Chapter 1 Introduction

1.1 Introduction

The papermaking industry is the third largest water-consuming industry in the world and generate enormous amount of wastewater which contains a large amount of recalcitrant materials, such as cellulose and lignin. It also has high pH because lime is added during the production process. Although biological treatment processes have been proved to be more efficient and less costly than many physicochemical processes for treating the pulp and paper wastewater, these recalcitrant compounds along with high solution pH will seriously reduce the efficiency of almost all biological treatment processes presently used for treating pulp and paper wastewater, such as the low BOD but high COD in effluent due to the amount of nonbiodegradable compounds in the raw wastewater. The effluent may meet current pulp and papermaking effluent regulation but will not meet more stringent limitation in the future.

In addition to conventional activated sludge process, several biological treatment systems have been developed for treating wastewater to meet the US EPA secondary effluent limitation, e.g. rotating biological contactors (RBC), fixed media submerged biofilters, fluidized bed reactors and so on. These systems generally retain more microbial mass that is better acclimated to the wastewater being treated thus lead to

better efficiency than the conventional system. The moving bed biofilm reactor (MBBR) originated from Norway is popular used to treat a pulp and paper wastewater for many years to improve the COD and suspended solids so that the effluent may meet more stringent limitations. In this study, the Kaldnes MBBR system was used to treat secondary-fiber papermaking wastewater. Additional benefits of using this system include the elimination of sludge return and elimination of dead space in the reactor. Additionally, the biofilm was enriched with a pure thermophilic species to find out whether the COD removal efficiency can be improved under thermophilic conditions.

In addition to COD and SS removal efficiencies, changes of microbial communities in the biofilm in response to different operational temperatures were also studied. Molecular biotechnology, developed rapidly in recent two decades, has been proved to be a suitable and faster tool to study the whole microbial community. Temperature gradient gel electrophoresis (TGGE) and denaturing gradient gel electrophoresis (DGGE) are the most common two molecular methods for carrying out such study. Both methods are based on the electrophoresis mobility of DNA fragments to separate them in a linear gradient gels. The only difference between these two systems is the type of linear gradient, i.e. the temperature gradient vs. the denaturing gradient which depended on the mixture of urea and formamide. The

DGGE method is easier to conduct for monitoring the microbial communities of the biofilm at different operational temperatures.

1.2 Study Objectives

This study established a thermophilic biological treatment system, which was more and more emphasized in recent years, to treat the papermaking wastewater and a molecular biotechnology was used to analyze the microbial communities in the system. Kaldnes MBBR system was used to treat the wastewater from a secondary-fiber papermaking plant in Taiwan. Different operation temperatures (at ambient temperature, 40 ^{o}C, 50 ^{o}C and 60 ^{o}C) were used to investigate the system removal efficiency for COD, TS and MLVSS. A thermophilic microorganism, strain BL11 was added to the system to see if the COD removal efficiency could be increased and also was a target bacterium for DGGE analysis. Traditional phenol-chloroform extraction and two commercial DNA extraction kits were used in this study for DNA extraction to compare their efficiency. The microbial communities were analyzed using PCR-DGGE to investigate the richness and diversity of microbial communities under different operation temperatures.

Chapter 2 Literatures Review

2.1 Secondary-fiber papermaking industry

In 1983, the United Nations Conference on Trade and Development (UNCTD) passed the International Tropical Timber Agreement (ITTA) to express concern to preserving forest resources (Huang *et al*., 1996). The globally, the pulp and paper industry, one of the major consumers of the forest resources, responded by initiating policies and measures to recover and recycled waste paper to cut down the consumption of natural resources. For recommending resource recovery policy, The Taiwan Environmental Protection Administration also implemented the policy requiring that all governmental offices use 60% of paper from secondary-fiber materials for office paper, toilet paper and packing paper. Hence, more and more paper mills use secondary-fiber paper instead of wood as the raw material. In addition to preserving natural resources, another benefit of using secondary-fiber materials is the reduction of the wastewater strength and water usage because wood pulping produces much more fiber, lignin, black liquid and various kinds of organic matters, but former needs more bleach chemical reagent to remove the dyestuff and printing ink, which results in release of heavy metals and precipitating in the sludge (Thompson *et al*., 2001). Recycled paper as raw materials for pulping has the advantage of releasing the fiber in the liquid and maintains homogeneous, but the

adhesive contaminants (increasing with the ratio of recycled paper in the raw materials) produced in the papermaking process affect both the paper quality and the operation of machine (Chai *et al*., 2006). Generally speaking, 60.0 kg of BOD are generated to produce1 ton of paper pulp generated along with 100.0 L of water used (Pokhrel and Viraraghavan, 2004). If using secondary-fiber paper for pulping, one-third of BOD can be reduced and only 15.0 L of water is needed. Besides, the secondary-fiber paper contains more short fiber, leading to lower stock consistency and higher fractionation to achieve savings in energy consumption (Grosmann and Salmén, 1997). The secondary-fiber paper was first cut up into small pieces; chemicals are then added to soften the paper. After flotation followed by straining, de-colorization and sieving, clean pulp is obtained that is ready for making new paper. The wastewater of this process must meet the limitations set by Taiwan EPA as 180.0 mg/L for COD and 6.0 to 9.0 for pH. Although using secondary-fiber paper for pulping can help to save the forest, it still has some limitations. For example, repeated recycling for pulping will consume more energy and more chemicals are needed.

2.2 The moving bed biofilm reactor system (MBBR)

The bio-treatment technology played an important role in pollution removal from

wastewater and development of biological treatment technology never stops. There are two major types of reactor systems, based on whether the bacterial growth in the reactor is suspended or fixed. The fixed system is generally superior to the suspended growth system in that it is capable of consuming less energy maintaining higher biomass and producing less sludge, so that it is easier and more cost effective to operate including better recovery from system shock (Loukidou and Zouboulis, 2001; Roders and Zhan, 2003). Additionally, simultaneous aerobic and anaerobic conditions can be maintained in the fixed microbial mass. The attached system, also known as the biofilm process, can be further classified as fixed-medium and moving-medium system. The former includes trickling filters and biological aerated filters, in which the biofilm is attached on immobile solid supporting media (Lu *et al*., 2004). In contrast, the fixed growth system may also allow the microbial mass to grown on a moving supporting medium. The moving-medium system is more likely to be free from clogging because the biofilm thickness can be controlled by adjusting the hydraulic shear force. In the conventional moving-medium system, e.g. the rotating biological contactor (RBC), the supporting medium is a set of rotating disks that are driven by mechanical force to move the microbial film grown on the disk surface. In the recently developed moving-medium system, e.g. moving bed biofilm reactor (MBBR), vertically moving biofilm reactor (VMBR) and the fluidized-bed

reactor (FBR), the microbial film is fixed on the surface of small suspended carriers which can be suspended in the liquid by mixing or rising air bubbles. Others advantages of the moving-medium process include more volume-effective, better mass and oxygen transfer efficiency and no backwashing needed (Rusten *et al*., 2006). Among the many moving-medium processes, the MBBR system has some unique characteristics that make it to other type of moving-medium process. Because of the carriers suspended in the reactor arising the retention time of the oxygen gas, made a better oxygen transmission efficiency than the activated sludge aerator (Yang *et al*., 2004).

2.2.1 Kaldnes MBBR system

One popular moving bed system is the Kaldnes moving bed system, which was developed in Norway by Kaldnes MiljØeknologi company. The Kaldnes system is widely used in treating industry wastewater or domestic sewage under aerobic or anaerobic conditions; several types of Kaldnes carriers (Fig. 2.1) are developed for treating wastewaters of various characteristics. Their characteristics are shown in Table 2.1 (Rusten *et al*., 2006). In this study, the original Kaldnes K1 type carrier was used. Under aerobic conditions, the carriers are moved and mixed in the reactor through aeration with rising bubbles. If anaerobic conditions are required, the

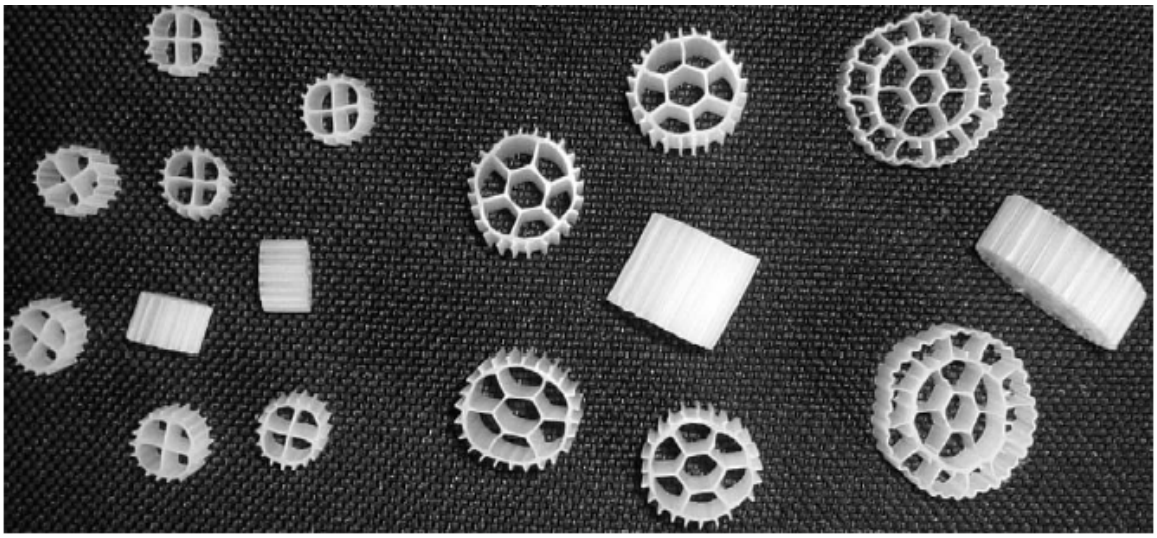


Fig. 2.1. Different kinds of Kaldnes biofilm carrier. From left to right: K1, K2, K3 type Kaldnes carrier.

Table 2.1 Data of different types of Kaldnes biofilm carriers

	Type of the Kaldnes moving bed biofilm reactor		
	K1	K2	K3
Material	Polyethylene (PE)		
Nominal diameter (mm)	9.1	15	25
Nominal length (mm)	7.2	15	12
Bulk density (kg/m^3)	150	95	100
Specific biofilm surface area (in bulk) (m^2/m^3)	500	350	500
Specific biofilm surface area at 60% fill (m^2/m^3)	300	210	300

(Rusten *et al*., 2006)

carriers are mixed with mechanical force (Fig. 2.2). The maximum filling ratio for carrier to move freely in the reactor is 70% such that the efficient biofilm growth area, calculated from the inside surface of carriers, is about 350.0 m^2/m^3. Because the outside surface is often made smooth; it cannot effectively support microbial growth thus using the carrier inside surface will be conservative in estimating the microbial mass (Rusten *et al*., 1997). The MBBR system can be satisfactorily operated with 60.0 kg COD/m^3d and has also been proved to be able to operate at high temperature up to 50 ^oC (Dalentoft and Thulin, 1997).

2.3 Thermophilic treatment system

A thermophilic biological treatment system is often operated at temperature equal or above 45^oC (LaPara and Alleman, 1999). Since the pulp and paper wastewater has a high temperature, it is suitable to be treated in a thermophilic biological treatment (Jahren and Ødegaard, 1999). Generally speaking, the thermophilic system has higher decay rate and lower sludge yield (Kim *et al*., 2002) than mesophilic system. In conventional wastewater treatment, the thermophilic process is often limited to anaerobic digestion of concentration waste such as the waste activated sludge. In recent years, aerobic thermophilic processes have been developed into practical method for treating a variety of wastewaters. Due to the

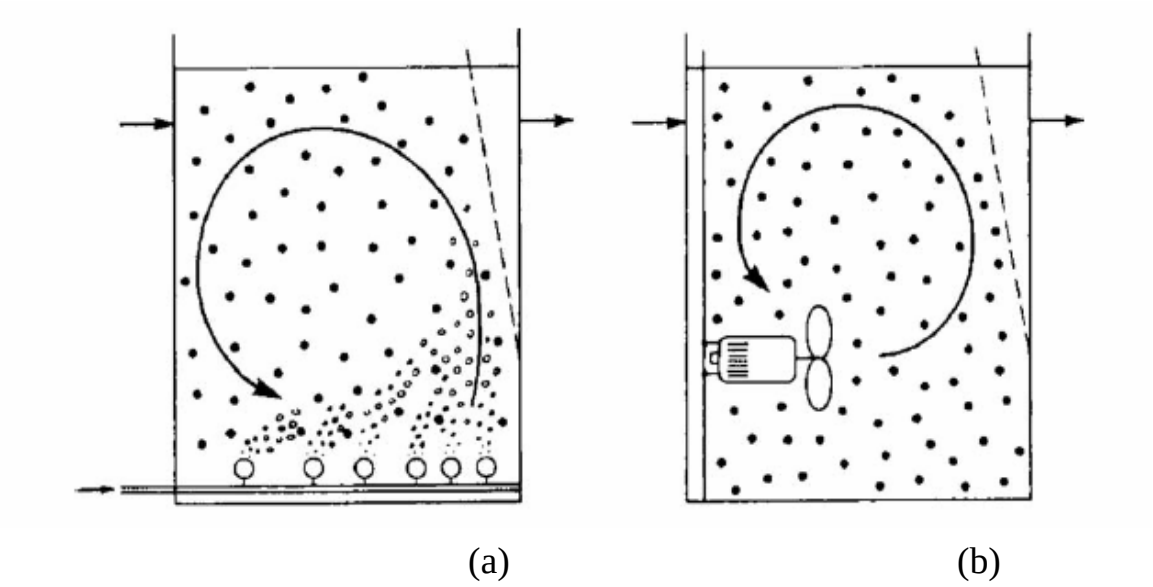


Fig. 2.2. Principle of Kaldnes moving bed biofilm reactor. (a) operation under aerobic condition, (b) operation under anaerobic condition.

higher temperature and lower sludge age, the thermophilic system exhibit faster reaction rate and produces smaller quantity of excess sludge than the mesophilic system (Suvilampi *et al*., 2003a). However, this system has a major draw back in that if used in the suspended growth, the bacterial mass cannot be effectively agglomerate into settleable flocs thus the sludge is difficult to be separated from the effluent. The poor solid-liquid separation often causes excessive quantities of suspended solids and organic matter in the treated effluent. Other researches indicate (Suvilampi *et al*., 2005; 2003b) that aerobic thermophilic process results in effluent with lower discharge quality which contains higher amount of dispersed particles than mesophilic process dose in COD concentration and turbidity due to the bed sludge setting under thermophilic condition. Therefore combined thermophilic-mesophilic system has been developed to remove soluble COD under thermophilic condition and decreasing total and colloidal COD residual concentration to improve the effluent quality during mesophilic period (Kurian *et al*., 2005; Tripathi and Allen, 1999). However, some other studies indicate that the poor sludge settling of thermophilic process is resulted from low sludge ages and high loading rate than higher operation temperature (Vogelaar *et al*., 2002). Another studies recommend when using the thermophilic aerobic biofilm processes the high loading rate and short hydraulic retention times (HRTs) can be controlled in order to maintain system stabilization due to the higher metabolism rate in the thermophilic system (Tiirola *et*

al., 2003). In recent years, more and more specific thermophilic bacteria were isolated from hot spring, high temperature wastewater, compost and so on. Combined with MBBR system, thermophilic bacteria can arise the capability of resistance from environment shock, this leads a potential to use of thermophilic pure culture in the wastewater treatment to improve the quality of effluent.

2.4 Microbial community analysis on biofilm

Traditional methods, i.e. examining the morphology, movement, physiological and chemical property, culture conditions, chemical composed if the incubated bacterial colonies from the isolated culture, to conduct the microbial community analyses on the biofilm are tedious and time-consuming. Unfortunately, almost 99% bacterial species cannot be cultured successfully in synthetic media. The merging molecular biotechnology, e.g. fluorescence in situ hybridization (FISH), restriction fragment length polymorphism (RFLP), temperature gradient gel electrophoresis (TGGE), denaturing gradient gel electrophoresis (DGGE) etc., become available for microbial community analyses. Both FISH and RFLP utilize specific probes to mark or cut on the DNA fragments to identify the different bacterial species. However, they are not capable of rapidly detecting changes of the microbial communities studied let alone the disadvantage of being costly. The electrophoresis is a

phenomenon of the DNA fragments migrated from negative to positive in a gel subject to an electrical field. The difference in mobility was based on to different DNA fragments. The distribution of the migrated DNA fragments for a mixed culture in the electrophoresis gel is defined as the "community fingerprinting", which was a basis to compare the diversity between two unknown samples. Generally speaking, each band in the DGGE gels that contributes to the richness and diversity of the community species is defined as the operational taxonomic unit (OTU) (Gafan and Spratt, 2005). In either the TGGE or DGGE method DNA fragments of different sizes are separated in a linear gradient gels based on their decreasing electrophoresis mobility. The only difference between these two systems is the type of linear gradient; TGGE uses the temperature gradient created with two water baths while DGGE uses the denaturing gradient depending on the mixture of urea and formamide. TGGE and DGGE have the advantages of simultaneous comparing the different operation conditions in the same and being inexpensive. The problem of co-migration is often experienced in the TGGE and DGGE methods. Hence, the OTU that contains more than one DNA fragment is removed and subject to additional electrophoresis analysis using the FISH and RFLP methods to solve the problem of co-migration biofilm (Muyzer, 1999). In this study, the DGGE method was using to analysis the microbial community on biofilm.

