Enhancement of Phototrophic Bacterial Growth by Fermented Plant Extract for Treating Latex Rubber Wastewater

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Thesis Title Enhancement of Phototrophic Bacterial Growth by Fermented Plant Extract for Treating Latex Rubber Wastewater

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ชื่อวิทยานิพนธ์ การกระตุ้นการเจริญของแบคทีเรียสังเคราะห์แสงด้วยน้ำหมักชีวภาพเพื่อบำบัดน้ำเสียจากการทำยางแผ่น

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บทคัดย่อ

งานวิจัยนี้เป็นการศึกษาการบำบัดน้ำเสียจากการทำยางแผ่นของสหกรณ์โรงรมยางด้วยแบคทีเรียสังเคราะห์แสงกลุ่มไม่สะสมซัลเฟอร์ (purple nonsulfur bacteria: PNSB) เพื่อเพิ่มประสิทธิภาพการบำบัด และกำจัดซัลไฟด์ (sulfide) ที่มาจากแก๊สไฮโดรเจนซัลไฟล์ (H_2S) [illegible] (fermented pineapple extract: FPE) [illegible] PNSB ที่อยู่ในน้ำเสียที่ไม่ผ่านการฆ่าเชื้อภายใต้สภาวะมีอากาศเล็กน้อย-[illegible] (microaerobic light conditions) [illegible] ใช้เป็นกล้าเชื้อ และเรียกกล้าเชื้อนี้ว่า stimulated indigenous purple nonsulfur bacteria (PNSBsi) [illegible] PNSB [illegible](PNSBsi) จึงใช้วิธีทางสถิติแบบพื้นที่[illegible] Box-Behnken design (BBD) [illegible] [illegible]FPE 2% ของน้ำเสียที่มีค่า chemical oxygen demand (COD) เท่ากับ 2000 มิลลิกรัม [illegible] [illegible]pH เริ่มต้นเป็น 7.0 สามารถทำให้ค่า oxidation reduction potential (ORP) [illegible] PNSBsi [illegible](6.31 x 10^7 CFU [illegible]) ในเวลา 2 วัน และเมื่อนำกล้าเชื้อ PNSBsi [illegible] [illegible]-[illegible] [illegible] central composite design (CCD) [illegible] PNSBsi 7% [illegible]FPE 0.8% และ ระยะ[illegible](retention time: RT) 4 [illegible] [illegible]COD [illegible] (suspended solids: SS) [illegible] (total sulfide: TtS) [illegible] 91 75 และ 61 [illegible] H_2S กรณีที่ใช้กล้าเชื้อ [illegible] *Rhodopseudomonas palustris* P1 [illegible] [illegible] PNSBsi [illegible] P1 เป็นกล้าเชื้อและเรียกกล้าเชื้อนี้ว่า PNSB P1 พบว่าสภาวะที่เหมาะสมในการบำบัดน้ำเสียจากการทำนายโดยการออกแบบที่ใช้ CCD [illegible] [illegible]PNSB P1 [illegible] 3 [illegible]FPE [illegible] 0.9 [illegible]4 [illegible] [illegible]COD SS [illegible] TtS [illegible] 98 79 และ 72 [illegible]

H_2S [illegible] และเมื่อทำการทดลองยืนยันผลที่ได้จาก CCD (verification test) [illegible] 4–8% [illegible] COD SS [illegible] TtS [illegible] 94 75 [illegible] 66% [illegible] ซึ่งมีประสิทธิภาพน้อยกว่าสภาวะการใช้กล้าเชื้อ [illegible] 2 [illegible] [illegible] FPE 0.75% [illegible] 4 วันจากชุดการทดลองที่ดีที่สุดของ CCD [illegible] [illegible] COD SS [illegible] TtS [illegible] 96 78 [illegible] 71 [illegible] [illegible] *R. palustris* P1 [illegible] ในการบำบัดน้ำเสียจากการผลิตยางแผ่น ที่ใช้กล้าเชื้อ PNSBsi [illegible] PNSB P1 [illegible] น้ำหมักสับปะรด ภายใต้สภาวะที่เหมาะสมของแต่ละกล้าเชื้อ โดยใช้เทคนิค nest-PCR-DGGE [illegible] 16S rDNA [illegible] [illegible] PNSB P1 มีค่าทั้งสองสูงกว่าประชากรแบคทีเรียในกล้าเชื้อ PNSBsi [illegible] [illegible] ($P < 0.05$) [illegible] *R. palustris* [illegible] หลักในกล้าเชื้อทั้งสอง และในการบำบ[illegible] 4 [illegible] PNSB P1 เท่านั้นที่มีค่าความเหมือนของ *R. palustris* [illegible] *R. palustris* P1 [illegible] 100% ซึ่งสอดคล้องกับประสิทธิภาพที่สูงในการบำบัดน้ำเสียที่เหลือซัลไฟด์น้อยและผ่านมาตรฐานน้ำทิ้งที่สามารถใช้เป็นน้ำเพื่อ[illegible]เกษตรได้ และในน้ำทิ้งมีชีวมวลที่มีปริมาณกรดอะมิโนจำเป็นสูงกว่าการบำบัดน้ำเสียโดยใช้กล้าเชื้อ PNSBsi ขณะที่กลุ่มประชากรแบคทีเรียอื่นๆ [illegible] บัดน้ำเสียด้วยกล้าเชื้อแบคทีเรียสังเคราะห์แสงทั้งสองชนิดในช่วงเริ่มต้น [illegible] γ-proteobacteria, ß-proteobacteria, negativicutes [illegible] flavobacteriia [illegible] 37.5 25.0 25.0 12.5 [illegible] 4 [illegible] α-proteobacteria, clostridia [illegible] mollicutes [illegible] 4 [illegible] ผลการทดลองที่ได้พิสูจน์ว่ากล้าเชื้อแบคทีเรียสังเคราะห์แสงทั้งสองกลุ่ม [illegible] [illegible] *R. palustris* P1 [illegible] [illegible] PNSBsi ในการบำบัดน้ำเสียจากการผลิตยางแผ่นโดยได้น้ำทิ้งที่สามารถใช้เพื่อ [illegible]

คำสำคัญ: [illegible]

[illegible] น้ำเสียยางพารา น้ำหมักสับปะร[illegible]

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Major Program Microbiology

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ABSTRACT

This research studied wastewater treatment of rubber sheet from a cooperative rubber sheet factory (CRSF) using purple nonsulfur bacteria (PNSB) for increasing efficiency and removal of sulfide from H_2S. Fermented pineapple extract (FPE) was used to stimulate the growth of PNSB in non sterile rubber sheet wastewater (RAW) under microaerobic light conditions to use as inoculum and this inoculum was named stimulated indigenous purple nonsulfur bacteria (PNSBsi). Use of response surface methodology with Box-Behnken design (BBD) found that the optimal conditions for stimulating the growth of indigenous PNSB were; addition of 2.0% FPE into RAW, which had a chemical oxygen demand (COD) of 2000 mg/L and an initial pH of 7.0 after adjustment, significantly decreased oxidation reduction potential value to be a reducing condition and this stimulated PNSBsi growth to reach a maximum of 6.31×10^7 cell/mL within 2 days. Consequently, a central composite design (CCD) was used to determine the optimal conditions for treating RAW by PNSBsi inoculum under microaerobic light conditions and the optimal conditions based on the predicted model and actual values from the CCD were 7% PNSBsi, 0.8% FPE and 4 days retention time (RT) by reducing 91% for COD, 75% for suspended solids (SS), 61% for total sulfide (TtS) and no detection of H_2S. In case of *Rhodopseudomonas palustris* P1 was prepared to be used an inoculum with the same method with PNSBsi by inoculating the strain P1 into RAW and this was called PNSB P1. Optimization conditions of RAW treatment using CCD on the basis of the predicted model found that a 3% PNSB P1, 0.9 % added FPE and a 4 day RT were the most suitable conditions as the removal of 98% COD (initial COD 3,005 mg/L), 79% of SS and 72% of TtS and no observation of H_2S.

Results of the verification test had an error of only 4–8 % confirmed removal of COD (initial COD 2,742 mg/L), SS and TtS at 94, 75 and 66% respectively. However, the efficiency was less than the best set obtained from the CCD experiment (2 % PNSB P1, 0.75 % FPE and 4 days RT); upon repeating, this set could reduce 96 % COD, 78 % SS and 71 % TtS. The biomass obtained after RAW treatment from the best set consisted mostly of *R. palustris* P1 and thus conditions of this set were used for further studied. The bacterial population dynamic including PNSB in RAW treated by inocula either PNSBsi or PNSB P1 and with FPE were investigated using DGGE of nested-PCR-amplified fragments of the 16S rRNA genes. The diversity and evenness of the community in the PNSB P1 inoculum was significantly higher ($P < 0.05$) than that the community in the PNSBsi inoculum. Only *R. palustris* was dominant throughout the RAW treatment over 4 days by either PNSBsi or PNSB P1 inocula; however, a 100% similarity index to the strain P1 was found only in the RAW treatment by PNSB P1 inoculum. This corresponded to a higher efficiency to treat RAW that met standard guidelines for using as irrigation water and biomass in the effluent contained higher amount of essential amino acids when compared to PNSBsi set. Bacterial communities in both RAW treatment processes, at starting point (t = 0) in addition of PNSB found 4 main bacterial populations belonging to 37.5% γ-proteobacteria, 25% ß-proteobacteria, 25% negativicutes and 12.5% flavobacteriia. Besides the 4 main bacterial groups; α-proteobacteria, clostridia and mollicutes were also detected in both communities later during the RAW treatment for 4 days. Overall results, it can be concluded that both inocula were able to compete with other heterotrophs and *R. palustris* P1 showed higher efficiency to treat RAW than that PNSBsi by producing the quality effluent with by-product as SCP for supplementation in animal feed.

Keywords: fermented plant extract, hydrogen sulfide, PCR-DGGE, purple nonsulfur bacteria, response surface methodology, rubber wastewater, single cell protein

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LIST OF ABBREVIATIONS AND SYMBOLS

ADS: Air dried sheets
BOD: Biochemical oxygen demand
BBD: Box-Behnken design
CCD: Central composite design
COD: Chemical oxygen demand
CRSFs: Cooperative rubber sheet factories
DCW: Dry cell weight
DGGE: Denaturing gradient gel electrophoresis
DsS: Dissolved sulfide
DO: Dissolved oxygen
DW: Dry weight
EC: Electrical conductivity
EM: Effective microorganisms
FPE: Fermented pineapple extract
GM: Glutamate-malate medium
HPC: Heterotrophic plate count
ICP-OES: Inductively couple plasma-optical emission spectrometer
LAB: Lactic acid bacteria
MRS: de Man Rogosa and Sharp
PCA: Plate count agar
PDA: Potato dextrose agar
PNSB: Purple nonsulfur bacteria
PNSBsi: Stimulated PNSB
OD: Optical density
ORP: Oxidation reduction potential
RAW: Non sterile rubber sheet wastewater

LIST OF ABBREVIATIONS AND SYMBOLS (continues)

RSM: Response surface methodology
RSS: Ribbed smoked sheets
RT: Retention time
SCP: Single cell protein
SS: Suspended solids
TKN: Total Kjeldahl nitrogen
TP: Total phosphorus
TtS: Total sulfide
UHS: Un-ionized hydrogen sulfide
VFAs: Volatile fatty acids

CHAPTER 1

INTRODUCTION

Rationale and Background

As Thailand is the largest latex rubber producer in the world (Vijayaraghavan *et al*., 2008), rubber has been one of the main agro-based industrial sectors that plays an important role in Thailand's economy. There are many cooperative rubber sheet factories (CRSFs) throughout Thailand (Kantachote *et al*. 2010), and there are over 700 CRSFs in Southern and Eastern Thailand (Chaiprapat and Sdoodee, 2007). Latex rubber sheet production consumes a large amount of water and produces wastewater that requires treatment before discharging to waterways. Lagoons or oxidation ponds are widely used to treat the wastewater from the CRSFs as they are inexpensive to construct and easy to operate (Chaiprapat and Sdoodee, 2007). However, these systems cause a major problem by producing hydrogen sulfide (H_2S: rotten-egg odor) and greenhouse gases such as methane (CH_4) and carbon dioxide (CO_2). The H_2S not only gives unpleasant smell but also harms human's health (Yalamanchili and Smith, 2008; Doujaiji and Al-Tawfiq, 2010). Therefore, alternative techniques have been researched in attempt to solve the serious problems and enable CRSFs to apply the appropriate technology by themselves.

Among microbes involve with anaerobic wastewater treatment, purple nonsulfur bacteria (PNSB) have the potential to solve the serious problem above due to the fact that they can grow not only photoautotrophically and photoheterotrophically under anaerobic light or microaerobic light conditions but also chemoorganotrophically under aerobic dark conditions (Madigan *et al*., 2009). In addition, PNSB have a commercial value as a source of Single Cell Protein (SCP) for supplemental feed stock or as a biofertilizer for fixing nitrogen gas (N_2) (Sasikala and Ramana, 1995; Kobayashi, 1995; Kim *et al*., 2004). Among the PNSB, *Rhodopseudomonas blastica* DK6 and *R., palustris* P1 have been proved for their abilities to reduce COD and sulfide in rubber sheet wastewater (Kantachote *et al*., 2005; Kantachote *et al*., 2010).

Nevertheless, blooming of PNSB in any wastewater is rare under natural operating conditions. Hence, any techniques that stimulate PNSB growth must be investigated.

Fermented plant extracts (FPEs) have been extensively used as a natural liquid fertilizer by agriculturist because they are easily produced by themselves from agricultural products or agricultural waste (Kantachote *et al*., 2009; Kantachote *et al*., 2010). In addition, FPEs have been applied in cleaning polluted lagoons or anaerobic wastewater system (Higa and Parr, 1994). Due to the fact that FPEs contain organic acids such as acetate and lactate (Kantachote *et al*., 2009) and these compounds are readily consumed by PNSB (Chen *et al*., 2008; Mohanakrishna and Mohan, 2013). Hence, FPEs like fermented pineapple extract (FPE) might be used to stimulate the growth of indigenous PNSB and a selected PNSB strain in non sterile latex rubber sheet wastewater (RAW) for using as inocula to treat RAW. Hence, optimized conditions for preparing inoculum in RAW were investigated using response surface methodology (RSM) because RSM as a statistical technique for designing experiment, building models, evaluating the effects of several factors and searching optimum conditions for desirable responses and reducing number of experiments (Montgomery, 2005; Bezerra *et al*., 2008; Steinberg and Bursztyn, 2010). Consequently, the use of stimulated inocula either from indigenous or the selected strain for treating RAW was also investigated by RSM for wastewater treatment. Additionally, the effluent from the most effective wastewater system was evaluated for its possible use as irrigation water and a source of SCP.

To understand the roles of PNSB and other heterotrophs in RAW treatment by stimulated PNSB inocula, bacterial communities should be studied by molecular techniques. This is because molecular techniques based on 16S rRNA/rDNA have been successfully applied to microbial ecology research. As a powerful culture-independent tool, polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) is based on a direct analysis of the extracted of DNA from the microbial communities and to determine the community dynamics in response to environmental variations (Mura *et al*., 2009). Mentioned using PNSB and FPE for treating wastewater from CRSFs would be good to provide appropriate technologies as the alternative treatment with higher efficiency than the conventional treatment such as lagoons and

oxidations. In addition, understanding the roles of bacterial populations in community may give information to improve the efficiency of RAW treatment. Therefore the aims of this thesis are given below.

Objectives

1. To optimize conditions for stimulating growth of indigenous PNSB in RAW by fermented plant extract to use as inoculum for wastewater treatment,

2. To optimize conditions for treating RAW by using stimulated PNSB inocula (indigenous or the selected strain) with FPE,

3. To study bacterial communities from the most effective wastewater treatment from each stimulated PNSB inoculum,

4. To investigate the possibility of the effluent from the most effective RAW treatment for use as irrigation water and a by product (SCP).

Scope of the study

To solve the problem of wastewater from CRSFs, appropriate techniques were developed as follows. Optimization conditions for producing stimulated indigenous PNSB in RAW by using FPE to use as inoculum were investigated using RSM. After that the stimulated PNSB inocula either from indigenous or a selected strain *R. palustris* P1 were used to treat RAW and RSM was used to determine the optimization conditions. In addition, the effluents containing biomass were evaluated to use as irrigation water and a source of SCP. Finally, bacterial communities between PNSB and heterotrophs were investigated by DGGE and 16S RNA genes to understand their roles for successful RAW treatment.

Anticipated Outcomes

Overall, the outcomes from this thesis work provide the appropriate technology for treating RAW by using the most effective PNSB inoculum that was

stimulated using FPE. The appropriate technique not only produces the effluent that meet standard guidelines to use as irrigation water in agriculture but also provides a source of SCP for animal feed. Hence, know how from this study will be transferred to CRSFs, and this will lead to sustainable and environmental friendly latex rubber sheet production by CRSFs.

CHAPTER 2

LITERATUREREVIEW

Processing of smoked rubber sheets

Production of the rubber in Thailand comes from the grass root farmers by a way of many small cooperative rubber sheet factories (CRSFs) mostly located in southern Thailand; each is capable of producing 2 T (Ton) of rubber sheet per day. There are over 700 of these factories in the country (Chaiprapat and Sdoodee, 2007) and nowadays expansion of CRSFsto other regions of Thailand following rubber plantation. In the production process, fresh rubber latex collected in a pool is diluted with water and mixed with formic acid to form solidified tofu-like slabs. These slabs are then transported in a water rail to a squeezing machine and are squeezed to form a thin sheet (2-3 mm). The raw rubber sheets are then hung on a cart to let the water evaporate and then are dried in a heating room. The only difference in ribbed smoked sheets (RSS) and air dried sheets (ADS) production is that the rubber sheets are in direct contact with smoke from rubber wood burning in RSS production, while in ADS production, only dry heat is used to dry the sheets (see details in Figure 2-1).

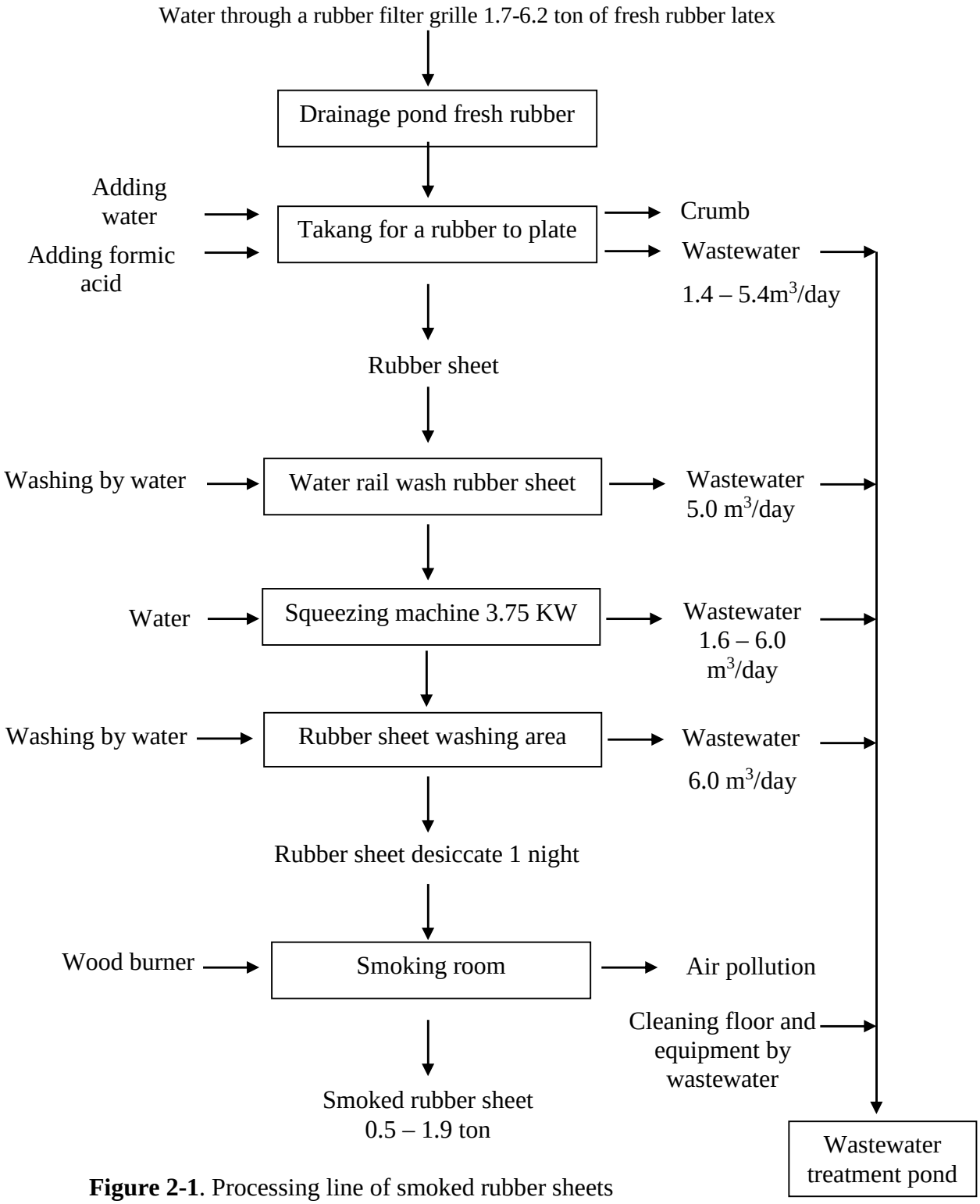

Figure 2-1. Processing line of smoked rubber sheets

Source: Pollution Control Department, (2005).

Wastewater treatment of cooperative rubber sheet factories (CRSFs)

Wastewater from rubber sheet production in the cooperatives comes from four sources; remainder of the water in the rubber sheet formation containers, transporting of the rubber sheets in a water rail to the squeezing machine, spraying of the rubber sheets during a squeezing process, and washing of the containers and factory floor.The wastewater from the four sources mentioned above of three rubber cooperatives was acidic because formic acid was used during the rubber sheet coagulation process as shown in Table 2-1.

Table 2-1. Average values of wastewater characteristic from rubber sheet production of three rubber cooperatives (Boonchuay, 1998).

Property	Wastewater Sources				
	A	B	C	D	E
pH	5.0	5.3	5.3	5.8	5.9
Temperature (°C)	26.0	26.7	26.7	27.1	26.3
DO (mg/L)	1.13	0.45	3.92	0.58	2.08
BOD_5 (mg/L)	9,433	3,433	7,016	1,391	4,783
COD (mg/L)	15,069	5,137	11,344	1,928	6,673
SS (mg/L)	164	93	195	525	167
TKN (mg/L)	162.1	79.5	190.9	60.2	132.0
NH_3-N (mg/L)	85.1	45.0	110.0	38.7	75.9
TP (mg/L)	21.6	20.0	17.8	19.4	14.9
Sulfate (mg/L)	472.6	225.8	445.2	136.0	188.1
Acidity (mg/L as $CaCO_3$)	986.5	347.8	581.8	130.1	391.7
BOD_5loading (kg BOD_5/d)	29.4	7.8	5.8	1.0	37.3

Notes: DO, dissolved oxygen; BOD, biochemical oxygen demand; COD, chemical oxygen demand; SS, suspended solids; TKN, total Kjeldahl nitrogen; and TP, total phosphorus.

A, remainder of the water in the rubber sheet formation containers.

B, water in the transport rail that moves rubber slabs to the squeezing machine.

C, water used in spraying the rubber sheets in the squeezing machine.

D, water used in washing the containers and factory floor.

E is the overall water from the rubber sheet production factory.

Although there is a treatment system in each CRSF, a survey of the current status indicates that it is minimally maintained, if at all, and not being operated according to the design parameter. There are two models of the treatment systems named according to the year of the factory construction, model 1994 and model 1995. Both types of the systems consist of four ponds (Figure 2-2) at sizes depending on the available donated land at the time of factory construction (Chaiprapat and Sdoodee, 2007). The original design employed a surface aerator to deliver oxygen to stimulate rapid organic substance degradation in the aerobic ponds; however, all of CRSFs had stopped using them due to high maintenance and energy costs. This situation affects on the quality of effluent from their treatment systems to degrade and unable to meet the industrial or irrigation effluent standards. Wastewater ponds are normally flooded in the rainy season and the wastewater flow to the neighboring agricultural areas that reason complaints from farmers in the vicinity area affected by the flood (Tekasakul *et al*., 2006).

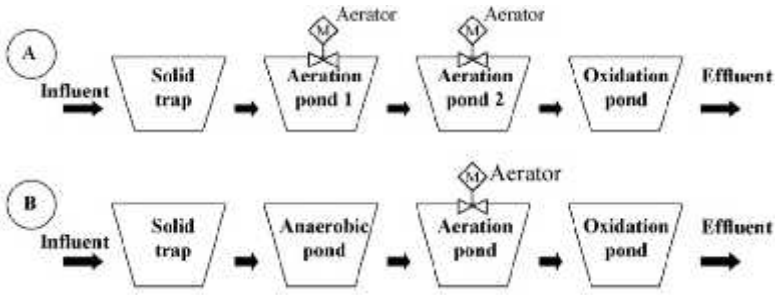

Figure 2-2. Schematic diagrams of the wastewater treatment system operate in the cooperative rubber sheet factories model 1994 (A) and 1995 (B).
Source: Chaiprapat and Sdoodee (2007).

Anaerobic wastewater treatment

In the anaerobic digestion system, roughly four groups of microorganisms sequentially degrade organic matter. Four categories of microorganisms are involved in the transformation of complex materials into simple molecules such as methane and carbon dioxide. These microbial groups operate in a synergistic relationship (Eckenfelder *et al.*, 2009).

Group 1: Hydrolytic bacteria

Consortia of anaerobic bacteria break down complex organic molecules (e.g. proteins, cellulose, lignin, lipids) into soluble monomer molecules such as amino acids, glucose, fatty acids and glycerol. The monomers are directly available to the next group of bacteria. Hydrolysis of the complex molecules is catalyzed by extracellular enzymes such as cellulases, proteases and lipases.

Group 2: Fermentative acidogenic bacteria

Acidogenic (i.e. acid-forming) bacteria (e.g. *Clostridium*) convert sugars, amino acids and fatty acids to organic acids (e.g. acetic, propionic, formic, lactic, butyric or succinic acids), alcohols and ketones (e.g. ethanol, methanol, glycerol, acetone), acetate, CO_2 and H_2. The products formed vary withthe bacterial type as well as with culture conditions (temperature, pH, oxidation reduction potential or redox potential).

Group 3: Acetogenic bacteria

Acetogenic bacteria (acetate and H_2-producing bacteria) such as *Syntrobacterwolinii* and *Syntrophomonaswolfei* convert short chain fatty acids (e.g. propionic acid, butyric acid) and alcohols into acetate, hydrogen and carbon dioxide which are consumed by methanogens. This group requires low hydrogen tensions for fatty acid conversion, necessitating a close monitoring of hydrogen concentration. Under relatively high H_2partial pressure, acetate formation is reduced; and the substrate is converted to propionic acid, butyric acid and ethanol rather than methane. There is a symbiotic relationship between acetogenic bacteria and methanogens. Methanogens help achieve the low hydrogen tension required by acetogenic bacteria.

Group 4: Methanogenicbacteria

Methanogens grow slowly in wastewater systems and their generation times from 3 days at 35°C to as high as 50 days at 10°C. Methanogens use a limited number of substrates that include acetate, H_2, CO_2, formate, methanol and methylamines. All of these substrates are reduced to methylCoM (CH_3-S-CoM) which is further converted to CH_4 by methylCoMreductase (Ritchie *et al*., 1997).Anaerobic digestion has long been used for the stabilization of wastewater sludge. Later on, however, it was successfully used for the treatment of industrial and domestic

wastewaters. This was made possible through a better understanding of the microbiology of this process and through improved reactor designs.

Phototrophic bacteria

Photosynthetic organisms use light energy to fuel their biosynthetic process. Oxygen is generated in oxygenic photosynthesis where water is used as the electron donor. In anoxygenic photosynthetic, organic or sulfur compounds are used as electron donors. Plant, algae and cyanobacteria carry out oxygenic photosynthetic, whereas the photosynthetic bacteria obtain energy from anoxygenic photosynthesis.

Anoxygenic photosynthetic bacteria

Photosynthetic bacteria are grouped according to their photosynthetic pigments and the electron donors used. These are purple bacteria, green bacteria and heliobacteria (see details Table 2-2) (Kim *et al*., 2008).

