Thesis for the Degree of Master of Fisheries Science

Characterization of anaerobic fermentation of rainbow trout *(Oncorhynchus mykiss)* effluent sludge using mixed anaerobes for reutilization

by

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KOICA-PKNU International Graduate Program of Fisheries Science

Graduate School of Global Fisheries

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무지개송어(*Oncorhynchus mykiss*) 양식장 슬러지 처리와 재이용을 위한 혐기성 혼합 균주의 특성

Advisor: Professor Joong Kyun Kim

by

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Abstract

Sludge degrading strains were screened and examined for their acidogenic ability. Two strains isolated from pond-bottom soil and identified as *Alcaligenes faecalis strain HCB2- A1* and *Alcaligenes faecalis A2* were determined to be useful sludge degrading strains. Strain A2 exhibited the highest acidogenic ability compared to A1 however the acidogenic ability of both strains were improved when cultured together producing a maximum of 6632 mg/L total volatile fatty acids. A gas chromatographic analysis done on the culture supernatant revealed that propionic and butyric acid were the dominant volatile fatty acids. The fermentation of rainbow trout sludge substrate revealed a steadily digesting system with total solids for the

substrates being reduced by between 42-58%. With 50PS:50SS mixture exhibiting the greatest reduction in total solids percentage. Chemical oxygen demand removal was also revealed in all substrates with removal efficiency in all the substrates ranging between 40-80% with 50PS:50SS having the highest (80%). This was also shown with the gradual decline in total nitrogen % and C:N ratio with digestion stabilizing around C:N ratio of 20:1. The volatile fatty acids production was also very high with toxicity levels being controlled by buffering thereby increasing and stabilizing the digestion process. This report is the first description of optimizing and balancing between secondary and primary sludge mixtures in an anaerobic digestion process. It demonstrates that a primary/secondary co-mixture digestion process is feasible and can eventually resolve the various environmental pollution problems associated with aquaculture sludge management.

1. Introduction

The production and processing of Rainbow trout (*Onchorhynchus mykiss*) is an important export oriented agro-industry in many tropical and subtropical countries. Currently, 25% of total production of farmed Salmonidae belongs to this species and affects an important portion of world food security, consumption and dissemination, and increases the fish consumption per capita in the world (FAO Fishery Statistics, 2012). Chile is currently the largest trout producer, and other major producing countries include Norway, France, Italy, Spain, Denmark, USA, Germany, Iran and the UK. The African continent also play an important role in trout production with total inland aquaculture for all species being about 1 485 367 tonnes of fish produced per year. In Zimbabwe trout aquaculture has risen to an estimated 1000 tonnes from subsistence and commercial aquaculture (FAO Fishery Statistics, 2012).

Commercial rainbow trout culture is mostly practiced as monoculture under intensive systems and it makes the operation economically attractive. Ground water can be used as trout culture water source where

pumping is not required, but aeration may be necessary in some cases. Supersaturated well-water with dissolved nitrogen can cause gas bubbles in the blood of fish and this affects circulation, a condition known as gas-bubble disease. Alternatively, river water can be used where it is diverted from its natural course, but temperature and flow fluctuations alter production capacity. Where these criteria are met, trouts are generally grown in raceways or ponds supplied with flowing water, but some are produced in cages and recirculating systems. The effluent water and the waste generated under these production systems are usually discharged as thickened sludge.

Sludge usually first accumulates at the bottom of settling sedimentation tanks before being discharged. It is mainly composed of dead fishes, fish faecal matter and unused feed in the water. The sludge is stored primarily in sedimentation tanks and then discharged into rivers or secondarily kept in waste stabilization ponds on the farm (Fig 1). Thus primary sludge is fresh at slight or no decomposition stage while secondary sludge is at an advanced stage of decomposition where it is kept in the stabilization ponds.

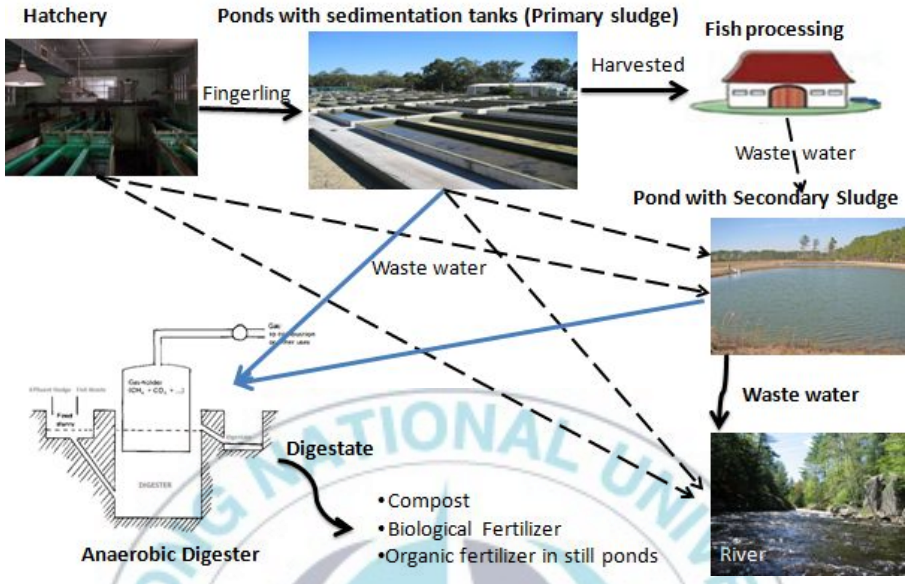


Fig. 1. A management model for rainbow trout wastes

Three primary pollutants discharged in sludge are pathogenic bacteria or parasites, therapeutic chemicals, antibiotics and metabolic products and food wastes (Beveridge *et al.,* 1991). Metabolic products (dissolved nutrients and dissolved oxygen uptake) and food wastes (solids and particulate nutrients) in trout farm effluents have received extensive research. Most raceway effluent also contains < 500 mg/L total suspended solids (TSS) (Fornshell, 2001). Cripps (1995) observed that a significantly large percentage of total particle volume is made up of larger particles (>60 μm), even though nutrient concentrations in smaller particles were greater in suspended solids and faeces.

Suspended solids and faeces per unit of feed in rainbow trout production averaged about 18-30% (Heinen *et al.,* 1996). Suspended solids and faeces have been observed to average 4.5 mg/l/day during normal operations while rising to 17.1 mg/l/day during cleaning and harvesting (Kelley *et al.,* 1997). Solids production of 30-50% of daily feed input is also common with a feed conversion ratio (kg feed: kg fish) gain of 0.9-1.0 (Cripps and Bergheim, 2000). Additional suspended solids generation can result from overfeeding which has been averaged as 150-200 g/kg feed (Bergheim, 2000). Combined,

50% or more of feed by weight can ultimately result in suspended solids that accumulate in raceways or are lost to receiving waters (Summerfelt, 1999). Studies have been conducted to characterize the nutrient composition of trout solids. Analysis of this effluent have identified that the majority of nitrogen, phosphorus and organic carbon are sediment bound (VWRC, 2002). Relative to total effluent loads reported filterable or settleable solids contain 30-80% of the effluent phosphorus (P) but only 15-32% of the total nitrogen (N) in effluent (Heinen *et al.,* 1996; Foy and Rosell, 1991; Bergheim *et al.,* 1993). Axler *et al.,* (1997) evaluated trout solids that had accumulated over a two week period and found that the nitrogen to phosphorus ratio (N:P) ranged from 1.1-3.1. Naylor *et al.,* (1999) defined a N:P ratio closer to 1.0 after evaluating solids from 12 commercial rainbow trout farms in Canada. Both studies indicate that waste solids are enriched in phosphorus relative to nitrogen given typical feed N:P ratios that range from 4-7 (Gatlin and Hardy, 2002). Stewart *et al.,* (2006) characterized trout sludge over a 7-day period on commercial trout farm. They found total solids averaging 67.8-79.5 g/l while total carbon averaged 3580-3690 mg/L. Total phosphorus content averaged 2325-2485 mg/L while ortho-phosphate content was between 283-233 mg/L. Total Kjeldahl nitrogen averaged 1990-

2010 mg/L while total ammonia nitrogen (400-433 mg/L), nitrite (2.33-2.67 mg/L), nitrate (67-100 mg/L) and total nitrogen (2059-2112 mg/L). The results from these studies indicate that trout sludge has significant solid and nutrient content thus their removal before discharge will greatly improve trout farm effluent water quality.

Trout farm effluent water quality varies with environmental factors such as influent water quality and flow rate, fish size and stocking density (Axler *et al.,* 1997). Other factors affecting effluent quality include facility management and feed types (Flimlin *et al.,* 2003), frequency of cleaning (IDEQ, 1998), and sedimentation (Bergheim *et al.,* 1991; Boardman *et al.,* 1998; IDEQ, 1998). Effluent pollutant levels can be many times higher during raceway harvesting or cleaning (Kelley *et al.,* 1997; Boardman *et al.,* 1998). Most of the aquaculture waste water is released directly into rivers or shallow coastal habitats thereby determining their organic and nutrient loads (mainly carbon, nitrogen and phosphorus as feed components, excreta and faeces). This causes bad smells and greatly pollutes the environment producing a significant adverse effect on ecosystems. These wastes sometimes become an environment threat due to their accumulation and slow degradation (Wang *et al.,* 2012). Elevated nutrients reduce water

quality (increasing biological oxygen demand, reducing dissolved oxygen and increasing turbidity) and increase the growth of algae and aquatic plants. Output restrictions require farms to have settling areas to remove solid wastes, though soluble phosphorous in the effluent cannot be removed economically - hence reductions in feed are needed to address the problem. To curtail these problems, in recent years, many countries have adopted national legislation that deals with waste disposal and have intensified environmental regulation. As a result of this, the benefit of economies of scale has driven most trout aquaculture farms to intensify production while reducing effluent flows and pollutant releases (Summerfelt and Wade, 1997). Chile, for example, requires aquaculture farms to adopt measures to prevent the dumping of solid or liquid waste and residue that could harm the surrounding environment (Chile, Environmental Regulation No. 320, Article 4 (2001). Most rainbow trout producing countries now allow the responsible departments to attach conditions to an aquaculture license to minimize the disposal of dead fish, the escape of waste products, and the pollution of the water in and around the aquaculture facility. Most aquaculture farms now prioritize solids removal from effluent streams as a means for reducing pollutant loading to receiving streams thus meeting licence requirements.

There is some potential for gaining more value from the solids removed, thus the need to develop economic but eco-friendly fish waste treatment methods for its safe disposal such as anaerobic digestion.