The microbial communities were first used as the bases for observing the the biofilm development on the carrier surface by Muyzer *et al*. (1993). The results show that DGGE is a powerful tool for rapidly observing the diversity microbial communities or the mutant bacteria because a large number of samples can be compared simultaneously to observe the time- or space-dependent changes (Mette and Ramsing, 2002). In recent years, the DGGE technology coupled with PCR to amplify DNA fragments becomes a popularly application for studying soils and sediments, plankton of lake and sea, and both biofilm and suspended broth (Lyautey *et al*., 2005). The principle of DGGE was based on adding 30 to 50 of GC-clamps, which are composed of G or C base pairs, into one-side of primer to prevent the double-stranded DNA from becoming single-stranded DNA by the denature gradient that may decrease the sensitivity of the diversity. The amount of hydrogen bond between A and T base pair was two, where in G and C was three. Each microbial community has a unique different base composition, especially the content of G and C; adding urea will interrupt the bonding between G and C bases thus causing a different denature level in the polyacrylamide gels. According to its melting behavior, DGGE can separate only the denatured DNA fragments, according to their GC content in a linear gradient gel (Fig 2.3) shows the DNA fragment migration in the DGGE gels; the GC clamp was opened by urea with increasing denaturant gradient. The OTUs

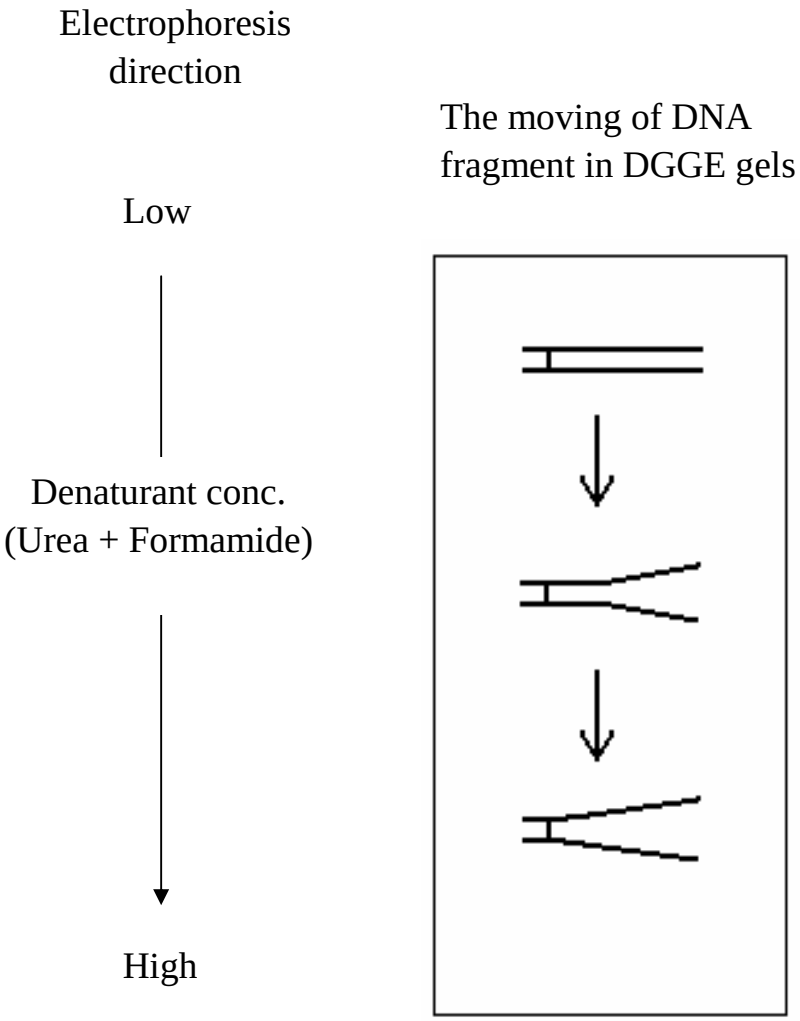


Fig. 2.3. Principle of denaturing gradient gel electrophoresis.

appearing near the bottom of the DGGE gel represented the higher GC content. The profiles of DNA bands in DGGE gels can be visualized under UV transillumination after stained with ethidium bromide or SYBR Green I; the latter has the higher sensitivity but is costly (Muyzer and Smalla, 1998).

Chapter 3 Materials and Methods

3.1 Experimental Design

In this study, a bench-scale Kaldnes MBBR system was built in which carriers developed by the Norwegian Kaldnes MiljØeknologi company was used and was fed with real wastewater. The wastewater was obtained from a secondary-fiber papermaking plant in Taiwan. For environmental consciousness and waste re-use, recycled paper was used as raw material for pulping in this factory. A thermophilic bacterium which preliminarily identified as *Paenibacillus sp.* BL11 was adding into the reactor to increase the COD removal efficiency and also for an index to investigate the feasibility of using denaturing gradient gel electrophoresis (DGGE) to study the difference in microbial communities between biofilm and suspended mixed liquor in MBBR. COD of influent and effluent was measured daily, while MLSS and MLVSS were measured every two days. The microbial characteristics of the biofilm under different operational temperatures were studied by using DGGE of the PCR-amplified bacterial 16S rDNAs. The flow chart of experimental design is shown in Fig. 3.1.

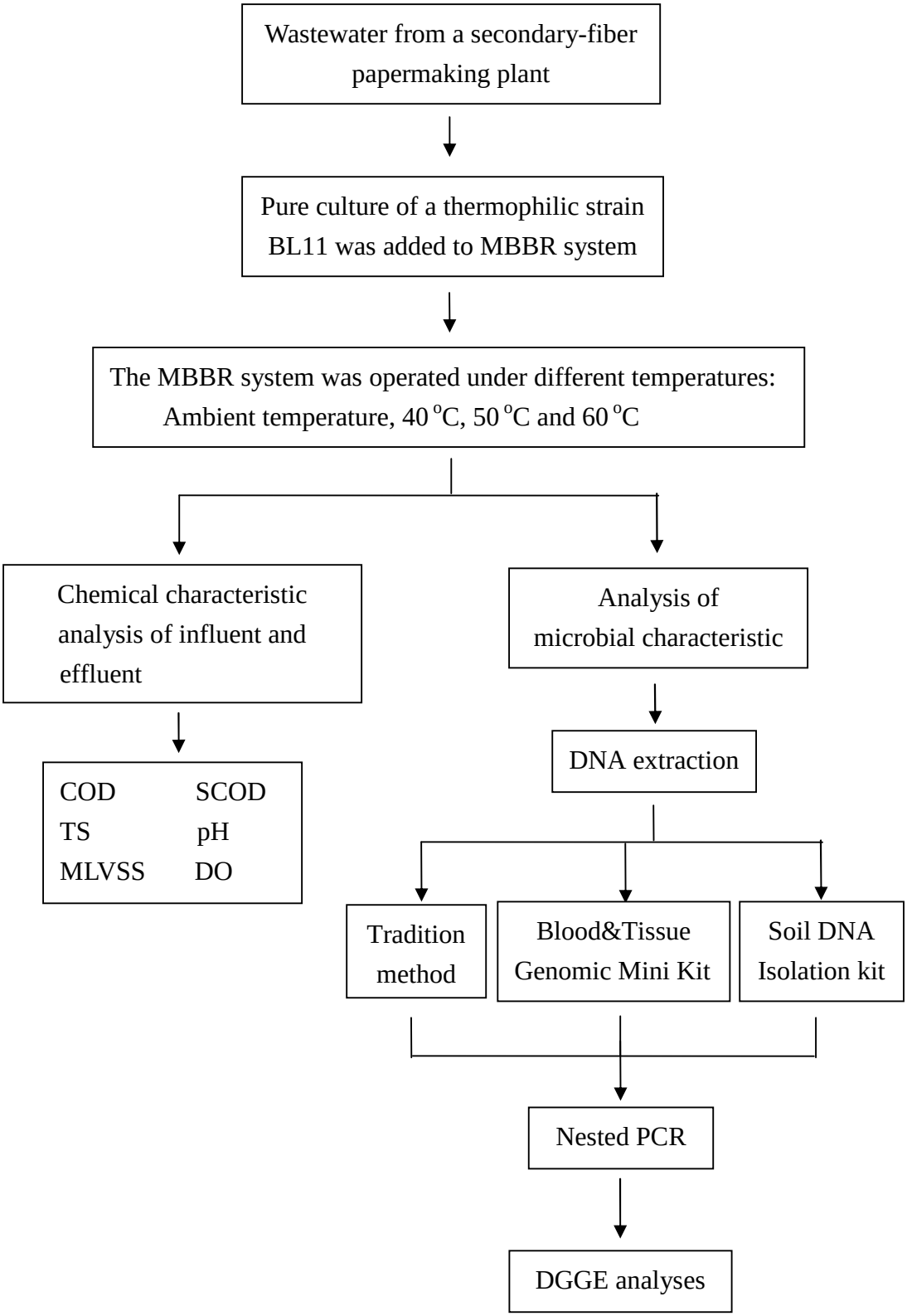


Fig. 3.1. Flow chart of experimental design.

3.2 Influent and seed sludge of the MBBR

The influent and seed sludge was obtained from real secondary-fiber papermaking wastewater. Wastewater was collected from the equalization tank of the wastewater treatment plant of the paper mill. pH, dissolve oxygen and temperature of the collected wastewater were measured immediately after sampling in each sampling period. The wastewater was collected in 20.0 L collection bucket and stored in 4 ^{o}C before used.

3.3 Inoculum

Strain BL11 is a thermophilic bacterium isolated from black liquor by the School of Forestry and Resource Conservation, National Taiwan University. The organism was able to grow at 25 ^{o}C to 60 ^{o}C and pH ranging from 4.5 to pH 8.5. The optimal growth temperature was found to be 55 ^{o}C, which the optimum pH was 7.0 (Ko *et al*., 2006). Strain BL11 was grown in LB medium at 28 ^{o}C for 72 hrs or 37 ^{o}C for 22 hrs to reach an OD_{600} of 0.50 before inoculating the MBBR system. Strain BL11 was able to utilize sterilized raw papermaking wastewater for growth on the spread plate test.

3.4 MBBR reactor

A laboratory scale MBBR with a total liquid volume of 34.5 L was used in this study (Fig. 3.2.). The reactor was divided into two parts; the first part (the carrier reactor, 4.5 L) was filled with 10% Kaldnes carrier (Fig. 3.3.), where the second part of reactor (13.0 L) was conventional aeration tank. The effluent flow rate was controlled in 30.0 L/day and the sludge return rate was 15.0 L/day. The reactor was kept in ambient temperature. The feed wastewater was mixed in a feed tank and the temperature of the wastewater was controlled with a submerged heating coil. The Kaldnes carrier was circulated within the bioreactor forced by aeration. The sludge was discharged every day to avoid clogging the primary clarifier. The DO concentration in carriers reactor was controlled at 2.5 to 3.0 mg/L and at 2.0 to 2.5 mg/L in activated sludge tank (Aeration pump: Medo Co., Japen; the max output air flow rate was 14.0 L/min and working air flow rate is controlled by flow meter). The temperature of fed wastewater was controlled at ambient temperature, 40 ^{o}C, 50 ^{o}C or 60 ^{o}C to observe the different performance of the MBBR system in different temperatures.

The size of primary clarifier and secondary clarifier were the same and were made of plastics with cone shape. Working heights of both clarifiers were 38.0 cm; diameter was 22.0 cm and total working volume was 8.5 L. The surface overflow

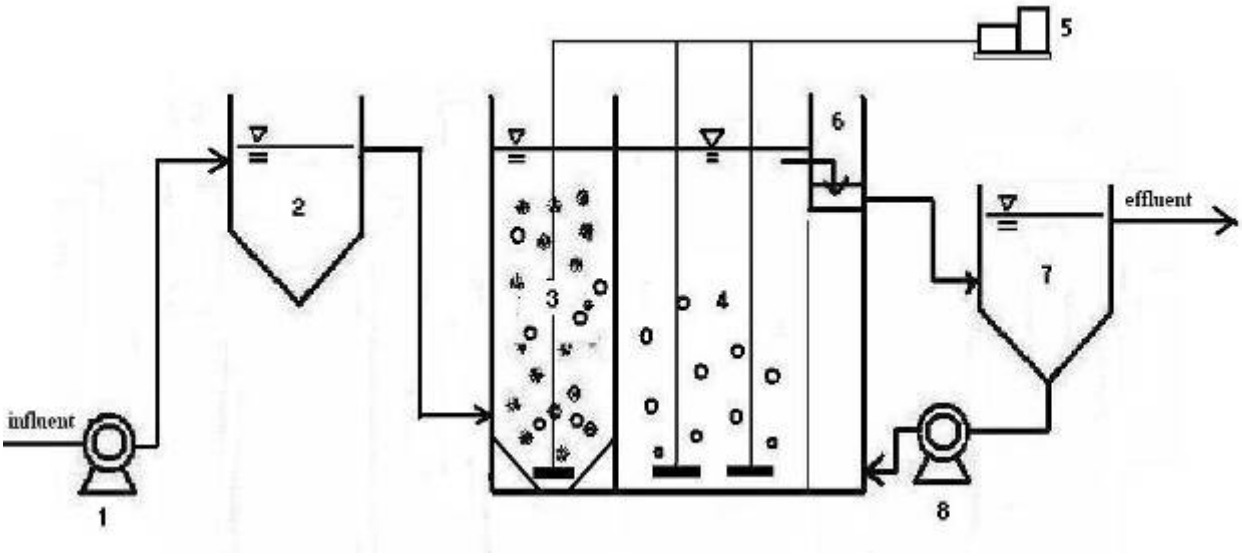


Fig. 3.2. Schematic diagram of experimental set-up. 1.feed pump; 2.primary clarifier; 3.carriers reactor; 4.activated sludge tank; 5.aeration pump; 6.spillweir; 7.secondary clarifier; 8.sludge return pump.

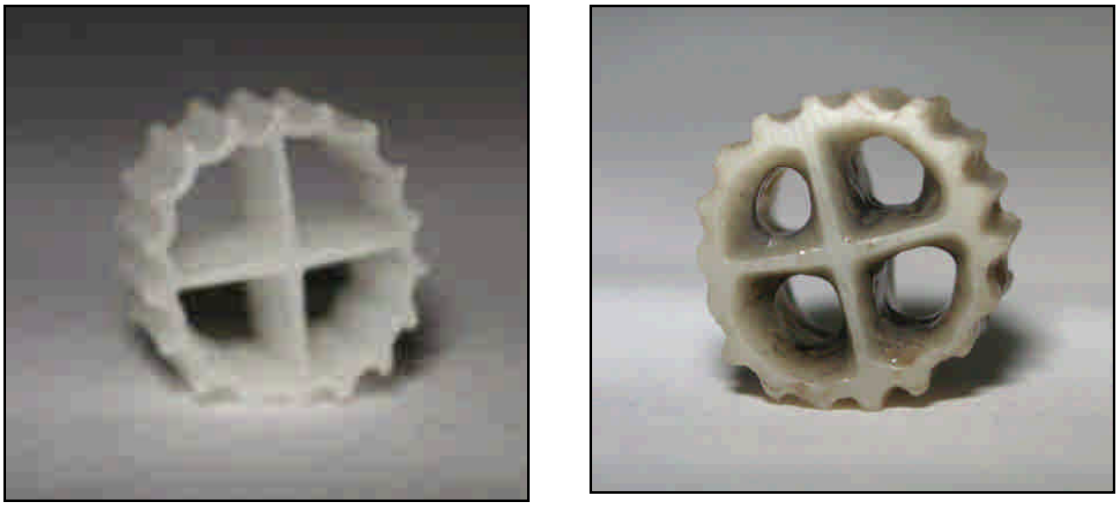


Fig. 3.3. Photo of Kaldnes K1 carrier. (a) new, (b) used.

rate (SOR) of clarifier was 1.34 m^3/m^2d. Carriers reactor and aerated reactor were made of acrylic fiber. Both of the reactors had same working height of 50.0 cm. The internal size of carrier reactor was 10.0 cm × 10.0 cm and 10.0 cm × 30.0 cm for the aerated reactor (included spillweir) with working volume of 4.5 L and 13.0 L, respectively. The carriers reactor was filled with 10% Kaldnes K1 bead (minimum limit). Kaldnes biofilm carriers K1 bead was made of polyethylene (PE). The data for the carrier were listed in Table 3.1. The spillweir was made of acrylic fiber. The working height is 15.0 cm, the internal diameters is 10.0 cm × 5.0 cm with working volume as 0.1 L. The hydraulic retention time (HRT) was 6.8 hrs in primary clarifier and secondary clarifier, 3.6 hrs in carriers reactor, 10.4 hrs in activated sludge tank (AST) and 0.08 hr in spillweir. Total HRT in this system was 1.15 day.

pH value of the mixed liquor was measured with pH meter (Suntex TS-2, Taiwan), combined with pH controller and glass membrane electrode (American Bantex Corp, USA). Electrode was calibrated with pH 4.0 and pH 7.0 standard solution before use. Concentration of dissolved oxygen was measured by dissolved oxygen meter (Oxi 315i) equipped with WTW CellOx 325 DO electrode (made in Germany) and was calibrated with internal aero-correction program.

Table 3.1 Data of K1 type Kaldnes biofilm carrier

Type	K1
Material	polyethylene (PE)
Nominal diameter (mm)	9.1
Nominal length (mm)	7.2
Density (g/cm^3)	0.95
Specific biofilm surface area (in bulk) (m^2/m^3)	500
Specific biofilm surface area at 60% fill (m^2/m^3)	300

(Rusten *et al*., 2006)

3.5 Experiment set-up

In started-up period, before adding strain BL11, the MBBR system was fed with raw wastewater and changes of COD, SCOD, TS and MLVSS concentration in the MBBR system were measured until the system was stabilized (the ratio of MLVSS and TS in both influent and effluent were about equal). The morphology of the microorganisms on the carrier was observed with a phase-contrast microscope equipped with a digital camera (Olympus BX40, Japan).

After started-up period, the study was separated for two sections. In first section, the affection of thermophilic strain BL11 added in COD removal efficiency was compared at 40 ^{o}C which was a suitable growth temperature for strain BL11. The experiment could be divided to three stages in first section, in first stage the reactor was operated under original without strain BL11, where as the incubated strain BL11 was directly added in the reactor with 1.0 L volume of medium, which had incubated at 37 ^{o}C for 18 hrs, during the second stage. The affected factor of DO controlled was observed in final stage where the DO concentration controlled at 2.0-3.0 mg/L.

In second section, the affected of different operated temperatures, ambient temperature, 40 ^{o}C, 50 ^{o}C and 60 ^{o}C were compared. During the ambient temperature a thermophilic bacteria strain BL11 was immobilized onto the Kaldnes

carriers, then these carriers were added to the reactor. The experiment was divided to four parts, which the MBBR reactor was run at ambient temperature in the first period.

3.6 Analytical methods

pH, DO, COD, TS and MLVSS of both influent and effluent were measured or analyzed every day during the operational period. Both pH and DO were measured from the reactor. COD, TS and MLVSS were determined according to standard analytical methods published by the Environment Analysis Laboratory, EPA, Executive Yuan, R.O.C. (Table 3.2). The samples were centrifuged at 1,500 rpm for 15 minutes and filtered through the 0.45 μm filter membrane for SCOD analysis. Samples were stored at 4 ^{o}C and analyzed within 7 days.

3.7 Microbial community analysis of the biofilm

3.7.1 DNA extraction methods

The biofilm on the carrier surface and microorganisms in the mixed liquor were collected for microbial community analysis under different operation temperatures. In this study, bacterial DNA in the biofilm was extracted using conventional phenol-chloroform extraction and two commercial DNA extraction kits,

Table 3.2 Analytical methods used in this study

Item	**Analytical methods**
pH	pH meter
DO	DO meter
COD	NIEA W515.53A
TS	NIEA W210.56A
MLVSS	*Standard Method 2540 E

*Based on American Public Health Association Standard Methods for the examination of waster and wastewater, 19th Edition, 1995.

Blood&Tissue Genomic mini-Prep (Viogen, R.O.C) and Soil DNA isolation kit (MOBIO, USA) to compare their efficiency on DNA extraction. All of the samples for DNA extraction were first pre-treatment washed with sterilized water to wash out and to eliminate residual substrate and retaining the cell pellet. 0.28 g to 0.32 g of biofilm pellet was collected in 1.5 mL eppendorf. After centrifuging at 13,000 rpm for 5 minutes, the supernatant was discarded and 1.0 mL of sterilized water was added to suspend the cell pellet. The same procedure was repeated at least twice times in order to avoid the interference from substrates in mixed liquor.