The purple sulfur bacteria include members of chromatiaceae and ectothiorhodospiraceae within the γ-*proteobacteria*. The former accumulate sulfur granules intracellularly and the latter extracellularly. The purple non-sulfur bacteria are more diverse, belonging to α and β-*proteoacteria*. They grow photosynthetically under anaerobic light conditions and many of them can grow chemoorganotrophically under aerobic dark conditions.The photosynthetic apparatus of purple bacteria consists of a reaction-center complex and light-harvesting complexes. These complexes contain bacteriochlorophylls and carotenoids to absorb light energy and convert it to electrochemical energy. The genes coding for the two core proteins of the reaction-center complex of purple bacteria, the L and M subunits, form an operon called *puf* with the genes for the two small hydrophobic proteins of the core light-harvesting (LH1) complex. The H subunit of the reaction-center complex is coded by the *puh*A gene and is included in another operon, *puh*gene (Bauer *et al*. 1991). The research uses primer detection of purple bacteria with *puf* gene (Okubo *et al*., 2006; Belila *et al*., 2009; Liang *et al*., 2010).

Table 2-2. Characteristics of purple and green bacteria.

	Purple bacteria		Green bacteria			
Character	Non-sulfur	Sulfur	Sulfur	FAPB	Heliobacteria	AAPB
BCHL	*a,b*	*a,b*	*a,c,d,e*	*a,c,d*	*g*	*a*
H_2S as e^- donor	±[a]	+	+	+	-	-
S accumulation	-	intracellular[b]	extracellular	-	-	-
H_2 as e^- donor	+	+	+	+	-	-
Organics as e^- donor	+	+	-	+	+	+
Carbon source	CO_2 organics	CO_2 organics	CO_2 organics	CO_2 organics	organics	organics
Aerobic respiration	+	-	-	+	-	+
CO_2 fixation	Calvin cycle	Calvin cycle	reductive TCA cycle	Calvin cycle[c]		

FAPB, filamentous anoxygenic phototrophic bacteria; AAPB, aerobic anoxygenic phototrophic bacteria; BCHL, bacteriochlorophyll

[a]Depending on the strain.

[b]Members of the family Ectothiorhodospiraceae accumulate sulfur granules extracellularly.

[c]Species of the genus *Chloroflexus* employ the 3-hydroxypropionate cycle.

Source: Kim *et al*., (2008).

The green bacteria are classified into two physiologically and phylogenetically distinct groups. These are the strictly anaerobic and obligatelyphotolithotrophic green sulfur bacteria and the filamentous anoxygenicphotolithotrophic bacteria that are facultatively anaerobic. These have different reaction centers. The latter have the pheophytin-quinone type while the former have the iron-sulfur type. The green sulfur bacteria cannot grow heterotrophically, while the filamentous anoxygenic phototrophic bacteria can grow heterotrophically under aerobic dark conditions. The latter members of chloroflexaceae, belong to a deep-branching lineage of bacteria. These are also called photosynthetic flexibacteria. They are Gram negative but lack of lipopolysaccharide.

The photoheterotrophic heliobacteria include three genera; *Heliobacterium*, *Helibacillus* and *Heliophium*. They do not grow aerobically in the dark and fix N_2. They do not have photosynthetic organelles and the photosynthetic pigments, including the unique bacteriochlorophyll*g*, are located in the cytoplasmic membrane. They have an iron-sulfur-type reaction center. Heliobacterial cells have several unusual features. They are extremely fragile and lyse when approaching the stationary phase. They are Gram negative but lack lipopolysaccharide like the filamentous anoxygenic phototrophic bacteria and do not fix CO_2.

Purple nonsulfur bacteria (PNSB) are affiliated with the *Alphaproteobacteria* and the *Betaproteobacteria*. Purple sulfur bacteria belong to the *Gammaproteobacteria*. The overwhelming evidence of molecular data led to major taxonomic reclassifications of these bacteria (Imhoff *et al*., 2005).

Phototrophic α-*proteobacteria*

Bacteria of this group are phototrophic PNSB, able to perform anoxygenic photosynthesis with bacteriochlorophylls and carotenoids as photosynthetic pigments. None of the described species contains gas vesicles. Internal photosynthetic membranes are continuous with the cytoplasmic membrane and consist of vesicles, lamellae, or membrane stacks. Cell suspensions are green, beige, brown, brown-red, red or pink. Photosynthetic pigments are bacteriochlorophyll*a*or *b* (esterified with phytol or geranylgeraniol) and various types of carotenoids. In most species, the formations of pigments and of the internal membrane systems are repressed under oxic conditions but become no repression at anoxic conditions.

Cells preferentially grow photoheterotrophically under anoxic conditions in the light. Photoautotrophic growth with molecular hydrogen and sulfide as photosynthetic electron donors may be possible, if growth factors are supplied. Most species are capable of chemotrophic growth under microoxic to oxic conditions in the dark. Some species are very sensitive to oxygen, whereas others grow equally well aerobically in the dark. Fermentation and anaerobic growth with appropriate terminal oxidants growth may occur. Polysaccharides, poly-β-hydroxybutyric acid and polyphosphate may be present as storage materials. One or more vitamins are generally required as growth factors, most commonly biotin, thiamine, niacin and *p*-

aminobenzoic acid. Growth of most species is enhanced by small amounts of yeast extract, and some species have a complex nutrient requirement.

Members of this group are widely distributed in nature and have been found in freshwater, marine and hypersaline environments that are exposed to light. These species live preferentially in aquatic habitats with significant amounts of soluble organic matter, low oxygen tension and moderate temperatures, but also in thermal springs and alkaline soda lakes. Colored blooms, which are characteristically formed by representatives of purple sulfur bacteria and phototrophic green bacteria, are rarely formed. These orders are classified with Rhodospirillales, Rhizobiales and Rhodobacterales of the γ-proteobacteria.

Phototrophic β-*proteobacteria*

Bacteria of this group are phototrophic PNSB, able to perform anoxygenic photosynthesis with bacteriochlorophylls and carotenoids as photosynthetic pigments. Cells are straight to curved rods, or circles, may be motile by means of polar flagella, divide by binary fission and do not have gas vesicles. Internal photosynthetic membranes are much less developed than in other phototrophic purple bacteria appearing as small finger-like intrusions and are not always evident.

Growth preferentially occurs under photoheterotrophic conditions anaerobically in the light. Reduced sulfur compounds are not used as photosynthetic electron donor and sulfide inhibits growth at low concentrations. Sulfate can be assimilated as a sulfur source. These orders are classified with Rhodocyclales and Burkholderiales of the β-proteobacteria.

Phototrophic γ-*proteobacteria*

Phototrophic purple sulfur bacteria that are able to perform photosynthesis under anoxic conditions without oxygen production, that preferentially use reduced sulfur compounds as photosynthetic electron donors and that grow photolithoautotrophically are Gammaproteobacteria. They are anoxygenic phototrophic bacteria and contain bacteriochlorophyll*a* or *b* and various types of carotenoids as photosynthetic pigments located in the cytoplasmic membrane and in internal membrane systems of different fine structure, which originate from and are

continuous with the cytoplasmic membrane. These species are classified with the *Chromatiaceae* and *Ectothiorhodospiraceae* of the Chromatiales.

Purple nonsulfur bacteria (PNSB) are versatile group of purple bacteria that can grow well both phototrophics and heterotrophics under light and dark conditions, respectively. These PNSB can use CO_2 and organic compounds as a carbon source for photosynthesis (Madigan and Gest, 1979). Various organic compounds can be used by PNSB such as formate, methanol and acetate (Qadri and Hoare, 1968; Quayle and Pfennig, 1975). These PNSB can utilize acetate under conditions of anaerobic-light and aerobic-dark. For instance, *R. palustris* and *R. vanneilii* have an incomplete TCA cycle, but they possess the enzymes isocitratelyase and malate synthase in glyoxylate shunt to convert acetyl-CoA into anapleurotic and gluconeogenic compounds (Alber and Gottschalk. 1976).

A new pathway for acetate assimilation in *R. sphaeroides* was purposed by Alber *et al*., (2006) which is called ethylmalonyl-CoA (EM) pathway. Assimilation begins with the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA, which undergoes reduction to β-hydroxybutyryl-CoA. The β-hydroxybutyryl-CoA is in turn activated and carboxylated to form the novel intermediate mesaconyl-CoA. Hydration of mesaconyl-CoA yields β-methylmalyl- CoA, which undergoes cleavage to glyoxylate and propionyl-CoA. Condensation of glyoxylate with acetyl-CoA yields malate, while the typical reactions of propionate metabolism result in carboxylation and conversion of propionyl-CoA to succinate. Thus, via this new pathway, CoA-activated esters of conventional and novel C3-C5 compounds serve as substrates for a series of condensation rearrangement and carboxylation reactions that form two C4 gluconeogenic precursors from three acetyl-CoA and two CO_2 molecules as summarized as follows:

$$3 \text{ Acetyl CoA} + 2\ CO_2 \longrightarrow \text{L-malate + succinate}$$

Application of PNSB in wastewaters

Kantachote *et al*. (2005) demonstrated that *Rhodopseudomonasblastica* DK6 had the best potential for use in treating latex rubber sheet wastewater. It was found that over 96 h a mixed culture (DK6 with indigenous microorganisms) under microaerobic-light conditions showed 90% removal of both COD and BOD and obtained 66.7% crude protein on the basis of dry weight.

Madukasi *et al*. (2010) designed a photobioreactor to treat pharmaceutical wastewater by *Rhodobactersphaerodies* Z08 under optimum conditions reduced 80% COD. The maximum dry cell weight and COD removal were 880 mg/L and 80% when using of Proximate analysis of the biomass after treatment of the enriched (with ammonium sulfate and yeast extract) and non-enriched wastewatershad crude protein 54.6 % and 38.0 %, respectively.

Prachanurak *et al*. (2014) demonstrated that photo-bioreactor with internal overflow recirculation was applied to treat real fermented starch wastewater and convert it to photosynthetic biomass for further utilization. The average BOD and COD removals were 95% and 88%, respectively during the operation of 154 days. Majority of photosynthetic bacteria was found attached on pipes as biofilm contributed to 82% of total biomass production. Photosynthetic biomass yield was 0.51 g dried solid/g BOD removed and crude protein content of 0.58 g/g dried solid.

Fermented plant extract and its application in wastewater treatment

Recently, fermented plant extracts (FPEs) or fermented plant juices have been extensively used as a natural liquid fertilizer by Thai farmers because they are easily produced from agricultural products or agricultural waste. These FPEs can promote plant growth and also act as bio-control agents depending on the type of plants being used (Kantachote and Charernjiratrakul, 2008). Moreover, it can be used in wastewater treatment such as latex rubber sheet wastewater (Kornochalert, 2008).

Response surface methodology; Central Composite Design (CCD) was used to investigate effects of inoculants (*Rhodopseudomonas palustris* P1) and fermented pineapple extract (FPE) for treating wastewater from a CRSF (Kornochalert, 2008). The best optimal condition for treatment wastewater was 3% inoculants and 1:750 FPE for 96 h by removals of 97% BOD, 92% COD, 87% SS, 58% sulfate, 71%

TS and 83% UHS. The effluent met both standards set by Industrial Factory Department and Irrigation Department (Kantachote *et al*., 2010).

Anwar et al. (2013) investigated the effectiveness of effective microorganisms (EM) in an Anaerobic Sequencing Batch Reactor (ASBR) system to treat rubber processing wastewater. It was found that the ratio of 1:1000 performed best to treat the sample (60% of COD, 62% of BOD5, 62% of turbidity, and 84% of TSS). This study suggested that EM technology in an anaerobic environment has the capability of elevating the quality of the treated rubber wastewater.

Molecular microbial ecology

Conventional microbiological techniques, based on isolation of pure cultures and morphological, metabolic, biochemical and genetic assays, have provided extensive information on the biodiversity of microbial communities in natural and engineering systems (Bitton, 2005). However, the drawbacks of the existing conventional methods, such as incomplete knowledge about their physiological (nutritional and physical–chemical) needs and the complex syntrophic and symbiotic relations, which are abundant in nature, make it is impossible to obtain pure cultures of most microorganisms in natural environments. Moreover, most culture media tend to favor the growth of certain groups of microorganisms, whereas others that are important in the original sample do not proliferate. It is therefore generally accepted nowadays that the number of known prokaryotic species (including the two domains: Bacteria and Archaea) is very small compared to the diversity of microorganisms and illustrates how difficult it is to get a full picture of the bacterial diversity of an ecosystem by relying only on conventional methodology. At present, a lot of bacteria species have been described but according to molecular and ecological estimates, the real number must be several orders of magnitude higher (Amann *et al*, 1995). This small known fraction does not reflect the composition and diversity of a microbial community.

One suitable solution to this problem is to use molecular biology approaches. The techniques are based on the RNA of the small ribosomal subunit (16S rRNA for prokaryotes) or their corresponding genes, considering it as a ‘‘molecular clock’’ or ‘‘evolutionary chronometer’’. This molecule was chosen because of its universality and abundance in all living beings (103 to 105 ribosomes/cell) and the fact

that it is a highly conserved molecule throughout evolution although bears some highly variable regions. These features allow comparison of organisms within the same domain, as well as differentiation of strains of the same species. Moreover, the gene sequence is sufficiently long to generate statistically relevant data and can be easily sequenced with current technology. The focus of this review is to present the main molecular biology techniques actually used to identify and quantify microorganisms in wastewater treatment systems, with its main advantages and disadvantages. These techniques have been developed very recently and mostly used at lab scale. Its real potential as tools for design or monitoring full-scale wastewater treatment plant must be evaluated in a near future.

Molecular biology techniques and their applicability to wastewater treatment

The possibility of identifying specific populations of microorganisms in their native habitat without the need to isolate them is revolutionizing microbial ecology and giving rise to various new applications in numerous research fields.

In wastewater treatment, microbial molecular ecology techniques have been applied mainly to the study of floc (activated sludge) and biofilm that grow in aerobic treatment systems (trickling filters). Based on the aim of this study the scope of the review, placing particular emphasis on anaerobic digestion and to the techniques unquestionably most broadly used: denaturant gradient gel electrophoresis (DGGE), Fluorescent in situ hybridization (FISH) and cloning of 16S rDNA. But polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) is based on powerful culture-independent tool a direct analysis of DNA from the microbial environment (Zhang *et al*., 2014).

Denaturant gradient gel electrophoresis (DGGE)

Denaturant gradient gel electrophoresis (DGGE) is reflected by the increasing number of studies that use this technique. It is based on the differing mobility on a gel of denatured DNA-fragments of the same size but with different nucleic acid sequences, thus generating band patterns that directly reflect the genetic biodiversity of the sample. The number of bands corresponds to the number of dominant species. Coupled with sequencing and phylogenetic analysis of the bands, this method can give

a good overview of the composition of a given microbial community (Figure 2-3).

DGGE is the method of choice when the desired information does not have to be as phylogenetically exhaustive as that provided by cloning, but still relatively precise to determine the dominant members of a microbial community with medium phylogenetic resolution. Since, its first use in the study of complex microbial populations (Muyzer and Smalla, 1998) this method has been employed in the characterization of a wide array of habitats, such as soil, hot springs and continental waters. The technique is less widely used in anaerobic wastewater treatment, though in recent years DGGE seems to be becoming increasingly popular. For example, DGGE has been used for the evaluation of the granular sludge's microbial diversity from UASB reactors treating brewery (Chan *et al*., 2001), alcohol distillery (Akarsubasi *et al*., 2006) and unbleached pulp plant wastewaters (Buzzini *et al*., 2006).

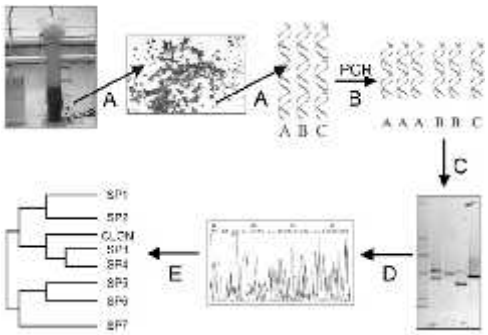

Figure 2-3. Schematic representation of DGGE. DNA is extracted from the original sample, in this case as granular sludge from a UASB reactor (A). The 16S rRNA gene is partially amplified by PCR usually with universal primers to give a mixture of DNA fragments, all of the same length. Each of the different product DNA molecules resulting from this step represents a different microorganism (B). The DNA mixture is then separated by denaturant gradient electrophoresis on an acrylamide gel with an increasing urea/formamide gradient. The DNA molecules migrate towards the positive pole and come to a halt on the gel on reaching their corresponding denaturant force (Tm), which depends on the DNA sequence. Every band on the gel corresponds to a different microorganism in the sample, providing sufficient information for many requirements (C). The bands can be cut from the gel and the DNA extracted and sequenced (D). Comparison of the sequences with a 16S rDNA database allows todetermine the phylogenetic affiliation of the microorganism (E).
Source: Sanz and Köchling, (2007).

In general, this technique is not used alone but rather as part of a combined approach with other methods, for example with in situ hybridization in the

study of sulfate reducing bacteria (Santegoeds *et al*., 1998) or phosphorous elimination (Oehmen *et al*., 2005). Both these are good examples of the advantages of combining fingerprinting with in situ hybridization. The authors managed to trace the most probable protagonist in the process by evaluating DGGE band intensity and then designing a specific probe with the help of the predominant band sequence, in turn enabling quantification of the candidate and confirmation of the results obtained by DGGE.

Belila *et al*. (2013) studied diversity of the purple photosyntheticbacteria during temporal pigmentation changes in wastewaterstabilization ponds treating domestic wastewater by DGGE method. This phototrophic bacterial assemblage was dominated by the purple non-sulfur bacteria group (59.3 %) with six different genera followed by the purple sulfur community (27.8 %) with four genera and finally 12.9 % of the *puf*M gene sequences were assigned throughout theaerobic anoxygenic phototrophic bacterial group.

Table 2-3 summarizes these techniques and their possible fields of application within the area of wastewater treatment.

Table 2-3. Comparison of the main molecular biology techniques and their application in bioreactors for wastewater treatment.

Limitation	Gene library	DGGE	FISH
Required skill level in molecular biology	High	Medium	Low
Specific facilities	PCR	PCR, DGGE	Fluorescence microscope, confocal laser scanning microscope (not mandatory)
Requires sequencing	Yes	Yes (conditional, band pattern could be sufficient)	No

Table 2-3. Comparison of the main molecular biology techniques and their application in bioreactors for wastewater treatment (continues).

Limitation	Gene library	DGGE	FISH
Quantitative	No	No	Yes
Time demands	High	Medium (low if sequences are not required)	Low
Phylogenetic precision	High	Medium	Low/medium
Structural analysis (3D-architecture)	No	No	Yes (requires confocal laser microscopy)
Sample throughput	Low	High	High
Observation of spatial temporal changes	No	Yes	Yes (only for a few key probes)
Detection of a particular taxonomic group or species	No	No	Yes
Apt for monitoring bioreactors	No	No/yes (only if band pattern shows obvious differences)	Yes, easy to standardize process for indicator species
Applicability in anaerobic digestion	Low	Medium	High
Quantitative	No	No	Yes
Time demands	High	Medium (low if sequences are not required)	Low
Phylogenetic precision	High	Medium	Low/medium

Table 2-3. Comparison of the main molecular biology techniques and their application in bioreactors for wastewater treatment (continues).

Limitation	Gene library	DGGE	FISH
Structural analysis (3D-architecture)	No	No	Yes (requires confocal laser microscopy)
Sample throughput	Low	High	High
Observation of spatial temporal changes	No	Yes	Yes (only for a few key probes)
Detection of a particular taxonomic group or species	No	No	Yes
Apt for monitoring bioreactors	No	No/yes (only if band pattern shows obvious differences)	Yes, easy to standardize process for indicator species
Applicability in anaerobic digestion	Low	Medium	High

Source: Sanz, and Köchling, (2007).

Wastewater reuse for irrigation

Wastewater reuse is the use of water for a beneficial goal such as crop and landscape irrigation or urban applications. This practice has gained importance worldwide because of water shortages, particularly in arid areas of USA (e.g. California, Arizona, Texas, Colorado) and wastewater disposal regulations that are becoming increasingly stringent. Essentially, water reuse is increasingly becoming an important increasingly becoming an important component of sustainable management of water resource (Levine and Asano, 2004). The reuse of effluent from wastewater treatment plants is possible in wide range of categories, as shown in Figure 2-4.

The irrigational use of treated effluent and sludge must be given the fullest consideration in planning any wastewater disposal scheme. There is sound logic

in returning the solids back to the soil and reusing the wastewater wherever feasible. From the agricultural point of view, treated wastewater and sludge could be used effectively provided certain quality constrains are met. Theadvantages over plain water such as: presence of fertilizing constituents (nitrogen, phosphorus and micro-nutrients) and favorable soil conditioning properties (e.g. aiding water retention).

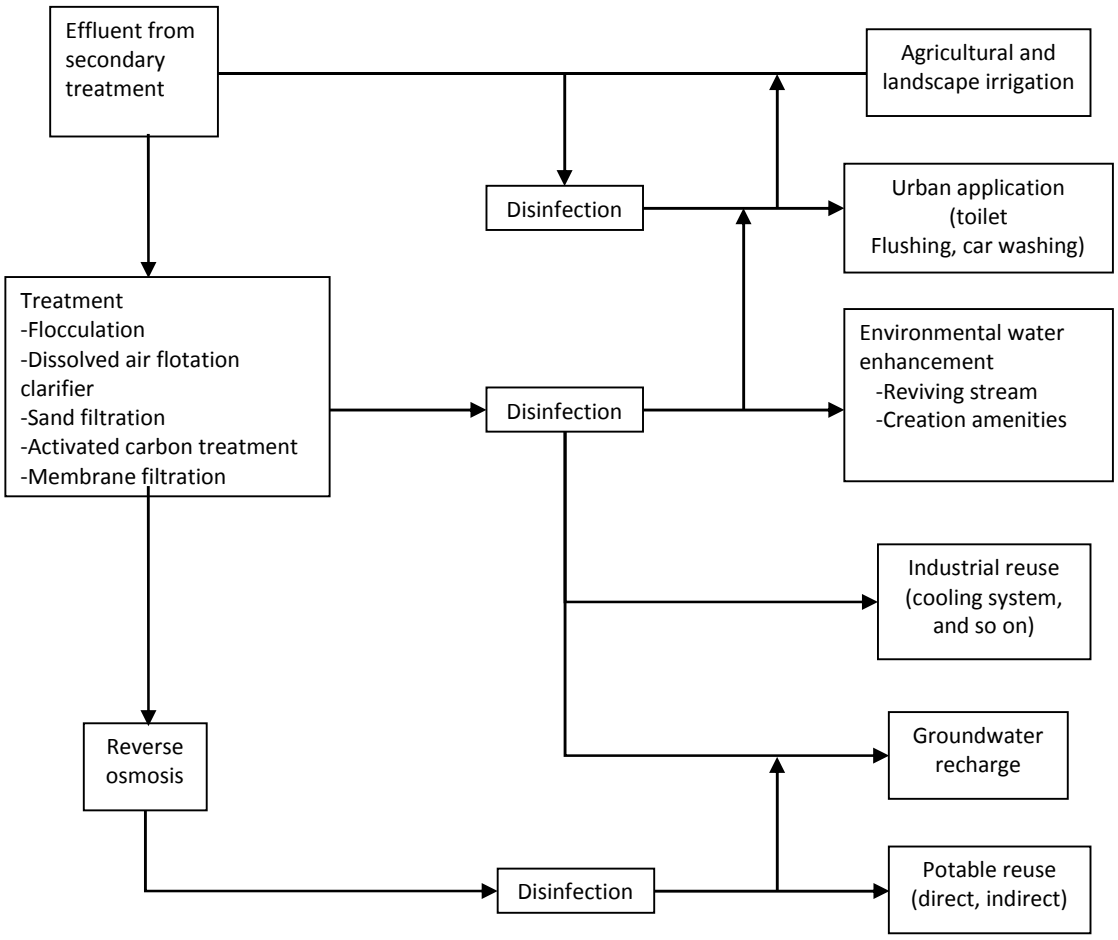

Figure 2-4. Wastewater treatment plant effluent reuse
Source: Eckenfelder *et al*., (2009).

Wastewater irrigation becomes a successful method of 'treatment' achieving primary, secondary, tertiary treatment in many instances, is capable of giving returns in the form of crops and reusable water. However, the main disadvantages associated with its irrigational use are; contamination and health hazards which likely harm agricultural workers and consumers of the crops (the food chain) due to, possible chemical effects of the wastewater or sludge on the soil and ground water.

Chantsavang *et al*. (1996) reported the use of effective microorganisms (EM) for treating pig wastewater and/or EM treated pig manure as sources of plant nutrients in comparison with chemical fertilizer and EM fermented compost. A series of pot experiments were conducted to evaluate yields of kale, chinese radish, chilli and marigold. The results showed that yields of kale grown with EM treated pig wastewater and/or EM treated pig manure were significantly lower than the chemical fertilizer. In contrast, no significant difference was found for the rest of plant tested between the effluent and chemical fertilizer.

Aiello *et al*. (2007) investigated the effects of reclaimed urban wastewater for irrigation on tomato fruit quality in Eastern Sicily, Italy. It was found that wastewater application increased microbial contamination on the soil. Tomato yield was increased although only slight accumulation of *E. coli* on tomato fruits irrigated with wastewater.

Gupta *et al*. (2008) studied effects of municipal wastewater irrigation on the accumulation of heavy metals (Pb, Zn, Cd, Cr, Cu and Ni) in soil and vegetables by monitoring wastewater-irrigated agricultural field of Titagarh, 24-Parganas (North), West Bengal, India. The mean concentrations of Pb, Ni, Cu in the irrigation water and the mean Cd content in soil were much above the recommended level. The concentrations of Pb, Zn, Cd, Cr and Ni in all the examined vegetables were beyond the safe limits.

Finley *et al*. (2009) demonstrated reuse of domestic greywater for irrigation of lettuce, carrots and peppers in a greenhouse. They found that no significant difference in contamination levels of fecal coliform and streptococci was observed between crops irrigation with tap water, untreated greywater and treated greywater. Moreover, contamination levels for all crops did not represent a significant health risk.

Response surface methodology (RSM)

Response surface method (RSM) is a collection of mathematical and statistical techniques that is useful for the modeling and analysis of problems (Montgomery, 2005). The technique is useful for developing, improving, and optimizing processes in which a response of interest is influenced by several variables and the objective is to optimize this response. In addition to analyzing the effects of the

independent variables, this experimental methodology also generates a mathematical model. The graphical perspective of the mathematical model has led to the term *Response Surface Methodology* (Bas and Boyacl, 2007). The relationship between the response and the input is given in Eq. (1):

$$\eta = f(x_1, x_2, \ldots, x_n) + \varepsilon \quad (1)$$

where η is the response, f is the unknown function of response, x_1, x_2, ..., x_n denote the independent variables, also called natural variables, n is the number of the independent variables and finally ε is the statistical error that represents other sources of variability not accounted for by f.

These sources include the effects such as the measurement error. It is generally assumed that ε has a normal distribution with mean zero and variance. The process of RSM is described for optimization study according to Bas and Boyacl, (2007) and Montgomery, (2005) as follows:

(1) Preliminary work: determination of independent variables and their levels

The first step in RSM is to find a suitable approximation for the truefunctional the relationship between response (y) and a set of independent variable (x). The factorial design may be used to screen the independent variables which main effect on response.

Coded variables are created to represent X for experimental design and regression analysis. The coded variables in the designed experiment of range -1 to 1 will be transformed to natural variables x (Myers *et al*., 2009).

$$X = \frac{x - [x_{max} + x_{min}]/2}{[x_{max} + x_{min}]/2}$$

Where x is the natural variable, X is the coded variable and x_{max} and x_{min} are the maximum and minimum values of the natural variable.

(2) Selection of the experimental design, prediction and verification of model equation

Experimental data of selected independent variable could be used to determine the relationship between response and selected independent variable by a low-order polynomial that the response is well modeled by a linear function of the independent variables, then the first-order model is the approximating function Eq. (2).

If the system is curvature, the second-order model is used for a polynomial of higher degree Eq. (3) (Montgomery, 2005).

The first-order model

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \ldots + \beta_k x_k + \varepsilon \quad (2)$$

Where β_0 is intercept, β is regression coefficient, β_k is liner and x_k is coded variable.

The second-order model

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x^2_i + \sum\sum_{i<j} \beta_{ij} x_i x_j + \varepsilon \quad (3)$$

Where β_{ij} is interaction and β_{ii} is quadratic coefficients

Response surface design is the design that uses a full quadratic equation or second-order equation for fitting response surface. The popular designs central composite design and three level design such as the Box-Behnken are used the optimum.

