การพัฒนาเครื่องหมายชีวภาพจากเชื้อประจำถิ่นเพื่อการตรวจสอบย้อนกลับปลานิลจากฟาร์มมหาวิทยาลัยเทคโนโลยีสุรนารี (มทส)

นางสาวดาราวรรณ ร่วมกุศล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
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DEVELOPMENT OF BIOLOGICAL PROBES FROM MICROFLORA TO ASSURE TRACEABILITY OF TILAPIA FROM SURANAREE UNIVERSITY OF TECHNOLOGY (SUT) FARM

Darawan Ruamkuson

A Thesis Submitted in Partial Fulfillment of the Requirements for the

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DEVELOPMENT OF BIOLOGICAL PROBES FROM MICROFLORA TO ASSURE TRACEABILITY OF TILAPIA FROM SURANAREE UNIVERSITY OF TECHNOLOGY (SUT) FARM

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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ดาราวรรณ ร่วมกุศล : การพัฒนาเครื่องหมายชีวภาพจากเชื้อประจำถิ่นเพื่อการตรวจสอบย้อนกลับ ปลานิลจากฟาร์มมหาวิทยาลัยเทคโนโลยีสุรนารี (มทส) (DEVELOPMENT OF BIOLOGICAL PROBES FROM MICROFLORA TO ASSURE TRACEABILITY OF TILAPIA FROM SURANAREE UNIVERSITY OF TECHNOLOGY (SUT) FARM) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.มารินา เกตุทัต-คาร์นส์, 56 หน้า

การศึกษานี้มีจุดมุ่งหมายในการพัฒนาเครื่องหมายชีวภาพ เพื่อการตรวจสอบย้อนกลับปลานิลจากฟาร์มมหาวิทยาลัยเทคโนโลยีสุรนารี (มทส) ด้วยเทคนิค denaturing gradient gel electrophoresis (DGGE) ตัวอย่างปลานิลจำนวน 5 ตัวต่อครั้งถูกเก็บมาจากบ่อเลี้ยงจากฟาร์มมทสและแหล่งอื่น พบประชากรแบคทีเรียจากตัวอย่างปลาจากฟาร์มมทส ในฤดูฝนมีค่าแปรผันอยู่ระหว่าง 1.6 x 10^6 ถึง 5.1 x 10^7 cfu g^{-1} ฤดูหนาว 8.9 x 10^5 ถึง 1.3 x 10^7 cfu g^{-1} และฤดูร้อน 6.8 x 10^6 ถึง 7.5 x 10^7 cfu g^{-1} ด้วยการเลี้ยงบนอาหารเลี้ยงเชื้อ ส่วนประชากรแบคทีเรียจากแหล่งอื่นๆมีปริมาณที่น้อยกว่าปริมาณแบคทีเรียจากฟาร์มมทส โดย 73% ของประชากรแบคทีเรียทั้งหมดเป็นแบคทีเรียแกรมลบ ตัวอย่างสารพันธุกรรมจากบริเวณเหงือกและลำไส้ปลาถูกสกัดและใช้เป็นแม่-แบบในการเพิ่มจำนวน 16S rDNA ของจุลินทรีย์ โดยใช้ไพรเมอร์ที่มี GC clamp ได้ทำการศึกษาเปอร์เซ็นต์โพลีอะคริลาไมด์เจล denaturant ระยะเวลา และค่าความต่างศักย์ไฟฟ้าที่เหมาะสมที่สามารถแยกความแตกต่างของสารพันธุกรรมของจุลินทรีย์จากปลานิล ให้ได้ประสิทธิภาพที่ดีที่สุด สภาวะที่เหมาะสมของ DGGE สำหรับการศึกษาประชากรจุลินทรีย์ในปลา พบว่าที่สภาวะเจลโพลีอะคริลาไมด์ 8% ความเข้มข้น denaturant 30-60% ความต่างศักย์ 120 โวลต์ และเวลา 12 ชั่วโมง เป็นสภาวะที่เหมาะสมสำหรับการศึกษาในครั้งนี้ แต่ได้ปรับเปลี่ยนสภาวะ DGGE อีกเล็กน้อย คือ ลดความต่างศักย์ที่ใช้เป็น 100 โวลต์ และเพิ่มระยะเวลาในการทดสอบเป็น 18 ชั่วโมง ซึ่งทำให้ได้ผลการทดลองที่คมชัดขึ้น และพบว่ามีแถบดีเอนเอ 3 แถบที่พบเฉพาะในตัวอย่างปลาจากฟาร์มมทส ในทุกฤดูแต่ไม่พบจากแหล่งอื่น เมื่อนำแถบดีเอนเอทั้ง 3 แถบไปหาลำดับนิวคลีโอไทด์และเปรียบเทียบข้อมูลจาก NCBI พบว่าทั้ง 3 แถบมาจากแบคทีเรียที่ไม่สามารถเพาะเลี้ยงในอาหารเลี้ยงเชื้อ (uncultured bacteria) ได้ และเป็นคนละชนิดกัน และเมื่อออกแบบไพรเมอร์จากลำดับนิวคลีโอไทด์ของทั้ง 3 ตัวอย่างนี้แล้ว ใช้ในการเพิ่มชิ้นดีเอนเอจากตัวอย่างทั้งจากฟาร์มมทส และแหล่งอื่น พบว่ามีเพียงชุดไพรเมอร์ D2 เท่านั้นที่สามารถเพิ่มชิ้นดีเอนเอจากเฉพาะตัวอย่างฟาร์มมทส ซึ่งมีขนาด 120 bp ได้ แต่ตัวอย่างจากแหล่งอื่นรวมทั้งเชื้อบริสุทธิ์ไม่สามารถ

ใช้ไพเมอร์คู่นี้ในการเพิ่มจำนวนดีเอนเอตามขนาดที่ถูกต้องได้ ดังนั้นชุดไพเมอร์ D2 จึงน่าจะใช้เป็นตัวบ่งชี้ตัวอย่างจากฟาร์มมทส ได้

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2552

ลายมือชื่อนักศึกษา _____
ลายมือชื่ออาจารย์ที่ปรึกษา _____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

DARAWAN RUAMKUSON : DEVELOPMENT OF BIOLOGICAL PROBES FROM MICROFLORA TO ASSURE TRACEABILITY OF TILAPIA FROM SURANAREE UNIVERSITY OF TECHNOLOGY (SUT) FARM. THESIS ADVISOR : ASST. PROF. MARIENA KETUDAT-CAIRNS, Ph.D., 56 PP.

TILAPIA/MICROBIAL COMMUNITY/MICROFLORA/GC-CLAMP/16S rDNA PCR-DGGE

The bacterial community of Suranaree University of Technology (SUT) tilapia was studied with the aim to develop biological markers for traceability using denaturing gradient gel electrophoresis (DGGE). Five fish per treatment were sampled from SUT farm and other sources. Total viable count (TVC) of bacteria from SUT farm varied between 1.6×10^6 and 5.1×10^7 colony forming units (cfu) g^{-1} in rainy season, 8.9×10^5 and 1.3×10^7 cfu g^{-1} in cool season and 6.8×10^6 and 7.5×10^7 cfu g^{-1} in hot season. The other sources found bacterial load less than sample from SUT farm. Seventy three percent of the bacteria was Gram-negative. Total DNA was extracted from the fish gills and intestine and then used as template to amplify bacterial 16S rDNA using GC clamp primer. Different bacteria have different DNA sequences that will be denatured at different percentage of polyacrylamide gel, denaturant, run time and voltage. The conditions of DGGE were optimized to screen the fish's bacterial community. The results indicated that 8% of polyacrylamide gel, 30-60% of denaturant concentration, running time of 12hr and voltage of 120V gave the best condition for this screening. However, the DGGE condition was modified by decreasing the voltage to 100V and increasing the running time to 18hr to obtain

better results. The results showed 3 DNA bands on DGGE gel being specific only to bacterial DNA of SUT tilapia when compared to the other sources. All of the 3 DNA bands were sequenced and aligned. The results indicated that they were uncultured bacteria of different species. Primers were designed from the 3 specific sequences and used to amplify DNA samples from four sources and pure cultured bacteria. The results indicate that only primer pair D2 can amplify DNA samples from SUT farm and give a specific band of about 120 bp, but it can not amplify other samples. Therefore, primer D2 can be used to specify samples from SUT farm.

School of Biotechnology

Academic Year 2009

Student's Signature_____

Advisor's Signature_____

Co-advisor's Signature_____

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Darawan Ruamkuson

CHAPTER I

INTRODUCTION

The aquaculture industry is probably the fastest growing food-producing industry in the world. Approximately 50% of all fish produced for consumption is aquaculture. Aquaculture has become an important part of the Thai fishing industry. Furthermore, the Thai fishing segment represents one of the largest national industries in terms of economic value. The export of traditional fish products especially, tilapia from Thailand is increasing and has very good further potential. Tilapia is a tropical fish, which is very well produced; both capture and culture fisheries in inland areas. It is one of the most sought-after fish both in the international and domestic market, especially in Malaysia and Thailand. The demand for tilapia products picked up in Thailand after the economic recession of 1997 and 1998. Currently, tilapia in Thailand is being exported to nearby markets like Singapore and Europe in live, chilled and frozen forms (FAO, 2008). However, fish is perishable product and is suitable substrate for bacterial growth especially pathogenic bacteria, which can cause consumers concerned. So, the food safety and security needed to be monitored throughout the supply chain. The regulations provide a greater degree of assurance in quality and safety that asked industries to trace their products and assure the safety of consumers (EU regulation 178/2002). The ability to identify and validate some pertinent biological markers (bacteria) from the environment of the fish to assure traceability would be of great benefit. Currently, there is no existing scientific

methods that can be followed or determined precisely to the food origin (Le Nguyen *et al*., 2008).

Researchers have been interested in the bacterial diversity of various environmental samples. The knowledge on bacterial diversity is useful for understanding the nature of the sample to be studied (Al-Harbi and Naim Uddin, 2004). For traditional analysis, microflora will be cultured on specific or non-specific growth media. This method consists of colony isolation, phenotypic characterization, morphology, and biochemical test (Huber *et al*., 2004). However, the weaknesses of phenotypic methods are poor reproducibility and laborious investigations are needed. Therefore, genotypic or molecular methods that study the bacterial DNA should be used. Molecular methods are important for detection, identification and characterization of microorganisms found in environmental samples, foods and other complex ecosystems (Murray *et al*., 1996). Investigation of the bacterial diversity in fish can be performed by polymerase chain reaction (PCR) amplification and the most common target region is the 16S rDNA of the bacterial genome. The denaturing gradient gel electrophoresis (DGGE) has been developed and applied for the identification of bacterial microflora in fish. Applications of the molecular methods are needed to improve the understanding of total bacterial community from cultivable technique, which can not cover all microbial diversity in samples (Le Nguyen *et al*., 2008).

In this research, bacterial microflora from tilapia were isolated and compared from each season and from other resources by cultivable technique and PCR-DGGE technique. The genetic markers of bacteria were also designed and used as biological probe to assure traceability.

Research objectives

This research focuses on tilapia, because they are very well produced in Thailand and exported to nearby markets and Europe. Bacterial microflora from tilapia were isolated and compared from each season and from other resources by cultivable technique and PCR-DGGE technique. The genetic markers of bacteria were also designed and used as biological probe to assure traceability. The objectives of this research were summarized below.

1. To optimize the PCR-DGGE condition for bacterial microflora of tilapia from SUT farm.

2. To compare cultivable method and PCR-DGGE method for screening bacterial microflora of tilapia from SUT farm in each season.

3. To apply the PCR-DGGE technique to separate the different bacterial species of tilapia from SUT farm and other resources.

4. To develop biological probes for traceability of tilapia from SUT farm.

CHAPTER II

LITERATURE REVIEW

2.1 Tilapia

Aquaculture plays an increasingly important role in food security and the economy of Thailand. In 2003, aquaculture production was around 1.064 million tonnes with the valued of US$ 1.46 billion contributing around one quarter of the total fisheries production (FAO, 2008).