3.7.1.1 Method I: Traditional phenol-chloroform DNA extraction

After sample wash process, each tube added 500.0 μl of solution I which placed at 4 ^{o}C before used. The components of solution I were 50.0 mM glucose, 25.0 mM Tris-HCl (pH = 8.0), 10.0 mM EDTA (pH = 8.0) and 0.02 g/mL lysozyme. Cells were resuspended and were sit for 30 minutes at 37 ^{o}C for cell wall breakage. Then, add 25.0 μl of 20% SDS and gentle vortex for 1 minute to lyse the cells. 20.0 μl of proteinase K (20.0 mg/mL) and 10.0 μl of RNase (50.0 mg/mL) were added to the mixture and the eppendorf was invented several times, then incubated at 60 ^{o}C for 30 minutes and vortex briefly every 5 minutes. Following three consecutive freeze-threw cycles at -70 ^{o}C and 60 ^{o}C, each steps for 10 minutes to break the cell completely. Centrifuge at 13,000 rpm for 5 minutes

to remove cell debris. Discard the pellet and transfer the supernatant to a new tube, the volume of supernatant was measured. Equal volume of 1:1(v:v) of phenol/chloroform-Isoamyl alcohol was added to the supernatant. Inverted several times to mix up throughoutly and centrifuge at 13,000 rpm for 5 minutes. Transfer the supernatant to a new tube. Add 1:1(v:v) of chloroform-Isoamyl alcohol. Inverted several times and centrifuged at 13,000 rpm for 5 minutes. Transfer the supernatant to a new tube. This step was repeated to remove the superfluous phenol. Add 2.5-time volume of 100% ethyl alcohol (kept at -20 ^{o}C before used) to the tube and inverted several times then centrifuge at 13,000 rpm for 5 minutes. Discard the supernatant, air-dried the DNA pellet. 50.0 to 100.0 μl of 70 ^{o}C sterilized water were added to dissolve DNA. Stored the genomic DNA solution at -20 ^{o}C.

Extracted genomic DNA was further purified by following procedures: Added equal volume of isopropanol and inverted several times. After incubating at -70 ^{o}C for 1 hour, centrifuge at 13,000 rpm for 15 minutes. Carefully remove the isopropanol and add 1.0 mL 70% ethanol to wash the pellet. After removing the 70% ethanol air-dried the DNA pellet. Add 50.0 μl of 70 ^{o}C sterilized water, then stored the genomic DNA solution at -20 ^{o}C.

3.7.1.2 Method II: Viogene Genomic DNA Miniprep Kit for Blood&Tissue

Bacterial DNA was extracted according to the user's guide and was summarized as the following: After sample during pre-treatment process, adding 200.0 μl of 20.0 mg/mL lysozyme buffer. The lysozyme was dissolved in TE buffer, the components of TE buffer were 10mM Tris-HCl and 1.0 mM EDTA, both of the solution was mark with pH 8.0. Suspend the pellet then adding 5.0 μl of RNase A (50.0 mg/mL) and the mixture was incubated at 37 ^{o}C for 30 minutes, vortex briefly once per 10 minutes. Then, 20.0 μl of proteinase K (10.0 mg/mL) and 200.0 μl of Buffer EX were added which included in the kit, vortex for 20 seconds. Put the mixture incubated at 60 ^{o}C for 30 minutes and vortex briefly every 5 minutes until the liquor clear. Next, incubated the upper tube at 70 ^{o}C for 30 minutes to remove the activity of proteinase K. After incubated at 70 ^{o}C, let the sample back to ambient temperature. Then, adding -20 ^{o}C 210.0 μl of 100% ethyl alcohol and invert several times. Connecting genomic DNA column onto collection tube, adding DNA sample in genomic DNA column. Centrifuge at 6,000 × g for 2 minutes. Discard the filtrate and re-put the genomic DNA column in collection tube. 500.0 μl of Wash Buffer (contain 98% ethanol) was added in Genomic DNA column. Centrifuge at 5,200 × g for 5 minutes. Discarded the filtrate and repeat this step. Follow upper step, put the empty

column in collection tube and centrifuge at maximum rotational speed to removal the residual ethanol in column. Put the column in new collection tube, then adding 200.0 μl pre-heated 70 ^{o}C of sterilized water. After placing around 2 minutes, centrifuge at 7,000 × g for 2 minutes. Store the genomic DNA solution at -20 ^{o}C.

3.7.1.3 Method III: UltraClean Soil DNA Isolation kit

Bacterial DNA was extracted according to the manufacturer instruction booklet and was summarized as the following: 0.28 g to 0.32 g pellet was added in Bead Solution tube and gently vortex to mix. Then, 60.0 μl of Solution S1 (contain SDS) and 200.0 μl of Solution IRS (Inhibitor Removal Solution) was added, vortex briefly in each added process. Secure bead tubes horizontally on a flat-bed with adhesive tape and vortex at maximum speed for 10 minutes. Centrifuge tubes at 10,000 × g for 30 seconds. Transfer the supernatant to a new microcentrifuge tube. 250.0 μl Solution S2 (contain a protein precipitation) was added and vortex for 5 seconds. After incubating the tube at 4 ^{o}C for 5 minutes, centrifuging the tube at 10,000 × g for 1 minute. Avoiding the sediment, transfer supernatant to a new microcentrifuge tube. Add 1.3 mL of Solution S3 (DNA binding salt solution) and vortex for 5 seconds. Load the liquor onto a spin filter

and centrifuge at 10,000 × g for 1 minute, repeated until all liquor has passed through the spin filter, each time add approximately 700.0 μl. Then, add 300.0 μl of Solution S4 (100% ethanol) and centrifuge for 30 seconds at 10,000 × g. Discard the filtrate and centrifuge again for 1 minute to avoid the Solution S4 remain onto the spin filter. Final, added 50.0 μl of Solution S5 (no EDTA) to the center of the filter membrane and centrifuge for 30 seconds. Discard the spin filter and preserve the genomic DNA solution at -20 ^{o}C.

The main differences of the three DNA extraction methods are summarized in Table 3.3.

3.7.2 DNA concentration and purity analysis

The extracted DNA concentration and purity analysis were measured with the UV absorption of the solution at 260 nm and 280 nm. Absorption at UV 260 nm measured the concentration of double stranded DNA, while absorption at 280 nm measured concentration of protein. The ratio of A_{260}/A_{280} can be used to illustrate the DNA purity in the solution. Generally speaking, the better purity ratio ranged from 1.5 to 1.9. The concentration of double stranded DNA was calculated by using the formula:

Table 3.3 Main steps of three DNA extraction methods used in this study

Methodology	Tradition	Blood & Tissue kit	Soil DNA kit
Cell lysis	Proteinase K Lysozyme SDS RNase Freeze-thawing	Proteinase K Lysozyme SDS RNase	Glass bead SDS Protein precipitation
DNA purification	Phenol, chloroform Isoamyl-alcohol, 100% alcohol	Genomic DNA column 98% alcohol	Spin filter 100% alcohol Tris (pH=8)

A_{260} of 1.0 = 50 μg/mL of double stranded DNA

Concentration of DNA (μg/mL) = A_{260} * 50 μg/mL * diluting multiple

3.7.3 Polymerase Chain Reaction, PCR

Polymerase Chain Reaction (PCR) was used for amplified the identical of genomic DNA. The whole process was divided into three parts with denaturing, annealing and extension. In denaturing period, the double stranded DNA was separated into two single stranded and the heating temperature usually used at 92 ^{o}C -96 ^{o}C. Then the primers were bound with the two short stretches of sequence, the region which utilized the characteristics of primers in annealing period with temperature ranged from 37 ^{o}C -65 ^{o}C. Expected to the higher species-specific, the selection of primers or genetic fingerprinting was shown significance (Fasoli *et al*., 2003). Utilized the characteristics of primers, polymerase, dNTP (components with dATP, dTTP, dGTP and dCTP) and temperatures control can amplify the fragment in which we wanted to observe (Liaw, 2004). Final part of PCR was extension usually heating at 72 ^{o}C, where template was used to extend to the bound sequence (Pritchard *et al*., 2005). The PCR has advantages on maintain the completeness of microbial communities and without cloning previous separation, because of the PCR can also amplified the dead cells which can visually as species

bands on the DGGE gels (Cocolin *et al*., 2002). The nested-PCR technique was necessary to use for amplify the most variable regions of 16S rDNA gene targeting V3-V5 region, because of the size limited of DNA fragments for DGGE. The primers used for nested PCR in this study are 341fGC: 5'-CGC CCG CCG CGC GCG GCC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3' and 926r: 5'-CCG TCA ATT CTT TGA GTT T-3' (Ritchie and Myrold., 1999). The chemical reagents for PCR were shown in Table 3.4 and the heating program of PCR was shown in Table 3.5.

Single DNA band on agarose gel electrophoresis indicated the optimal PCR conditions. The agarose electrophoresis was run in 1X TAE buffer. Components of 50X concentrated TAE buffer was listed in Table 3.6. After electrophoresis the gel was dyed by Ethidium Bromide (EtBr) for 15 minutes and observed under UV light. The components of agarose electrophoresis are listing in Table 3.7.

3.7.4 Denature Gradient Gel Electrophoresis, DGGE

DGGE technique was used to analyze microbial community in both biofilm and suspended broth. Each kind of microbial community has different base composition, especially the content of G and C. DGGE can separate DNA

Table 3.4 Chemical components for PCR with total volume of 50.0 μl

Components	**Volume**	**Final Concentration**	**Source**
10X PCR buffer	5.0 μL	1X (contain 20.0 mM Mg^{2+})	PROTECH
dNTP (10.0 mM)	1.0 μL	200.0 μM	FINNZYMES
341fGC (5.0 μM)	2.0 μL	0.5 μM	MEG-biotech
926r (5.0 μM)	2.0 μL	0.5 μM	MEG-biotech
Tag DNA Polymerase (2.0 U/μL)	1.0-2.0 μL	2.0-4.0 U/μL	PROTECH
Template DNA	--	< 500.0 ng	
Distilled water	--	--	
Total Volume	50.0 μL		

Table 3.5 Heating program of PCR in this study

Temperature	Time	Cycle numbers	Reactions
94 ^{o}C	10 min	1	Activation
94 ^{o}C	30 sec.	30	Denaturation
55 ^{o}C -61 ^{o}C	30 sec.	30	Annealing
72 ^{o}C	20 sec.	30	Extension
72 ^{o}C	2 min	1	Final extension

Table 3.6 Reagent and concentration of stocked 50X TAE buffer

Item	**Amount**	**Final concentration**
Tris base	242.0 g	2.0 M
Acetic acid, glacial	57.1 mL	1.0 M
0.5 M EDTA (pH=8.0)	100.0 mL	50.0 mM
dH_2O	To 1000.0 mL	--

Table 3.7 Components of agarose electrophoresis

Item	**Components**	**Source**
Agarose gel	1.5 g/ 100.0 mL 1X TAE buffer	Sigma
Gel-6X loading dye buffer	--	Yeastern Biotech
Electrophoresis buffer	1X TAE buffer	--
Dye reagent (EtBr)	200.0 µl / 1.0 L 1X TAE buffer (25.0 mg EtBr/ 1.5 mL DH_2O)	Sigma

fragments with different GC content from lower to higher in proper order. Each size of the base pair had the optimal perform with different gel percentage, the relationship was shown in Table 3.8.

The matters needing attention and the method of casting parallel gradient gel with DGGE equipments (DCode Universal Matation Detection System, Bio-Rad, USA) were described in the following: Before cast parallel gradient gel the glass should wash with 70% ethanol and air-dried to avoid the inaccurate analysis. The equipment of cast gel is constructed with two glasses and clips, put spacer between the glass and the bottom should be sealed by tape to avoid gel leak. According to previous experiment, the gradient range of denaturant was decided from 30% to 60%. The reagent and concentration of DGGE gel was shown in Table 3.9. A gradient delivery system (Bio-Rad Model 475 Gradient Delivery System) was used to deliver gel mixture into the mold. After pouring into the gel, put in the comb carefully to avoid the bubble produced and sit for at least 60 minutes for gel hardening. 1X TAE buffer was added to each well after the comb taken off, because when the gel put in pre-heated electrophoresis tank, the temperature will make the urea release that impeded DNA loading. 10.0 μl DNA samples will mix with 5.0 μl 6X loading dye buffer, then run the electrophoresis at 60V for 18 hours at 60 ^{o}C. After electrophoresis, the gel will stained with 1X SYBR for 40-60

Table 3.8 Relationship between gel percentage and base pair separation

Gel percentage	**Base pair separation**
6%	300-1000 bp
8%	200-400 bp
10%	100-300 bp

.

Table 3.9 Reagent and concentration of DGGE

Reagent	**Amount**	
A. 40% Acrylamide/Bis (37.5:1)		
Acrylamide	38.9 g	
Bis-acrylamide	1.1 g	
dH_2O	To 100.0 mL	
B. Denaturing Solution (6% gel)		
	0%	100%
40% Acrylamide/Bis	15.0 mL	15.0 mL
50X TAE Buffer	2.0 mL	2.0 mL
Formamide (deionized)	0.0 mL	32.0 mL
Urea	0.0 g	33.6 g
dH_2O	To 100.0 mL	To 100.0 mL
C. Clot reagent		
10% APS	1000.0 µl / 100.0 mL denaturing solution	
TEMED	100.0 µl / 100.0 mL denaturing solution	

minutes, the SYBR was mixed with 1X TAE buffer, make sure the buffer liquor was covered the gel.

3.7.5 Microbial community similarity analysis

Microbial community of the biofilm was analyzed using DGGE method, each of the different DNA bands on denaturing gels took as independent microbial community. The analyzed of community similarity was using the Dice index (Cs):

$$Cs = \frac{2j}{a+b}$$

where j is the sum of common bands in samples A and B, and a and b are stand for the number of bands in samples A and B (Dice, 1945; LaPara *et al*., 2001). The value was defined as Cs = 1.0 for identical samples and Cs = 0 for completely distinct between the two samples.

Chapter 4 Results and Discussion

4.1 The characteristics of raw wastewater

The characteristics of influent wastewater were shown in Table 4.1. It was unnecessary to treat the nitrogen because ammonium concentration was below 3.0 mg/L. The low inorganic nitrogen content in the secondary-fiber papermaking wastewater is similar to that reported by Järvinen(1997). The BOD was measured in the start-up period but stopped measuring after start-up period due to its high variation. Feed wastewater was considered as biodegradable because the BOD/COD ratio was about 0.6, which indicated the biodegradability of the wastewater (Tchobanoglous and Burton, 2004). According to some studies, MLVSS could be regarded as biomass (Raposo *et al*., 2004; Le Bihan and Lessard, 2000), and the change of MLVSS in the mixed liquor could indicate the condition of cell immobilization onto the carrier. The ratio of MLVSS to TS is higher than 0.5, indicating that the biomass concentration in the TS is high. This datum suggested that the abundance of bacteria in the raw wastewater. The pH of raw wastewater was near to neutral.

4.2 Experiment started-up period

4.2.1 COD removal

The reactor was operated for 16 days continuously at ambient temperature for

Table 4.1 Characteristics of raw wastewater

Item	Concentration (mg/L)	Item	Concentration (mg/L)
COD	1630.1 ± 542.1	**TS**	3087.0 ± 511.1
SCOD	437.4 ± 104.2	**MLVSS**	1686.8 ± 390.0
BOD	970.7 ± 274.0	**DO**	1.2 ± 0.6
NH_4^+	< 3.0	**pH**	6.8 ± 0.2
		Temperature	35.9 ± 1.7 ^oC
BOD/COD	≒0.6	**MLVSS/TS**	≒0.6

reactor starting up. The change of COD concentrations in both influent and effluent during started-up period were shown in Fig. 4.1. On the sixth day, the influent COD concentration was dramatically dropped because the raw wastewater was diluted by a heavy rain during sampling period.

Table 4.2 showed the performance of the MBBR system during the started-up period. Although the average COD removal efficiency reached 72%, the COD concentration in the effluent did not meet the regulation (180.0 mg/L). The pH value in the effluent ranged from 7.3-7.6, which conformed to the regulated discharged limits of 6.0-9.0.

4.2.2 Microbiology of the biofilm on the carrier

The microbiology of the biofilm was observed using phase-contrast microscope and the interpretation was based on the book “Biological diagnosing technology of wastewater treatment functioning” published by the Industrial Development Bureau/Ministry of Economic Affairs (MOEA), R.O.C. When DO concentration was below 1.0 mg/L in the reactor, the microorganisms were mostly dispersed in the mixed liquor, where filamentous bacteria had the advantage of growing in low DO concentration. This explained why filamentous bacteria were mostly observed by phase-contrast microscopy since the DO concentration was controlled at 2.5 mg/L in

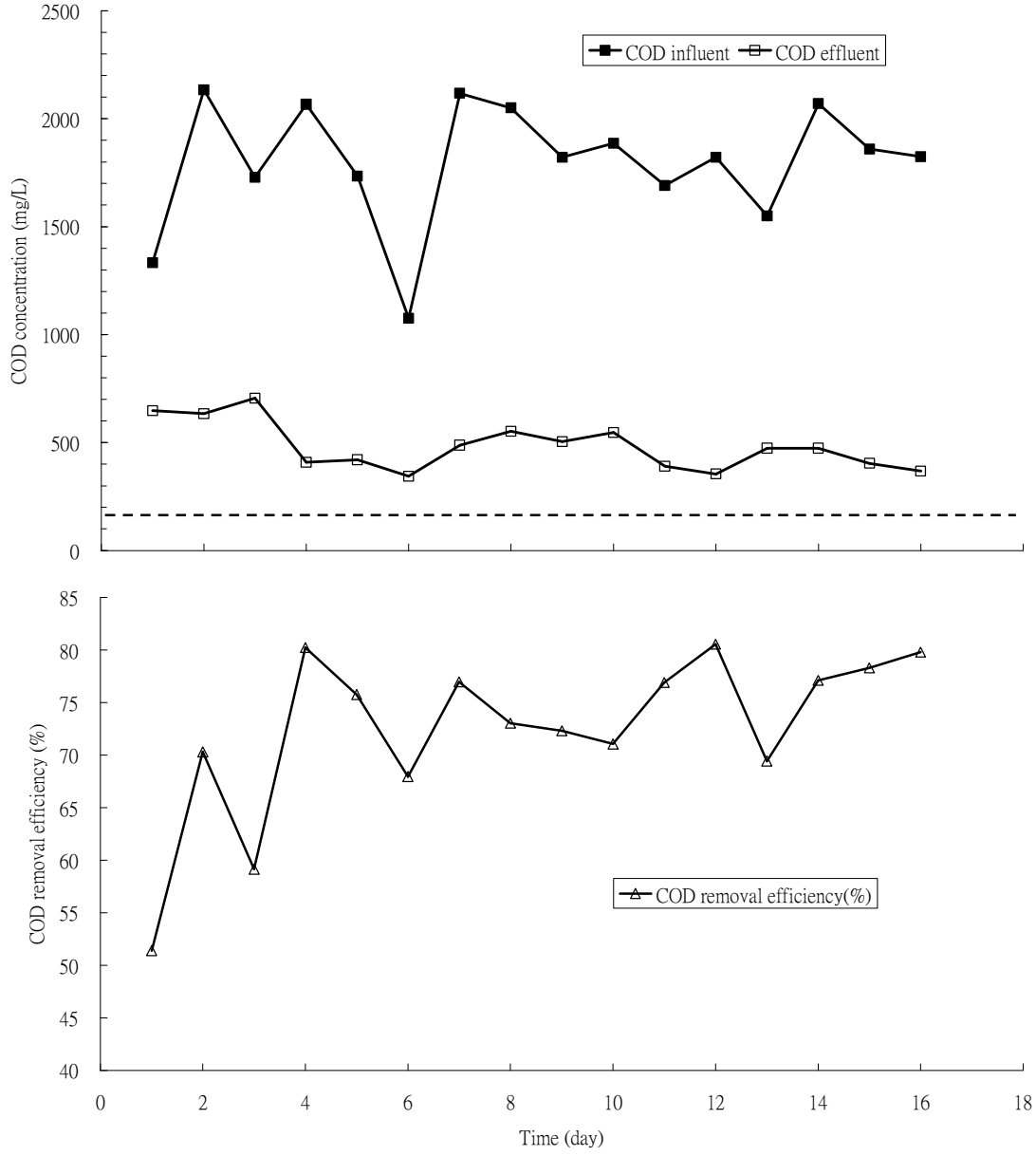


Fig. 4.1. Change of COD concentration during started-up period at ambient temperature. (■): influent COD; (□): effluent COD; (△): COD removal efficiency. The dotted line indicates discharge standard of secondary-fiber papermaking wastewater, which is 180.0 mg/L.