Box and Behnken (1960) developed a family of efficient three level designs for fitting second-order response surfaces. The methodology for design construction is interesting and quite creative. The class of designs is based on the construction of balanced incomplete block designs which this design by combining two level factorial designs with incomplete block designs. These designs are, in this way, more efficient and economical then their corresponding 3^k designs, mainly for a large number of variables (Bezerra *et al*., 2008).

In Box–Behnken designs, the experimental points are located on a hypersphere equidistant from the central point, as exemplified for a three-factor design in Figure 2-5. Its principal characteristics are:

(1) requires an experiment number according to $N= 2k(k-1) + c_p$ where k is the number of factors and (c_p) is the number of the central points.

(2) all factor levels have to be adjusted only at three levels (−1, 0, +1) with equally spaced intervals between these levels.

Its 13 experimental points present the Box–Behnken design for three-variable optimization (Figure 2-5a). In comparison with the original 3^3 design with 27 experiments (Figure 2-5b), it is noted that this design is more economical and efficient.

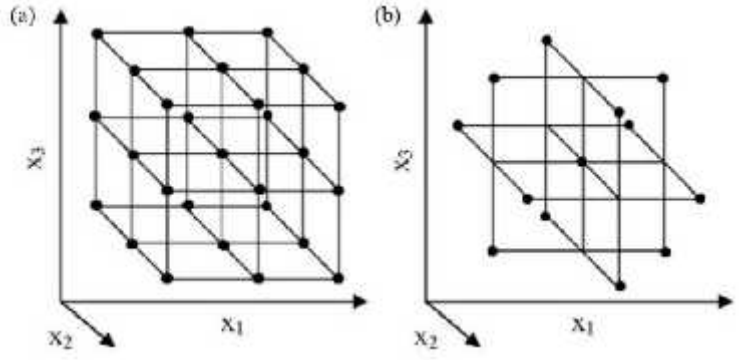

Figure 2-5. Experimental designs based on the study of all variables in three levels: three-level factorial design for the optimization of (a) three variables and (b) Box–Behnken design for the optimization of three variables.

After selection of the design, the next experiments data would be displayed as surface plot and regression equation that is second-order equation. The optimum of response would be verified after analyze model.The more reliable way to evaluate the quality of the model fit is by the application of analysis of variance (ANOVA). It is to compare the variation due to the treatment (change in the combination of variable levels) with the variation due to random errors inherent to the measurements of the generated responses (Montgomery, 2005). From this comparison, it is possible to evaluate the significance of the regression used to foresee responses considering the sources of experimental variance. The significant of each coefficient present in regression model is determined by the F-value (Fisher variation ratio) and values of probability >F. F-value is used to test the significant of regression model. Prob>F is the probability of seeing the observed F-value if this value is less than 0.05 indicate that the model terms are considered to be statistically significant. R^2 (coefficient of determination) is the proportion of the total variation in the response variable that is explained by independent variable. It is used to verify the quality of the fit of polynomial model. Coefficient of variance (CV) is the ratio of the standard error of estimate to the mean value of the observed response (express in percentage) that is a measure of reproducibility of the model and as a general rule a model can be considered reasonably reproducible if its CV is less than 10% that well precision and reliability of the experiments. Adequate precision is a measure of the range in predicted response relative to its associated error. The lack of fit F-test is used to test the model adequacy.

If insignificant, the P-value was greater than 0.05.

(3) Graphical presentation of the model equation and determination of optimal operating conditions

The response surface plot and contour plot can be obtained the visualization of the predicted model equation (Figure 2-6). The response surface plot is the theoretical three-dimensional plot showing the relationship between the response and the independent variables. The two-dimensional display of the surface plot is called contour plot.

Characterize the shape of the surface and the optimum with reasonable precision can be used to the contour plot. Stationary point; maximum, minimum and saddle point is called the point level of the independent variables that optimize the predicted response (Montgomery, 2005). The plane of the independent variables is drawn in the contour plot. The maximum or minimum response is present the center of the system that the contour plot shows ellipses or circles.

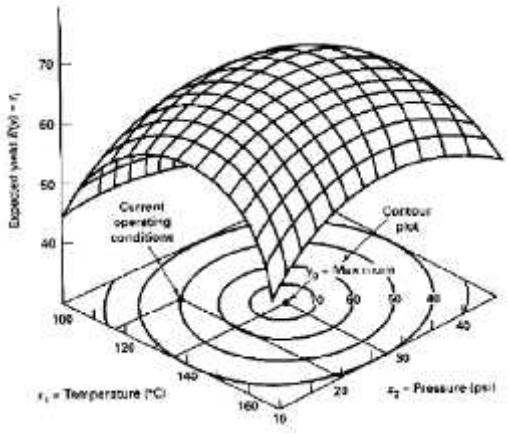

Figure 2-6. A three dimensional response surface showing the expected yield with a contour plot of a response surface.
Source: Montgomery, (2005).

The advantage of RSM, large information from a small of experiments provides RSM that saved time and cost of experiment. It can be possible to observe both main effect and interaction of independent variable on responses. The second order model that related the response to independent variables is employed to receive information about the system.

The disadvantage of RSM is to fit the data to a second order polynomial. Some cases, the data could not be curveted, so there is not fit with this model. Preliminary work becomes more critical for the determination of the independent parameter range.

CHAPTER 3

Bioaugmentation of latex rubber sheet wastewater treatment with stimulated indigenous purple nonsulfur bacteria by fermented pineapple extract

Abstract

Treating latex rubber sheet wastewater often leads to the generation of a rotten-egg odor from toxic H_2S. To increase the treatment efficiency and eliminate H_2S, purple nonsulfur bacteria (PNSB), prepared by supplementing non sterile rubber sheet wastewater (RAW) with fermented pineapple extract (FPE), were used to treat this wastewater under microaerobic light conditions. The following 3 independent variables; chemical oxygen demand (COD), initial pH and FPE dose were investigated using the Box-Behnken design to find optimal conditions for stimulating the growth of indigenous PNSB (PNSBsi). The addition of 2.0% FPE into RAW, which had a COD of 2000 mg/L and an initial pH of 7.0, significantly decreased oxidation reduction potential (ORP) value and stimulated PNSBsi to reach a maximum of 7.8 log CFU/mL within 2 days. Consequently, these PNSBsi, used as inoculants were investigated for their ability to treat the wastewater under microaerobic light conditions. A central composite design was used to determine the optimal conditions for the wastewater treatment. These proved to be 7% PNSBsi, 0.8% FPE and 4 days retention time (RT) and this combination resulted in a reduction of 91% for COD, 75% for suspended solids, 61% for total sulfide while H_2S was not detected. Results of an abiotic control and treatment sets indicated that H_2S was produced by heterotrophic bacteria present in the RAW and FPE and it was then effectively deactivated by the PNSBsi.

Key words: bioremediation; fermented plant extract; purple nonsulfur bacteria; response surface methodology; rotten-egg odor; rubber wastewater.

บทคัดย่อ

การบำบัดน้ำเสียจากการแปรรูปยางแผ่นก่อให้เกิดแก๊สไข่เน่าจาก H_2S การเพิ่มประสิทธิภาพการบำบัด และกำจัด H_2S ด้วยแบคทีเรียสังเคราะห์แสงกลุ่มไม่สะสมซัลเฟอร์ (purple nonsulfur bacteria: PNSB) ที่เตรียมในน้ำเสียที่ผ่านการฆ่าเชื้อเติมด้วยน้ำหมักสับปะรด (fermented pineapple extract: FPE) ถูกนำไปใช้ในการบำบัดน้ำเสียภายใต้สภาวะมีอากาศเล็กน้อย-มีแสง การติดตาม 3 ปัจจัยอิสระ ได้แก่ chemical oxygen demand (COD) pH เริ่มต้น และ ปริมาณ FPE ในการหาสภาวะที่เหมาะสมของการกระตุ้นการเจริญของ PNSB ในธรรมชาติ (PNSBsi) โดยวิธีทางสถิติแบบพื้นที่การตอบสนอง Box-Behnken design การเติม FPE 2% ของน้ำเสียที่มีค่า COD เท่ากับ 2000 มก./ล. pH เริ่มต้น 7.0 สามารถทำให้ค่า (oxidation reduction potential: ORP) ลดลงอย่างมีนัยสำคัญ และกระตุ้นการเจริญของ PNSBsi ได้สูงสุด (7.8 log CFU/ml) ในเวลา 2 วัน กล้าเชื้อ PNSBsi ที่ถูกกระตุ้นถูกติดตามประสิทธิภาพในการบำบัดน้ำเสีย ภายใต้สภาวะมีอากศเล็กน้อย-มีแสง วิธีทางสถิติแบบพื้นที่การตอบสนอง central composite design ได้นำมาใช้ศึกษาสภาวะที่เหมาะสมของการบำบัดน้ำเสียคือ 7% PNSBsi 0.8% FPE และ ระยะการบำบัด (retention time: RT) 4 วัน โดยมีการลดลงของ COD SS (suspended solids) และ TS (totoal sulfide) ได้ 91 75 และ 61% ตามลำดับ และตรวจไม่พบ H_2S ผลการทดลองในชุดควบคุม (abiotic) และชุดทดลองบ่งชี้ว่าปริมาณ H_2S ถูกผลิตโดยแบคทีเรียในกลุ่ม heterotrophic ในน้ำเสีย และ FPE ซึ่งจะถูกกำจัดอย่างมีประสิทธิภาพด้วย PNSBsi

คำสำคัญ: การบำบัดทางชีวภาพ น้ำหมักสับปะรด แบคทีเรียสังเคราะห์แสงกลุ่มไม่สะสมซัลเฟอร์ วิธีการตอบสนองพื้นที่ผิว กลิ่นไข่เน่า น้ำเสียยางพารา

Introduction

Since their founding in years 1994 and 1995 by the central government of Thailand, many of these small cooperative rubber sheet factories (CRSFs) are still functioning throughout Thailand (Chaiprapat and Sdoodee, 2007). One serious problem arising from the treatment of their wastewater is the associated noxious smell of rotten-eggs gas (hydrogen sulfide, H_2S). Open lagoons or natural oxidation ponds used for this treatment now are commonly operated in these factories (Chaiprapat and Sdoodee, 2007; Kantachote *et al*., 2010). It is well recognized that H_2S is a toxic gas which at high level is dangerous to human health and at low level causes nuisance odor (Yalamanchili and Smith, 2008; Doujaiji and Al-Tawfiq, 2010). Devising a means to solve this problem by removing H_2S requires the cooperative efforts of engineers and microbiologists. It is well recognized that phototrophic bacteria, especially purple nonsulfur bacteria (PNSB) are well capable of treating wastewaters due to their versatile metabolic pathways. They have a capability to grow as both photoautotrophs and photoheterotrophs under conditions of anaerobic or microaerobic-light, and chemoheterotrophs under conditions of anaerobic or aerobic-dark (Kantachote *et al*., 2005; Okubo *et al*., 2006; Liang *et al*., 2010; Madukasi *et al*., 2010). Some members of the PNSB such as *Rhodopseudomonas*, *Rhodobacter* and *Rhodospirillum* are known to reduce the H_2S odor from facultative waste stabilization ponds (Veenstra *et al*., 1995; Kim *et al*., 2004).

In our previous work, a combination of fermented pineapple extract (FPE) as growth stimulator and a selected purple nonsulfur bacterium *Rhodopseudomonas palustris* P1 showed a high efficiency for treating latex rubber sheet wastewater to meet the Thai standard guidelines within only 3 days. It was also found that the mix ratio of bacterial culture as inoculum had a great influence during the reaction period (Kantachote *et al*., 2010). It is, however, difficult for CRSFs to have access to the functional PNSB inoculants to treat their wastewater. It was also observed that FPE, when added at an optimal amount, i.e. 0.13%, facilitated the treatment of non sterile latex rubber sheet wastewater (RAW). This relatively small amount of FPE stimulated the growth of indigenous PNSB under conditions of anaerobic or microaerobic-light by providing simple substrates such as acetate and lactate that were

easily consumed by PNSB (Shi and Yu, 2006; Chen *et al*., 2008; Kantachote *et al*., 2010). This stressed the feasibility of preparing the PNSB inoculant culture with the help of a simple and easy to find FPE. However, successful bioaugmentation depends on the use of the effective inoculants, either single culture or consortium, which are usually well adapted to each individual wastewater. This is mainly related to the survival and degrading ability of microorganisms introduced to a wastewater system as dictated by various biotic and abiotic factors within the system. Therefore, autochthonous bioaugmentation is attractive to use for the enhancement of efficiency of wastewater system due to the fact that foreign microbes when used as inocula, are unable to avoid competition with normal flora.

The amount of added FPE into the wastewater medium decreases initial pH and increases COD value, thereby optimum initial pH and COD values should be investigated as these are the key factors for stimulating growth of indigenous or native PNSB (PNSBsi) to make good inocula. The response surface methodology (RSM) which is a collection of mathematical and statistical techniques for the modeling and analysis of multivariate problems has been used to optimize the desired outputs (Bezerra *et al*., 2008). This methodology is more practical as it arises from experimental methodology that includes interactive effects among the variables and eventually, it depicts the overall effects of the parameters in the process (Bas and Boyaci, 2007). In the process of the wastewater treatments, RSM was reportedly effective in evaluating the interactive effects of operating parameters (Ahmadi *et al*., 2005; Wang *et al*., 2005).

The aims of this study were firstly to stimulate indigenous PNSB (PNSBsi) in a RAW lagoon with FPE for re-inoculation in the treatment of rubber sheet wastewater, and secondly to determine the optimal values of the factors affecting the growth of indigenous PNSB and their efficiency to treat the rubber sheet wastewater.

MATERIALS AND METHODS

Rubber sheet wastewater medium

Rubber sheet wastewater was collected from a lagoon of a CRSF at Pichit suburb, Songkhla Province, Thailand. The collected wastewater was filtered through cheesecloth into a 25 liter non transparent plastic tank and stored in a cold room at 6 ± 2°C until use. The wastewater without autoclaving was supplemented with 0.05% NH_4Cl as a supplementary nitrogen source to allow a reasonable growth of PNSB (based on our preliminary work). This medium was named RAW since the non-sterile rubber sheet wastewater was used.

Monitoring parameters

The methods used in this study are described in the Standard Methods (APHA, 1998). All effluent samples including RAW were placed in a cold room for 2 hrs to allow sedimentation. The supernatant (clear liquid near the water surface) was sampled for the measurement of settleable COD by the dichromate reflux method. Sulfide was measured in 3 forms, as total sulfide (TtS), dissolved sulfide (DsS) and un-ionized hydrogen sulfide (UHS: H_2S) using the iodometric method, while sulfate was measured using the turbidimetric method. A portable multi gas detector (MX 2100, Oldham, France) was used to measure H_2S in headspace of the treatment bottles. In this work, volatile fatty acids (VFAs) were analyzed by distillation method. A pH meter (Seven multi, Metller Toledo, USA) was used to measure pH and electrical conductivity (EC). The oxidation-reduction potential (ORP, Eh) probe (La Motte, USA) was used to measure the Eh values and the data were recorded after obtaining a constant value. The probe was checked frequently in a quinhydrone buffer solution following the method described by the manufacturer. Total acidity was determined by a titration method and presented as a 'lactic acid amount'. The actual amounts of lactic and acetic acids were determined using gas chromatography according to the method of Yang and Choong (2001). Viable cells count of PNSB was performed on GM (glutamate-malate agar) and incubated in anaerobic light conditions for 5 days (Kantachote *et al*., 2005) whereas yeasts, heterotrophic plate count (HPC) and lactic acid bacteria (LAB) were enumerated on potato dextrose agar (PDA), plate count agar (PCA) and de Man Rogosa and Sharp

(MRS) agar for 3 days. Both LAB and yeasts were counted because these microbial groups play important roles in the production of the FPE.

Fermented pineapple extract

FPE was produced in our laboratory according to the method described by Kantachote *et al*., (2009). The fermentation process lasted for 2 months. At that time, it composed of 1.90% total acidity, 0.58% lactic acid and 0.15% acetic acid with a pH of 3.61 and an EC value of 3.51 mS/cm. In addition to the nutrients, the population of HPC, yeasts and LAB were estimated in the region of 10^6 CFU/mL for each group. FPE was kept in a cold room until used.

Experimental design for studying the stimulation of indigenous PNSB from a lagoon with FPE to make an inoculum for treating RAW

RSM using Box-Behnken Design (BBD) was chosen because relatively fewer experimental combinations of the variables were required to estimate potentially complex response functions with an acceptable reliability (Annadurai and Sheeja, 1998). The BBD was used to optimize three input independent variables on the response of the amount of PNSBsi in RAW. The significant variables; initial COD, initial pH and amount of FPE were selected as the critical independent variables and designated as X_1, X_2 and X_3, respectively. The low, middle and high levels of each variable were coded as -1, 0, +1, respectively (Table 3-1) with the design matrix of a 17-trial experiment, established using a Design Expert 6 software (Stat Ease Inc. Minneapolis, USA). Variations on the values of each independent variable were designed according to the results of our preliminary work. The behavior of the system was explained by the following quadratic equation model.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

Where Y is the predicted response; β_0 is the intercept; β_1, β_2 and β_3 are the linear coefficients, β_{11}, β_{22} and β_{33} are the square coefficients and β_{12}, β_{13} and β_{23} are the interaction coefficients. To predict the optimal point, a second order polynomial function was fitted to correlate the relationship between the independent variables and the response values (dependent variables; PNSBsi and ORP). The optimal conditions

for stimulating PNSB (PNSBsi) were obtained by solving the regression equations and also by analyzing overlay interaction plots.

Table 3-1. The Box–Behnken design matrix for the real variables along with the actual and predicted responses of the oxidation reduction potential (ORP) and stimulated indigenous purple nonsulfur bacteria (PNSBsi) values in non sterile rubber sheet wastewater (RAW) under microaerobic light conditions for 2 days after adding fermented pineapple extract (FPE).

Run no.	Code of variables and levels			ORP (mV)		PNSBsi (log CFU/mL)	
	X_1	X_2	X_3	Actual	Predicted	Actual	Predicted
1	1500	8	2	-188.5	-186.4	7.09	7.25
2	2000	8	1.5	-203.9	-205.12	7.53	7.52
3	2000	7	2	-230.7	-228.2	7.79	7.8
4	2000	7	2	-226.7	-228.2	7.79	7.8
5	2500	7	2.5	-205.3	-204.45	7.53	7.67
6	2500	7	1.5	-190.7	-190.13	7.2	7.2
7	2500	8	2	-188.9	-188.22	6.94	6.95
8	1500	7	1.5	-177.1	-177.95	7.19	7.05
9	2000	8	2.5	-206.9	-208.46	7.46	7.3
10	1500	7	2.5	-173	-173.5	7.1	7.1
11	2000	7	2	-227.3	-228.2	7.83	7.8
12	2000	7	2	-228.6	-228.2	7.8	7.8
13	2500	6	2	-198.7	-200.8	6.94	6.78
14	2000	7	2	-227.7	-228.2	7.81	7.8
15	1500	6	2	-158.8	-159.48	5.79	5.78
16	2000	6	1.5	-197.9	-196.34	6.06	6.21
17	2000	6	2.5	-204.1	-202.88	6.94	6.96

X_1 = initial Chemical Oxygen Demand, COD (mg/L), X_2 = initial pH and X_3 = FPE (%, V/V)

The experimental runs were performed in 120 mL serum glass bottles, containing 100 mL of RAW medium per bottle to achieve microaerobic conditions and all serum bottles were incubated under tungsten light conditions of 3500 lux for 2 days. A Denki light meter (model DK-211) was used to measure light intensity. Three replicates were set for each experimental run. The following parameters; PNSB, COD, pH and ORP, were monitored at the start and at the end of the experiment (2 days

incubation). The ORP was also monitored as a dependent variable as it might help explain the role of FPE on stimulating PNSB growth. To validate the optimum conditions of the 3 independent variables, confirmatory experiments were carried out under microaerobic light conditions. To determine the effect of FPE on PNSBsi growth in RAW, there were 2 sets of verified tests, one with sterile and one with non sterile FPE, beside a control set without FPE addition. Parameters; pH, EC, ORP, VFAs, HPC, LAB and PNSBsi, were monitored at the beginning and end of the 2 day incubation. These experiments were conducted in triplicate and ANOVA (Tukey HSD post-hoc test) was used to analyze data in this verification test with the significance level of 0.05.

Experimental design for studying the treatment of rubber sheet wastewater using stimulated PNSB inoculum and FPE

To determine the optimum conditions for treatment of CRSF wastewater, the key factors most likely to affect the efficiency were investigated: PNSBsi numbers (X_1), FPE concentrations (X_2) and retention times (RT) (X_3). To fit a second order response surface, central composite design (CCD) is used. The levels of these 3 independent variables (X_1, X_2 and X_3) were studied at 5 coded levels; -1.682, -1, 0, +1 and + 1.682 establishing the design matrix for a 20 trials experiment. Variable levels were selected based on the results obtained from preliminary work. Design Expert (version 6, Stat Ease Inc. Minneapolis, USA) was used for experimental design and statistical analysis. The quadratic model equation (Eq.1) was also used to explain the behavior of the system.

The optimum wastewater treatment conditions by PNSBsi were obtained by solving the regression equations and also by analyzing the response surface overlay contour plots. The quality of the fit of the model equations was expressed by the coefficient of determination, R^2, while regression coefficients were used to generate a contour map of the regression model. All experimental runs were conducted in 120 mL serum glass bottles and incubated in microaerobic light conditions previously described, and the following parameters were determined: COD, pH, PNSB, SS and TtS. The effectiveness of the treatments was evaluated from the response values (COD, SS and TtS).

The results from the CCD were selected for obtaining the optimal percentage of FPE, inoculums size of PNSBsi and RT. The optimum conditions based on calculation from the CCD were confirmed. In order to explain the roles of PNSBsi, FPE, and a combination of starter cultures plus FPE, the experimental design was as follows: RAW (native control), RAW with optimal amount of FPE, RAW with optimal dose of PNSBsi, RAW with optimal amounts of both FPE and starter PNSBsi, and the other 4 sterile sets of each corresponding treatment were also conducted in the same way. After 4 days, the efficiency was assessed by the loss of COD, SS, TDS, sulfate ion (SO_4^{2-}), TtS, DsS, UHS (H_2S in wastewater) and H_2S in the head space. The amounts of HPC, LAB and PNSB were also counted. All data in this experiment were analyzed by ANOVA (Tukey HSD post-hoc test) with a significance level of 0.05.

RESULTS

Stimulation of indigenous PNSB from a lagoon with FPE for use as an inoculum

The experimental results of BBD were analyzed by regression analysis consisting of the linear, quadratic and interaction effects which generated the following regression equations with increasing PNSBsi and reducing ORP values as a function of COD (X_1), pH (X_2), and FPE (X_3).

$$Y_{PNSBsi} = - 51.48 + 0.01\ X_1 + 12.02\ X_2 + 4.46\ X_3 - (1.64 \times 10^{-6})\ X_1^2 - 0.67\ X_2^2 - (6.51 \times 10^{-4})\ X_1X_2 - 0.48\ X_2X_3 \quad (2)$$

$$Y_{ORP} = 1421.52 - 0.61\ X_1 - 240.77\ X_2 - 156.34\ X_3 + (1.22 \times 10^{-4})\ X_1^2 + 13.89\ X_2^2 + 44.43\ X_3^2 + 0.019\ X_1X_2 - 0.018\ X_1X_3 \quad (3)$$

Population of PNSBsi (Y_{PNSBsi}) and value of ORP (Y_{ORP}) at specific combination of three independent variables could be predicted by substituting the corresponding values of each variable in equations (2) or (3), respectively. The

predicted values from both equations for PNSBsi and ORP of each experimental run are presented in Table 3-1. ANOVA for the response surface model is summarized in Table 3-2. The statistical significance of model equation was evaluated by *F*-test. As the greater *F*-value indicates that the factors explain adequately the data variation about its mean and the estimated factors are real. In this study, *F*-values of 24.17 for PNSBsi model and 197.89 for ORP model, signify that the models were significant (Table 3-2). ANOVA at the low probability value ($P > F$) also demonstrated that the quadratic model of PNSBsi value ($P > F = 0.0002$) and ORP value ($P > F < 0.0001$) were significant. However, a lack of fit *F*-values of 6.5775 and 2.64 of these models suggested that it was not significant relative to the pure error. Non significant lack of fit would be appropriate for this experiment. These are 5% and 19% chances that a lack of fit *F*-value could occur due to the experimental errors in PNSBsi and ORP models, respectively. Based on these results, PNSBsi model was chosen to produce the response surface contour plots for the determination of optimal conditions. In addition, the coefficients of variation (R^2) were 0.9688 for PNSBsi population and 0.9961 for ORP value indicating a high correlation between the observed and predicted values from model equations (2) and (3). Thus, both equations could be used for predicting the amount of PNSBsi population and ORP value under conditions varied with only three independent variables in the experimental range.

Table 3-2. ANOVA of the quadratic models for the values of stimulated indigenous purple nonsulfur bacteria (PNSBsi) and oxidation reduction potential (ORP) in non sterile rubber sheet wastewater (RAW) incubated under microaerobic light conditions for 2 days after adding fermented pineapple extract (FPE).

Source	Sum of squares	DF	Mean square	*F*-Value	Prob > *F*	R^2
PNSBsi value						
Model	5.2744	9	0.5860	24.1706	0.0002	0.9688
Residual	0.1697	7	0.0242			
Lack of Fit	0.1411	3	0.0470	6.5775	0.0502	not significant
Pure Error	0.0286	4	0.0072			
Corrected Total	5.4441	16				
ORP value						
Model	7296.50	9	810.72	197.89	< 0.0001	0.9961
Residual	28.68	7	4.10			
Lack of Fit	19.05	3	6.35	2.64	0.1858	not significant
Pure Error	9.62	4	2.41			
Corrected Total	7325.18	16				

The regression coefficient in the response surface model for the linear, quadratic and interaction effects of the variables are presented along with *P*-value in Table 3-3 The linear effect of COD ($P < 0.05$), pH ($P < 0.01$) and FPE ($P < 0.05$) were statistically significant for increasing PNSBsi population, whilst, the linear effect of COD ($P < 0.01$), pH ($P < 0.01$) and FPE ($P < 0.05$) were also statistically significant for reducing ORP value. Both regression equations show significant effects of interacting relationships between the COD vs. initial pH on the PNSBsi population ($P < 0.01$) and the ORP values ($P < 0.01$). However, a significant effect of interacting

relationships between the initial pH versus the FPE concentration was observed only on the PNSB population ($P < 0.05$) while a significant effect of the interacting relationship between the COD versus the FPE concentrations was found only on the ORP ($P < 0.01$).

The interacting relationship between 2 factors using the overlaid contour plot is presented by designation of another factor as zero level or mid-point. A 2% FPE was designed for consideration of the interacting relationship between the COD and the initial pH on PNSBsi and ORP. It was found that the increase of the PNSBsi population with the decrease of ORP value relied on the following ranges of the examined variables: COD 1750-2400 mg/L and initial pH 6.5-8.0 (Figure 3-1a). In a similar manner, the initial pH of 7 was used to consider the effect of COD and FPE showing that PNSBsi increased with the decrease of ORP when the COD was in the range of 1800-2400 mg/L and FPE was between 1.65-2.5% (Figure 3-1b). When the COD was around 2000 mg/L, an increase of the PNSBsi with the decrease of ORP was observed at an initial pH and FPE in a range of 6.5-8.0 and 1.51-2.50% (Figure 3-1c), respectively. The overlaid contour plot (Figure 3-1) was also used to indicate the region where the optimization routine was searched for optimal points. The optimum conditions determined were COD concentration 2103 (mg/L); initial pH 7.0 and FPE 2.0%. The predicted response of PNSBsi was 7.82 log CFU/mL while the actual value was between 7.79-7.83 log CFU/mL (runs numbers 3, 4, 11, 12 and 14) (Table 3-1). These results were considered to be adequate and acceptable as the desirability obtained was 0.999. Hence, the optimal conditions for stimulating PNSB were 2000 mg/L COD, pH 7 and 2.0% FPE, and were therefore further verified, whilst the actual ORP values for those runs numbers were in a range of -227 mV to -231 mV with the predicted value of -228 mV.