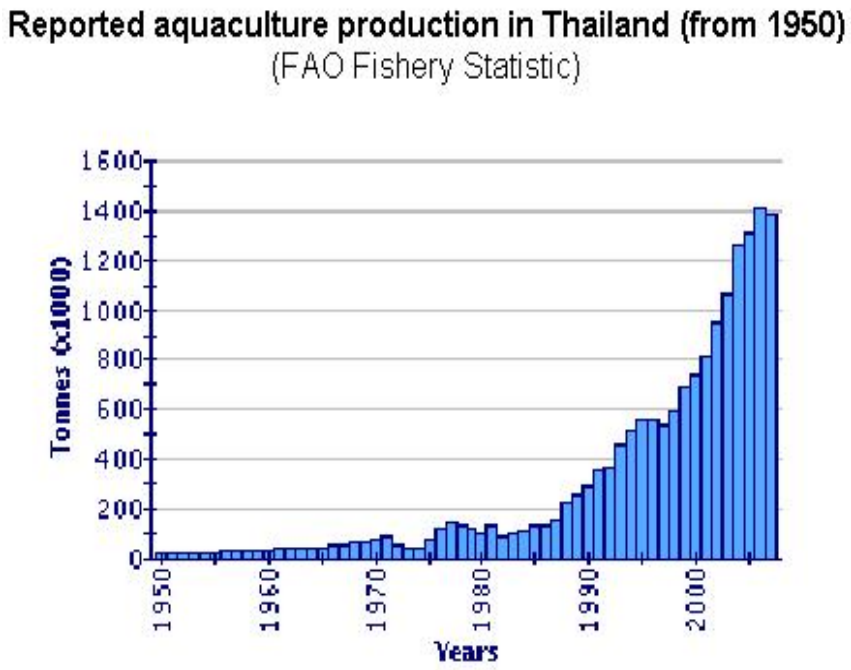


Figure 2.1 Total aquaculture production in Thailand according to FAO statistics.

In 2003, total Thai freshwater aquaculture production was estimated at around 0.32 million tonnes, the top five species consist of Nile tilapia (*Oreochromis niloticus*), hybrid catfish (*Clarias macrocephalus* x *C. gariepinus*), Java barb (*Barbodes gonionotus*), giant river prawn (*Macrobrachium rosenbergii*) and snakeskin gourami (*Trichogaster pectoralis*) but the main freshwater species cultured were Nile tilapia (FAO, 2008). World tilapia production has been booming during the last decade, with output doubling from 0.83 million tonnes in 1990 to 1.6 million tonnes in 1999 and over 2.5 million tonnes in 2005. Figure 2.2 showed that aquaculture was the main responsible for the increase, while capture fisheries of tilapia stayed more or less stable over the years (Josupeit, 2007).

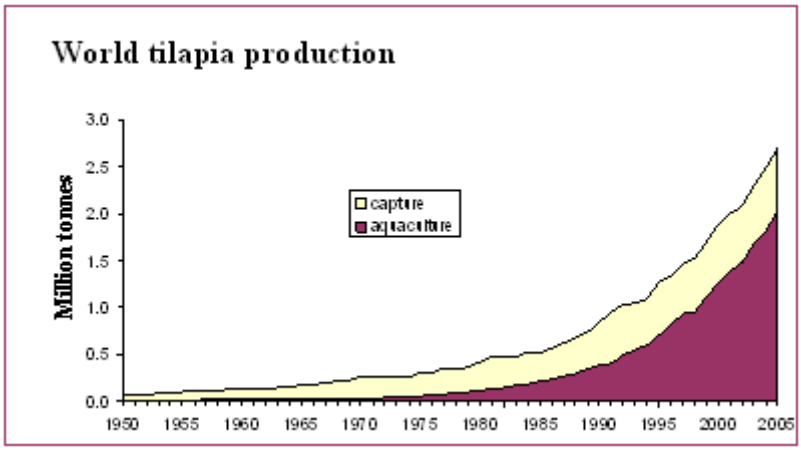


Figure 2.2 The growth of world tilapia production (Josupeit, 2007).

The production of tilapia from Thailand contributes around 29 percent (83,780 tonnes) of total freshwater aquaculture production. Freshwater products are mainly for domestic consumption with around 72 to 75 percent consume in fresh/live form and 18 to 19 percent in several processed forms. Export accounts for only around 6

percent of the cultured freshwater fish production, mostly in fresh/chilled and frozen forms. Thailand has discovered and opened the EU market for tilapia products (Josupeit, 2007). However, EU concerned about pathogenic bacteria and stated that the food safety and security needed to be monitored throughout the supply chain. The regulations provide a greater degree of assurance in quality and safety that asked industries to trace their products and assure the safety of consumers (EU regulation 178/2002). The ability to identify and validate some pertinent biological markers (bacteria) which came from the environment of the fish to assure traceability would be great benefit (Le Nguyen *et al*., 2008).

2.2 Cultivable technique

Cultivable technique has been used to identify bacterial microflora in fish. Generally, microflora is related to habitat of fish, which varies with several factors such as salinity of the habitat and bacterial load in water, which lead to the colonization of predominant microflora. The samples such as skin surface, gills and intestine of fish have been selected to screen bacterial microflora. Many researches studied bacterial microflora in skin and gills using cultivable technique such as Colwell (1962) studied microflora of Puget Sound fish caught by different fishing methods. The genus *Pseudomonas*, *Vibrio*, *Achromobacter, Flavobacterium* and Enterobacteriaceae were found on skin surface (Colwell, 1962). Horsley studied bacterial microflora on Atlantic salmon skin surface, genus *Acinetobacter, Pseudomonas, Aeromonas, Vibrio* and Enterobacteriaceae were found (Horsley, 1973). The study of gills, bacterial microflora recorded genus *Pseudomonas, Vibrio, Aeromonas, Achromobacter, Micrococcus* and others in both marine and freshwater

fish (Colwell, 1962; Simidu *et al*., 1969; Mudarris and Austin, 1988). However, bacteria from skin surface and gills may be more transient than resident depend on the catching methods, which might lead to the decrease of the microflora (Colwell, 1962; Gillespie and Macrae, 1975). Storage time and methods can also change the microflora and increase the contaminants (Shewan, 1971). They may be contaminated from equipments and/or handling so, most researches studied the microflora in the intestinal tract. Bacteria existed in intestinal tract are mainly related with the environment or diet and they can survive and vary with the complexity of fish digestive system (Sakata *et al*., 1980). Some bacteria isolated from intestinal tract of sick fish can also be isolated from healthy fish (Trust and Sparrow, 1974). Nevertheless, the association between intestinal microflora and fish disease remain unclear, because some microflora synthesized vitamins that are lacking in the feed and demonstrated to enhance the growth of fish (Trust and Sparrow, 1974; Robinson and Lovell, 1978; Limsuwan and Lovell, 1981). They can cause diseases when fish are under stress condition e.g. stocked at high density or subjected to poor environmental conditions. More understanding and studying of bacteria in their environment and host's physiology led to the conclusion that bacterial diseases are mainly related to stress (Molinari *et al*., 2003). The bacterial microfloras of intestinal tract from marine and freshwater fish have been investigated by many researchers. Genus *Pseudomonas, Vibrio, Bacillus, Aeromonas, Micrococcus* and Enterobacteriaceae have been found in fish intestinal tracts (MacFarlane *et al*., 1986; Sakata and Koreeda, 1986; Austin and Al-Zahrani, 1988). Moreover, some researches also found lactic acid bacteria (LAB) and fungi in samples (Newman *et al*., 1972; Sugita *et al*., 1983; Nair and Surendran, 2005). However, bacterial

communities established within environmental ecology are not easily isolated and cultivated on media. Only very small amount, typically 1% of the total bacteria present are cultivable on agar. Especially bacterial community from skin surface, it has been reported that lower than 0.01% can be cultivated on agar (Bernadsky and Rosenberg, 1992; Amann *et al.*, 1995). Therefore, molecular method (PCR-DGGE) becomes important because it can identify both the cultivable and uncultivable bacteria.

2.3 Uncultivable technique

Investigation of the bacterial diversity in fish can be performed by genetic amplification method such as PCR, because it takes short time and shows high level of specificity (Amann *et al.*, 1995). The most common target region for PCR amplification is the 16S ribosomal DNA (rDNA). The 16S rDNA has several attributes that make it suitable for bacterial identification. It occurs in all bacteria and consists of both variable and conservative regions that can be used to separate the different species. Furthermore, the 16S rDNA shows high degree of functional constancy (Li *et al*., 2006). The 16S rDNA can be easily and rapidly sequenced. Online databases of available sequences, e.g. the EMBL database from the European Bioinformatics Institute (http://www.ebi.ac.uk/embl/) and the BLAST databases at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/), allow direct comparison of the achieved sequences. The 16S rDNA sequencing is a powerful tool to identified unknown bacterial populations and species (Vandamme *et al.*, 1996). Researchers have been able to design primers for detection of bacterial species. Several primers have been used to amplify variable regions of the rDNA using universal primers

intended for population analyses (Ercolini, 2004). Theoretically, the amplification of the 16S rDNA can be done with universal primers for all bacterial samples and the universal primers flanking the variable region V3 are commonly used (Cocolin *et al.*, 2007).

Many molecular methods have been developed for identification of microorganisms in complex samples. One of them, the most favor is the denaturing gradient gel electrophoresis (DGGE), which is a molecular fingerprinting method that separate similar size (bp), but different sequences of PCR products. DGGE used polyacrylamide gel that contains denaturing gradient of urea and formamide to separate the DNA of bacterial population and the diversity in the samples can be detected. In 1983, the theoretical aspects of this method was described for the first time (Fischer and Lerman, 1983). Separation of PCR products in DGGE is based on the electrophoretic mobility of partially melted doubled-stranded DNA molecules of differences in the GC content at constant temperature (60^{o}C) (Muyzer and Smalla, 1998). To prevent the complete denaturation of the double strand DNA, a 30-50 GC-rich sequence is added at the 5'-end of one primer. This GC-clamp acts as a melting restrictive domain (Sheffield *et al*., 1989). Molecules with different sequences would have a different melting behaviour and would stop migrating at different position in the gel (Muyzer *et al.*, 1993). PCR-DGGE had already been used to investigate several patterns of distribution of marine bacterial assemblages (Murray *et al.*, 1996; Øvreås *et al.*, 1997; Moeseneder *et al.*, 1999; Riemann *et al.*, 1999). Application for the traceability studies in freshwater fish has also been performed (Le Nguyen *et al.*, 2008). The specific advantages of this technique have been used for the analysis of cultivable and uncultivable, anaerobic and aerobic bacteria. DGGE also provides a

rapid method to observe the changes in community structure in response to different environmental factors (Yang *et al.*, 2001).

Many researchers used this technique, such as Yang *et al.,* 2001 used this DGGE technique with 8% (w/v) polyacrylamide gel, and a denaturing gradient ranging from 30% to 70% at 200V for 5hr to identify soil bacteria (Yang *et al.*, 2001). In 2007, Hovda *et al.* used 8% (w/v) polyacrylamide gel, with a denaturing gradient ranging from 30% to 55% at 70V for 18hr (Hovda *et al.*, 2007) and Le Nguyen *et al.,* 2008 used 8% (w/v) polyacrylamide gel, with a denaturing gradient ranging from 30% to 60% at 120V for 12hr (Le Nguyen *et al.*, 2008) to identify bacterial community in fish. In 2008, Liew and Jong used 10% (w/v) polyacrylamide gel, with a denaturing gradient ranging from 30% to 70% at 120V for 5hr to identify microbial diversity in a Malaysian crude oil (Liew and Jong, 2008).

The samples in each location will have different bacterial community so; the optimal condition of DGGE for the sample in some location may be not suitable for other locations. In some cases, several DGGE conditions were followed but no good results were obtained. Therefore, the screening DGGE conditions for bacterial community in each location should be optimized.

In Thailand, tilapia is very well produced and exported to nearby markets and Europe. The traceability is needed to follow fish origin. Microflora can be used as biological probes. The PCR-DGGE analysis of fish bacterial community could be applied to differentiate geographical location. In 2008, Le Nguyen *et al*. showed that biological markers for the specific locations stayed stable among the different seasons and that they show sufficient statistical specificity per farm. Therefore, markers for bacterial DNA can be used as probes for traceability fish products will be developed.

CHAPTER III

MATERIALS AND METHODS

3.1 Fish sampling

The tilapia samples and water in the pond were collected (five fish / time) five times during 12 months (for all seasons) from SUT farm and 3 other resources (2 farms, tilapia were raised and fed in floating baskets that water flow all the time and Nakhon Ratchasima moat, tilapia grown in nature) in only rainy season. The samples were transferred to storage bags and maintained on ice until transported to laboratory. Then, the fish were measured and weighed. Gills and intestine were aseptically removed from each tilapia then, water and some of the gills and intestine were suspended in 0.85% NaCl to isolate bacterial microflora. The remnant of gills and intestine were used for genomic DNA (gDNA) extraction.