Table 4.2 Mean values and standard deviation of COD, SCOD, TS and MLVSS concentration changes in the MBBR reactor during start-up period at ambient temperature

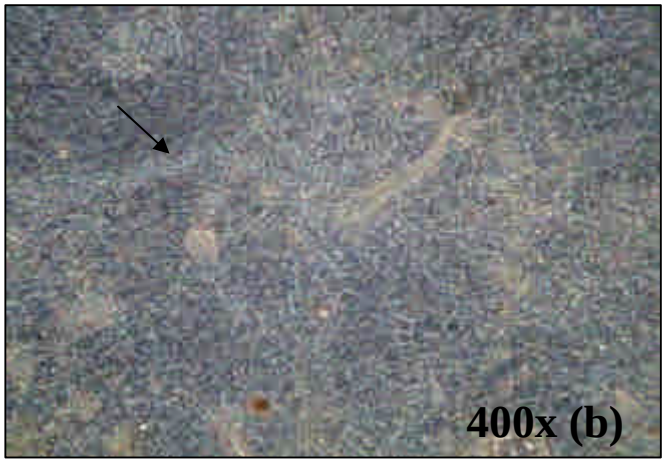
Item	Influent Concentration (mg/L)	Effluent Concentration (mg/L)	Removal Efficiency (%)
COD	1822.9 ± 300.8	489.3 ± 115.4	72.4 ± 8.4
SCOD	672.3 ± 139.4	418.8 ± 75.4	37.1 ± 7.3
TS	2762.5 ± 396.2	1746.3 ± 263.2	36.4 ± 6.8
MLVSS	1727.5 ± 378.8	792.5 ± 233.2	53.5 ± 9.8

carriers reactor during the started-up period.

After the MBBR system had run for one week, the white-limpid biofilm was formed on the carrier as shown in Fig. 4.2a. Presence of *Beggiatoa* sp. (Fig. 4.2b) and protozoan *Vorticella* sp. (Fig. 4.2c) in the biofilm indicated a higher and suitable organic loading, respectively in the influent. However the numbers of these two protozoa were not found predominantly under the microscope. This microscopic observation explained that the biofilm on the carrier had not developed well, resulting in the unstabilization of the MBBR system during the first 7 days in start-up period.

The biofilm on the carrier turned gray after the MBBR system had run for two weeks (Fig. 4.3a). Number of *Vorticella* sp. increased and turned into the dominant species (Fig. 4.3b), which indicated the microbial community of the biofilm was developed almost completely in MBBR system. The organic loading decreased from high to suitable due to the increased biomass on the carrier and was diagnosed by the presence of *Vorticella* sp. on the biofilm. Fig. 4.3c showed the presence of another protozoan, *Paramecium* which indicated a high organic loading rate into the biofilm, but its presence was rare. Number of dispersed bacteria in the mixed liquor also decreased during this stage. This microbiological analysis, along with COD analysis of the effluent indicated that the whole MBBR system reached

(a)



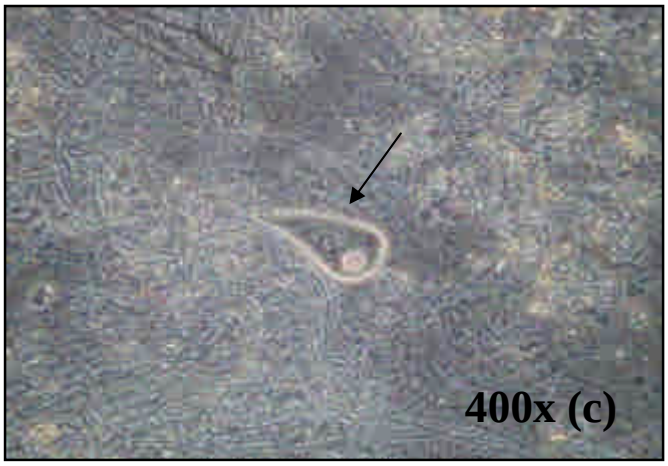


Fig. 4.2. Photographs of Kaldnes carrier after 1 week of start-up period (a) and the phase-contrast photomicrographs of microorganisms on the biofilm (b and c). Arrows indicates the *Beggiatoa* sp. (b) and *Vorticella* sp.(c).

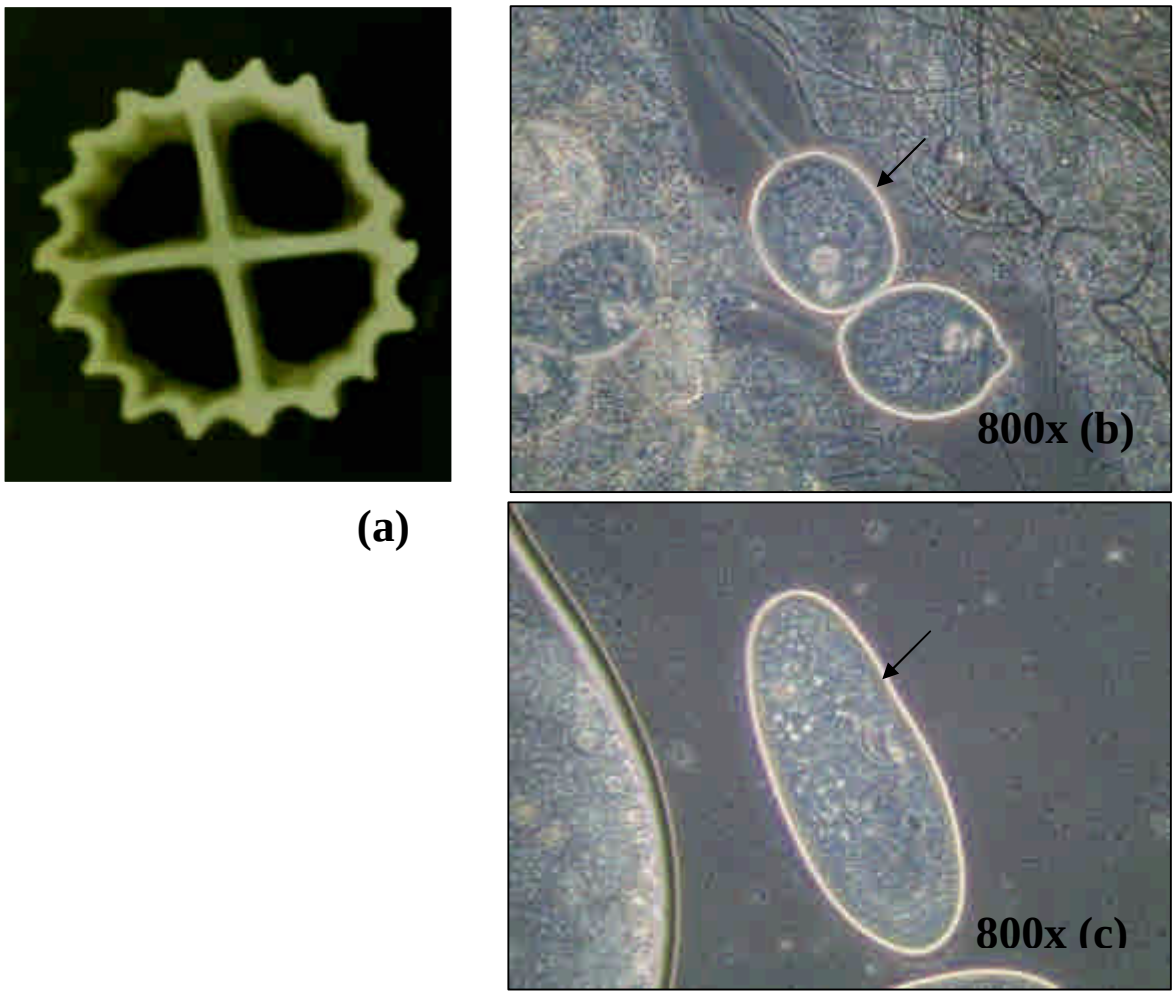


Fig. 4.3. Photographs of Kaldnes carrier after 2 weeks of start-up period (a) and the phase-contrast photomicrographs of microorganisms on the biofilm (b and c). Arrows indicates the *Vorticella* sp. (b) and *Paramecium* (c).

stabilization after two weeks in the start-up period at ambient temperature.

After the MBBR system had run for three weeks, the biofilm became gray-black and the thickness of biofilm in the central of carrier increased (Fig. 4.4a). *Paramecium* was found to the dominant protozoan species during this period, which indicated the last phase of biofilm development on the carrier. Some literatures reported that the biofilm structure at conventional aeration was arranged in order of aerobic, anoxic and anaerobic form outer layer to inner layer on the carrier (Walter *et al*., 2005; Rodgers *et al*., 2003; Semmens *et al*., 2003). In this stage, thick biofilm resulted in forming of an anaerobic inner area for anaerobes to live. An aerobic combining anaerobic biotreatment system was established during this stage. The COD removal efficiency increased during the experiment period when formation of biofilm on the carrier was gradually completed. However, the presence of *Paramecium* became a dominant protozoan species indicated the high organic loading into the reactor. The phenomenon might be explained by decrease of biomass in the anaerobic zone in the biofilm, but the COD removal rate in anaerobic was higher than that in aerobic area (Jahren and Ødegaard., 1999). If the DO concentration was controlled in the range of 2.0 to 3.0 mg/L, this situation might be also helpful to the growth of *Paramecium* in the carriers reactor during the whole experiment period.

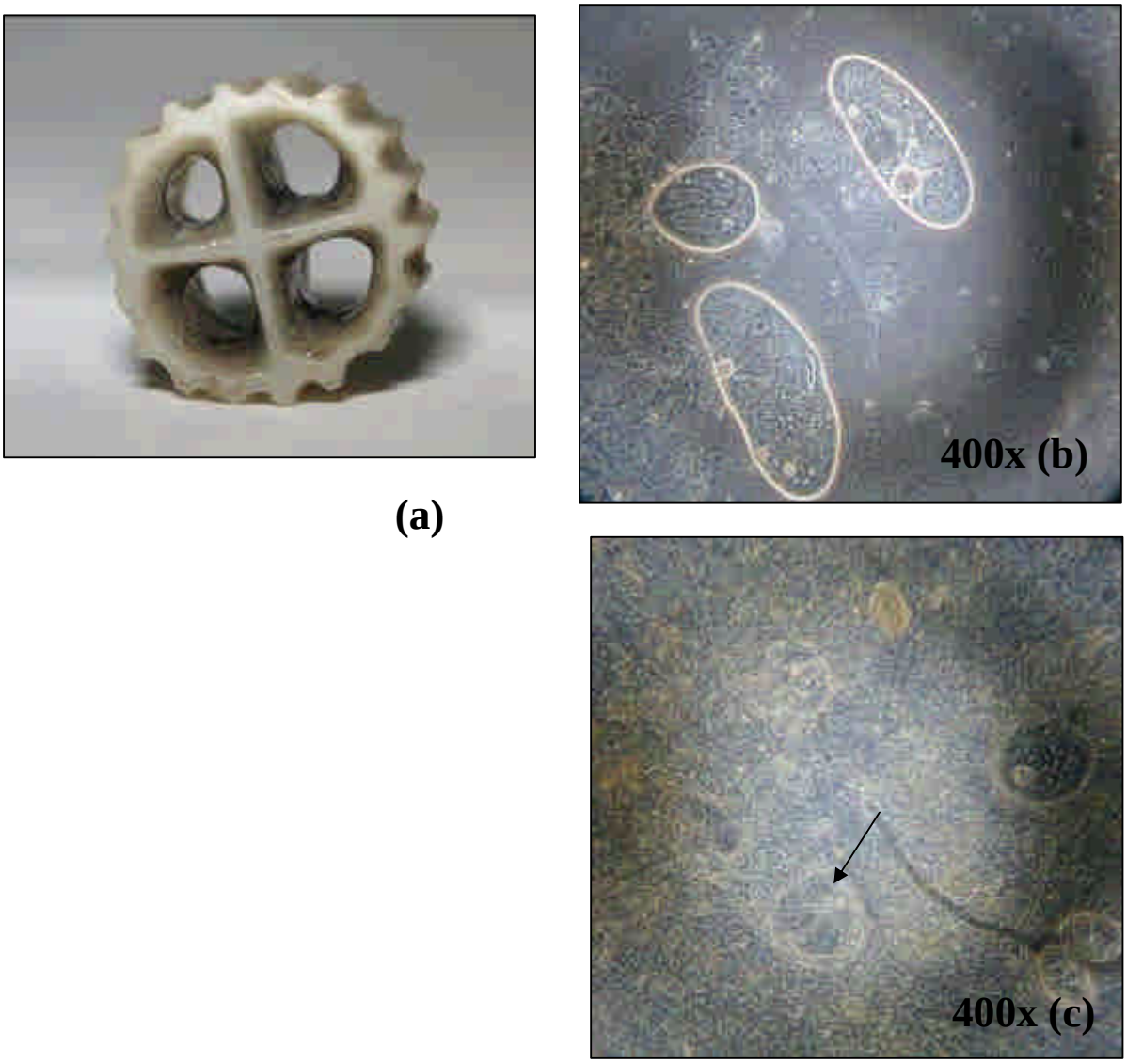


Fig. 4.4. Photographs of Kaldnes carrier after 3 weeks of start-up period (a) and the phase-contrast photomicrographs of microorganisms on the biofilm *Paramecium* (b). Arrows indicates the filamentous organism (c).

4.3 COD removal with inoculation of a thermophilic strain BL11

The influent COD varied due to the characteristic of raw wastewater. Wastewater was collected from equalization tank where fresh and old fed wastewater were mixed. Characteristic analysis of the raw mixed wastewater showed that the COD differed significantly among each sampling. The effluent COD of the MBBR system was reduced to below 180.0 mg/L in the early phase of the first experimental period and started to increase in the middle phase (Fig. 4.5). According to MLVSS removal efficiency decreased (Table 4.3), these results can explain the competition between strain BL11 and original microbial communities in the biofilm reactor, which might made biomass varied, such situation was found at the latter two periods of the experiment after strain BL11 was added. When operating at 40 ^{o}C, the MBBR system performed a well-effect on COD removal. When comparing to first and second period, addition of strain BL11 did not show the capability of increasing COD removal efficiency. On the contrary, COD removal efficiency decreased after adding strain BL11 into the system. This result might be due to the competition or even the wastewater could not supply growth substrate to the thermophilic strain BL11. The changed of COD in the effluent between second and third period indicated that a consistent air flow rate could not maintain a stable DO concentration

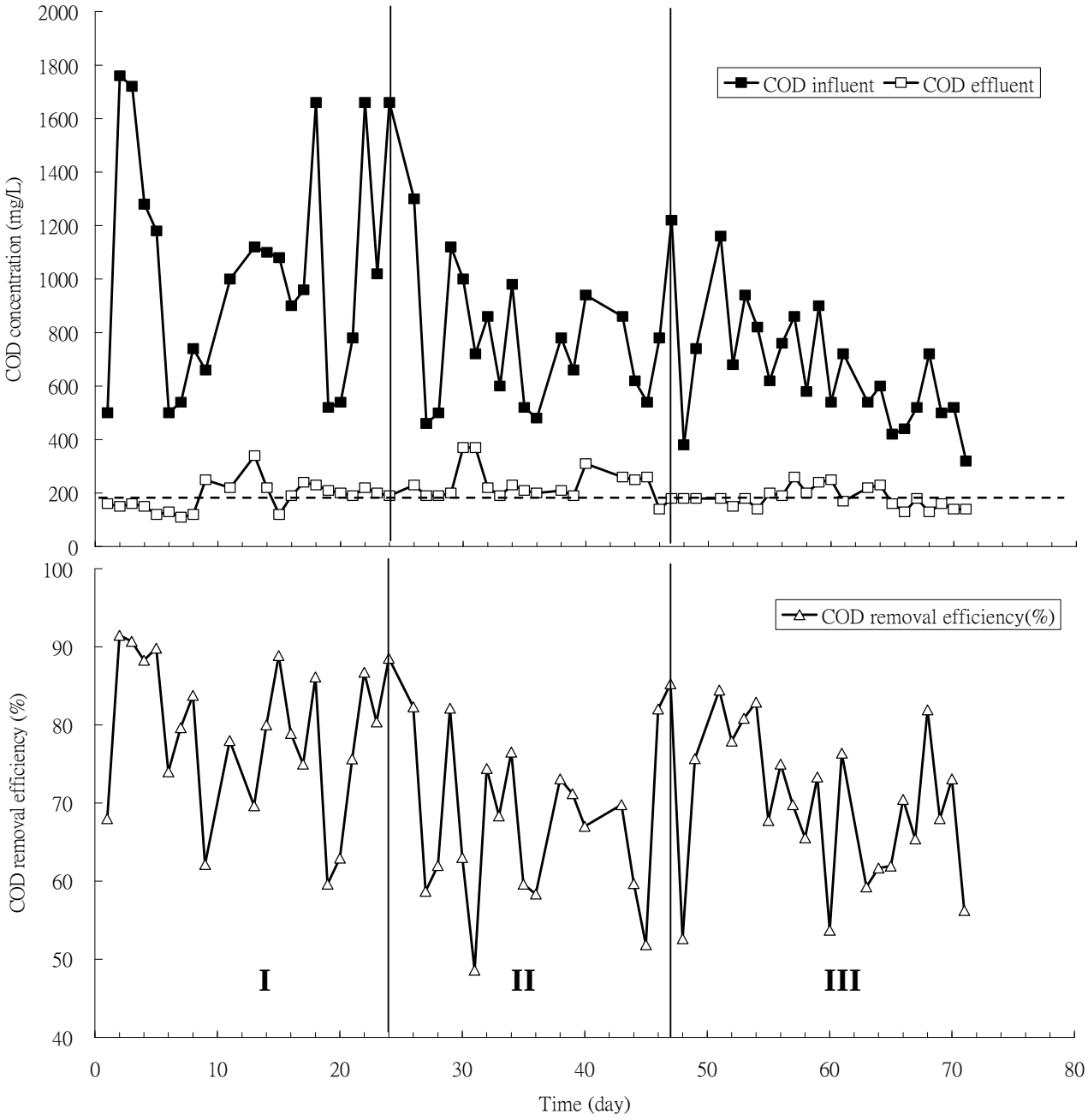


Fig. 4.5. Change of COD concentration during three different operation periods. I: operated temperature at 40 °C; II: addition of strain BL11; III: controlled DO concentration, the DO concentration in carriers reactor was controlled at 2.5 to 3.0 mg/L and in activated sludge reactor was at 2.0 to 2.5 mg/L. (■): influent COD; (□): effluent COD; (△): COD removal efficiency. The dotted line indicates discharge standard for secondary-fiber papermaking wastewater which is 180.0 mg/L.