Anaerobic digestion as a natural process enables the re-entering of organic materials into the environmental cycle. As an industrial process it can be helpful to solve problems of environmental pollution and recovery of natural resources but needs special efforts for stable and efficient operation. Anaerobic digestion is a sequence of complicated metabolic procedures of a great number of populations of microorganisms living more or less in symbiosis. The initial stage named hydrolysis involves the decomposition of solid organic matter into water-soluble compounds by extra-cellular bacterial enzymes. The second stage called acidogenesis involves utilization of the soluble high molecular substrate by different species of microorganisms which set free intermediates like alcohols, sugars and primarily C_3- to C_5- organic acids such as acetic acid, butyric acid and propionic acid. The last stage called methanogenesis involves usage of these intermediates by end-of-metabolic chain methanogens with the gaseous end products of CH_4 and CO_2. With the help of experimental results and the

analytical description of complex organic compounds it is possible to develop models of energy and mass balances for the digestion of wastes like rainbow trout sludge as well as heterogeneous bulk biowastes. The worldwide revival of the anaerobic process for the treatment of biowastes and organic residues has enhanced the general interest especially to find out the capabilities and limits of this technology. Anaerobic digestion offers improved energy conservation with potential reduction in greenhouse gas emissions. It results not only in sludge mass reduction, but also in water and energy savings, as well as in biogas production which can serve as an alternative energy source and partially cover the energy demand (Natella *et al.,* 2010). When various organic wastes either un-treated or pre-treated are digested together, this has proved to overcome the inhibitory technological problems and improve digestion efficiency. This is usually performed with at least two different but occasionally complementary substrates with many possible ecological, technological and economical benefits (Alvarez and Liden, 2008). Despite the well known reported co-digestion benefits, it is not clear whether some co-substrates have adverse impact when they are co-digested with another waste in particular if there is synergisms or antagonisms among the co-digested substrates and if several co-substrates of

similar biochemical composition can be co-digested (Malta-Alvarez *et al.,* 2004; Callaghan *et al.,* 2002). Therefore, it is critical to obtain an optimal mixture of the available co-substrates as well as the optimum operating conditions, which allow high benefits without compromising the efficiency of the digestion process (Alvarez *et al.,* 2009; Thirumurugan and Gopalakrishnan, 2012). These operating conditions are critical for the efficient anaerobic treatment by the responsible microorganisms.

A wide variety of microbial communities have been reported to be involved in the anaerobic decomposition process. Fricke *et al.* (2007) reported that organic material is most likely decomposed by heterotrophic microorganisms. Lee *et al*. (2009a) also reported that Clostridium species are most common among the degraders under anaerobic condition. However, it is very unusual for a biological treatment to rely solely on a single microbial strain and generally a microbial consortium is responsible for the anaerobic digestion process (Fantozzi and Buratti, 2009). According to Ike *et al.* (2010), a group of microorganisms such as Actinomycetes, Thermomonospora, Ralstonia and Shewanella are involved in the degradation of food waste into volatile fatty acids, but Methanosarcina and

Methanobrevibacter/Methanobacterium mainly contribute in methane production. An increase in methane content was also observed with the increase in the number of hydrogenotrophic species (Trzcinski and Stuckey, 2010). However, high concentration of organic acid like acetic acid (>5000 mg/L) and butyric acid (>3000 mg/L) in the bio-digester has been found to inhibit the growth of microorganisms and consequently the production of energy rich compounds (Kim *et al.,* 2008). The use of these microorganisms has proved that waste reutilization is possible after anaerobic sludge-mass reduction.

A reduction in sludge-mass minimizes the potential environment hazard and economic burden stemming from its disposal. Removing solids from effluent streams can provide an effective means for reducing pollutant loading to receiving streams (Cripps, 1992; Schwartz and Boyd, 1994; Boyd *et al.,* 1998; VWRC, 2002). In addition, potential utilization techniques and ecologically acceptable means of reutilization of waste sludge can be established, not only to solve the environment pollution problem created by the waste but also to provide energy needs at farm level. There is some potential for gaining more value from fish sludge, thus the need to develop

an economic but eco-friendly strategy for its safe disposal becomes imperative in fisheries waste management.

Fisheries waste management, specifically solids, has become an integral part of facility maintenance to reduce effluent loads (Bergheim *et al.,* 1991). Modern waste management systems aims at incorporating the idea of using waste from one system as inputs for another and also seek benefits from economies of scale. Fishery sludge waste has huge potential for reutilization; it mainly consists of protein, lipids, carbohydrates, ash, chitin etc; the overall percentage of each vary with type of feed or degree of decomposition. Sludge can be used to feed other fish directly such as catfish but continued use might cause undesirable high BOD. When the sludge is used as a biofertilizer without treatment there is a risk of introducing pathogens and heavy metals which can be eliminated first by biological treatment. Traditionally waste water and sludge is deposited into rivers or used as a biofertilizer without treatment but recent studies have focused on biological treatment such as bacterial fermentation as an alternative to aquaculture sludge digestion, stabilization and reutilization (Natella *et al.,* 2010).

The reutilization of waste material collected from concrete-lined ponds of a rainbow trout farm was evaluated as a fertilizer, on a strongly acidic and phosphorous deficient soil, in combination with a liming agent. Lucerne was used as the test crop. The trout farm waste was a source of available nitrogen and phosphorus and had a moderate neutralizing capacity. It did not cause changes in the composition of the herbage which would be detrimental to crop growth or grazing animals. The results should encourage the utilization of the waste as a fertilizer, rather than its disposal by discharge to water courses where it may lead to pollution. (Willett and Jakobsen, 1986). The neutralizing and fertilizing capacity of the trout waste is highly likely to be improved with biological treatment. The utilization of the digestate from biological treatments also improves pathogen control and veterinary safety when compared to untreated manure and slurries. The standardization of the digestate is by the retention of some thermopiles digestion temperature, pasteurization or pressure control. In all these cases the aim is to inactivate pathogens, weed seeds and control of other biological hazards by microbial fermentation.

Microbial fermentation is advantageous over enzyme hydrolysis as this process omit the procedure for purifying enzymes and decreases the cost. Thus anaerobic treatment methods are well accepted because of their environment compatibility, low cost and reproducibility (Wang *et al.,* 2012). There is need to develop an appropriate way of dealing with relatively small waste streams from geographically dispersed farming operations in developing countries. Most fishing and fish processing locations are usually in remote and undeveloped locations lacking such basic amenities like electricity. So energy sourcing and production become a major challenge. The challenge is greatly seen in the fish processing and storage lapses. The integrated fish and fish waste biogas projects will generate the much needed energy for fish preservations. The gas energy in its potential form can be used for fish processing and storage (Salam *et al.,* 2009). Nowadays, experiments and experience have shown that when the mass and energy balances have been properly collaborated and optimized that nothing is a waste. However, currently there is a new global paradigm where environmental conservation can be the new economic driver e.g. by developing new energy alternatives to the increased global energy demands. With environmental issues such as the greenhouse effect and correct waste

disposal methods gaining much attention throughout the fishing community, the concept of controlled anaerobic digestion is perhaps a much overlooked example of a way to reduce greenhouse gas emissions and provide a better waste disposal method for organic fish waste in fish farms, processing and packaging centers (Mata-Alvarez *et al*., 2004). Nevertheless, there is scant information available on the biodegradation of sludge from aquaculture.

This study seeks to develop an on-farm, economic but eco-friendly anaerobic biological treatment strategy for the safe disposal of primary and secondary sludge from trout farms in tropical and subtropical countries. To date, anaerobic digestion has mainly focused on fisheries processing waste instead of the huge volumes of waste water and sludge produced during fish production. In this context, anaerobic digestion of sludge has been suggested as an effective waste management alternative to the disposal and efficient management of rainbow trout sludge waste. The screening of suitable anaerobic bacteria for the effective anaerobic digestion of sludge from a rainbow trout farm is therefore necessary.

Thus the aims of this study are:

i) Isolation of acidogenic strains for the anaerobic digestion of rainbow trout sludge substrate (RTSS)

ii) Evaluate the feasibility of using rainbow trout sludge as a substrate for anaerobic digestion

iii) Evaluate the possible yield from the anaerobic digestion of RTSS

iv) Find the optimum mixture between primary and secondary sludge for the maximization of anaerobic digestion yield

To meet the above objectives we hypothesized that:

i) Null hypothesis – Co-mixture of primary and secondary sludge does not enhance acidogenic potential

ii) Alternative hypothesis – Co-mixture of primary and secondary sludge enhance acidogenic potential

2. Materials and Methods

2.1 Preparation of the rainbow trout sludge substrate (RTSS)

Primary (raw) sludge and secondary sludge was collected from Ihwajeong trout farm (Sangju, Gyeongbuk Province, South Korea). The farm is producing rainbow trout using concrete lined recirculating aquaculture system producing an average of 420 tonnes per year using underground water. The farm has been operating for ten years while the waste sampled from earthen ponds was between 1-12 months old. Primary sludge was collected at 0.5 m depth (one month old) at the mouth of the pipe from the sedimentation tanks while secondary sludge (more than one month old) was collected 10 m away in the waste stabilization pond at 2 m depth. The sludge was transferred in ice boxes to the laboratory and then stored at -20^{o}C until further use.

2.2 Pretreatment and Characterization of the RTSS

The sludge was shredded and sieved to ensure particle size of less than 2 mm and ensure homogeneity. The rainbow trout sludge substrate was then characterized to determine the following: total solids (TS), volatile solids (VS), total nitrogen content and chemical oxygen demand (Table 1a) and chemical characterization (Table 1b). The sludge was also autoclaved for 10 min at 121^{o}C and it was then preserved at -70^{o}C prior to use in the experiments.

Table 1a. Characteristics of the rainbow trout sludge substrate.

Characteristic	Primary Sludge	Secondary Sludge
pH	5.793	6.512
ORP	-160.4	-183.5
Total Solids (%)	13.65	10.40
Volatile Solids (%)	64.20	35.03
Fixed Solids (%)	35.80	64.97
COD (mg/L)	127264	70646
Total N (mg/L)	4271	2926
C:N	30:1	24:1

Table 1b. Chemical characteristics of the rainbow trout sludge substrate.

Element	Primary	Secondary
Lipid	1.23 ± 0.000	0.16 ± 0.000
Protein	3.36 ± 0.020	1.90 ± 0.000
Carbohydrate	95.41 ± 0.000	97.94 ± 0.000
Ca (%)	1.65 ± 0.023	1.47 ± 0.018
P (%)	0.80 ± 0.006	0.62 ± 0.006
K (%)	0.02 ± 0.000	0.03 ± 0.001
Mg (%)	0.04 ± 0.000	0.05 ± 0.001
Na (%)	0.02 ±0.002	0.03 ± 0.001
Zn (ppm)	200 ± 0.000	200 ± 0.000
Fe (ppm)	700 ± 10.00	900 ± 20.00
Mn (ppm)	100 ± 0.000	100 ± 0.000
Cd (ppm)	1.09 ± 0.077	0.67 ± 0.102
Cu (ppm)	n.d.	n.d.
Hg (ppm)	n.d.	n.d.
Se (ppm)	n.d.	n.d.

