การศึกษาคุณลักษณะของแบคทีเรียที่ผลิตก๊าซมีเทนจากดินนาและรากข้าว

จิรศักดิ์ คงเกียรติขจร [1] วินัย แก้วสวัสดิ์ [2] และ สมเกียรติ เตชกาญจนรักษ์ [3]

มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าธนบุรี ท่าข้าม บางขุนเทียน กรุงเทพฯ 10150

บทคัดย่อ

ส่วนหนึ่งของแหล่งปลดปล่อยที่ก๊าซมีเทนที่ทำให้โลกร้อนคือนาข้าวซึ่งมีแบคทีเรียที่ผลิตก๊าซมีเทน ศึกษาสมบัติของแบคทีเรียที่ผลิตก๊าซมีเทนในตัวอย่างดินบริเวณรากข้าวและรากข้าวได้จากศูนย์วิจัยข้าวปทุมธานี ตัวอย่างได้ถูกนำมาบ่มในอาหารเลี้ยงเชื้อในขวดซีรั่มภายใต้สภาวะไร้อากาศที่อุณหภูมิ 37 องศาเซลเซียสเป็นเวลา 40 วัน มีเทนในขวดถูกวิเคราะห์โดยก๊าซโครมาโตกราฟี ผลการทดลองพบว่าการปลดปล่อยก๊าซมีเทนจากตัวอย่างดินบริเวณรากข้าวของข้าวสายพันธุ์หอมสุพรรณบุรี และสุพรรณบุรี 90 ในปริภูมิของขวดซีรั่มมีปริมาณก๊าซมีเทนสูงสุดที่อัตรา 21.55 และ 24.67 ไมโครโมลต่อกรัมต่อวันในวันที่ 24 และวันที่ 22 ของการบ่ม ตามลำดับ ส่วนรากข้าวพันธุ์หอมสุพรรณบุรี และ สุพรรณบุรี 90 พบก๊าซมีเทนที่ผลิตจากจุลชีพที่รากมีปริมาณสูงสุดที่อัตรา 19.92 และ 23.23 ไมโครโมลต่อกรัมต่อวันในวันที่ 28 ของการบ่มตามลำดับ จากผลวิเคราะห์ชนิดแบคทีเรียที่ผลิตก๊าซมีเทนโดยวิธี Fluorescent In Situ Hybridization (FISH) พบว่าแบคทีเรียจากรากข้าวอยู่ในจีนัส Methanosarcina ในขณะที่แบคทีเรียจากดินอยู่ในจีนัส Methanosaeta จากการวิเคราะห์จำนวนแบคทีเรียที่ผลิตก๊าซมีเทนโดยวิธี most probable number (MPN) ในตัวอย่างดินและรากข้าว แสดงให้เห็นว่าปริมาณการผลิตก๊าซมีเทนขึ้นกับจำนวนแบคทีเรียที่ผลิตก๊าซมีเทน

คำสำคัญ : มีเทน / แบคทีเรียที่ผลิตก๊าซมีเทน / ข้าว / ราก

---

[1] อาจารย์ สาขาวิชาเทคโนโลยีชีวเคมี คณะทรัพยากรชีวภาพและเทคโนโลยี

[2] นักศึกษาบัณฑิตศึกษา คณะทรัพยากรชีวภาพและเทคโนโลยี

[3] นักวิจัย ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ

Characterization of Methane Producing Bacteria from Ricefield Soil and Rice Roots

Jirasak Kongkiattikajorn [1], Winai Keawsawat [2], and Somkiatti Tachakanchanarag [3]

King Mongkut's University of Technology Thonburi, Thakham Bangkhuntian, Bangkok 10140

Abstract

One of the emission sources of methane in the atmosphere causing the world warming comes from paddy fields which are the habitats of methane producing bacteria. Rhizospheric soil samples and rice roots cv. Supanburi 90 and Homsupanburi grown at the Rice Research Center, Pathumthani Province were analyzed for the presence of methane producing bacteria. The samples were incubated in basal broth medium in serum vials under anaerobic condition at 37°C for 40 days. Methane concentration in the head space vials was determined by gas chromatography. The results showed that the methane was released from the producing bacteria from the rhizospheric soil of the paddy field of the rice root samples cv. Homsupanburi and Supanburi 90 could produce methane at the maximum concentration of 21.55 and 24.67 micromole/g dry weight /day at 24 and 22 days of incubation, respectively. The microorganisms from the rice root cv. Supanburi 90 and Homsupanburi could produce methane at maximum rate of 19.92 and 23.23 micromole/g dry weight/day at 28 days of incubation, respectively. From the experiments of determination on the type of methane producing bacteria by Fluorescent In Situ Hybridization (FISH) technique, it was found that the microbacteria from rice root was characterized to be *Methanosarcina* sp. while the microbacteria from the soil was characterized to be *Methanosatae* sp. The number of the methanogenic bacteria from the soil and rice root of the rice was determined by most probable number (MPN) method. The results showed that methane production rate depended on the number of the methanogenic bacteria.