Table 4.3 Results of COD, TS and MLVSS concentration changes from operated at 40 ^{o}C, adding thermophilic strain BL11 and controlling DO concentration. The DO concentration in carriers reactor was controlled at 2.5 to 3.0 mg/L and 2.0 to 2.5 mg/L in activated sludge tank

Item	Influent Concentration (mg/L)	Effluent Concentration (mg/L)	Removal Efficiency (%)
40 ^{o}C for 24days			
COD	1014.0 ± 431.3	187.3 ± 54.8	79.0 ± 9.8
TS	1968.0 ± 555.3	1014.0 ± 255.6	47.2 ± 11.9
MLVSS	1214.0 ± 390.3	492.0 ± 178.9	58.6 ± 11.2
After addition of thermophilic strain BL11 for 22days			
COD	786.3 ± 255.8	231.6 ± 61.2	68.0 ± 10.7
TS	1339.0 ± 280.8	1310.0 ± 223.0	24.4 ± 14.4
MLVSS	471.8 ± 271.8	506.0 ± 202.0	43.0 ± 21.3
Controlled DO concentration for 24days			
COD	649.1 ± 204.5	182.3 ± 38.7	69.7 ± 9.5
TS	1780.0 ± 441.6	1194.0 ± 273,9	31.1 ± 14.4
MLVSS	928.0 ± 359.8	470.0 ± 217.9	49.0 ± 15.0

in the reactor. As a consequence the DO concentration in the reactor was controlled at 2.0-3.0 ppm to improve COD removal. The pH value in effluent ranged from 7.0-7.6 which met the discharged limits of 6.0-9.0. The average values of COD, TS and MLVSS concentration in influent, effluent and removal efficiency were shown in Table 4.3.

The summarized results of COD, TS and MLVSS removal efficiency under three different operational conditions were shown in Fig. 4.6. The best COD removal efficiency was occur when the operational conditions rising at 40 ^{o}C and the average COD removal efficiency was 79%. Compared MLVSS concentration in the first and second period in the decreased MLVSS removal efficiency (from 58% to 43%) was possibly due to the slough-off biofilm from carrier after addition of strain BL11 and the COD removal efficiency also declined from 79% to 68%. The same tendency was appeared to TS and MLVSS removal efficiency. Removal efficiency of all measured parameters (COD, TS, MLVSS) suggested that a fixed DO in both carrier tank and AS tank could improve removal efficiency.

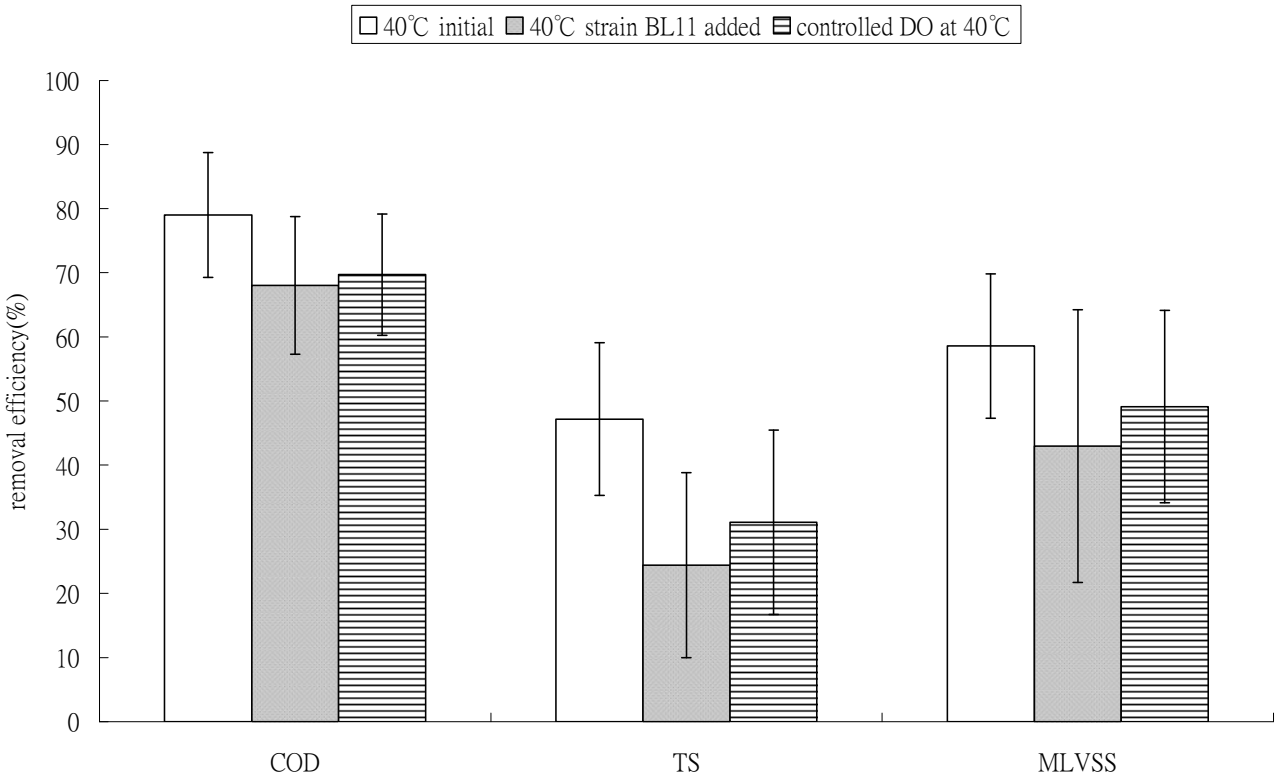


Fig. 4.6. Comparison of COD, TS and MLVSS removal efficiency in three different operational conditions.

4.4 Effect of different operational temperatures on reactor performance

The Kaldnes MBBR system was run at different operational temperatures (ambient temperature, 40 ^{o}C, 50 ^{o}C and 60 ^{o}C) to observe the temperature effect on the reactor performance. The Kaldnes carriers utilized were form the preceding experiment during which strain BL11 was immobilized on the carrier. Although addition of strain BL11 to the reactor did not show the capability of increasing COD removal efficiency in the preceding experiment, it was used as a target microorganism for determining the feasibility of using DGGE for microbial community study on the biofilm. In some studies indicated that the most important thing was to maintain the biomass in the reactor during transition phase from mesophilic (25 ^{o}C-40 ^{o}C) to thermophilic (50 ^{o}C-60 ^{o}C), since high temperature might killed mesophilic bacteria and decreased the biomass concentration in the system (Cecchi *et al*., 1993).

Inoculation of strain BL11 to the whole system was found to decrease COD removal efficiency. Comparing Fig. 4.5. and Fig. 4.7. during which the systems were both operated at 40 ^{o}C, the effluent COD was lower during inoculation periods in both runs. There were two reasons for the difference during this operation period, one was the sampling time in the influent and the other was the breakage of stirrer from day 33 to 41 during the period of operation temperature at 40 ^{o}C, which made

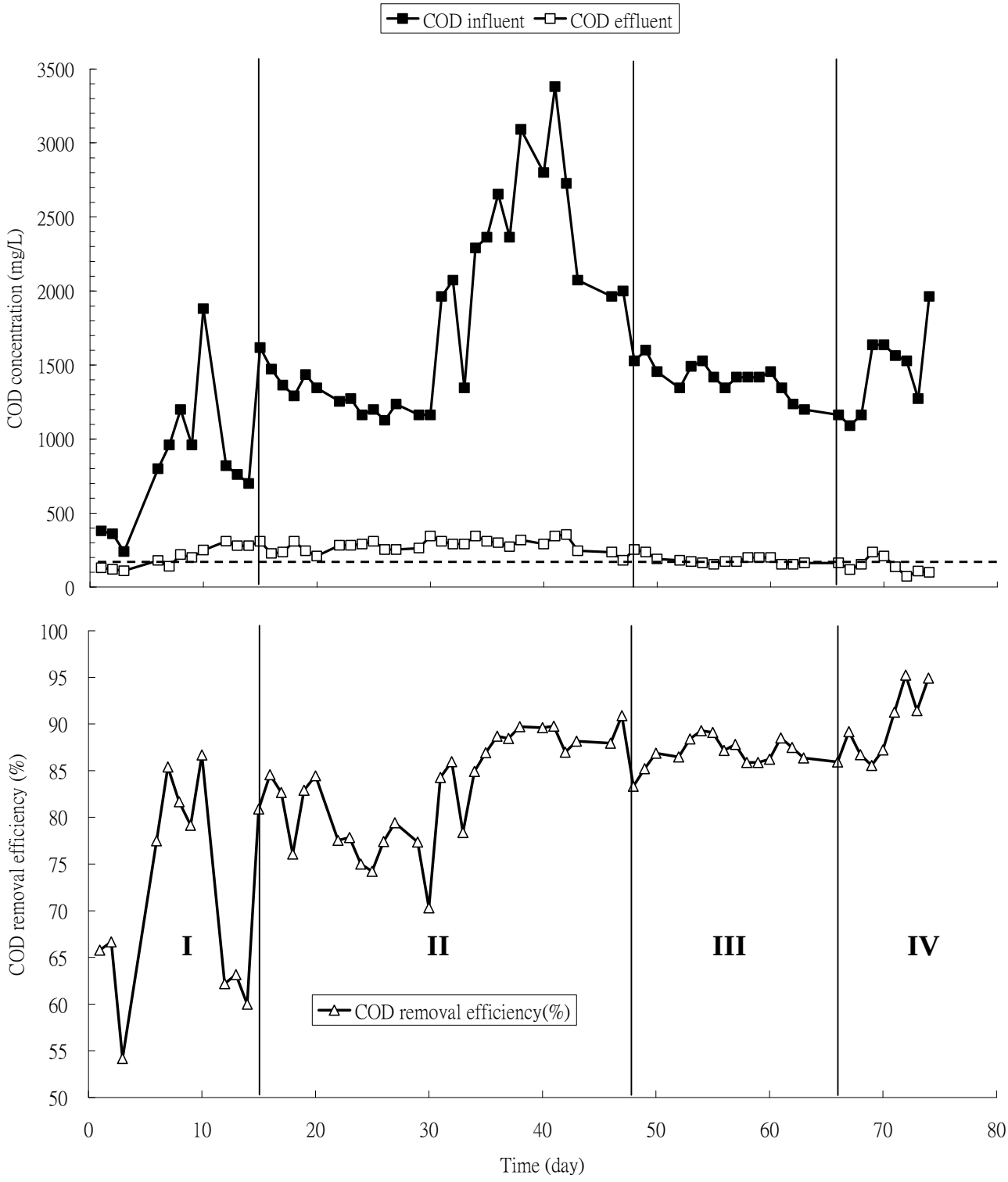


Fig. 4.7. Change of COD concentration at different operational temperatures. I: ambient temperature; II: 40 °C; III: 50 °C, IV: 60 °C. (■): influent COD; (□): effluent COD; (△): COD removal efficiency. The dotted line indicates discharge standard which is 180.0 mg/L.

the influent wastewater had high concentration in every measured characters. In Fig. 4.7., the lower effluent COD was noticeable with the increasing operational temperatures. At 60 ^{o}C, the average effluent COD was 144.0 mg/L which met the regulatory discharged limits. The pH value in effluent ranged from 7.3-7.6 also met to the discharged limit ranging from 6.0-9.0. The average mean values of COD, SCOD, TS and MLVSS in influent, effluent and removal efficiency were shown in Table 4.4.

The summarized results of COD, SCOD, TS and MLVSS removal efficiency under different operational temperatures were shown in Fig. 4.8. The COD and SCOD removal efficiency increased with the rising temperature, the removal efficiency was 89.7 % in COD and 54.5% in SCOD. However, the TS removal efficiency decreased due to poor sludge settling with the raising temperature. The highest removal efficiency of MLVSS was occurred at 50 ^{o}C, while the lowest occurred at 60 ^{o}C. The sudden increase of the temperature might be the reason for decreased MLVSS removal due to detach of biofilm from the carrier. The relationship between temperature and measured items removal efficiency (including COD, SCOD and TS) was subjected to statistical analysis with single factor analysis of variance (ANOVA) using Microsoft Excel 2003. The P values were 2.2×10^{-8}, 0.0314 and 0.4743 for COD, SCOD and TS, respectively. According to the

Table 4.4 Change of COD, SCOD, TS and MLVSS concentration during different operational temperatures: ambient temperature, 40 ^{o}C , 50 ^{o}C and 60 ^{o}C

Item	Influent Concentration (mg/L)	Effluent Concentration (mg/L)	Removal Efficiency (%)
Ambient temperature for 14days			
COD	823.6 ± 384.9	201.8 ± 57.8	71.1 ± 11.0
TS	1983.3 ± 660.6	836.7 ± 322.8	55.6 ± 15.2
MLVSS	1216.7 ± 580.0	388.3 ± 308.7	69.7 ± 16.0
40 ^{o}C for 33days			
COD	1799.4 ± 670.9	283.1 ± 42.4	82.4 ± 6.1
SCOD	496.6 ± 68.9	256.4 ± 37.7	48.0 ± 6.1
TS	3155.6 ± 938.2	1515.6 ± 197.4	48.5 ± 13.9
MLVSS	1894.8 ± 787.5	614.8 ± 145.5	53.6 ± 13.7
50 ^{o}C for 18days			
COD	1413.3 ± 107.2	184.8 ± 29.7	86.0 ± 1.6
SCOD	379.4 ± 89.8	166.7 ± 18.4	54.1 ± 10.0
TS	2932.9 ± 246.7	1536.5 ± 227.5	47.6 ± 6.9
MLVSS	1537.6 ± 228.9	428.2 ± 244.3	72.8 ± 13.2
60 ^{o}C for 11days			
COD	1446.5 ± 290.8	144.4 ± 52.7	89.7 ± 3.7
SCOD	343.4 ± 104.2	150.5 ± 32.8	54.5 ± 9.2
TS	3164.0 ± 547.4	1716.0 ± 272.7	45.0 ± 9.6
MLVSS	1964.0 ± 173.8	926.0 ± 297.6	52.5 ± 15.3

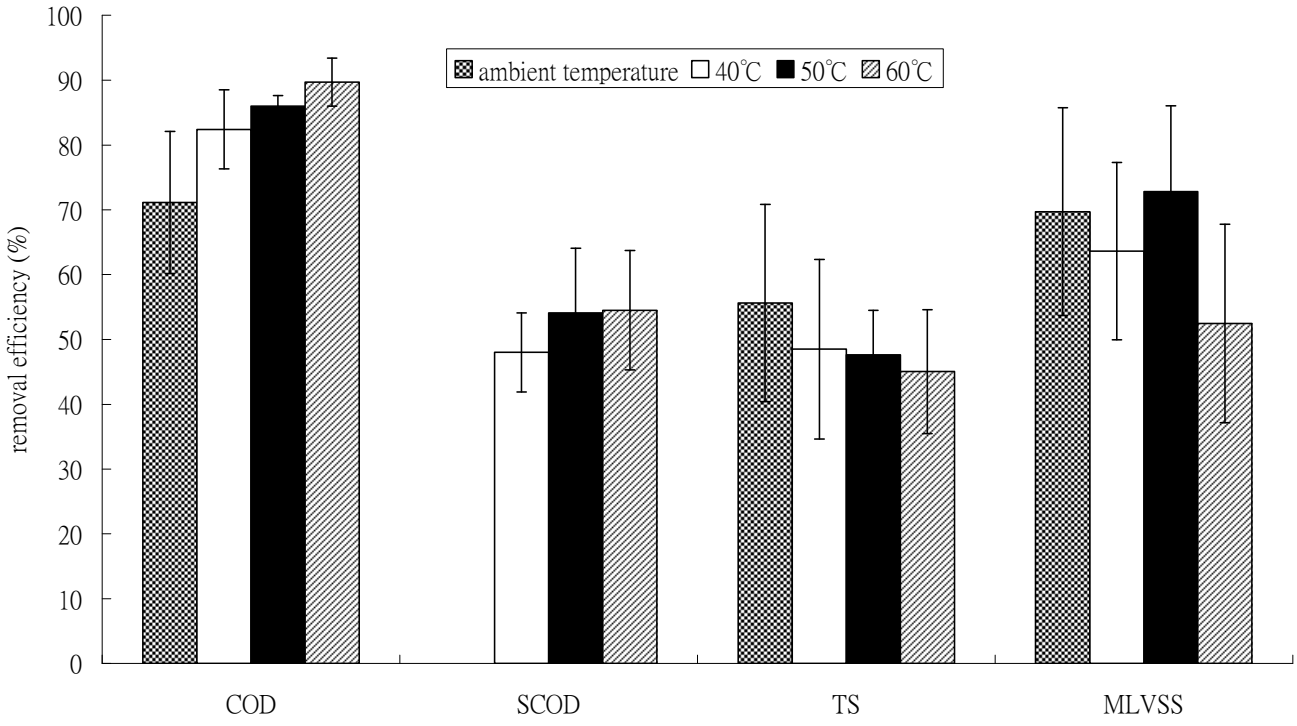


Fig. 4.8. Comparison of COD, SCOD, TS and MLVSS removal efficiency with four different operational temperatures: ambient temperature, 40 °C, 50 °C and 60 °C.

definition, when the P value was <0.005 the relationship between the single variable factor and the result was significant. This study showed the rising temperature had the most effect on COD removal, but no significant effect on SCOD and TS removal. Compared the COD removal efficiency between each operating temperatures, the results indicated that the temperature factor affect the COD removal was significant between ambient temperature and others operating temperatures. After the temperature raised above ambient temperature into thermophilic system the temperature effect was no significant for each ten-degree intervals.

The COD removal efficiency versus volume loading rate at different operated temperatures were shown in Fig 4.9. Because of the trend line between COD removal efficiency and volume loading rate at 50 ^{o}C and 60 ^{o}C was non-linear ($R^2 <$ 0.25), only the result of relationship for ambient temperature and 40 ^{o}C was discussed here. In Fig 4.9, the results indicated that different volume loading rates had more influence to COD removal efficiency at ambient temperature; in other words, the MBBR system had better recover capability from the loading shock at 40 ^{o}C. This might be due to higher growth and reaction rate of thermophilic bacteria, which made faster response to different volume loading rate and resulted in increasing the COD removal efficiency.