2.3 Isolation and identification of acidogenic strains

Pond bottom surface soil samples were collected from Pukyong University Aquaculture Farm. Approximately one gram of soil sample was inoculated into two 150 ml of acidogenic broth medium in a 250 ml conical flask (Cheong and Hansen, 2006). The medium was adjusted to pH 7.3-7.4 before autoclaving and flashed with sterile filtered 95% N_2-5% CO_2 gas atmosphere after adding the soil sample. The flasks were anaerobically sealed and then incubated at 37°C and shaken at 160 rpm for three weeks. The medium used for the isolation and screening of the acidogens was composed of glucose (21300 mg/l), meat extract (2000 mg/l), NH_4Cl (2125 mg/l), K_2HPO_4 (420 mg/l), KH_2PO_4 (1000 mg/l), $FeCl_2.4H_2O$ (180 mg/l), $CaCl_2.2H_2O$ (375 mg/l), $MgSO_4.7H_2O$ (312.5 mg/l), KCl (250 mg/l), Trace nutrients solution (1 ml/l) (composed of H_3BO_3 (50 mg/l), $ZnCl_2$ (50 mg/l), $CuCl_2$ (30 mg/l), $MnSO_4H_2O$ (500 mg/l), $(NH_4)_6Mo_7O_{24}.4H_2O$ (50 mg/l), $AlCl_3$ (50 mg/l), $CoCl_26H_2O$ (50 mg/l), $NiCl_2$ (50 mg/l), HCl (36%) (1 ml)) and $NaHCO_3$ (8000 mg/l) was added to maintain initial buffering capacity (Cheong and Hansen, 2006). Distilled water was used as dilution water.

After three weeks of incubation, 100 µl of the broth were poured onto agar plates with the above medium and bromocresol purple (0.01%). To extend the screening possibility, bromocresol purple indicator was used to test the acid producing ability of the strains on agar. After incubating at 37°C for 3 days, all types of colonies showing yellowish color around colonies were subcultured separately in glass tubes containing 3 ml of nutrient broth. The strains obtained from this screening were subcultured repeatedly in nutrient agar plate to obtain pure cultures. The agar plates were incubated in air tight gas pack containing palladium pellets to catalyze reaction removing O_2. And an anaerobic pack producing hydrogen gas and carbon dioxide was also put inside. Methylene blue was used as the anaerobic indicator for anaerobic condition. A gas pack containing sterile filtered 95% N_2-5% CO_2 gas was also attached to the gas pack, as shown in Fig 2.

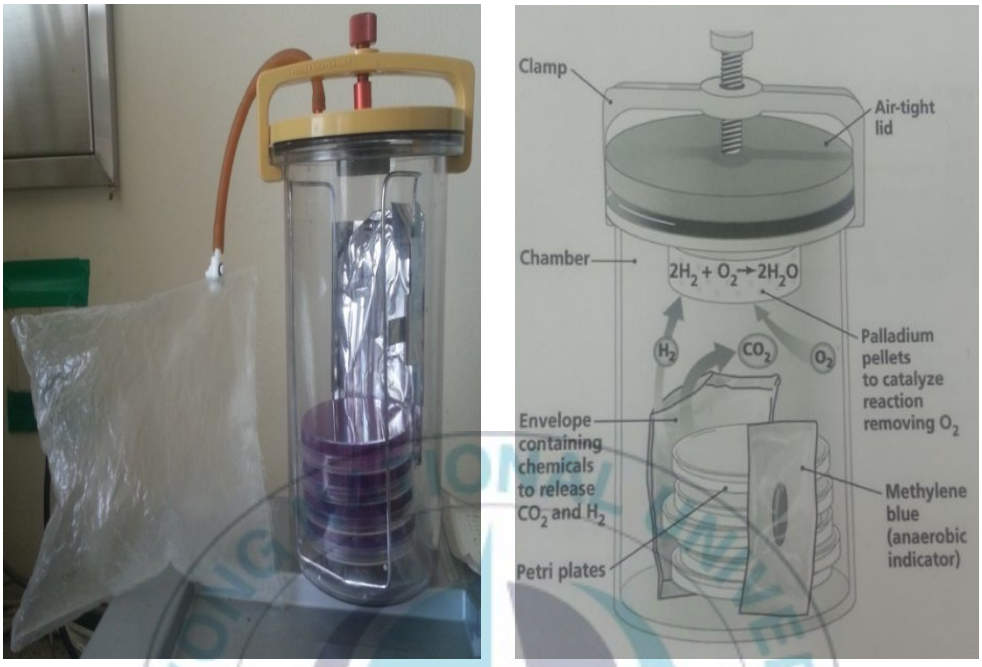


Fig. 2. Anaerobic gas pack used for incubation of petri dishes.

2.3.1 Catalase test

The acidogenic isolates taken from the soil were investigated for the presence of the catalase enzyme and identify anaerobic activity. This was done by placing 1 drop of 3% H_2O_2 onto the organism on the microscope slide using a dropper. The slide was immediately covered with a petri-dish lid to limit aerosols and observe for immediate bubble formation. The test was done in triplicates for both strains. Quality control was performed by using organisms known to be positive and negative for catalase.

2.3.2 Anaerobic activity test

The anaerobic activity and mobility of the isolated strains were tested in 15 ml tubes containing acidogenic medium (Cheong and Hansen, 2006). Acidogenic medium (10 ml) with 10% agar (pH 7.3-7.4) was put in tubes. The tubes were stab-inoculated with a tip containing the bacterial strain and were incubated for three weeks at 37°C. The growth behavior of the strains in the tubes was observed. The tubes were incubated in triplicates for both strains.

2.3.3 Acidogenic activity test

The selected strains were also tested for acidogenic activity by measuring the amount of volatile fatty acids produced in anaerobically sealed tubes containing acidogenic medium. The tubes were filled 2/3 and the remaining headspace was gassed with sterile filtered N_2/CO_2 and anaerobically sealed with butyl rubber stoppers. Culture supernatants (30%) were collected by centrifuging at 10000 rpm for 10 min at 4°C, after 24 h and inoculated into the tubes. The tubes were incubated for 60 h with samples taken every 6 h for volatile fatty acids (VFA) analysis. The mixed liquor was collected on a time interval basis, and the supernatant was roughly separated by centrifugation, and then filtered through a 0.45 μm filter into soluble components. The VFAs such as acetic acid (HAc), propionic acid (HPr), butyric acid (HBu) and valeric acid (HVa) concentration were determined by gas chromatography (Acme-6000 series, Younglin) equipped with a cross-linked polyethylene glycol (Rtx-WAX) capillary column (30 m x 250μm x 0.25μm) and an FID detector. The temperature of the injection port and detector were 220°C and 230°C, respectively. The oven temperature was set at 200°C at a speed of 15°C min^{-1}. The carrier gas was Nitrogen, with a pressure of 30.4 kPa, injected at a rate of 1.0 mL min^{-1}

(Qigui et al., 2014). The change in pH and oxidation-reduction potential (ORP) together with cell growth was also measured to monitor the strain activity.

2.3.4 Test of synergistic ability of selected strains

The antagonistic behavior of the selected strains against each other was first tested by the perpendicular streak technique as described by Alippi and Reynaldi (2006). The isolates were streaked perpendicular to each other and plates incubated at 37^{o}C in a tight gas pack and then checked for any growth inhibition of each isolate. To check the synergistic ability of the selected acidogenic strains, they were cultured together according to the above procedure. The volatile fatty acids produced by the strains combined together were also determined by gas chromatography to determine the synergistic ability.

2.3.5 Identification of isolated useful strains

After screening of isolates by the catalase test and volatile fatty acids measurements, potentially acidogenic strains were primarily characterized by colony and cell morphology, motility and gram staining. For final

identification of screen isolates, 16S rDNA sequence analysis was conducted. Genomic DNA was extracted with an AccuPrep® Genomic DNA extraction kit (Bioneer, Korea), according to the manufacturer's instructions. PCR amplification of the DNA using the universal 16S rDNA primer sets, 27F (5' – AGAGTTTGATCMTGGCTCAG-3') and 1492R (5' – TACGGYTACCTTGTTACGACTT-3') was performed with a PCR thermal cycler DICE model TP600, TaKaRa, Japan. Reaction mixture contained 10 µl of 2 x Prime Taq Premix (Prime Taq DNA polymerase 1 unit/10 µl, 2 x reaction buffer, 4mM $MgCl_2$, enzyme stabilizer, sediment, loading dye, pH 9.0, 0.5mM of each dNTPs) (GENET BIO), 1 µl each of forward and reverse primers (10 pmol µl-1), 4 µl template DNA and 4 µl sterilized distilled water to achieve a final volume of 20 µl. PCR was performed under the following conditions: initial denaturation at 94 ^{o}C for 5 min; 30 cycles of denaturation at 94 ^{o}C for 30 s, annealing at 55 ^{o}C for 30 s, and extension at 72^{o}c for 30 s; and a final extension step at 72 ^{o}C for 5 minutes. Five microliters of amplification products were separated by electrophoresis on 1 % agarose gel (Seakem LE Agarose, Cambrex BioScience, Rockland, Inc. USA) in 1 x TAE buffer at 100 V for 18 min. One kb DNA marker was loaded alongside to identify the PCR product. Gels were stained with

ethidium bromide (Bioshop Canada Inc.) and photographed under UV light. Gel images were recorded using a Polaroid camera. The sequencing of the PCR products was performed by Macrogen Ltd. (Seoul, Korea). The sequences of the 16S rDNA were compared with the available sequences in the GenBank (National Center for Biotechnology Information, Rockville Pike, Bethseda, MD) using the Advanced Basic Local Alignment Search Tool (BLAST) similarity search option accessible from the homepage at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). ClustalW program of BioEdit Sequence Alignment Editor Version 7.0.9 was used to check alignment. After identification, the isolated strains were stored on the agar slants at -70^{o}C until used and transferred to a fresh agar slant every two weeks.

2.4 Analytical Methods

Cell growth of the strains was measured using a VIS/UV spectrophotometer at 390 nm. The pH and ORP was measured by an Istek pH/ORP meter.

Total Solids, fixed solids and volatile solids were measured according to waste water analytical methods (APHA, 1998). The Chemical oxygen demand (COD_{Cr}) and total nitrogen concentrations were measured using the

spectrophotometric method using a Water-quality Analyzer (Humans Co., Ltd, Korea) (ISO, 2002). All measurements were done in triplicates.

2.5 Acidogenic digestion of rainbow trout sludge substrate

The strains exhibiting the highest acidogenic ability were used to digest primary and secondary sludge in different mixtures as outlined in Table 2. The proportions of primary and secondary sludge and distilled water appropriate to achieve 300 ml working volume mixture were calculated to achieve 10% total solids with a fixed amount of inoculum (30%). The different wastes were mixed thoroughly before entering the digester to ensure a sufficient homogeneity. The conditions of the biodigesters are outlined in Table 3. The digestion was conducted in 500 ml conical flasks with butyl rubber stoppers. The flasks were filled 2/3 and the remaining headspace was gassed with sterile filtered N_2/CO_2 and anaerobically sealed with butyl rubber stoppers. Distilled water was used to fill-up to the working volume to achieve 70% moisture content. The tests were carried out in triplicates including the control with inoculums and distilled water only. The acidogenic yield was measured everyday for a digestion period of 7 days.

