Dark Black	Dark Black	Dark Black	Dark Black	Dark Black
Odor	Smelly	Smelly	Smelly	Smelly	Smelly	Smelly
pH	4.65 – 4.70	4.20 – 4.30	4.26 – 4.62	4.20 – 4.30	4.50 – 4.70	4.26 – 4.62
Temperature(°C)	66 - 78	41 - 48	45 - 72	41 - 48	31 - 38	34 - 45
COD (mg/L)	86,400 – 103,950	57,600 – 76,545	93,850 – 65,915	57,600 – 76,545	33,600 – 48,000	65,915 – 39,200
BOD (mg/L)	63,840 – 77,200	40,320 – 45,780	70,520 – 36,760	40,320 – 45,780	18,000 – 24,000	36,760 – 21,000
TS (mg/L)	44,700 – 53,460	21,430 – 33,200	49,080 – 27,315	21,430 – 33,200	12,500 – 15,107	27,315 – 13,804
SS (mg/L)	15,489 - 16,850	13,054 – 14,640	16,170 – 13,847	13,054 – 14,640	8,470 – 9,972	13,847 – 9,221
VS (mg/L)	47,900 – 52,403	42,667 - 46,580	50,152 – 44,624	42,667 - 46,580	26, 094 - 24,053	44,624 – 25,074
TKN (mg/L)	161 - 280	96 - 125	111 – 221	96 - 125	61 - 64	63 – 111
Grease & Oil (mg/L)	27,671 - 30,785	13,523 - 16,786	15,155 – 29,228	13,523 - 16,786	4,517 - 4,902	4,710 – 15,155
Sulfate (mg/L)	432 - 450	353 - 368	360 - 441	353 - 368	198 - 255	227 - 360

The formation of the scum layer on the top of the settler compartment could also reduce overall efficiency if floating solids were detached from the scum matrix and released with the effluent. On the other hand, the maintenance of a solid scum layer in open settler compartments could mitigate odor problems by entrapping and/or treating sulfurous gases that otherwise would escape to the atmosphere. The results of the physical and chemical characteristics of scum of the first and the second anaerobic ponds are presented in Table 4-6. The scum covered on surface of the first and the second anaerobic ponds suitable for the existing of microorganisms community, the scum had composited of carbon source as the energy source of MOB (40 – 45 percents for the first pond and 39 – 42 percents for the second pond). The oxygen content in scum layer was about 20 percent. Whittenbury and Dalton (1981), Lidstrom (1991) and Dijkhuizen *et al.* (1992) reported that the most methylotrophic bacteria were those aerobic bacteria that utilized one-carbon compounds as sole carbon and energy source. In addition, Metcalf and Eddy (2004) reported that optimum temperatures for bacterial activity were in range from 25 to 35˚C and pH (6.45 – 6.97).

4.3.3 Biological characteristics of samples

4.3.3.1 Microorganisms' community existed in scum

The scum samples from the first anaerobic pond were spread on the media consists of NA, YMA (incubated 35˚C) and PDA (incubated 27˚C) and cultured under aerobic condition. It was found that the total bacteria, yeast and fungi were 7.075 x 10^6, 9.6 x 10^6 and 0.123x 10^6 Colony-Forming Units per gram (CFUs/g), respectively. For the second anaerobic pond, the total bacteria mostly, yeast and fungi were 4.13 x 10^6, 3.8 x 10^5 and 2.3 x 10^4 CFUs/g, respectively.

Table 4-6 The physical and chemical characteristics of scum in the first and the second anaerobic ponds.

Parameters	The first anaerobic pond		The second anaerobic pond	
	*Min - max	Average	*Min - max	Average
Color	Black-dark	Black-dark	Black-dark	Black-dark
Oder	Smelly	Smelly	Smelly	Smelly
pH	6.50	6.50	6.60 - 6.70	6.65
Temperature (˚C)	62 - 65	62	36 - 42	39
Grease and Oil (% g/g)	32 - 36	35	19 – 23	21
%C	40 – 45	43	39 - 42	41
%H	7	7	6 - 7	7
%O	23 – 24	24	19 – 22	21
%N	0.38 - 0.53	0.45	0.63 – 0.71	0.67
%S	< LOQ	< LOQ	< LOQ	< LOQ

Remark: * Minimum to maximum of the physical and chemical characteristics analysis for three times.
LOQ; Limit of Quantification (LOQ) = 0.01 percent.

The colonies grown on NA, YMA and PDA cultured from the scum samples in the first anaerobic pond could be separated into 25 different types. They consisted of fungi (F_1), yeast (Y_1) and bacteria (B_1) which were 9, 10 and 6 types of colonies, respectively. The colonies on YMA and PDA mostly consists of white, yellow, irregular, circular, whereas on NA were white cloud, gray cloud, white, pink, yellow, irregular. The culture and morphological characteristics were further resolved on the basis of microscopic examination (1000X). Yeast and fungi mostly detected in the shape of rods, cocci and filament. For bacteria, they had rods, cocci and filament shaped that were all Gram-negative (see in Appendix D).

For the second anaerobic pond, microorganisms' community in scum including fungi (F_1), yeast (Y_1) and bacteria (B_1) could be separated into 25 different types were 8, 11 and 6 types of colonies, respectively. Characteristics of bacteria and yeast, the colonies were found white, pink, yellow, irregular and circular. In part of fungi, characteristics of the colonies were white cloud, gray cloud and white. The cultural and morphological characteristics were further resolved on the basis of microscopic examination. The morphology characteristics of bacteria were rods, cocci and filament shape and were stained, they were all gram-negative (please, see in Appendix D).

4.3.4 Sulfate reducing bacteria in wastewater and scum

To study SRB group in the wastewater and scum samples of the first and the second anaerobic ponds, the samples were cultured in selective medium at 25˚C for 5 weeks under anaerobic condition. It was found that the medium color changed from white to black as shown in Figure 4.13 to 4.14 (see additional data in Appendix D) and hydrogen sulphide gas. It can be concluded that SRB was grown in wastewater and scum layer of the first and the second ponds. It may have the activity for reducing the GHG. Lindsey and Creaser (1975) and Orphan *et al.,* (2003) reported that the degradation of organic compounds under anaerobic condition by SRB occurred by the small organic acid molecules degradation in acidogenes stage and it was used as an energy source, and then produce methane and carbondioxide. In addition, the cooperation of SRB group and the methanogens (anaerobic oxidation of methane; AOM group) can use the sulfate compounds as the electron receptor which it was called "Sulfate Reduction" (Visser, 1994) to produce carbondioxide and emit into the atmosphere. Therefore, it may help to decrease the methane.

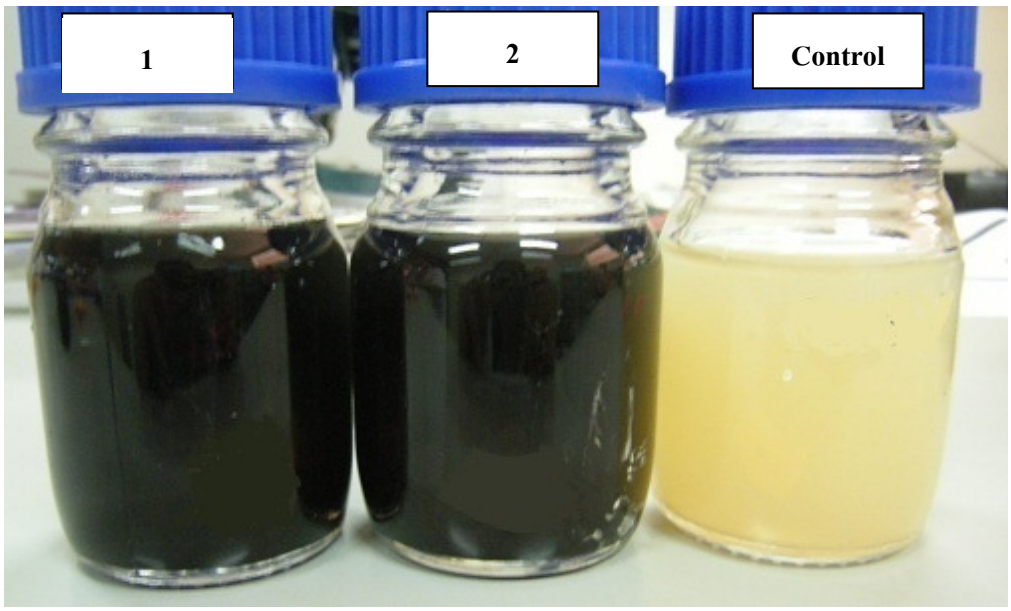


Figure 4.13 The characteristic of medium from scum samples in the first anaerobic pond.

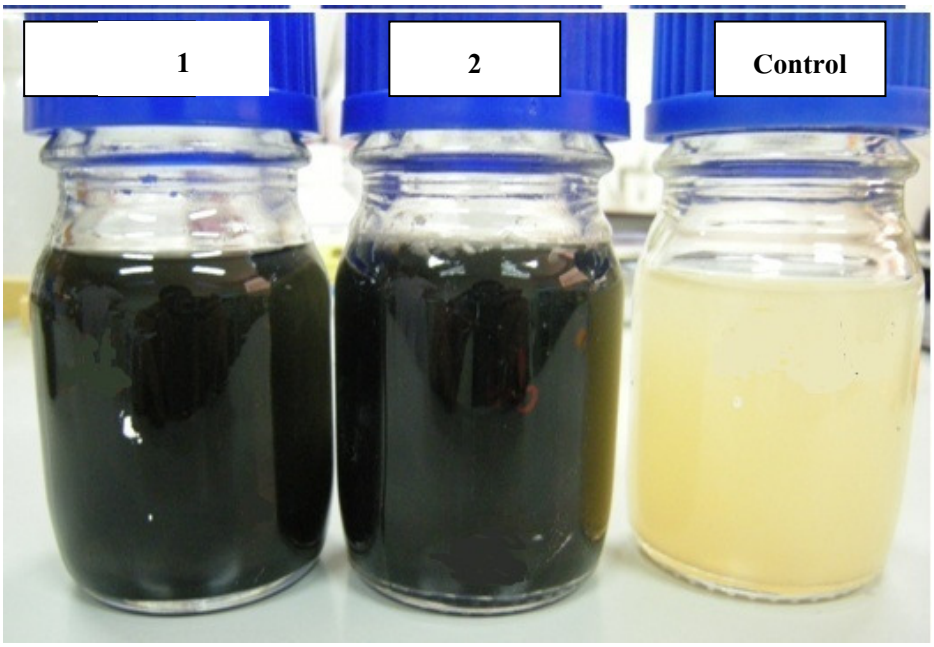


Figure 4.14 The characteristic of medium from wastewater samples in the first anaerobic pond.

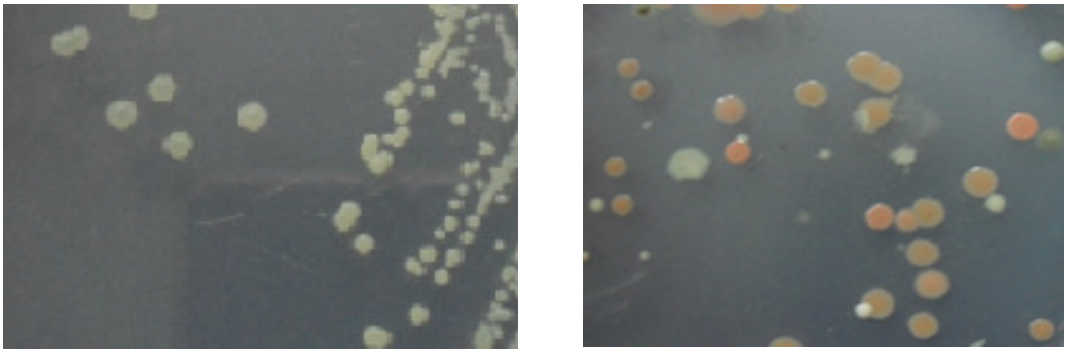
4.3.5 Methane oxidation bacteria (MOB) in wastewater and scum

4.3.5.1 Morphological characteristics of MOB in the wastewater and scum

The average of colonies in the wastewater and scum from the first anaerobic pond grown on AMS agar without methanol were 2.6 CFUs/mL and 0.12 CFUs/g, respectively. The average colonies number in the wastewater and scum samples of the second anaerobic pond were 3.2 CFUs/mL and 28 CFUs/g, respectively. The results show the MOB number existed in the second anaerobic pond was higher than that of in the first anaerobic pond. The scum in the second anaerobic pond that formed on the surface of the pond is in the suitable conditions for growth of MOB. This experiment focused on the colonies that grown in scum layer.

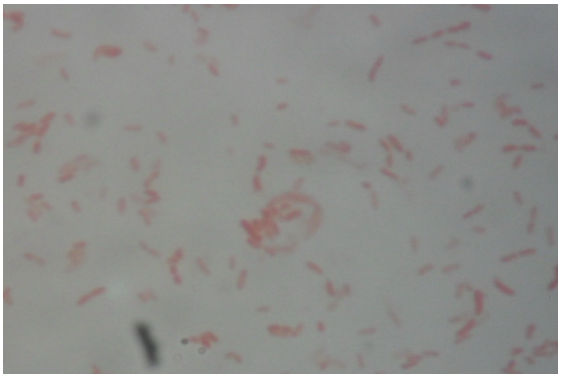
The study of morphological characteristics of the colonies on AMS agar without methanol with visual aspect was conducted. It was found that after conducting pure culture more than three times, there were two groups of colonies including white and pink colonies. Their diameters were 2 – 3 mm. and in the shape was of round, with even edges and smooth (Figure 4.15).

The morphological characteristics under the microscope of the white and pink colonies were rod and cocci, respectively. They were Gram-negative bacteria (Figure 4.16). Hanson and Hanson (1996) reported that the colonies characteristic of the MOB were pink but sometime was orange and red, Gram-negative bacteria.

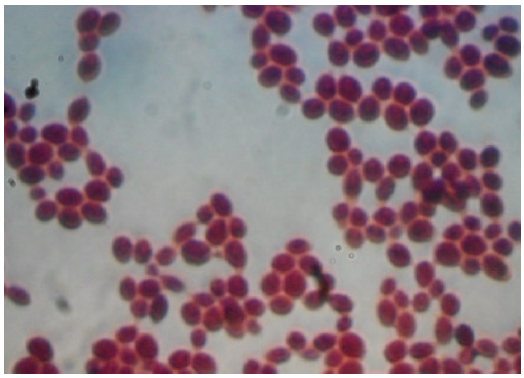


White colonies Pink colonies

Figure 4.15 The morphological characteristic of the colonies on AMS agar with visual aspect.



Rod-shape (White colonies)



Cocci-shape (Pink colonies)

Figure 4.16 The morphological characteristic with microscope (Olympus 1000X).

4.3.5.2 Methane oxidizing ability of MOB isolated

The white and pink colonies from the first and the second ponds were purified. They were used to study methane consumption in the polythene container. Headspace gas of the polythene container was contained with the mixed gas that composted of 60 percent of methane, 20 percent of oxygen and 20 percent of other gas in headspace at 25 °C for one week. Everyday, the gas samples in headspace were collected by using gas tight syringe and storage in vacuum tube and the methane concentration was analyzed with GC-TCD. It was found that the concentration of methane decreased while the concentration of carbondioxide increased as shown in Appendix E. Cells of the white and pink colonies increased on the plate while they were incubated.

Whittenbury *et al*. (1970) and Anthony (1982) reported that more than 100 types of methane-oxidizing bacteria used oxygen, methane and ethanol as sole carbon and energy source to produce hydrogen and carbon-dioxide. Hutton and ZoBell (1949) and Haber *et al.* (1983) reported that the growth of MOB can occurred by the consumption of methane for use as a carbon and energy source. In addition, Patt *et al.* (1974), Haber *et al.* (1983), Urakami *et al.* (1993), Hanson and Hanson (1996) and Green (2005) reported that most methane oxidizing bacteria were in the genus *Methylobacterium*. The optimum growth-temperature for all *Methyloba*cter*ium* strain was in the range of 25 to 30 ºC.

The consumption of methane per day of the white and pink colonies that grew on AMS agar was determined. The reaction order and the reaction rate coefficient were also investigated. The reaction order of methane consumption by MOB in first and second anaerobic ponds was the first order. From Figure 4.17 to 4.20, the reaction rate coefficient of the white and pink colonies from the first anaerobic pond was 0.27 and 0.33 day^{-1}, respectively (haft life of 2.56 and 2.09 day^{-1}, respectively). The reaction rate coefficient of 0.13 and 0.34 day^{-1} of the white and pink colonies from the second anaerobic pond was found, respectively (haft life = 5.32 and 2.03 day^{-1}, respectively).