Key Words : Methane / Methanogenic Bacteria / Rice / Root

[1] *Lecturer, Division of Biochemical Technology, School of Bioresources and Technology.*

[2] *Graduate Student, School of Bioresources and Technology.*

[3] *Researcher, National Center for Genetic Engineering and Biotechnology.*

1. Introduction

Anaerobic decomposition of landfilled solid waste generates significant amounts of greenhouse gas comprising 60% methane and 40% carbon dioxide (v/v), together with numerous trace gases. Flooding paddy field is a source of methane emission [1]. Methane is one of the major greenhouse gases and it is widely known that its concentration in the atmosphere has been increasing, with about 25 times more infrared absorption capacity per molecule than CO_2 [2]. Wetland rice agriculture is a major anthropogenic source of atmospheric methane [3], and this source has increased in recent years due to the expansion of rice cultivation. The amount of methane emission from wetland paddy fields accounts for 10-20% of total methane emission, amounting to 50-100 Tg $year^{-1}$ [4]. It is projected that the methane emission from rice cultivation may increase to 145 Tg $year^{-1}$ in 2025 [5]. Methane emission from paddy fields is the net effect of methane production by methanogens and oxidation by methanotrophs eventually to carbon dioxide [6]. The soil of the field is anaerobic. It was found that acetate is substrate for methanogenesis by different kinds of methanogen [7].

Mira *et al.* 1999 [8] studied the relationship between 6 cultivar of rice Pusa, methane emission in the range of 0.65-1.12 $mg/m^2/h$ were reported. During growing in the field where the total methane emission as the following, 27.2, 26.9, 26.3, 24.0, 16.9 and 15.6 kg/ha, for growth duration 125, 125, 135, 70-90, 70-90 and 120 days, for Pusa 933, Pusa 1019, Pusa Basmnti, Pusa 834, Pusa 677, Pusa 169, respectively.

In Thailand, rice paddy has been estimated as one of the important sources of atmospheric CH_4 with annual emission of 1.8 Tg $year^{-1}$, representing 65% of total emission from all sources [9]. The strength of this source may be increased in recent decades due to expansion of rice cultivation. There is evidence that more than 90% of methane emission from the anoxic paddy soils is through the rice plant [10].

Chidthaisong *et al.* [11] studied methanogens in soil and rice root in California USA and inoculated into the culture medium for multiplying the methanogen. It was found that 80% of methanogen associated with rice root was *Methanospirillum* sp. and 60% of methanogen in soil was *Methanobacterium* sp. However, it was found that methanogen in the paddy field around the world was that mostly *Methanobacterium* sp. and *Methanospirillum* sp. was rarely found at rice root if it was not amplified a though it was mostly found at rice root. So, knowing the causes and the factors involving of methane production and emission might bring to solving to control or repetative emission from the paddy fileds.

Methane is produced by the activity of methanogens. Although the mechanism of methanogenesis in paddy soils has been studied [7, 12], analysis of methanogenic flora in paddy field in Thailand has never been reported. The aim of this study was to characterise the population of methanogen associated with rice root and rhizospheric soil of paddy rice cv. Supanburi 90 and Homsupanburi by most probable number (MPN) and Fluorescent In Situ Hybridization technique and study methane and carbon dioxide production pattern from the methanogenic bacteria by cultivation of the microoganisms in culture medium to probably be used to predict the methane release rate by estimating the number of acetate-utilizing methanogens in the paddy soils.

2. Materials and Methods

2.1 Soil and rice root

The soil samples were collected from rice fields of the Rice Research Institute in Prathum Thani province. Soil samples were collected from a flooded rice paddy. All samples were utilized immediately (i.e. within 24 h) after transportation to the laboratory.

Soil from paddy field before growing rice, rice root and rhizospheric soil of rice cv. Supanburi 90 and Homsupanburi 70 days of age were from the Rice Research Center, Prathum Thani Province, kindly provided by the Head of the Rice Research Center. The fields were given applications of 100-50-100 kg ha^{-1} N-P-K for rice.

2.2 Media for isolation and culture of methanogens

Enrichment cultures were grown in defined basal medium prepared as described in Table 1 [13]. The pH of the medium was adjusted to 7.3 with 2.0 N HCl. Acetic acid 0.1% was added to the medium as a carbon source or growth-stimulating reagent.

2.3 Gas analysis

1 g of Soil or rice root (wet weight) was added with 7.5 ml of basal medium in serum vial as described by Zhang and Noike [14]. Each sample was done four replicates. The vials were sealed with rubber cap and dawn out the air inside to be anaerobic and incubated at 37 °C. The headspace content of the vials were sampled with 1.0 ml gas-tight syringes (Hamilton, USA), and were analy sed for methane and carbon dioxide production at different time intervals using gas chromatography (Shimadzu Model GC 9A) using Parapak-N column by using Thermal Conductivity Detector (TCD) as detector. The temperature of the column, injector and TCD was 70, 120, 120 °C, respectively, with current Bridge 100 mA using helium as carrier gas with flow rate of 50 ml/min. Calibrated standards (2.0 ppm, 3.0 ppm and 11.0 ppm for CH_4; 360 ppm, 1,250 ppm and 4,000 ppm for CO_2)(Barascientific Company) were used for CH_4 concentration determination.

Table 1 Medium composition of batch culture

Components	Concentration
KH_2PO_4	0.4 g/l
K_2HPO_4	0.4 g/l
NH_4Cl	1.0 g/l
$MgCl_2.6H_2O$	0.21 g/l
Mineral solution[a]	10 ml
Vitamin solution[b]	10 ml
$NaHCO_3$	4.0 g/l
Cysteine $HCl.H_2O$	0.5 g/l
$Na_2S.9H_2O$	0.25 g/l
Resazurine	0.002 g/l

[a]Contains, in grams per liter of distilled water: nitriotriacetic acid, 4.5; $FeCl_24H_2O$; $CoCl_26H_2O$, 0.01; NaCl, 1.0; $CaCl_2$, 0.02; Na_2MoO_4, 0.01; $MnCl_24H_2O$, 0.10; H_3BO_3, 0.01; $CuSO_4$ $5H_2O$, 0.01; $NiCl_26H_2O$, 0.02.