The following parameters were measured everyday: pH, ORP, chemical oxygen demand, total nitrogen, total solids (%) and volatile fatty acids.

Table 2. Rainbow trout sludge substrate mixtures

Biodigester	Mixture
1	Primary sludge only (PS)
2	Secondary sludge only (SS)
3	Primary (33%) + Secondary (67%)
4	Primary (67%) + Secondary (33%)
5	Primary (50%) + Secondary (50%)
6	Control (Inoculum only)

Table 3. Conditions for acidogenic digestion

Characteristic	Digester Condition
Working volume	300 ml
Seed	Screened Acidogens
Inoculum size	100 ml (30%)
Agitation speed	160 rpm
Temperature	37^{o}C
C:N ratio	20-30:1
COD/N	45-60
Total solids (%)	10%
pH	7.3-7.4
Sampling time	Every 24 h
Running time	7 days

3. Results

3.1. Screening of acidogenic strains

After three weeks of incubation in acidogenic medium, six different types of colonies were isolated from the pond bottom mud. Two isolates from the pond bottom mud designated A1 and A2 displayed positive growth and acid producing characteristics on acidogenic agar with bromocresol purple indicator after three days of incubation. The strains showed yellowish colour around colonies to show acidogenic properties.

3.1.1. Catalase activity test

For the six strains isolated from the pond bottom mud the catalase activity test was evaluated on 24 hour colonies using 15% H_2O_2. The catalase test is valuable for differentiating aerobic and obligate anaerobic bacteria, as anaerobes are generally known to lack the catalase enzyme. Two isolates A1 and A2 displayed a negative reaction with no bubble formation against a dark background. The two strains proved to be anaerobes since they showed no presence of catalase enzyme to hydrolyze the hydrogen peroxide. Quality

control performed using known aerobic strains in the laboratory showed positive reactions.

3.1.2 Anaerobic activity of selected strains

The two strains which showed a negative catalase reaction were tested for their anaerobic activity and mobility of the isolated strains were tested in tubes containing acidogenic medium. The tubes were stab inoculated with a tip containing the bacterial strain and were incubated for three weeks at 37^{o}C. The growth behavior of the strains in the tubes was observed. The tubes displayed anaerobic activity with both strains showing positive growth away from surface of tube which had traces of oxygen. The strain growth was indicated by colour change away from the surface as shown on Fig 3. The growth away from the stabbed axis also proved the mobility of the two strains.

Fig. 3. Culture tubes showing anaerobic growth of selected strains at the bottom of the tubes. A dark colour change of about 10 mm at the bottom of the tubes showed positive anaerobic and motile activity

3.1.3. Production of fatty acids

The two strains A1 and A2 were tested for their acidogenic ability in 150 ml anaerobically sealed flasks. The acidogenic ability was revealed by the production of volatile fatty acids. All strains produced volatile fatty acids in 150 ml acidogenic medium. There was a decrease in pH due to active acidification up to 20 hours (Fig. 4) with pH falling below 5.5. This also led to a rapid drop in oxidation-reduction potential (ORP) within a 20 hour period. Oxidation-reduction potential fell to almost -200 mV with the mixed culture showing higher biochemical activity due to the lowest ORP achieved during incubation. During this period when there is active acidification there was also active cell growth as shown by the rapid increase in optical density (Fig. 6.). After 45 hours active biochemical reactions had ended for both strains with an observed increase in oxidation reduction potential.

Both strains produced high amount of volatile fatty acids. They were found to be potential candidates that could degrade sludge based on the amount of volatile fatty acids reaching almost 8000 mg/L. During the 60 hour incubation period, A2 produced the greatest quantity of VFA (7786 mg/L) within 30 hours (Fig 8). However when the strains (A1 and A2) were grown

together the total VFA production was enhanced to 7844 within 24 hours (Fig 9, 11). Similarly acetic acid production was enhanced in a mixed culture with concentration reaching a maximum of 2198 mg/L within 18 hours (Fig 10).

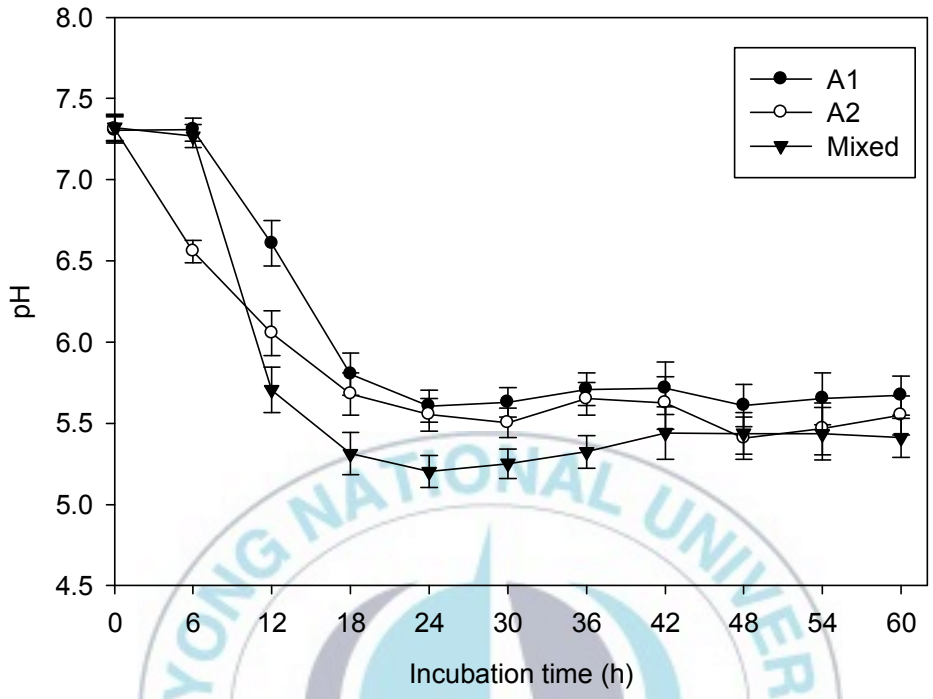


Fig. 4. Time courses of pH change during incubation of A1, A2 and mixed (A1 and A2) cultures in acidogenic medium at 37°C in a shaking incubator (160 rpm). The data are presented as the mean ± SD (n=3)

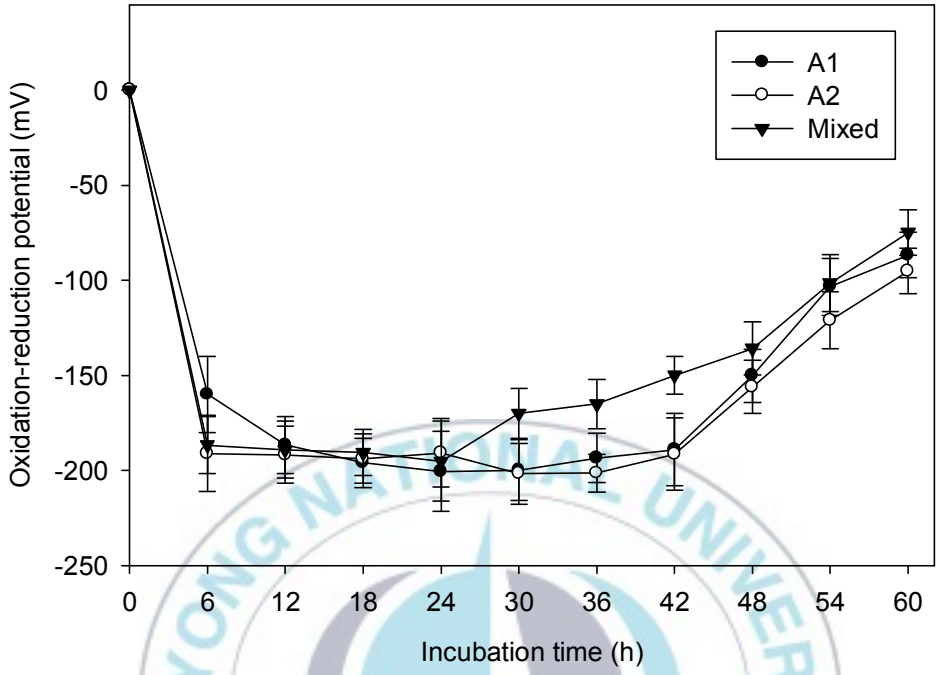


Fig. 5. Time courses of change in oxidation-reduction potential during incubation of A1, A2 and mixed (A1 and A2) cultures in acidogenic medium at 37°C in a shaking incubator (160 rpm). The data are presented as the mean ± SD (n=3)

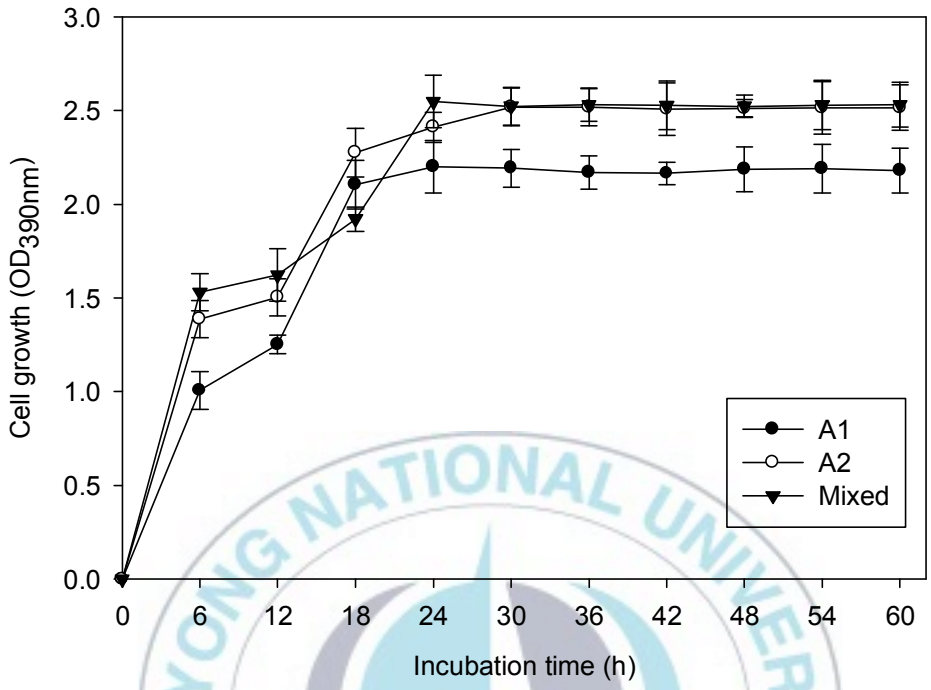


Fig. 6. Time courses of cell growth during incubation of A1, A2 and mixed (A1 and A2) cultures in acidogenic medium at 37°C in a shaking incubator (160 rpm). The data are presented as the mean ± SD (n=3)

3.1.4. Synergistic ability test on selected strains

Synergistic ability of strains A1 and A2 to degrade anaerobically was checked in terms of producing volatile fatty acids. The strains were cultured individually and also when mixed and the change in production of volatile fatty acids was observed. There was a positive synergistic ability observed by an increased amount of total volatile fatty acids (Fig 11) and acetic acid (Fig 10) when the strains were cultured together. There was also no antagonistic behavior between the two strains, when they were streaked perpendicular to each other; colonies of both strains were observed.