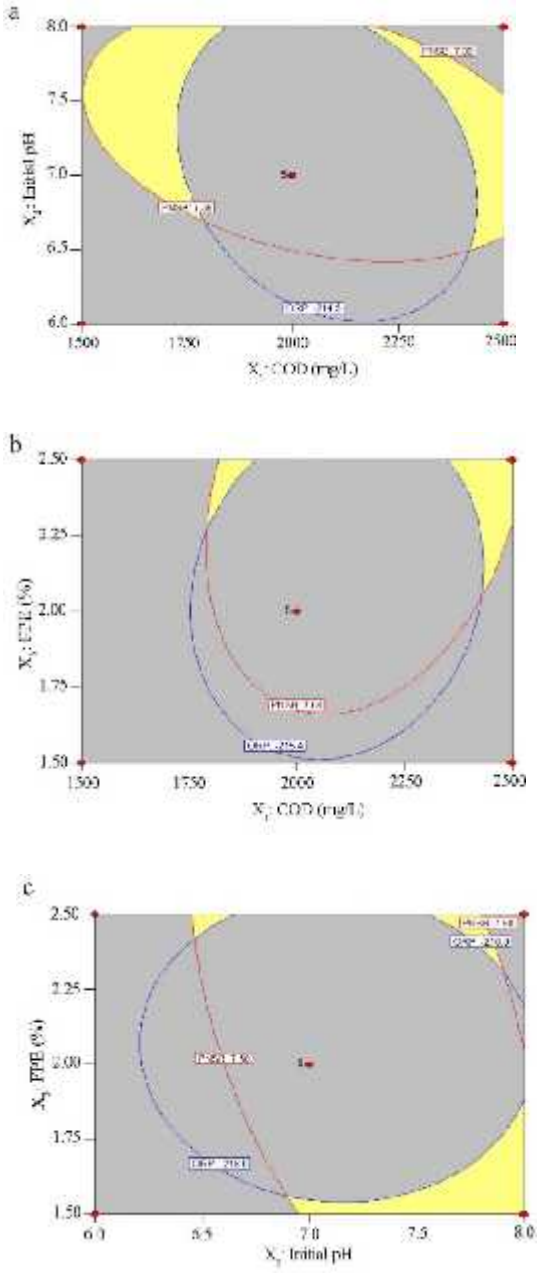

Figure 3-1. The overlay interaction plots of chemical oxygen demand (COD), initial pH and fermented pineapple extract (FPE) on oxidation-reduction potential (ORP) value and stimulating purple nonsulfur bacteria (PNSB) growth in non sterile rubber sheet wastewater (RAW) under microaerobic light conditions over 2 days incubation.

Table 3-3. Estimated regression coefficient and corresponding *P*-value for the values of stimulated indigenous purple nonsulfur bacteria (PNSBsi) and oxidation reduction potential (ORP) in non sterile rubber sheet wastewater (RAW) incubated under microaerobic light conditions for 2 days after adding fermented pineapple extract (FPE).

Terms	PNSBsi value		ORP value	
	Coefficient	*P*-value	Coefficient	*P*-value
Constant	-51.47809	0.0002**	1421.52	< 0.0001**
COD (X_1)	0.010655	0.0141*	-0.61	< 0.0001**
pH (X_2)	12.02	0.0001**	-240.77	0.0015**
FPE (X_3)	4.46422	0.0487*	-156.34	0.0107*
COD x COD	-1.64E-06	0.0010**	0.00	< 0.0001**
pH x pH	-0.66787	< 0.0001**	13.89	< 0.0001**
FPE x FPE	-0.42108	0.2079	44.43	< 0.0001**
COD x pH	-6.51E-04	0.0041**	0.02	< 0.0001**
COD x FPE	4.17E-04	0.2228	-0.02	0.0024**
pH x FPE	-0.47868	0.0180*	1.60	0.4552

* $P < 0.05$, ** $P < 0.01$

Results of the verification experiments under microaerobic light conditions over 2 days test period with sterile and non sterile FPE are shown in Table 3-4. Under the optimized conditions as calculation, significant differences were found for the amounts of HPC and PNSBsi, i.e. in a non sterile FPE set, PNSBsi were 8.13 log CFU/mL and 7.69 log CFU/mL in a sterilized FPE set and no PNSB were detected in the control set. The lowest amount of the HPC was observed in a non sterile FPE set which had the highest amount of PNSBsi. In contrast, there was no significant difference found in both verification sets for the following parameters; pH and LAB

with the exception of ORP, VFAs and EC. The values of pH, VFAs and ORP including HPC in the control set were significantly higher than those found in both verification sets. However, no population of LAB was detected in any set.

Treatment of rubber sheet wastewater using stimulated PNSB and FPE

Results of CCD experiment are shown in Table 3-5 along with experimental and predicted values using RSM. COD, SS, and TtS were used as key responses to evaluate the efficiency of the treatment and the data obtained from all runs were analyzed by fitted regression models. The fitted regression models are as follows.

$$Y_{COD} = 4372.87 - 145.87X_1 - 957.70X_3 - 127.72X_1X_2 + 26.27X_1X_3 + 7.18X_1^2 + 2863.12X_2^2 + 84.17X_3^2 \quad (4)$$

$$Y_{SS} = 102.05 - 2.11X_1 - 21.61X_3 + 0.52X_1X_3 + 1.41X_3^2 \quad (5)$$

$$Y_{TtS} = 17.44 - 0.27X_1 - 0.36X_1X_2 + 0.61X_2X_3 + 0.03X_1^2 + 8.65X_2^2 + 0.17X_3^2 \quad (6)$$

Table 3-4. Verification experiments of the stimulation of PNSB by adding sterile FPE and non sterile FPE to RAW and incubating under microaerobic-light conditions for 2 days.

Parameters	Treatments			
	RAW, 2000 mg/L COD, pH 7, t = 0	Control No addition of FPE	Verify test (2.0%FPE)	Verify test (sterilized 2.0%FPE)
pH	7.0 ± 0.0^{a}	7.5 ± 0.0^{c}	7.2 ± 0.0^{b}	7.2 ± 0.0^{b}
EC (mS/cm)	2.69 ± 0.05^{b}	2.47 ± 0.09^{a}	2.78 ± 0.05^{b}	2.97 ± 0.06^{c}
ORP (mV)	73 ± 1.1^{d}	-78 ± 1.7^{c}	-201 ± 0.4^{a}	-163 ± 0.1^{b}
VFAs (mg/L)	53 ± 6^{d}	47 ± 6^{c}	23 ± 6^{a}	37 ± 6^{b}
HPC (log CFU/mL)	5.42 ± 0.07^{a}	5.88 ± 0.08^{c}	5.27 ± 0.09^{a}	5.65 ± 0.08^{b}
LAB (log CFU/mL)	0	0	0	0
PNSBsi (log CFU/mL)	0^{a}	0^{a}	8.13 ± 0.06^{c}	7.69 ± 0.07^{b}

Different lowercase letters in each row indicate significant differences ($P < 0.05$).
EC, electrical conductivity; FPE, Fermented pineapple extract; HPC, Heterotrophic plate count; LAB, lactic acid bacteria; ORP, oxidation reduction potential; PNSB, purple nonsulfur bacteria; PNSBsi, stimulated indigenous purple nonsulfur bacteria; RAW, non sterile rubber sheet wastewater; VFA, volatile fatty acids.

The models for removals of COD, SS and TtS (Y_{COD}, Y_{SS} and Y_{TtS}) were significant by the *F*-test, The ANOVA for the quadratic models were highly significant as all had low *p*-value($P < 0.0001$, 0.0008, < 0.0001 for COD, SS and TtS, respectively). The goodness of fit for COD, SS and TtS was confirmed by R^2 for COD, SS and TtS of 0.9369, 0.8947 and 0.9352, respectively, suggesting that only 6.31%, 10.53% and

6.48% of the total variation could not be explained by each respective model. In this study, the adj. R^2 values for COD, SS and TtS were 0.8801, 0.8000 and 0.8769, respectively, which are close to R^2 values. This is due to that if there are many terms in the model and the sample is not very large, the adusted. R^2 might be noticeably smaller than the R^2. Based on the above results, the COD multiple regression model was selected to build the surface overlay contour plots and to derive the optimal condition for verification test.

Considering equations 4 and 5, RT had a significant effect on reducing the COD and SS values ($P < 0.05$), and it also implies that the growth of the PNSBsi is dependent on the RT. Consequently, the PNSBsi had a significant influence on reducing the TtS (equation 6). The regression equation 4 shows significant effects of interacting relationships between PNSBsi versus FPE and PNSBsi versus RT on the COD value. However, equation 5 shows that the interaction between PNSBsi vs. RT had a substantial impact on the SS value, whilst the TtS value was sensitive to the interaction between PNSBsi vs. FPE and FPE vs. RT (equation 6).

Overlaid contour plots were used to present interacting relationships between 2 factors by providing another factor at a mid-point for determination of optimum conditions. The interacting relationship between PNSBsi and FPE when the treatment was 4 days had decreasing values of COD, SS and TtS, when the levels of PNSBsi and FPE were in a range of 7.2-10% and 0.75-0.94%, respectively (Figure 3-2a). When 0.75% FPE was the mid point, the interacting relationship between PNSBsi and RT showed that when both factors were in a range of 7.2-8.9% and 3.7-4.3 days, there was a significant decrease of COD, SS and TtS values (Figure 3-2b). The interacting relationship between FPE and RT when PNSBsi was 6% showed that values of COD, SS and TtS decreased when the FPE and RT levels were 0.79-0.81% and 4.2-4.3 days (Figure 3-2c). The overlaid contour plot (Figure 3-2) was also used to indicate the region where the optimization routine was found. The optimum conditions were determined as 7% PNSBsi population, 0.81% FPE and 4.22 days for the RT. The predicted responses of COD, SS and TtS were 241, 22 and 6.66 mg/L (corresponded to 88, 61 and 51%, respectively) and these were close to actual values of run number 7 as COD, SS and TtS were 288, 24 and 6.89 mg/L with the conditions of 6% PNSBsi,

0.75% FPE and 4 days (Table 3-5). The results were considered to be adequate and acceptable as the desirability was 0.910. However, for practical work, the optimal conditions were adjusted to 7% PNSBsi, 0.8% FPE and 4 days of RT with little changes of FPE and RT in the verification test.

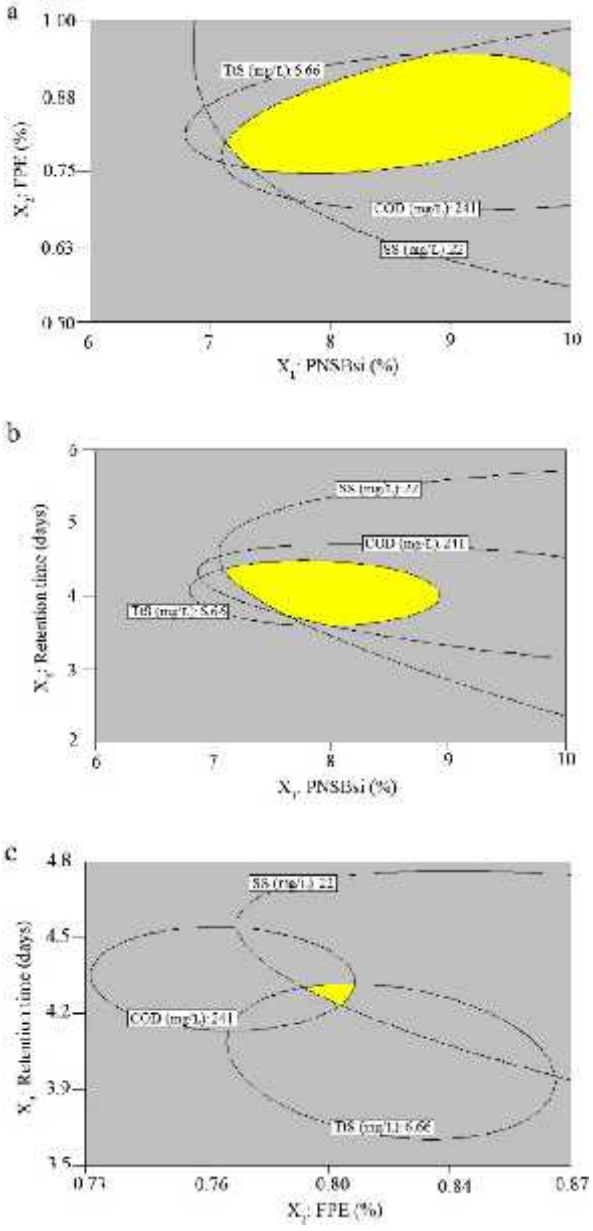

Figure 3-2. The overlay interaction plots of stimulated indigenous purple non sulfur bacteria (PNSBsi), fermented pineapple extract (FPE) and retention time (RT) on the efficiency to treat non sterile rubber sheet wastewater under microaerobic light conditions.

Table 3-5. The central composite design for treating non sterile rubber sheet wastewater (RAW) with initial values in mg/L of 2024 COD, 57 suspended solids (SS) and 13.56 total sulfide (TtS) by stimulated indigenous purple nonsulfur bacteria (PNSBsi) inoculum under microaerobic light conditions.

Run	%PNSBsi	%FPE	RT (day)	COD (mg/L)		SS (mg/L)		TtS (mg/L)	
no.	(X_1)	(X_2)	(X_3)	Actual	Predicted	Actual	Predicted	Actual	Predicted
1	2	0.75	4	770	953	32	38	8.67	8.98
2	4	0.625	5	673	596	30	25	8.67	8.36
3	8	0.625	3	724	825	32	33	8.89	8.88
4	4	0.875	3	1061	914	40	35	9.56	9.3
5	8	0.875	3	472	503	17	17	7.11	7.15
6	8	0.875	5	537	676	25	24	8	7.8
7[a]	6	0.75	4	288	289	24	24	6.89	6.77
				(16)		(1.15)		(0.26)	
8	6	0.5	4	660	797	27	30	8.44	8.63
9	6	0.1	4	867	794	23	25	7.78	7.97
10	10	0.75	4	394	275	15	14	7.33	7.39
11	4	0.625	3	1578	1393	52	49	9.33	9.27
12	8	0.625	5	976	868	28	26	8.44	8.3
13	6	0.75	2	1533	1504	48	48	8.89	8.85
14	4	0.875	5	1520	1582	47	45	9.11	8.99
15	6	0.75	6	886	979	27	33	8.22	8.63
R^2					0.9369		0.8947		0.9352
Adj. R^2					0.8801		0.8		0.8769

[a] The experiment was repeated 6 times and the responses represented average values with their standard deviation in parenthesis.

The role of FPE and PNSBsi to treat rubber sheet wastewater

The wastewater treatment efficiencies of FPE, PNSBsi and combination of FPE and PNSBsi (verification test) in a non sterile set under microaerobic light conditions over 4 days are shown in Table 3-6. A native control set without the addition of FPE and PNSBsi produced the least efficient reduction of COD, SS and TtS levels and in this set PNSB were not detected. However, the addition of 0.8% FPE stimulated

PNSB growth to 7.3 log CFU/mL and provided a higher efficiency for the reduction of COD than the native control. On the other hand, with the addition of only 7% PNSBsi, 8.11 log CFU/mL were achieved, with reduction percentages of 85 for COD, 63 for SS and 52 for TtS. The best treatment efficiency was observed in a verification test with the addition of both 0.8% FPE and 7% PNSBsi as the reduction percentages were then 91 for COD, 75 for SS and 61 for TtS. However, H_2S was not detected in any set except that RAW had an initial H_2S level of 10 mg/L. The highest amount of HPC was found in the native control set followed by a FPE set and the least in sets with PNSBsi. No detection of LAB was observed in any set. According to the results above, the verification test produced significantly higher efficiency than the predicted values derived from CCD experiment for COD (88%), SS (61%) and TtS (51%).

Results of a parallel sterile set to treat sterile RAW over 4 days are presented in Table 3-7. The abiotic control showed little degradation in RAW; however, with the addition of 0.8% FPE, the amount of HPC was 4.51 log CFU/mL. Therefore, higher efficiencies for reducing COD, SS and TtS were found, but the most marked decrease of these levels were found in sets of verified and only addition of PNSBsi. A 7% PNSBsi inoculum provided PNSBsi (7.93 log CFU/mL) and HPC (4.19 log CFU/mL), and this effectively reduced COD 84%, SS 51% and TtS 49%. There were significant differences for the amounts of HPC and PNSBsi between the PNSBsi set and a verified set. Therefore, the verifying set produced the highest efficiency by reducing COD, SS, and TtS at 88%, 70% and 56%, respectively. The results of H_2S in this study indicated that this gas was produced by HPC from 4 mg/L to 15 mg/L in a FPE set and it was completely removed by PNSBsi. However, a loss of H_2S was also observed in an abiotic control.

Table 3-6. Characteristics of the effluents from the non sterile sets after by fermented pineapple extract (FPE), stimulated indigenous purple nonsulfur bacteria (PNSBsi) inoculants and results of the verification test.

Parameter mg/L[a]	Non sterile RAW	Control Native Control	Addition 0.8% FPE	Addition 7% PNSBsi	Verification test, 0.8% FPE + 7% PNSBsi
	T = 0	Day 4	Day 4	Day 4	Day 4
pH	7.03 ± 0.01^{a}	7.46 ± 0.03^{d}	7.31 ± 0.03^{b}	7.39 ± 0.01^{c}	7.35 ± 0.02^{bc}
COD	2722 ± 11^{e}	1196 ± 11^{d}(56)	1033 ± 11^{c}(62)	414 ± 11^{b}(85)	239 ± 11^{a}(91)
SS	43 ± 3^{d}	32 ± 3^{c} (26)	29 ± 5^{c} (33)	16 ± 6^{b} (63)	11 ± 3^{a} (75)
TDS	547 ± 12^{c}	464 ± 12^{b}	433 ± 42^{ab}	367 ± 31^{a}	380 ± 20^{a}
Sulfate	5.1 ± 0.2^{d}	3.9 ± 0.1^{c}	2.7 ± 0.1^{b}	2.1 ± 0.1^{a}	2.0 ± 0.2^{a}
TtS	13.78 ± 0.38^{d}	8.44 ± 0.38^{c}(39)	7.67 ± 0.67^{bc}(44)	6.65 ± 0.77^{ab}(52)	5.33 ± 0.67^{a}(61)
DsS	12.89 ± 0.38^{d}	10.22 ± 0.38^{c}	6.22 ± 0.38^{b}	5.78 ± 0.38^{ab}	5.11 ± 0.38^{a}
UHS	5.67 ± 0.17^{d}	2.45 ± 0.09^{c}	1.74 ± 0.11^{b}	1.39 ± 0.09^{a}	1.23 ± 0.09^{a}
H_2S	10 ± 2^{b}	0^{a}	0^{a}	0^{a}	0^{a}
HPC	8.3 ± 0^{d}	7.83 ± 0.1^{c}	7.63 ± 0.1^{b}	7.09 ± 0^{a}	7.13 ± 0^{a}
LAB	0	0	0	0	0
PNSB	0^{a}	0^{a}	7.3 ± 0^{b}	8.11 ± 0^{c}	8.39 ± 0^{d}

[a] Unless otherwise stated and unit for microbial population is log CFU/mL

Numbers in parentheses are reduction percentages.

Different letters in each row indicate the significant differences ($P < 0.05$).

TDS, total dissolved solids; TtS, total sulfide; DsS, dissolved sulfide; UHS, unionized hydrogen sulfide (H_2S in wastewater); HPC, heterotrophic plate count; LAB, lactic acid bacteria; PNSB, purple nonsulfur bacteria

Table 3-7. Characteristics of effluents from sterile sets after treatment by FPE, PNSBsi inoculum and the verification test.

Parameter mg/L[a]	Sterile RAW	Abiotic Control	Addition 0.8% FPE	Addition 7% PNSBsi	Verification test, 0.8% FPE + 7% PNSBsi
	T = 0	Day 4	Day 4	Day 4	Day 4
pH	7.06 ± 0.01^{c}	7.33 ± 0.02^{d}	6.85 ± 0.02^{a}	6.96 ± 0.02^{b}	7.73 ± 0.02^{e}
COD	2684 ± 11^{e}	2632 ± 11^{d}(2)	1558 ± 11^{c}(42)	443 ± 11^{b}(84)	326 ± 11^{a}(88)
SS	37 ± 6^{b}	35 ± 5^{b}(3)	33 ± 3^{b}(11)	18 ± 3^{a}(51)	11 ± 5^{a}(70)
TDS	467 ± 12^{b}	459 ± 21^{b}	447 ± 12^{b}	440 ± 20^{b}	387 ± 12^{a}
Sulfate	4.8 ± 0.1^{d}	4.7 ± 0.1^{d}	2.5 ± 0.1^{c}	2.1 ± 0.2^{b}	1.9 ± 0.1^{a}
TtS	13.11 ± 0.38^{c}	12.65 ± 0.38^{c}(3)	10.22 ± 0.38^{b}(22)	6.67 ± 0.67^{a}(49)	5.78 ± 0.38^{a}(56)
DsS	12.44 ± 1.02^{b}	12.22 ± 0.38^{b}	7.78 ± 0.38^{a}	7.33 ± 0.67^{a}	6.44 ± 0.77^{a}
UHS	4.73 ± 0.39^{d}	3.42 ± 0.11^{c}	4.13 ± 0.47^{cd}	1.76 ± 0.16^{b}	0.84 ± 0.10^{a}
H_2S	4 ± 1^{b}	0^{a}	15 ± 3^{c}	0^{a}	0^{a}
HPC	0^{a}	0^{a}	4.51 ± 0.03^{d}	4.19 ± 0.03^{c}	4.10 ± 0.06^{b}
LAB	0	0	0	0	0
PNSB	0^{a}	0^{a}	0^{a}	7.93 ± 0.05^{b}	8.10 ± 0.02^{c}

[a] Unless otherwise stated and numbers in parentheses are reduction percentages.

Different letters in each row indicate the significant differences ($P < 0.05$).

For identification of abbreviations see Table 3-6.

DISCUSSION

The role of FPE for stimulating PNSB growth

It is well recognized that PNSB prefer to grow under a low oxygen tension with light conditions (Izu *et al*., 2001) and in this study it was confirmed that the lowest ORP (-228 mV) strongly promoted the growth of PNSB (Table 3-1). This result was supported by Izu *et al*., (2001) who reported that maximum PNSB ratios of up to 80% of the total microbes were obtained using non-aeration conditions with ORP values of less than -200 mV. The ORP in the sediments of paddy fields is in the range of -200 to -250 mV was found as the most suitable condition for enhancing PNSB growth (Overmann and Garcia, 2006). COD, initial pH and FPE are independent variables each, having significant influences on the ORP value (Table 3-3). This is because both COD and FPE factors are a source of nutrient and optimal pH that could stimulate the growth of normal flora and the consequent depletion of oxygen (ORP decreased). Hence, anaerobic conditions under light stimulated indigenous PNSB growth so they became the dominant organism. No PNSB were detected in a control set, without FPE, while under light conditions, 2.0% FPE had enough nutrients to stimulate PNSB growth (Table 3-4). This was because the FPE consisted of 1.90% organic acids and other nutrients with an EC value of 3.51 mS/cm. In contrast, lower nutrients in the control set without added FPE in the light was enough to support some algal growth. However, why would FPE stimulate PNSB but have little effect on the heterotrophic plate count (HPC). It is possible that the VFAs in FPE in both verified sets were used preferentially as electron donors for the photosynthesis in the partially anaerobic light conditions as a lower amount of VFAs was found in both sets when compared with the control set (Table 3-4). It was found that LAB were not detected in any set (Table 3-4). This might be a result of very low number of LAB in the stored FPE since they could not survive the high competition for growth substrates from the heterotrophs and PNSB in the RAW under light conditions.

According to BBD, the optimal conditions for promoting PNSB growth were a COD of 2000 mg/L, initial pH 7 and 2% FPE (Figure 3-1 and Table 3-4). This can be explained by the fact that PNSB are photoorganotrophs therefore they prefer to

grow in a condition that has a relatively high organic matter (Kim *et al*., 2004; Kantachote *et al*., 2005; Kantachote *et al*., 2010). The 2.0% FPE addition in this study provided an optimal concentration of organic matter for proliferation of PNSB because a lower organic content would facilitate the growth of cyanobacteria as previously described in the control set. In contrast, too much organic matter may promote the growth of HPC and repress the growth of PNSB (Kantachote *et al*., 2010). It is generally known that a neutral pH supports the growth of most microorganisms including PNSB (Kantachote *et al*., 2010; Panwichian *et al*., 2010). Therefore, it was not surprising that PNSB became the dominant organism due to the provision of the most suitable conditions provided for the growth of photoheterotrophs under microaerobic light condition, which quickly switched to anaerobic conditions with the addition of FPE (ORP = -228 mV) (Table 3-1).

The role of PNSBsi for treating rubber sheet wastewater

Efficiency of the rubber sheet wastewater treatment is dependent on PNSBsi, FPE and RT. The results demonstrated that RT had a significant effect on the reduction of COD and SS values whereas the PNSBsi strongly reduced TtS (equations 4-6). RT is one of the major factors that controls the degradation of carbonaceous wastes as it represents the length of time the microbial cells are in contact with the substrate, which directly dictates the efficiency of wastewater treatment (Bitton 2005). In this case, it ensures that the PNSBsi had enough time to consume most of the substrate and nutrients over the RT of 4 days. There was a significant reduction of TtS caused by the PNSBsi because some members of the PNSB can use sulfide or other reduced forms of sulfur as an extra source of an electron donor to support photosynthesis (Kim *et al*., 2004; Kantachote *et al*., 2005). COD value was also dependent on the interactive relationship between PNSBsi vs. FPE and PNSBsi vs. RT (equation 4), this again illustrated that FPE stimulated PNSB growth as previously described.

The SS values were significantly governed by the interactive relationship between PNSBsi vs. RT as their biomass increased with time after consuming nutrients (equation 5). However, the PNSBsi gave a higher efficiency to

reduce SS value than the HPC (Tables 3-6 and 3-7). The reason is that PNSB have a growth yield ($Y_{biomass/substrate\ (COD)}$) in a range of 0.28-0.45 (He *et al*., 2010; Lu *et al*., 2011), while, usually, HPC growth yields are around 0.40-0.60 (Heijnen and Roles, 1981). The interactive relationships between PNSBsi vs. FPE significantly decreased TtS while the FPE vs. RT interaction increased TtS (equation 6). This suggested that PNSBsi removed TtS while the FPE was involved with producing TtS as it supported the growth of the HPC. This corresponded with the results in Table 3-7. In contrast, H_2S was not detected in the abiotic control after 4 days, although it was observed at time zero at 4 mg/L (Table 3-7). This indicated that the loss of rotten gas odor may be caused by the change of pH from 7.06 to 7.33 because a higher pH promotes the conversion of sulfide into its HS^- and also some may be lost by precipitation with small amount of metal ions existed in the medium.

According to the overlaid contour plot, the adjusted optimal conditions for treating RAW were 7% PNSBsi, 0.8% FPE and 4 days treatment time, and this condition was verified with non sterile and sterile FPE, including controls (Tables 3-6 and 3-7). Results of the native control show that addition of FPE stimulated PNSB growth which is consistant with the results shown in Table 3-4. As there was little change of most of the measured parameters in the abiotic control set (Table 3-7). The efficiency to treat RAW in the sets of non sterile and sterile with no/addition of FPE/PNSBsi appeared to be governed by the microbes and the efficiencies were in the following order: the verified sets > PNSBsi > FPE > native control. As only 0.8% FPE was added into the sterile RAW, this allowed HPC growth to increase to 4.51 log CFU/mL (Table 3-7) and this could also treat RAW, although their activity was significantly lower than the treatments with the PNSBsi inoculum (Tables 3-6 and 3-7). Addition of PNSBsi as inoculum also included some HPC population (Table 3-7). The removal efficiency to treat RAW was positively related to the additions of FPE and PNSBsi into RAW, although some normal flora present in the RAW may have involved (Tables 3-6 and 3-7). The verified set in this study (Table 3-6) had higher efficiencies (91% COD, 75% SS and 61% TtS) to treat RAW that had an initial COD of 2722 mg/L than those predicted values (88% COD, 61% SS 51% TtS), with an initial COD of 2024 mg/L (Table 3-5). A possible reason for that is that the different batches of the

wastewater used generally show high variations in both biotic and abiotic properties. The results in Table 3-7 showed that the sterile verified set had the removal efficiency of 88% for COD, 70% for SS and 56% for TtS, all of which were close to the predicted values. This means that biotic component in non sterile verified set also supported RAW treatment.