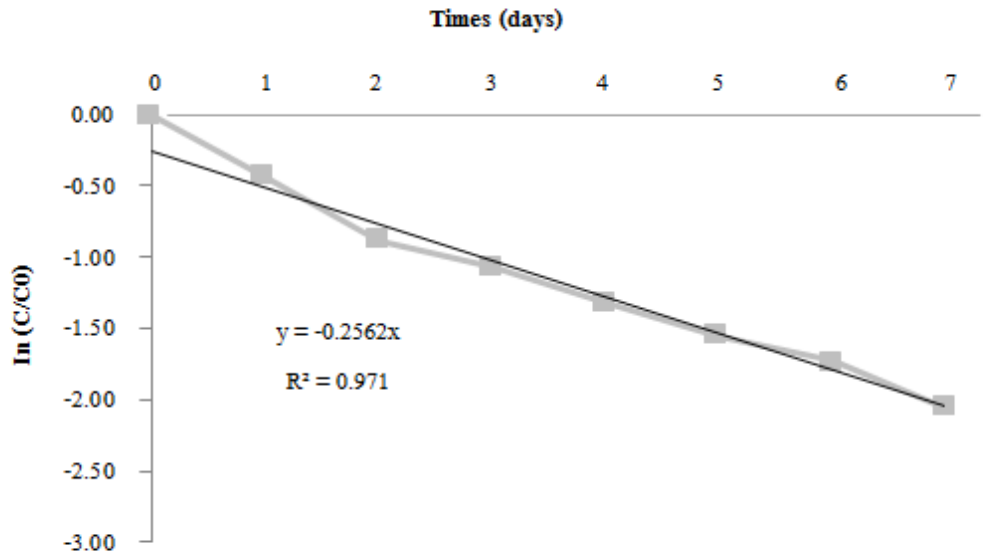


Figure 4.17 Consumption of methane per day by the white colonies isolated from the scum sample in the first anaerobic pond.

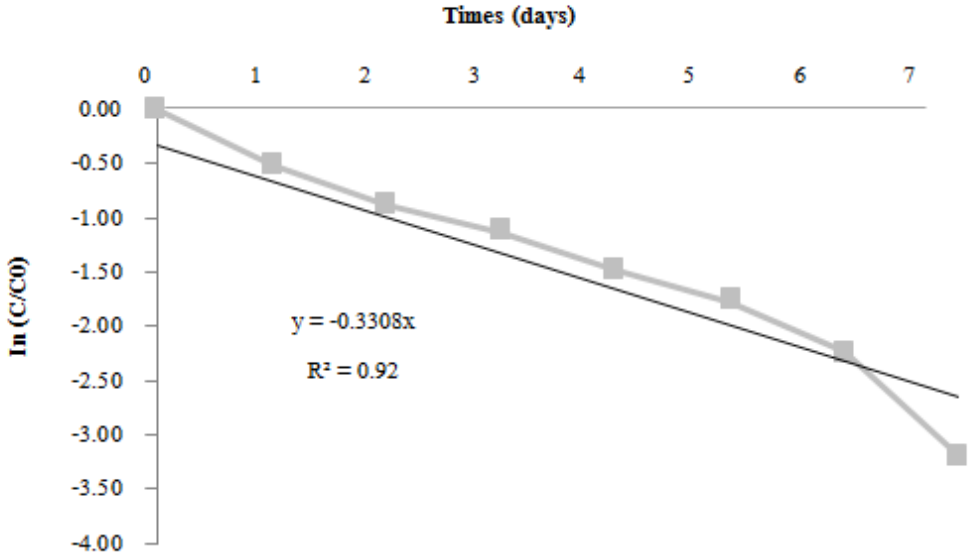


Figure 4.18 Consumption of methane per day by the pink colonies isolated from the scum sample in the first anaerobic pond.

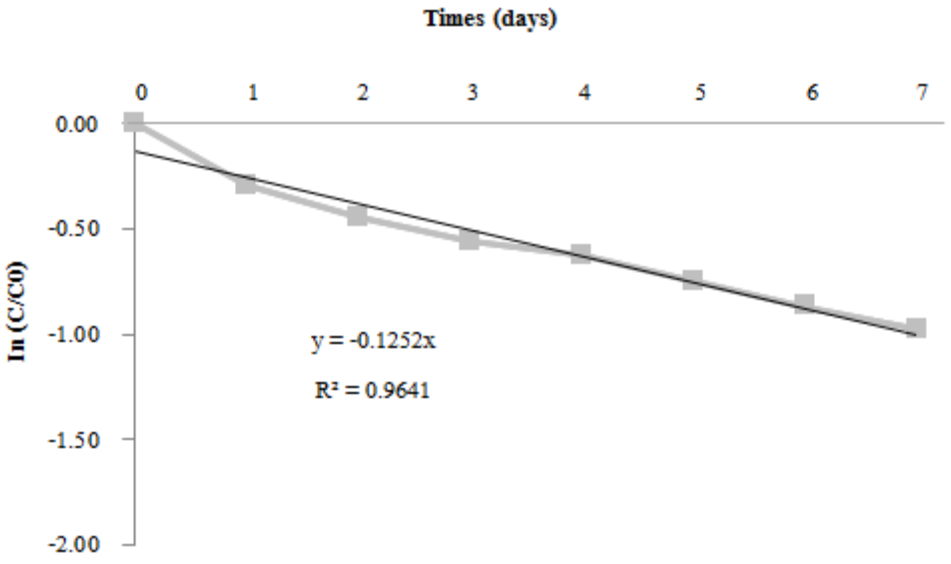


Figure 4.19 Consumption of methane per day by the white colonies isolated from the scum sample in the second anaerobic pond.

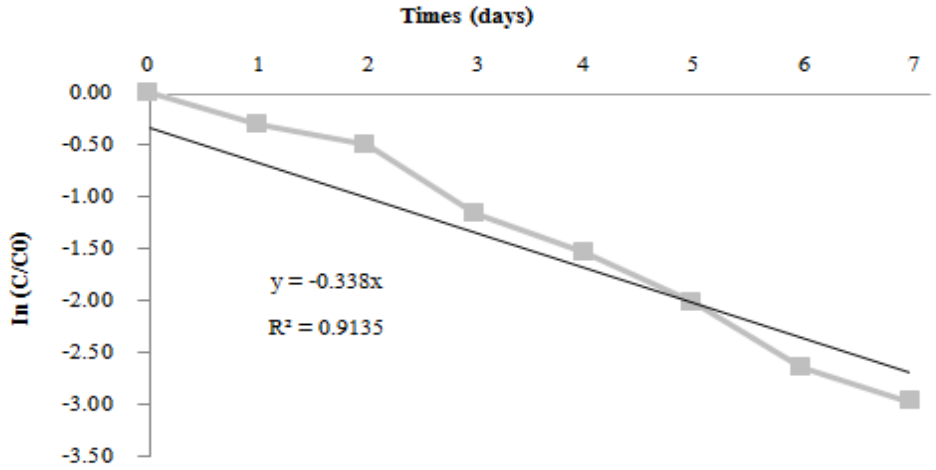


Figure 4.20 Consumption of methane per day by the pink colonies isolated from the scum sample in the second anaerobic pond.

From the results in Figure 4.18 to 4.21, it was found that the average reaction rate coefficient (slope) of white and pink colony were 0.20 and 0.34 day^{-1}, respectively (haft life 3.46 and 2.03 day^{-1}, respectively). The pink colony could reduce the methane higher than the white colony.

4.3.3.3 Identification of white and pink colonies with polymerase chain reaction (PCR) technique

The white and pink colonies grown on specific medium (AMS agar) were identified by PCR with 16s rRNA gene in order to confirm the type of microorganisms at Department of Microbiology, Kasetsart University**.** The primer sequences used to identify the white and pink colonies are shown in Table 4-7 and 4-8, respectively.

(1) White colonies: The nucleotide sequence of the 16S rRNA genes amplified by mean of PCR was determined by direct automated sequencing. The determined sequence 1,473 base had 97.43 – 99.19% similarity to the 16S rRNA genes sequence. Phylogenic analysis based an 16S rRNA gene sequence indicated that this bacterium was a member of the genus *Rahnella aquatilis*as and was closed to *Rahnella aquatilis*as with 99.19% similarity (DSM4594(T)) as shown in Figure 4.21 and Table 4-7. *Rahnella aquatilis* is a member of the family *Entero- bacteriaceae* (Lizzi, Miriam and Caroline, 1989). *R. aquatilis* is an enteric bacterial species that occurs widely in water and soil environments (Herie *et. al*., 1985, Berge *et al*., 1991 and Heulin *et. al*., 1994 and Brenner *et al*., 1998) and is a small gram-negative rod (Maraki *et al.,* 1994).

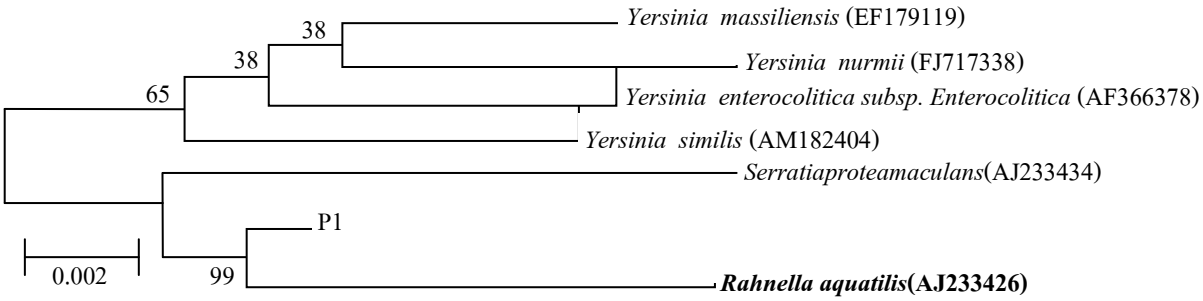


Figure 4.21 Alignment and phylogenic tree by neighbor – joining method of white colony.

Table 4-7 Identification of white colony by partial 16s rRNA gene sequence analysis.

Rank	Name/Title	Authors	Strain	Accession	Pairwise Similarity
1	*Rahnella aquatilis*	Sproer *et al.*(1999)	DSM 4594(T)	AJ233426	99.188
2	*Yersinia enterocolitica* subsp. *enterocolitica*	Kim *et al.*(2003)	ATCC 9610(T)	AF366378	97.970
3	*Yersinia massiliensis*	Merhej *et al.*(2008)	CCUG 53443(T)	EF179119	97.970
4	*Serratia proteamaculans*	Sproer *et al.* (1999)	DSM 4543(T)	AJ233434	97.700
5	*Yersinia nurmii*	Murros-Kontiainen *et al.* (2009)	APN 3a-c(T)	FJ717338	97.429
6	*Yersinia similis*	Sprague *et al.*(2008)	Y228(T)	AM182404	97.429

(2) Pink colonies : The nucleotide sequence of the 16S rRNA gene amplified by mean of PCR was determined by direct automated sequencing. The determined sequence 1,528 bases had 97.32 - 98.73 percent similarity to the 16S rRNA genes sequence of 26 strains of *Pseudomonas* species. Phylogenic analysis based an 16S rRNA gene sequence indicated that this bacterium is member of the genes *Pseudomonas psychrophila* and was closed to *Pseudomonas psychrophila* (E-3(T)) and *Pseudomonas fragi* (ATCC4973 (T)) with 98.73 percent similarity as shown in Figure 4.22 and Table 4-8.

Pseudomonas is methanotroph group that it species of methanogenicarchaea (Marina *et al.*, 1993 and Pikuta and Richard, 2007). *Pseudomonas psychrotphilic* is a mesophilic microorganism in the methanotroph group (Saruyama *et al.*, 1978, Gounot, 1986, and Yumoyo *et al.*, 2001). It utilized methanol in addition to methane, but no other substrates, that is isolated from the cold room, can grow at -1 to 35°C (Pikuta and Richard, 2007) but no growth was observed at 40°C or higher in aerobe condition and shows a maximum growth at 25°C (Mastuo *et al.,* 2010). The cells of the methanotroph were minute (0.5 – 2.0 mm.), round – shaped, pinkish, with even edges, smooth, and of water consistency that are gram – negative cocci resembling *Methylococcus*. (Saruyama *et al.*, 1978, Omelehenko *et al.,* 1996, and Yumoyo *et al.*, 2001).

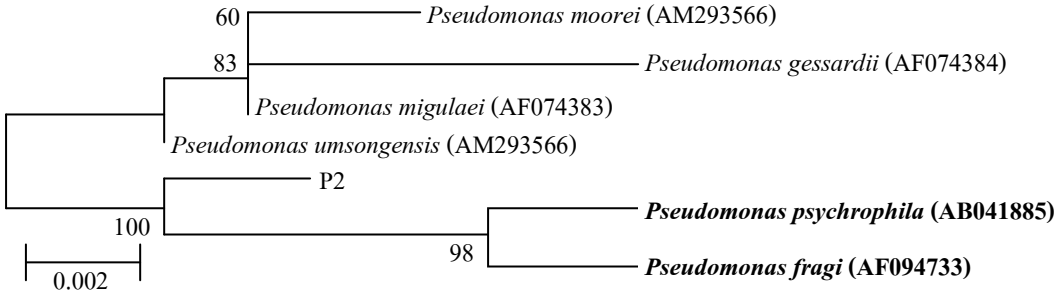


Figure 4.22 Alignment and phylogenic tree by neighbor – joining method of pink colony.

Table 4-8 Identification of pink colony by partial 16s rRNA gene sequence analysis.

Rank	Name/Title	Authors	Strain	Accession	Pairwise Similarity
1	*Pseudomonas psychrophila*	Yumoto *et al.*(2002)	E-3(T)	AB041885	98.731
2	*Pseudomonas fragi*	Galdzicka *et al.*(1902)	ATCC 4973(T)	AF094733	98.731
3	*Pseudomonas umsongensis*	Kwon *et al.*(2003)	Ps 3-10(T)	AF468450	98.590
4	*Pseudomonas moorei*	Camara *et al.*(2007)	RW10(T)	AM293566	98.553
5	*Pseudomonas migulae*	Verhille *et al.*(1999)	CIP 105470(T)	AF074383	98.449
6	*Pseudomonas gessardii*	Verhille *et al.*(1999)	CIP 105469(T)	AF074384	97.320

4.4 Concluding remarks

The open anaerobic pond system is one source that emits the methane into the atmosphere. The evaluation of methane emission of the wastewater treatment system of the palm oil mill shows that the first and the second anaerobic ponds were the major methane emission source. The first and the second anaerobic ponds were covered by scum layer. The measurement of methane emission at the ponds with/without scum layer by actual measurement found that the area without scum layer emitted the methane higher than the area covered with scum layer. The thickness of the floating scum layer in the anaerobic pond was about 2.5 - 3 cm. The scum layer in the first anaerobic pond at influent and effluent locations can decrease the methane emission 39 and 52 percents, respectively while that of in the second anaerobic pond can decrease 60 and 62 percents of methane at influent and effluent locations, respectively. The methane generated from the second anaerobic pond was higher than that of the first anaerobic pond. The second pond has the optimum temperature for growth of methanogenic bacteria. Therefore, the scum of the second anaerobic pond has optimized condition for growth of microorganism more than the first anaerobic pond.

The number of MOB which grew in the second anaerobic pond scum (28 CFUs/g) was more than that of the first anaerobic pond (0.12 CFUs/g). Two dominant colonies found included the white and pink colonies, round, with even edges, smooth. Their morphological characteristics were rods and cocci shapes, Gram-negative and they consumed methane in the headspace. The reaction order for methane reduction was the first order reaction with rate coefficient of 0.21 and 0.43 day^{-1} for the white and pink colonies, respectively. PCR technique with 16S ribosome RNA gene, was used to identify the white and pink colonies. The white colony had 99.19 percent similar to *Rahnell aquatilis*. The pink colony had 98.73 percent similar to *Pseudomonas psychrophila*.

The types of MOB in scum layer and methane reduction rate were identified in this works. The future works should be focused on the condition and circumstance that could enhance methane reduction rate by MOB in the scum layer in the lab scale prior to apply for the practical work.