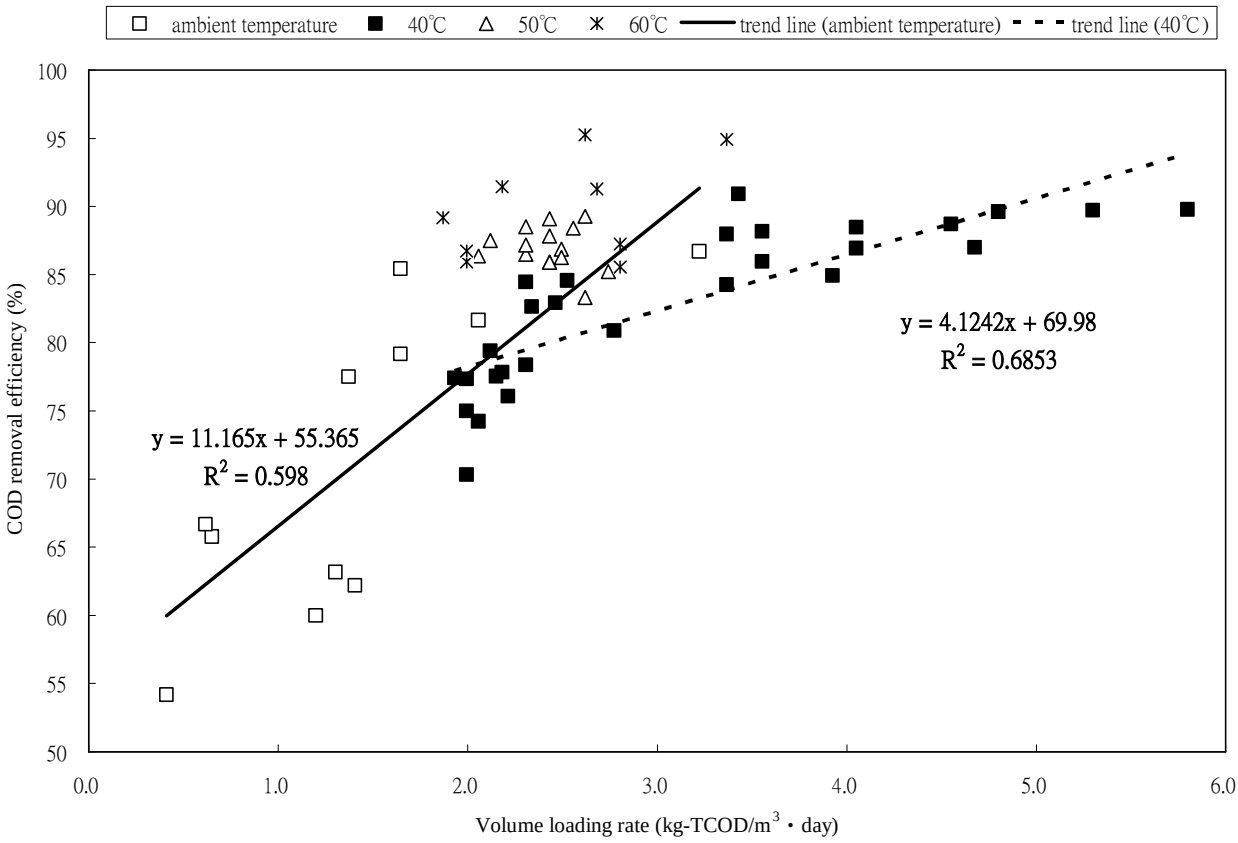


Fig. 4.9. Removal efficiency versus volume loading rate at different operating temperatures, ambient temperature (□), 40 °C (■), 50 °C (△) and 60 °C (∗).

At present, there were many kinds biological treatment processes for paper and pulp mill wastewater. Most of the studies (Jahren and Ødegaard., 1999; Jahren *et al*., 2002; Tardif and Hall., 1997; Tripathi and Allen, 1999; Vogelaar *et al*., 2002), as well as this study, focused on the feasibility of using thermophilic system for treatment of such wastewater. Comparison of different studies on paper and pulp mill wastewater treatment at different temperatures was showed in Table 4.5. Compared to other MBBR systems, Kaldnes carrier used in this study had a minimum surface area but COD removal rate was the highest. Although this study showed a better SCOD removal rate, which depended on the higher SCOD influent loading, the removal efficiency was lower than the results of Broch-Due *et al*. (1997) or Jahren and Ødegaard (1999), while the former study combined a tertiary chemical treatment and the latter used an anaerobic-aerobic system. Studies of Jahren and Ødegaard (1999) indicated that anaerobic treatment of their system had better SCOD removal rate than that of aerobic part, while the removal efficiency was on the contrary. Such result was due to different biomass on the carrier. During the experiment in their study, the biomass increased in aerobic part was 300.0% and 65.0% in anaerobic part. According to this result, it suggests that the aerobic MBBR system can be established with less time than anaerobic MBBR system, and also had better recovery efficiency from loading shock. Compared to other systems such as sequencing batch reactors

(SBRs), ultrafiltration (UF) or membrane bioreactor (MBR), both COD and SCOD removal rate of the Kaldnes MBBR combined with aerobic sludge tank in this study were higher than those of other types of reactors. All of the studies had sludge settling problem at high operation temperature which affected TS removal efficiency. Only the study of Vogelaar *et al*. (2002) operated the activated sludge system at 55 ^{o}C without sludge settling problem, but this is due to the calcium precipitation in the aeration tanks which made temperature became less significance in TS removal. Tripathi and Allen (1999) indicated that the COD removal efficiency decreased (75% to 63%) with increased operational temperatures (35 ^{o}C to 60 ^{o}C) for an SBR system. Phenotypic finger-printing analysis of the sludge in the same report showed a deceased microbial community structure when operation temperature increased from 35 ^{o}C to 60 ^{o}C. Result of PCR-DGGE analysis of microbial community in the biofilm also indicated that microbial community changed with operation temperature at an interval of 10 ^{o}C. However, the COD and SCOD removal rate increased with rising operation temperature. Bioaugmentation of an aerobic granule system by seeding a superior mixed flora successfully increased its COD removal efficiency (Wang *et al*., 2006). Although the influent loading of that study was low, results of Wang *et al*. (2006) and this study showed that bioaugmentation can enhance the efficiency of a wastewater treatment, while the parameters, such as inoculum quantity,

temperature and DO, were under control.

Table 4.5 Comparison of system efficiency of various treating systems for papermaking wastewater

Temp. range (^{o}C)	**Influent**	**System**	**loading rate (kg/m^{3}day) and DO concentration (mg O_2/L)**	**Carrier Area (m^2/m^3)**	**Observations**	**TS and COD removal rate (kg/m^{3}day)**	**Reference**
40, 50 and 60	Secondary -fiber papermaking wastewater	Aerobic MBBR (10% K1 filled)	COD Loading: 43.4 SCOD Loading: 10.3 DO: 2.0-3.0	50.0	90% COD and 55% SCOD removal; removal efficiency increases with arising temperature	**COD:** 39.1 **SCOD:** 5.8 at 60 ^{o}C	In this study
23-35	Integrated newsprint mill wastewater	Aerobic MBBR (70% K1 filled)	COD Loading: 2.7-17.8 SCOD Loading: 2.1-13.5 DO: 2.5-5.7	350.0	65-75% COD removal; 95% COD removal when combined with a tertiary chemical treatment	**COD:** 2.0-13.3 **SCOD:** 1.6-10.1	Broch-Due *et al.*, 1997
55	TMP whitewater	Anaerobic-Aerobic MBBR (60% K1 filled)	COD Loading: unknown SCOD Loading: 7.0 DO: 2.0-3.0 (in aerobic reactor)	300.0	30% and 40% SCOD removal in an anaerobic-aerobic MBBR with higher removal rate in anaerobic reactor	**COD:** unknown **SCOD:** 3.4 in anaerobic; 1.8 in aerobic, total is 5.2	Jahren and Ødegaard., 1999

55	TMP whitewater	Aerobic MBBR (58% K1 filled)	COD Loading: 25.5 SCOD Loading: 2.7 DO: 2.0-3.0	290.0	60-65% SCOD removal; removal rate increases with raised loading rates	**COD:** 11.3 **SCOD:** 1.7	Jahren *at al*., 2002
20-55	Recirculated newsprint whitewater	SBR, UF, SBR+UF and MBR	COD Loading: 6.3-25.1 SCOD Loading: 5.0-20.0 DO: unknown	No carrier	SBR+UF (89%) and MBR (78%) have better removal efficiency at high operation temperature (up to 40 ^{o}C); MBR had better capability to maintain the system under different conditions	**COD:** unknown **SCOD:** 5.0 in SBR+UF system; 15.6 in MBR system	Tardif and Hall., 1997
35, 45, 55, 60	Bleached kraft pulp mill effluent	SBRs	COD Loading: 1.5-2.0 SCOD Loading: unknown DO: 4.8 at 35 ^{o}C, while 2.5 at 60 ^{o}C	No carrier	COD removal efficiency decreases with increasing temperatures (75% at 35 ^{o}C, 63% at 60 ^{o}C); bed sludge setting at high temperature; Phenotypic finger-printing results shows different microbial community between mesophilic and thermophilic conditions	**COD:** 1.3 at 35 ^{o}C; 1.1 at 60 ^{o}C **SCOD:** unknown	Tripathi and Allen, 1999

30, 55	Recycled paper mill process water	Activated sludge system	COD Loading: 16.9 SCOD Loading: 10.6 DO: above 2.0	No carrier	Higher COD removal efficiency at 30^{o}C (58%) than at 55 ^{o}C (48%); no sludge settling problem occurred in both systems	**COD:** 10.1 in mesophilic; 9.5 in thermophilic **SCOD:** 3.5 in mesophilic; 2.9 in thermophilic	Vogelaar *et al*., 2002
33	Papermaking wastewater	SBR, aerobic granules (both of the systems combines with superior mixed flora seeding)	COD Loading: 6.0 SCOD Loading: unknown DO: 2.5-3.5	No carrier	84% COD removal; successful bioaugmentation by seeding superior mixed flora into aerobic granules system; granulation process affects by filamentous microbes; inoculum quantity, temperature, DO, BOD:N:P ratio, pH and shear force are the control parameters	**COD:** 4.7 **SCOD:** unknown	Wang *et al*., 2006

Note: MBBR: moving bed biofilm reactors; TMP: thermomechanical pulping, SBRs: sequencing batch reactors, UF: ultrafiltration, MBR: membrane bioreactor

4.5 Microbial community analysis of the biofilm with PCR-DGGE

4.5.1 Comparison of different DNA extraction methods

DNA extraction is known to be important and have greet influence on the following PCR-DGGE analysis of microbial community (Lyautey *et al*., 2005). Three routine extraction procedures were tested in this study. The extracted DNA concentration and purity with three different extraction methods were shown in Table 4.6. Conventional phenol-chloroform method can extract the most amount of DNA but accompanied with less purity of DNA. Both commercial DNA extraction kits, Blood&Tissue Genomic mini-Prep (Viogen, R.O.C) and Ultra Clean Soil DNA isolation kit (MOBIO, USA) showed a better purity of extracted DNA but with lesser DNA concentration. The Viogene Genomic DNA Miniprep Kit had better extraction efficiency than UltraClean Soil DNA Isolation kit did. Commercial DNA extract kits use ion-exchange column and spin filter techniques to remove interferent compound, therefore they give a higher purity of DNA than traditional phenol-chloroform technique does. However, after isopropanol purification process, the purity of extracted DNA by using traditional phenol-chloroform method was higher than DNA extracted with two commercial kits, but DNA amount decreased from 824.5 ng/μL (before purification) to 93.5

Table 4.6 Extracted DNA concentration and purity by using three different extraction methods

	OD_{260}	OD_{280}	purity	DNA concentration(ng/μL)
BL11 T1	0.044	0.039	1.13	374.00
50 °C T1	0.097	0.06	1.62	824.50
50 °C T2	0.011	0.008	1.38	93.50
60 °C T1	0.034	0.029	1.17	340.00
60 °C T2	0.002	0.001	2.00	20.00
BL11 B	0.003	0.002	1.50	25.50
50 °C B	0.021	0.014	1.50	178.50
60 °C B	0.006	0.004	1.50	60.00
BL11 S	0.002	0.002	1.00	17.00
50 °C S	0.002	0.002	1.00	17.00
60 °C S	0.003	0.001	3.00	30.00

Note: T1: traditional phenol-chloroform method; T2: traditional phenol-chloroform method followed by isopropanol DNA purification process; B: Viogene Genomic DNA Miniprep Kit for Blood&Tissue; S: UltraClean Soil DNA Isolation kit.

ng/μL (after purification).

4.5.2 PCR and nested PCR amplification

4.5.2.1 Amplification of 16S rDNA from biofilm with co-culture with strain BL11

The optimal heating program of PCR in this section was shown in Table 4.7. The annealing temperature for amplifying 16S rDNA from biofilm operated at ambient temperature and at 40 ^{o}C was higher than others to increase the specificity of amplicons. Nonspecific amplicons will affect the separation of microbial DNA bands on the denaturing gel of DGGE. The extracted DNA concentrations and purity (OD_{260}/OD_{280} ratio) of the nested PCR products were shown in Table 4.8. Most of the purity was ranged from 1.5 to 1.9 which indicated a suitable quality of the amplicons. The products of nested PCR were then check with the electrophoresis to confirm the size of the amplicons (Fig 4.10). Each nested PCR product showed a single DNA band with a size of ca. 600 bp.

The microbial community of the biofilm grown at different temperatures and the mixed liquor were analysed by 16S rDNA PCR-DGGE approach. Three sets of denaturant range (20-80, 25-60 and 30-60%) were tested for optimally separating the amplified 16S rDNA fragments from the community, and a better separation of

Table 4.7 Optimal conditions of PCR amplification of bacterial 16S rDNA

Item	Activation temp.	Denaturation temp.	Annealing temp.	Extension temp.	Final extension temp.
Ambient temperature			61 °C, 30 sec.		
40 °C			60 °C, 30 sec.		
Strain BL11			58 °C, 30 sec.		
Mixed liquor	94 °C, 10 min	94 °C, 30 sec.	57 °C, 30 sec.	72 °C, 20 sec.	72 °C, 2 min
Carrier			59 °C, 30 sec.		
50 °C			57.5 °C, 30 sec.		
60 °C			57.5 °C, 30 sec.		

Note: ambient temperature : extraction of DNA from MBBR system when operated at ambient temperature ; mixed liquor: extraction of DNA from liquid after adding pure culture at 40 °C; carrier: extraction of DNA from biofilm after adding pure culture at 40 °C

Table 4.8 DNA concentrations and their purity of nested PCR products

	OD_{260}	OD_{280}	purity	DNA concentration(ng/μL)
Ambient temperature	0.046	0.026	1.77	460.00
40 °C	0.050	0.035	1.43	500.00
Strain BL11	0.045	0.022	2.05	450.00
Mixed liquor at 40 °C	0.047	0.027	1.74	470.00
Carrier at 40 °C	0.052	0.027	1.93	520.00
50 °C	0.050	0.025	2.00	500.00
60 °C	0.063	0.037	1.70	630.00

Note: ambient temperature : extraction of DNA from MBBR system when operated at ambient temperature ; mixed liquor: extraction of DNA from liquid after adding pure culture at 40 °C; carrier: extraction of DNA from biofilm after adding pure culture at 40 °C

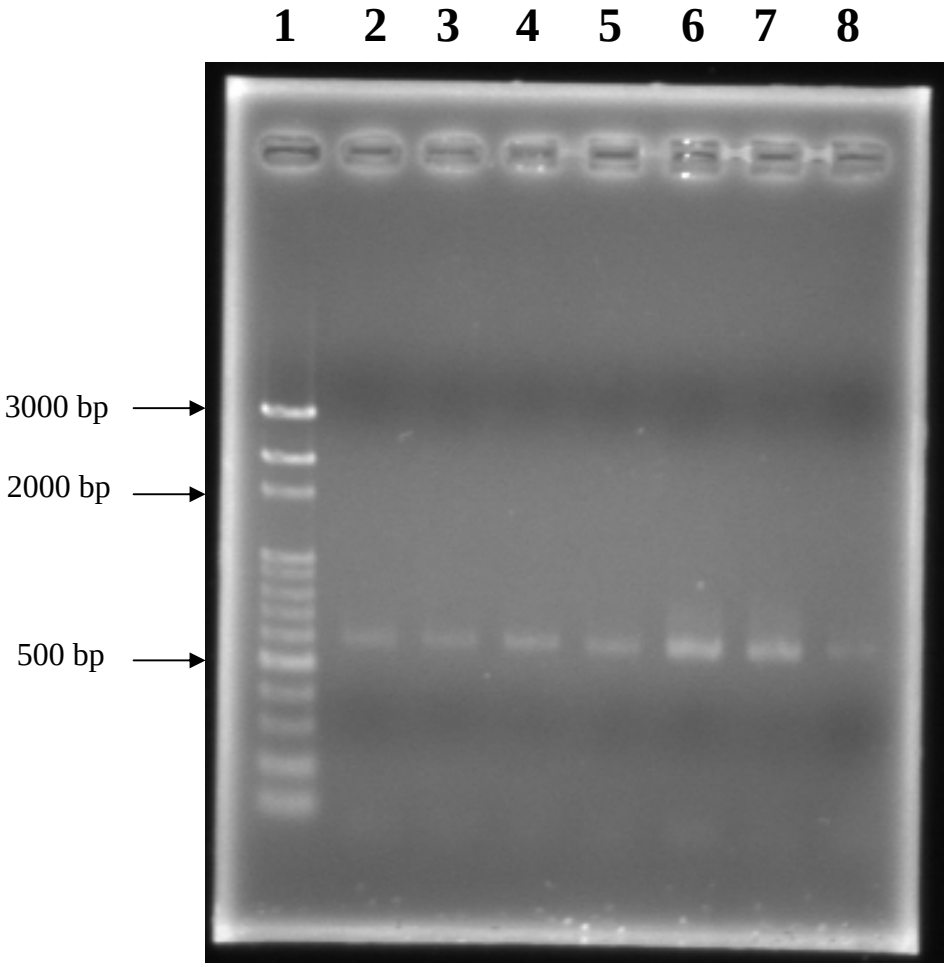


Fig. 4.10. Agarose gel electrophoresis of the nested PCR products. Line 1: Molecular weight marker, 0.1kb-3.0kb; Line 2: ambient temperature; Line 3: extraction of DNA from biofilm at 40 ^{o}C; Line 4: pure culture BL11; Line 5: extraction of DNA from liquid after adding pure culture at 40 ^{o}C; Line 6: extraction of DNA from biofilm after adding pure culture at 40 ^{o}C; Line 7: extraction of DNA from biofilm at 50 ^{o}C, and Line 8: extraction of DNA from biofilm at 60 ^{o}C.