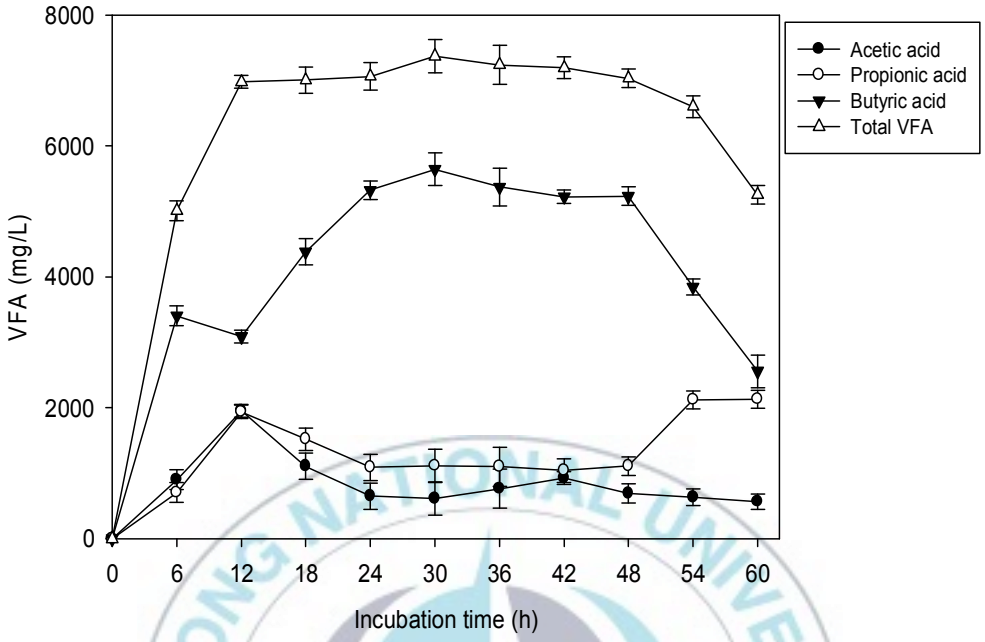


Fig. 7. Time courses of volatile fatty acids production by A1 strain cultured in acidogenic medium at 37°C in a shaking incubator (160 rpm). The data are presented as the mean ± SD (n=3)

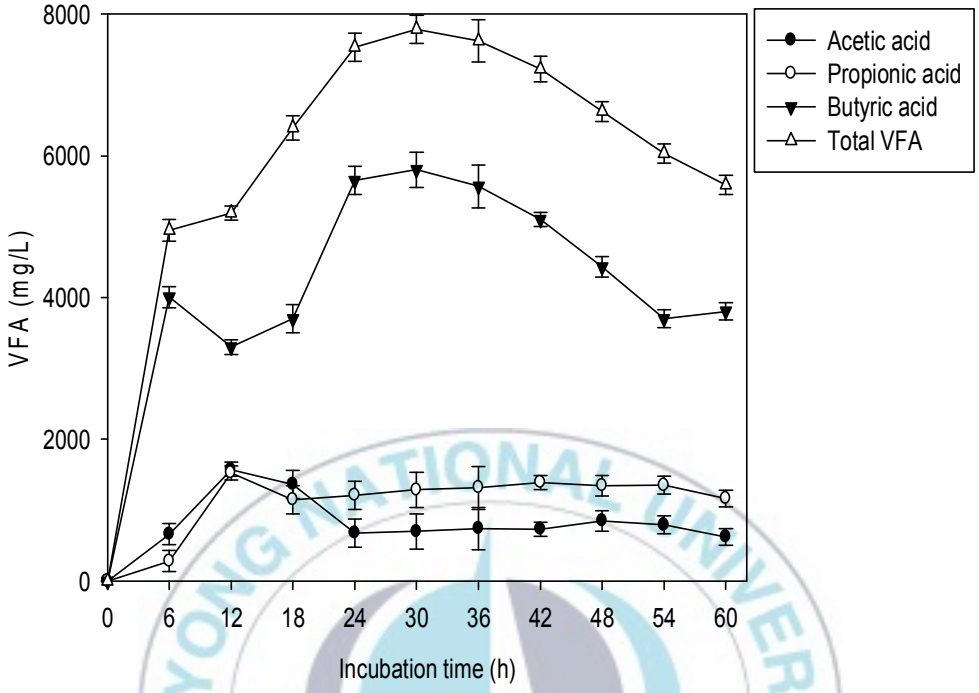


Fig. 8. Time courses of volatile fatty acids production by A2 strain cultured in acidogenic medium at 37°C in a shaking incubator (160 rpm). The data are presented as the mean ± SD (n=3)

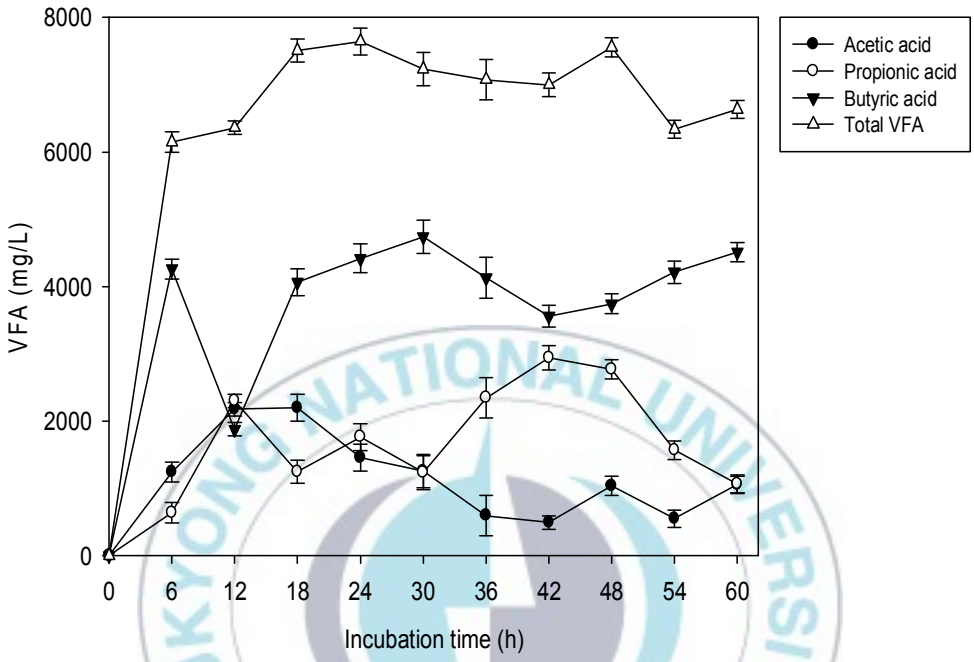


Fig. 9. Time courses of volatile fatty acids production by mixed (A1 and A2) strains cultured in acidogenic medium at 37°C in a shaking incubator (160 rpm). The data are presented as the mean ± SD (n=3)

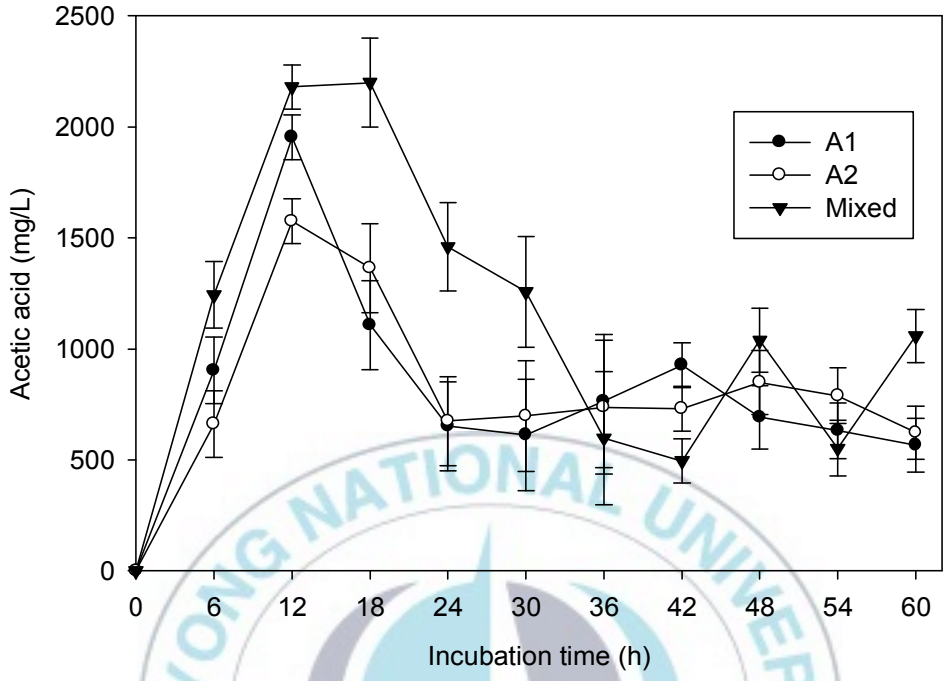


Fig. 10. Time courses of acetic acid production by A1, A2 and mixed (A1 and A2) strains cultured in acidogenic medium at 37°C in a shaking incubator (160 rpm). The data are presented as the mean ± SD (n=3)

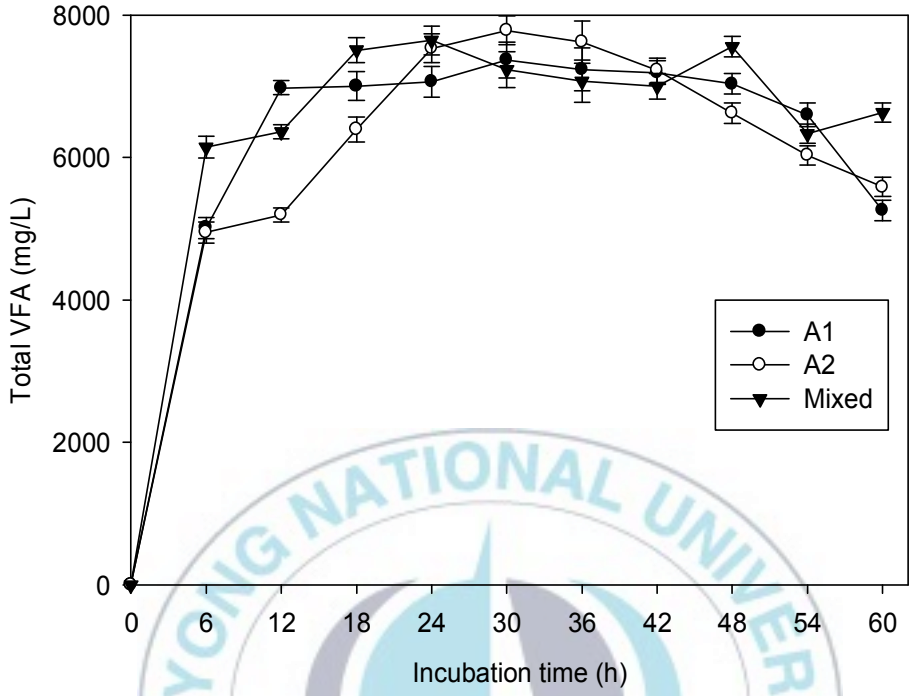


Fig. 11. Time courses of total volatile fatty acids production by A1, A2 and mixed (A1 and A2) strains cultured in acidogenic medium at 37°C in a shaking incubator (160 rpm). The data are presented as the mean ± SD (n=3)

3.2 Identification and characterization of useful acidogenic strains

When both strains were grown on acidogenic medium with bromocresol purple indicator they showed yellowish colonies due to the production of fatty acids (Fig 12). After 48 hours of incubation A1 strain showed light cream cloudy colonies which were flat and circular in shape. The A1 colonies were also slimy, soft and wet (Fig 12). A2 strain also showed cream colonies after 48 hours of incubation and were fast growing as compared to A1. The colonies for A2 were also circular, flat, soft, wet and sticky (Table 5). Microscopic examination of the A1 and A2 cells revealed that both strains were motile, gram positive rods (Table 6 and Fig 13). Under favourable anaerobic conditions short chains (2-3 cells) were formed. The two strains however did not show any spore formation.