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APPENDICES

Appendix A: Paper I

Lactobacillus species and genotypes associated with dental caries in Thai preschool children

S. Piwat[1], R. Teanpaisan[1], S. Thitasomakul[2], A. Thearmontree[2] and G. Dahlén[3]

1 Department of Stomatology, Faculty of Dentistry, Prince of Songkla University, Songkhla, Thailand

2 Department of Preventive Dentistry, Faculty of Dentistry, Prince of Songkla University, Songkhla, Thailand

3 Department of Oral Microbiology, Institute of Odontology, The Sahlgrenska Academy at University of Gothenburg, Gothenburg, Sweden

This manuscript was published in Mol Oral Microbiol. 2010;25(2):157-64.

molecular oral microbiology

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S. Piwat[1], R. Teanpaisan[1], S. Thitasomakul[2], A. Thearmontree[2] and G. Dahlén[3]

1 Department of Stomatology, Faculty of Dentistry, Prince of Songkla University, Songkhla, Thailand
2 Department of Preventive Dentistry, Faculty of Dentistry, Prince of Songkla University, Songkhla, Thailand
3 Department of Oral Microbiology, Institute of Odontology, The Sahlgrenska Academy at University of Gothenburg, Gothenburg, Sweden

Correspondence: Rawee Teanpaisan, Department of Stomatology, Faculty of Dentistry, Prince of Songkla University, Hat-Yai, Songkhla, 90112, Thailand Tel.: + 66 74 429878; fax: + 66 74 212922; E-mail: rawee.t@psu.ac.th

Keywords: dental caries; genotyping; *Lactobacillus salivarius;* 16S rRNA
Accepted 27 October 2009

SUMMARY

Lactobacilli have been associated with the presence and progression of dental caries. Nevertheless, the relation between certain species or genotypes of *Lactobacillus* and caries is unclear and there are no data available for the Thai population. This study aimed to examine the distribution of species and genotypes of oral *Lactobacillus* among children with rather high caries prevalence, and to investigate whether certain species or genotypes were more related to caries activity than others. One hundred and sixty-five children were examined for caries status. Saliva samples were collected and the numbers of lactobacilli were counted. A total of 357 *Lactobacillus* isolates from 59 children were identified to species level by 16S ribosomal RNA genes polymerase chain reaction (PCR) –restriction fragment length polymorphism and 16S ribosomal RNA gene sequencing. Furthermore, 304 isolates from 56 children were genotyped using arbitrarily primed PCR. Significant correlation was found between levels of lactobacilli and dental caries ($P < 0.001$). Among the 10 identified species of *Lactobacillus*, *L. salivarius* was more prevalent in children with moderate to high caries prevalence compared with children with low caries prevalence, while *L. fermentum* was the most predominant species in all study groups. Moreover, a genetic heterogeneity of *Lactobacillus* species was found among the children and those with high caries prevalence tended to be colonized with more than one clonal type. In summary, *L. salivarius* may be a putative caries pathogen among preschool Thai children.

INTRODUCTION

Lactobacillus is part of the normal oral microflora, and it has been recognized for decades as a major contributor in the caries process (van Houte, 1994). Our previous reports have shown that *Lactobacillus* species are strongly associated with the presence and progression of dental caries in Thai children and adults (Teanpaisan *et al.*, 2007, 2009). Nevertheless, some *Lactobacillus* species have been introduced as probiotics in caries prevention, mainly because of their inhibitory activities against cariogenic *Streptococcus* spp. (Nase *et al.*, 2001; Chung *et al.*, 2004; Simark-Mattsson *et al.*, 2007). Although there is a strong association between lactobacilli and caries, less is known of the relationship at species level because of difficulties in identifying *Lactobacillus* species. It is important to understand the role of various lactobacilli, whether they are harmful, beneficial or neutral for the development of dental caries.

Genotypic studies of bacterial species are of interest in the search for more pathogenic clones. Recent

findings indicate that specific clones of *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* from cases of severe periodontitis can be associated with higher virulence (Enersen *et al.*, 2008). It has also been demonstrated that caries-associated bacteria such as *Streptococcus mutans* and *Streptococcus sobrinus*, are usually presented as one single or a very limited number of genotypes in the predominant oral flora at a given time (Kilian *et al.*, 2006). However, genetic studies that relate the severity of caries with *Lactobacillus* genotypes are diverse and controversial.

As a consequence, it is important to define the roles of various species or genotypes of *Lactobacillus* in the caries process, because this will lead to better understanding of the natural habitat of various *Lactobacillus* species. The aims of the present study were to investigate the distribution of species and genotypes of oral *Lactobacillus* among Thai children with rather high caries prevalence (Teanpaisan *et al.*, 2007), and to determine whether certain species or genotypes were more related to caries activity than others.

METHODS

Subjects and clinical examination

One hundred and sixty-five Thai children aged 2–5 years old were recruited from children who attended a well-baby clinic at the hospital and health centers in Thepa district, Songkhla province, Thailand. The study protocol was approved by the National Ethical Committee, at the Ministry of Public Health, Thailand, and parental informed consent was obtained. The individual's caries status was recorded as dmft/dmfs indices (decayed, missing, filled teeth/decayed, missing, filled tooth surfaces) according to the criteria adapted from the World Health Organization's 1997 criteria (World Health Organization, 1997).

Bacterial sampling

A modified spatula method (Kohler & Bratthall, 1979) was used to obtain bacterial samples. A spatula was inserted into the mouth to moisten it with saliva. Each side of spatula was then placed directly on the surfaces of Rogosa SL agar (Difco, Sparks, MD) for recovery of lactobacilli and incubated anaerobically (80% N_2, 10% H_2, and 10% CO_2) at 37°C for 72 h. The numbers of lactobacilli colonies on two predetermined areas, approximately 1.5 cm^2 of each spatula-pressed area, were counted as colony-forming units (CFU). For further analysis, colonies were collected from plates that contained average numbers of lactobacilli of more than 5 CFU per 1.5 cm^2.

A random sampling method was used for all culture plates. At least three colonies of either the same or different colonial appearance were collected from the culture plates. The colonies were tentatively identified as *Lactobacillus* based on their growth on Rogosa SL agar, colonial morphology, Gram staining and by being catalase negative (Felis & Dellaglio, 2007). After pure culture, all isolates were kept at –80°C until use.

Lactobacillus species identification

DNA samples were prepared using a Genomic DNA Extraction Kit (RBC Bioscience, Taipei, Taiwan), following the manufacturer's protocol for gram-positive bacteria. The *Lactobacillus* isolates were identified to species level by restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR) -amplified 16S ribosomal RNA (rRNA) genes by the method of Teanpaisan & Dahlen (2006). Briefly, the 16S rRNA genes were amplified by PCR using the universal primers 8UA (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGGTACCTTGTTACGACTT-3′) (Sato *et al.*, 2003). The PCR 50-μl reaction mixture contained 100 ng of DNA template, 1.0 μM of each primer, 5 μl 10 × buffer with 2.0 mM $MgCl_2$, 1.0 U of *Taq* DNA polymerase, and 0.2 mM of each dNTP. Amplification proceeded using a GeneAmp PCR System 2400 (Applied Biosystems, Foster, CA) programmed as follows: initial heat activation at 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, primer extension at 72°C for 1.5 min and a final extension step at 72°C for 10 min. The PCR products were individually digested with *Hpa*II or *Hae*III (New England Biolab, Ipswich, MA) according to the manufacturer's instructions. Digestion products were separated through 7.5% polyacrylamide and stained with silver nitrate. Discriminations between *L. casei* and *L. rhamnosus*, and between *L. acidophilus* and *L. crispatus*, which were not possible from

the PCR-RFLP pattern, were performed using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) (Teanpaisan & Dahlen, 2006). Initially 14 type strains of *Lactobacillus* were included in the panel: *L. acidophilus* ATCC 4356^T, *L. brevis* ATCC 14869^T, *L. casei* ATCC 393^T, *L. crispatus* ATCC 33820^T, *L. curvatus* ATCC 25601^T, *L. delbrueckii* ATCC 9649^T, *L. fermentum* ATCC 14931^T, *L. gasseri* ATCC 33323^T, *L. paracasei* CCUG 32212^T, *L. plantarum* ATCC 14917^T, *L. reuteri* CCUG 33624^T, *L. rhamnosus* ATCC 7469^T, *L. salivarius* ATCC 11741^T, *Olsenella* (formerly *Lactobacillus*) *uli* CCUG 31166^T. Three other clinical isolates, *L. mucosae* CCUG 43179^T, *L. oris* CCUG 37396^T, and *L. vaginalis* CCUG 31452^T, were identified by 16s rDNA sequencing and were included in the panel. The isolates that did not fit to the panel above were identified by 16S rRNA gene sequencing. Also, several strains of the same species, identified by PCR-RFLP of 16S rRNA genes, were chosen for sequencing of 16S rRNA genes to confirm the results.

Sequencing was performed using an ABI PRISM Big Dye Terminator Kit and ABI PRISM 377 genetic analyzer (Applied Biosystems). In a 50-μl volume, the PCR mixture consisted of 500 ng template, 0.8 μl Terminator Ready Reaction Mix (Applied Biosystems), and 3.2 pmol each universal primer (8UA and 1492R primers). PCR was performed at 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min for a total of 25 cycles using the Gene Amp® PCR System 2400 (Applied Biosystems). Analysis of the alignment of % homology for the sequences was performed using the blast programs (http://www.ncbi.nlm.nih.gov/BLAST/).

Lactobacillus paracasei CCUG 32212^T and all clinical strains identified as *L. paracasei* showed minor bands of PCR-RFLP and SDS–PAGE patterns different from *L. casei* ATCC 393^T. Therefore, these isolates were presented as *L. casei/paracasei* group.

Genotyping

After identification, three or more colonies of the same *Lactobacillus* species from the same child were collected for genotyping using arbitrarily primed PCR (AP-PCR) with the primers; ERIC1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) (Matsumiya *et al.*, 2002). The reaction mixture in a 50-μl reaction mixture contained 100 ng of DNA template, 1.0 μm of each primer, 5 μl 10 × buffer with 2.0 mM $MgCl_2$, 1.0 U *Taq* DNA polymerase, and 0.2 mM of each dNTP. The mixture was subjected to 35 cycles of denaturation at 95°C for 1 min; ramping to 35°C in 3 min; annealing at 35°C for 1 min; extension at 74°C for 2 min, and a final extension at 74°C for 5 min. PCR products were separated through 7.5% polyacrylamide and silver-stained.

Analysis of data

Because there were no missing teeth and only one filling among the children investigated, the numbers of decayed teeth/surfaces (dt/ds) were depicted. The children were divided into three groups according to the first and third quartile cut-off points of dt; low caries was dt range 0–4, moderate caries was dt range 5–10, and high caries was dt more than 10. The average numbers of lactobacilli were categorized as: 0 CFU/1.5 cm^2, 1–10 CFU/1.5 cm^2, and >10 CFU/1.5 cm^2. The Kruskal–Wallis and chi square tests were used to evaluate the relationships between caries status and levels of salivary lactobacilli. The distribution of *Lactobacillus* species and genotypes was calculated as a percentage. The associations between frequency of isolation of each *Lactobacillus* species and caries group were compared using chi square test and Fisher's exact test. The analyses were performed with the SPSS statistical program (SSPS Inc., Chicago, IL). The differences were considered significant when $P < 0.05$.

RESULTS

A total of 165 children aged 2.2 ± 0.8 years had mean dt and ds of 5.7 ± 4.8 and 12.3 ± 13.3, respectively. In this study, the prevalence of caries-free children was quite low, with 22/165 of children (13.3%) being caries-free and 15/22 (68.2%) of children being negative for lactobacilli detection. Ninety-two out of 165 children (55.8%) carried salivary lactobacilli. Among these, 59 children produced more than five colonies of lactobacilli per 1.5 cm^2 and these were retained for species identification (total 357 isolates). The species frequency analysis of lactobacilli was therefore performed only for those 59 children with a predominant presence of lactobacilli.

Salivary lactobacilli levels in children with low caries prevalence were significantly different from those in children with moderate to high caries prevalence ($P < 0.001$) (Table 1). There were significantly higher mean dt and ds as numbers of lactobacilli increased ($P < 0.001$) (Table 1). The distribution of *Lactobacillus* species between different frequencies of caries lesions in children is shown in Table 2. *L. fermentum* (83% of the children) and *L. salivarius* (25% of the children) were the predominant species found. *L. salivarius* was the only species found in significantly higher numbers in the moderate to high caries group (35.9%) compared with the group with low caries (5%) ($P = 0.01$). The presence of other species was not related to the caries status. *L. fermentum* was the most frequently found (more than 80% of children) species in all groups. *L. plantarum* and *L. mucosae* were found only in the moderate to high caries group, while *L. gasseri*, *L. vaginalis*, and *L. oris* were identified more frequently in the low-caries group. The significance of these differences could not be further evaluated because too few subjects harbored these species. Most children (79.6%) harbored only one or two species of *Lactobacillus* with the maximum of five species detected in one individual. However, the diversity of *Lactobacillus* species was not statistically different between the groups ($P = 0.17$) All 10 *Lactobacillus* species from 56 children were further investigated by AP-PCR. The numbers of subjects and isolates of each species are shown in Table 3. Generally, isolates from each individual showed a distinct genotypic pattern, and between one and five different genotypes could be detected in a single child (Fig. 1). It was noted that children who had high caries prevalence tended to be colonized by more than one genotype. This distribution was statistically significant for *L. fermentum* ($P < 0.01$), but was not significant for *L. salivarius* because too few isolates were recovered (Table 3).

Table 1 Mean of decayed teeth/surfaces and prevalence of oral lactobacilli in low-caries group (dt ≤ 4, $n = 84$) and in moderate to high-caries group (dt > 4, $n = 81$)

	Mean ± SD of decayed		Number of children (%)	
Lactobacilli (CFU/1.5 cm^2)	Teeth	Surfaces	Low-caries group	Moderate to high- caries group
0	3.9 ± 3.5	7.7 ± 8.7	49 (58.3)	24 (29.6)
1–10	6.2 ± 4.9	12.7 ± 13.7	20 (23.8)	25 (30.9)
> 10	8.2 ± 5.4	19.0 ± 15.8	15 (17.9)	32 (39.5)
P-values	< 0.001[1]	< 0.001[1]	< 0.001[2]	

[1]Kruskal–Wallis test.
[2]Chi-square test.
CFU, colony-forming units; dt, decayed teeth score.

Table 2 Distribution of *Lactobacillus* isolated from children in low-caries group (dt ≤ 4) and children in moderate to high-caries group (dt > 4)

	All subjects		Low-caries group		Moderate to high-caries group	
Species	No. of subjects (%)	No. of isolates (%)	No. of subjects (%)	No. of isolates (%)	No. of subjects (%)	No. of isolates (%)
L. fermentum	49 (83.1)	195 (54.6)	17 (85)	74 (59.7)	32 (82.1)	121 (51.9)
L. salivarius	15 (25.4)	53 (14.8)	1 (5)	2 (1.6)	14[1] (35.9)	51 (21.9)
L. casei/paracasei	11 (18.5)	32 (8.9)	5 (25)	14 (11.3)	6 (15.4)	18 (7.7)
L. mucosae	6 (10.2)	12 (3.4)	0	0	6 (15.4)	12 (5.2)
L. rhamnosus	5 (8.5)	14 (3.9)	2 (10)	4 (3.2)	3 (7.7)	10 (4.3)
L. oris	5 (8.5)	12 (3.4)	3 (15)	6 (4.8)	2 (5.1)	6 (2.6)
L. gasseri	4 (6.8)	18 (5)	3 (15)	14 (11.3)	1 (2.6)	4 (1.7)
L. plantarum	4 (6.8)	11 (3.1)	0	0	4 (10.3)	11 (4.7)
L. vaginalis	2 (3.4)	10 (2.8)	2 (10)	10 (8.1)	0	0
Total	59 (100)	357 (100)	20 (100)	124 (100)	39 (100)	233 (100)

[1]Fisher's exact test: $P = 0.01$.

Table 3 Genotypes of 304 *Lactobacillus* strains (with genotype = 1 or >1) of 56 children

Species (no. of children/isolates)	No. of genotypes	No. (%) of children/isolates (%)		
		Low-caries group	Moderate-caries group	High-caries group
L. fermentum (38/180)[1]	1	12 (85.7)/60 (88.2)	13 (92.9)/52 (92.9)	4 (40)/17 (30.4)
	>1	2 (14.3)/8 (11.7)	1 (7.1)/4 (7.1)	6 (60)/39 (69.6)
L. salivarius (10/45)	1	0	5 (100)/19 (100)	1 (20)/3 (11.5)
	>1	0	0	4 (80)/23 (88.5)
L. casei/paracasei and *L. rhamnosus* (9/34)	1	4 (100)/13 (100)	1 (25)/4 (22.2)	1 (100)/3 (100)
	>1	0	3 (75)/14 (77.8)	0
Others[2] (10/45)	1	2 (75)/11 (57.9)	4 (100)/15 (100)	3 (100)/11 (100)
	>1	1 (25)/8 (42.1)	0	0

[1]Chi-square test only for *L. fermentum*: $P = 0.02$.
[2]Others species included *L. mucosae* (1/5), *L. oris* (3/10), *L. gasseri* (2/12), *L. plantarum* (3/10), *L. vaginalis* (1/8).

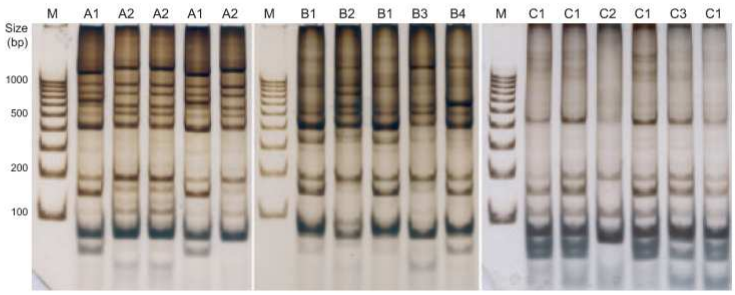

Figure 1 Arbitrarily primed polymerase chain reaction profiles of 16 *Lactobacillus fermentum* isolates from saliva of three children (A–C) with high caries, which showed A1–A2, B1–B4, and C1–C3 genotypes, respectively. M, molecular size markers – 100 base pairs (bp) DNA Ladder; Bio-Rad, Hercules, CA.