The PNSBsi inocula not only easily adapted themselves to their original source (RAW) but they were also able to compete with the normal flora for treating RAW. This was evidenced by their higher efficiencies in the non sterile condition than those in the sterile condition (Tables 3-6 and 3-7). The results indicated that the use of autochthonous bioaugmentation (re-inoculation) for treating rubber sheet wastewater was a practical technique to be used by farmers who desire to use the treated RAW for the preparation of PNSB inoculum. The inoculum can be carried out onsite with a simple procedure of adding the appropriate amount of FPE into a portion of wastewater in a shallow pond nearby the plant under light condition. This application can sustain the need for inoculation and support a long term operation of this community level wastewater treatment ponds. However, the amount of UHS remained in the effluent must be solved and the use of selected PNSB strains might be possible to solve the problem, and this will be further studied.

CONCLUSIONS

This work showed that the role of FPE in the stimulation of PNSB growth under light condition was to lower ORP leading to the reducing environment in the culture. A new approach for the preparation of indigenous PNSBsi inoculum from rubber sheet wastewater with the addition of optimal amount of FPE under microaerobic/anaerobic light conditions was developed. In addition, autochthonous bioaumentation using PNSBsi proved to be effective for treating the wastewater.

CHAPTER 4

Use of *Rhodopseudomonas palustris* P1 stimulated growth by fermented pineapple extract to treat latex rubber sheet wastewater to obtain single cell protein

Abstract

Latex rubber sheet wastewater (non sterile wastewater: RAW) was treated efficiently using a stimulated *Rhodopseudomonas palustris* P1 inoculum with added fermented pineapple extract (FPE) under microaerobic light conditions. Optimization of wastewater treatment conditions using CCD found that a 3% stimulated P1 inoculum with 0.9 % added FPE and a 4-day retention time (RT) were the most suitable conditions. Calculations from CCD experiments predicted that a COD of 3,005 mg/L could be 98 % removed, together with 79% of SS and 72% of TtS. No H_2S was detected, production costs were low and single cell protein (SCP) was a by-product. The results of the verification test had an error of only 4–8 % and confirmed removal of COD (initial COD 2,742 mg/L), SS and TtS at 94 %, 75 % and 66 %, respectively. These values were less than the best set obtained from the CCD experiment (2 % stimulated P1 inoculum, 0.75 % FPE and 4 days RT); upon repeating, this set could reduce 96 % of the COD, 78 % SS and 71 % TtS. The treated wastewater met the standard guidelines for irrigation use and no H_2S was detected. The biomass obtaining after wastewater treatment from the best set consisted mostly of *R. palustris* P1; the biomass of this set had 65 % protein, 3 % fat, 8 % carbohydrate, 14 % ash and 10% moisture. The results demonstrated that an inoculum of stimulated P1 grew well in RAW supplemented with FPE and could be considered to be an appropriate technology for effectively treating wastewater, with SCP as a by-product.

Keywords: Fermented pineapple extract, Hydrogen sulfide, Latex rubber sheet wastewater, Purple nonsulfur bacteria, Single cell protein, Response surface methodology

บทคัดย่อ

น้ำเสียจากการทำยางพาราแผ่น (non sterile wastewater: RAW) ได้รับการบำบัดอย่างมีประสิทธิภาพด้วยกล้าเชื้อ *Rhodopseudomonas palustris* P1 ที่ถูกกระตุ้นด้วยน้ำหมักสับปะรดภายใต้สภาวะมีอากาศเล็กน้อย-มีแสง จากการใช้ central composite design (CCD) พบว่าสภาวะที่เหมาะสมในการบำบัดน้ำเสียคือการใช้กล้าเชื้อ 3% ปริมาณน้ำหมัก 0.9% และระยะเวลาบำบัด 4 วัน โดยสภาวะดังกล่าวสามารถบำบัดน้ำเสียที่ (มีค่า chemical oxygen demand (COD) 3005 มก/ลิตร) โดยลดลงค่า COD suspended solids (SS) และ total sulfide (TtS) ได้ 98% 79% และ 72% ตามลำดับ นอกจากนี้ตรวจไม่พบแก๊สไฮโดรเจนซัลไฟล์ มีค่าใช้จ่ายในการบำบัดต่ำ และได้โปรตีนเซลล์เดียวหลังการบำบัดเป็นผลพลอยได้ การยืนยันผลการทดลองที่ได้จาก CCD พบว่ามีค่าความคลาดเคลื่อนอยู่ในช่วง 4–8% โดยสามารถลดค่า COD (COD เริ่มต้น 2742 มก/ลิตร) SS และ TtS ได้ 94%, 75% และ 66% ตามลำดับ แต่สภาวะดังกล่าวมีประสิทธิภาพน้อยกว่าชุดการทดลองที่ดีที่สุดของการทดลองที่ออกแบบโดยใช้ CCD คือสภาวะการใช้กล้าเชื้อ 2% ปริมาณน้ำหมัก 0.75% และระยะเวลาบำบัด 4 วัน ซึ่งสามารถลดค่า COD SS และ TtS ได้ 96%, 78% และ 71% ตามลำดับ น้ำเสียที่ผ่านการบำบัดไม่พบแก๊สไฮโดรเจนซัลไฟล์ และมีค่าอยู่ในเกณฑ์มาตราฐานสำหรับนำไปใช้ในการเกษตร ปริมาณชีวมวลหลังการบำบัดด้วยสภาวะของชุดที่ดีที่สุดมี *R. palustris* P1 [illegible] ไขมัน คาร์โบไฮเดรต เถ้า และ ความชื้น 65% 3% 8% 14% และ 10% ตามลำดับ จากผลการทดลองดังกล่าวพบว่าการใช้น้ำหมักกระตุ้น การเจริญของกล้าเชื้อ P1 ได้ผลดีและกล้าเชื้อเพิ่ม [illegible]

คำสำคัญ: น้ำหมักสับปะรด แก๊สไฮโดรเจนซัลไฟด์ น้ำเสียจากยางพาราแผ่น แบคทีเรียสังเคราะห์ แสงกลุ่มไม่สะสมซัลเฟอร์ โปรตีนเซลเดียว วิธีการตอบสนองพื้นที่ผิว

Introduction

Nowadays there are many cooperative rubber sheet factories (CRSFs) throughout all parts of Thailand (Kantachote *et al*., 2010).Wastewater from CRSFs contains organic and inorganic matter including the ammonia, formic acid, sodium metabisulfite and sodium sulfite used during the manufacturing process (Kantachote *et al*., 2005; Chaiprapat and Sdoodee 2007). Open lagoons or natural oxidation ponds are used for treatment of wastewater, with little attention due to the lack of skilled personnel and budgets to look after the system. The systems are, thus, unable to consistently produce effluent that can comply with industrial effluent or irrigation standards. In addition, serious problems arise from such lagoons due to incomplete oxidation of wastes, causing a rotten-eggs smell of hydrogen sulfide (H_2S) and emission of greenhouse gases such as methane (CH_4) and carbon dioxide (CO_2) (Nakajima *et al*., 1997). H_2S gas is toxic to human health at high levels (Yalamanchili and Smith 2008) and causes nuisance odor at low concentrations. Therefore, an appropriate technology for treatment of CRSF wastewater should ideally have low maintenance and operating costs while still being effective. In this regard, the use of indigenous microbes to consume organic matter and sulfides would meet these objectives and could be maintained by CRSFs themselves.

An interesting group of microbes that meet the above requirements is the anoxygenic phototrophic bacteria, the purple nonsulfur bacteria (PNSB), which have been studied extensively for the treatment of various wastewaters as they are versatile organisms able to grow photoorganotrophically under anaerobic and microaerobic light conditions, and chemoorganotrophically under aerobic dark conditions (Kim *et al*., 2004; Okubo *et al*., 2006; Lu *et al*., 2011; Luo *et al*., 2012). PNSB not only show high efficiency in wastewater treatment while releasing fewer greenhouse gases and less odor, but also produce biomass that can be utilized as single cell protein (SCP) for animal feed or biofertilizer (Kantachote *et al*., 2005; He *et al*., 2010; Kantha *et al*., 2012). The role of PNSB such as *Rhodopseudomonas*, *Rhodobacter* and *Rhodospirillum* to remove the odorous H_2S has been reported (Kim *et al*., 2004; Belila *et al*., 2009; Kantachote *et al*., 2010). However, blooms of PNSB in lagoons of CRSFs are rare, suggesting that stimulating native/indigenous PNSB or applying inoculant

PNSB is required. Our previous studies showed that indigenous PNSB (PNSBsi) stimulated by fermented pineapple extract (FPE) were able to treat latex rubber sheet wastewater (RAW) (in chapter 3); however, the efficiency was less than that obtained using inoculant *Rhodopseudomonas palustris* P1 prepared by sterile RAW (Kantachote *et al*., 2010). Hence, it would be worth investigating the possibility of stimulating the growth of inoculant P1 by FPE in RAW for treating wastewater at lower cost and obtaining SCP as a by-product.

Effective microorganisms (EM) have been used widely in various countries including Thailand since first developed by Dr. Teuro Higa in the 1970s at the University of Ryukyus, Okinawa, Japan (Okuda and Higa, 1999). However, few scientific reports have been published on the use of EM although the product has been used heavily in some areas of agriculture and the environment, particularly in wastewater treatment systems (Shrivastava *et al*., 2012). In Thailand, EM products have been replaced by fermented plant extracts (FPlEs), especially in agricultural applications as farmers can produce FPlEs by themselves (Kantachote *et al*., 2009) from various organic plant or animal residues. The main products in FPlEs are organic acids (lactic acid and acetic acid), which are the preferred substrates of PNSB (Kantachote *et al*., 2010). However, high amounts of FPlEs will also increase the organic matter in wastewater; therefore an optimal concentration should be determined for successful wastewater treatment. In addition, FPlEs could be used to stimulate the growth of indigenous PNSB for treating RAW; however, efficiency is limited by the need for a high inoculum sizes for successful treatment (in chapter 3). Hence, in this work, we examined the use of the selected *R. palustris* P1 with growth stimulated by FPE as an inoculum for treating wastewater.

To determine an effective method for wastewater treatment, the concentrations of inoculant P1 and FPE, and retention time (RT) should be optimized. Response surface methodology (RSM)—a collection of mathematical and statistical techniques—is useful for analyzing the effects of several independent variables (Bas and Boyaci, 2007). The eventual objective of RSM was to determine the optimal operating conditions for the treatment of CRSFs wastewater, and to determine a region that satisfies the operating specifications, and where the stimulating effect was most

effective. The growth of the selected strain, *R. palustris* P1 (P1), was correlated to FPE concentration and RT, with the goal of finally producing P1 cells as SCP alongside achieving wastewater treatment.

Materials and methods

Latex rubber sheet wastewater used

Latex rubber sheet wastewater was collected from a lagoon pond of a CRSF at Pichit in Hat Yai district, Songkhla province, Thailand. The collected wastewater was filtered through cheesecloth into a 25 L plastic tank until nearly full to prevent aerobic conditions and stored in a cold room at 6 ± 2 °C while not in use. Based on our preliminary work, the wastewater was supplemented with 0.05 % (w/v) NH_4Cl as an extra nitrogen source to support microbial growth; specifically, PNSB and this wastewater was used as the medium for all the experiments in this work. The wastewater medium was named RAW because it was not sterilized; therefore indigenous microbes were still present.

Fermented pineapple extract preparation

Fermented pineapple extract (FPE) was used as a medium for preparing the P1 inoculum and also for treating RAW due to the large amount of waste from the coring process of pineapple canning. The FPE was produced in our laboratory (Kantachote *et al*., 2009). The fermentation process was stopped after 2 months and the FPE was kept in a cold room until use. After 2 months the FPE had no sugar but contained 1.90 % total acidity (0.58 % lactic acid, 0.15 % acetic acid, etc.) with 3.51 mS/cm electrical conductivity (EC) and a pH of 3.61. In addition to nutrients, FPE contained populations of heterotrophs that were counted by a heterotrophic plate count (HPC), lactic acid bacteria (LAB) and yeasts, at roughly 10^6 cfu/mL for each group.

Inoculant preparation

Due to its ability to utilize H_2S, *Rhodopseudomonas palustris* P1 was used to treat latex rubber sheet wastewater (Kantachote *et al*., 2010). One loopful of isolate P1 from a stab culture was inoculated into a screw cap test tube

(20×150 mm: 30 mL) containing 28 mL GM (glutamate-malate) broth (Kantachote *et al*., 2005), leaving a small space at the top of the medium to provide microaerobic conditions. The culture was incubated with a light intensity of 3,500 lux, generated by a 60 W incandescent lamp for 48 h. The light intensity was measured using a Denki light meter Model DK-211. The culture broth was centrifuged at 6,000 rpm (Sorvall RC 5C Plus, Du-pont,Wilmington, DE) for 15 min and the cell pellet was washed twice with 0.85%NaCl, then adjusted to obtain a 0.5 OD660 nm in sterile distilled water. Distilled water was used instead of NaCl to provide the same conditions between the control and treatment sets with no extra NaCl. With regard to our preliminary work, the optimal conditions for stimulation the growth of the isolate P1 was as follows: RAW in which the initial chemical oxygen demand (COD) had been adjusted to 2,000 mg/L then supplemented with 2 % FPE (v/v); the final pH was adjusted to 7 using 5N NaOH because the addition of FPE decreased the pH value, and a 2 % cell suspension of P1 (v/v) was transferred into the adjusted RAW, and incubated under the same conditions as above for 48 h to yield stimulated P1 inoculum (P1) for treating wastewater.

Analytical methods

The standard methods used in this study are described in APHA (1998). The dichromate reflux method was used to determine the COD and the settleable COD was determined by placing all effluent samples including RAW in a cold room for 2 h to allow sedimentation prior to determination of COD. The amount of sulfate was examined by a turbidimetric method. Sulfide in wastewater was measured in three forms—total sulfide (TtS), dissolved sulfide (DsS) and un-ionized hydrogen sulfide (UHS: H_2S)—using an iodometric method. However, H_2S in the air space of the treatment bottles (bioreactors) was measured using a portable multi gas detector (MX 2100, Oldham, France). As settleable COD was measured, the supernatant (clear liquid near the water surface) was sampled and analyzed. Hence, the microbial cells had settled and were not present in the SS measurement. SS and total dissolved solids (TDS) were determined after filtration using a standard glass fiber filter and then the residue retained on the filter was dried to a constant weight at 103–105 °C to obtain SS while the filtrate was dried to constant weight at 180 °C for determination of TDS. Values of

pH and EC were measured using a pH meter (Seven multi, Metler Toledo, Columbus, OH). An oxidation-reduction potential (ORP or redox) probe was used to measure the redox values with the data recorded after obtaining a constant value. The phosphate content of the wastewater was measured photometrically using a test kit (Spectroquant® 1.14842.0001, Merck, Darmstadt, Germany) according to the manufacturer's instructions. Elements such as Mn, Cu and Cd were measured by inductively coupled plasma-optical emission spectroscopy (ICP-OES; 4300 DV, Perkin-Elmer, Uberlingen, Germany). Samples of RAW before and after treatment were analyzed directly and the protocol used for the ICP-OES followed the instruction manual for the instrument.

Total acidity was presented as lactic acid and determined by a titration method, whereas the actual amounts of lactic acid and volatile fatty acids such as acetic acid were determined using gas chromatography according to the method of Yang and Choong (2001). Viable cell counts of PNSB were enumerated by spreading on GM agar and incubating under anaerobic light conditions for 5 days (Kantachote *et al*., 2005) and were assumed to be the inoculant P1 based on morphology (colony appearance: size and shape) and cell shape under a light microscope after Gram staining. Yeasts, HPC and LAB were counted on potato dextrose agar (PDA), plate count agar (PCA) and de Man Rogosa and Sharp (MRS) agar, respectively, for 3 days. All plates were incubated at 30 °C in an incubator to match the wastewater temperature in the bioreactors. HPC, LAB and yeasts were also counted because they were part of the initial flora of the FPE while HPC were also indigenous microbes found in the wastewater. Proximate analysis was performed according to methods described in AOAC (2000). The moisture content was measured by drying the samples overnight at 100 °C to constant weight. Crude protein content was determined by the Kjeldahl method, and crude lipid content was determined by the acid hydrolysis method. The ash content was determined by burning samples overnight at 550 °C. The carbohydrate content was calculated from the difference (carbohydrate =100 − % protein − % fat − % ash − % moisture) while total energy content was calculated from the sum of energy obtained from the energy sources.

Experimental design and data analysis

Central composite design (CCD) was chosen as the experimental design in this study because this method is suited to fitting a quadratic surface and helps optimize the effective parameters with a minimum number of experiments. It also enabled an analysis of the interaction between the parameters (Montgomery 2001). The independent variables studied were the amounts of stimulated inoculant P1 (X_1), FPE (X_2) andRT (X_3), and the levels of each independent variable investigated (Table 4-1). These three independent variables together with their respective ranges were chosen based on our preliminary studies (data not shown). The experimental sequence was randomized in order to minimize the effect of light intensity and temperature by the distance from the light source and bioreactors.

Table 4-1. Experimental range and coded levels of independent variables for treating latex rubber sheet wastewater (RAW)

Variables	Code	Units	Coded variable levels				
			-α	-1	0	+1	+α
Inoculum P1	X_1	% (v/v)	1	1.5	2	2.5	3
Fermented pineapple extract (FPE) [A]	X_2	% (v/v)	0.5	0.625	0.75	0.875	1
Retention time (RT)	X_3	days	2	3	4	5	6

[A] Low concentrations are designed to prevent any significant increase of the COD in the wastewater.

The CCD consists of 2^n factorial runs with 2n axial runs and nc center runs. For each categorical variable, a 2^3 full factorial CCD for the three independent variables, consisting of eight factorial points, six axial points and six replicates at the center point were employed. The total number of experiments with three variables was 20 (= 2^n + 2n + 6), where n is the number of independent variables. The center point with six runs was used to determine the experimental error and the reproducibility of the data. To evaluate the efficiency of the wastewater treatment; COD, SS and TtS were the important key parameters for monitoring and were considered as responses, in particular for the latex rubber sheet wastewater. The responses (dependent variables)

were reductions of COD (Y_1) SS (Y_2) and TtS (Y_3) with the statistics program. For the three factors, the following equation was used.

$$Y_N = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_1X_2 + b_5X_2X_3 + b_6X_1X_3 + b_7X_1^2 + b_8X_2^2 + b_9X_3^2 \quad (1)$$

Where Y_N is the predicted response; b0 intercept; b_1, b_2, b_3 linear coefficient; b_4, b_5, b_6 interaction coefficients, and b_7, b_8, b_9 square coefficient.

The initial COD of RAW used in this study was 3,005 mg/L while the SS and TtS were 42 and 11.11 mg/L. All experimental runs as shown in Table 4-2 were carried out in 120 mL serum glass bottles (bioreactors) and three replicates were used in each run. All bottles were incubated in microaerobic light conditions as described previously for varying the RT. The parameters COD, SS and TtS were determined. In addition to RSM, ANOVA (Tukey HSD post-hoc test) was also used to analyze data in this work.

Verification test

The CCD results were used to calculate the optimal conditions for levels of FPE, inoculumsize of the stimulated P1 inoculum (P1) and RT. Optimal conditions based on CCD calculations were then confirmed. Moreover, the experimental conditions at the center point that produced the highest efficiency for treatment of RAW based on Table 4-2 were also confirmed in this work and named “best run”. A control set without addition of P1 and FPE was included in the experimental design in order to help explain the roles of the treatment sets by using a combination of P1 and FPE according to the calculation and actual results of the best run. After 4 days, the loss of COD, SS, TDS, sulfate ion (SO_4^{2-}), phosphate ion (PO_4^{3-}) TtS, DsS, UHS (H_2S in wastewater) and H_2S in the head space were monitored to assess the efficiency of the treatment. The amounts of HPC, LAB and PNSB (mostly P1) were also enumerated to confirm the efficiency of wastewater treatment with an initial COD of 2,742 mg/L.

Biomass was separated from the effluents of the best set and the control set in this study and weighed. The measured values were used to calculate the biomass yield and also to examine the cell composition. The biomass yield or the cell yield

(Yx/s) was calculated based on the consumed COD whereas the cell composition as an approximate analysis was conducted to assess their biomass as SCP. In order to compare the efficiency of RAW treatment by P1 or PNSBsi, the biomass obtained from effluent treated by PNSBsi from our previous work (in chapter 3) was used to determine biomass yield and also cell composition. Moreover, all completed treatments as mentioned above were also used to evaluate their potential as effluents for use as irrigation water based on Thai standard guidelines (Pollution Control Department 1994; Royal Irrigation Department 1989) and the amounts of heavy metals were also determined. ANOVA (Tukey HSD post-hoc test) was also used to analyze data in this verified test. The mean of three determinations and its standard deviation are reported.

Table 4-2. Removal of COD, suspended solids (SS) and total sulfide (TtS) from RAW (initial COD 3,005 mg/L) by treatment with a combination of stimulated P1 inoculum and FPE under microaerobic light conditions.

Run no.	%P1	%FPE	RT (day)	COD(mg/L)		SS(mg/L)		TtS(mg/L)	
	(X_1)	(X_2)	(X_3)	Actual	Predicted	Actual	Predict	Actual	Predicted
1	1	0.5	6	307^{b}	190	18.33^{cd}	16.53	5.11^{h}	4.75
2	1	0.5	2	$1,895^{m}$	1,837	53.33^{h}	50.80	7.56^{n}	7.24
3	2	0.75	7	405^{d}	445	13.33^{ab}	13.75	5.11^{h}	5.10
4	3	0.5	2	$1,483^{k}$	1,481	31.67^{e}	33.19	5.78^{j}	5.66
5	0	0.75	4	634^{g}	744	20.00^{d}	25.12	6.44^{m}	6.86
6	1	1	2	$1,679^{l}$	1,669	40.00^{f}	37.47	6.00^{k}	5.88
7	2	1.17	4	810^{i}	723	18.33^{cd}	19.61	3.56^{c}	3.25
8*	2	0.75	4	170^{a} (13)	169	13.33^{ab}	13.29	3.45^{b}	3.44
9	3	1	2	673^{h}	781	16.67^{bcd}	17.36	3.16^{a}	3.44
10	2	0.75	1	$2,215^{n}$	2,188	45.00^{g}	46.16	6.22^{l}	6.34
11	3	0.5	6	464^{e}	465	15.00^{abc}	16.42	4.44^{f}	4.48
12	1	1	6	621^{f}	613	18.33^{cd}	15.70	5.56^{i}	5.59
13	4	0.75	4	327^{c}	229	11.67^{a}	8.12	4.89^{g}	4.58
14	2	0.33	4	856^{j}	956	33.33^{e}	33.62	4.00^{d}	4.41
15	3	1	6	307^{b}	356	11.67^{a}	13.09	4.22^{e}	4.46

*The experiment was repeated 6 times and the responses represented average values with their standard deviation in parenthesis

Different lowercase letters in each column indicate significant differences with ANOVA (Tukey HSD post-hoc test, P <0.05).

Results

Efficiency of wastewater treatment using FPE to stimulate the growth of the P1 inoculum

In this study, CCD was used to determine the optimal conditions for treating RAW. Actual and predicted values of COD, SS and TtS in wastewater after treatment using varying levels of stimulated P1 inoculum, FPE and RT under microaerobic light conditions are shown in Table 4-2. The actual data obtained from the experiments were analyzed by multiple linear regression to provide predicted values. Among the experimental runs, the actual values averaged from six runs at the center point to be run no. 8 had the lowest COD of 170 mg/L, while the SS of 13.33 and TtS of 3.45 (in mg/L) were a little higher than in run nos. 9 (TtS, 3.16), 13 and 15 (SS, 11.67), respectively. However, there was no significant difference in SS value in run nos. 8, 13 and 15. The conditions of run no. 8 were: 2% stimulated P1 inoculum, 0.75 % FPE and 4 days RT, while the conditions of run no. 9 were (3 % P1, 1 % FPE and 2 days), run no. 13 (4 % P1, 0.75 % FPE and 4 days) and run no. 15 (3 % P1, 1 % FPE and 6 days) were different. The predicted values of COD, SS and TtS in run no. 8 were 169, 13.29 and 3.44 mg/L (corresponding to a reduction percentage of 94, 68 and 69, respectively). Run no. 8 produced the same removal percentages for COD, SS and TtS in both actual and predicted values. Among runs 8, 9, 13 and 15, run no. 8 was considered to be the best run as the minimal dose of inoculant P1 was used with best removals of COD and SS.

According to actual data, as shown in Table 4-2, COD values were in the range of 170–2,215 mg/L, while values of SS and TtS were between 11.67 and 53.33 mg/L and 3.16–7.56 mg/L. Design Expert software was used to analyze the relationship of the variables to the responses using the regression model with the significance level $\alpha = 0.05$. The *P*-value is a tool for evaluating the significance and thus quadratic models were appropriate by considering the *P*-value ($P < 0.5$), lack of fit ($P \geq 0.05$) and the test statistics (Std. Dev PRESS lower and higher R^2 and adjusted R^2) combination. The F-value was high and P-value was low, which indicated that the model was good.

Table 4-3. ANOVA analysis for the full quadratic equations; COD, suspended solids (SS) and total sulfide (TtS).

Source	Degree of freedom	Sum of squares	Mean square	*F*-value	*P*-value (Prob > *F*)
Model (COD)	7.54E+06	9	8.38E+05	6.88E+01	<0.0001
X_1	3.20E+05	1	3.20E+05	2.63E+01	0.0004
X_2	6.55E+04	1	6.55E+04	5.38E+00	0.0429
X_3	3.66E+06	1	3.66E+06	3.01E+02	<0.0001
X_1^2	1.81E+05	1	1.81E+05	1.49E+01	0.0032
X_2^2	8.09E+05	1	8.09E+05	6.64E+01	<0.0001
X_3^2	2.37E+06	1	2.37E+06	1.94E+02	<0.0001
X_1X_2	1.42E+05	1	1.42E+05	1.16E+01	0.0067
X_1X_3	1.99E+05	1	1.99E+05	1.63E+01	0.0024
X_2X_3	1.75E+05	1	1.75E+05	1.43E+01	0.0036
Model (SS)	2.84E+03	9	3.15E+02	2.47E+01	<0.0001
X_1	3.49E+02	1	3.49E+02	2.74E+01	0.0004
X_2	2.37E+02	1	2.37E+02	1.86E+01	0.0015
X_3	1.27E+03	1	1.27E+03	9.95E+01	<0.0001
X_1^2	2.00E+01	1	2.00E+01	1.57E+00	0.2391
X_2^2	3.20E+02	1	3.20E+02	2.51E+01	0.0005
X_3^2	5.00E+02	1	5.00E+02	3.92E+01	<0.0001
X_1X_2	3.12E+00	1	3.12E+00	2.45E-01	0.6312
X_1X_3	1.53E+02	1	1.53E+02	1.20E+01	0.0061
X_2X_3	7.81E+01	1	7.81E+01	6.13E+00	0.0328

Table 4-3. ANOVA analysis for the full quadratic equations; COD, suspended solids (SS) and total sulfide (TtS) (continues).

Source	Degree of freedom	Sum of squares	Mean square	*F*-value	*P*-value (Prob > *F*)
Model (TtS)	3.04E+01	9	3.38E+00	2.10E+01	<0.0001
X_1	6.25E+00	1	6.25E+00	3.89E+01	<0.0001
X_2	1.62E+00	1	1.62E+00	1.01E+01	0.0099
X_3	1.85E+00	1	1.85E+00	1.15E+01	0.0069
X_1^2	9.35E+00	1	9.35E+00	5.82E+01	<0.0001
X_2^2	2.73E-01	1	2.73E-01	1.70E+00	0.2212
X_3^2	9.35E+00	1	9.35E+00	5.82E+01	<0.0001
X_1X_2	3.76E-01	1	3.76E-01	2.34E+00	0.1572
X_1X_3	8.60E-01	1	8.60E-01	5.35E+00	0.0432
X_2X_3	2.42E+00	1	2.42E+00	1.51E+01	0.003

wastewater treatment conditions, the *P*-value of COD reduction; X_1, X_2, X_3, X_1^2, X_2^2 X_3^2, X_1X_2, X_1X_3 and X_2X_3 were less than 0.05 (Table 4-3). In addition, the *P*-values of dependent variables for SS and TtS that were less than 0.05 can be found in the following equations.

$$\text{COD: } Y_1 = 169.50 - 153.11X_1 - 69.26X_2 - 518{:}03X_3 - 133.12X_1X_2 + 157.62X_1X_3 + 147.82X_2X_3 + 112.13X_1^2 + 236.87X_2^2 + 405.49X_3^2 \quad (2)$$

$$\text{SS: } Y_2 = 13.29 - 5.05X_1 - 4.17X_2 - 9{:}64X_3 + 4.37X_1X_3 + 3.12X_2X_3 + 4.71X_2^2 + 5.89X_3^2 \quad (3)$$

$$\text{TtS: } Y_3 = 3.44 - 0.68X_1 - 0.34X_2 - 0.37X_3 + 0.33X1X_3 + 0.55X_2X_3 + 0.81X_1^2 + 0.81X_3^2 \quad (4)$$

The experimental results were analyzed by regression analysis, which consisted of the effect of linear, quadratic and interactions that provided regression equations for COD, SS and TtS as a function of the stimulated inoculant P1 (X_1), FPE

(X_2), and RT (X_3) with each response. The equations were used to predict the removal of COD, SS and TtS values. The fit of the models was further checked by the coefficient of determination, R^2. According to the ANOVA results, the COD model showed a high R^2 value of 98.4%, which implied that only 2 % variation could not be explained by this model. A higher R^2 value indicated a higher representing capability of the full quadratic equation for COD under the given experimental domain. The adjusted R^2 value of 97.0 % indicated that the model was meaningful and it was in agreement between the actual and predicted values of wastewater treatment. The models of SS and TtS had R^2 values of 95.7 % (adjusted R^2 91.8 %), and 95 % (adjusted R^2 90.5 %), respectively. These results indicate that the accuracy of the polynomial models was good as those equations could be used to predict the value of COD, SS and TtS. However, the polynomial model for COD was selected to use in a verified test as this model gave the highest R^2 and adjusted R^2 values. The results also showed that the stimulated P1 inoculum (X_1), FPE (X_2) and RT (X_3) were the main factors that affected the COD, SS and TtS values. In contrast, the interaction of the terms of X_1X_3 and X_2X_3 was minor.