3.2 Screening of bacterial community

For each gills and intestine samples, 1 g of intestine or gills were transferred to a test tube containing 9 ml of sterile 0.85% NaCl prepared in water. One milliliter of the solution was serially diluted to 10^{-6}. Zero point one milliliter of the dilutions was spreaded onto plate count agar (PCA, Himedia, India), potato dextrose agar (PDA, Himedia, India) supplemented with 5 mg/ml chlortetracycline·HCl and 5 mg/ml chloramphenical (Oxoid, UK), thiosulfate citrate bile salts sucrose agar (TCBS, Oxoid, UK), Aeromonas agar base supplemented with 5 μg/ml amplicilin (Oxoid,

UK), Pseudomonas agar base contained 5 µg/ml Cetrimide-Fucidin-Cephalosporin (C-F-C) supplement (Oxoid, UK) and de Man, Rogosa and Sharpe (MRS) contained $CaCO_3$ (Oxoid, UK), in duplicate. Aeromonas agar base plates were incubated for 18-24hr, MRS plates for 48hr and PDA plates for 3-5 days at 30°C. PCA plates were incubated for 24hr and TCBS agar plates for 18-24hr at 37°C and Pseudomonas agar plates at 25°C for 18-48hr. Colony forming units (cfu) were counted with a colony counter and readings obtain with ≥ 30 to 300 colonies on plate were used to calculate bacterial population results and recorded as cfu per gram sample.

3.3 Purification and Gram's staining

The colonies were re-streaked to confirm the purification and kept as glycerol stock at -80°C. The purified colonies were Gram's staining followed protocol of Rollins *et al*., 2003 and the morphology was visualized under microscope.

3.4 Total DNA extraction

The protocol of Le Nguyen *et al*., 2008 was followed with some minor modification briefly, gills and intestine were separately crushed in liquid nitrogen and 0.2 g were transferred into 1.5 ml microcentrifuge tube, then 720 µl extraction buffer (1M Tris-HCl pH8, 0.5M EDTA, 5M NaCl and 10% SDS) was added. Samples were incubated at 65°C in heat box and vortex every 5 min 3 times and 225 µl of 5M potassium acetate was added and mixed then, incubated on ice and shaken for 20 min. The suspensions were then centrifuged at 16,000 x g for 15 min and 750 µl of supernatant was transferred to new microcentrifuge tubes. The DNA was precipitated with 500 µl of cold isopropanol then, centrifuged at 16,000 x g for 10 min. The

supernatant was removed and washed with 300 µl of 70% cold ethanol and centrifuged at 8,160 x g for 10 min then, the supernatant was removed and air dried at room temperature. Finally, the DNA was re-suspended in 30 µl of TE buffer and stored at -20^{o}C until analysis.

3.5 PCR-DGGE analysis

3.5.1 16S rDNA amplification

Bacterial 16S rDNA from tilapia was amplified using primers GC338f (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACG GGAGGCAGCAG-3') and 518r (5'-ATTACCGCGGCTGCTGG-3') (Øvreås *et al.*, 1997; Ampe *et al.*, 1999). Each mixture (final volume 50 µl) contain approximately 2 ng template DNA, 0.4 µM primers, 0.2 mM dNTP, 2 mM $MgCl_2$, 1x of $MgCl_2$ free buffer (promega, USA) and *Taq* polymerase (homemade @ SUT). First stage; 1 cycle at 94^{o}C for 5 min, second stage; 35 cycles at 94^{o}C for 30 sec, 55^{o}C for 30 sec and 72^{o}C for 30 sec and the last stage; at 72^{o}C for 7 min. PCR products (3 µl) were analyzed first by conventional electrophoresis on 2% (w/v) agarose gel with 1x TAE buffer and quantified using a standard (DNA mass ladder 100 bp, promega, USA).

3.5.2 Optimal condition for DGGE of 16s rDNA

The PCR products were then analyzed by DGGE using DCode^{TM} universal mutation detection system (Bio-Rad Laboratories, USA). PCR products were separated onto several gel conditions. The percent of polyacrylamide gel (8% and 10% (w/v)), percent of denaturing gradient (ranging from 30% to 55%, 30% to 60% and 30% to 70%), voltage (70V, 120V and 200V) and running time (5hr and 12hr)

were varied. All electrophoresis experiments were performed at 60^{o}C in 1x TAE buffer. The gels were stained for 1 min with 1.0 μg/ml ethidium bromide and rinsed for 15 min in water and then photographed on an UV transilluminator.

3.5.3 Sequencing and alignment

The DNA bands specific for only SUT samples from all seasons were eluted from polyacrylamide gel and purified and then sent to Macrogen for sequencing. The sequencing results were aligned using NCBI blast with databases.

3.6 Specific primers development

The 16S rDNA sequences of specific bacteria from SUT farm were aligned and specific oligonucleotide primers for these sequences were designed using DNASTAR Lasergene v7 program. The extracted DNA samples were used as template to amplify using these newly designed specific primers. Firstly, PCR products were analyzed by conventional electrophoresis on 2% (w/v) agarose gel with 1x TAE buffer and quantified by using a standard (DNA mass ladder 100 bp, promega) and finally, confirm by polyacrylamide gel electrophoresis (PAGE). DNA samples from other resourcess were amplified also to make sure that these primers were specific only for samples from SUT.

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Screening of bacterial community using media

Five tilapia were collected each time (five times in all seasons from SUT farm, twice from farm #1, and once from farm #2 and Nakhon Ratchasima moat). In the past, SUT farm sold adult tilapia but, the farm got some problems about water and pond space to cultivate them so, SUT farm change to cultivate only small tilapia (fingerling) to sell to farmers. Therefore, only fingerlings were obtained from SUT farm. For the two other farms, tilapia were adult sized. The tilapia were bought from the farms and tilapia from Nakhon Ratchasima moat were caught by ourselves. Only fingerlings were caught. Bacterial community from fish skin surface, gills and intestine were investigated using 6 media (PCA, MRS, Pseudomonas agar medium, Aeromonas agar medium, TCBS and PDA). When compared bacterial load in samples from SUT in all seasons the results showed that, total viable count of bacterial load were not different although temperature is quite different (Table 4.1 and Figure 4.1). When compared bacterial load from SUT and three other resources in rainy season, the results indicated that, SUT farm showed more bacterial load than the three other resources (Figure 4.2). This is due to the tilapia from SUT farm is raised in close system that the feed and everything remained there, which may lead to more colonization of bacteria in the system. For the two other farms, tilapia are raised and fed in the open system in the float baskets that water flow all the time so, colonization

of bacteria are not much. For Nakhon Ratchasima moat, less bacterial load than SUT farm were found because, they are grown in nature that do not have feed remain in water. When compared with the two other farms, Nakhon Ratchasima moat have more bacterial load than the two other farms because, it is more of a close system and water was not as clean as the farms, with the water flow all the time so, more colonization of bacteria than two other farms was observed (Table 4.2 and Figure 4.3). The results from PDA medium indicated that small number of yeast and fungi were found in samples from SUT farm and Nakhon Ratchasima moat. But, sample from the two other farms no yeast and fungi were found.

Table 4.1 Show the temperature in the pond, weight and length of tilapia and bacterial load of tilapia from SUT farm in different seasons of the year on different media.

Seasons	Weight (g)	Length (cm)	Samples	PCA (cfu/g)	MRS (cfu/g)	Ps agar (cfu/g)	Aero agar (cfu/g)	TCBS (cfu/g)	PDA (cfu/g)
Rainy	27.2±1.5	12.4±1.1	Water	$4.8\pm1.1\times10^{5}$	$3.7\pm1.1\times10^{3}$	$1.2\pm1.3\times10^{4}$	$2.8\pm1.9\times10^{4}$	$1.7\pm1.1\times10^{3}$	$6.0\pm1.8\times10^{1}$
(27-30 °C)			Gill	$7.4\pm1.1\times10^{6}$	$7.4\pm1.1\times10^{4}$	$6.8\pm1.1\times10^{4}$	$8.6\pm1.7\times10^{5}$	$7.4\pm1.6\times10^{3}$	nd
			Intestine	$1.6\pm1.5\times10^{6}$	$1.5\pm0.9\times10^{5}$	$2.6\pm1.1\times10^{4}$	$3.6\pm2.1\times10^{5}$	$3.6\pm2.4\times10^{3}$	nd
Cool	30.2±2.4	13.4±1.7	Water	$2.3\pm1.5\times10^{5}$	$4.3\pm2.1\times10^{3}$	$1.8\pm1.4\times10^{4}$	$1.1\pm0.9\times10^{3}$	$1.3\pm1.1\times10^{2}$	$4.0\pm1.7\times10^{1}$
(25-27 °C)			Gill	$3.6\pm1.3\times10^{6}$	$1.9\pm1.5\times10^{5}$	$9.6\pm2.2\times10^{4}$	$9.3\pm2.5\times10^{4}$	$2.5\pm1.3\times10^{3}$	nd
			Intestine	$8.9\pm1.5\times10^{5}$	$5.6\pm2.3\times10^{5}$	$5.7\pm1.5\times10^{4}$	$1.9\pm1.6\times10^{4}$	$2.0\pm1.1\times10^{3}$	nd
Hot	21.7±1.7	11.5±1.0	Water	$3.6\pm1.4\times10^{5}$	$1.3\pm1.1\times10^{3}$	$3.1\pm1.5\times10^{3}$	$1.6\pm1.2\times10^{4}$	$3.6\pm2.4\times10^{4}$	$3.0\pm1.7\times10^{1}$
(29-35 °C)			Gill	$2.4\pm1.5\times10^{7}$	$4.2\pm1.1\times10^{4}$	$7.4\pm3.1\times10^{3}$	$4.6\pm2.1\times10^{5}$	$8.4\pm1.5\times10^{4}$	nd
			Intestine	$6.8\pm2.3\times10^{6}$	$7.1\pm1.5\times10^{4}$	$5.5\pm2.5\times10^{3}$	$8.8\pm1.5\times10^{4}$	$4.5\pm1.8\times10^{4}$	nd

**nd = non detected at lowest dilution (10^{-1})

Table 4.2 Show the temperature in the pond, weight and length of tilapia and bacterial load of tilapia from three other resources in the rainy seasons only on different media.

Farm	Weight (g)	Length (cm)	Samples	PCA (cfu/g)	MRS (cfu/g)	Ps agar (cfu/g)	Aero agar (cfu/g)	TCBS (cfu/g)	PDA (cfu/g)
#1	661.8 ±	32.7±4.8	Water	$3.2\pm1.5\times10^{2}$	$2.0\pm1.6\times10^{2}$	$1.3\pm0.9\times10^{2}$	$1.1\pm0.9\times10^{2}$	$7.0\pm2.4\times10^{1}$	nd
(25 °C)	112.5		Gill	$5.5\pm1.1\times10^{4}$	$2.0\pm1.3\times10^{4}$	$7.6\pm1.1\times10^{3}$	$6.8\pm1.4\times10^{3}$	$2.5\pm1.7\times10^{3}$	nd
			Intestine	$2.2\pm1.3\times10^{4}$	$1.5\pm1.2\times10^{4}$	$3.3\pm1.3\times10^{3}$	$3.4\pm0.9\times10^{3}$	$1.4\pm1.1\times10^{3}$	nd
#2	648.4 ±	35.8±3.8	Water	$3.6\pm2.1\times10^{2}$	$2.1\pm1.1\times10^{2}$	$1.5\pm1.2\times10^{1}$	$1.7\pm0.9\times10^{1}$	$9.0\pm2.1\times10^{1}$	nd
(25 °C)	125.2		Gill	$4.5\pm2.0\times10^{4}$	$1.7\pm1.1\times10^{4}$	$5.9\pm2.1\times10^{3}$	$4.4\pm0.9\times10^{3}$	$2.3\pm1.3\times10^{3}$	nd
			Intestine	$3.7\pm1.9\times10^{4}$	$1.2\pm1.1\times10^{4}$	$3.1\pm1.8\times10^{3}$	$2.5\pm1.5\times10^{3}$	$1.8\pm1.5\times10^{3}$	nd
NR moat	35.6 ±	16.8±2.6	Water	$2.2\pm1.1\times10^{3}$	$1.7\pm1.2\times10^{2}$	$4.0\pm2.2\times10^{2}$	$7.7\pm1.8\times10^{2}$	$3.0\pm1.5\times10^{2}$	$3.0\pm1.3\times10^{1}$
(26 °C)	7.7		Gill	$5.7\pm1.1\times10^{4}$	$7.2\pm1.5\times10^{3}$	$4.3\pm1.9\times10^{3}$	$3.9\pm1.8\times10^{3}$	$2.4\pm1.7\times10^{3}$	nd
			Intestine	$5.0\pm1.7\times10^{4}$	$3.4\pm1.5\times10^{4}$	$5.1\pm1.8\times10^{3}$	$3.0\pm1.9\times10^{3}$	$1.6\pm1.2\times10^{3}$	nd