DISCUSSION

There are few studies on species and genotypes of *Lactobacillus* in relation to dental caries in young children. This study included a greater number of subjects and strains than previous reports (Milnes & Bowden, 1985; Marchant *et al.*, 2001), and also included children with low caries levels. A limitation of this study is related to the unexpected high caries prevalence (86.7%) in our children aged 2.2 ± 0.8 years. There were only five caries-free children who carried sufficient lactobacilli for species identification. As the patterns of *Lactobacillus* species distribution between caries-free children ($n = 5$) and other children in the low-caries group ($n = 15$) were similar (data not shown), those subjects were included in the low-caries group to gain sufficient numbers of children for statistical analyses. The prevalence of salivary lactobacilli in our subjects was found to be 55.8%, which was similar to culture-based studies in other populations (Nancy & Dorignac, 1992). Moreover, the children in the moderate to high-caries groups were frequently and heavily colonized by lactobacilli compared with the low-caries children.

This is the first attempt to speciate and genotype a substantial number of oral lactobacilli from Thai children. The application of PCR-RFLP and SDS–PAGE for comparison of isolates with type strains representative of known *Lactobacillus* species is suitable for discrimination of oral lactobacilli, simple to perform, and reproducible (Teanpaisan & Dahlen, 2006). Ten different *Lactobacillus* species were found in saliva samples from the children. Byun *et al.* (2004), using 16S rDNA sequence analysis and real-time PCR, found 18 different phylotypes of lactobacilli in carious dentine. About 50% of these were novel and it was concluded that diversity among lactobacilli was much greater than previously thought. *L. fermentum* was present in only 22% of the samples. The present study might have underestimated the diversity of lactobacilli by relying on culture on Rogosa agar plates, but the predominant species in carious dentine could be different from those in saliva. Ultimately, *Lactobacillus* taxonomy is still complex and conclusions about species relationships with clinical conditions should be made with caution.

When the distributions of *Lactobacillus* species of the group with low caries prevalence and the group

with moderate to high caries were compared, it was found that most children were colonized by several species, as observed by others (Marchant *et al.*, 2001; Caufield *et al.*, 2007). Interestingly, *L. salivarius* was found to be more highly associated with caries than the other lactobacilli in the present study. Caufield *et al.* (2007) showed that *L. salivarius* was one of nine taxa commonly found in subjects with active caries. *L. salivarius* may play a role in the caries process because it is acidogenic and can produce lactate, acetate and hydrogen peroxide (Martin *et al.*, 2006). *L. salivarius* is also aciduric and is reported to survive 4 h incubation at pH 2.5 (Strahinic *et al.*, 2007). Generally, lactobacilli have low affinity for the sound tooth surface and are recovered in low numbers in plaque samples, although they can be presented in high levels in saliva (van Houte *et al.*, 1972; van Houte, 1980). It has also been shown that *L. salivarius* adheres to saliva-coated hydroxyapatite *in vitro* (Matsumoto *et al.*, 2005). It is possible that *L. salivarius* is more likely to be incorporated into dental plaque than other lactobacilli. Fitzgerald *et al.* (1981) reported that *L. salivarius* isolates from human dental plaque could induce severe caries in the fissures of molars in germ-free rats receiving either the glucose-containing or sucrose-containing diet. This cariogenic capacity was further supported by the findings that *L. salivarius* strains were more cariogenic than *S. mutans* Ingbritt in gnotobiotic rats (Seppa *et al.*, 1989). In the presence of sucrose and low pH, *L. salivarius* further lowered the pH and this resulted in changes in the bacterial community within oral biofilms (Pham *et al.*, 2009). Therefore, the closer association of *L. salivarius* with caries prevalence found in the present study, together with other evidence above, indicates strongly that *L. salivarius* may be cariogenic.

The relationship of *L. fermentum* to the caries process is not clear. This was the most predominant species found in saliva of caries-free subjects in some studies (Colloca *et al.*, 2000; Ahumada Mdel *et al.*, 2003), while others have reported a high prevalence of *L. fermentum* in subjects with caries (Milnes & Bowden, 1985; Marchant *et al.*, 2001; Caufield *et al.*, 2007). In the present study, *L. fermentum* was the most common species presented in saliva of both the low-caries and the moderate to high-caries groups. Strains of this species have been previously isolated from Thai traditional foods (Klayraung *et al.*, 2008). Possibly food-associated lactobacilli survive within the normal resident microflora of the human mouth, and so may not associate with caries.

Other species that have been associated with caries such as *L. casei*, *L. paracasei* and *L. rhamnosus* (Nancy & Dorignac, 1992; Marchant *et al.*, 2001), were infrequently found in this study and were not related to caries in our subjects. The prevalence of *L. gasseri*, *L. plantarum* and *L. vaginalis* was relatively low and they may be uncommon in the oral cavity.

Genotypic studies of oral *Lactobacillus* species are limited, and only two studies reported on relationship to caries (Marchant *et al.*, 2001; Caufield *et al.*, 2007). Marchant *et al.* (2001) showed in a genotypic study on 39 *Lactobacillus* strains isolated from carious dentine of three children that diverse genotypes of *Lactobacillus* species were found within and between carious lesions in the same child as well as between children. Furthermore, Caufield *et al.* (2007) reported genetic heterogeneity among 180 isolates of salivary lactobacilli from six women with active caries. Our study using AP-PCR, with ERIC primers set to reveal the *Lactobacillus* intra-species variability, gave high discrimination with the polymorphic AP-PCR patterns reflecting differences within the species at the subspecies level. Marchant *et al.* (2001) and Matsumiya *et al.* (2002) showed that ERIC-PCR methodology was efficient and practical for discriminating genotypes within species of *Lactobacillus*. We found a genetic heterogeneity among 304 *Lactobacillus* strains from 56 children, and neither individual was colonized with the same genotypes. The high-caries prevalence children were found to be frequently colonized with more than one genotype compared with the low-caries group, and from one up to five genotypes could be found in individuals. The reason why a greater genotypic diversity was found in subjects with high caries is unknown. It has previously been postulated that environmental stress in the oral cavity could lead to a reduced number of genotypes that are best adapted to colonize and proliferate in a particular environment (Bowden & Hamilton, 1998). Conversely, high sugar availability in the carious environment could lead to growth of increased numbers of different *Lactobacillus* clones compared with less supportive conditions (Beighton *et al.*, 1996). There is, however, limited knowledge available

regarding the importance of genetic diversity and the impact of such diversity on the ecology of the oral microflora.

In conclusion, this study showed that salivary *Lactobacillus* isolated from Thai preschool children exhibited wide species and genotype heterogeneity. *L. salivarius* was predominant in children with high levels of caries, which may indicate an association with the cariogenic process. *L. fermentum* on the other hand was the most predominant species in all study groups. Children with high caries levels were often colonized with more than one clonal type. Further studies of the biological properties of these bacteria are necessary to determine their role in caries processess.

ACKNOWLEDGEMENTS

This study was supported by The Thailand Research Fund (TRF) through the Royal Golden Jubilee PhD program (PHD/0091/2549/A.1) and National Research council of Thailand. We would like to thank all subjects and their parents for their kind cooperation.

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Appendix B: Paper II

Acid production and growth by oral *Lactobacillus* spp *in vitro*

Piwat S.[a], Teanpaisan R.[a], Dahlén G [b] , Thitasomakul S.[c] and Douglas C.W.I.[d]

[a]Department of Stomatology, [c]Department of Preventive Dentistry, Faculty of Dentistry, Prince of Songkla University, Songkhla, Thailand

[b]Department of Oral Microbiology, Institute of Odontology, Sahlgrenska Academy at Gothenburg University, Gothenburg, Sweden

[d]Department of Oral & Maxillofacial Pathology, School of Clinical Dentistry, University of Sheffield, Sheffield, UK

This manuscript was submitted in Arch Oral Biol.

Abstract

The ability of bacteria to grow and produce acid at the tooth surface is an important cariogenic factor. Our previous study in Thai preschool children indicated that certain species of *Lactobacillus* may play a more important role in caries development than others. The aim of the present study was to analyze the acid producing and growth abilities of 39 oral clinical strains and type strains of *Lactobacillus*, representing 9 species including *L. casei/paracasei*, *L. fermentum*, *L. gasseri*, *L. mucosae*, *L. oris*, *L. plantarum*, *L. rhamnosus*, *L. salivarius* and *L. vaginalis*. Overnight, anaerobically grown bacterial cells were inoculated in MRS broth containing 2% glucose at pH 7. Acid production and growth were measured at 0, 1.5, 3, 5, 7 and 24 h. Most *Lactobacillus* species were able to lower the pH below the critical pH for tooth demineralization (pH 5.5) within 7 h. *L. salivarius*, *L. rhamnosus*, *L. casei/paracasei* and *L. plantarum* grew more rapidly and reached an optical density higher than other species and they produced more acid than the others. *L. vaginalis* showed the lowest rate of growth and acid production. These findings showed that all the strains were acidogenic but could be categorized into three groups, strong, moderate and weakly acidogenic. Four species, *L. salivarius*, *L. rhamnosus*, *L. casei/paracasei* and *L. plantarum*, were the strongest acid producers and suggests that they may play a more important role in caries development than the others.

Key words: Acid production, growth, oral, *Lactobacillus*

Introduction

Lactobacillus species are a large group of gram-positive, facultative and anaerobic bacteria which are acid-producing and are widely acknowledged as being cariogenic pathogens [Tanzer et al., 2001; Teanpaisan et al., 2007; van Houte, 1994]. Our previous study of *Lactobacillus* species in young children also indicated the association between the oral lactobacilli and dental caries and although a variety of *Lactobacillus* species were isolated from the subjects, only *L. salivarius* was found to be more associated with caries than other lactobacilli [Piwat et al., 2010]. However, it is not known whether this species is truly more cariogenic than other species of lactobacilli.

Caries develops as a result of imbalance between de- and remineralization of enamel and dentine and the critical pH for enamel to be demineralized is considered to be approximately 5.5 [Larsen and Pearce, 1997]. Acidogenesis is one of the two most important virulence factors that differentiate the more cariogenic microorganisms from those that are less cariogenic and lactobacilli can generate the lowest pH from fermentable carbohydrates [Badet et al., 2001; Klinke et al., 2009; Matsuyama et al., 2003; Takahashi and Nyvad, 2008]. Moreover, increased caries progression is associated with increased proportions of organisms with higher rates of acid production and greater ability to metabolise and grow at low pH (aciduricity) [Bowden and Hamilton, 1998; van Houte et al., 1996]. Lactobacilli can metabolize many different sugars; including glucose, sucrose, lactose, sorbitol and xylitol, to lactic acid [Haukioja et al., 2008] and they are aciduric [Badet et al., 2001,. However, in similarity to non-mutans streptococci, lactobacilli show heterogeneity in acidogenicity between species and strains [de Soet et al., 2000; Klinke et al., 2009; Matsuyama et al., 2003]. Although, several studies have reported on the acid production ability of *Lactobacillus* species, not many species of oral lactobacilli were included [Klinke et al., 2009; Matsuyama et al., 2003; Moynihan et al., 1998]. Consequently, the aim of the present study was to analyze the growth and acid producing capabilities of oral lactobacilli with particular focus on clinical isolates obtained from preschool children in Thailand.

Materials and Methods

Bacterial Strains and Culture Conditions

A total of 39 *Lactobacillus* strains, 29 clinical isolates and 10 type strains were examined. The number of tested strains, the designation of type strains and clinical association of the *Lactobacillus* strains are shown in Table 1. Each tested strain was isolated from a different child to avoid a possible clonal relationship between strains and were chosen at random from our collection. The details of isolation and identification of the clinical *Lactobacillus* strains have been described in a previous study [Piwat et al., 2010]. Briefly, strains were isolated from saliva of children on Rogosa SL agar and identified according to 16S-rRNA gene profiles by restriction fragment length polymorphism analysis (PCR-RFLP) and protein profiles by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [Teanpaisan and Dahlén, 2006]. The strains were stored at -80^{o}C until used.

Initially strains were grown as starter cultures anaerobically (80% N2, 10% H2, and 10% CO2) in filter sterilized (pore size 0.22 μm, Nalgene, NY, USA) de Man, Rogosa and Sharpe (MRS) broth (Lab M, Bury, UK) at 37oC for 16-18 h, which brought them into exponential growth phase. From these, cells were harvested by centrifugation at 3000 rpm for 5 min at 4oC, washed twice in phosphate buffered saline (PBS; Oxoid, Basingstoke, UK) and inoculated into fresh, pre-warmed MRS broth (50ml) containing 2% (w/v) glucose, pH 7.0, to give an optical density of 1.0 at 650 nm using a spectrophotometer (Pharmacia Ltd, UK). Cultures were then incubated in an anaerobic chamber (miniMacs Anaerobic Workstation, Don Whitley Scientific Ltd, UK) for 24 h.

Measurement of cell growth and pH

Two milliliters of each culture was removed and measured for growth and pH at the start (0) and at 1.5, 3, 5, 7 and 24 h after inoculation. Bacterial growth was monitored by measuring the OD of cultures at 650 nm (OD650). The growth of each strain was expressed as the growth rate constant which was determined from the slope of a logarithmic line of best fit through the data points for the exponential phase of growth of the culture according to the formula:-

growth rate constant = (ln OD2 - ln OD1)/ t2-t1,

where OD1 and OD2 are OD value at time point 1 (t1) and time point 2 (t2), respectively.

In each case purity and viability of each *Lactobacillus* strain was assessed at the final sampling time (24 h) by plate counting on MRS agar anaerobically for 48 h.

Acid production was studied by recording pH change using a pH electrode and meter (Hanna pH 211, Hanna Instrument, UK) during the incubation period. The rate of acidification by each strain (acid production rate) was determined from the change in pH (δpH) divided by the OD650 per hour at the logarithmic growth period. The overall acidogenicity of each stain was expressed as the "pH area", which is the integration of the area bounded by the pH curve and the line of pH 7, as described by [Moynihan et al., 1998]. The "pH area" indicates, therefore, how much the medium was acidified by the bacteria within a certain period of time and was calculated using ImageJ software. Also, the final pH reached was recorded for each strain. Each strain was tested twice, using separately grown cultures. All measurements were performed in triplicate.

Statistical Analysis

The average value of each parameter is presented as mean $\pm$ standard error (SE). The correlation between growth and pH change was assessed using Pearson's correlation coefficient at the significant level $p<0.01$. The analyses were performed with the SPSS statistical program (SSPS Inc., Chicago, IL, USA).

Results

The growth and acid production from glucose among oral lactobacilli varied between species (fig. 1 and table 2). *L. salivarius*, *L. rhamnosus* and *L. plantarum* grew more rapidly than the other species, having growth rate constants of 0.21, 0.19 and 0.18 h^{-1}, respectively. They reached stationary phase by the 5^{th} hour and reached a higher optical density than the other species (fig. 1a). *L. gasseri*, *L. oris* and *L. vaginalis* were the slowest growers in our system, with growth rate constants of 0.10, 0.08 and 0.08 h^{-1}, respectively.

The rate at which the *Lactobacillus* species acidified their growth medium was consistent with their growth rate (fig. 1). There were positive and statistically significant correlation between the growth and the pH decrease among the *Lactobacillus* strains (Pearson correlation coefficients r = 0.86-0.999, all significant at $p<0.01$). As expected the highest rate of acid production occurred during the period of most rapid growth, which was within 1.5-3 h for most species, although *L. gasseri* and *L. vaginalis* strains took longer to start growing quickly (fig. 1). Table 2 shows the acid production characteristics of the *Lactobacillus* species studied and there were marked differences in acidogenicity among them. *L. rhamnosus*, *L. salivarius*, *L. casei/paracasei* and *L. plantarum* showed the highest acid production rate at the logarithmic growth period. They were able to drop the pH to the critical pH (5.5) within 2.2-3 h and reach a lower minimal pH sooner than the other species. The "pH area" was greater for these species and was particularly different after 24 h incubation (fig. 2) and thus they have been termed the "strong acidogenic" group. Also, these species were still viable after 24 h culture, even when the pH had dropped as low as 3.9. Although the species other than the strong acidogenic group also dropped the pH below the critical pH, the rate of acid production was always lower and the time to reach the critical pH was markedly extended. From the overall data, these bacterial species can be divided to the other two groups; a moderate acidogenic group i.e. *L. fermentum*, *L. gasseri*, *L. mucosae* and *L. oris*, and a weakly acidogenic group i.e. *L. vaginalis*.