Design-Expert software was used to build the 3D surface plots shown in Figure 4-1 and to analyze the interaction effects of the three variables—inoculum P1, FPE and RT—on wastewater treatment efficiency. This figure shows that COD and SS decreased significantly as influenced by RT (Figure 4-1b, c, e, f), but decreased only slightly for TtS (Figure 4-1h, i). It was also observed that P1 and FPE individually had less impact on COD, SS, and TtS removal as the response surface did not show much change with their variations (Figure 4-1a, d, g). However, there was a strong interaction between P1 and FPE (Figure 4-1a). FPE may have stimulated the activity of our inoculum. The TtS contour plot versus inoculum P1 and RT (Figure 4-1h) shows the zone of minimum response located in the middle of the figure, suggesting that minimum effluent values of TtS could be found in our parameter ranges. The optimal conditions that minimized COD reduction were calculated by setting the partial derivatives of the function to zero, with respect to the corresponding variables. The optimum condition for COD removal was found at 3 % stimulated P1inoculum, 0.90% FPE and 4 days RT.

Based on our model, this optimal condition gives the removal of COD, SS and TtS at 98 %, 79 % and 72 %, respectively.

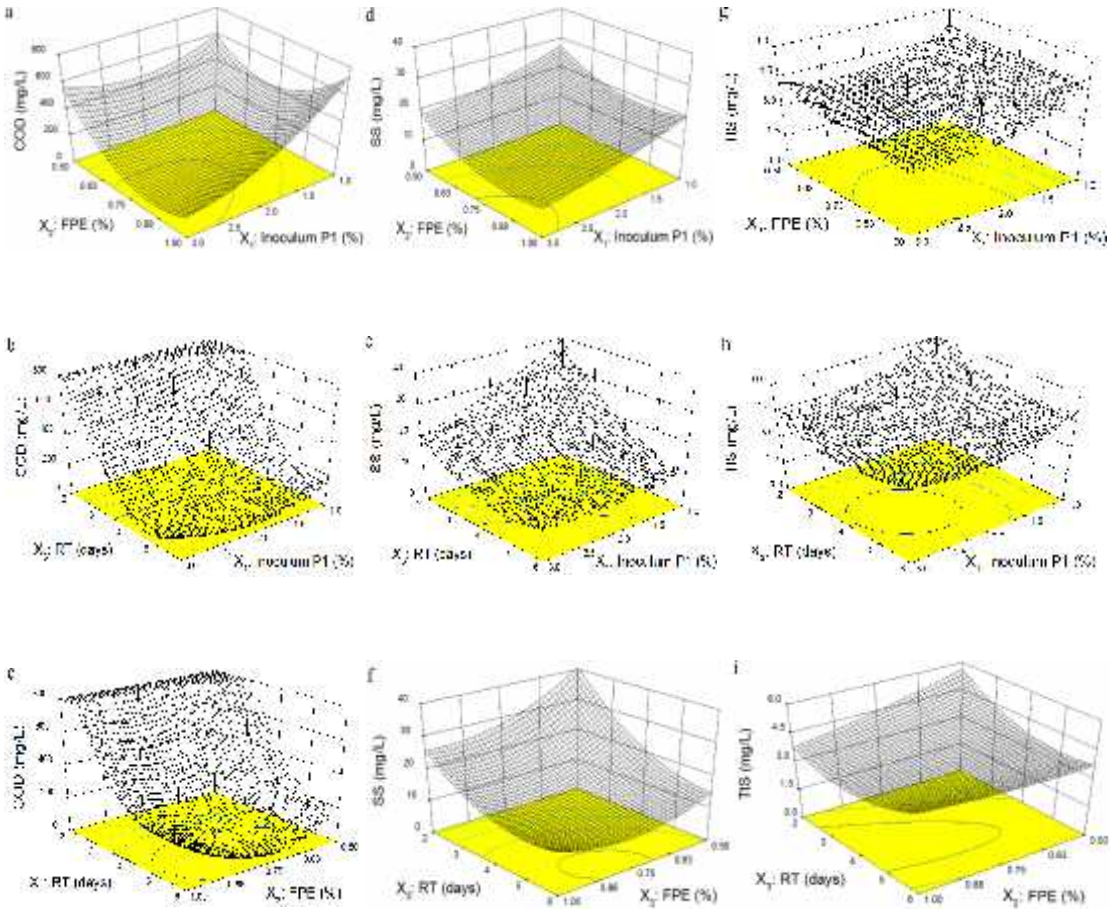

Figure 4-1. Three-dimensional (3D) response surfaces illustrating the values of chemical oxygen demand (COD) (a, b, c); suspended solids (SS) (d, e, f) and total sulfide (TtS) (g, h, i) as functions of inoculum P1, fermented pineapple extract (FPE) and retention time (RT). Each graph displays the interaction effect of two variables while the third variablewas fixed at its central level shown in Table 4-2.

Verification of the model and optimum conditions

Based on the results in Table 4-2 using a numerical optimization method as previously described (Figure 4-1), the optimum operating conditions calculated (3 % stimulated inoculant P1, 0.9 % FPE and 4 days of RT) were confirmed in RAW (initial COD 2,742 mg/L) under microaerobic light conditions (verified test) and the removal

percentages for COD, SS and TtS under the designed experiment were 94 %, 75 % and 66 %, respectively (Table 4-4). It should be noted that the experimental values obtained were in good agreement with the values predicted from the models, with relatively small errors between predicted and actual values, of only 4 %, 5 % and 8 %, for COD, SS and TtS removals, respectively. Therefore, it can be concluded that the generated model has sufficient accuracy to predict the efficiency of rubber sheet wastewater treatment as the error is less than 10 %.

Table 4-4. Verification test based on the optimal conditions (3% inoculum P1, 0.9% FPE and 4 days RT) predicted by the CCD for treating latex rubber sheet wastewater (initial COD 2,742 mg/L) under microaerobic light conditions.

Removal (%)	Experimental	Predicted	Error (%)
COD	94	98	4
Suspended solids (SS)	75	79	5
Total sulfide (TtS)	66	72	8

Results of the verification experiments under microaerobic light conditions with optimal prediction of design (verified set) and the best conditions from experimental run no. 8 (Table 4-2) are shown in Table 4-5 and Figure 4-2. Under the verified set, the removal percentages of COD, SS and TtS were 94 %, 75 % and 66 % as previously described, whereas in the best condition run, the removal percentages of COD, SS and TtS were 96 %, 78 % and 71 %, respectively. Again, removal of 68 % of the sulfate and 32 % of the phosphate were found in the best condition run, but in the verified set removals of sulfate and phosphate were only 66 % and 30 %, respectively. A control set without addition of the stimulated P1 inoculum and FPE produced the lowest efficiency of RAW treatment as the removal percentages for COD, SS, TtS, sulfate and phosphate ions were 45, 24, 31, 24 and 20, respectively. No significant differences were found for the numbers of HPC and stimulated P1 inoculum in the verified set (7.20 and 8.46 log CFU/mL) when compared with the best run (7.18 and 8.41 log CFU /mL). In contrast, for the control set, no PNSB were detected whereas the

amount of HPC was higher at 7.68 log CFU /mL. No LAB were found in any treatment sets or the control set. Based on the above results, the best condition run was called the best set and this was studied further for its yield of biomass and cell composition and to consider its use as an SCP.

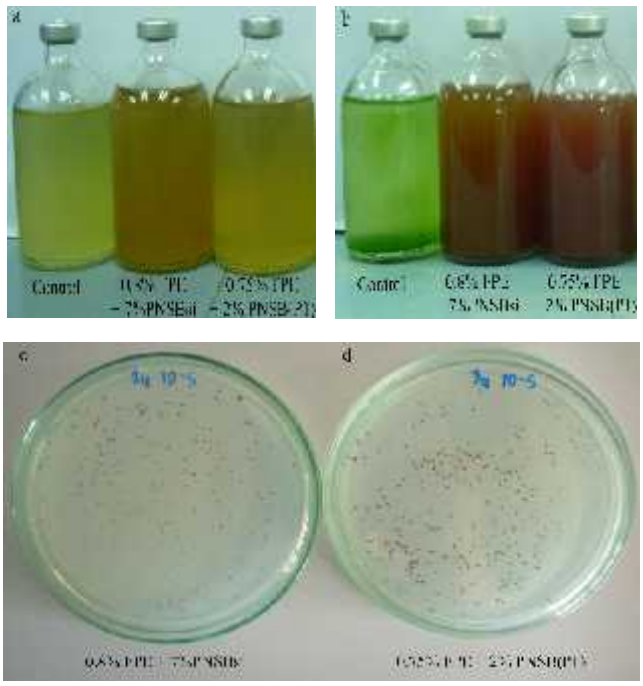

Figure 4-2. Photographs showing the treatment process for latex rubber sheet wastewater (RAW) using a combination of stimulated indigenous purple nonsulfur bacteria (PNSBsi) or stimulated inoculant of P1 (P1) and FPE with optimal conditions under microaerobic light conditions (a) at the start of the experiment, (b) at the end (day 4), (c) PNSB from a set of PNSBsi at day 4 and (d) PNSB from a set of P1 that was presumed to be isolate P1.

The amounts of some heavy metals and cations found in the wastewater sets of the control and treatment sets (best set, P1 and PNSBsi) are shown in Table 4-6. According to the results, only the effluent treated by P1 passed the standard guidelines set by the Pollution Control Department and Royal Irrigation Department, Thailand. However, the PNSB sitreated wastewater passed in almost all parameters, with the exception of UHS. In contrast, the control set did not pass standard guidelines

for levels of COD, SS and UHS. The biomass at day 4 was 865 mg/L for the best set of the stimulated P1 inoculum, and the corresponding wastewater COD removal was 2,626 mg/L. Hence, the calculated cell yield was 0.33 (Table 4-6). A lower cell yield was obtained (0.30) in the PNSBsi set, but a higher cell yield (0.42) was found in the control set. The biomass obtained after 4 days treatment of RAW had the maximum protein content in the biomass from the best P1 treatment followed by the biomass from a control set; the lowest biomass was from PNSBsi (Table 4-6). The biomass from the best P1 condition set had 65%protein, 8%carbohydrate, 3%crude fat, 14%ash, 10% moisture and 319 kcal (see details of cell composition of other biomass in Table 4-6).

Table 4-5. Efficiency of latex rubber sheet wastewater treatments using a combination of FPE and stimulated P1 inoculum under microaerobic light conditions.

Property		Percentage reduction		
mg/L	[RAW]	Control	0.9% FPE + 3% P1 (Verified set)	0.75% FPE + 2% P1 (The best set)
	T = 0	Day 4	Day 4	Day 4
COD	2742 ± 11	45.3 ^{a}	94 ^{b}	96 ^{b}
SS	42 ± 3	23.8 ^{a}	75 ^{b}	78 ^{c}
TDS	540 ± 20	18.5 ^{a}	55 ^{b}	56 ^{b}
Phosphate	185 ± 1	20 ^{a}	30 ^{b}	32 ^{b}
Sulfate	5.0 ± 0.2	24.0 ^{a}	66 ^{b}	68 ^{b}
TtS	13.11 ± 0.38	30.5 ^{a}	66 ^{b}	71 ^{c}
DsS	12.22 ± 0.38	21.2 ^{a}	63 ^{b}	66 ^{c}
UHS	5.38 ± 0.17	57.4 ^{a}	96 ^{b}	94 ^{b}
H_2S	8 ± 2	100	100	100
Numbers of organisms (log CFU/mL)				
Parameter	T = 0	Day 4	Day 4	Day 4
pH	7.03 ± 0.01	7.46 ^{a} ± 0.03	8.15 ^{b} ± 0.01	8.07 ^{b} ± 0.01
HPC	8.15 ± 0.02	7.68 ^{b} ± 0.03	7.20 ^{a} ± 0.01	7.18 ^{a} ± 0.02
LAB	0	0	0	0
PNSB (P1)	0	0	8.46 ^{a} ± 0.01	8.41 ^{a} ± 0.02

Different lowercase letter in the same row indicates a significant difference ($P < 0.05$).

Table 4-6. Proximate analysis of biomass obtained from effluents after 4 days treatment by a combination of stimulated P1 inoculum or stimulated indigenous PNSB with FPE under microaerobic light conditions.

Parameter (mg/L)[A]	Control	7% indigenous PNSB + 0.8%FPE (PNSBsi)	2% inoculant P1+ 0.75%FPE (P1)	Guideline level [B], [C]
pH	7.46 ± 0.03 [a]	7.35 ± 0.02 [a]	8.07 ± 0.01 [b]	5.5 - 9.0, 6.5-8.5
COD	1500 [c] ± 10	239 [b] ± 11	116[a] ± 19	120 - 400, na
SS	42 [b] ± 3	11 [a] ± 3	10 [a] ± 2	≤ 50, ≤ 30
UHS	2.29 [c] ± 0.07	1.23 [b] ± 0.09	0.32 [a] ± 0.01	≤ 1.0, ≤ 1.0
Cd	0.001 ± 0.0001	0.004 ± 0.001	0.001 ± 0.000	≤ 0.03, ≤ 0.03
Cr (Hexavalent)	0.001 ± 0.0001	0.003 ± 0.001a	0.001 ± 0.000	≤ 0.25, ≤ 0.30
Pb	< 0.0001	< 0.0001	< 0.0001	≤ 0.2, ≤ 0.1
Mn	0.199 ± 0.001	0.174 ± 0.001	0.246 ± 0.001	≤ 5, ≤ 0.5
Cu	0.007 ± 0.001	0.018 ± 0.001	0.005 ± 0.001	≤ 2, ≤ 1
Zn	0.016 ± 0.001	0.058 ± 0.001	0.012 ± 0.001	≤ 5, ≤ 5
Effluent quality	Exceed	Nearly pass	Pass	
Biomass (Yx/s)	0.42 [c]	0.30 [a]	0.33 [b]	na
% Protein	64.4 [b]	55.5 [a]	64.7 [b]	na
%Carbohydrate	4.5 [a]	11.2 [c]	8.0 [b]	na
% Fat	0.8 [a]	12.6 [c]	3.1 [b]	na
% Ash	23.3 [c]	17.1 [b]	14.1 [a]	na
% Moisture	7.0 [b]	3.6 [a]	10.1 [c]	na
Energy (kcal)	283 [a]	380 [c]	319 [b]	na

[A] Unless otherwise stated, [B] Criterion of the Pollution Control Department and [C] Royal Irrigation Department, Thailand. na = not available. Different lowercase letter in the same row indicates a significant difference ($P < 0.05$).

Discussion

According to the CCD experiment, run no. 8 (Table 4-2) provided the best conditions for treating RAW based on the COD and SS removals by using a 2 % stimulated P1 inoculum, 0.75 % FPE and 4 days RT. The result of using the stimulated

P1 inoculum and the 2 % FPE with the non sterile wastewater (RAW) resulted in the inoculant P1 becoming the major bacterium involved in the treatment process. It is not surprising that the P1 inoculum became the dominant organism both in the preparation of the inoculum and in the RAW treatment due to the provision of the most suitable conditions for its growth as a photoheterotroph under microaerobic light conditions. To explain why FPE stimulated the growth of PNSB but had little effect on HPC in RAW, our previous studies had clearly shown that the FPE contained organic acids that stimulated the growth of PNSB including the isolate P1 under low dissolved oxygen (DO) and the availability of good light (Kantachote *et al.,* 2010). This is because the role of FPE stimulating the growth of PNSB under light condition is to lower the ORP value and to provide reducing conditions. In our previous study (Kornochalert *et al.,* 2011), the ORP value reportedly was the most effective parameter for stimulating growth of PNSB, and was −340 mV in the treatment with 2.5 % FPE and 235 mV in the control set (RAW). This proved that the lactic acid and acetic acid in the FPE were being used preferentially as electron donors for photosynthesis in the partially anaerobic light conditions. These results are in agreement with those of Okubo *et al.* (2006), who reported that the lower chain length fatty acids such as acetate and propionate in a swine wastewater ditch stimulated the growth of PNSB, particularly *Rhodopseudomonas* and *Rhodobacter* spp., to form visible microbial mats. One explanation of why microaerobic light conditions promote PNSB growth is that these organisms are anoxygenic photosynthetic bacteria; this is due to the PNSB exhibiting higher oxygen tolerance and being able to perform aerobic respiration at full atmospheric oxygen tension (Okubo *et al.,* 2005), unlike purple sulfur bacteria, which fail to grow in the presence of even a low concentration of oxygen.

Based on the removal efficiencies of COD and SS, the RT had the biggest influence (Eqs. 2, 3) due to the organisms having enough time to hydrolyze and consume nutrients (COD and SS) in the wastewater, and 4 days RT was confirmed to be the optimal time for PNSB including *R. palustris* P1 to treat RAW (Kantachote *et al.,* 2005, 2010). However, the RT varied depending on the type of wastewater and PNSB present in the wastewater systems. For example, treating pharmaceutical wastewater by PNSB under microaerobic light conditions took 3–5 days (Madukasi *et*

al., 2010). However, treatment of swine wastewater with an initial COD of 18,700 mg/L by *R. palustris* required 6 days to reduce the COD by 90 % (Kim et al. 2004). With regard to H_2S removal, the stimulated P1 inoculum was the key factor to reduce all forms of sulfide in RAW (Eq. 4, Figure 4-1g–h). This is due to sulfide also being used as an electron donor for photosynthesis by PNSB, including isolate P1. The results were supported by our previous work showing that isolate P1 can use sulfide under good microaerophilic light conditions (Kantachote *et al.*, 2010). In addition, *Rhodobacter* and *Rhodopseudomonas* are able to use inorganic electron donors such as sulfide or H_2 as reductants for NAD(P)+ enabled by redoxactive enzymes that are able to accept electrons from these substrates and subsequently donate them to the cyclic electron transport chain (Sinha and Banerjee, 1997).

This study has demonstrated clearly that the stimulated P1 inoculum performed highly effectively to treat CRSF wastewater with the complete removal of any odor of H_2S as there was no detection of this gas in the head space of the bioreactors (Table 4-5). This is because light was applied for treating RAW under microaerobic conditions, which quickly became anaerobic conditions based on redox values (−50 to −110 mV) in the wastewater systems (data not shown). These conditions allow organisms, either microalga in the control set or PNSB in the treatment sets, to grow as photoautotroph/photoorganotroph in RAW and this sulfide may be used as an electron donor by PNSB. In addition, as H_2S was not detected in the head space, this was also related to the pH values as the pH altered the sulfide (<7: H_2S, 7–8: HS^- and> 8: S^-) (Markl, 1999). Hence, any H_2S in the head space was changed to HS^- and S^- due to the pH of the wastewater being higher than 7 in both the control and treatment sets (Table 4-6) and this might be a reason why no H_2S was detected in the control set with the microalgae.

Based on the results of Table 4-5, the use of 0.75%FPE and 2 % stimulated P1 inoculum led to higher efficiency treatment of RAW than that found in the verification set with 0.9 % FPE and a 3 % stimulated P1 inoculant. It might be that the former condition was more suitable than the latter in the case that the initial COD that was 2,742 mg/L and this indicated that inoculums of only 2 % allowed the P1 inoculum to become the dominant organism (8.41 log CFU/mL), as was also the case

for the 3%inoculum size (8.46 log CFU/mL). Moreover, the use of a lower inoculum size meant that a lower amount of FPE was needed for stimulating growth. In this work, the stimulated P1 inoculum was prepared in non-sterile wastewater (RAW) and it was claimed to be the dominant organism as previously described; this was supported by the evidence from checking morphology on a GM agar plate and cell shape (data not shown). Again, when using P1 for treating non-sterile wastewater, the same kind of colonies were found on GM agar (Figure 4-2d) and rod-shaped cells were observed, although the colonies were no different from other PNSBsi (Figure 2c). In this work, a 2 or 3 % inoculum of P1 added to the RAW as previously described was able to compete with other microbes by using the RAW (initial COD 2,742 mg/L) with a high efficacy similar to the inoculum P1 prepared with sterile RAW to treat RAW with an initial COD of 1,457 mg/L (Kantachote *et al.,* 2010) due to the effluent meeting the standard guidelines set by the Pollution Control Department and Royal Irrigation Department, Thailand.

In addition, the stimulated P1 inoculum with lower inoculum size, such as only 2 or 3 %, performed with a higher efficiency to treat RAW than 7 % PNSBsi (in chapter 3) (Table 4-6). This supports the concept that most of the organisms in the stimulated inoculant P1 were R. palustris P1; this isolate is a useful strain previously isolated from latex rubber sheet wastewater (Kantachote *et al.,* 2010). Therefore, it could compete with other microbes in both the preparation of an inoculum and for highly efficient treatment of wastewater. The phosphate removal results (Table 4-5) indicated that almost all the organisms found in the stimulated P1 inoculum are likely to be isolate P1 as it has a higher efficiency (32 %) to remove phosphate when compared with the control set that produced only a 20 % reduction. This is in accordance with Liang *et al*. (2010), who reported that PNSB found in activated sludge has the potential to accumulate phosphorus and thereby, to remove phosphorus from the wastewater. According to the above results, a study using molecular methods of the dynamics of bacteria during wastewater treatment should be conducted in the future for a better understanding of the efficacy of the system.

The lowest efficacy of treating RAW was observed in a control set as this set had no additions of either FPE or stimulated P1 inoculum. In the control set,

microalgal growth appeared as a green color while the treatment sets had the red color of PNSB (Figure 4-2a, b). This can be explained by the lower level of nutrients in the control set under light conditions with microaerobic conditions stimulating microalgal growth. Moreover, this is also the reason why the green color observed in the control set was from microalga not purple sulfur bacteria because the latter organisms cannot survive in O_2. This result was in agreement with previous work that had shown that low nutrients under light condition with a little O_2 supports the growth of microalga (Valderramaa *et al.*, 2002). LAB were not detected in any sets (Table 4-5) although LAB are microaertolerant; it is presumed that the initial numbers of LAB in the wastewater that came from FPE were low as only a small volume was used and substrates in the RAW were not suitable for supporting LAB growth when compared with PNSB.

The amounts of heavy metals such as Cd, Cr and Pb were very low in the wastewater treated by PNSBsi or P1, including the control set (Table 4-6); however, the PNSBsi effluent had a slightly higher amount of UHS than the acceptance level. A lower efficiency of UHS removal in the PNSBsi-treated set might be caused by the lower pH (7.35) compared to the value of 8.07 in the P1 set, as the amount of PNSB was not significantly different (Tables 4-5, 4-6). This also indicated that most of the PNSB in the P1 set was isolate P1 because of the higher pH being attributed to the active consumption of sulfide when compared with the PNSBsi. Therefore, only P1-treated wastewater can be considered for use as irrigation water for agriculture, particularly in the dry season. In addition, the PNSB biomass obtained should be considered for use as SCP for animal feed as previously mentioned. Among the effluents, the control set produced maximum biomass yield. The highest biomass yield (Yx/s=0.42) was found in the control set was due to the growth of the microalgae, and this also had the highest HPC (Table 4-5). In general, the biomass yield of HPC is in the range of 0.45–0.77 (Majone et al. 1999), whereas the biomass yield of PNSB such as *Rhodobacter sphaeroides* Z08 grown in soybean wastewater was 0.28 (He et al. 2010). However, in this study, a higher biomass yield of 0.30 was obtained for the PNSBsi and 0.33 for the P1 set. The amount of crude protein was 65 %; 56 % was found in the biomass of effluents in the best condition set with the use of *R. palustris* P1 and

PNSBsi could be considered as SCP. The results of our work are in accordance with those of Honda *et al*. (2006), which showed that the crude protein of PNSB biomass in mixed culture was between 56 % and 68 %, whereas
Kantachote *et al*. (2005) using *R. blastica* DK6 to treat RAW under microaerobic light conditions produced biomass with 65 % crude protein. However, treatment of soybean wastewater by *Rhodobacter sphaeroides* Z08 under natural conditions produced a biomass of 52 % crude protein (He *et al.*, 2010). This is because the amount of the crude protein depends on the organism used.

Conclusions

Application of PNSB for treating RAW could be possible as this study has successfully developed appropriate technology by using FPE to stimulate the growth of *R. palustris* P1 under light conditions first to use as an inoculum for treating RAW under anaerobic treatment with high efficiency and with no detection of H_2S. The RAW treatment with P1 not only produced effluent that met the standard guidelines for use as irrigation water but also obtained a biomass as a by-product to utilize as SCP.

CHAPTER 5

Dynamics of bacterial communities treating latex rubber sheet wastewater by stimulated indigenous purple non sulfur bacteria or *Rhodopseudomonas palustris* P1 inocula with fermented pineapple extract

Abstract

In this work, the dynamics of bacterial members including the purple nonsulfur bacteria (PNSB) in non sterile rubber sheet wastewater (RAW) treated by stimulated indigenous PNSB (PNSBsi) or *Rhodopseudomonas palustris* P1 (PNSB P1) inocula with fermented pineapple extract (FPE) were investigated using denaturing gradient gel electrophoresis (DGGE) of nested-PCR-amplified fragments of the 16S rRNA gene. The diversity and evenness of the communities in the stimulated PNSB P1 inoculum was significantly higher than for the community in the stimulated PNSBsi inoculum ($P < 0.05$). Only *R. palustris* was dominant throughout the RAW treatment over 4 days by either PNSBsi or PNSB P1 inocula; however, a 100% similarity index to the strain P1 was found only in the RAW treatment by PNSB P1 inoculum. This also corresponded to a higher efficiency to treat RAW of the strain P1 as there was little of the H_2S remaining in RAW and a major by product was their biomass that had higher amounts of essential amino acids for use as an animal feed. Bacterial communities in both RAW treatment processes, at starting point (t = 0) in addition of PNSB found 4 main bacterial populations belonging to 37.5% γ-proteobacteria, 25% ß-proteobacteria, 25% negativicutes and 12.5% flavobacteriia. Besides the 4 main bacterial groups; α-proteobacteria, clostridia and mollicutes were also detected in both communities later during the RAW treatment for 4 days. Overall the results proved that both stimulated PNSB inocula with FPE could out compete other heterotrophs in their community and *R. palustris* P1 was the most effective strain to treat RAW.