** NR moat = Nakhon Ratchasima moat; nd = non detected at lowest dilution (10^{-1})

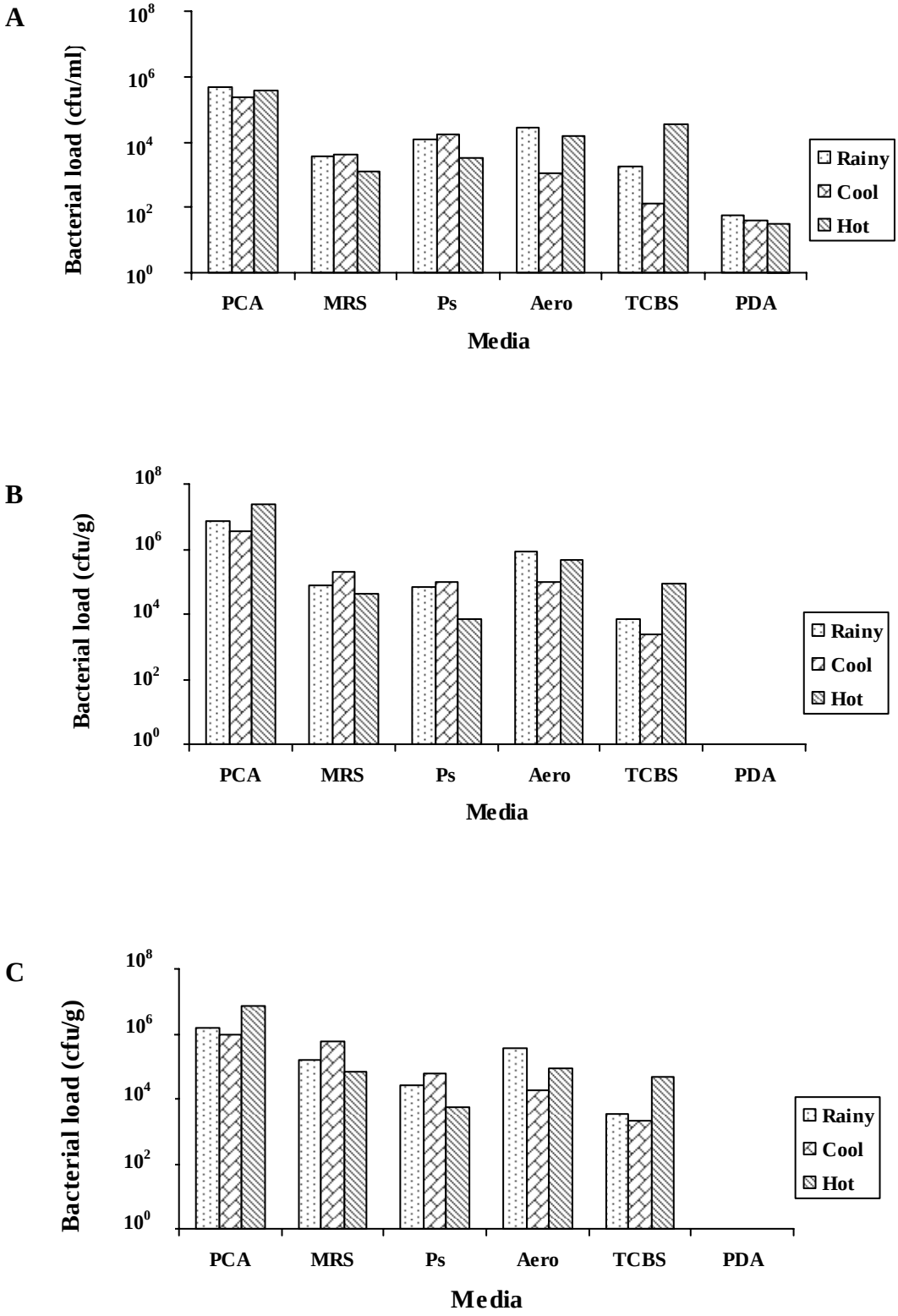


Figure 4.1 Bacterial load of tilapia from SUT farm in different seasons on different media in A; water, B; gills and C; intestine.

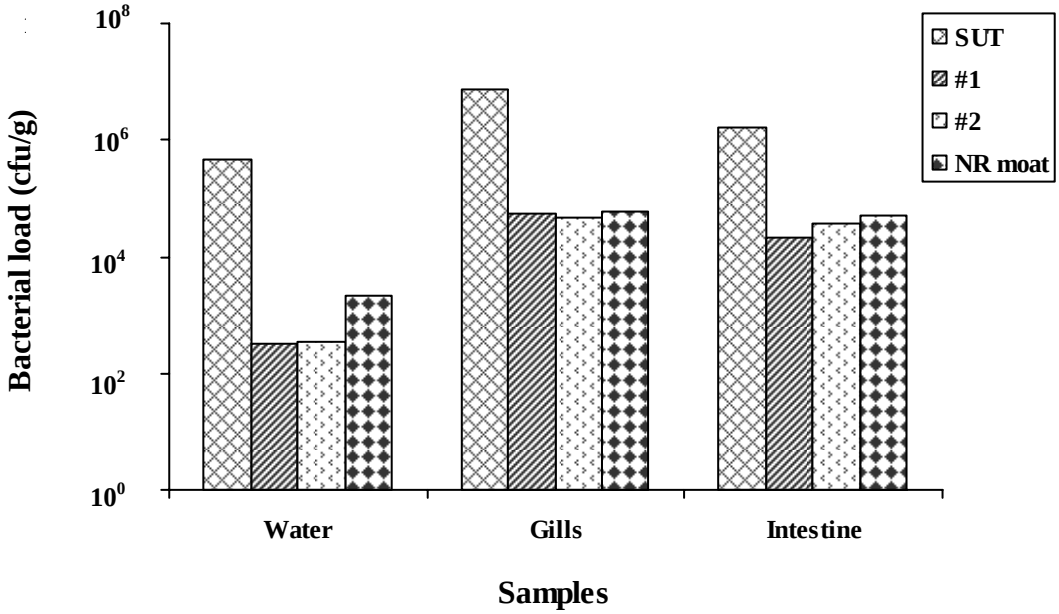


Figure 4.2 Bacteria load on PCA medium of four resources in rainy season.

4.2 Purification and Gram's staining

Total 249 bacterial isolates from all samples were Gram stained. The results indicated that 73% (Figure 4.4A) of the isolates from PCA, Pseudomonas agar base, Aeromonas agar base and TCBS media were Gram-negative rod. Actually, some Gram-negative rod should be Gram-negative crescent-rod of genus *Vibrio*, because the colonial morphology on TCBS medium indicated that, they are this genus but, the photographs were not clear. Therefore, separation between Gram-negative rod and Gram-negative curved-rod were not identified. The figures just looked like Gram-negative rod. The genera *Pseudomonas* and *Aeromonas* grew on Pseudomonas agar and Aeromonas agar media, respectively. After Gram stained, they were Gram-negative rod (Figure 4.4A) and other isolates that isolated from PCA medium also showed cells morphology similar to Figure 4.4A. Eighteen percent of isolates were from MRS medium. They were Gram-positive rod (Figure 4.4B and 4.4C) and 6%

were Gram-positive cocci (Figure 4.4D). Three percent of all isolates were Gram-negative short rod (Figure 4.4E). Identification of genus and species of all isolates should be done both by conventional method and sequencing together to confirm each result. But, it is not necessary because the aim of this research is to find specific bacteria to identify and trace tilapia from SUT farm.

Most of the bacteria were Gram-negative bacteria. Gram-negative bacteria are normal flora of healthy fish. However, some of them can be isolated from unhealthy fish also. Actually, they are opportunistic pathogen that can not harm healthy fish moreover, they synthesized vitamins that are lacked in feed and demonstrated to enhance the growth of fish (Trust and Sparrow, 1974). However, they can cause diseases when fish are under stress condition e.g. stocked at high density or subjected to poor environmental conditions.

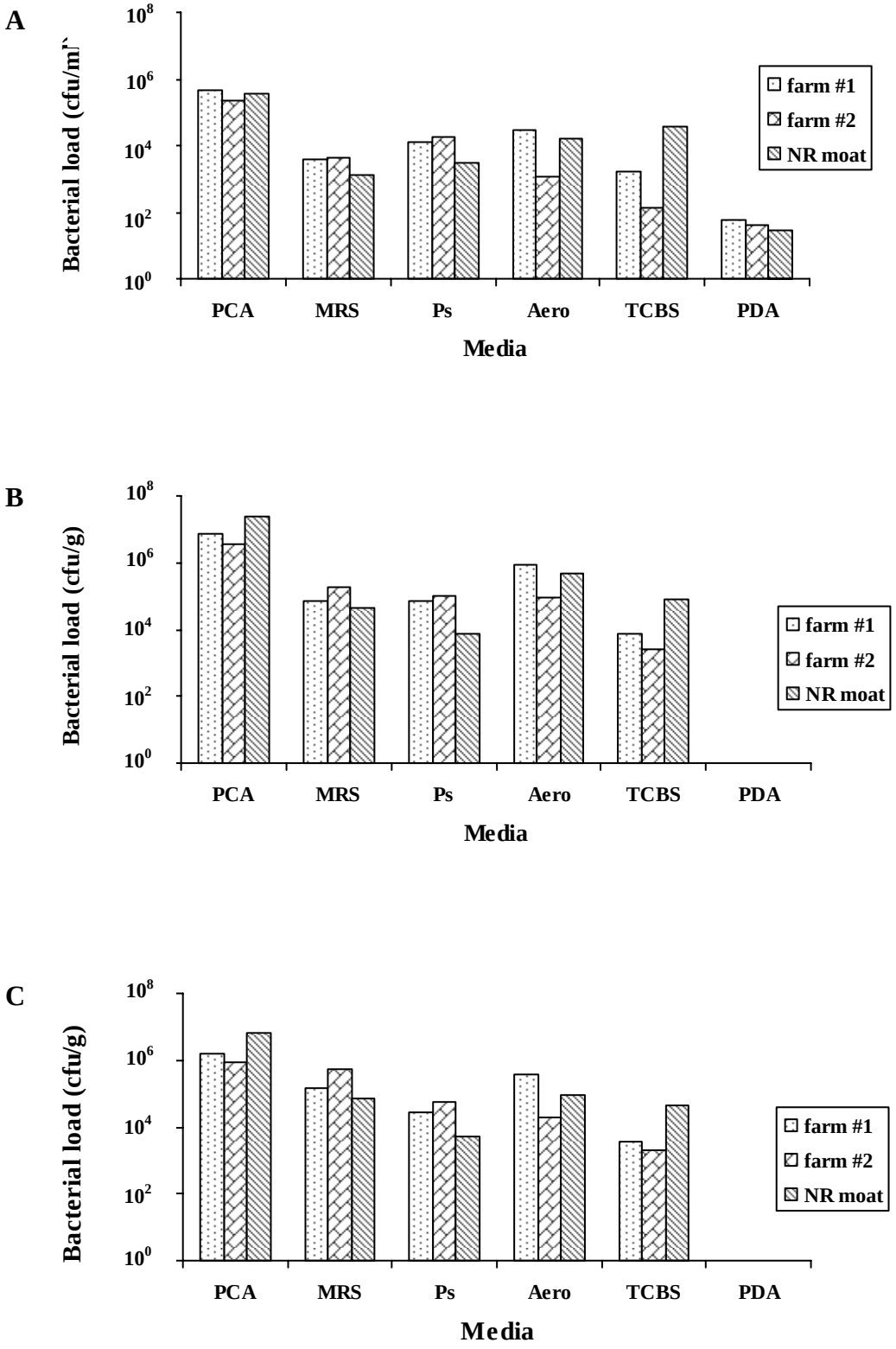
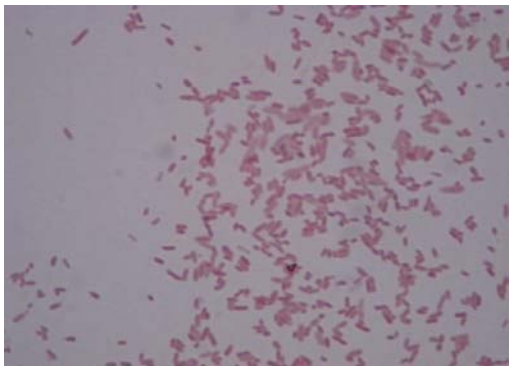
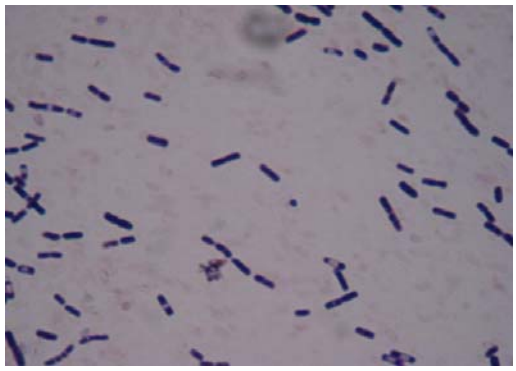


Figure 4.3 Bacterial load of tilapia from three other resources in rainy season on different media in A; water, B; gills and C; intestine).