Discussion

A positive correlation between the growth and the pH decrease of the *Lactobacillus* strains was found in general, although the rate of growth and acid production of each species differed. The ability to grow and to produce acid at a fast rate, in addition to their aciduric properties provides an environmental advantage for cariogenic bacteria when excess sugar is presented [Bradshaw and Marsh, 1998; Takahashi and Nyvad, 2008]. Lactobacilli are one of the major bacterial groups thought to be involved in the progression of caries [Badet and Thebaud, 2008] and high counts of lactobacilli often indicate a high sugar consumption [Bowden, 1997]. This is probably due to the fact that the majority of the *Lactobacillus* species are able to derive almost all of their energy from the homolactic fermentation of glucose, with 85-90% of the sugar utilized being converted to lactic acid [Carlsson, 1989]. In this study the majority of strains of lactobacilli were able to metabolize glucose to generate a final pH below 5.5, the critical pH for the demineralization of enamel and dentin. However, the group of "strong acidogenic" species, (*L. salivarius, L. rhamnosus, L. casei/paracasei* and *L. plantarum*), were able to reach pH 5.5 within only 2.2-3 h (mean 2.64 h) compared with a mean of 4.7 h for the "moderate acidogenic group (*L.fermentum, L.gasseri, L.mucosa* and *L.oris*). Moreover, the strong acidogenic group grew faster than the other species in the culture system used here and it is tempting to speculate that they may also grow to a higher proportion in plaque.

Indeed our earlier study showed that one of the "strong acidogenic" group, *L. salivarius*, is the predominant species associated with caries in Thai children [Piwat et al., 2010]. The present findings further support a role for *L. salivarius* as a cariogenic pathogen and is in accordance with findings of Pham et al.[2009], which showed that *L. salivarius* could significantly lower the pH and change the community profiles of oral biofilms in the presence of sucrose. The other members of the "strong acidogenic" group, (*L. rhamnosus, L. casei/paracasei* and *L. plantarum*) have also been associated with caries, even if the predominant species among various populations differed [Badet and Thebaud, 2008; Marchant et al., 2001; Nancy and Dorignac, 1992]. Cariogenicity in vivo is a product of several factors, including ability to colonize and compete with other microorganisms as well as acidogenicity and aciduricity [Bowden and Hamilton, 1998]. A study of the cariogencity of individual strains will be required to confirm the importance of the "strong acidogenicity" group of lactobacilli.

In our previous study, the "moderate acidogenic" species, *L. fermentum*, was commonly isolated in both the low- and moderate to high-caries groups, while the other species in this group were found less frequently (*L. gasseri, L. oris* and *L. vaginalis*) [Piwat et al., 2010]. Although in this study *L. fermentum, L. gasseri, L. oris* and *L. vaginalis* were not able to present a great acidic challenge, they were nonetheless able to drop the final pH to below the critical pH and so may contribute to the caries process. However, the presences of the strains with high acidogenicity are presumably more likely to be important in higher rates of caries progression than strains with lower acidogenicity.

Variation in acidogenicity among strains of the same species was also noticeable, particularly during the first 7h of incubation (Table 2, Fig. 2). It is not unexpected that we found variation within the *L. casei/paracasei* group, since these two species may possess the different acidogenicity but we were not able to differentiate *L. casei* from *L. paracasei* using our identification scheme. However, others have reported similar strain-to-strain variation in growth and ability to produce acid among strains of other bacteria e.g. *S. sanguinis* and *Actinomyces* spp [van Houte et al., 1996]. The importance of this is not clear at this stage, but it may also reflect strain-to-strain variation in cariogenicity. Khoo et al. [2005] reported that strains of mutans streptococci isolated from caries subjects were more acidogenic than those isolated from caries-free subjects. In this study, it seemed that species identity related more strongly to acidogenicity than to caries status of the individual from whom the strain was isolated. However, unfortunately, the study reported here contained too few caries-free subjects to be able to draw statistically valid conclusions about any relationship between caries status and relative acidogenicity of the *Lactobacillus* isolates. The study in the future will elucidate the unclear point mentioned.

In conclusion, this study has demonstrated that oral *Lactobacillus* species can be categorized into three groups according to their acidogenicity (strong, moderate and weak). It is speculated that the strong acidogenic species may present a greater acidogenic challenge in vivo and so contribute to a faster caries progression rate than the less acidogenic species.

Acknowledgements

This study was financially supported by The Thailand Research Fund (TRF, PHD/0091/2549) and The Swedish Research Council (Grant 348-2007-6650).

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Legends:

Table 1. Distribution of *Lactobacillus* strains (total = 39) evaluated in the study, the designation of type strains and the number of selected clinical strains with caries score

Table 2. Growth and acid production characteristics by *Lactobacillus* species

Fig. 1. Growth and acidification of *Lactobacillus* species after 3-, 7- and 24-h incubation in MRS broth containing 2% glucose. (a) Bacterial growth shown as the increase of OD650 from the initial at OD650 = 1.0. (b) Decrease of pH from initial (0h) of pH 7. Number of strains tested is in parenthesis.

Fig. 2. Acidogenicity of *Lactobacillus* species expressed by the area bounded by the pH curve and a horizontal line of pH 7 (pH area) after 7-h (a) and 24-h (b) incubation period. Note the dissimilar y-axis.

Table 1. Distribution of *Lactobacillus* strains (total = 39) evaluated in the study, the designation of type strains and the number of selected clinical strains with caries score

Species	Number of tested strains	*Lactobacillus* strains			
		Type strains	Number of clinical strains from children with		
			caries free	dt 2 to7	dt >10
L. casei / paracasei	9	*L. casei* ATCC 393, *L. paracasei* CCUG 32212	2	2	3
L. fermentum	4	*L. fermentum* ATCC 14931	1	0	2
L. gasseri	3	*L. gasseri* ATCC 33323	0	1	1
L. mucosae	5	*L. mucosae* CCUG 43179	0	1	3
L. oris	4	*L. oris* CCUG 37396	0	1	2
L. plantarum	4	*L. plantarum* ATCC 14917	0	1	2
L. rhamnosus	4	*L. rhamnosus* ATCC 7469	1	2	0
L. salivarius	4	*L. salivarius* ATCC 11741	0	1	2
L. vaginalis	2	*L. vaginalis* CCUG 31452	0	1	0

dt = Number of decayed teeth

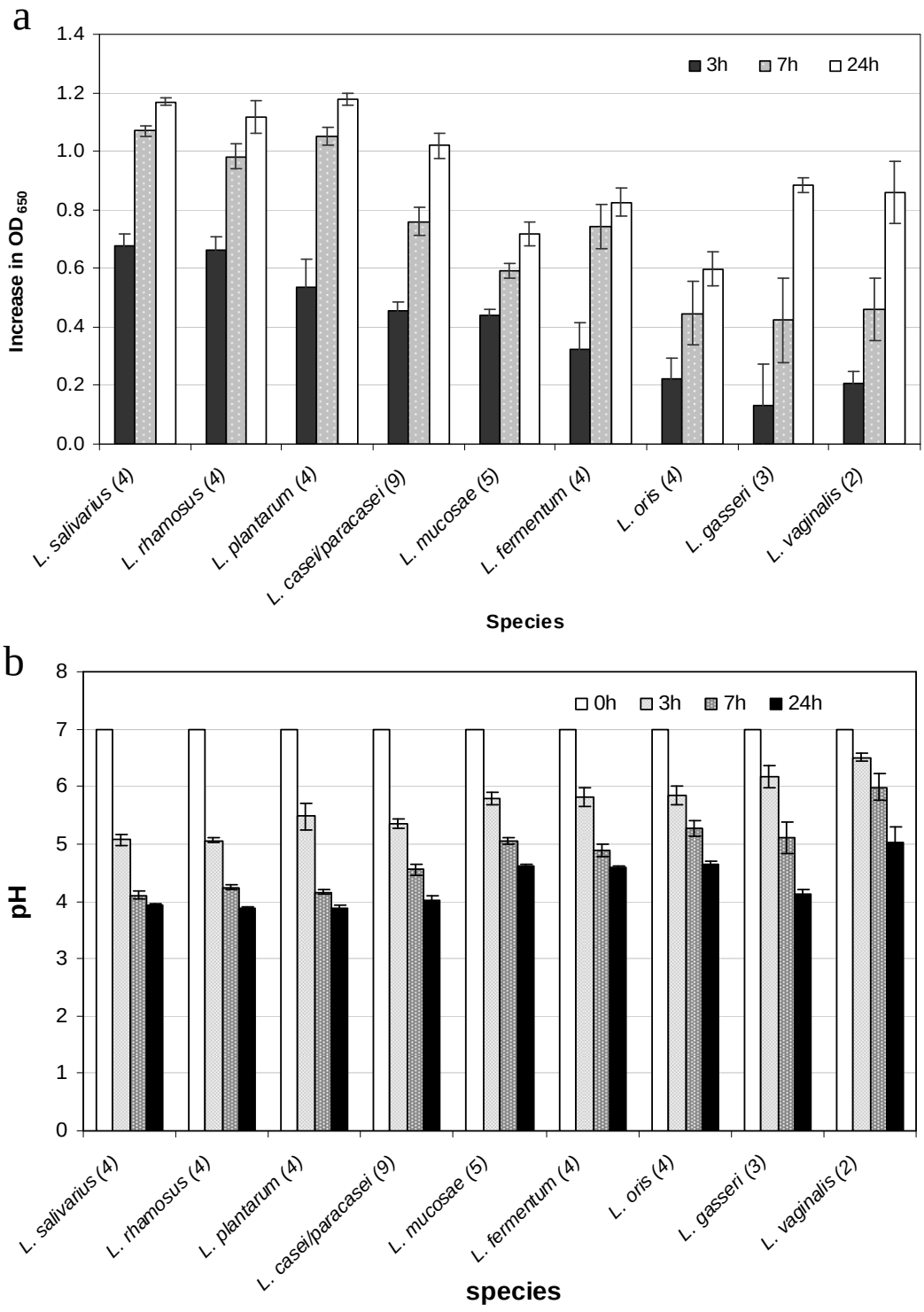

Fig. 1. Growth and acidification of *Lactobacillus* species after 3-, 7- and 24-h incubation in MRS broth containing 2% glucose. **(a)** Bacterial growth shown as the increase of OD_{650} from the initial at OD_{650} = 1.0. **(b)** Decrease of pH from initial (0h) of pH 7. Number of strains tested is in parenthesis.

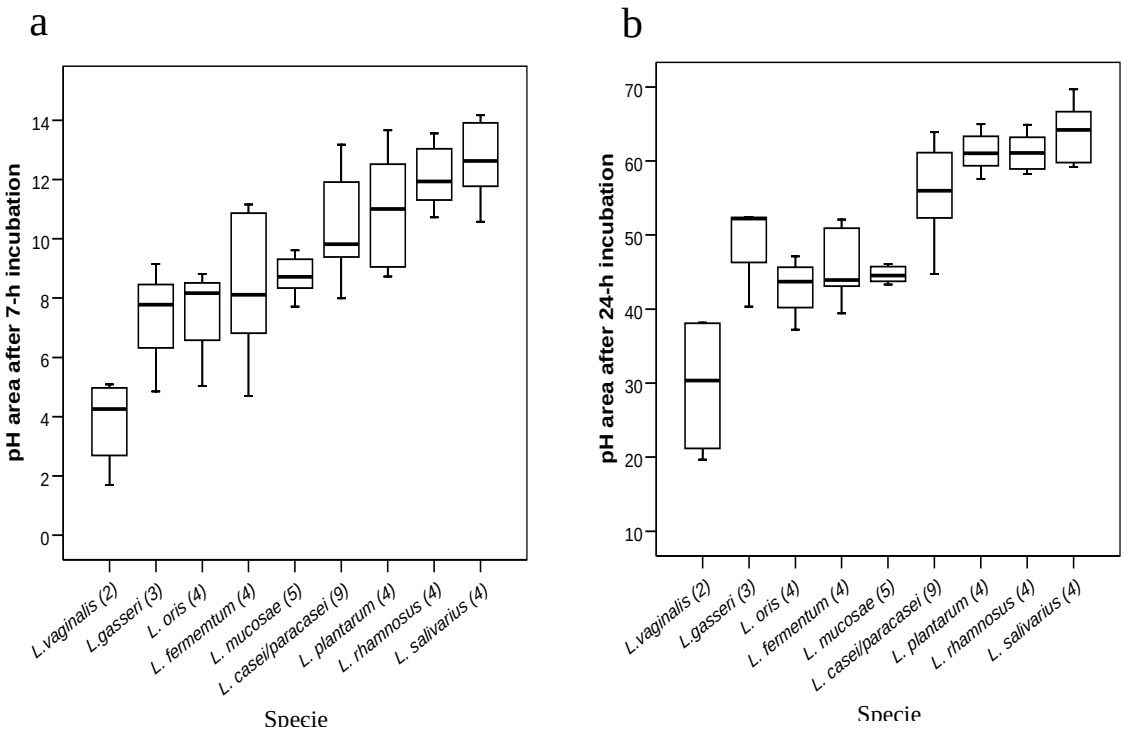

Fig. 2. Acidogenicity of *Lactobacillus* species expressed by the area bounded by the pH curve and a horizontal line of pH 7 (pH area) after 7-h (**a**) and 24-h (**b**) incubation period. Note the dissimilar y-axis and the number of strains tested is in parenthesis.

Table 2. Growth and acid production characteristics by *Lactobacillus* species

Lactobacillus species (n)	Growth characteristics		Acid production characteristics		
	Growth rate constant* (h^{-1}) ± SE	Max OD increase ± SE	Acid production rate# (OD^{-1} h^{-1}) ± SE	Time to pH 5.5 (h) ± SE	Final pH at 24-h ± SE
L. casei/paracasei (9)	0.14 ± 0.01	1.02 ± 0.04	0.41 ± 0.02	2.87 ± 0.18	4.02 ± 0.06
L. fermentum (4)	0.12 ± 0.02	0.82 ± 0.05	0.34 ± 0.05	4.18 ± 0.47	4.58 ± 0.02
L. gasseri (3)	0.10 ± 0.02	0.88 ± 0.03	0.27 ± 0.04	5.70 ± 1.40	4.13 ± 0.08
L. mucosae (5)	0.15 ± 0.01	0.72 ± 0.04	0.40 ± 0.03	4.10 ± 0.27	4.62 ± 0.03
L. oris (4)	0.08 ± 0.02	0.60 ± 0.06	0.37 ± 0.04	5.50 ± 1.35	4.65 ± 0.04
L. plantarum (4)	0.18 ± 0.01	1.18 ± 0.02	0.39 ± 0.05	2.98 ± 0.34	3.89 ± 0.03
L. rhamosus (4)	0.19 ± 0.02	1.12 ± 0.05	0.47 ± 0.02	2.27 ± 0.06	3.89 ± 0.02
L. salivarius (4)	0.21 ± 0.01	1.17 ± 0.01	0.42 ± 0.03	2.45 ± 0.12	3.92 ± 0.04
L. vaginalis (2)	0.08 ± 0.01	0.86 ± 0.11	0.18 ± 0.02	16.39 ± 4.40	5.02 ± 0.28

* $(\ln OD_2 - \ln OD_1)/ t_2 - t_1$

δpH / OD/ h

Appendix C: Paper III

Longitudinal study of the presence of mutans streptococci and lactobacilli in relation to dental caries development in 3-24 month old Thai children

Teanpaisan R, Thitasomakul S, Piwat S, Thearmontree A, Pithpornchaiyakul W, Chankanka O.

Prince of Songkla University, Hatyai, Songkhla, Thailand

This manuscript was published in Int Dent J 2007; 57(6):445-451.

International Dental Journal (2007) 57, 445-451

Longitudinal study of the presence of mutans streptococci and lactobacilli in relation to dental caries development in 3-24 month old Thai children

R Teanpaisan, S Thitasomakul, S Piwat, A Thearmontree, W Pithpornchaiyakul and O Chankanka
Songkhla, Thailand

Objective: To study the acquisition of mutans streptococci (MS) and lactobacilli in relation to dental caries development in 3-24 month old Thai children. **Methods:** Salivary samples were collected from 169 children using sterile wooden tongue depressors at the ages of 3, 9, 12, 18 and 24 months. The blades were pressed onto selective media for MS or lactobacilli. After incubation, the colony forming units of MS/lactobacilli were counted. Dental status was recorded from 9 months old using modified WHO criteria. **Results:** The number of children with caries and number and severity of decayed teeth significantly increased with age. The presence of MS/lactobacilli was detectable at an early age and the cumulative prevalence of MS/lactobacilli increased with age. Children who were colonised early by MS or lactobacilli showed a higher number of decayed teeth than of those who were colonised later. The children with no MS/lactobacilli had significantly fewer decayed teeth and there was a significant correlation between MS/lactobacilli level and tooth decay. **Conclusions:** This longitudinal study shows early colonisation of the mouths of Thai children by MS/lactobacilli and where there are persistently high levels of the bacterias increased risk of development of dental caries.