[b]Contains, in milligrams per of distilled water: biotin, 2; folic acid, 2; pyridoxine HCl, 10; thiamine HCl, 5; riboflavin, 5; nicotinic acid, 5; DL-calcium pantothenate, 5; vitamin B12, 0.1; *p*-aminobenzoic acid.

2.4 Counts of methanogens

The methanogens of each sample was cultivated under anaerobic condition using the basal medium as described by Zhang and Noike [14]. The medium was added with 0.1% acetic acid. The population density of the groups of methanogens were estimated by the most probable number (MPN) method (4 tubes per dilution). Successive 10-fold serial soil suspension dilutions were inoculated in the media described above. Then 1 ml of each dilution was added with 7.5 ml basal medium. Methanogen growth was assayed by measuring CH_4 produced after 30 days of incubation at 37°C. Inoculated tubes containing medium supplemented with 0.1% acetic acid, where no 0.1% acetic acid was added, served as control. The gas produced in the headspace content of each vial was drawn out for 0.2 ml to determine gas methane by comparing with reference standard CH_4 and converted the amount of methane gas using the MPN Table to be the number of methanogen. A tube was considered positive when CH_4 produced was as least 5% higher than in the control (tube contained medium without adding sample). Populations were expressed as MPN per g dry matter of soil or root.

All determinations were done in four replicates to estimate mean values and standard errors.

2.5 Study of morphology and classification of methanogen by Fluorescent In Situ Hybridization technique (FISH)

Samples from serum vials were fixed with 3% paraformaldehyde and resuspended in Tris buffer and ethanol as described by Raskin *et al.* [15]. The cell suspensions were stored at -20 °C. Hybridization was performed on poly-L-Lysine-coated slides as described by Amann *et al.* [16]. The following oligonucleotide probes complementary to specific regions of 16S rRNA were used (i) ARC915, specific for the domain *Archaea* [17] and (ii) MSMX860, specific for family *Methanosarcinaceae* [17]. Oligonucleotide probes were synthesized and 5' labeled with CY3 dye by Thermo Hybrid (Germany). Probe concentration was 50 ng in 10 μl of hybridization solution. The samples were viewed under an Olympus Microscope BX60 with appropriate filters. Images were captured with an Olympus DP50 digital camera system and final images were prepared with Adobe PhotoShop 7.0 software (Adobe, Mountain View, CA, USA).

3. Results

3.1 Determination of methane production

Methane production of methanogen from paddy soil, rhizospheric soil and rice root cv. Homsupanburi was shown in Fig. 1. It was shown that the methanogen from paddy soil before growing rice (MBS.H) and rice root (MRoot.H) produced maximum rate of methane on the 26^{th} and 28^{th} day of incubation for 19.00 and 19.92 micromole/g dry weight/day, respectively, after that the volume of gas decreased to 9.19 and 14.54 micromole/g dry weight/day at the 40^{th} day of incubation, respectively. The methanogen from rhizospheric soil (MAS.H) produced methane more than paddy soil before growing rice and rice root. The maximum rate of methane produced on the 24^{th} day of incubation at 21.55 micromole/g dry weight/day till on the 34^{th} day, the methane decreased to 14.73 micromole/g dry weight/day on the 40^{th} day of incubation.

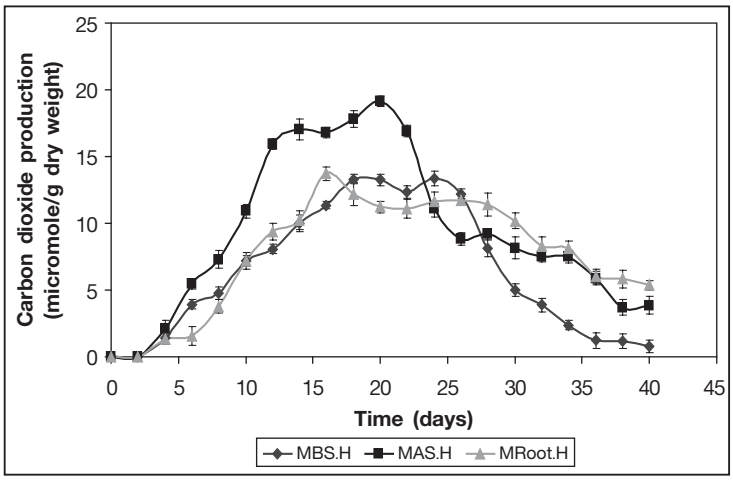

Fig. 1 Methane production of enrichment cultivation from paddy soil before growing rice cv. Homsupanburi (MBS.H), rhizospheric soil (MAS.H) and rice root (MRoot.H)

Methane production of methanogen from paddy soil, rhizospheric soil and rice root cv. Supanburi 90 was shown in Fig. 2. It was shown that the methanogen from paddy soil before growing rice (MBS.S90) produced maximum rate of methane on the 34th day of incubation at 21.78 micromole/g dry weight /day after that the volume of gas decreased to 14.73 micromole/g dry weight/ day on the 40th day of incubation. The methanogen from rice root (MRoot.S90) produced maximum rate of methane on the 26th - 30th day of incubation at 23.23 micromole/g dry weight /day and then the rate of methane production decreased to 18.68 micromole/g dry weight /day on the 40th day after incubation. The methanogen from rhizospheric soil (MAS.S90) produced methane more than paddy soil before growing rice and rice root. The maximum rate of methane produced on the 22th day of incubation at 24.67 micromole/g dry weight /day till on the 28th day, the methane decreased to 15.66 micromole/g dry weight /day on 40th day of incubation.