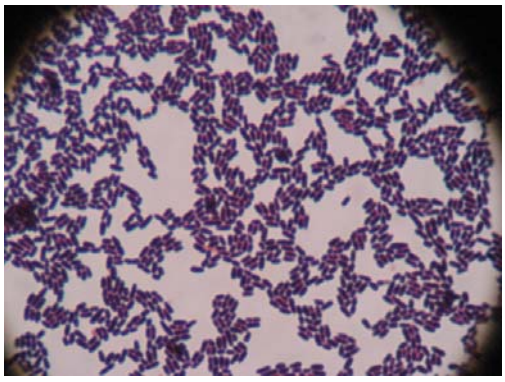
A



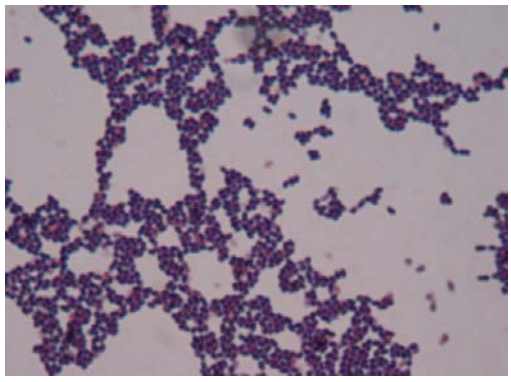
B



C



D



E

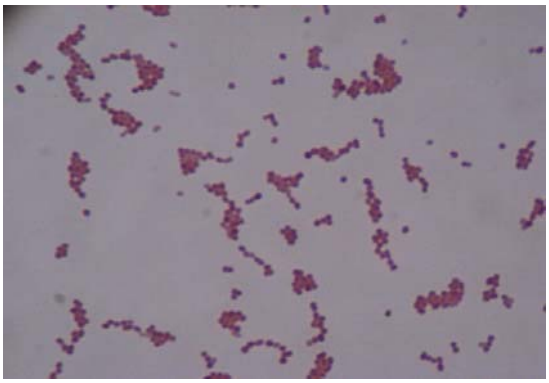


Figure 4.4 Light micrograph magnification: 1000 (A: Gram-negative rod; B, C: Gram-positive rod; D: Gram-positive cocci; E: Gram-negative short rod)**.**

4.3 Total DNA extraction

Genomic DNA samples were separately extracted from intestine and gills of each tilapia from all samples. They were diluted 50x before used as templates for PCR amplification to decrease contaminants such as RNA and salt that can cause trouble in PCR amplification reactions and may led to the decrease of PCR products. Figure 4.5 showed gDNA samples diluted 50x. It was found that the amount of gDNA are quite similar and with good quality.

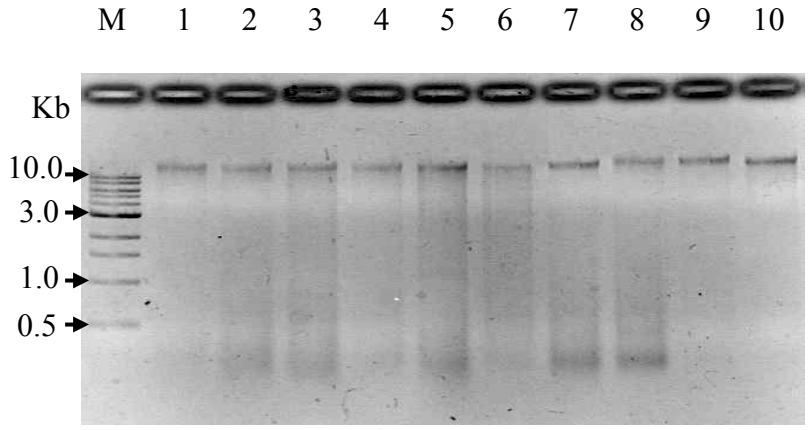


Figure 4.5 Genomic DNA (gDNA) extracted from gills and intestine diluted 50x on 1% agarose gel (M: 1Kb marker; 1-5: gDNA samples from five tilapia gills no.1-5, respectively; 6-10: gDNA samples from five tilapia intestinal tract no.1-5, respectively).

4.4 PCR-DGGE analysis

4.4.1 16S rDNA amplification

PCR amplification was done using GC clamp primer and 50x diluted DNA templates (Figure 4.5) to amplify bacterial 16S rDNA. The results showed that, the PCR products were about 200 bp on agarose gel (Figure 4.6)

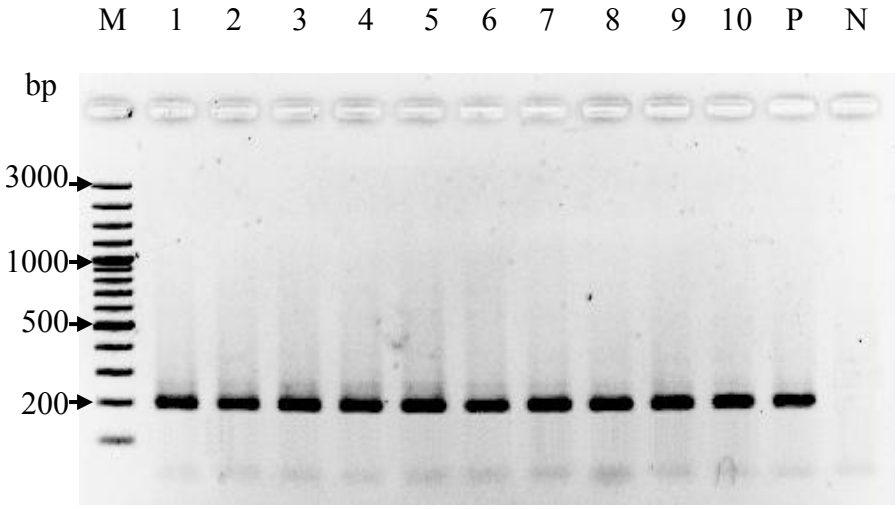


Figure 4.6 PCR products on 2% agarose gel (M: 100bp marker; 1-5, 6-10: DNA samples from gills and intestine of five tilapia no.1-5, respectively; P: positive control (*E. coli*); N: negative control).

4.4.2 Optimal condition for DGGE of 16S rDNA

Many researchers used different conditions of DGGE for bacterial community analysis. Therefore, to obtain good results, optimal condition is important and should be considered. So, the DNA samples extracted from intestinal tract of five tilapia were used as template for optimal condition tests. The results on 2% agarose gel showed that, the PCR products were about 200 bp (Figure 4.6). Then, the rest of the samples were loaded on several conditions of DGGE. When compare the percent of polyacrylamide gel (Figure 4.7) the 8% polyacrylamide gel showed better results than the 10% polyacrylamide gel indicating that the 200 bp GC clamp PCR products were better separated in this condition. This result was in agreement with the results of Le Nguyen *et al*., 2008 that also used 8% polyacrylamide gel to separate bacterial community from fish. However, this result contradicts the results of Liew and Jong, 2008 that use 10% polyacrylamide gel to identify microbial community in crude oil.

This may indicated that separating of bacterial community from fish by DGGE is better in 8% polyacrylamide gel. Kawai *et al*., 2002 used 6.5% polyacrylamide gel to separate bacterial community from water, so we also wanted to try these conditions. However, the gels we prepared were not able to polymerize in all percentage of denaturant (45-55%, 30-60% and 30-70%) that we tested. Even though increased amount of ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) were used, the gels still did not polymerize. Therefore, the 6.5% gel was not tested.

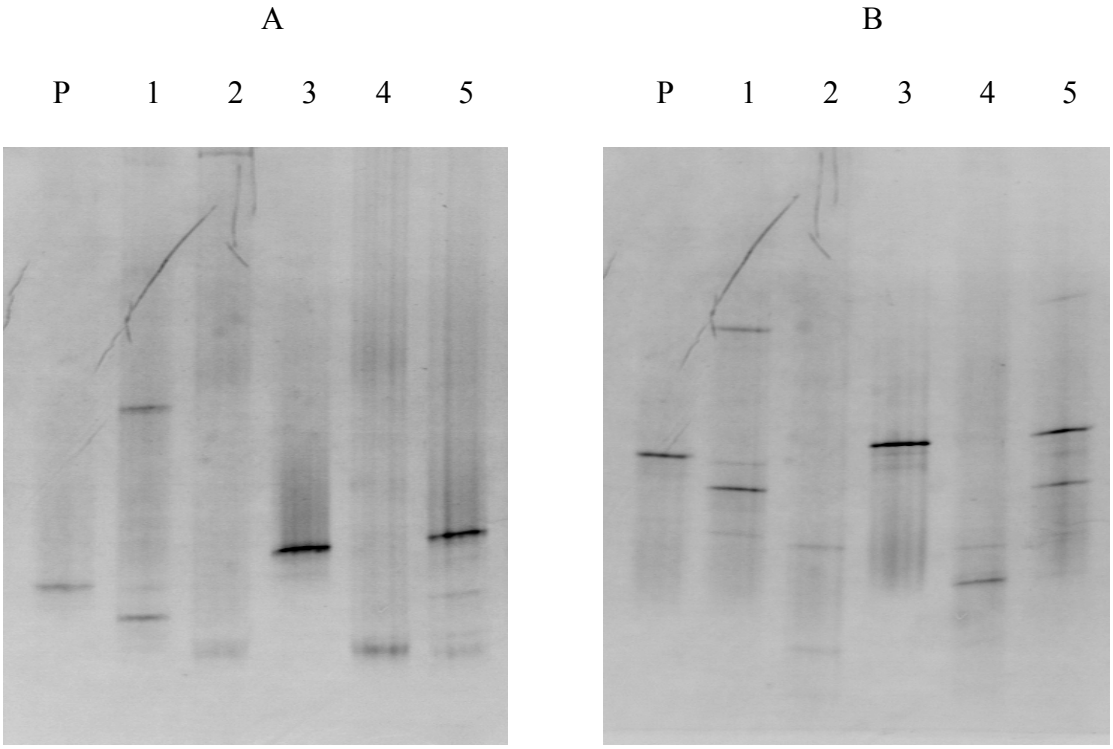


Figure 4.7 DGGE conditions: 30-60% denaturant, 120V and 12hr; A: 10% polyacrylamide gel; B: 8% polyacrylamide gel (1-5: DNA samples from five tilapia intestinal tract no.1-5, respectively; P: positive control (*E. coli*)).

In Figure 4.8 the running time of 12hr showed better results than 5hr running time when 120V was used. In these conditions, 5hr is not enough to separate the PCR products obtained from fish intestinal microflora. However, in the lane P (Figure 4.8A) using *E. coli* as a positive control, we can see that the running time of 5hr (Figure 4.8A lane P) and 12hr (Figure 4.8B lane P) does not show much different.

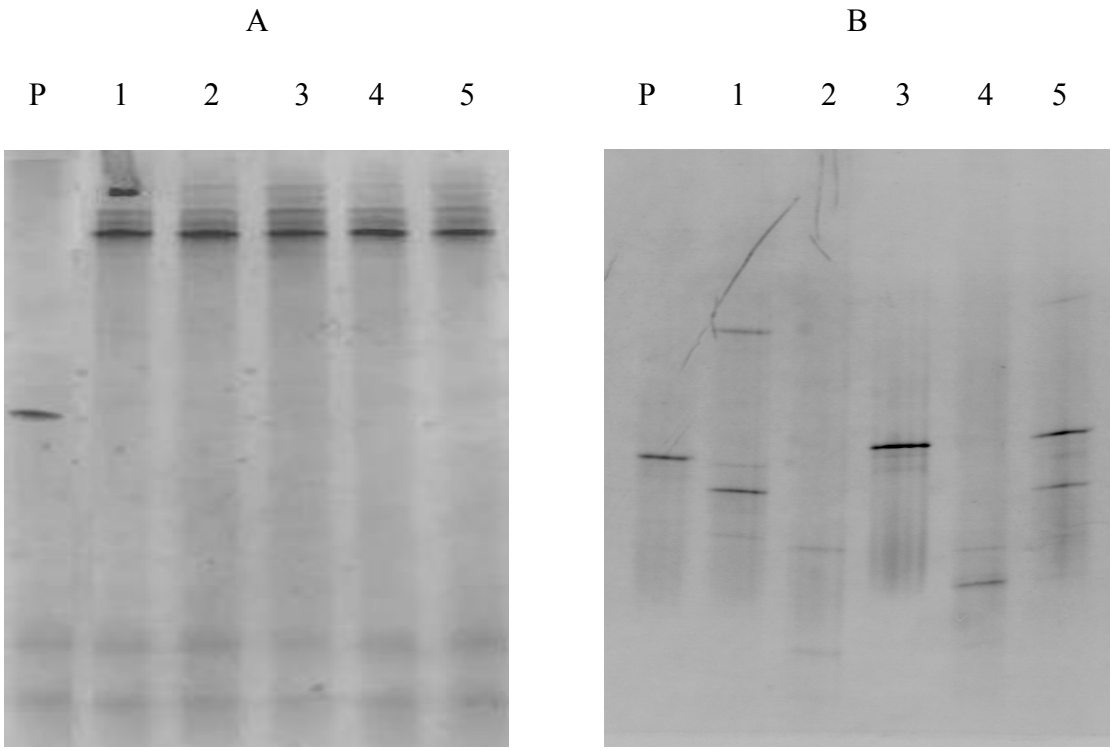


Figure 4.8 DGGE conditions: 8% polyacrylamide gel, 30-60% denaturant and 120V; A: 5hr; B: 12hr (1-5: DNA samples from five tilapia intestinal tract no. 1-5, respectively; P: positive control (*E. coli*)).