Key words: Mutans streptococci/lactobacilli, dental caries, young children

Mutans strepcocci (MS) are considered to be causative agents of dental caries[1,2]. The degree of colonisation by these organisms correlates with the prevalence of dental caries in children and experimental animals[3,4]. Moreover, MS are found to be more closely associated with the initiation of caries than any other organisms[1,5].

Unlike MS, lactobacilli have been claimed to be related to the progression of caries and also to be an indicator of the content of fermentable carbohydrates in the diet[6]. Positive correlations have been shown to exist between lactobacilli counts and caries prevalence and activity[7-9].

Based on such findings, MS and lactobacilli have been recommended and used as predictors of caries in an attempt to identify individuals with a high risk of acquiring the disease. The time of MS/lactobacilli acquisition may also be important since caries risk is found to be dependent on early colonisation of organisms[10]. Some studies have found MS in children at or before their first birthday[11-13], while others report later acquisition[14,15]. However, similar information on lactobacilli is sparse.

Thailand has a high and an increasing prevalence of dental caries among young children; however, there are relatively few data on the time of acquisition of MS/lactobacilli. Therefore, the objective for the present study was to determine the basal MS/lactobacilli carriage among Thai children age 3-24 months, and the

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relationship of time of acquisition and level of colonisation to dental caries development.

Material and Methods

Subjects

The present study was a longitudinal, observational, community-based study. It was designed in association with the national Prospective Cohort Study of Thai Children (PCTC), which aims to follow infants from birth to 24 years of age. The study site, Thepa district, Songkhla province has a population of 66,990. Of its seven sub-districts, six could be categorised as rural, and the other sub-district is urban, although 90% of the population live in the rural areas. A total of 1,076 children born between November 2000 and October 2001 were selected as the study population. For dental examination, 795 children were recruited by cluster sampling, which covered all seven sub-districts and among these, 25% (198 children) were randomly selected for bacterial study. Details of the study site and the recruitment of the study sample have been published elsewhere[16]. This study was approved by the National Ethical Committee, at the Ministry of Public Health. All eligible guardians of the infants in the study area were invited to join the PCTC and provide their consent for the infants to participate in a series of assessments including the oral health study.

Oral examination

The dental examination started at the age of 9 months with further examinations at 12, 18, and 24 months. The clinical examination of dental status was conducted by five dentists using standardised methods at health centres or hospital depending on convenience for the subjects' carers. The range of the Cohen's Kappa coefficients of intra-examiner calibration ranged from 0.75 and 0.91 and the inter-examiners' coefficients ranged from 0.68 to 0.89. The clinical examinations were carried out using a WHO probe (#621) under natural light, and a scoring system adapted from the WHO's criteria, 1997. All teeth present were examined for dental status using this coding system as follows:

d_1 = initial decay/decay limited in enamel; the lesion demonstrates whitish/yellowish opaque with/without micro-cavity but no softened floor/wall

d_2 = decay to dentine; cavitated lesion is seen to extend beyond enamel that certainly catches the probe with softened floor/wall of determined enamel

d_3 = decay involving pulp; a deep lesion with probable pulpal involvement or deep lesion with present/history of spontaneous pain/fistula opening.

Bacteriological examination

Salivary samples from each child were collected by inserting a sterile wooden tongue depressor into the oral cavity until the blade was visibly moistened. The blade was then pressed immediately onto contact petri dishes that contained Mitis Salivarius Bacitracin agar[17] and Rogosa agar[9], which are selective media for MS and lactobacilli, respectively. The plates were incubated in an anaerobic jar at 37°C for 72h, after which the number of colony forming units (CFU) of MS and lactobacilli within the impression were determined based on their typical morphological appearance under a microscope[9,17].

Data analysis

The data of dental status is described as prevalence of dental caries (number and percentage of children with decayed teeth, mean and sd of decayed teeth), number of erupted teeth and severity of decay (d_1, d_2 and d_3). The prevalence of bacterial presence is presented as cumulative prevalence according to the age of the children at examination. The count of MS and lactobacilli was used as the main independent variable. The presence of 1CFU/1.5cm^2 was taken as positive presence of bacteria. The number of MS and lactobacilli were categorised as: 0CFU/1.5cm^2, 1-50CFU/1.5cm^2, and >50CFU/1.5cm^2. The mean age of first detectable acquisition of each organism was used as a cut-off point to dichotomise the children as either early or late colonisation. The Mann-Whitney-U test was used to determine the association of the mean number of decayed teeth with early or late bacterial colonisation. All related variables were further used for logistic regression analysis, the odds ratios and their 95% confidence interval were calculated. During the logistic regression analysis the dependent variables, number of decayed surfaces, was dichotomised at different cut-off points according to the prevalence of decayed surfaces and age. At the age of 9 months the presence of $\geq$ 1 decayed surface was counted as belonging to the decayed group while decay-free children were set as the reference group. At the age of 12 months the decayed group was defined as children with $\geq$ 2 decayed surfaces, while at 18 and 24 months of age, $\geq$ 4 decayed surfaces defined the decayed group. The low decay-group, children with < 2 decayed surfaces at the age of 12 months and children with < 4 decayed surfaces at the age of 18 and 24 months, was set as a reference group. A critical level of significance of all analyses was set at $p<0.05$. The analyses were carried out using SPSS statistical software (SPSS, release 10.0 for Windows)

Results

Of 198 recruited children, 29 (14.6%) failed to attend because either the family moved out of the study area or because the children were uncooperative. Of the 169 children who participated in the bacterial study, 52 children completed 5 salivary examinations whereas 62, 36, 12 and 7 children had 4, 3, 2, and 1 salivary examinations, respectively.

The prevalence of decayed teeth at different age intervals is shown in *Table 1*. The number of children with caries and the number and severity of decayed teeth significantly increased with age.

The cumulative prevalence of MS/lactobacilli detection is shown in *Figure 1*. By the age of 24 months, 86.98% and 66.86% of children harboured MS and lactobacilli, respectively. The temporal pattern of colonisation by MS differed from that of the lactobacilli. The lactobacilli were found more frequently during the first year of age than MS. The frequency of detection of lactobacilli was higher than MS from base line to 9 months of age but MS was detected with a higher frequency between 18-24 months.

Of the 169 children studied, MS was detected in 147 and lactobacilli in 113. The mean ages of first detection were 16.74±6.71 months and 13.88±8.43 months

Table 1 Dental caries status of children at different ages

Age in months	Mean number of erupted teeth	Number (%) of children		Mean ± SD of decayed teeth (surfaces):			
		without decayed teeth	with decayed teeth	Total	d_1	d_2	d_3
9	2.55±2.24	136 (95.8)	6 (4.2)	0.14±0.70 (0.20±1.05)	0.13±0.64 (0.18±0.98)	0.01±0.17 (0.01±0.17)	0.00±0.00 (0.00±0.00)
12	5.99±2.70	94 (70.1)	40 (29.9)	1.05±1.84 (1.57±3.38)	0.90±1.54 (1.26±2.32)	0.15±0.64 (0.31±1.71)	0.00±0.00 (0.00±0.00)
18	10.56±3.66	55 (36.9)	94 (63.1)	2.81±3.09 (5.34±7.16)	1.89±2.16 (3.36±4.24)	0.87±1.68 (1.81±4.17)	0.05±0.29 (0.17±1.06)
24	16.80±2.14	24 (15.5)	131 (84.5)	5.35±4.34 (10.89±10.42)	2.65±2.37 (4.35±4.19)	2.43±2.67 (5.52±6.73)	0.26±0.91 (1.01±3.50)

d_1 = initial decay/decay limited in enamel
d_2 = decay to dentine
d_3 = decay involving pulp

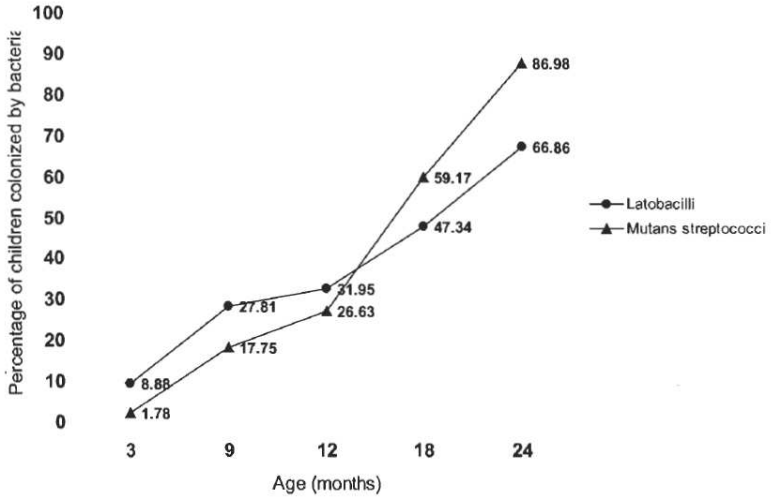

Figure 1. Cumulative prevalence of MS/lactobacilli at different ages

for MS and lactobacilli, respectively. Children who had detectable MS/lactobacilli before the population mean age of when MS/lactobacilli were detected had a higher number of decayed teeth at all ages, which was statistically significant by 24-month of age (*Table 2*). Moreover, children who harboured both MS and lactobacilli tended to have higher numbers of decayed teeth, while children who had none or only one of the two had a significantly lower number of decayed teeth at age of 12, 18, 24 months (*Table 3*).

When the main variables were analysed at once (*Table 4*), MS was found to be the factor significantly associated with caries at ages of 12, 18, 24 months. The odds ratios of developing caries at these ages when having MS > 50CFU/1.5cm^2 were 13.0, 8.8 and 7.5, respectively. The lactobacilli count was associated with the number of decayed teeth only at the age of 24 months and children who harboured lactobacilli counts of 1-50 CFU/1.5 cm^2 and of > 50 CFU/1.5 cm^2 had significantly higher risk of developing caries (odds ratios of 3.1 and 13.3, respectively).

Discussion

Longitudinal data on the development of the oral microflora in young children are still scarce. This study based on a community cohort focuses on the time for MS and lactobacilli colonisation in relation to dental caries development in 3-24 month old Thai children. Dental caries related to socio-economic factors in the same group of children has been reported separately[16].

In this study the rate of caries development was extremely high in comparison to other studies performed with 24-month old children. In our study's caries was detected soon after first tooth eruption at 8 months of age and more decayed teeth were found the older the children became. For example, 4.2% of 9 month old children had a mean total caries score of 0.14±0.7, whereas 84.5% of children at 24 months old had a mean total caries score of 5.35±4.34 and most of these were in categories of d1 and d2. In the study of Roeters *et al.*[18] only 5.9% of children had the mean dmfs of 0.14 ± 0.72, and in another study 26.3% of Japanese children showed a mean dmft of 3.19 ± 0.77 [19].

The number of children with detectable MS entering this investigation was also higher than previous studies[18,19]. However, the result of the present study is consistent with earlier reports which have indicated the prevalence of MS colonisation increases with age and number of teeth[11,15,19-21]. Most studies have reported that MS first appears in the mouth at the time of tooth eruption, explained by the assumption that MS require non-shedding surfaces for colonisation[22,23]. However, our findings would suggest that MS present prior to tooth eruption in 4% of Thai children. This is in keeping with other studies that have noted the presence of MS in predentate infants, although on small numbers of children[24-26]. This could be explained by the find-

Table 2 Decayed teeth among early- and late- detection of MS/lactobacilli at different ages

Age in months	Mean ±SD of decayed surfaces of children (number) whom found MS			Mean ±SD of decayed surfaces of children (number) whom found lactobacilli		
	≤ 16 m	> 16 m	p-value	≤ 13 m	> 13 m	p-value
9	0.31±1.28 (52)	0.15±0.94 (81)	0.408	0.38±1.48 (52)	0.10±0.70 (81)	0.198
12	2.00±3.19 (49)	1.26±3.57 (74)	0.241	2.21±4.71 (43)	1.17±2.43 (82)	0.181
18	6.53±8.67 (47)	4.63±6.19 (89)	0.186	6.87±9.44 (47)	4.38±5.50 (90)	0.100
24	13.90±12.85 (48)	9.24±8.38 (91)	0.026	13.08±12.36 (50)	9.47±8.86 (89)	0.048

Table 3 Mean of decayed teeth and presence of MS or lactobacilli at different ages

Age in months	Mean ±SD of decayed surfaces and children (number) who presented of			
	MS only	lactobacilli only	both MS/lactobacilli	no MS/lactobacilli
9	0.13±0.50 (16)	0.33±1.41 (18)	0.00±0.00 (6)	0.05±0.45 (78)
12	2.50±4.20 (14)	1.71±3.73 (7)	2.33±2.07 (6)	0.75±7.95[a] (51)
18	6.00±2.49 (48)	4.14±5.49 (7)	9.50±3.91 (28)	2.51±4.16[b] (49)
24	9.05±8.89 (65)	9.10±0.50 (10)	16.41±10.99[d] (56)	3.05±4.67[c] (10)

a, statistically significant difference of no MS/lactobacilli group to MS only group at 12 months
b, statistically significant difference of no MS/lactobacilli group to both MS/lactobacilli and to MS only groups at 18 months
c, statistically significant difference of no MS/lactobacilli to both MS/lactobacilli, to MS only and to lactobacilli only groups at 24 months
d, statistically significant difference of both MS/lactobacilli to MS only and to lactobacilli only groups at 24 months

Table 4 Logistic regression analyses, odds ratios (95%CI), on risk of getting decay* by MS count, lactobacilli count and the age of bacterial colonisation

Age in months	Variable		n	β	Odds ratios (95%CI)
9	Lactobacilli	0	86		
	(CFU/1.5cm²)	1-50	26	0.87	2.39 (0.20-28.41)
		> 50	5	-6.42	0.002 (0.00-9.9E+49)
	MS	0	93		
	(CFU/1.5cm²)	1-50	18	0.39	1.49 (0.13-17491)
		> 50	7	-6.36	0.002 (0.00-5.9E+59)
12	Lactobacilli	0	58		
	(CFU/1.5cm²)	1-50	7	0.65	1.92 (0.41 - 8.94)
		> 50	13	7.67	2148.16 (0.00 - 3.44E+46)
	MS	0	63		
	(CFU/1.5cm²)	1-50	12	-13.92	0.00 (0.00 - 1.37E+56)
		> 50	78	2.57	13.01 (2.89 - 58.52)**
18	Colonisation age of lactobacilli	early (≤ 13 months)	47		
		late (> 13 months)	90	0.25	1.28 (0.52 - 3.14)
	Colonisation age of MS	early (≤ 16 months)	47		
		late (> 16 months)	89	0.29	1.34 (0.54 - 3.32)
	Lactobacilli	0	55		
	(CFU/1.5cm²)	1-50	27	0.49	1.63 (0.53 - 5.03)
		> 50	49	0.75	2.12 (0.49 - 9.19)
	MS	0	87		
	(CFU/1.5cm²)	1-50	31	0.15	1.16 (0.41 - 3.25)
		> 50	14	0.22	8.79 (3.1 - 24.77)**
24	Colonisation age of lactobacilli	early (≤ 13 months)	50		
		late (> 13 months)	89	-3.69	0.69 (0.25 - 1.91)
	Colonisation age of MS	early (≤ 16 months)	48		
		late (> 16 months)	91	-0.75	0.93 (0.37 - 2.34)
	Lactobacilli	0	25		
	(CFU/1.5cm²)	1-50	20	1.13	3.09 (1.27 - 7.54)**
		> 50	101	2.59	13.29 (1.53 - 115.31)**
	MS	0	73		
	(CFU/1.5cm²)	1-50	53	0.07	1.070 (0.28 - 4.12)
		> 50	24	2.01	7.46 (2.54 – 21.89)**

*, The cut-off points to dichotomise the dependent variable for age 9, 12, 18 and 24 months was 1, 2, 4 and 4 decayed surfaces, respectively (see text).
** $p<0.05$

ing that *Streptococcus mutans* has the ability to adhere to keratinised and non-keratinised human oral epithelial cells[27]. Therefore, the colonisation of MS can occur before tooth eruption.

Unlike studies on MS, cohort studies on the presence of lactobacilli in young children are sparse. Our study appears be the first to determine the presence of lactobacilli and the caries prevalence pattern in children in the age range 3-24 months, since previous studies have been restricted to children of 2 years or older[18,28]. It is generally quoted that lactobacilli are late colonisers of the mouth[14,29,30]. Lactobacilli are assumed to be preferentially recovered from carious lesions, although usually after those lesions were colonised by the MS[31,32]. Some data has suggested that lactobacilli colonisation may be favoured by prior colonisation by the MS. Indeed, lactobacilli seem to be rarely found alone and are often isolated together with MS from plaque and saliva[29]. In contrast, the present study showed that lactobacilli were found in up to 7.2% of the children at 3 months of age and 40% of those children had lactobacilli at a high level (>100 CFU, data not shown). Therefore, early acquisition of lactobacilli is possible. However, differences between reports of lactobacilli acquisition in infants may reflect true differences between the populations being studied or may reflect differences in sampling methods.