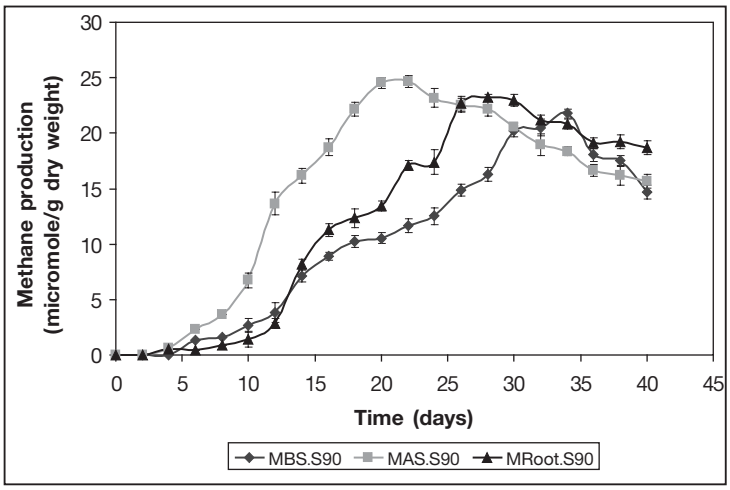

Fig. 2 Methane production of microorganisms from paddy soil before growing rice cv. Supanburi 90 (MBS.S90), rhizospheric soil (MAS.S90) and rice root (MRoot.S90)

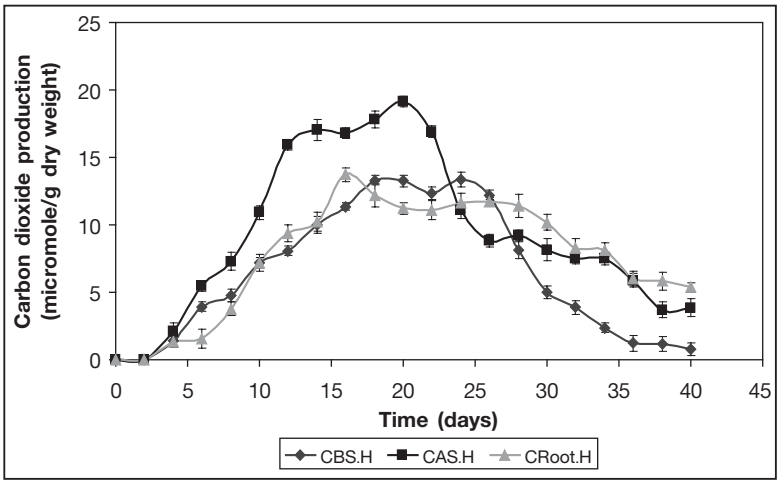

Fig. 3 Carbon dioxide production of enrichment cultivation from paddy soil before growing rice cv. Homsupanburi (CBS.H), rhizospheric soil (CAS.H) and rice root (CRoot.H)

Carbon dioxide production of methanogen from paddy soil, rhizospheric soil and rice root cv. Homsupanburi was shown in Fig. 3. It was shown that the methanogen from paddy soil before growing rice (CBS.H) and rice root (CRoot.H) produced maximum rate of carbon dioxide on the 18th and 16th day of incubation for 13.30 and 13.72 micromole/g dry weight /day, respectively, after that the volume of gas decreased to 0.77 and 5.36 micromole/g dry weight /day at the 40th day of incubation, respectively. The methanogen from rhizospheric soil (CAS.H) produced more carbon dioxide than paddy soil before growing rice and rice root. The maximum rate of carbon dioxide produced on the

20th day of incubation at 19.12 micromole/g dry weight /day till on the 32th day, the carbon dioxide decreased to 3.85 micromole/g dry weight /day on the 40th day of incubation.

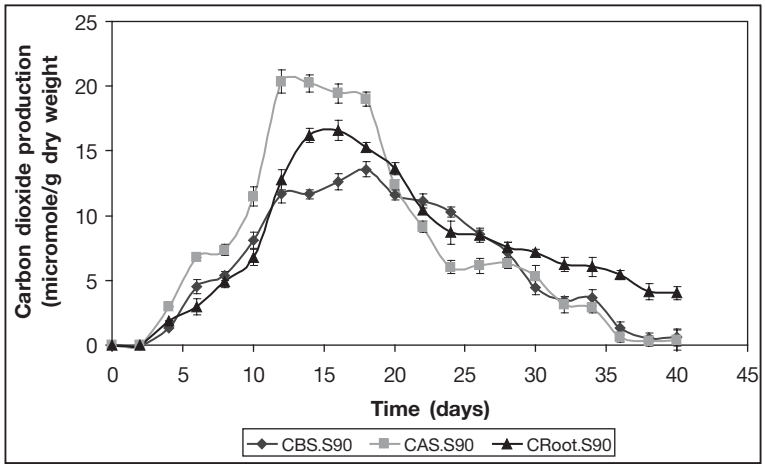

Fig. 4 Carbon dioxide production of microorganisms from paddy soil before growing rice cv. Supanburi 90 (CBS.S90), rhizospheric soil (CAS.S90) and rice root (CRoot.S90)

Carbon dioxide production of methanogen from paddy soil, rhizospheric soil and rice root cv. Supanburi 90 was shown in Fig. 4. It was shown that the methanogen from paddy soil before growing rice (CBS.S90) and rice root (CRoot.S90) produced maximum rate of carbon dioxide on the 18th and 16th day of incubation for 13.57 and 19.43 micromole/g dry weight/day, respectively, after that the volume of gas decreased to 0.59 and 4.02 micromole/g dry weight/day at the 40th day of incubation, respectively. The methanogen from rhizospheric soil (CAS.S90) produced more carbon dioxide than paddy soil before growing rice and rice root. The maximum rate of carbon dioxide produced on the 12th day of incubation at 20.36 micromole/g dry weight/day till on the 30th day, the carbon dioxide decreased to 0.41 micromole/g dry weight/day on the 40th day of incubation.