PCR-amplified 16S rDNA fragments were shown in the gel with 30-60% denaturant gradient (Fig 4.11). As concerns the number of visualized bands, excluding the DNA of strain BL11 (Line 3), 24, 26, 13, 25, 32 and 19 bands were respectively seen from both mixed liquor and biofilm at different temperatures (Fig 4.12). Though most studies showed that microbial diversity decreased with raising temperatures (LaPara *et al*., 2001), such phenomenon only occurred when operational temperature raised from 50 ^{o}C to 60 ^{o}C in this study. The microbial community of the biofilm showed a highest richness (32 bands) at 50 ^{o}C, resulting in the highest MLVSS removal efficiency at this temperature. Higher annealing temperatures in PCR amplification of the microbial community at ambient temperature and 40 ^{o}C may result in the specificity of priming, which decrease the richness of community. Compared DNA bands on Line 4 to those on Line 5, the capability of increasing biomass on the carrier was proved with the richness microbial community distinguished sampling from water and the biofilm collected on carrier. The target DNA (strain BL11) was found in biofilm community when operated at ambient temperature, 40 ^{o}C (not observed in mixed liquor), 50 ^{o}C and disappeared at 60 ^{o}C (Fig. 4.12b). Although strain BL11 was a thermophilic bacterium, 60 ^{o}C was its maximal growth temperature, which explain its disappearance in the DGGE gel at 60 ^{o}C due to the poor growth environment. On

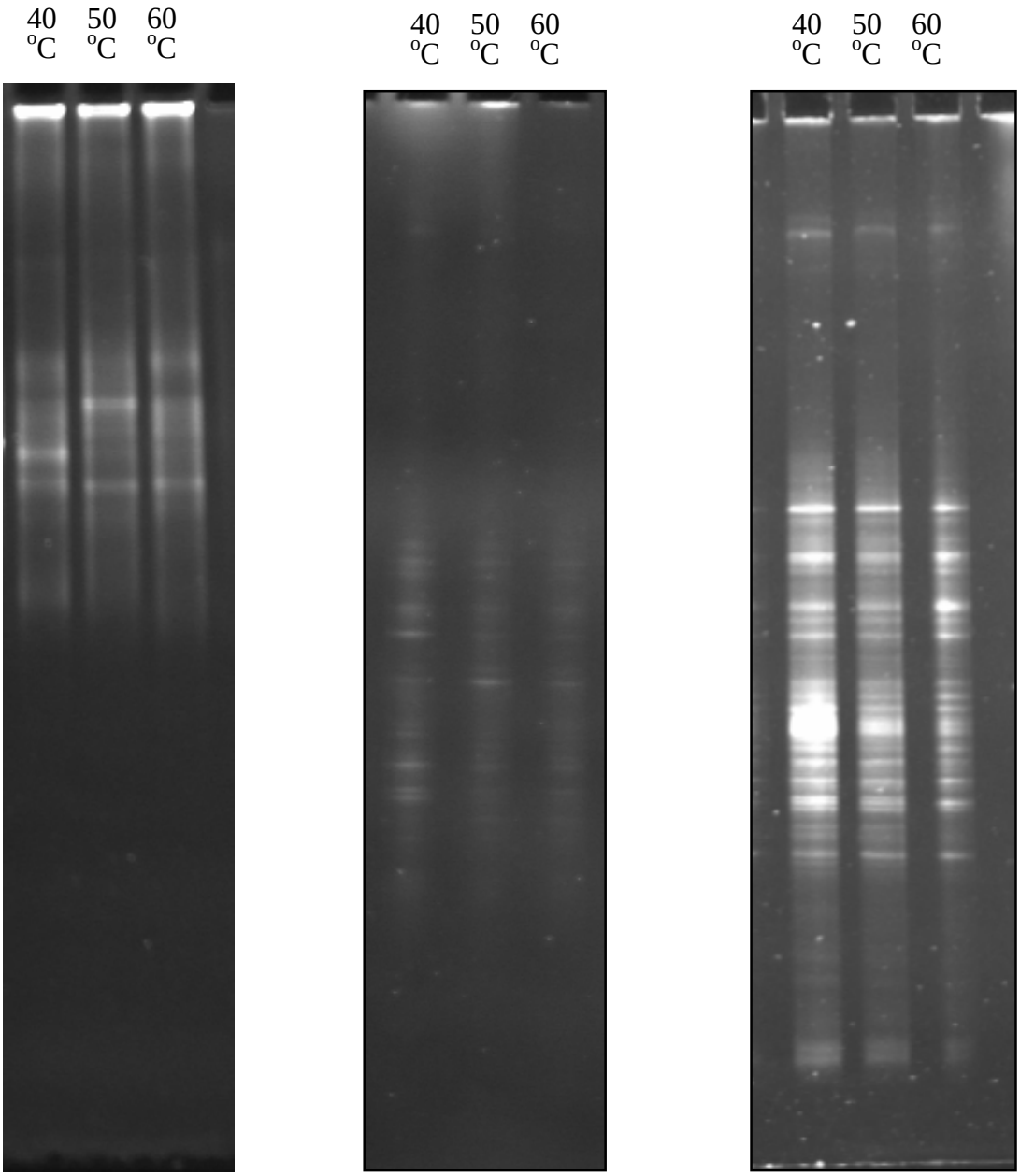


Fig. 4.11. Results of DGGE at different operational temperatures (40 °C, 50 °C and 60 °C) under different denaturant gradient. (a) gradient range from 20-80%; (b) gradient range from 25-60%; and (c) gradient range from 30-60%

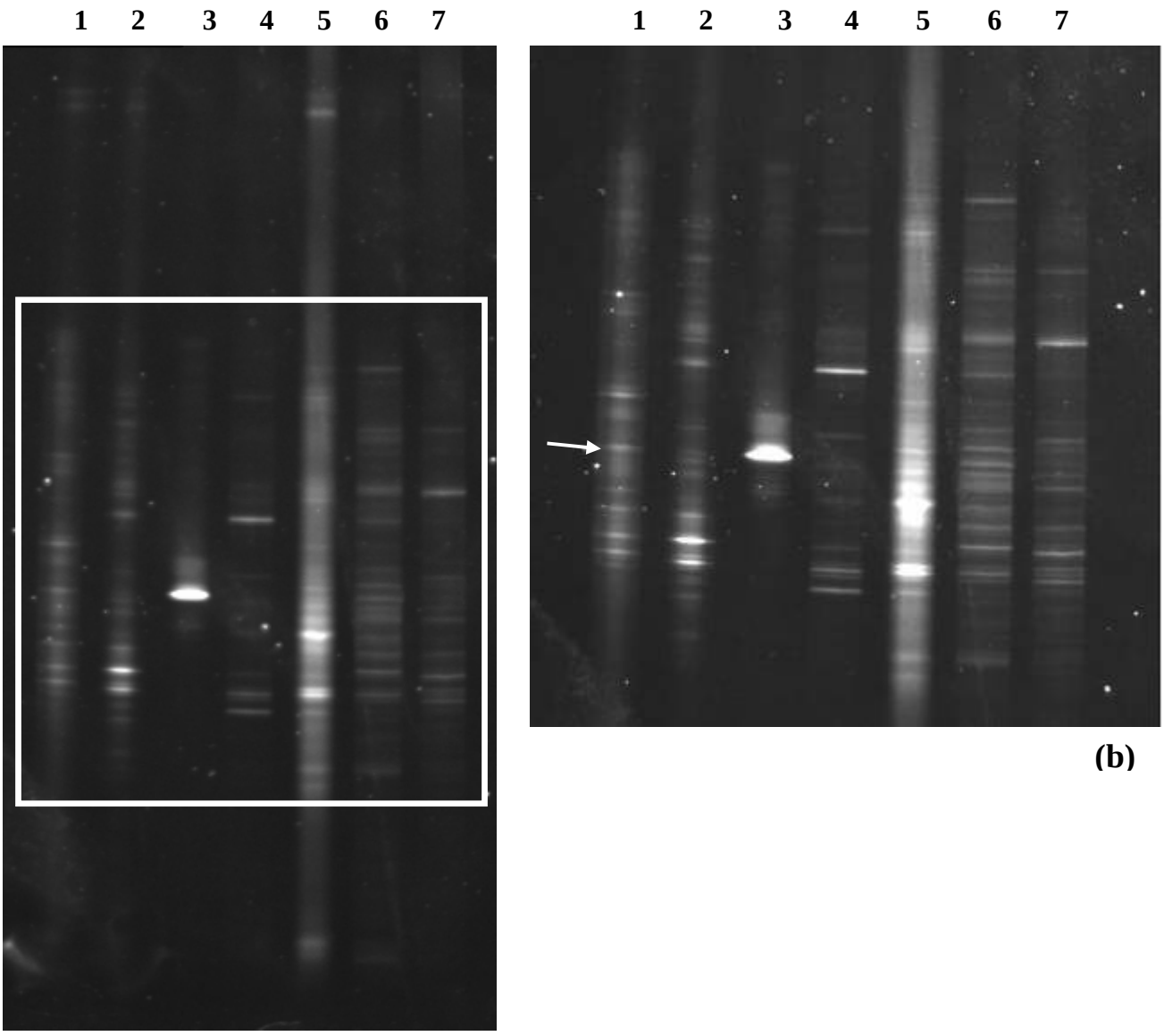


Fig. 4.12. (a) Picture shown the PCR-DGGE profile of microbial community of the biofilm with strain BL11 inoculation at different operational conditions, the gradient range of denaturant range was from 30% to 60%. (b) amplified from the area select in picture a, the arrow shows the site of the strain BL11. All of these samples were using traditional phenol-chloroform method. Line 1: ambient temperature; Line 2: extraction of DNA from biofilm at 40 ^{o}C; Line 3: pure culture BL11; Line 4: extraction of DNA from liquid after adding pure culture at 40 ^{o}C; Line 5: extraction of DNA from biofilm after adding pure culture at 40 ^{o}C; Line 6: extraction of DNA from biofilm at 50 ^{o}C; and Line 7: extraction of DNA from biofilm at 60 ^{o}C.

the same distance of the denatured gel, the target DNA also found in Line 2 with a light band, there were two explanations of the results, one is the residual strain BL11 in the carrier reactor when the condition changed, another explanation was that a bacterial had similar GC content to strain BL11 existed in the raw water. The raising annealing temperature also had the potential to lose some of the microbial communities.

The similarity analysis for PCR-DGGE during different operational conditions was shown in Table 4.9. Microbial communities had a moderate relatedness of similarity between ambient temperature and 40 ^{o}C, ambient temperature and 50 ^{o}C, 40 ^{o}C and 50 ^{o}C, 50 ^{o}C and 60 ^{o}C, where the Cs values were ranged from 0.4 to 0.7. The moderate relatedness between those not listed here (such as ambient temperature and 60 ^{o}C, 40 ^{o}C and 60 ^{o}C... etc.), the Cs values were all below 0.4 which indicates a lower similarity index. The Cs value was be defined as if Cs = 1.0, the two microbial communities would be identical and Cs = 0 would be completely distincted between the two samples. The significance analysis of microbial community at different conditions was determining by chi-square analysis (Table 4.10). In the chi-square analysis, the assumption of one degree of freedom and a probability of lower than 0.1 ($p<0.1$) were made. According to the chi-square distribution, a significant difference

Table 4.9 Similarity analysis of microbial community on the biofilm using the Dice Index (Cs) for PCR-DGGE during different operational conditions

	Line 1	**Line 2**	**Line 4**	**Line 5**	**Line 6**	**Line 7**
Line 1	1	-	-	-	-	-
Line 2	0.28	1	-	-	-	-
Line 4	0.22	0.36	1	-	-	-
Line 5	0.53	0.31	0.47	1	-	-
Line 6	0.46	0.34	0.36	0.60	1	-
Line 7	0.33	0.27	0.38	0.32	0.47	1

Table 4.10 Significance analysis of microbial community on the biofilm using chi-square analysis for PCR-DGGE during different operational conditions

	Line 1	**Line 2**	**Line 4**	**Line 5**	**Line 6**	**Line 7**
Line 1	-	-	-	-	-	-
Line 2	5.95	-	-	-	-	-
Line 4	6.20	1.65	-	-	-	-
Line 5	9.69	5.93	0.00	-	-	-
Line 6	0.23	3.37	0.40	1.96	-	-
Line 7	2.72	7.06	1.66	4.60	0.00	-

between two compared samples was suggested when the total chi-square value was greater than 2.706. The results indicated that microbial community in the biofilm changed with operation temperature at an interval of 10 ^{o}C, except for 50^{o}C and 60^{o}C. This might be due to the reason that both 50^{o}C and 60^{o}C were thermophilic system.

4.5.2.2 Comparison of different DNA extraction methods

The optimal heating program of PCR in this section was shown in Table 4.11. The annealing temperature for each sample was same (58 ^{o}C) except 57.5 ^{o}C for traditional phenol-chloroform method with isopropanol purification process for biofilm at 50 ^{o}C. The extracted DNA concentrations and purity of the nested PCR products were shown in Table 4.12 with only the method of using UltraClean Soil DNA Isolation kit at 60 ^{o}C had the purity below the standard quality. Comparing the results of Table 4.8 to those of Table 4.12, different DNA extraction methods had higher effect on extracted DNA concentration than on purity of extracted DNA. DNA templet without purifying process gave more products during nested PCR, only the extraction method of UltraClean Soil DNA Isolation kit at 50 ^{o}C perform the special case. Gel electrophoresis of nested PCR products from biofilm under different operating temperatures showed a

Table 4.11 Optimal conditions of PCR amplification of bacterial 16S rDNA

Item	Activation temp.	Denaturation temp.	Annealing temp.	Extension temp.	Final extension temp.
50 °C T1			58 °C, 30 sec.		
50 °C T2			57.5 °C, 30 sec.		
50 °C B			58 °C, 30 sec.		
50 °C S	94 °C, 10 min	94 °C, 30 sec.	58 °C, 30 sec.	72 °C, 20 sec.	72 °C, 2 min
60 °C T2			58 °C, 30 sec.		
60 °C B			58 °C, 30 sec.		
60 °C S			58 °C, 30 sec.		

Table 4.12 DNA concentrations and their purity of nested PCR products

	OD_{260}	OD_{280}	purity	DNA concentration (ng/µL)
50 °C T1	0.05	0.025	2.00	500.00
50 °C T2	0.012	0.006	2.00	120.00
50 °C B	0.014	0.008	1.75	140.00
50 °C S	0.064	0.034	1.88	640.00
60 °C T2	0.012	0.007	1.71	120.00
60 °C B	0.013	0.006	2.17	130.00
60 °C S	0.013	0.012	1.08	130.00

Note: T1: traditional phenol-chloroform method, T2: traditional phenol-chloroform method with isopropanol sublimating process, B: Viogene Genomic DNA Miniprep Kit for Blood&Tissue, S: UltraClean Soil DNA Isolation kit.

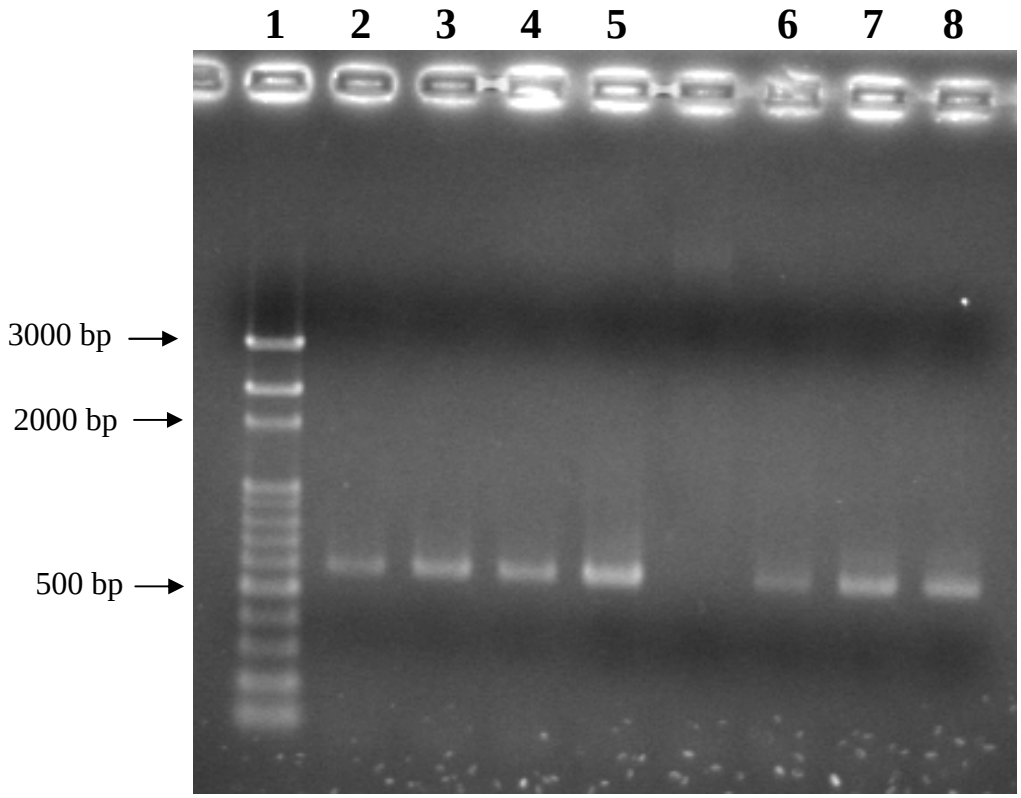


Fig. 4.13. Agarose gel electrophoresis of the nested PCR products of the biofilm DNA. Line 1: Molecular weight marker, 0.1kb-3.0kb; Line 2: at 50 ^{o}C, with phenol-chloroform method; Line 3: at 50 ^{o}C, with phenol-chloroform method combined isopropanol purification process; Line 4: at 50 ^{o}C, with chemical extraction method combined column purified process; Line 5: at 50 ^{o}C, with glass bead extraction method combined spin filter purified process; Line 6: at 60 ^{o}C, with phenol-chloroform method combined isopropanol sublimating process; Line 7: at 60 ^{o}C, with chemical extraction method combined column purified process; and Line 8: at 60 ^{o}C, with glass bead extraction method combined spin filter purified process.

single band for each sample (Fig. 4.13). This result allowed identification and confirmation of bacterial 16S rDNA amplicons of the first PCR.

To compare the extraction efficiency between different extraction methods, the DGGE gel was loaded the same amount of DNA with 2,400 ng of DNA in each well. The results of DGGE, using different extraction methods at 50 ^{o}C and 60 ^{o}C were shown in Fig. 4.14. and the gradient of denaturant range was from 30% to 60%. 26, 36, 35, 31, 22, 33 and 30 bands were seen from biofilm at different extraction methods at 50 ^{o}C and 60 ^{o}C, respectively (Fig.4.14). The isopropanol purification process was remarkable, compared with the number of visualized bands in Line 1 and Line 2. Effect of temperature was only found on traditional phenol-chloroform method because the number of the visualized bands decreased from 36 to 22.