In Figure 4.9, when higher percentage of polyacrylamide gel was used (10%) with narrower percent denaturant (44-55%), 5hr running time with either 200V (Figure 4.9A) or 120V (Figure 4.9B), the PCR products from fish intestinal microflora can not be separated, but the PCR products from *E. coli* can be seen clearly

(Figure 4.9A and 4.9B lane P). These results indicated that 10% polyacrylamide gel, 45-55% denaturant and 5hr running time are not good conditions to separate PCR products from fish intestinal microflora.

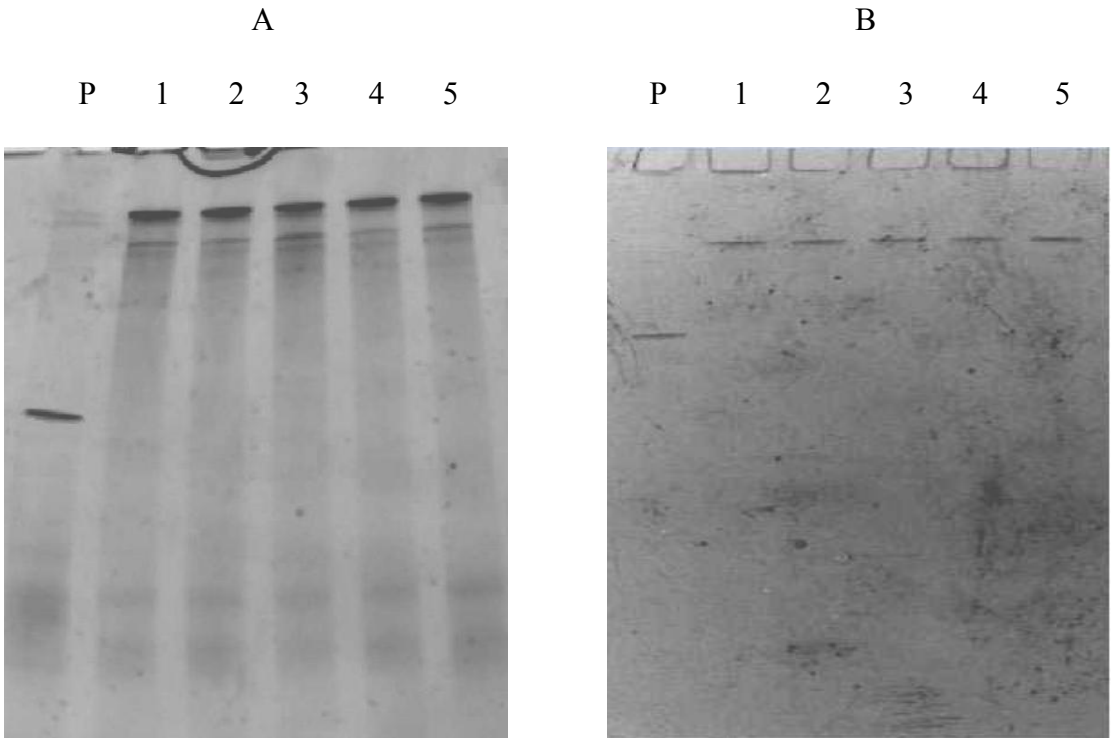


Figure 4.9 DGGE conditions: 10% polyacrylamide gel, 45-55% denaturant and 5hr; A: 200V; B: 120V (1-5: DNA samples from five tilapia intestinal tract no. 1-5, respectively; P: positive control (*E. coli*)).

The comparison of percent denaturant in Figure 4.10A and 4.10B showed that, 30-60% denaturant (Figure 4.10B) give better separation of the PCR products than the 45-55% denaturant (Figure 4.10A). The 30-70% denaturant was also tried; however, at this condition the separation was not better than using 30-60% denaturant. From these experiments, the optimal condition for analysis of bacterial community in tilapia intestinal samples indicated that, the condition of 8% polyacrylamide gel, 30-

60% denaturant, 120V and 12hr (Figure 4.7B, 4.8B and 4.10B) is the best condition for these samples. In the beginning, when we tried to use this optimal condition to analyze DNA bands pattern on DGGE gel, we found that the bands were not sharp, so we tried to adjust some parameters to obtain better results. The DGGE condition was modified by decreased the voltage to 100V and increased the running time to 18hr, so the best DGGE condition (Figure 4.11 - Figure 4.15) to analyze bacterial community in samples from SUT and other resources in this research was 8% polyacrylamide gel, 30-60% denaturant, 100V and 18hr of running time.

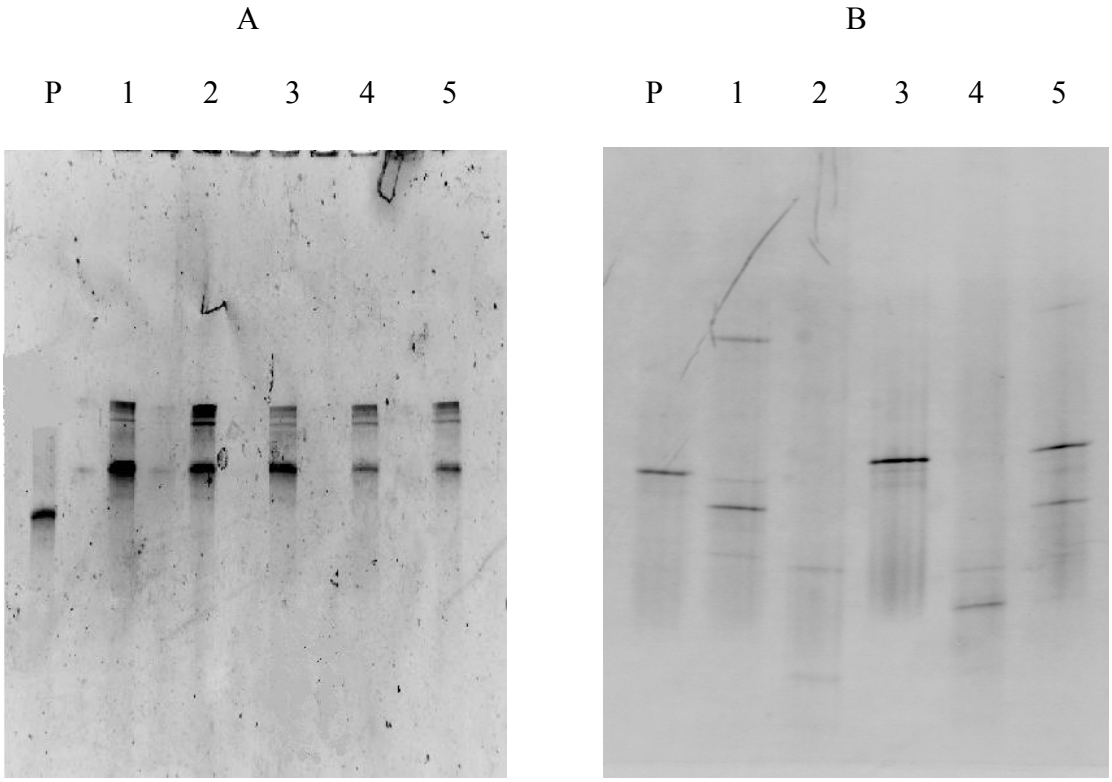


Figure 4.10 DGGE conditions: 8% polyacrylamide gel, 120V and 12hr; A: 45-55%; B: 30-60% denaturant (1-5: DNA samples from five tilapia intestinal tract no. 1-5, respectively; P: positive control (*E. coli*)).

4.4.3 DGGE patterns of 16S rDNA samples from each farm

The total DNA extracted from tilapia gills and intestine were used as template to amplify bacterial 16S rDNA using GC clamp primer and PCR products were loaded on 2% agarose gel to check. Then, the remnant of PCR products were loaded on 8% polyacrylamide gel, 30-60% denaturant, 100V and electrophorese for 18hr. Figure 4.11-4.14 show the DGGE results of samples from SUT farm in three seasons, farm #1, farm #2 and Nakhon Ratchasima moat in only rainy season, respectively. The same pattern of DNA bands on the polyacrylamide gel can be seen from the samples of the same resource even though the DNA samples were from different tilapia (Figure 4.12-4.14). The patterns of DNA bands on the polyacrylamide gel are quite similar in all seasons of samples from SUT farm (Figure 4.11). Figure 4.15 shows the comparison of DNA bands from samples of SUT farm, farm #1, farm #2 and Nakhon Ratchasima moat.

P 1 2 3 4 5 6 P

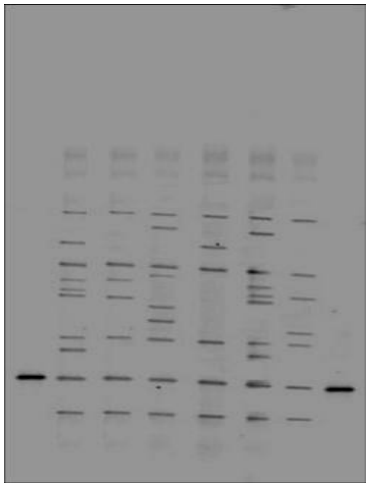


Figure 4.11 The pattern of DNA bands in all seasons of samples from SUT farm (1, 3, 5: DNA samples from intestine; 2, 4, 6: DNA samples from gills; 1-2: DNA samples from hot season; 3-4: DNA samples from cool season; 5-6: DNA samples from rainy season; P: positive control (*E.coli*)).

P 1 2 3 4 5 6 7 8 9 10

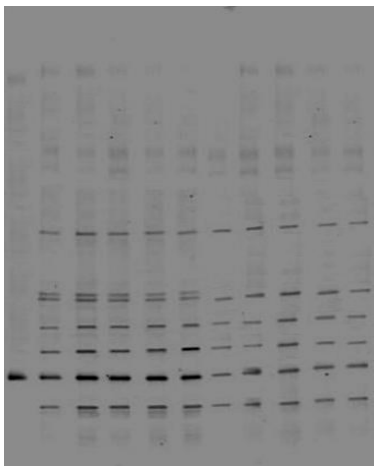


Figure 4.12 The pattern of DNA bands from farm #1 in rainy season (1-5: DNA samples from intestine of fish no.1-5 respectively; 6-10: DNA samples from gills of fish no.1-5, respectively; P: positive control (*E.coli*)).

1 2 3 4 5 6 7 8 9 10 P

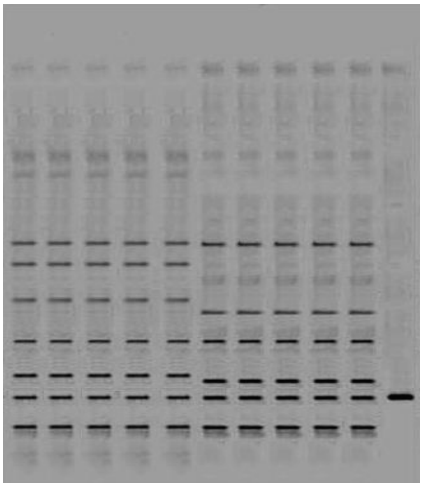


Figure 4.13 The pattern of DNA bands from farm #2 in rainy season (1-5: DNA samples from intestine of fish no.1-5, respectively; 6-10: DNA samples from gills of fish no.1-5, respectively; P: positive control (*E.coli*)).