The results indicated that the paddy soil, rhizospheric soil and rice root cv. Supanburi 90 produce both methane and carbon dioxide more than the other cultivar Homsupanburi. The results from the fraction of CO_2 and CH_4 showed that CH_4 was produced from CO_2 reduction exclusively (Fig. 5, 6). In the initial time of incubation (0-6 day) the fraction of CO_2 and CH_4 was increased. Then, the fraction of CO_2 and CH_4 decreased, presumably since acetoclastic methanogens became activated by the increasing acetic acid concentrations and CO_2 was reduced to CH_4 until CO_2 was depleted. (Fig. 3, 4). The fraction of CO_2 and CH_4 had decreased when acetic acid became depleted. Integration of the fractions over the incubation time until acetic acid was depleted (40 day).

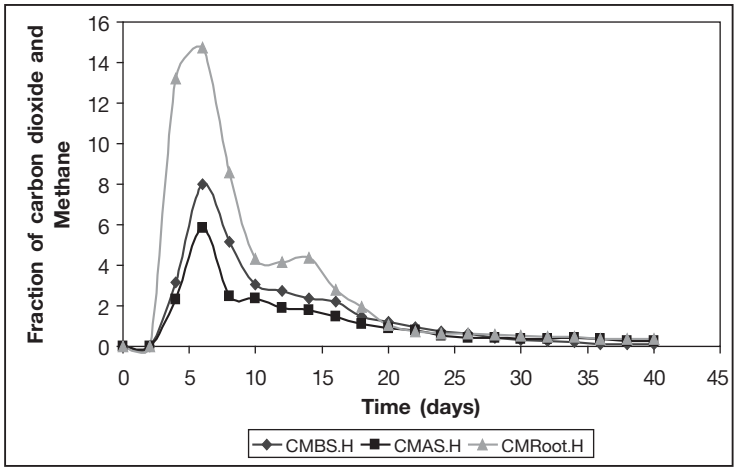

Fig. 5 Time course of the fractions of CH_4 and CO_2 in anoxicially incubated paddy soil before growing rice cv. Homsupanburi (CMBS.H), rhizospheric soil (CMAS.H) and rice root (CMRoot.H)

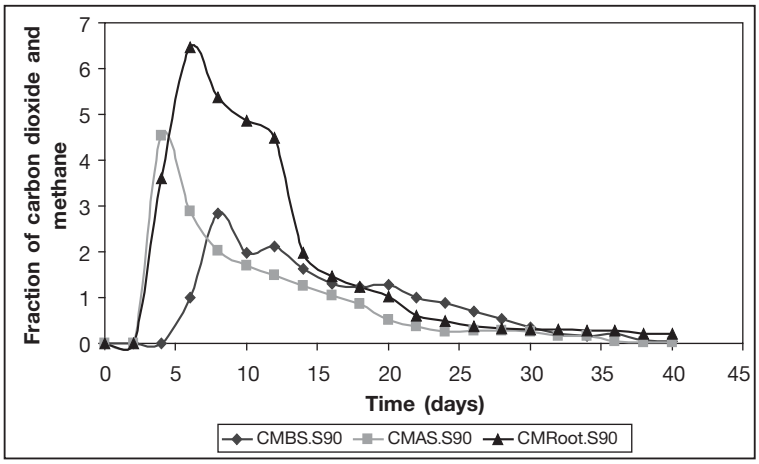

Fig. 6 Time course of the fractions of CH_4 and CO_2 in anoxicially incubated paddy soil before growing rice cv. Supanburi 90 (CMBS.S90), rhizospheric soil (CMAS.S90) and rice root (CMRoot.S90)

3.2 Fluorescent In Situ Hybridization technique (FISH)

From the results as shown in Fig. 7, microorganisms enriched by 0.1% acetic acid specific for probe ARC 915 were different morphology. It was found that enriched acetoclastic methanogens from soils before growing rice, rhizospheric soils and rice root were divided into 2 groups, bacilli in single and chain morphology, and multicellular clusters or cell packet morphology. For soil samples before growing rice, cells hybridized with ARC915 were long chain rod morphology and aligned within a sheathed filament, similar to *Methanosaeta* sp. (Fig. 7). For soil samples around the rice root, cells hybridized with ARC915 were long chain rod morphology, similar to *Methanosaeta* sp. and cells forming tetrads, similar to *Methanosarcina* species (Fig. 7). The use of ARC915 and *Methanosarcinaceae*-specific MSMX860 probes in hybridization showed that *Methanosarcina*-like cells were the predominant group presented on the rice root.