The early detection of lactobacilli in this study may be explained by the finding that maternal microflora may be transferred to the infant during childbirth and then subsequently survive in the oral cavity[33]. Those authors found that the oral cavity in 40% of the infants was colonised by *Lactobacillus* spp. already at birth. The number of the colonised infants declined to 2% during first two weeks, however, the number increased to 10% one month later. In another study using the genotyping method[34], it was reported that one-quarter of infants acquired vaginal lactobacilli from their mother at birth, but that the acquired lactobacilli do not remain for long and are replaced by other genotypes from milk or unknown sources after birth. Our findings are in agreement with those results, and showed that 7.2% of children harboured lactobacilli at an early age. An increasing rate of lactobacilli in a second period (18 to 24 months) may derive either from environmental sources or relate to caries progression.

It is not clear whether the bacterial species detected are part of the indigenous flora at any point in time or whether they are transient strains. It has been generally assumed that the persistence of bacterial strains in the mouth is associated with their capacity not only to adhere and grow, but also to withstand a range of stresses, such as the presence of the other competing bacteria and inhibiting substances. A certain strain can be more adapted, once it can cope with physical, chemical, biological and environmental challenges, to dominate and to become established permanently, while other strains are not suited to the host and thus form a transient population. In the past, the method of identifying strains of lactobacilli to species level was limited; therefore, little information is available concerning which species of lactobacilli dominate in the oral cavity. We have recently proposed a PCR method of 16S rRNA and SDS-PAGE to identify oral lactobacilli[35]. Further studies of species and genotyping of lactobacilli will allow us to understand the persistence and colonisation in early childhood.

Many reports have shown that dental caries is strongly associated with the number of salivary MS and lactobacilli[3,7-9,18,19], and suggested that the earlier the colonisation of *S. mutans*, the higher the caries risk[10]. The present findings strongly support this but extend the findings to include lactobacilli. Children who developed caries tended to acquire MS/lactobacilli at a high level and/or at an earlier age than those who had a low caries experience. Thus, caries risk was associated with the age and level of acquisition of MS/lactobacilli. Furthermore, our study has confirmed that the number of MS is the primary risk factor for caries initiation, while lactobacilli were more significant as carious lesions progress. It was found that MS was significantly associated with clinically detectable caries from 12 months old; whereas, lactobacilli were only of significance by the age of 24 months (*Table 4*). Of course, by at the age of 24 months more children had decayed teeth developed to involve dentine (*Table 1*). However, the effect of early- or late-bacterial colonisation was eliminated when the bacterial count was included into the model (*Table 4*). This showed that the bacterial count was a more important contributor to caries than the age of colonisation. Thus, to reduce caries risk, measures to control the bacterial number would be more effective than measures to delay the time of colonisation.

In conclusion, data from our longitudinal study have shown that the presence of MS/lactobacilli among Thai children was detectable at an early of age and that there was a positive association between salivary MS/lactobacilli and the prevalence and severity of dental caries. Acquisition of MS/lactobacilli at high level is a significant risk factor for caries development; and so appropriate preventive programmes are urgently needed for such high risk children.

Acknowledgements

This study was supported by the National Research Council of Thailand 2549, the Research Funding Agency, Prince of Songkla University (PSU), the Thailand Research Fund: MRG4780013 and Thai Health Promotion Foundation: 48-00-0286.

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Correspondence to: Dr Rawee Teanpaisan, Department of Stomatology, Faculty of Dentistry, Prince of Songkla University, Hat-yai, Songkhla, 90112, Thailand. Email: rawee.t@.psu.ac.th

Appendix D: Paper IV

A longitudinal study of early childhood caries in 9- to 18-month-old Thai infants

Thitasomakul S[1], Thearmontree A[1], Piwat S[1], Chankanka O[1], Pithpornchaiyakul W[1], Teanpaisan R[2], Madyusoh S[3].

[1]Department of Preventive Dentistry, Faculty of Dentistry, Prince of Songkla University, Hatyai, Songkhla, Thailand, [2]Department of Stomatology, Faculty of Dentistry, Prince of Songkla University, Hatyai, Songkhla, Thailand, [3]Dental Division, Thepa Hospital, Thepa, Songkhla, Thailand

This manuscript was published in Community Dent Oral Epidemiol 2006; 34(6):429-36.

Community Dent Oral Epidemiol 2006; 34: 429–436

Community Dentistry and Oral Epidemiology

A longitudinal study of early childhood caries in 9- to 18-month-old Thai infants

Songchai Thitasomakul[1], Angkana Thearmontree[1], Supatcharin Piwat[1], Oitip Chankanka[1], Wattana Pithpornchaiyakul[1], Rawee Teanpaisan[2] and Sorae Madyusoh[3]

[1]Department of Preventive Dentistry, Faculty of Dentistry, Prince of Songkla University, Hatyai, Songkhla, Thailand, [2]Department of Stomatology, Faculty of Dentistry, Prince of Songkla University, Hatyai, Songkhla, Thailand, [3]Dental Division, Thepa Hospital, Thepa, Songkhla, Thailand

Thitasomakul S, Thearmontree A, Piwat S, Chankanka O, Pithpornchaiyakul W, Teanpaisan R, Madyusoh S. A longitudinal study of early childhood caries in 9- to 18-month-old Thai infants. Community Dent Oral Epidemiol 2006; 34: 429–436. © Blackwell Munksgaard, 2006

Abstract – *Objective:* To examine the rate and pattern of early childhood caries (ECC) development and to investigate the transitional changes of the carious lesions during a follow-up period of 3–9 months. *Methods:* A longitudinal observational community-based survey of 599 children, 9–18 months old. The children's dental examinations were first carried out at the age of 9 months with re-examination at 12 and 18 months by five dentists using standardized methods. The affected rates of dental caries were determined for prevalence, incidence density for risk of caries per person (ID_p) and risk by surface (ID_s). Changes in dental status over time were explored from unerupted (U) to sound (S), including enamel caries (D1), dentine caries (D2) and caries involving pulp (D3) by computing transitional probabilities. *Results:* The prevalence of caries was 2.0%, 22.8% and 68.1% among 9-, 12- and 18-month olds, respectively. The ID_p observed for newly affected children 9–12 and 12–18 months old was 10.32 and 15.70 persons/100 person-months, respectively. The ID_s for children 9–12 months old was 2.17 newly affected surfaces/100 surface-months whereas it was 2.22 surfaces/100 surface-months for children 12–18 months old. The buccal surface of maxillary incisors was the most affected (44.9%) followed by lingual, mesial and distal surfaces, respectively. The transitional probability of caries progression ranged between 1.79% and 15.38% during the follow-up period from 9 to 12 months old. It was 3.43–39.60% from 12 to 18 months old. *Conclusions:* An extremely high caries-affected rate was found among the study children even before the age of 18 months. The buccal surface of the maxillary incisors was the most affected. The teeth acquired caries at 3–6 months after initial eruption and carious lesions developed continuously over time.

Key words: early childhood caries; epidemiology; incidence density; prevalence; Thailand; transitional probability

Songchai Thitasomakul, Department of Preventive Dentistry, Faculty of Dentistry, Prince of Songkhla University, Hatyai, Songkhla 90112, Thailand
Tel: +66 7428 7678
Fax: +66 7442 9883
e-mail: songchai.t@psu.ac.th

Submitted 21 June 2005;
accepted 7 October 2005

It is widely accepted that deciduous teeth are important, as they can influence the growth and development of children (1, 2). The most obvious oral disease among young children is dental caries, now known as early childhood caries (ECC) (3–5). The etiology of ECC is known to be multi-factorial (6–8). In developed countries, ECC is most prevalent among infants from deprived groups (9–11). The ECC development in children under 3 years of age could lead to long-term caries risk (12, 13). Among children with a high prevalence of ECC, especially in developing countries, ECC mostly develops early in life (14–16). Most of the previous studies of ECC development have been cross-sectional in design and restricted to a single observation of subjects to determine caries prevalence. Although they contained some descriptions of the incremental rate of caries development, the prevalence was calculated at different chronological ages, and the time-period of observation of each individual tooth was not included in the analyses. A longitudinal study is better able to follow the changes of dental caries progression, which addresses clearly understanding the rate of caries

progression of each individual tooth and the probability of transitional changes over time in order to find an appropriate access-time for ECC prevention. The aims of the present study were to examine the rate and pattern of ECC development and to investigate the transitional changes of the carious lesions during a follow-up period from 9 to 18 months of age.

Materials and methods

The study was a longitudinal observational community-based study. It was designed in association with the national child health research project, Prospective Cohort Study of Thai Children (PCTC). The PCTC intends to follow infants from birth to 24 years of age, and study changes in their physical and behavioral development. Among the five study regions participating in the PCTC, the present study was confined to a southern cohort, which is located in Thepa district of Songkhla province, Thailand.

Population and sample

The study site in Thepa district, Songkhla province is located about 1000 km south of Bangkok. It has a population of 66 990. Of its seven sub-districts, six could be categorized as rural. The other sub-district is urban. Ninety percent of the population lives in the rural areas. They are predominantly Muslims (68%), 32% are Buddhists. The average annual household income is 68 931 Baht (1681 US$). Forty-five percent of the population is employed in rubber plantations, rice growing and fishing. The remaining, 20% are employees of non-agricultural businesses, 19% are farmers working on their own land and 16% are merchants and government officers. The fluoride concentration of drinking water in this area is low, ranging from 0.1 to 0.2 ppm (17). Each sub-district is served by one to two of the total 11 health centers and a district hospital. Dental care is only available at the district hospital; all dental services are provided by two general dental practitioners and two dental nurses. A total of 1076 children born between November 2000 and October 2001 and registered at one of these health centers or at the district hospital were set as the study population. During the sampling process, seven health centers and the hospital were selected to cover all of the seven sub-districts. The 795 infants registered at these faculties were recruited. This study has been approved by the National Ethical Committee, at the Ministry of Public Health. All eligible guardians of the infants in the study area were invited to join the PCTC and provide their consent for the infants to participate in a series of assessments including the oral health study.

Clinical examination

Dental examination appointments were made for all eligible subjects at the ages of 9, 12, and 18 months old. A second appointment was arranged within 1 month if the first appointment was missed. As a result the children were examined at a ±1-month interval around the intended examination age. The examination took place at the health centers, the hospital or in the village depending on convenience for the subject's caretakers. The subjects were examined in a knee to knee position. The teeth were examined with a WHO probe (no. 621) under natural light using a scoring system adapted from the WHO's criteria, 1997. The dental status of each examined surface was categorized as:

U = Unerupted surface; no part of the surface emerges to the oral cavity.

S = Normal enamel surface/texture and no restoration.

D1 = Initial caries/caries limited in enamel; the lesion demonstrates whitish/yellowish opaque with/without micro-cavity but no softened floor/wall.

D2 = Caries to dentine; cavitated lesion is seen to extend beyond enamel that certainly catches the probe with softened floor/wall of undermined enamel.

D3 = Caries involving pulp; a deep lesion with probable pulpal involvement or deep lesion with present/history of spontaneous pain/swelling/fistula opening.

The dental status data were collected by five dentists using standardized methods. Prior to the data collection, all examiners and recorders participated in a meeting to discuss the process of data collection and to study the dental examination criteria. Later a practical standardization was carried out at the university daycare center. The standardization was performed at tooth surface level. The range of the Cohen's Kappa coefficients of overall intra-examiner standardization ranged from 0.75 and 0.91 and the overall inter-examiners coefficients ranged from 0.68 to 0.89. The Kappa coefficients of intra-examiner standardization, only in surfaces with S and D1, ranged from 0.66 to 0.79 whereas the inter-examiner coefficients fell

between 0.49 and 0.78. The level of reliability was maintained over the study period.

Statistical analysis

The data were checked for accuracy and entered in the computer using the SPSS® statistical program (SPSS Inc., Chicago, IL, USA). Frequency counts and cross-tabulations of the data were used to check for errors in data entry. Analysis first resulted in the reporting of descriptive statistics. The rates of dental caries were presented in prevalence, incidence density and transitional probability of carious lesions from one stage to another. The prevalence was the number of persons with carious teeth divided by the population at a specified time. The incidence was the number of new caries-affected teeth in a defined population, within a specified period of time. Incidence in the present study was calculated as incidence density for risk of caries of a person (ID_p) and of a tooth surface (ID_s) summarized by the formula below:

$$ID_p = \frac{\text{Number of new caries-affected subjects}}{\text{Total person} - \text{time at risk for having at least one caries lesion (person} - \text{month)}}$$

$$ID_s = \frac{\text{Number of new carious surfaces}}{\text{Total surface-time at risk (surface-month)}}$$

Person-time in ID_p and surface-time in ID_s was calculated by summing the observation-time of individuals/surfaces having no caries. For simplicity, we assumed that the teeth had a uniform hazard rate within each follow-up interval. For teeth that were present as sound at t_0 and t_1, the time at risk was $= t_1 - t_0$. For teeth which were absent at t_0, the time of eruption was assumed to be $(t_1 - t_0)/2$. For teeth that were present as sound at t_0 and became carious teeth at t_1, the onset of caries was assumed to take place at $(t_1 - t_0)/2$. Similarly if the teeth were absent at t_0 and became carious teeth at t_1, the eruption time was calculated to be at $(t_1 - t_0)/2$ whereas the onset of caries was assumed to be at $t_0 + 3/4(t_1 - t_0)$. In other words, the caries-free duration of these teeth was one-fourth of the interval. When a carious lesion was detected that person/surface was considered to be a new case for the period. It was then excluded from the at-risk status.

In addition to the ID analysis, we employed transitional probabilities computation dividing each tooth into five stages as unerupted tooth (U), sound tooth (S), enamel caries (D1), dentine caries (D2) and caries involving pulp (D3) in a series from U > S > D1 > D2 > D3. In this analysis, all the durations were ignored and the calculation was carried out for the whole mouth and broken down by tooth type, that is, incisors, canines and molars. The distribution of carious surfaces is presented in bar charts.

Results

Of the 795 recruited subjects, 196 (24.7%) were absentees. This was due to the unwillingness of their parents to participate in the study, inconvenience, the family moved out of the study area or the uncooperativeness of the children. Out of 599 attending the dental examination, 42 (7.0%) had only one examination; therefore this data was used only for the prevalence analysis. The remaining 557 (93.0%) were used for incidence density analysis. For transitional analysis only, 406 (67.8%) subjects who completed all follow ups were used (Table 1).

The prevalence of caries, which included all D1, D2, and D3 was 2.0%, 22.8%, and 68.1% among 9, 12 and 18 month olds, respectively. The average number of teeth in children of 9, 12 and 18 months was 2.2 ± 2.1, 5.5 ± 2.6 and 10.4 ± 3.6 teeth/child, respectively. The number of caries-affected teeth was 0.05 ± 0.39, 0.73 ± 1.6, and 2.82 ± 2.69 teeth/child, respectively. The incidence of caries, D1, D2 and D3, affected persons (ID_p) observed from the age of 9 to 12 months was 10.32 persons/100 person-months of observation whereas it was 15.70 person/100 person-months from the period of 12 to 18 months. The rate of new caries-affected tooth surfaces (ID_s) in the period from 9 to 12 months was 2.17 surfaces/100 surface-months of observation, whereas the ID_s in the period from 12 to 18 months was 2.22 surfaces/100 surface-months (Table 2).

Figure 1 shows the percentage of unerupted, sound and decayed lesions by tooth surfaces and tooth types. Decayed surfaces included all D1, D2 and D3. At the age of 9 months a few carious surfaces (0.3%) were observed on the buccal surface of upper incisors. At 12 months, 10% of the erupted surfaces were affected by caries. The majority of the lesions were located on the upper incisors, where 60% of the buccal surface and 30% of lingual surfaces were affected. By 18 months, 35.0% of the teeth and 14.9% of the surfaces were affected by caries. Buccal surfaces were the most affected surfaces (44.9%), followed by lingual

Table 1. Distribution of children by examination ages

Examination age (months)			Children attending examination		
			N	%	Data analysis
9	12	18	406	67.78	Incidence density, transitional probability of caries attacked and prevalence of dental caries
9	12		75	12.52	Incidence density and prevalence of dental caries
9		18	62	10.52	Prevalence of dental caries
9			23	3.84	Prevalence of dental caries
	12	18	14	2.34	Incidence density and prevalence of dental caries
	12		5	0.83	Prevalence of dental caries
		18	14	2.34	Incidence density and prevalence of dental caries
		Total	599	100.00	

Aggregate totals for 9, 12 and 18 months = 566, 500 and 496, respectively.

Table 2. Frequency, incidence and rates of risk of caries-affected children/surfaces

	Observation period (months)	
	9–12	12–18
N (persons)	463	396
No. of new caries-affected cases	105	249
Person-months of observation	1 017.25	1 585.50
ID_p/100 persons at risk	10.32	15.70
N (surfaces)	11 315.00	23 159.00
No. of new caries-affected surfaces	504.00	2 107.00
Surface-months of observation	23 271.00	94 774.50
ID_s/100 surfaces at risk	2.17	2.22

(24.2%), mesial (20.0%) and distal surfaces (8.9%). The only posterior teeth present were first molars, 10.7% of them were carious teeth. Occlusal surfaces were the most affected surfaces (50.7%), followed by buccal (39.6%), lingual (8.4%) and distal surfaces (1.3%).