1 2 3 4 5 6 7 8 9 10 P

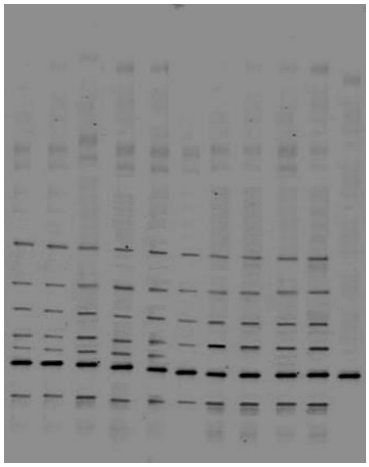


Figure 4.14 The pattern of DNA bands from Nakhon Ratchasima moat in rainy season (1-5: DNA samples from intestine of fish no.1-5, respectively; 6-10: DNA samples from gills of fish no.1-5, respectively; P: positive control (*E.coli*)).

When compared the pattern of DNA bands on polyacrylamide gel from all four resources, the results indicate that, SUT farm showed more bands than the other two farms (Figure 4.15). This is due to the tilapia from SUT farm is raised in close system which may lead to more bacteria in the system. For the other two farms, tilapia are raised and fed in the open running water system that water flow all the time. Therefore, the samples from SUT farm showed more DNA bands assuming more bacterial diversity than the other two farms. For samples from Nakhon Ratchasima moat, even though it not really running water system, but there was no regular feeding of the fish so, no contamination of bacteria from leftover feed were found in the system. Therefore, the samples from Nakhon Ratchasima moat showed less DNA bands assuming less bacterial diversity than SUT farm. Moreover, we found three specific bands of the samples from SUT farm. The three bands showed up at different positions and we assumed that they are different bacterial species (Figure 4.15, arrows).

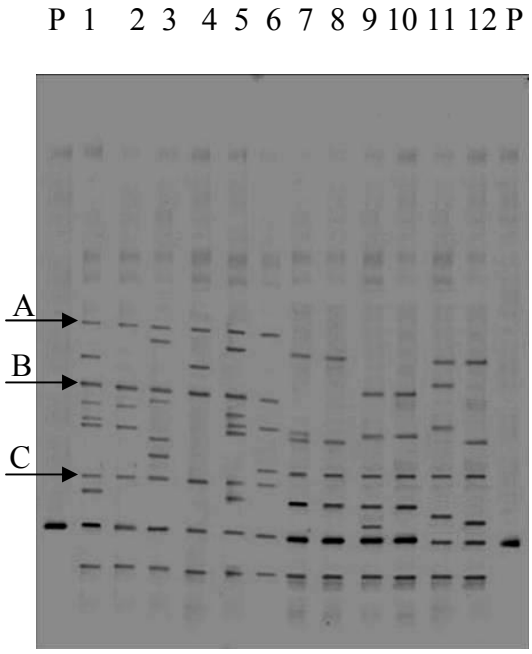


Figure 4.15 Comparison DNA bands between samples from SUT farm, farm #1, Nakhon Ratcharima moat and farm #2 (1-2: DNA samples from SUT farm in hot season; 3-4: DNA samples from SUT farm in cool season; 5-6: DNA samples from SUT farm in rainy season; 7-8: DNA samples from farm #1; 9-10: DNA samples from Nakhon Ratchasima moat; 11-12: DNA samples from farm #2; odd lanes: DNA samples from intestine; even lanes: DNA samples from gills; A-C: specific DNA bands from SUT farm only; P: positive control (*E.coli*)).

4.4.4 Sequencing and alignment

Three specific DNA bands; A, B and C from samples of SUT farm in Figure 4.15 were named D1, D2 and D3, respectively. The bands were eluted and were sequenced (Table 4.3) and analyzed; the results indicated that they were all uncultured bacteria shown in Figure 4.16A-C, respectively.

Table 4.3 Sequences of specific DNA samples D1, D2 and D3.

DNA samples	Sequences of samples
D1	5’-GAAAGCCTGATGGAGCAATGCCGCGTGAGGGATGAAGGATCGTGGTCTGTAAACCTCTTTTCTTAAGGAAGATAGTGACGGTGCTTAAGGAAAAAACGCCGGCTAACTCTGTGCCAGCCGCCGCGGTAATATA-3'
D2	5’-CCACAAGCCTGATCCAGCAATTCTGTGTGCACGATGAAGGTCTTCGGATTGTAAAGTGCTTTCAGTTGGGAAGAAGAAAGTGACGGTACCAACAGAAGAAGCGACGGCTAAGTACGTG-3’
D3	5’-ATGCCGCGTGTGAGAGGAAGGCCTTCGGGTTGTAGAGTACGGAAGGAGGGCAGGAAGGGACCTTAGCTAAGGATCCGGGGGAGTTGACGTTACCTGCAGGAGAAGCCGCGGTAATATCCGGG-3’

A

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
GU305832.1	Uncultured bacterium clone YHW7 16S ribosomal RNA gene, partial	188	188	86%	5e-45	91%
EU255684.1	Uncultured bacterium clone Sed-BAC-P2B11 16S ribosomal RNA ge	188	188	86%	5e-45	91%
AJ548894.1	Uncultured Scenedesmus sp. chloroplast partial 16S rRNA gene, clo	188	188	86%	5e-45	91%
AJ548893.1	Uncultured Scenedesmus sp. chloroplast partial 16S rRNA gene, clo	188	188	86%	5e-45	91%
AJ242752.1	Chlorella pyrenoidosa chloroplast 16S rRNA gene, partial, strain IAI	188	188	86%	5e-45	91%

B

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU460633.1	Uncultured bacterium clone PB2_aai23f09 16S ribosomal RNA gene,	241	241	100%	8e-61	99%
GU301238.1	Uncultured bacterium isolate DGGE gel band B22 16S ribosomal RN	237	237	100%	1e-59	98%
GU293224.1	Uncultured bacterium clone SM17 16S ribosomal RNA gene, partial s	237	237	100%	1e-59	98%
GU293197.1	Uncultured bacterium clone SC34 16S ribosomal RNA gene, partial s	237	237	100%	1e-59	98%
GU293194.1	Uncultured bacterium clone SC30 16S ribosomal RNA gene, partial s	237	237	100%	1e-59	98%

C

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ203007.1	Uncultured bacterium clone SHFG585 16S ribosomal RNA gene, part	114	114	100%	8e-23	80%
EU097176.1	Uncultured bacterium clone 144 16S ribosomal RNA gene, partial se	111	111	100%	1e-21	78%
GU208326.1	Uncultured prokaryote clone Se2-23 16S ribosomal RNA gene, parti	109	109	100%	4e-21	79%
EU245199.1	Uncultured organism clone MAT-CR-H4-A06 16S ribosomal RNA gen	109	109	100%	4e-21	79%
AM158423.1	Uncultured bacterium partial 16S rRNA gene, clone T314D	109	109	100%	4e-21	78%

Figure 4.16 Sequences analysis of three specific DNA bands from SUT farm using NCBI blast (A: aligned D1 sequence; B: aligned D2 sequence; C: aligned D3 sequence).

4.5 Specific primers development

All three specific DNA sequences were used to design specific primers using DNASTAR Lasergene v7 program to amplify DNA samples from SUT and other resources to confirm specificity for samples from SUT farm only.

Table 4.4 Sequences of three specific primers, Tm and the size of PCR products.

Name of primers	**Sequences of primers**	**T_m (^oC)**	**Expected product size (bp)**
D1_NimF	5'-GATGAAGGATCGTGGTC-3'	52.8	90
D1_NimR	5'-CGGCTGGCACAGAGTTAG-3'	58.2	
D2_NimF	5'-CCACAAGCCTGATCCAGC-3'	58.2	122
D2_NimR	5'-CTGGCACGTACTTAGC-3'	51.7	
D3_NimF	5'-CTTCGGGTTGTAGAGTAC-3'	53.7	77
D3_NimR	5'-CTGCAGGTAACGTCAACTC-3'	56.7	

These specific primers (Table 4.4) were used to amplify DNA samples from SUT farm and compared with DNA samples from two other farms and Nakhon Ratchasima moat to confirm specificity of these primers to samples of SUT farm. Firstly, these primers were used to amplify samples from all four resources. The results in Figure 4.17-4.19 show a lot of non specific bands. Using primer D1 (Figure

4.17), the expected band should be about 90 bp, however on a 2% agarose gel the 90 bp products can not be distinguished from primer dimer. Samples from farm #1 also show an unexpected sharp strong band at about 1100 bp. Samples from SUT farm did not show any strong specific band. Therefore, this primer pair was discarded.

Primer pair D3 (Figure 4.19) amplified samples from all four resources showed several bands non at the expected size of about 80 bp (Table 4.4), or if there is any, it was not clear. Also, a size of about 80 bp is not easy to separate from primer dimer. So, this primer pair was also discarded.

For the primer pair D2, the target band from SUT samples have the expected size of about 120 bp (Figure 4.18 lane 1-10). However, in samples from other resources also contain several other sizes of PCR products, but not the size of about 120 bp as expected from SUT samples (Figure 4.18 lane 11-18). More detail study was done using this primer pair. Optimizations of the PCR conditions were done. The annealing temperature was optimized using gradient PCR of annealing temperature of 50-60^{o}C with 1^{o}C integral. The results indicated that, lower non specific bands can be seen at higher annealing temperature. However, the intensity of the specific band of 120 bp was also lower. Finally, the annealing temperature of 57^{o}C was used (Figure 4.20). Figure 4.20, lanes 1-10 showed specific band of about 120 bp, which is not seen in lane 11-22 or in the negative control. However, in lanes 11-22 high intensity of primer dimmer can be seen. Anyway, this primer pair D2 can be used to distinguish bacteria from SUT farm when compared to other resources.

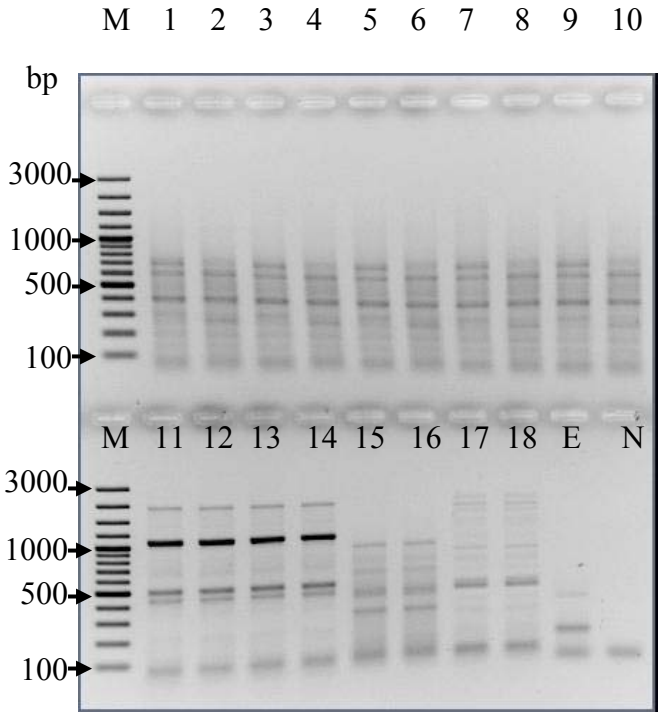


Figure 4.17 Amplified DNA samples from fish of all four resources using primers D1_NimF and D1_NimR (M: 100bp marker; 1-2: DNA samples from SUT farm in rainy season in first time of fish collection; 3-4: DNA samples from SUT farm in rainy season in second time of fish collection; 5-6: DNA samples from SUT farm in cool season in first time of fish collection; 7-8: DNA samples from SUT farm in cool season in second time of fish collection; 9-10: DNA samples from SUT farm in hot season; 11-14: DNA samples from farm #1; 15-16: DNA samples from Nakhon Ratchasima moat; 17-18: DNA samples from farm #2; odd lanes: DNA samples from intestine; even lanes: DNA samples from gills; E: DNA of *E. coli*; N: negative control).