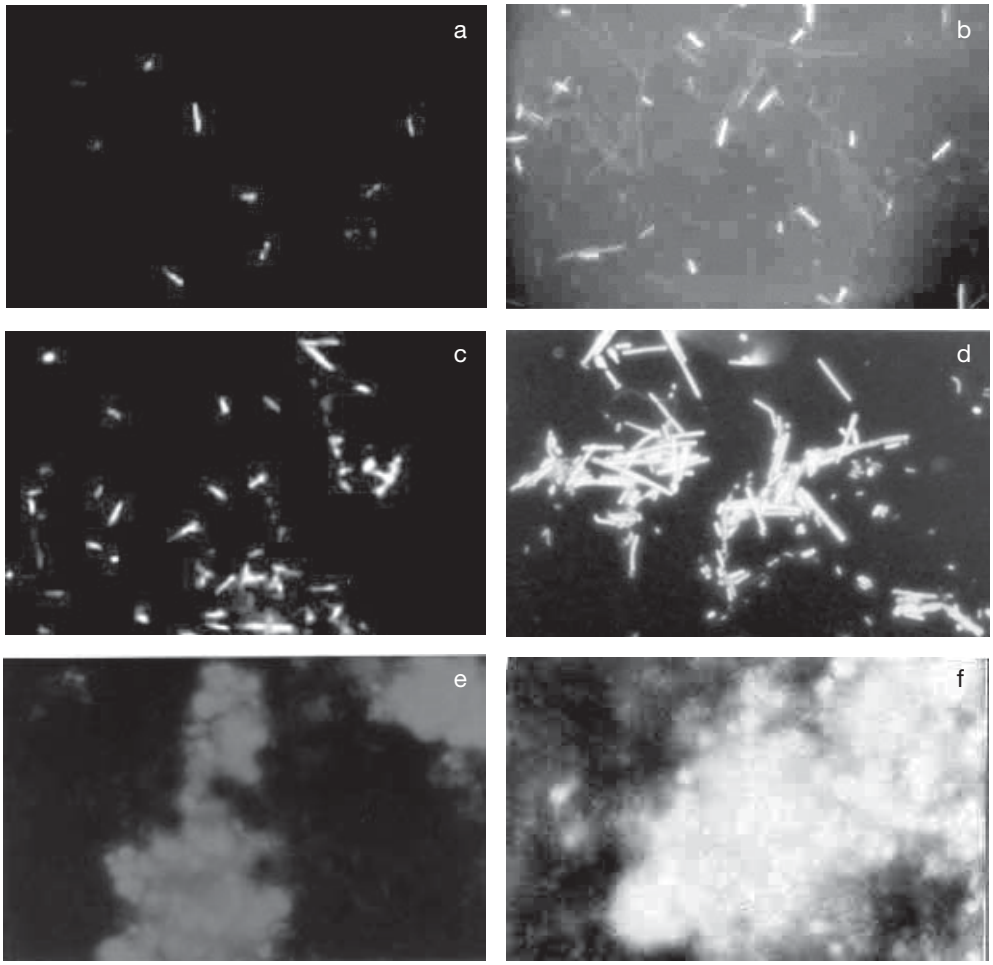

Fig. 7 Methanogen staining by Fluorescent In Situ Hybridization technique;
methanogen from paddy soil before growing rice cv. Homsupanburi (a), Supanburi 90 (b),
methanogen from rhizospheric soil of rice cv. Homsupanburi (c), Supanburi 90 (d),
methanogen from rice root cv. Homsupanburi (e) and Supanburi 90 (f).

It has been observed that enriched acetoclastic methanogens from soil and rice root was divided into 2 groups; by cell morphology firstly the bacilli, in single and chain this group may be *Methanosaeta* sp., and secondly, the cocci in single, dicocci, and cluster, indicating that this group may be *Methanosarcina* sp.

Further characterization of the microorganism from paddy soil before rice growing cv. Homsupanburi (a) and Supanburi 90 (b) enriched by 0.1% acetic acid by FISH to specific probe was bacilli, so it should be *Methanosaeta* sp. The microorganism from rhizospheric soil of rice cv. Homsupanburi (c) and Supanburi 90 (d) enriched by 0.1% acetic acid specific to probe was cocci, and bacilli, so it should be *Methanosarcina* and *Methanosaeta* sp., respectively. The microorganism from rice root cv. Homsupanburi (e) and Supanburi 90 (f) enriched by 0.1% acetic acid specific to probe was cocci, so it should be *Methanosarcina* sp.

3.3 Number of methanogen determined by MPN after 0.1% acetic acid enrichment

The numbers of acetoclastic methanogen from soil before growing rice, rhizospheric soil and rice root were determined by MPN technique as shown in Table 2.

Table 2 MPN counts in the soil and rice roots of rice cv. Homsupanburi and Supanburi 90

Sample	Homsupanburi (cell/g dry matter)	Supanburi 90 (cell/g dry matter)
Paddy soil before rice growing	$8.91 \times 10^3 (1.4 \times 10^2)$	$1.02 \times 10^4 (1.6 \times 10^3)$
Rhizospheric soil	$3.75 \times 10^5 (5.2 \times 10^4)$	$3.49 \times 10^7 (4.7 \times 10^6)$
Rice root	$5.22 \times 10^3 (2.2 \times 10^2)$	$2.01 \times 10^3 (3.3 \times 10^2)$

95% confidence levels of MPN counts are given in brackets.

4. Discussion

Both gas-type gas chromatography serum vial method have been successfully used to measure the greenhouse gas concentrations of methane and carbon dioxide production from soil and rice root samples. The highest methane production was 21.55 and 24.67 micromole/g dry weight/day for rhizospheric soil of rice cv. Homsupunburi and Supunburi 90, respectively. Similarly, highest carbon dioxide production was 19.12 and 20.36 micromole/g dry weight/day for rhizospheric soil of rice cv. Homsupunburi and Supunburi 90, respectively. The methanogenic activity originated from the acetate utilizing methanogens was attributed, because the acetoclastic methanogens were supposed to be predominated in the acidic conditions than the other methanogens.