The similarity analysis for PCR-DGGE using different extraction methods at 50 ^{o}C and 60 ^{o}C was shown in Table 4.13. Although purification of PCR products with isopropanol did not improve the separation of DNA fragments by DGGE, it did improve the resolution of the analysis at 50 ^{o}C (Line 1 and Line 2 in Fig. 4.14). The result indicted the moderate relatedness showed a higher similarity between the samples which used the purity processes at same operational temperature ($Cs \geqq 0.7$). The significance analysis was determining

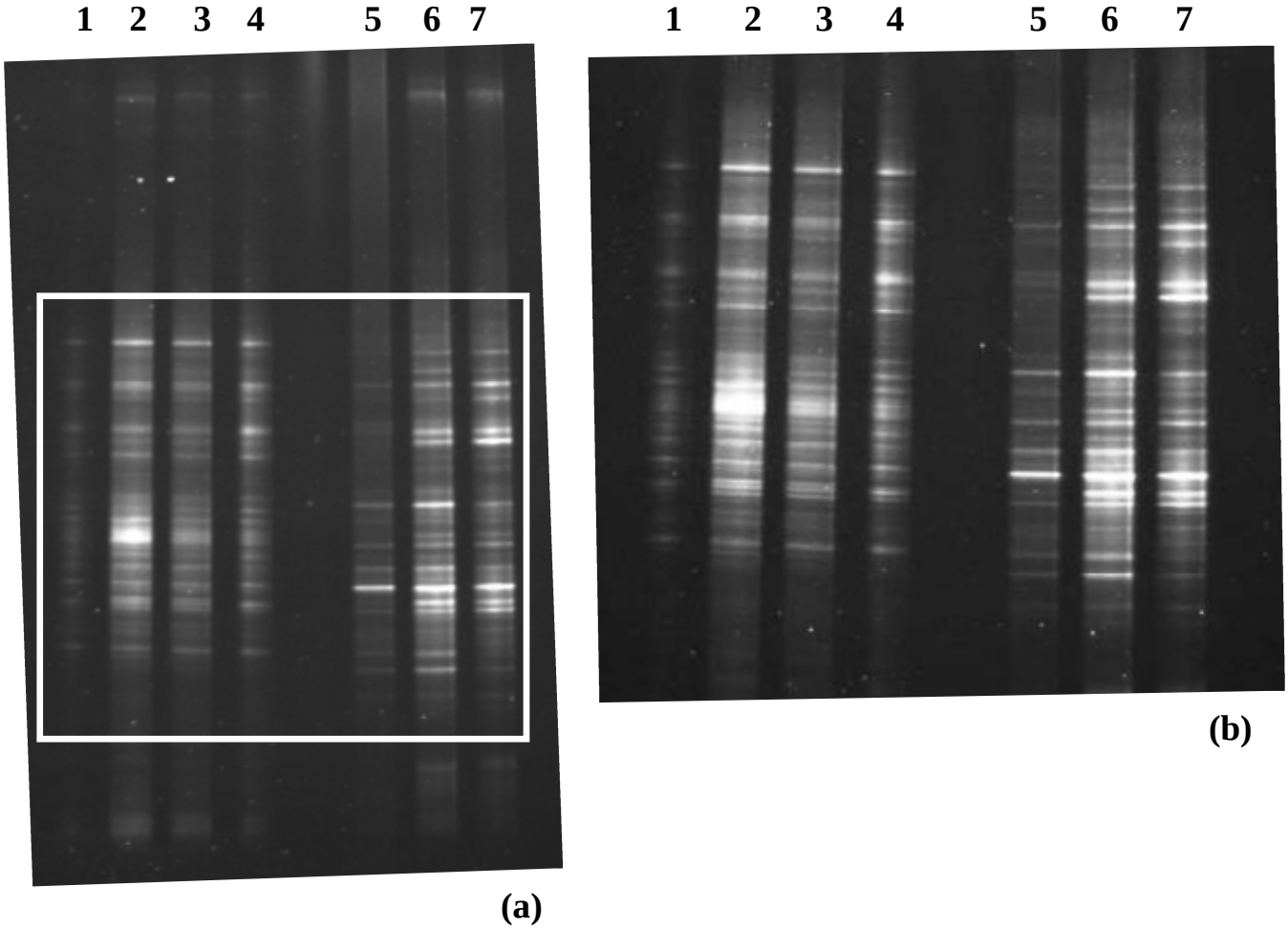


Fig. 4.14. (a) Picture shown the PCR-DGGE profile of bacterial 16S rDNA of the biofilm extracted by using different extraction methods and the gradient of denaturant range was decided from 30% to 60%. (b) amplified from the area select in picture a. Line 1: at 50 ^{o}C, with phenol-chloroform method; Line 2: at 50 ^{o}C, with phenol-chloroform method combined isopropanol purification process; Line 3: at 50 ^{o}C, with chemical extraction method combined column purified process; Line 4: at 50 ^{o}C, with glass bead extraction method combined spin filter purified process; Line 5: at 60 ^{o}C, with phenol-chloroform method combined isopropanol sublimating process; Line 6: at 60 ^{o}C, with chemical extraction method combined column purified process; and Line 7: at 60 ^{o}C, with glass bead extraction method combined spin filter purified process.

Table 4.13 Similarity analysis of microbial community on the biofilm using the Dice Index (Cs) for PCR-DGGE using different extraction methods at 50 ^{o}C and 60 ^{o}C

	Line 1	**Line 2**	**Line 3**	**Line 4**	**Line 5**	**Line 6**	**Line 7**
Line 1	1	-	-	-	-	-	-
Line 2	0.61	1	-	-	-	-	-
Line 3	0.62	0.85	1	-	-	-	-
Line 4	0.63	0.84	0.76	1	-	-	-
Line 5	0.29	0.41	0.35	0.45	1	-	-
Line 6	0.41	0.52	0.53	0.53	0.73	1	-
Line 7	0.39	0.55	0.52	0.52	0.69	0.89	1

Table 4.14 Significance analysis of microbial community on the biofilm using chi-square analysis for PCR-DGGE using different extraction methods at 50 ^{o}C and 60 ^{o}C

	Line 1	**Line 2**	**Line 3**	**Line 4**	**Line 5**	**Line 6**	**Line 7**
Line 1	-	-	-	-	-	-	-
Line 2	3.28	-	-	-	-	-	-
Line 3	3.32	24.47	-	-	-	-	-
Line 4	3.42	22.13	14.73	-	-	-	-
Line 5	6.37	0.97	3.90	0.24	-	-	-
Line 6	1.92	0.17	0.18	0.19	11.66	-	-
Line 7	1.96	0.74	0.19	0.20	6.17	27.76	-

by chi-square analysis, results was shown in Table 4.14. In this section, the assumption of chi-square with one degree of freedom and probability was lower than 0.05 ($p<0.05$). In the chi-square analysis, the assumption of one degree of freedom and a probability of lower than 0.05 ($p<0.05$) were made. According to the chi-square distribution, a significant difference between two compared samples was suggested when the total chi-square value was greater than 3.841. The results indicated that traditional phenol-chloroform method combined isopropanol purification process was affect the performance of microbial communities and the method of Viogene Genomic DNA Miniprep Kit has more richness than another commercial kit, while the relationship between two commercial DNA extraction kits was signified ether at 50 ^{o}C or at 60 ^{o}C.

Chapter 5 Conclusions and Suggestions

5.1 Conclusions

The MBBR system is shown to be successfully operated with 60.0 kg COD/m^3d and has also been proved to be able to operate at high temperature up to 50 ^oC (Dalentoft and Thulin, 1997). Results from this study showed that the Kaldnes MBBR can be operated at temperature up to 60 ^oC to efficiently remove COD from secondary-fiber papermaking wastewater. In this study, an aerobic thermophilic activated sludge process combined with Kaldnes MBBR system is proved to be feasibility in treating secondary-fiber papermaking wastewater with increasing removal efficiency of COD and SCOD following the rising operated temperature but with decreasing in TS removal. The average effluent COD is 144.0 mg/L at 60 ^oC which met the discharged effluent limits without chemical addition. The rising temperature affects the COD removal without significant effects on both SCOD and TS removal efficiency determining by using single factor analysis of variance (ANOVA). Addition of a thermophilic bacterium to the MBBR system does not show effect on increasing COD removal.

3 different DNA extraction methods are used in this study to compare their efficiency of DNA extraction. The results shows the conventional phenol-chloroform extraction has the highest efficiency for extracting genomic DNA

from the biofilm on Kaldnes carrier than the other two commercial DNA extraction kits do, but with lower purity. After the isopropanol purification process, the purity of extracted DNA by using traditional phenol-chloroform method improved and gave more microbiological richness than other extraction kits from the biofilm at 50 ^{o}C. According to the chi-square analysis, the method of Viogene Genomic DNA Miniprep Kit has more richness than another commercial kit. The results of DGGE analysis of microbial community indicates that the richness of the biofilm complies with rising temperature, and high amount of bacterial species is existed at 50 ^{o}C, who may be helpful for COD removal. Based on Dice index (Cs) and chi-square analyses, the results indicated that the microbial community of the biofilm changed with operation temperature at an interval of 10 ^{o}C, expect for 50^{o}C and 60^{o}C. In this study, the DGGE method has the potential to be developed as a monitoring method for optimal operation stratagem of the MBBR system.

5.2 Suggestions

The removal efficiency in this study may be improved with higher carrier fill rate with more powerful aeration equipment. The strain BL11 in this study does not show the capability to increase COD removal efficiency larger amount of pre-grown culture may be used and added to the system to improve COD removal. Oxygen

Uptake Rate (OUR) can be used to calculate the amount of biomass or even detect the development of biofilm onto carrier with a suitable calibration curve.

The bands on DGGE gel are too close that make the gel re-extraction more difficult. A lower denaturant gradient of the polyacrylamide gel, along with more electrophoretic time may be merit to test in order to improve separation of each DNA fragments.

References

Broch-Due, A., R. Andersen and B. Opheim. 1997. Treatment of integrated newsprint mill wastewater in moving bed biofilm reactors. *Water Science &Technology* **35**(2-3): 173-180.

Cecchi, F., P. Pavan, A. Musacco, J. M. Alvarez and G. Vallini. 1993. Digesting the organic fraction of municipal solid waste: moving from mesophilic (37 ^{o}C) to thermophilic (55 ^{o}C) conditions. *Waste Management & Research* **11**: 403-414.

Chai, X.-S., J. C. Samp, Q.F. Yang, H.N. Song, D.C. Zhang and J.Y. Zhu. 2006. Determination of microstickies in recycled whitewater by headspace gas chromatography. *Journal of Chromatography A* **1108**: 14-19.

Cocolin, L., A. Daniele, M. Marisa, C. Carlo and C. Giuseppe. 2002. An application of PCR-DGGE analysis to profile the yeast populations in raw milk. *International Dairy Journal* **12**: 407-411.

Dalentoft, E. and P. Thulin. 1997. The use of the Kaldness suspened carrier process in treatment of wasterwaters from the forest industry. *Water Science & Technology* **35**: 123-130.

Dice, L. R. 1945. Measures of the amount of ecological association between species. *Ecology* **26**: 297–302.

Fasoli, S., M. Marzotto, L. Rizzotti, F. Rossi, F. Dellaglio and S. Torriani. 2003. Bacterial composition of commercial probiotic products as evaluated by PCR-DGGE analysis. *International Journal of Food Microbiology* **82**: 59-70.

Gafan, G. P. and D. A. Spratt. 2005. Denaturing gradient gel electrophoresis gel expansion (DGGEGE) - An attempt to resolve the limitations of co-migration in the DGGE of complex polymicrobial communities. *FEMS Microbiology Letters* **253**(2): 303-307.

Grosmann, H. and L. Salmén. 1997. Exploration of new scientific ideas for significant electricity savings in mechanical upgrading of pulp fibres for papermaking. *Applied Thermal Engineermg* **17**(8-10): 947-954.

Jahren, S. J., J. A. Rintala and H. Ødegaard. 2002. Aerobic moving bed biofilm reactor treating thermomechanical pulping whitewater under thermophilic conditions. *Water Research* **36**: 1067-1075.

Jahren, S. J. and H. Ødegaard. 1999. Treatment of thermomechanical pulping (TMP) whitewater in thermophilic (55 ^{o}C) anaerobic-aerobic moving bed biofilm reactors. *Water Science & Technology* **40**(8): 81-89.

Järvinen, R.. 1997. Nitrogen in the effluent of the pulp and paper industry. *Water Science & Technology* **35**: 139-145.

Kim, M., Y. H. Ahn and R.E. Speece. 2002. Comparative process stability and efficiency of anaerobic digestion; mesophilic vs. thermophilic. *Water Research* **36**: 4369-4385.

Ko, C. H., W. L. Chen, C. H. Tsai, W. N. Jane,C. C. Liu and J. Tu. 2006. *Paenibacillus campinasensis* BL11: A wood material-utilizing bacterial strain isolated from black liquor. *Bioresource Technology* (in print).

Kurian, R., C. Acharya, G. Nakhla and A. Bassi. 2005. Conventional and thermophilic aerobic treatability of high strength oily pet food wastewater using membrane-coupled bioreactors. *Water Research* **39**: 4299-4308.

LaPara, T. M., C. H. Nakatsu, L. M. Pantea and J. E. Alleman. 2001. Aerobic biological treatment of a pharmaceutical wastewater: effect of temperature on COD removal and bacterial community development. *Water Research* **35**(18): 4417-4425.

LaPara, T. M. and J. E. Alleman. 1999. Review paper-Thermophilic aerobic biological wastewater treatment. *Water Research* **33**(4): 895-908.

Le Bihan, Y. and P. Lessard. 2000. Monitoring biofilter clogging: biochemical characteristics of the biomass. *Water Research* **34**(17): 4284-4294.

Loukidou, M. X. and A. I. Zouboulis. 2001. Comparison of two biological treatment processes using attached-growth biomass for sanitary landfill leachate treatment. *Environmental Pollution* **111**: 273-281.

Lyautey, E., B. Lacoste, L. Ten-Hage, J. L. Rols and F. Garabetian. 2005. Analysis of bacterial diversity in river biofilms using16S rDNA PCR-DGGE: methodological settings and fingerprints interpretation. *Water Research* **39**: 380-388.

Mette, H. N. and N. B. Ramsing. 2002. Denaturing gradient gel electrophoresis (DGGE) approaches to study the diversity of ammonia-oxidizing bacteria. *Journal of Microbiological Methods* **50**: 189- 203.

Muyzer, G. 1999. DGGE/TGGE a method for identifying genes from natural ecosystems. *Current Opinion in Microbiology* **2**: 317-322.

Muyzer, G. and K. Smalla. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek* **73**: 127-141.

Muyzer, G., E.C. de Waal and A. G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis and polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* **59**: 695-700.

Pokhrel, D. and T. Viraraghavan. 2004. Treatment of pulp and paper mill wastewater-a review. *Science of the Total Environment* **333**: 37-58.

Pritchard, L., D. Corne, D. Kell, J. Rowland and M. Winson. 2005. A general model of error-prone PCR. *Journal of Theoretical Biology* **234**: 497-509.

Raposo, F., R. Borja., E. Sáchez., M. A. Martí and A. Martí. 2004. Performance and kinetic evaluation of the anaerobic digestion of two-phase olive mill effluents in reactors with suspended and immobilized biomass. *Water Research* **38**: 2017-2026.

Ritchie, N. J. and D. D. Myrold. 1999. Phylogenetic placement of uncultured *Ceanothus* microsymbionts using 16S rRNA gene sequences. *Canadian Journal of Botany* **77**: 1208-1213.

Rodgers, M. and X.-M. Zhan. 2003. Moving-medium biofilm reactors.

Reviews in Environmental Science and Bio/Technology **2**: 213-224.

Rodgers, M., X.-M. Zhan and B. Gallagher. 2003. A pilot plant study using a vertically moving biofilm process to treat municipal wastewater. *Bioresource Technology* **89**: 139–143.

Rusten, B., B. Eikebrokk, Y. Ulgenes, and E. Lygren. 2006. Design and operations of the Kaldnes moving bed biofilm reactors. *Aquacultural Engineering* **34**: 322-331.

Rusten, B., O. Kolkinn and H. Ødegaard. 1997. Moving bed biofilm reactors and chemical precipitation for high efficiency treatment of wastewater from small communities. *Water Science & Technology* **35**(6): 71-79.

Semmens, M. J., K. Dahm, J. Shanahan, A. Christianson. 2003. COD and nitrogen removal by biofilms growing on gas permeable membranes. *Water Research* **37**: 4343–4350.

Suvilampi, J., A. Lehtomäki and J. Rintala. 2005. Comparative study of laboratory-scale thermophilic and mesophilic activated sludge processes. *Water Research* **39**: 741-750.

Suvilampi, J., A. Lehtomäi and J. Rintala. 2003a. Comparison of laboratory-scale thermophilic biofilm and activated sludge processes integrated with a mesophilic activated sludge process. *Bioresource Technology* **88**: 207-214.

Suvilampi, J. E., J. A. Rintala and J. M. K. Nuortila-Jokinen. 2003b. On-site aerobic treatment at high and varying process water temperatures. *TAPPI Journal*, Online Exclusive **2**(5).

Tardif, O. and E. R. Hall. 1997. Alternatives for treating recirculated newsprint whitewater at high temperatures. *Water Science & Technology* **35**(2-3): 57-65.

Tchobanoglous, G. and F. L. Burton. 2004. Wastewater engineering-Treatment, Disposal and Reuse by Metealf and Eddy, Inc, 4th ed. Mc Graw Hill, New York, USA.

Thompson, G., J. Swain, M. Kay and C.F. Forster. 2001. The treatment of pulp and paper mill effluent: a review. *Bioresource Technology* **77**: 275-286.

Tiirola, M. A., J. E. Suvilampi, M. S. Kulomaa and J. A. Rintala. 2003. Microbial diversity in a thermophilic aerobic biofilm process: analysis by length heterogeneity PCR (LH-PCR). *Water Research* **37**: 2259-2268.

Tripathi, C. S. and D. G. Allen. 1999. Comparison of mesophilic and thermophilic aerobic biological treatment in sequencing batch reactors treating bleached kraft pulp mill effluent. *Water Research* **33**(3): 836-846.

Vogelaar, J. C. T., E. Bouwhuis, A. Klapwijk, H. Spanjers and J. B. van Lier. 2002. Mesophilic and thermophilic activated sludge post-treatment of paper mill process water. *Water Research* **36**: 1869-1879.

Walter, B., C. Haase and N. Räbiger. 2005. Combined nitrification/denitrification in a membrane reactor. *Water Research* **39**: 2781-2788.

Wang, H., G. Yu, G. Liu and F. Pan. 2006. A new way to cultivate aerobic granules in the process of papermaking wastewater treatment. *Biochemical Engineering Journal* **28**: 99-103.

廖經裕。2004。以分子生物方法與培養方式分析台灣兩處溫泉之嗜熱菌族群。東海大學環境科學系研究所碩士論文。

盧重興，白曛綾，張國財與許世杰。2004。操作績效自我評估管理制度手冊-生物濾床。

經濟部工業局。1997。廢水處理功能生物診斷技術。

黃宗煌，蔡攀龍，李顯峰與蕭代基。國際環保公約、貿易組織與協定之概述。1996。農業金融論叢，第36輯，第43-92頁。

楊錦麟，鍾引驊，許添順，陳正一與曾治乾。2004。生物流動床技術(MBBR)結合生物活性碳系統(BAC)處理綜合性工業廢水之模廠應用實例。產業環保工程實務技術研討會論文集。第 278-294 頁。