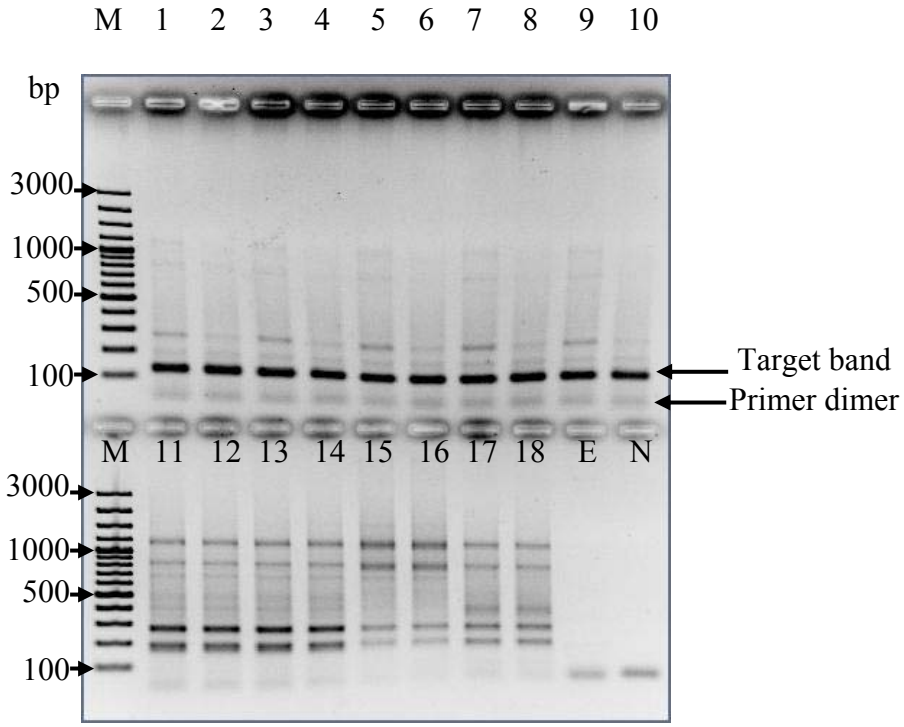


Figure 4.18 Amplified DNA samples from fish of all four resources using primers D2_NimF and D2_NimR (M: 100bp marker; 1-2: DNA samples from SUT farm in rainy season in first time of fish collection; 3-4: DNA samples from SUT farm in rainy season in second time of fish collection; 5-6: DNA samples from SUT farm in cool season in first time of fish collection; 7-8: DNA samples from SUT farm in cool season in second time of fish collection; 9-10: DNA samples from SUT farm in hot season; 11-14: DNA samples from farm #1; 15-16: DNA samples from Nakhon Ratchasima moat; 17-18: DNA samples from farm #2; odd lanes: DNA samples from intestine; even lanes: DNA samples from gills; E: DNA of *E. coli*; N: negative control).

M 1 2 3 4 5 6 7 8 9 10

bp

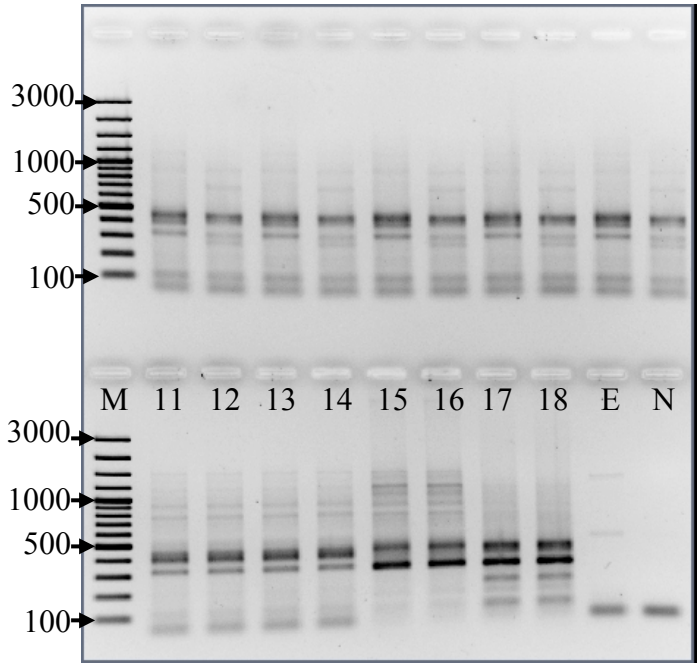


Figure 4.19 Amplified DNA samples from fish of all four resources using primers D3_NimF and D3_NimR (M: 100bp marker; 1-2: DNA samples from SUT farm in rainy season in first time of fish collection; 3-4: DNA samples from SUT farm in rainy season in second time of fish collection; 5-6: DNA samples from SUT farm in cool season in first time of fish collection; 7-8: DNA samples from SUT farm in cool season in second time of fish collection; 9-10: DNA samples from SUT farm in hot season; 11-14: DNA samples from farm #1; 15-16: DNA samples from Nakhon Ratchasima moat; 17-18: DNA samples from farm #2; odd lanes: DNA samples from intestine; even lanes: DNA samples from gills; E: DNA of *E. coli*; N: negative control).

M 1 2 3 4 5 6 7 8 9 10 E N

bp

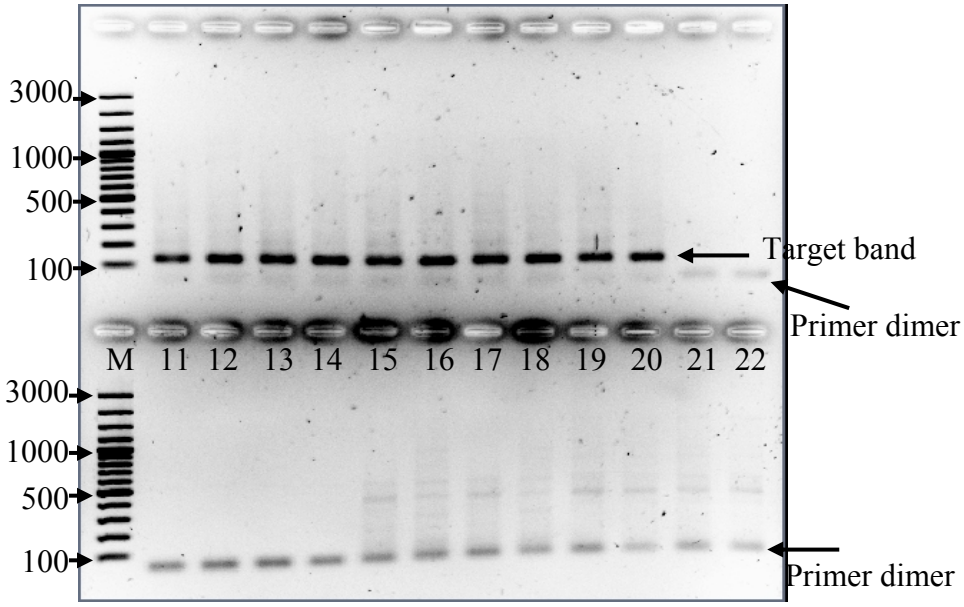


Figure 4.20 Amplified DNA samples from fish of all four resources and pure cultured samples using primers D2_NimF and D2_NimR (M: 100bp marker; 1-2: DNA samples from SUT farm in rainy season in first time of fish collection; 3-4: DNA samples from SUT farm in rainy season in second time of fish collection; 5-6: DNA samples from SUT farm in cool season in first time of fish collection; 7-8: DNA samples from SUT farm in cool season in second time of fish collection; 9-10: DNA samples from SUT farm in hot season; 11-12, 13-14: DNA samples from pure cultured of Gram-positive and Gram-negative bacteria, respectively; 15-18: DNA samples from farm #1; 19-20: DNA samples from Nakhon Ratchasima moat; 21-22: DNA samples from farm #2; odd lanes: DNA samples from intestine; even lanes: DNA samples from gills, 11-12, 13-14: DNA of pure cultured bacteria from intestine and gills of SUT tilapia, respectively; E: DNA of *E. coli*; N: negative control).

CHAPTER V

CONCLUSION

Bacterial community in fish is related to habitat and management of the farm. Although, most of the bacteria are opportunistic pathogens but they do not harm healthy fish. Moreover, they synthesized vitamins that are lack in feed and have been demonstrated to enhance the growth of fish (Trust and Sparrow, 1974). Specific bacteria from fish can be used to identify and trace the location of fish.

PCR-DGGE technique was used to specify bacterial DNA. Many researchers use different conditions of DGGE for bacterial community analysis. Therefore, to obtain good results, optimization of condition used is important and should be considered. Several parameters including the size of PCR products, percent denaturant, percent polyacrylamide gel, running time and voltage used need to be optimized to obtain good sharp results. The best condition for tilapia intestinal microflora analysis in this research was 8% polyacrylamide gel, 30-60% denaturant, 100V and 18hr of running time. In this study, three specific DGGE bands of partial 16S rDNA of bacteria from SUT fish were sequenced. They were identified as uncultured bacteria of different species. Three specific primer pairs were designed from these sequences. Only primer pair named D2 can be used to specify and trace samples from SUT farm. This research can be used as a model to develop traceability marker for the products of interest.

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APPENDIX

APPENDIX

MEDIA AND REAGENTS

1. Aeromonas agar medium (Oxoid, UK)

Dissolved 29.5 g of medium in 500 ml of distilled water, bring gently to the boil. Do not autoclave. Cool to 50°C and aseptically add one vial of Ampicillin Selective Supplement (2.5mg/vial) reconstituted as directed. Mix well and pour into sterile Petri dishes.

2. de Man, Rogosa and Sharpe (MRS) agar (Oxoid, UK)

Dissolved 62.0 g of medium and 0.5% $CaCO_3$ in 1 liter of distilled water, bring to the boil to dissolve completely, sterilize by autoclaving at 110°C for 20 min. Allow the medium cool to 50°C and pour into sterile Petri dishes.

3. Plate count agar (PCA, Himedia, India)

Dissolved 23.5 g of medium in 1 liter of distilled water, bring to the boil to dissolve completely, sterilize by autoclaving at 121°C for 15 min. Allow the medium cool to 50°C and pour into sterile Petri dishes.

4. Potato dextrose agar (PDA, Himedia, India)

Dissolved 39.0 g of medium in 980 ml of distilled water, bring to the boil to dissolve completely, sterilize by autoclaving at 121°C for 15 min. Allow the medium cool to 50°C and then add 20 ml of antibiotic solution (Chlortetracyclin•HCl 0.5g and Chloramphenicol 0.5g/100ml). Mix well and pour into sterile Petri dishes.

5. Pseudomonas agar medium (Oxoid, UK)

Suspend 24.2 g of the agar medium in 500 ml of distilled water. Add 5 ml of glycerol. Bring to the boil to dissolve completely, sterilize by autoclaving at 121°C for 15 min. Allow the medium cool to 50°C, then add the contents of 1 vial of Pseudomonas C-F-C Supplement (Cetrimide 5.0mg, Fucidin 5.0mg and Cephalosporin 5.0mg) rehydrated as directed. Mix well and pour into sterile Petri dishes.

6. Thiosulfate citrate bile salts sucrose (TCBS, Oxoid, UK)

Suspend 88 grams in 1 liter of distilled water. Boil to dissolve the medium completely. Do not autoclave. Pour plates without further heating and dry before use.

7. Extraction buffer

1.0M Tris-Cl pH 8.0	1.00ml
0.5M EDTA pH 8.0	1.00ml
5.0M NaCl	1.00ml
10% SDS	1.25ml
DI water	5.75ml

Pipette all above solutions into sterile focal tube. Mix well and incubate at 65^{o}C for 5 min before use.

8. 40% (w/v) Acrylamide/bis

Acrylamide	38.93g
N-N-methylene-bis acrylamide	1.07g

Dissolve all above in 50 ml DI water. Adjust into 100 ml using volumetric flask. Prepare and keep in dark condition.

9. 50X TAE buffer (per liter)

Tris base	242.0g
Acetic acid glacial	57.1ml
0.5M EDTA pH 8.0	100.0ml

Weigh and pipette all above chemical, then add DI water. Adjust into 1 liter using volumetric flask. Do not autoclave.

10. 30 % denaturant (for 8% polyacrylamide gel per 100ml)

40% Acrylamide/Bis	20.0ml
50X TAE buffer	2.0ml
Formamide	12.0ml
Urea	12.6g

Weigh and pipette all above chemical, then add DI water. Adjust into 100 ml using volumetric flask. Keep at 4^{o}C in dark condition.

11. 60 % denaturant (for 8% polyacrylamide gel per 100ml)

40% Acrylamide/Bis	20.0ml
50X TAE buffer	2.0ml
Formamide	24.0ml
Urea	25.2g

Weigh and pipette all above chemical, then add DI water. Adjust into 100 ml using volumetric flask. Keep at 4^{o}C in dark condition.

BIOGRAPHY

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Miss Darawan Ruamkuson was born on 15 May 1982 in Khon Kaen, Thailand. She graduated with bachelor's degree of Science in the Department of Microbiology, Faculty of Science, Khon Kaen University (KKU), Khon Kaen in March 2005. After graduation, she worked for 3 years at the Department of Biotechnology, KKU and School of Biotechnology, SUT. In 2008, she had an opportunity to study Master degree in School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima. Her thesis is "Development of biological probes from microflora to assure traceability of tilapia from Suranaree University of Technology (SUT) farm". She has present part of her thesis work at The Annual Meeting and International Conference of the Thai Society for Biotechnology (TSB 2007) at Thummasat University (TU) and (TSB 2008) at Maha Sarakham, The 15th National Graduated Research Conference 2009, Rajaphat Nakhon Ratchasima and 2nd SUT Graduate Conference 2009, SUT. Part of her work has been published in SUT Science and Technology Journal (Ruamkuson and Ketudat-Cairns, 2009).