Table 4. Identification of microorganisms isolated from the bottom mud soil

Isolate	GenBank accession no.	Identification	Similarity (%)
A1	KF534470.1	*Alcaligenes faecalis strain HCB2*	99%
A2	AF155147.1	*Alcaligenes faecalis*	98%

Table 5. Colony characteristics of newly isolated potential sludge degrading strains

Characteristic	A1	A2
Colour	Light cream/cloudy	Cream
Size	Small	Medium
Diameter (cm)	0.30-0.50	0.40-0.60
Shape	Circular	Circular
Edge	Entire	Entire
Elevation	Flat	Flat
Texture	Soft, wet and little sticky	Soft, wet and sticky

Table 6. Microscopic characterization of newly isolated potential sludge degrading strains

Characteristic	A1	A2
Shape	Rod	Rod
Length of rod (μm)	3-4	2-3
Width of rod (μm)	1-1.2	1.0-1.2
Gram reaction	Negative	Negative
Catalase reaction	Negative	Negative
Motility	Positive	Positive
Chain formation	Short chain (2-3) is common	Short chain (2-3) is common
Spore formation	No spore formation	No spore formation

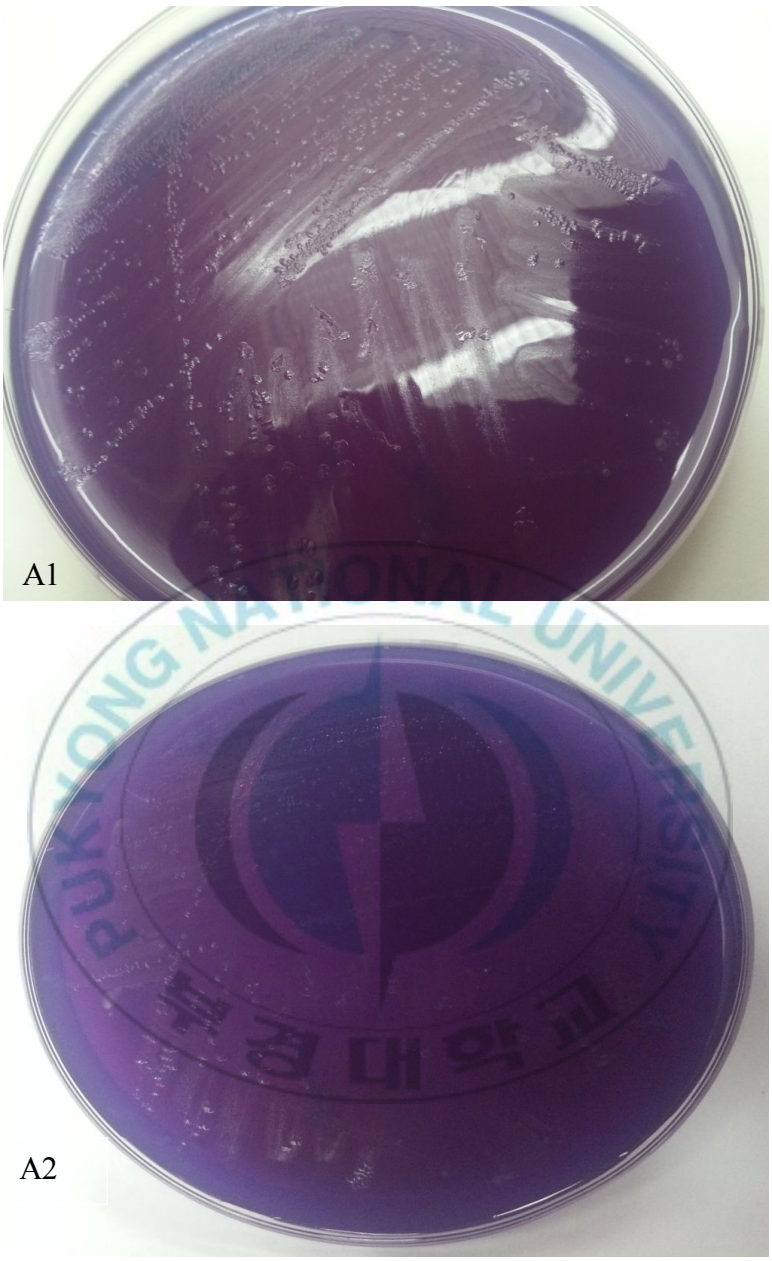
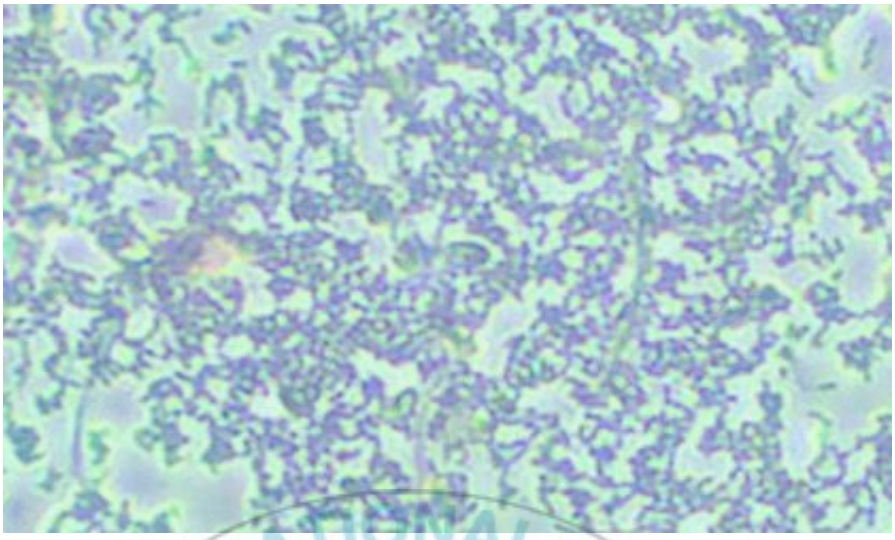


Fig.12. Pure culture of A1 and A2 after 48 hour incubation at 37°C on acidogenic agar with bromocresol purple indicator.

A1



A2

Fig. 13. Gram stained microscopic view of newly isolated potential sludge degrading strains A1 and A2 isolated from pond bottom soil

3.3 Anaerobic digestion of rainbow trout sludge substrate

3.3.1 Effect of anaerobic digestion of rainbow trout substrate mixtures on pH

After checking the acidogenic ability of the two strains, a mixed culture of them (A1 and A2) was identified as the most promising candidate for degrading sludge. The mixed culture was put as an inoculum in the different substrate mixtures and during the 7 day incubation period there was a gradual drop from the adjusted pH range of 7.3-7.4. With the biggest drop on pH occurring with 24 hours to between 6.4-6.6 in all substrate mixtures (Fig. 14). However the change in pH gradually decreased and remained almost constant after 6 days.

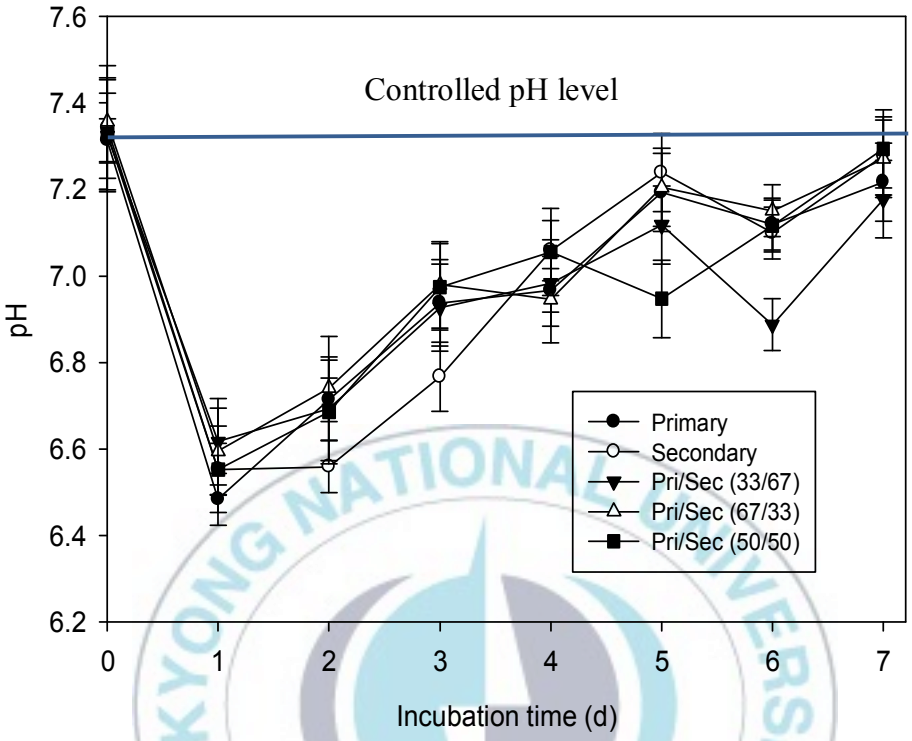


Fig. 14. Time courses of pH change during degradation of sludge substrates using mixed culture (A1 and A2) in a 300-ml flask incubated at 37°C in a shaking incubator (160 rpm). The data are presented as the mean ± SD (n=3)

3.3.2 Effect of anaerobic digestion of rainbow trout substrate mixtures on oxidation reduction potential

The change in oxidation-reduction potential (ORP) was also monitored throughout the 7 day incubation period. The ORP drastically dropped to below -250 mV for all the substrates within 48 hours and began to slowly increase throughout the incubation period. The ORP for Primary/Secondary (50/50) mixture still remained lowest as compared to all the other substrates throughout the 7-day incubation period. On the other hand primary sludge only showed the sharpest increase in ORP after 7 days with ORP rising from a lowest of -274 mV to a highest of -112 mV (Fig. 15).