Keywords: bacterial community, hydrogen sulfide, PCR-DGGE, purple nonsulfur bacteria, rubber wastewater, single cell protein

บทคัดย่อ

งานวิจัยนี้พลวัตของประชากรแบคทีเรียรวมทั้งแบคทีเรียสังเคราะห์แสงกลุ่มไม่สะสมซัลเฟอร์ (PNSB) ในการบำบัดน้ำเสียจากการผลิตยางแผ่นโดยใช้กล้าเชื้อแบคทีเรียสังเคราะห์แสงที่ถูกกระตุ้นการเจริญด้วยน้ำหมักสับปะรด จากจุลินทรีย์ประจำถิ่น (PNSBsi) หรือแบคทีเรียสังเคราะห์แสง *Rhodopseudomonas palustris* P1 (PNSB P1) ถูกวิเคราะห์โดยใช้เทคนิค nest-PCR-DGGE และดีเอ็นเอบริเวณ 16S rDNA พบว่าความหลากหลาย และการกระจายตัว ของประชากรแบคทีเรียในกล้าเชื้อ PNSB P1 มีค่าสูงกว่าประชากรแบคทีเรียในกล้าเชื้อ PNSBsi อย่างมีนัยสำคัญ ($P < 0.05$) แบคทีเรียสังเคราะห์แสง *R. palustris* เป็นกลุ่มประชากรหลักในการบำบัดน้ำเสียตลอดระยะเวลา 4 วัน ทั้งในกล้าเชื้อ PNSBsi และ กล้าเชื้อ PNSB P1 อย่างไรก็ตามเฉพาะการบำบัดน้ำเสียด้วยกล้าเชื้อ PNSB P1 เท่านั้นที่ *R. palustris* มีค่าความเหมือนกับสายพันธุ์ P1 100% ซึ่งสอดคล้องกับประสิทธิภาพที่สูงกว่าในการบำบัดน้ำเสียด้วยกล้าเชื้อ PNSB P1 ที่มีแก๊สไฮโดรเจนซัลไฟด์เหลือเพียงเล็กน้อยในน้ำเสีย และ มีชีวมวลเป็นผลพลอยได้จากการบำบัดโดยมีปริมาณกรดอะมิโนจำเป็นสูงกว่ามีศักยภาพใช้เสริมในอาหารสัตว์ได้ เมื่อเริ่มต้นการบำบัดกลุ่มประชากรแบคทีเรียในน้ำเสียของทั้งสองชุด (PNSBsi และ PNSB P1) ประกอบด้วย γ-proteobacteria 37.5% ß-proteobacteria 25% negativicutes 25% และ flavobacteriia 12.5% นอกจากแบคทีเรีย 4 กลุ่มหลักนี้แล้วในระยะเวลา 4 วัน ของการบำบัดของทั้งสองชุดยังพบแบคทีเรียกลุ่มอื่นๆ ได้แก่ α-proteobacteria, clostridia และ mollicutes ภาพรวมของผลการทดลองที่ได้ พิสูจน์ว่ากล้าเชื้อแบคทีเรียสังเคราะห์แสงทั้งสองชุดที่ถูกกระตุ้นด้วยน้ำหมักสับปะรดมีความสามารถในการแข่งขันกับจุลินทรีย์ประจำถิ่นกลุ่มบริโภค (heterotrophs) และ *R. palustris* P1 เป็นสายพันธุ์ที่มีประสิทธิภาพสูงสุดในการบำบัดน้ำเสียจากการผลิตยางแผ่น

คำสำคัญ: ประชากรแบคทีเรีย, แก๊สไฮโดรเจนซัลไฟด์, PCR-DGGE, แบคทีเรียสังเคราะห์แสงสีม่วงกลุ่มไม่สะสมซัลเฟอร์, น่ำเสียจากการผลิตยาง,โปรตีนเซลล์เดียว

Introduction

Many cooperative rubber sheet factories (CRSFs) that operate throughout all parts of Thailand, use open lagoons or natural oxidation ponds to treat their wastewater because they are low cost operations (Chaiprapat and Sdoodee 2007; Kantachote *et al*., 2005). However, such systems are unable to produce effluents that comply with the industrial effluent or irrigation standards and are also faced with serious problems such as the 'rotten-egg odor' of hydrogen sulfide (H_2S) (Kantachote *et al*. 2010). Hence, there is a need to develop effective technology to treat this CRSF wastewater without increasing operating costs. Purple nonsulfur bacteria (PNSB) have been extensively studied for their ability to treat various wastewaters with by products such as hydrogen gas (Liao *et al*., 2010) and effluents containing biomass or single cell protein (SCP) with high in proteins and vitamins but low in uric acid (Azad *et al*., 2001; Kantachote *et al*., 2005; Honda *et al*., 2006).

PNSB are versatile organisms that can grow in various metabolic modes such as photoheterotrophic and heterotrophic processes under anaerobic/microaerobic light and aerobic dark conditions, respectively. However, competition between PNSB with other bacteria to enable them to become the dominant organisms is rare under natural operating conditions. In our previous studies, we found that the growth of PNSB either as indigenous or selected strains of *Rhodopseudomonas palustris* P1 was stimulated by adding fermented pineapple extract (FPE) to such an extent that they became the dominant organism(s) in a non sterile rubber sheet wastewater (RAW) and consequently used as inoculum for RAW treatment process. The stimulated inoculum P1 and a little FPE were used for RAW treatment considerably improved the quality of the effluent so that it could be used as irrigation water (in chapter 4). To try to explain the roles of the bacterial members in the communities of the RAW treated by stimulated

indigenous PNSB (PNSBsi) or stimulated strain P1 (PNSB P1) we had carried out the following investigations.

As there are limitations on using cultivation techniques to evaluate the dynamics of bacterial communities, molecular tools have been used to target a wide variety of 16S rRNA genes to identify the variety of bacteria present in a given system from which they originated without cultivation and isolation (Dar *et al*., 2007; Lücker *et al*., 2007). One of the main molecular techniques used for monitoring the dynamics of bacterial community members is denaturing gradient gel electrophoresis (DGGE) which is an effective tool for the characterization of bacterial communities (Liang *et al*., 2010; Muuyzer *et al*., 1993; Okubo *et al*., 2006). Universal primers such as EUB8f and U1492r are normally used to study the variety of eubacteria in any ecology (Amann *et al*., 1995; Orphan *et al*., 2001). On the other hand, the *puf*M gene is frequently utilized for genetic surveys of anoxygenic phototrophs (Achenbach *et al*., 2001; Béjà, *et al*., 2002; Karr *et al*., 2003; Okubo et al. 2006; Yutin et al. 2005). The aim of this study was to compare bacterial members in communities of stimulated indigenous PNSB (PNSBsi) and stimulated PNSB P1 that bloomed in their PNSB populations during RAW treatment. Thus we hoped to gain information to help us to understand their roles on the efficiency of RAW treatment by both types of stimulated PNSB inocula with FPE.

Materials and Methods

Wastewater samples and effluents for treatment by PNSB stimulated with FPE

Samples used in this study were collected from RAW treatments inoculated with FPE stimulated indigenous PNSB (PNSBsi) or *R. palustris* P1 (PNSB P1) under their optimal conditions that had been determined in our previous studies (in chapter 4). To prepare stimulated PNSB inocula, 2% FPE was added into RAW (COD, 2000 mg/L) and then adjusted pH to 7 and this called adjust RAW. The adjusted RAW was incubated under microaerobic light conditions for 2 days to obtain PNSBsi while PNSB P1 was prepared by adding 2% cell suspension of strain P1 into the adjusted RAW and incubated under the same condition for 2 days (see detail in chapter 4).

Samples were taken at the start of the treatment and every day for 4 days. Initial RAW samples were collected from a lagoon pond at a CRSF at the Pichit suburb in Songkhla province, Thailand and used to investigate the efficiencies of the stimulated PNSB either from PNSBsi or PNSB P1 under their optimal conditions as follows: stimulated indigenous PNSB (7% PNSBsi and 0.80% FPE) and stimulated PNSB strain P1 (2% PNSB P1 and 0.75% FPE). All experiments were conducted in 120 mL serum glass bottles (bioreactors) and sacrificed samples were used for each sampling. Each experiment was carried out in triplicate and all bottles were incubated in microaerobic light conditions. The following parameters; COD, SS, TtS, pH, TS, TDS and UHS (H_2S in wastewater) and sulfate were monitored. For bacterial enumeration, a fraction of the samples was separated and immediately processed using plate count agar and GA medium for mesurements of heterotrophs (HPC) and PNSB, while the rest of the samples in each bottle were used to extract DNA. The characteristics of the RAW used and the effluents are presented in Table 5-1 (in chapter 4).

Table 5-1. Physicochemical properties of rubber sheet wastewater before and after 4 days treatment by using inoculants of purple non sulfur bacteria (PNSB) stimulated with fermented pineapple extract (FPE) under microaerobic-light conditions.

Measurements mg/L	Before treatment	After treatment 0.75% FPE + 2% P1	Before treatment	After treatment 0.8% FPE + 7% PNSBsi
	T = 0	Day 4	T = 0	Day 4
COD	2742 ± 11	110 ± 11 (96) [A]	2722 ± 11	239 ± 11(91)
Suspened solids	42 ± 3	9 ± 2 (79)	43 ± 3	11 ± 3 (74)
Total dissolved solids	540 ± 20	238 ± 11 (56)	547 ± 12	380 ± 20 (31)
Sulfate	5.0 ± 0.2	1.6 ± 0.1 (68)	5.1 ± 0.2	2.0 ± 0.2 (61)
Total sulfide	13.11 ± 0.38	3.8 ± 0.67(71)	13.78 ± 0.38	5.33 ± 0.67(61)
Dissolved sulfide	12.22 ± 0.38	4.15 ± 0.38 (66)	12.89 ± 0.38	5.11 ± 0.38 (60)
Un-ionized H_2S, UHS	5.38 ± 0.17	0.32 ± 0.05 (94)	5.67 ± 0.17	1.23 ± 0.09 (78)
H_2S	8 ± 2	0 (100)	10 ± 2	0 (100)
Effluent [B]		Pass		Exceed, UHS

Table 5-1. Physicochemical properties of rubber sheet wastewater before and after 4 days treatment by using inoculants of purple non sulfur bacteria (PNSB) stimulated with fermented pineapple extract (FPE) under microaerobic-light conditions (continues).

Measurements mg/L	Before treatment	After treatment 0.75% FPE + 2% P1	Before treatment	After treatment 0.8% FPE + 7% PNSBsi
Numbers of organisms (log CFU/mL)				
Parameter	T = 0	Day 4	T = 0	Day 4
pH	7.03 ± 0.01	8.07 ± 0.01	7.03 ± 0.01	7.35 ± 0.02
Heterotrophs (HPC)	8.15 ± 0.02	7.18 ± 0.02	8.30 ± 0.01	7.13 ± 0.01
PNSB	0	8.41 ± 0.02	0	8.39 ± 0

[A] Numbers in parentheses are reduction percentages; [B] Based on guideline levels by Pollution Control Department and Royal Irrigation Department, Thailand
HPC, Heterotrophic plate count; PNSB, Purple nonsulfur bacteria

Sampling and preparing the wastewater treatments for analysis of the bacterial communities

As previously described, wastewater samples from each bioreactor were collected initially (t= 0) and every day for 4 days. Samples were centrifuged at 12,000 rpm for 10 min at 4 °C and washed twice pellets with phosphate buffer (pH 7). The pellets were stored at -20 °C for molecular analysis. Sodium dodecyl sulfate (SDS) was used to extract total DNA from the biomass pellets of the wastewater samples by following the method of Zhou *et al*. (1996) with some modification. Electrophoresis on a 1% (w/v) agarose gel followed by UV visualization after ethidium bromide (EB) staining were used to determine the yield and fragmentation of DNA. DNA was purified with a Gel/PCR DNA fragment extraction kit (Geneaid, Taiwan) according to the manufacturer's instructions and stored at -20 °C before PCR–DGGE analysis.

For amplification of DNA by PCR, in order to increase the sensitivity and to facilitate DGGE by analysis of fragments of the same length, a nested PCR

technique was applied and primers used in this study are shown in Table 4-2. DNA was amplified as follows: the PCR reaction mixture for each set had a total volume of 50 µL with 50 mg DNA template, 20 pmol of each primer, 200 µM deoxynucleoside triphosphates (dNTP), 1X PCR buffer, 3 mM $MgCl_2$, and 1 U of Taq DNA polymerase (KAPA Biosystems, USA). In the first round, approximately 1,500-bp fragments of the bacterial 16S rRNA genes were generated by amplification using universal bacterial primers EUB8f (Amann et al. 1995) and U1492r (Orphan *et al*., 2001). The PCR program for the primer set EUB8f/U1492r included an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min and a final extension at 72 °C for 7 min. In the second round, PCR products for DGGE analysis were generated by the 16S rDNA product using primers 338GC-F and 518r (Amann *et al.,* 1995; Moyer *et al.,* 1994; Orphan *et al.*, 2000). The GC-camp was attached on the ‘‘59 end’’ of the forward primer 338GC-F and 344GC-F to make a higher melting domain and to prevent DNA fragments from denaturing completely (McCammon *et al.,* 2005). The PCR program for the primer set 338-GC-F/518r were; initial denaturation at 95 °C for 5 min, followed by 25 cycles of denaturation at 95 °C for 50 s, annealing at 60 °C for 30 s, and extension at 72 °C for 50 s and a final extension at 72 °C for 7 min. The primer set *puf*M557F and *puf*M750R were used to amplify the photosynthetic unit-forming gene (*puf*M) of the purple phototrophic bacteria (Achenbach *et al*., 2001). PCR was performed using an initial denaturation step at 95 °C for 15 s, followed by 35 amplification cycles of denaturation (95 °C for 1 min), annealing (54 °C for 30 s), elongation (72 °C for 1 min), and a final extension step at 72 °C for 10 min. All PCR reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystem). Products from triplicate PCR amplifications were combined for each sample. The size and amount of the PCR products were visualized and estimated after electrophoresis on a 1% agarose gel using ethidium bromide staining.

Table 5-2. Oligonucleotide primers used in this study.

Primer	Target group	Sequence (5' → 3')
EUB8f	universal bacterial	AGAGTTTGATCCTGGCTCAG
U1492r	universal bacterial	GGTTACCTTGTTACGA CTT
338GC-F	Bacterial domain	[a]GC-ACTCCTACGGGAGGCAGC
518r	Bacterial domain	ATTACCGCGGCTGCTGG
pufM557GC-F	Purple phototrophic bacteria	[a]GC-CGCACCTGGACTGGAC
pufM750R	Purple phototrophic bacteria	CCCATGGTCCAGCGCCAGAA

[a]GC = -CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG.

Denaturing gradient gel electrophoresis analysis

DGGE analysis was used to separate DNA fragments of the same size that had different compositions to obtain the operational taxonomic units (OTUs), and this analysis was performed as described by Muyzer *et al.* (1993) using a DGGE-2000 system apparatus (CBS Scientific Company, Del Mar, CA). Briefly, samples containing equal amounts of PCR products were loaded onto an 8% (w/v) polyacrylamide gel in 1X Tris-acetate-EDTA (TAE) with a denaturing gradient that ranged from 35 to 60% denaturants (100% denaturant contains 7 M urea and 40% (v/v) formamide in 1X TAE). Electrophoresis was performed at 60 °C and at a constant voltage of 200 V for 5 h. Following electrophoresis, the gel was stained with SYBR Green nucleic acid stain (Molecular Probes, Eugene, OR) for 30 min. The images were visualized on a UV transilluminator and captured using Biovision CN 1,000/26M (Vilber Lourmat, France). DGGE analysis was repeated three times to confirm the results of the PCR products.

Sequencing and phylogenetic analysis

To identify the DGGE bands, individual DGGE bands were excised, resuspended in 20 µL of MilliQ water, and stored at 4 °C overnight. After this period the DNA diffused out of the acrylamide gel and the solution was used as a template in a reamplification PCR. For sequencing, reamplification was performed using the primer set without the GC-clamp. The purified products were then sent for sequencing (1st Base, Malaysia). Analysis of the sequence was built by a BLAST search (site http://www.ncbi.nlm.nih.gov/BLAST/) for identification to the nearest related species in the database. A phylogenetic tree was constructed using the neighbor joining method (Tamura *et al*., 2007) in MEGA version 4.1.

Amino acids analysis

In this study, biomass pellets collected from the effluents that had been treated using the FPE stimulated inocula of either the PNSBsi or the PNSB P1 were used to analyze the amino acid composition. To analyze amino acids, 100 g biomass of each sample was hydrolyzed by 6N HCl at 110 °C for 22 h to obtain amino acid hydrolysate for determining amino acids. Amino acid analysis was performed with a LC-6A HPLC amino acid analysis (Shimadzu, Tokyo, Japan) equipped with a fluorescence detector. Amino acid hydrolysate was separated on a Shim-pack ISC-07/S 1504 Na column (packed with cation exchanger consist of sulphonate syrene divinyl benzene copolymer). The mobile phase consisted of solvent A (0.2 N sodium citrate containing 7% EtOH, pH 3.2), solvent B (0.6 N sodium citrate with 0.2 N boric acid, pH 10) and solvent C (0.2 N sodium hydroxide), except for tryptophan when we used 0.6 N sodium citrate containing the 0.2 N boric acid (pH 9), and was purified with a Milli-Q filtration system (Millipore, Bedford, MA). Calibration was performed using an amino acid mixture standard.

Results

Bacterial communities in stimulated PNSB inocula

Analysis of the triplicate DGGE profiles (repeated 3 times) derived from the bacterial communities, PNSBsi and PNSB P1 inoculums, detected a total of 59 bands. Of these 59 bands, 37 were reproducibly detected across triplicate samples from either the PNSBsi or PNSB P1 inocula. Of these, 37 bands; 8 bands were common to inocula (PNSBsi and PNSB P1), 14 bands only in PNSBsi, and 7 bands only in PNSB P1. There were only 6 bands from the 14 bands of PNSBsi inoculum appearing across all three replicates from the profiles of PNSBsi while the PNSB P1 inoculum had only 2 bands from 7 bands appearing across all three replicates. A Simpson's diversity index and evenness that was based on the DGGE profiles were calculated for each replicate of each inoculum. It was found that bacterial members in the stimulated PNSB P1 inoculum showed a higher significant difference for both the diversity and evenness than that of bacterial communities in the stimulated PNSBsi inoculum ($P < 0.05$) (Figure 5-1).

A total of 8 DGGE bands (6 + 2) that were unique for the stimulated PNSBsi and PNSB P1 inoculums were excised and subsequently sequenced to describe the phylogenetic diversity of the amplified 16S rDNA for showing the relationships of the various members of bacterial communities present in both the PNSB inocula and a positive PNSB (proteobacterium G11: *R. palustris* HaA2) was included (Figure 5-2). In addition to the PNSB inocula, other heterotrophs found for the unique bacterial members in PNSBsi on the basis of band intensity in percent were 64.7, 57.3, 46.0, 42.1, 33.3 and 36.2 for bands 1 to 6, respectively (Figure 5-2a). Identification results after DNA amplification and sequencing found that they were very close to *Anaeroarcus burkinensis*, *Anaeromusa acidominophila*, *Simplicispira metamorpha* for band nos. 1, 2, 3; *Acidovorax caeni* and *A. temperans* for band nos. 4 and 6; and *Flavobacterium resistens* for band no. 5 (Figure 5-2a). In contrast, the PNSB P1 inoculum consisted of only 2 unique populations of *Pseudomonas trivialis* and *P. cuatrocienegasensis* as their band intensities were 42.6 and 57.4% for band nos. 7 and 8 (Figure 5-2b).

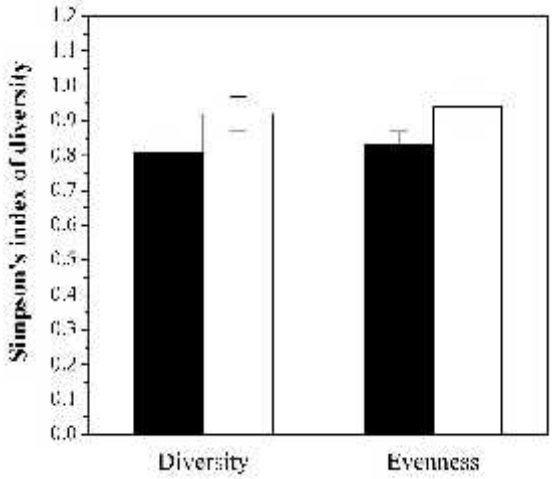

Figure 5-1. Diversity and evenness of the bacterial communities between the stimulated inocula of PNSBsi and PNSB P1, as derived from DGGE profiles and analysis by Simpson's index. Black bars stand for the PNSBsi community while the white bars stand for the PNSB P1 community. Diversity and evenness indices were determined from triplicate DGGE profiles and averaged. Error bars represent standard deviations across the triplicates.

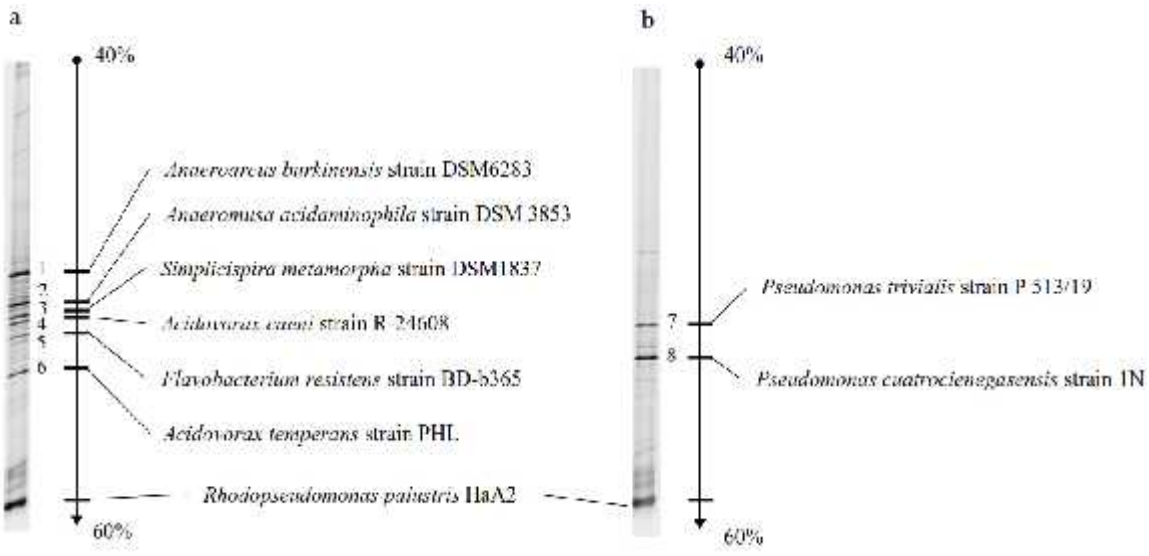

Figure 5-2. Unique bacterial members in the community profiles determined with PCR-DGGE of partial 16s rRNA gene fragments from the stimulated indigenous PNSB inoculum (a) and stimulated PNSB P1 inoculum (b). Percent indicates gradient gel electrophoresis.

Bacterial dynamics in RAW treatments by the FPE stimulated PNSB inocula

The DGGE analysis was performed to confirm the presence of PNSB in the RAW wastewater treatment based on the amplified PCR products of the *puf*M gene for an anoxygenic photosynthetic bacterial community. There was only *R. palustris* with a similarity index in the range of 95-97% to *R. palustris* P1 that was used as a positive control observed throughout the RAW treatment by PNSBsi inoculum during the 4 days including the PNSBsi inoculum, lane 1 (Figure 5-3a). These were based on the intensity of band nos. 1-8; 39k, 44k, 36k, 43k, 42k, 42k, 0 and 37k (arbitrary units: arb.) with the highest intensity being found at day 1, lane 3 (Figure 5-3b). A similar result was observed in the PNSB P1 inoculum and its RAW treatment for 4 days as only *R. palustris* was found (Figure 5-3c). Band intensities of this organism were 57k, 0, 50k, 48k, 45k, 48k, 0 and 42k (arb.) and these corresponded to a 97-100% similarity index with *R. palustris* P1. A 100% similarity index or the highest intensity was found in the PNSB P1 inoculum, lane 1 and at day 3 of RAW treatment, lane 5 (Figure 5-3d). This demonstrated that the DNA sequences were shown to be 100% identical to the DNA sequence of the control strain, *R. palustris* P1.

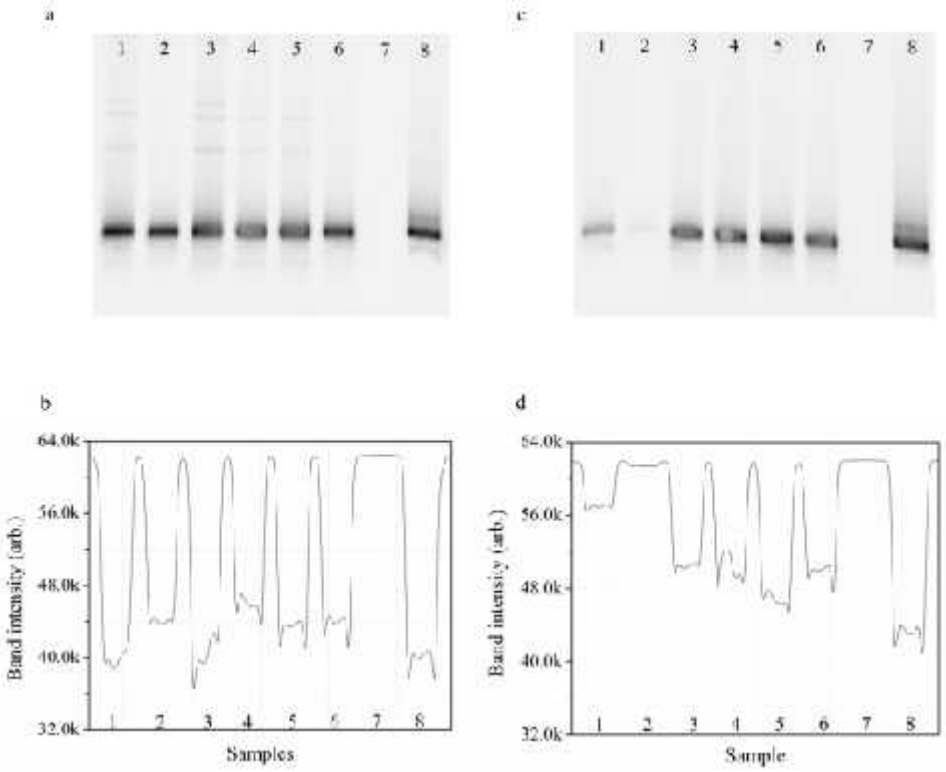

Figure 5-3. DGGE profile generated by PCR amplification of the *puf*M gene from the FPE stimulated PNSB inocula and their RAW treatment from days 0, 1, 2, 3 and 4 of PNSBsi (a, b) and PNSB P1 (c, d); Lane 1: inoculum Lanes 2-6: wastewater treatment by PNSB at days 0-4; Lane 7: negative control and Lane 8: positive control (P1: *R. palustris* JX876953). Band intensities (peak height) of dsDNA and ssDNA were estimated by ImageJ software.

These results have demonstrated that the bacterial communities in addition to the PNSBsi or P1 inoculums, had other members that also were found in both the RAW treatment processes, at the starting point (t = 0) were 4 main bacterial members belonging to 37.5% γ-proteobacteria, 25% ß-proteobacteria, 25% negativicutes and 12.5% flavobacteriia (Figure 5-4a). More varieties of bacterial groups; clostridia, mollicutes and α-proteobacteria could be observed during treatments at days 1, 2-3 and 4, respectively. A 20% clostridia (*Carboxydocella thermautotrophica*, *Desulfitobacterium dehalogenans*; band nos. 17 and 18) was observed in treatment process at day 1 (Figure 5-4b). An increase of clostridia to 30% as *Peptoniphilus lacrimalis* (band no.19) was found and 10% mollicutes (*Acholeplasma vituli*, band no. 20) was also found at day 2. However, γ-proteobacteria (*P. plecoglossicida* and *Acinetobacter haemolyticus* from bands; 15 and 16) decreased to 10% of total population (Figure 5-4c). At day 3 members of 25% γ-proteobacteria such as *P. alcaligenes* and *Acinetobacter radioresistens* (band nos. 21 and 23) were observed (Figure 5-4d). The genera of 6.67% α-proteobacteria (*Maricaulis virginensis*, band no. 22), ß-proteobacteria (*Pseudorhodoferax soli* and *A. temperans* band nos. 24 and 6) were found at day 4 of treatment (Figure 5-4e).