Schwarz and Frenzel [18] found that methane production of soil sampled in spring before flooding was low at the beginning with 0.020 micromole CH_4 g dry weight soil/day, then increased transiently to 0.210 micromole CH_4 g dry weight soil/day. Finally, methanogenesis remained stabled at 0.100 micromole CH_4 g dry weight soil/day until the end of the experiment. However, the experiment was done with soil that was sampled in autumn and showed a higher methanogenic activity with a peak rate of 0.840 micromole CH_4 g dry weight soil/day. Methane production rates on rice roots stayed relatively stable over the season at around 2.4-3.6 micromole CH_4/g dry weight/day. A short-term peak in activity was observed around 80 days after flooding, where 16.8 $\pm$ 3.62 and 11.47 $\pm$ 12.72 micromole CH_4/g dry weight/day [19].

The methane production correlated to the number of methanogen. As shown in Fig. 1 to Fig. 4 and Table 1, the more number of methanogen, the more production of both gas-types production. Methane production related to other microorganisms that degraded the large macromolecule to acetic acid which is necessary for the growth of methanogen. In addition, the rate of methane production is related to soil, the organic contents and the number of microorganism [20].

On the basis of phenotypic studies, all the isolated rods were affiliated to the genus *Methanosarcina* and the bacilli were affiliated to the genus *Methanosaeta*. Our results seem to indicate that, in ricefields, *Methanosarcina* and *Methanosaeta* sp. are mostly responsible for CH_4 production from acetate. These genera are probably ubiquitous. Microorganisms in acetate enriched culture specific for probe ARC 915 were different morphology both from rice cv. Homsupanburi and Supanburi 90, those were *Methanosarcina* and *Methanosaeta* sp. corresponding to the studies of Chin *et al.* [21] and Grobkopf *et al.* [22].

Methanosarcina sp. only found at rice root is probably indicates the microorganism being growth associated of rice using root exudate for growth and rice might intake certain byproducts from the microorganism. It was found that the microorganism was intercalated at the split of the root. However, *Methanosaeta* sp. was not found at rice root. It might be due to the large site of the morphology.

Although results from classical isolation of methanogens from ricefields suggest the ubiquity and dominance of *Methanosarcina* sp. among culturable organisms, Kudo *et al.* [19], using PCR amplification of archaeobacterial 16S ribosomal DNA from extracted soil DNA ricefield soils, reported the presence of *Methanobacterium* in only one soil, where it was not dominant. On the other hand, similar to our results, this study also reported the presence of members of the genera *Methanosarcina*. From their results, Kudo *et al.* [23] concluded to the dominance of *Methanosarcina* in soil samples, to that of *Methanogenium* in two soils and *Methanosaeta* in two soils.

Enumeration of methanogens in dry soil samples originating from 2 ricefields representing a different range of physico-chemical properties origins. Since acetate was demonstrated to be a major methanogenic substrate in wetland ricefields [7, 12, 24], enumerations were performed on selective media containing this energy sources. MPN counts of methanogens on acetate ranged from 10^3 to 10^7 g^{-1} dry weight. Populations were in a range similar to those reported in Senegalese ricefields (10^2-10^7 g^{-1} dry weight). In an Italian ricefield, Schutz *et al.*, [7] and Mayer and Conrad [25] counted 10^4-10^5 acetotrophs g^{-1} dry weight. Acetotrophs were mostly sarcinae. Sarcinae are known to develop as dense aggregates, difficult to separate into individual cells, thus their populations are underestimated by MPN counts, which mostly record the number of aggregates [26]. The MPN method we used is possibly selective for acetoclassic methanogens and might lead to the erroneous conclusion of its dominance when it is only present. The results indicate that methanogen biodiversity in ricefields is not yet elucidated.

However, from this study, it might be concluded that the methanogen from paddy soil before cultivation of rice cv. Supanburi 90 was found to be *Methanosaeta* sp. while *Methanosarcina* and *Methanosaeta* sp. were found from rhizospheric soil and *Methanosarcina* sp. was only be found from the rice root. The methanogen from paddy soil before cultivation of rice cv. Homsupanburi was found to be *Methanosaeta* sp. while *Methanosarcina* and *Methanosaeta* sp. were found from the rhizospheric soil but *Methanosaeta* sp being greater. For the rice root, the methanogen was only *Methanosarcina* sp. It was found that the methane produced from the soil was more than from the root, this is due to the number of the methanogen from the soil being more than from the rice root.

From the results, methane production depended on the number of methanogen, so to decrease the methane emission from the paddy field, a decrease in methanogen is indicated.

5. References

1. Minami, K., Mosier, A., and Sass, R., 1994, "CH_4 and NO_2 : Global Emission and Controls from Rice Fields and other Agricultural and Industrial Sources", *NIAES* series 2, Yokendo, Tokyo.

2. Rath, A. K., Mohanty, S. R., Mishra, S., Kumaraswamy, S., Ramakrishnan, B., and Sethunathan, N., 1999, "Methane Production in Unamended and Rice-Straw-Amended Soil at Different Moisture Levels", *Biology and Fertility of Soils*, Vol. 28, pp. 145-149.

3. Conrad, R., 1996, "Soil Microorganisms as Controllers of Atmospheric Trace Gases, (H_2, CO, CH_4, OCS, N_2O and NO)", *Microbiology Review*, Vol. 60, pp. 609-640.

4. Sass, R. L., Fisher, F. M., Lewis, S. T., Jund, M. F., and Turner, F. T., 1994, "Methane Emission from Rice Fields: Effects of Soil Properties", *Global Biogeochemical Cycle*, Vol. 8, pp. 135-140.

5. Anastasi, C., Dowding, M., and Simpson, V. J., 1992, "Future CH_4 Emission from Rice Production", *Journal of Geophysical Research*, Vol. 97, pp. 7521-7525.