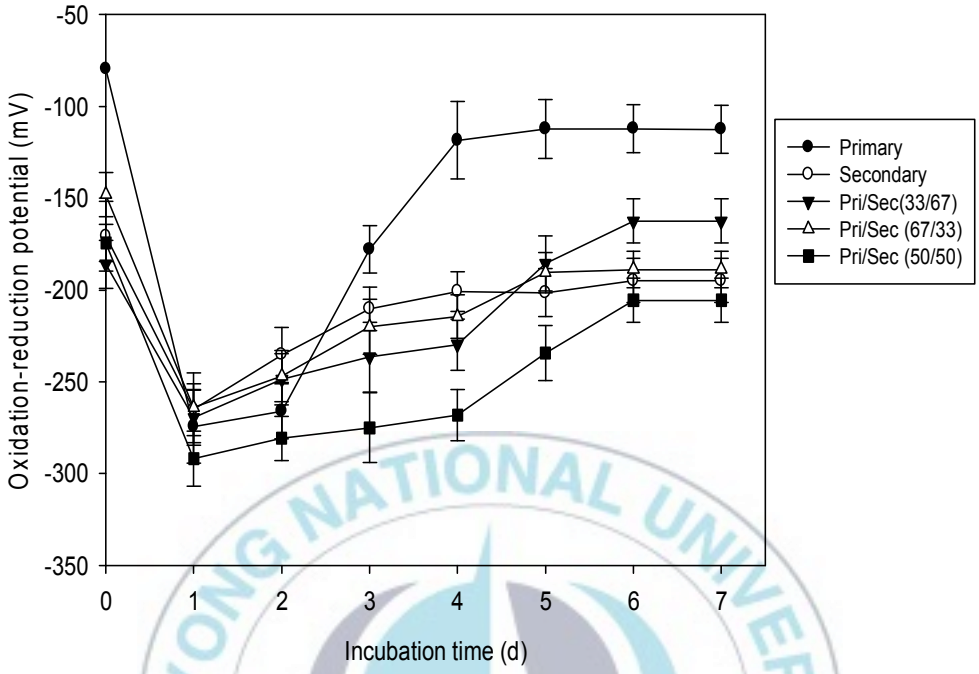


Fig. 15. Time courses of ORP change during degradation of sludge substrates using mixed culture (A1 and A2) in a 300-ml flask incubated at 37°C in a shaking incubator (160 rpm). The data are presented as the mean ± SD (n=3)

3.3.3 Effect of anaerobic digestion of rainbow trout substrate mixtures on total solids

There was gradual decline in total solids (%) in all substrates throughout the seven day incubation period. The most significant change in total solids (%) was observed in primary/secondary (50/50) mixture which showed a 58% reduction (Fig. 16). Half of the total solids (%) reduction in 50/50 mixture was observed within 4 days. The changes in total solids change in other mixtures primary only, secondary only, primary/secondary (33/67) and primary/secondary (67/33) were 55%, 42%, 47% and 50% respectively. The least change in total solids % was observed in secondary sludge.

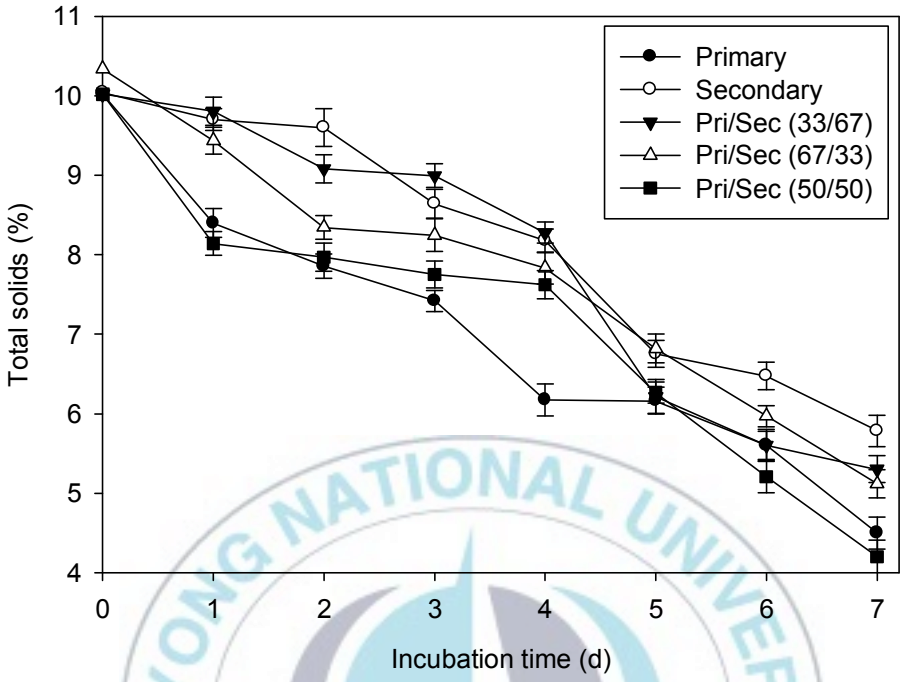


Fig. 16. Time courses of total solids (%) change during degradation of sludge substrates using mixed culture (A1 and A2) in a 300-ml flask incubated at 37°C in a shaking incubator (160 rpm). The data are presented as the mean ± SD (n=3)

3.3.4 Effect of anaerobic digestion of rainbow trout substrate mixtures on chemical oxygen demand

There was a general decrease in chemical oxygen demand (COD) across all the substrates. Secondary sludge had the smallest percentage change (39.8%) throughout the 7-day incubation period. On the other hand the highest decrease in chemical oxygen demand was observed in primary/secondary (50/50) mixture (79.3%). The other mixtures primary only, primary/secondary (33/67) and primary/secondary (67/33) had 67%, 62.8% and 60.4% decrease in COD respectively (Fig. 17).

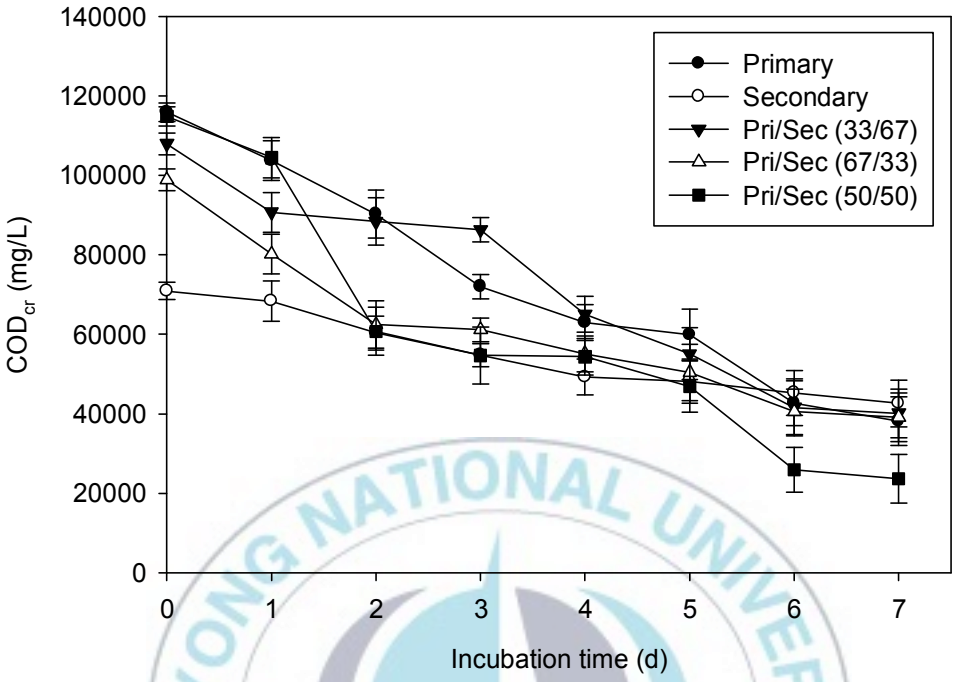


Fig. 17. Time courses of chemical oxygen demand change during degradation of sludge substrates using mixed culture (A1 and A2) in a 300-ml flask incubated at 37°C in a shaking incubator (160 rpm). The data are presented as the mean ± SD (n=3)

3.3.5 Effect of anaerobic digestion of rainbow trout substrate mixtures on total nitrogen

The percentage in total nitrogen across all substrates ranged between 43-55% throughout the 7-day incubation period. There was a notable decrease in total nitrogen across all the substrates. Primary/secondary (50/50) sludge had the smallest percentage change (42.7%) throughout the 7-day incubation period. On the other hand the highest decrease in total nitrogen was observed in primary/secondary (33/67) mixture (55.2%). The other mixtures primary only, secondary only, and primary/secondary (67/33) had 48.4 %, 48.2 % and 50.5% decrease in total nitrogen respectively (Fig. 18).

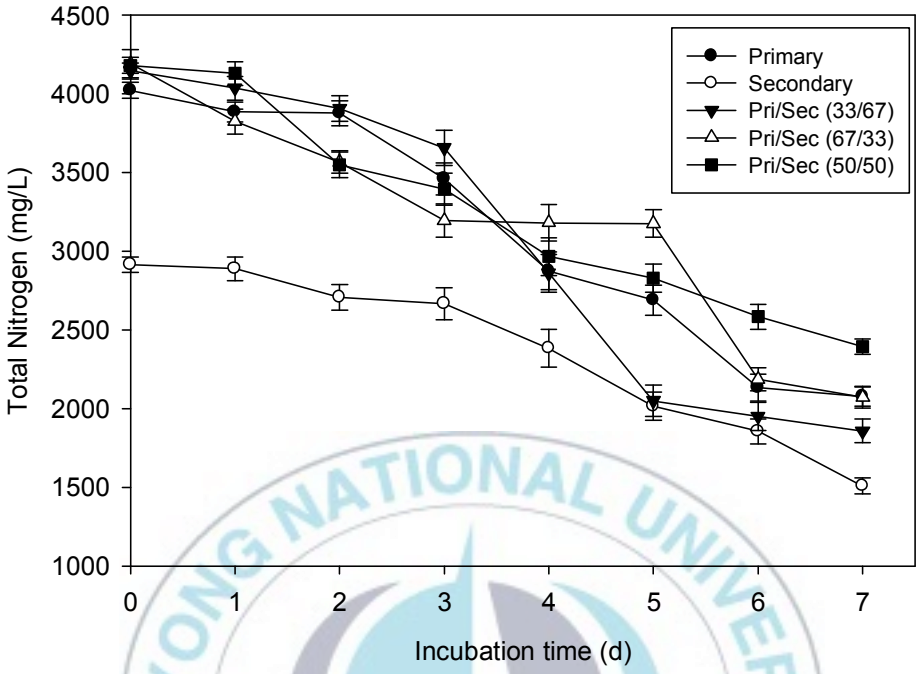


Fig. 18. Time courses of total nitrogen change during degradation of sludge substrates using mixed culture (A1 and A2) in a 300-ml flask incubated at 37°C in a shaking incubator (160 rpm). The data are presented as the mean ± SD (n=3)