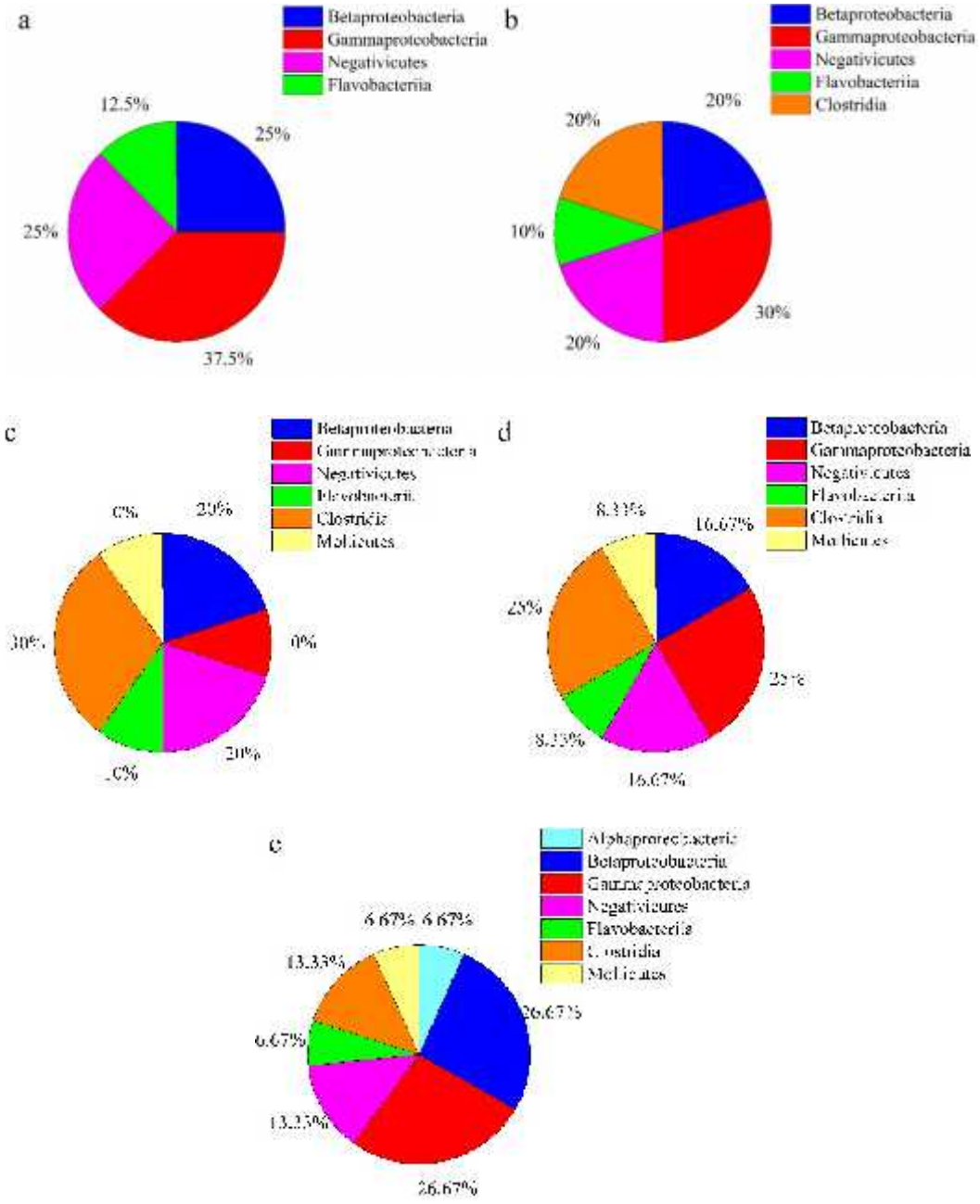

Figure 5-4. Proportion of the different major bacterial phylogenetic lineages in the DGGE profile of partial 16S rRNA gene sequences obtained after PCR amplification of 16S rRNA genes retrieved from RAW treatment by FPE stimulated PNSB inoculum either PNSBsi or PNSB P1 at day 0 (a), day 1 (b), day 2 (c), day 3 (d) and day 4 (e).

Amino acid composition in biomass of PNSB communities

The amount of the essential amino acids with the exception of phenylalanine and histidine in the biomass obtained from the effluent of the RAW treatment of PNSB P1 community was significantly higher than that found in the biomass of the PNSBsi community (see detail in Table 5-3). Among the essential amino acids found in both biomasses of PNSBsi and PNSB P1 communities had the same trend as the maximum amount was phenylalanine (2.84-3.08 % dry weight) followed by leucine (1.36-1.74% dry weight) and the least amount was isoleucine (0.21-0.28% dry weight). In contrast, most of the non essential amino acid in the biomass of PNSBsi community was significantly higher than that found in the biomass of the PNSB P1 community. One of the essential amino acids (methionine) and two of the non essential amino acid (tryptophan and cysteine) were not detected in either of the PNSB biomasses.

Cost analysis for treating RAW

RAW treatment cost was estimated from the costs of inoculum preparation and inoculum size used for treating RAW (see details in Table 5-4). Although other costs such as electricity should be considered, while pineapple used for producing FPE will be a by-product from a pineapple can factory; thus, the estimated cost of RAW treatment in this study would be closely to the real cost. It was found that with the use of FPE to stimulate PNSB growth either PNSBsi or PNSB P1 in RAW to use as inoculum provided a lower cost (roughly 5669 times) than that of GM broth (Sigma-Aldrich Co, USA). The use of PNSB P1 inoculum to treat RAW produced a higher efficiency at a lower cost (8.64 baht/m^3) compared to PNSBsi inoculum (10.24 baht/m^3).

Table 5-3. Amino acid composition of single cell protein from purple nonsulfur bacteria in effluents of rubber sheet wastewater (RAW) treatment by the FPE stimulated PNSB inocula under their optimal conditions.

Compositions	Effluent of RAW treatment Biomass of PNSBsi	Effluent of RAW treatment Biomass of PNSB P1	Effluent of sterile poultry slaughterhouse Biomass of *R. gelatinosus* R_1 [B]	FAO guideline [A]
Crude protein (%)	55.5[C]	64.7 [C]	67.6	
Amino acid (g/100 g dry cell)				
Essential amino acid				
Lysine	1.75[a]	1.91[b]	3.61	4.2
Histidine	2.12[b]	1.78[a]	1.83	NA
Threonine	1.89[a]	2.03[b]	3.52	2.8
Valine	0.81[a]	0.90[b]	4.56	4.2
Methionine	ND	ND	1.4	2.2
Isoleucine	0.38[a]	0.43[b]	3.18	4.2
Leucine	2.45[a]	2.70[b]	6.8	4.8
Phenylalanine	5.54[b]	4.40[a]	3.03	2.8
Non essential amino acid				
Aspartic acid	5.62[b]	5.53[a]	5.74	NA
Tyrosine	ND	ND	2.9	NA
Serine	2.97[b]	2.67[a]	2.63	NA
Glutamic acid	5.38[b]	5.21[a]	6.85	NA
Proline	1.93[a]	1.91[a]	3.27	NA
Glycine	2.86[b]	1.67[a]	4.18	NA
Alanine	4.27[b]	3.26[a]	6.98	NA
Tryptophan	ND	ND	1.74	NA
Cysteine	ND	ND	0.59	NA

Different lowercase letters in each row indicate significant differences with ANOVA (Tukey HSD post-hoc test, $P < 0.05$).

[A] Data from FAO (1980).

[B] Data from Ponsano et al. 2003 and [C] Data from in chapter 4.

Table 5-4. Cost estimation for obtaining 1 liter of PNSB inoculum based on only media used for treating RAW.

Cost	Baht/L Pineapple fruit[A]	Baht/L Pineapple core[B]
GM broth	181.41	181.41
1 L inoculum	181.41	181.41
Inoculum cost using FPE and NH_4Cl		
0.05% NH_4Cl[C]	0.012	0.012
2% FPE (pineapple and molasses)	0.08	0.02
1 L inoculum	0.092	0.032
Ratio GM/FPE	1972 times	5669 times
1 L RAW treatment by PNSBsi		
0.8% FPE	0.03	0.008
7% PNSBsi	0.006	0.00224
Total cost	0.036	0.01024
Treatment cost for 1 m^3 (1000 L)	36 (1170 USD)	10.24 (333 USD)
1 L RAW treatment by P1		
0.8% FPE	0.03	0.008
2% PNSB P1	0.002	0.00064
Total cost	0.032	0.00864
Treatment cost for 1 m^3 (1000 L)	32	8.64

[A]1 liter FPE (pineapple fruit, molasses and water) = 4 baht
[B]1 liter FPE (pineapple as a by-product, molasses and water) = 1 baht
[C]1000 g NH_4Cl = 24 baht

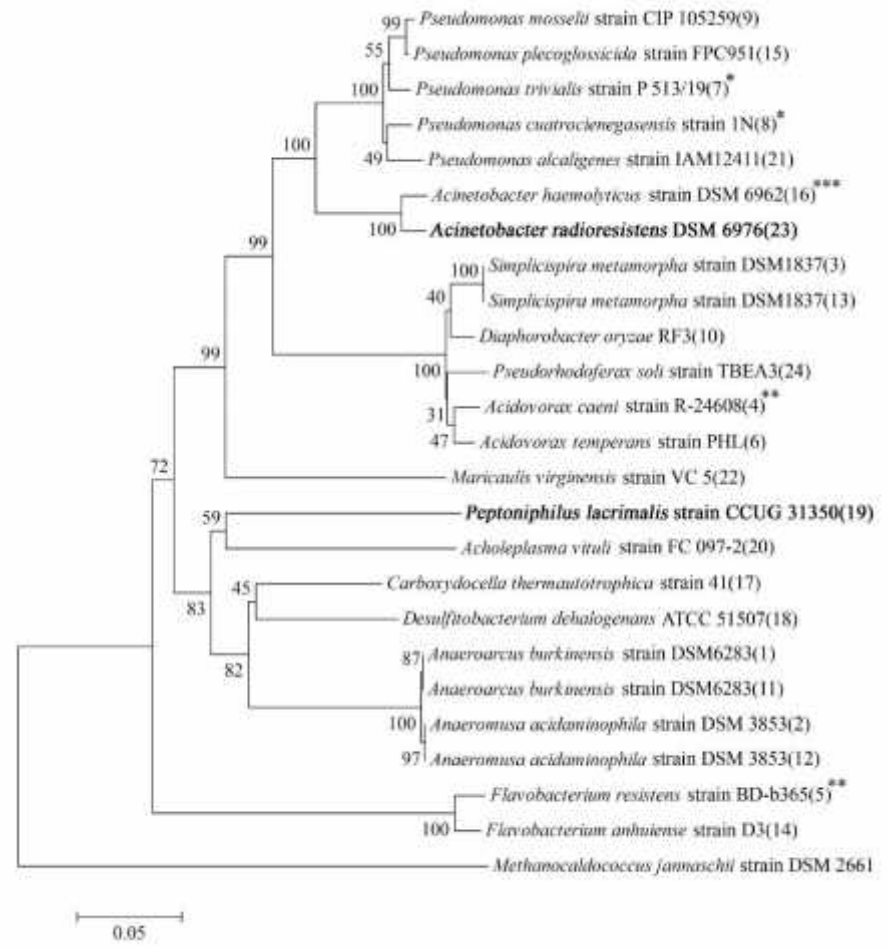

Figure 5-5. Phylogenetic tree of bacterial communities from the FPE stimulated PNSB inocula and their RAW treatments with neighbor joining analysis showing the relationship between DGGE bands that were amplified using primer 338FGC and 518R (16S rDNA) and there nearest neighbors held on-line. The tree is based on a final alignment of 170 bases. The distance scale indicates 0.05 substitutions/site. Numbers represent the number of times the clade to the right of the node was recovered in 1000 bootstrap re-samplings of the data. *Methanocaldococcus jannaschii* DSM 2661 (Accession No.L77117.1) was used to root the tree. The asterisk indicates the organisms found only in the stimulated PNSB inocula; *PNSB P1, **PNSBsi, and *** Only at day 0 of RAW treatments. The bold type indicates the organisms found only in RAW treated by PNSB P1.

Discussion

The PNSB P1 population was much more diverse than the PNSBsi population, even for the heterotrophs as only 2 species of *Psudomonas* were found across the triplicate DGGE and the rest of the members were different (Figure 5-1 and 5-2). As the same RAW was used for preparation of the stimulated PNSB inocula by the same batch of FPE thus the difference was only on the source of the PNSB population that originated from the indigenous PNSB or a selected strain, *R. palustris* P1 for PNSB P1 inoculum. This indicates that *R. palustris* P1 might be a good candidate to allow only the pseudomonad population (dense bands) dominance. This is because *Pseudomonas* spp. (bands 7-8) such as *P. cuatrocienegasensis* are normally found in lagoons (Escalante *et al.*, 2009) while *P. trivialis* may arise from the FPE as it is normally part of the bacterial flora of plants (*Behrendt et al*., 2003). In contrast, PNSBsi allowed those heterotrophs (6 bands) to be dominant this was because they originated from the same habitat that differed from the PNSB P1 as a selected strain.

With the use of the stimulated PNSB inocula to treat RAW under their optimal conditions, the bacterial community of PNSB P1 was able to reduce COD 96%, SS 79%, total sulfide 71% and unionized hydrogen sulfide (UHS) 94%, while the bacterial community associated with the PNSBsi reduced COD 91%, SS 74%, total sulfide 61% and UHS 78% (Table 5-1). The amount of UHS was still 1.23 mg/L in the effluent of the PNSBsi community and this would not pass the guideline level (≤1.0 mg/L) (Pollution Control Department, 1994; and Royal Irrigation Department, 1989). The results showed that the PNSB P1 community was more efficient at treating the RAW than that PNSBsi community although the amounts of the total PNSB (8.39 and 8.41 CFU/mL) and the total heterotrophic plate count (HPC) (7.13 and 7.18 log CFU/mL) were not different for both communities. In addition, H_2S was not detected in any headspace of the bioreactors, and thus there was no sulfide odor from either of the RAW treatments by the PNSB inocula although the UHS found in the RAW treatment by PNSBsi exceeded the guidelines (Table 5-1). According to the above results it seemed that the efficiency to treat RAW was governed by the PNSB population more than by the HPC population. This was due to the finding that during

RAW treatment both PNSB inocula had an ability to reduce the amount of HPC by roughly one log cycle.

This was also supported by the DGGE analysis of the PNSB with the *pufM* gene that was used to explain the results, and it was found that only *R. palustris* was dominant in both the RAW treatment systems throughout the 4 days (Figure 5-3). However, *R. palustris* found in the PNSBsi community might be different strains from the P1 strain (accession number JX876953) as the similarity index was 95-97 compared to 97-100 for the PNSB P1 community. This confirmed that the strain P1 is an effective strain to use to treat RAW as it reduces the UHS by 94% compared to 78% by PNSBsi, and this result is in agreement with our previous study (Kantachote *et al.,* 2010) that the strain P1 was very effective in its ability to reduce the sulfide levels. Our results also agree with those of Okubo *et al.* (2006) who reported that on the basis of 16S rRNA and the *pufM* gene, the dominant PNSB of the microbial mats in a swine wastewater ditch was *Rhodobacter* and *Rhodopseudomonas*, and the latter organism had a wider spectrum for its source of carbon with a higher affinity for acetate than did the former organism. A similar result was also reported by Liang *et al.* (2010) who used PCR-DGGE and cloning methods based on their characteristic *pufM* genes and found that *R. palustris* and *Rhodobacter* sp. were the dominant PNSB in activated sludge. This is a possible reason why both our RAW treatments using the PNSB inocula detected only *R. palustris* as the dominant PNSB.

The PNSB population could bloom in both the RAW treatment systems from day 1 to day 4 as previously described because RAW treatment had high concentrations of short chain fatty acids that were initially produced by the HPC populations (Kantachote *et al.,* 2010). Hence, in addition to the most prominent PNSB population other bacterial populations were also involved in the efficient treatment of RAW. Bacterial members of the HPC in both the RAW treatment systems changed dramatically during the treatment process, as at the starting time of the RAW treatment, many bacterial members were missing from the RAW treatment of the PNSBsi community such as *Acidovorax caeni*, *A. temperans* and *Flavobacterium resistens* (Figure 5-5). After day 0 of RAW treatment, the missing members were replaced by the following; *Pseudomonas mosselii, P. plecoglossicida, Acinetobacter haemolyticus,*

Diaphorobacter oryzae and *Flavobacterium anhuiense*. This indicated that bacterial members in RAW treatment by PNSBsi community were more diverse than those found in the PNSBsi inoculum. This is because these heterotrophs were indigenous bacteria in RAW that initially were present in the lagoon. Those heterotrophs were also observed in the PNSB P1 community at the start of the treatment; that meant that there was no difference in heterotrophs population in both RAW treatment systems at the starting point. It was the same for the PNSBsi community that *P. trivialis* and *P. cuatrocienegasensis* disappeared in the RAW treated by PNSB P1 community; this might be explained that they could not compete with the other heterotrophs associated with the PNSB populations of the RAW because *Pseudomonas* spp. are aerobes normally found at the beginning of a wastewater treatment whereas the conditions used for treating the RAW were microaerophilic/anaerobic.

There was only *Acinetobacter haemolyticus* was lost from both bacterial communities during RAW treatment by either PNSBsi or PNSB P1 from days 1-4 as found only at day 0 (Figure 5-5). This is a good sign because this organism is normally found in various wastewaters; and it is a causative agent of resistant nosocomial infections, and is also a water and food borne pathogen (Doughri *et al.,* 2012). The results indicated that the PNSB were able to eliminate *A. haemolyticus* from the RAW wastewater treatment. In contrast, the following genera were found during treatment; *Maricaulis virginensis, P. alcaligenes, Acinetobacter radioresistens, Pseurhodoferax soli, Carboxydocella thermautotrophica, Desulfitobacterium dehalogenans, Peptoniphilus lacrimalis, Acholeplasma vituli.* Moreover, *Acidovorax temperans*, a member of the PNSBsi inoculum, was lost at the starting time; so it became one of the heterotrophic members in both bacterial communities. Those heterotrophs are acidogenic fermentative bacteria with an ability to produce short chain fatty acids such as acetic and butyric in wastewater (Heylen *et al.*, 2008; Utkin *et al.,* 1994) and consequently stimulated the growth of PNSB as previously described.

As there was no difference found among the heterotroph members of both RAW treatment communities, and the amount of PNSB was dominated by *R. palustris* present at much higher numbers than the heterotrophs by about 1 log cycle in both the RAW treatment communities. This is more evidence that the ability to treat

RAW was mainly due to the PNSB population with help from some heterotrophs (in chapter 4). In addition, in our unpublished data we have found that heterotrophs produced H_2S during RAW treatment and this was related to members of heterotrophs found in this study such as *Desulfitobacterium dehalogenans and Peptoniphilus lacrimalis* that released H_2S to the environment (Utkin *et al.,* 1994; Ezaki *et al.,* 2001). It is well recognized that H_2S is a toxic gas that occurs by anaerobic respiration using sulfate ion as a final electorn acceptor, and leading to the rotten egg odor in anaerobic treatments such as from anaerobic digesters and lagoons (Kantachote *et al.,* 2005; Kantachote *et al.,* 2010). Fortunately, under microaerobic light conditions there was a stimulated proliferation of the PNSB and the strain P1 does utilize sulfide as an electron donor for photosynthesis; thereby H_2S was not detected and only a small amount of UHS was left so this allowed the effluent to pass the standard guidelines for use as irrigation water (Table 5-1). The result is in agreement with Kantachote *et al.* (2010) and also Okubo *et al.* (2006) who reported that *R. palustris* and *Rhodobacter* can use sulfide as an electron donor for their photosynthesis.

Among the essential amino acids found in the biomass of PNSBsi or PNSB P1 community that were collected from the effluent of RAW treatment by both PNSB inocula, only phenylalanine was present in amounts (2.84-3.08% dry weight) that met FAO guidelines (2.80% dry weight). The amino acid composition in the PNSB biomass obtained from the microaerobic light conditions in our study was less than for the biomass of *Rhodocyclus gelatinosus* as R1 is grown in the sterile poultry slaughterhouse under anaerobic light conditions (Ponsano *et al.,* 2003). This might be that a different wastewater and culture was used and included conditions to obtain biomass between a mixed culture with other heterotrophs (our study) and a pure culture. Based on the above information, a pure culture can probably provide a better source of essential amino acids; and a type of wastewater and used culture conditions that had improved amino acids content. Moreover, no methionine was detected in either PNSB biomasses in our study and it is well recognized that SCP also has a low level of this amino acid (Shipman *et al.,* 1975). However, both PNSB biomasses obtained from the effluents of RAW treatment could be considered for using in animal feeds.

According to cost analysis for treating RAW (Table 5-4) in a step of inoculum preparation if include the electricity cost; the cost for treating 1 m^3 RAW should be higher than 8.64 baht by PNSB P1 inoculum. Therefore, use of sunlight in the step of inoculum preparation should be further investigated. The operating cost for treating the wastewater in this study may be higher than other conventional treatments such as the use of commercial EM ball as one EM ball (10 baht) could be used to treat wastewater 3-5 m^3 (http://www.rubberthai.com/board/index.php?topic=2017.0;wap2). However, the use of PNSB for treating wastewater produced the effluent that met the standard guidelines to use as irrigation water and also provided biomass as SCP for animal feed. In addition, pineapple core should be replaced by agricultural wastes to make a lower cost of FPE and PNSB inoculum for treating rubber sheet wastewater and this would be attractive to use by CRSFs.

Conclusions

Bacterial communities in RAW treatment using microaerobic or anaerobic light conditions and FPE stimulated PNSB inocula were dominated by *R. palustris*. However, a superior blooming of the selected *R. palustris* P1 is required due to its higher activity to reduce sulfide and control other heterotrophs that produced H_2S for successful RAW treatment, and then the effluent could be used as irrigation water with a biomass as by product of SCP although the essential amino acid profile was not high.

CHAPTER 6

CONCLUSION

The final chapter of this thesis concludes the findings of the research study by integrating the conclusions drawn from each chapter and identifying future research requirements. Finally, some general conclusions are drawn from the work as follows.

Based on BBD experiment, the addition of 2.0% FPE into RAW (COD, 2000 mg/L and pH 7.0) significantly decreased ORP value to obtain a reducing condition, and this with microaerobic light conditions stimulated PNSBsi growth to reach a maximum of 7.8 log CFU/mL within 2 days. Consequently, PNSBsi was used as the inoculum to treat RAW under microaerobic light conditions. According to CCD experiment, the optimal conditions were 7% PNSBsi, 0.8% FPE and 4 days retention time, and these conditions reduced 91% for COD, 75% for SS, and 61% for TtS. The amount of sulfide in RAW exceeded Thai standard guidelines (Pollution Control Department and Royal Irrigation Department) although H_2S was not been detected in the headspace. Compared with sets of abiotic control and treatment found that, H_2S was produced by HPC present in the RAW and also FPE; however, it was effectively deactivated by PNSBsi. The work carried out with indigenous PNSB raises the question of how to remove sulfide in RAW to meet Thai standard guidelines and this points out that a selected PNSB should be used for successful RAW treatment.

Like indigenous PNSB using as inoculum, *R. palustris* P1 was prepared by stimulating with FPE. The use of CCD experiment found that a combination of 3% PNSB P1, 0.9 % FPE and 4 day RT were the most suitable conditions for wastewater treatment under microaerobic light conditions. The removal of COD (initial COD 3,005 mg/L), SS, TtS and H_2S were 98, 79, 72 and 100%, respectively and there was only 4–8 % error in the verification test as resulted in the removal of COD (initial COD 2,742 mg/L), SS and TtS at 94 %, 75 % and 66 %

respectively. However, these values were less than the best set that obtained from the CCD experiment (2 % PNSB P1, 0.75 % FPE and 4 days RT) as this set reduced COD, SS and TtS by 96, 78 and 71%, respectively. The effluent of RAW treated by PNSB P1 met the Thai standard guidelines to use as irrigation water and a by-product from biomass could be considered as a source of SCP. The biomass collected from the effluent of PNSB P1 had 65 % protein, 3 % fat, 8 % carbohydrate, 14 % ash and 10% moisture. The results suggested that stimulated *R. palustris* P1 by FPE had the ability to compete with other organisms for achieving RAW treating under microaerobic light conditions. To understand why stimulated PNSB P1 inoculum showed a higher efficiency to treat RAW than that found in stimulated PNSBsi inoculum, bacterial communities between both RAW treatments should be investigated.

As mentioned above the finger print of dynamics of bacterial members including PNSB in RAW treated by inocula either PNSBsi or PNSB P1 and FPE were investigated using DGGE of nested-PCR-amplified fragments of the 16S rRNA gene. The results showed that diversity and evenness of bacterial population in PNSB P1 inoculum were significantly higher than the PNSBsi inoculum. Bacterial communities in both RAW treatment processes, at starting point (t = 0) in addition of PNSB population found 4 main bacterial populations belonging to 37.5% γ-proteobacteria, 25% ß-proteobacteria, 25% negativicutes and 12.5% flavobacteriia. Besides the 4 main bacterial groups; α-proteobacteria, clostridia and mollicutes were also detected in both communities later during the RAW treatment for 4 days. In addition of PNSB population other heterotrophs found in both RAW treatments were similar. However, for *R. palustris* observed in PNSB P1 matched to the strain P1 that differed from indigenous *R. palustris* in PNSBsi. This indicated that the strain P1 had a higher efficiency to treat RAW for producing the quality effluent that could be used as the irrigation water and by product of SCP although the essential amino acid profile was not high.

Overall the results demonstrated that the inoculum of stimulated *R. palustris* P1 with a little amount of FPE under microaerobic light conditions could be considered to be the appropriate technology for effective treating RAW with SCP as a

by-product in the treatment process. This appropriate technology should be scaled up prior to applying in the field.

Future research needs

There are many questions that remain unanswered and some specific suggestions that arising out of the current studies are given below.

1. RAW treatment by stimulated PNSB under light-dark cycle should be studied for applying in the wastewater treatment plants or at CRSFs.

2. RAW treatment by stimulated PNSB under sun light and night time should be studied for applying in the wastewater treatment plants or at CRSFs.

3. Any methods to reduce sulfide in RAW for increasing efficiency of PNSBsi should be investigated.

4. Removal of particulate matters in RAW should be studied in order to increase light penetration for higher efficiency of RAW treatment by PNSB.

5. Other metabolites of PNSB in the effluent such as 5-aminolevulinic acid (ALA), exopolymeric substances and plant hormone should be investigated for applying in agriculture.

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APPENDIX

A. Medium

GM medium

Sodium L- glutamate	3.8	g
DL-malic acid	2.7	g
Yeast extract	2.0	g
KH_2PO_4	0.5	g
$KHPO_4$	0.5	g
$(NH_4)_2HPO_4$	0.8	g
$MgSO_4.7H_2O$	0.2	g
$CaCl_2.\ 2H_2O$	0.053	g
$MnSO_4.5H_2O$	0.0012	g
$CoCl_2.6H_2O$	0.00095	g
Ferric Citrate	0.0025	g
Nicotinic acid	0.001	g
Thiamine hydrochloride	0.001	g
Biotin	0.00001	g
Deionized water	1000	ml
pH	6.8	

B. Report of Microbial Identification by 16S rDNA sequence analysis

LOCUS	JX876953 1331 bp DNA linear BCT 30-SEP-2013
DEFINITION	*Rhodopseudomonas palustris* strain P1 16S ribosomal RNA gene, partial sequence.
ACCESSION	JX876953
VERSION	JX876953.1
KEYWORDS	-
SOURCE	*Rhodopseudomonas palustris*
ORGANISM	*Rhodopseudomonas palustris* Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Bradyrhizobiaceae; *Rhodopseudomonas*.
REFERENCE	1 (bases 1 to 1331)
AUTHORS	Kornochalert,N.
TITLE	Use of fermented plant extract to enhance the growth of phototrophic bacteria for use in the treatment of latex rubber wastewater
JOURNAL	Kornochalert, N., Kantachote, D., Chaiprapat, S., and Techkarnjanaruk, S. 2014. Use of *Rhodopseudomonas palustris* P1 stimulated growth by fermented pineapple extract to treat latex rubber sheet wastewater to obtain single cell protein. Annals of Microbiology: 1-12.

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FEATURES Location/Qualifiers

source 1..1331

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/mol_type="genomic DNA"

/strain="P1"

/isolation_source="shrimp farm sediment"

/db_xref="taxon:1076"

/country="Thailand: Pattani Province"

rRNA <1..>1331

/product="16S ribosomal RNA"

BASE COUNT 329 a 317 c 412 g 273 t

ORIGIN

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121 cgccgaaaga tcggcccgcg tctgattagc tagttggtga ggtaatggct caccaaggcg

181 acgatcagta gctggtctga gaggatgatc agccacattg ggactgagac acggcccaaa

241 ctcctacggg aggcagcagt ggggaatatt ggacaatggg cgaaagcctg atccagccat

301 gccgcgtgag tgatgaaggc cctagggttg taaagctctt ttgtgcggga agataatgac

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541 gaggtgagtg gaactgcgag tgtagaggtg aaattcgtag atattcgcaa gaacaccagt

601 ggcgaaggcg gctcactggg ccattactga cgctgaggca cgaaagcgtg gggagcaaac

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