6. Watanabe, I., Hashimoto, T., Shimoyama, A., 1997, "Methane Oxidizing Activities and Methanotrophic Populations Associated with Wetland Rice Plants", *Biology and Fertility of Soils*, Vol. 24, pp. 261-265.

7. Schutz, H., Seiler, W., and Conrad, R., 1989, "Processes Involved in Formation and Emission of Methane in Rice Paddies", *Biogeochemistry*, Vol. 7, pp. 33-53.

8. Mira, S., Jain, M. C., Kumar, S., Bandyopadhyay, S. K., and Kalra, N., 1999, "Effect of Rice Cutivars on Methane Emission", *Agriculture Ecosystems and Environment*, Vol. 73, pp. 177-183.

9. Phantumvanit, D., 1997, *Thailand's National Greenhouse Gas Inventory 1990*, Thailand Environment Institute, 130 p.

10. Inubushi, K., Muramatsu, Y., and Umerayasi, M., 1992, "Influence of Percolation on Methane Emission from Flooded Paddy Soil", *Japanese Journal of Soil Science Plant Nutrition*, Vol. 63, pp. 184-189.

11. Chidthaisong, R. B. A. and Conrad, R., 1999, "Measurement of Monosaccharides and Conversion of Glucose to Acetate in Anoxic Rice Field Soil", *Applied and Environmental Microbiology*, Vol. 65, pp. 2350-2355.

12. Conrad, R., Bak, F., Seitz, H. J., Thebrath, B., Mayer, H. P., and Schutz, H., 1989, "Hydrogen Turnover by Psychrotrophic Homoacetogenic and Mesophilic Methanogenic Bacteria in Anoxic Paddy Soil and Lake Sediment", *FEMS Microbiology Ecology*, Vol. 62, pp. 285-294.

13. Li Y. Y. and Noike T., 1992, "Upgrading of Anaerobic Digestion of Waste Activated Sludge by Thermal Pretreatment", Water Science Technology, Vol. 26, pp. 857-866.

14. Zhang, T. C. and Noike, T., 1991, "Comparison of One-Phase and Two-Phase Anaerobic Digestion Process in Characteristics of Substrate Degradation and Bacterial Population Level", *Water Science Technology*, Vol. 23, pp. 1157-1166.

15. Amann, R., Snaidr, J., Wagner, M., Ludwig, W., and Schleifer, K. -H., 1996, "In Situ Visualization of High Genetic Diversity in a Natural Microbial Community", *Journal of Bacteriology*, Vol. 178, pp. 3496-3500.

16. Stahl, D. A., Flesher, B., Mansfield, H. R., and Montgomery, L., 1988, "Use of Phylogenetically Based Hybridization Probes for Studies of Ruminal Microbial Ecology", *Applied and Environmental Microbiology*, Vol. 54, pp. 1079 -1084.

17. Raskin, L., Stromley, J. M., Rittmann, B. E., and Stahl, D. A., 1994, "Group-Specific 16S rRNA Hybridization Probes to Describe Natural Communities of Methanogens", *Applied and Environmental Microbiology*, Vol. 60, pp. 1232-1240.

18. Schwarz, M. V. J. and Frenzel, P., 2005, "Methanogenic Symbionts of Anaerobic Ciliates and Their Contribution to Methanogenesis in an Anoxic Rice Field Soil", *FEMS Microbiology Ecology*, Vol. 52, pp. 93-99.

19. SuB, J., Engelen, B., Cypionka, H., and Sass, H., 2004, "Quantitative Analysis of Bacterial Communities from Mediterranean Sapropels Based on Cultivation-Dependent Methods", *FEMS Microbiology Ecology*, Vol. 51, pp. 109-121.

20. Lloyd, D., Thomas, K. L., Hayes, A., Hill, B., Hales, B. A., Edwards, C., Saunders, J. R., Ritchie, D. A., and M. Upton, 1998, "Micro-ecology of Peat: Minimally Invasive Analysis using Confocal Laser Scanning Microscopy, Membrane Inlet Mass Spectrometry and PCR Amplification of Methanogen-Specific Gene Sequences", *FEMS Microbiology Ecology*, Vol. 25, pp. 179-188.

21. Chin, K.-J., Hahn, D., Hengstmann, U., Liesack, W., and Jassen, P. H., 1998, "Characterzation and Identification of Numerically Abundant Culturable Bacteria from Anoxic Bulk Soil of Rice Paddy Microcosms", *Applied and Environmental Microbiology*, Vol. 65, pp. 5042-5049.

22. Grobkope, P. H. J. R. and Liesack, W., 1998, "Diversity and Structure of the Methanogenic Community in Anoxic Rice Soil Microcosms as Examined by Cultivation and Direct 16S rRNA Gene Sequence Retrieval", *Applied and Environmental Microbiology*, Vol. 64, pp. 960-969.

23. Kudo, Y., Nakajima, T., Miyaki, T., and Oyaizu, H., 1997, "Methanogen Flora of Paddy Soils in Japan", *FEMS Microbiology Ecology*, Vol. 22, pp. 39-48.

24. Takai, Y., 1970, "The Mechanism of Methane Fermentation in Flooded Paddy Soil", *Soil Science and Plant Nutrition*, Vol. 16, pp. 238-244.

25. Mayer, H. P. and Conrad, R., 1990, "Factors Influencing the Population of Methanogenic Bacteria and the Initiation of Methane Production upon Flooding of Paddy Soil", *FEMS Microbiology Ecology*, Vol. 73, pp. 103-112.