Table 3 shows the transitory changes in dental status over the 9- to 12- and 12- to 18-month periods. The calculation was based on a total number of 20 deciduous teeth. The dental status of the majority of cases in both periods remained unchanged while the probability of one-step progression from 9 to 12 months ranged between 12% and 18% but doubled at second follow-up at 18 months. The major transitory change with a two-step progression was observed from S at 12 months to D2 at 18 months (8.38%). A three-step progression was rare.

The transitional probability calculation by tooth type shows that from 9 to 12 months, 69.8% and 44.2% of maxillary and mandibular incisors had erupted, whereas the percentage of erupted canines and molars of both jaws ranged between 0.9% and 2.6%. Among newly erupted maxillary incisors, 9.5% had changed from U to D1 whereas 0.6% had changed to D2. In respect of sound teeth (S) at 9 months, 68.0% were still sound teeth whereas 27.1% and 4.9% had changed to D1 and D2, respectively. Of D1 teeth at 9 months, 77.8% had no progression, whereas 22.2% had changed to D2. For mandibular incisors, 0.9% of unerupted teeth were later seen to be D1 whereas 3.1% with normal enamel surface developed to D1.

The transitory changes during the second follow-up period at 12–18 months were in line with those from 9 to 12 months (Table 4). The majority of transitional changes were observed among maxillary and mandibular incisors. One-step changes among incisors ranged from 24.1% to 78.1%. Maxillary incisors suffered the highest caries attack rate. One-step caries progressions, S to D1 and D1 to D2, were observed in approximately 40% of the cases. The probabilities of two-step progressions among maxillary incisors were also high; and the transitions from U to D1 and S to D2 were 28.0% and 15.8%, respectively.

Discussion

The results of this study on prevalence of dental caries correspond with the results of many previous epidemiological studies (14–16). However, detailed dental caries development measurements in particular deciduous teeth have previously been less available. The rate of caries in the study population was extremely high. The prevalence of ECC rose sharply from 2.0% at 9 months to 68.1% at 18 months. These results raise similar concerns from a previous epidemiological study in Thailand where the prevalence of ECC among children in Suphan Buri province was 83% (14). As previously stated, early eruption may pose a higher risk of

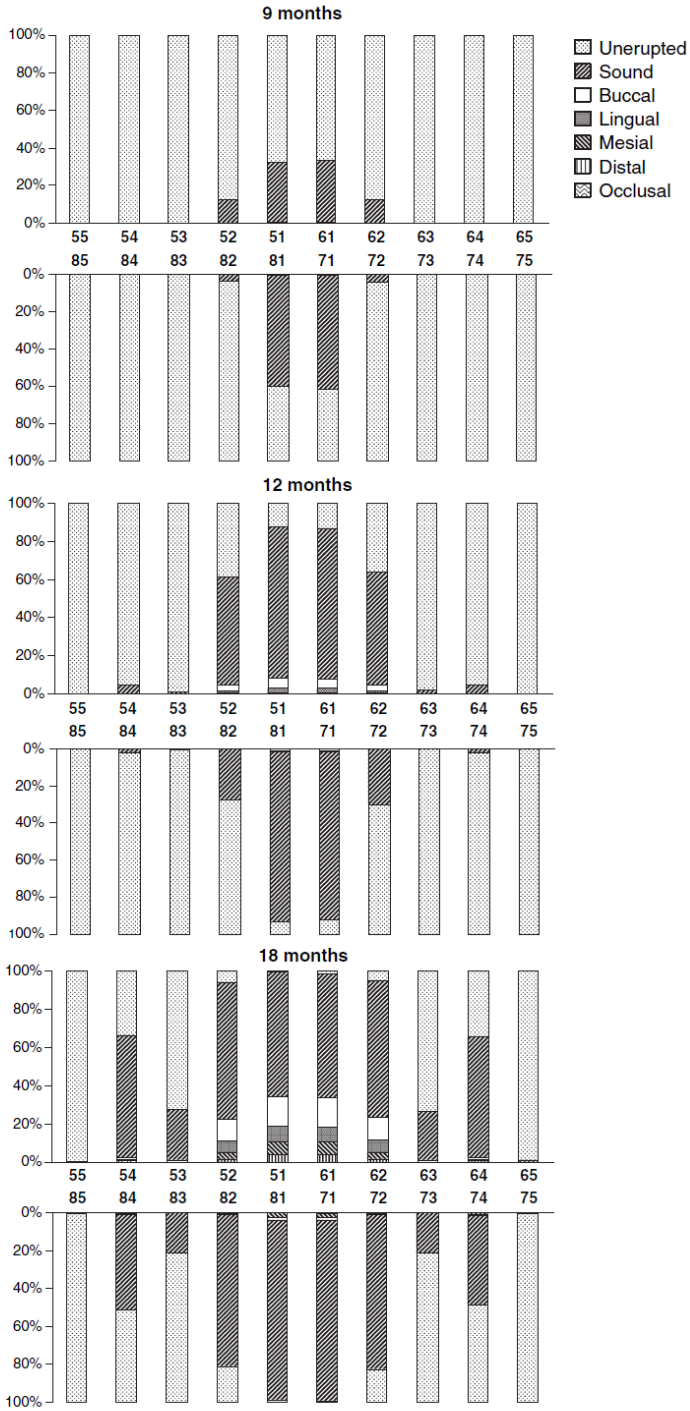

Fig. 1. The distribution of carious surfaces at the ages of 9, 12 and 18 months.

ECC (18). However, in this study population the average age of first tooth eruption was 8.53 months, whereas the average age of first tooth eruption among children in Bangkok is 7.5 months (19). Thus, tooth eruption did not occur early but the caries attacked rate was still very high.

Table 3. Transitional probability (%) of dental status of the whole mouth observed in the same children aged 9–12 and 12–18 months ($N = 406$)

Transitional status	Observation period (months)	
	9–12	12–18
U → U	79.96	60.39
U → S	18.13	35.51
U → D1	1.79	3.43
U → D2	0.11	0.67
U → D3	0.00	0.00
S → S	84.93	67.99
S → D1	12.86	23.62
S → D2	2.20	8.38
S → D3	0.00	0.00
D1 → D1	84.61	55.60
D1 → D2	15.38	39.60
D1 → D3	0.00	2.00
D2 → D2	100.00	77.42
D2 → D3	0.00	22.58
D3 → D3	0.00	0.00

U, unerupted; S, sound; D1, enamel caries; D2, dentine caries; D3, caries involving pulp.

Therefore, the higher risk is not explained by early eruption. By the age of 9 months an average of 2.2 teeth had already erupted; among these 0.05 teeth were affected by caries. The increasing number of caries-affected teeth was considerably higher than the increasing number of newly erupted teeth in the same period. This implies that the caries process starts as soon as the teeth emerge into the oral cavity. This is of significant interest and needs further investigation as a factor.

A cohort study is suitable for the calculation of an incidence rate and better characterizes the incremental rate of disease events. Incidence density was used in the study, as this more precisely estimates the rate of disease occurrences as it accounts for the varying times of follow up where the prevalence and incidence rates do not (20, 21). This is of importance as the length of follow up was not consistent for all participants. Some participants lost contact during the follow-up period, and some entered the study later than others. Moreover, the length of time between the two examinations was also different (3 versus 6 months) for the two periods.

The incidence density of caries-affected children sharply increased from 9 to 18 months. Among children unaffected by caries at 9 months, 22.7% were affected at the age of 12 months. Further, for those who were caries free at 12 months, 62.9% had acquired caries 6 months later at the 18-month follow up. This shows that susceptibility to caries in study children occurred in the first 3–6 months after the teeth had erupted into the oral cavity. To counteract the high caries rate among this high-risk population, a preventive program must be implemented early in life. To obtain the greatest benefit from the preventive program, it should commence at the time of tooth eruption or even before.

The analysis of carious surface distribution revealed that buccal surfaces of maxillary incisors were most affected. Lingual surfaces were next most affected, followed by mesial and distal surfaces. This confirmed the results of previous studies of caries manifestation of ECC (12, 13, 22). The high rate of caries attacks on buccal surfaces of maxillary incisors is known to be associated with both biological mechanisms and psychosocial behaviors (7, 8, 23). The mandibular teeth were less affected which is consistent with the findings of previous studies (14, 16).

Table 4. Transitional probability (%) categorized by tooth types observed in the same children aged 12–18 months ($N = 406$)

Transitional status	Maxillary teeth			Mandibular teeth		
	Incisors	Canines	Molars	Incisors	Canines	Molars
U → U	10.40	64.01	65.53	19.39	74.94	73.56
U → S	5.52	33.97	31.39	78.09	24.31	24.98
U → D1	28.00	1.90	2.50	1.65	0.76	1.32
U → D2	6.40	0.13	0.58	0.33	0.00	0.13
S → S	43.06	84.62	79.07	92.23	1.00	73.68
S → D1	41.12	15.38	16.28	6.63	0.00	15.79
S → D2	15.82	0.00	4.65	1.14	0.00	10.53
D1 → D1	52.73	0.00	0.00	76.67	0.00	0.00
D1 → D2	43.64	0.00	0.00	10.00	0.00	0.00
D1 → D3	2.27	0.00	0.00	0.00	0.00	0.00
D2 → D2	75.86	0.00	0.00	100.00	0.00	0.00
D2 → D3	24.14	0.00	0.00	0.00	0.00	0.00

U, unerupted; S, sound; D1, enamel caries; D2, dentine caries; D3, caries involving pulp.

Analysis of the transitional changes probability of caries susceptibility for the whole mouth shows that among study children there is a tendency for all teeth to demonstrate a rapid rise in caries susceptibility. These results demonstrate that new lesions develop not only as the child ages but more importantly, that the progression of caries lesions continues in the child's early years. Although the observation period of the subjects was limited to only 18 months of age, it is noteworthy to observe that during the 6-month follow up, a third of the enamel caries (D1) developed to be dentine caries (D2) and a fifth of dentine caries (D2) developed further to involve pulp tissue (D3). Based on this pattern, we would expect to see some of these D3 lesion teeth to be missing in the near future. As this study was carried out among children of 9–18 months, unerupted teeth accounted for the majority of the probability.

Maxillary teeth are more susceptible than mandibular teeth. Analysis by tooth type demonstrated that a rapid rise in caries susceptibility occurred in a rather short time as seen with maxillary incisors where around 9% of those unerupted teeth (U) at 9 months were enamel caries (D1) affected at 12 months. Although the nature of all caries development cannot be inferred from the present analysis, investigation is certainly needed to identify important associated factors in this rapid caries progression. A previous cross-sectional study among pre-school children in southern Thailand found that the high caries rate was related to oral hygiene, consumption of sweet milk, use of non-fluoride toothpaste, whether mothers or caretakers examined the child's teeth and socioeconomic status (24). Early colonization with mutans streptococci seems also to be an important risk factor as shown in a study by Bratthall et al. (25). It demonstrated that children in Bangkok who had oral streptococcus mutans colonization were more caries affected than remote hill tribe children who were mutans-free. The major route of the mutans streptococci transmitted to the children is from mothers (26, 27). Therefore, preventing cariogenic bacteria transmission from mothers to children would prevent ECC (28, 29).

An extremely high caries-affected rate was found among study children. The buccal surfaces of the maxillary incisors were the most affected. Generally, the teeth acquired caries at 3–6 months after initial eruption into the oral cavity and the carious lesions developed continuously over time. By the age of 18 months, a large proportion of children were affected by caries. Further investigation to identify risk factors of ECC development is needed.

Acknowledgements

This study received financial support from the Research Funding Agency, Prince of Songkla University (PSU), the Thailand Research Fund: MRG4780013 and Thai Health Promotion Foundation: 48-00-0286. We would like to acknowledge Prof. Chongsuvivatwong, V. Epidemiology Unit, PSU for his academic advice. We thank Dr Chuprapawan, C. the former director and the founder of PCTC and Assoc. Prof. Mo-suwan, L. Department of Pediatric, PSU for their generosity in the provision of background data of the study cohort. We would also like to thank Dr Ungchusak, C. Department of Dental Health, Ministry of Public Health for the provision of survey advice and facilities. The voluntary cooperation of the children, parents, caretakers and health workers at the health centers is also greatly appreciated.

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Appendix E: Additional information

Table 12. Distribution of *Lactobacillus* isolated from caries free children and children with dt 1-4

Species	Low-caries group (dt 0-4)		Caries free group		Children with dt 1-4	
	No. of subjects (%)	No. of isolates (%)	No. of subjects (%)	No. of isolates (%)	No. of subjects (%)	No. of isolates (%)
L. fermentum	17 (85)	74 (59.7)	4 (80)	13 (48.2)	13 (86.7)	61 (62.9)
L. salivarius	1 (5)	2 (1.6)	0	0	1 (6.7)	2 (2.1)
L. casei/paracasei	5 (25)	14 (11.3)	1 (20)	3 (11.1)	4 (26.7)	11 (11.8)
L. mucosae	0	0	0	0	0	0
L. rhamnosus	2 (10)	4 (3.2)	1 (20)	3 (11.1)	1 (6.7)	1 (1)
L. oris	3 (15)	6 (4.8)	0	0	3 (20)	6 (6.2)
L. gasseri	3 (15)	14 (11.3)	0	0	3 (20)	14 (14.4)
L. plantarum	0	0	0	0	0	0
L. vaginalis	2 (10)	10 (8.1)	1 (20)	8 (29.6)	1 (6.7)	2 (2.1)
Total	20 (100)	124(100)	5 (100)	27 (100)	15 (100)	97 (100)

VITAE

Name Mrs. Supatcharin Piwat

Student ID 4863001

Educational Attainment

Degree	Name of Institution	Year of Graduation
DDS.	Chulalongkorn University	1991
MDSc. in Pedodontics	Chulalongkorn University	1999

Scholarship Awards during Enrolment

1. The Royal Golden Jubilee Ph.D. Grant (RGJ) (grant No. PHD/0091/2549). The Thailand Research Fund (TRF), Thailand, 2006-2009.
2. The National Research Council of Thailand, Thailand, 2551.
3. The Swedish Research Council (grant No. 348-2007-6650), Sweden, 2007.

Work – Position and Address

Lecturer in Division of Pediatric Dentistry, Department of Preventive Dentistry, Faculty of Dentistry, Prince of Songkla University, Hat Yai, Songkhla 90112 Thailand

List of Publication and Proceeding

1. Dahlen G, Konradsson K, Eriksson S, Teanpaisan R, Piwat S, Carlen A. A microbiological study in relation to the presence of caries and calculus. ***Acta Odontol Scand*** 2010; 68(4):199-206.
2. Piwat S, Teanpaisan R, Thitasomakul S, Thearmontree A, Dahlén G. Lactobacillus species and genotypes associated with dental caries in Thai preschool children. ***Mol Oral Microbiol*** 2010; 25(2):157-64.
3. Kiatwateeratana T, Kintarak S, Piwat S, Chankanka O, Kamaolmatyakul S, Thearmontree A. Partial pulpotomy on caries-free teeth using enamel matrix derivative or calcium hydroxide: a randomized controlled trial. ***Int Endod J*** 2009; 42(7):584-92.

4. Thitasomakul S, Piwat S, Thearmontree A, Chankanka O, Pithpornchaiyakul W, Madyusoh S. Risks for early childhood caries analyzed by negative binomial models. ***J Dent Res*** 2009; 88(2):137-41.

5. Teanpaisan R, Thitasomakul S, Piwat S, Thearmontree A, Pithpornchaiyakul W, Chankanka O. Longitudinal study of the presence of mutans streptococci and lactobacilli in relation to dental caries development in 3-24 month old Thai children. ***Int Dent J*** 2007; 57(6):445-51.

6. Thitasomakul S, Thearmontree A, Piwat S, Chankanka O, Pithpornchaiyakul W, Teanpaisan R, et al. A longitudinal study of early childhood caries in 9- to 18-month-old Thai infants. ***Community Dent Oral Epidemiol*** 2006; 34(6):429-36.

7. Musekapan M, Teanpaisan R, Piwat S, Faroongsarng D, Ratanasathien S. Development of chlorhexidine sandarac varnish for dental caries. ***J Dent Assoc Thai*** 2005;55(1-2):88-99.

8. Trairatvorakul C, Piwat S. Comparative clinical evaluation of slot versus dovetail Class III composite restorations in primary anterior teeth. ***J Clin Pediatr Dent*** 2004;28(2):125-130.

9. Kamolmattyakul S, Vongvatcharanon S, Thearmontree A, Chankanka O, Piwat S, Paiboonwarachart D, Leewiboonsilp W. Effectiveness of midazolam / hydroxyzine compared to chloral hydrate / hydroxyzine in sedating pediatric dental patients. ***J Dent. Res*** 2002;81(special issue): 3703.