3.3.6 Effect of anaerobic digestion of rainbow trout substrate mixtures on C: N ratio

The C: N ratio change across the substrates was calculated using total nitrogen and chemical oxygen demand. There was a slight decrease in C: N ratio on the sludge substrates except for secondary sludge which showed a slight 16% increase at the end of the 7-day incubation period. The highest decrease in C: N was noted in primary/secondary (50/50) which showed a 63.95% decline from a ratio of 27:1 to 10:1. The other substrates primary sludge only, primary/secondary (33/67) and primary/secondary (67/33) C: N ratio decreased by 36.1 %, 17.02 % and 19.93 % respectively (Fig. 19)

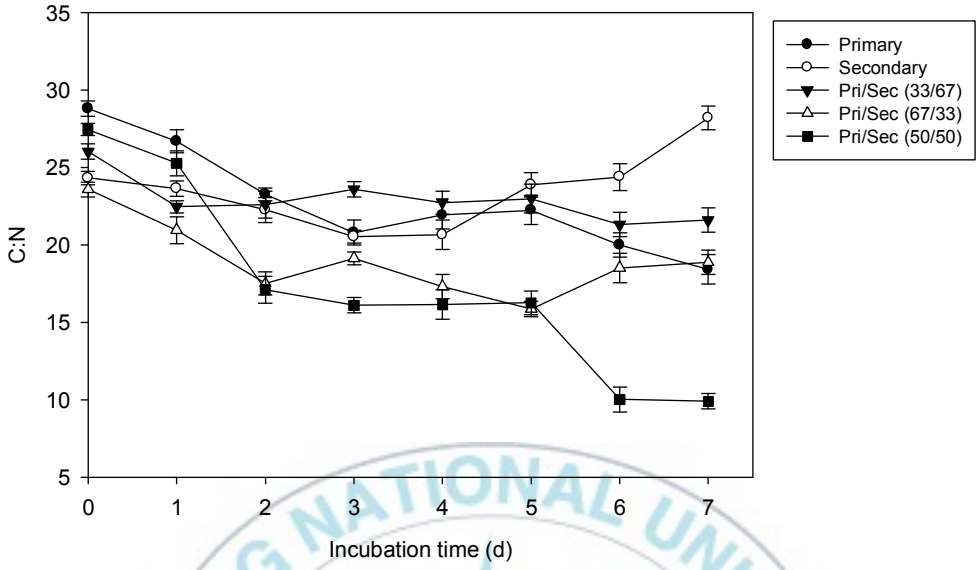


Fig. 19. Time courses of C:N ratio change during degradation of sludge substrates using mixed culture (A1 and A2) in a 300-ml flask incubated at 37°C in a shaking incubator (160 rpm). The data are presented as the mean ± SD (n=3)

Table 7. Total Nitrogen and COD removal efficiency during digestion

Substrate	Total N		COD	
	Removal efficiency (%)	Rate of removal (mg/L/day)	Removal efficiency (%)	Rate of removal (mg/L/day)
Primary	48.4	278	67	11094
Secondary	48.2	201	39.8	4047
33PS:67SS	55.2	327	62.8	9686
67PS:33SS	50.5	303	60.4	8530
50PS:50SS	42.7	255	79.3	13017

3.3.7 Effect of anaerobic digestion of rainbow trout substrate mixtures on volatile fatty acids production

There was a notable increase in volatile fatty acids production across all the substrates during the 7-day incubation period. The accumulation in the concentrations of the volatile fatty acids were however depressed due the pH adjustments done to maximize anaerobic digestion with a maximum VFA of only 6119 mg/L reached in primary sludge substrate (Fig. 20). Butyric acid was the major volatile fatty acid produced in all the substrates (Fig. 20-24) with concentration ranging between 1000-2000 mg/L.

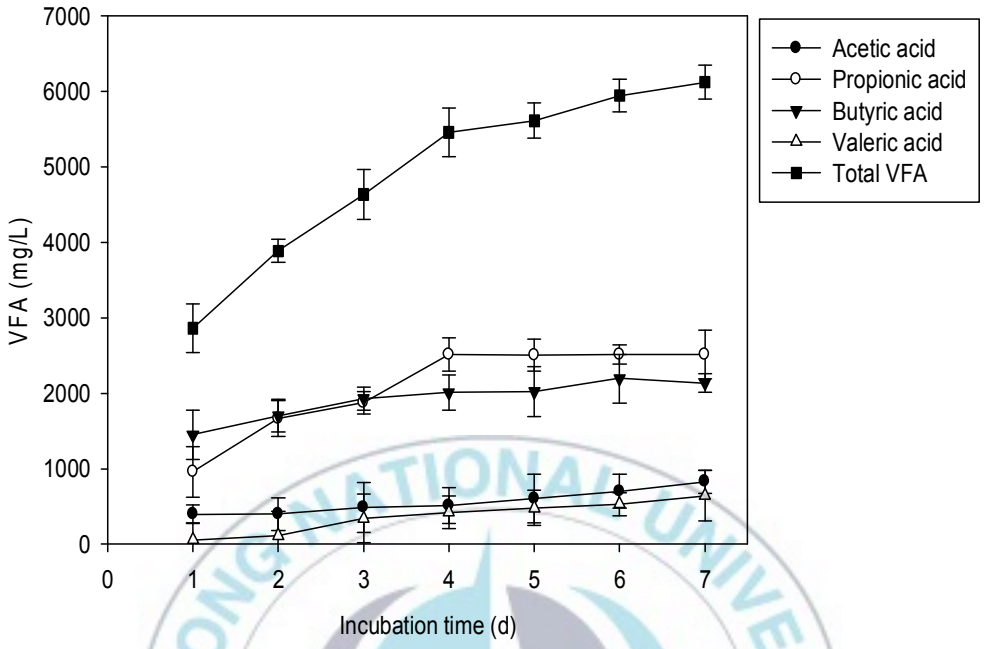


Fig. 20. Time courses of volatile fatty acids production during degradation of primary sludge substrate using mixed culture (A1 and A2) in a 300-ml flask incubated at 37°C in a shaking incubator (160 rpm). The data are presented as the mean ± SD (n=3)

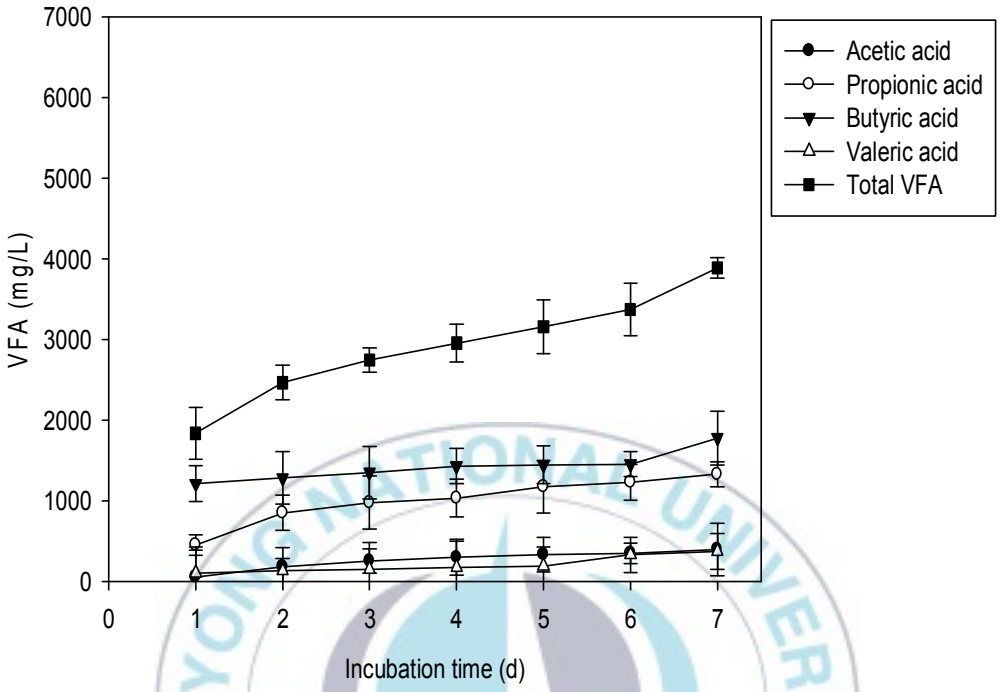


Fig. 21. Time courses of volatile fatty acids production during degradation of secondary sludge substrate using mixed culture (A1 and A2) in a 300-ml flask incubated at 37°C in a shaking incubator (160 rpm). The data are presented as the mean ± SD (n=3)

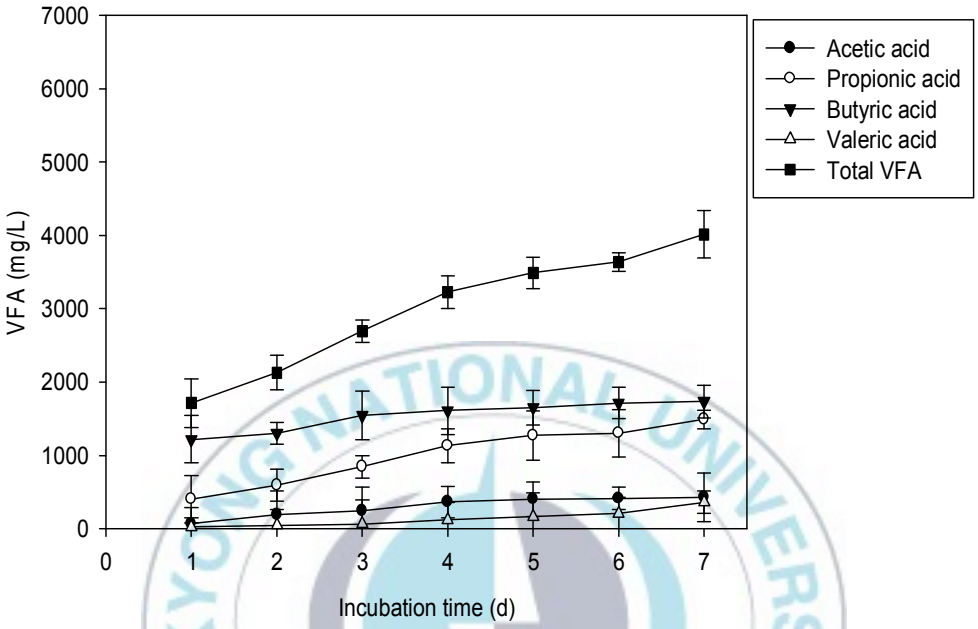


Fig. 22. Time courses of volatile fatty acids production during degradation of primary/secondary (33/67) sludge substrate using mixed culture (A1 and A2) in a 300-ml flask incubated at 37°C in a shaking incubator (160 rpm). The data are presented as the mean ± SD (n=3)

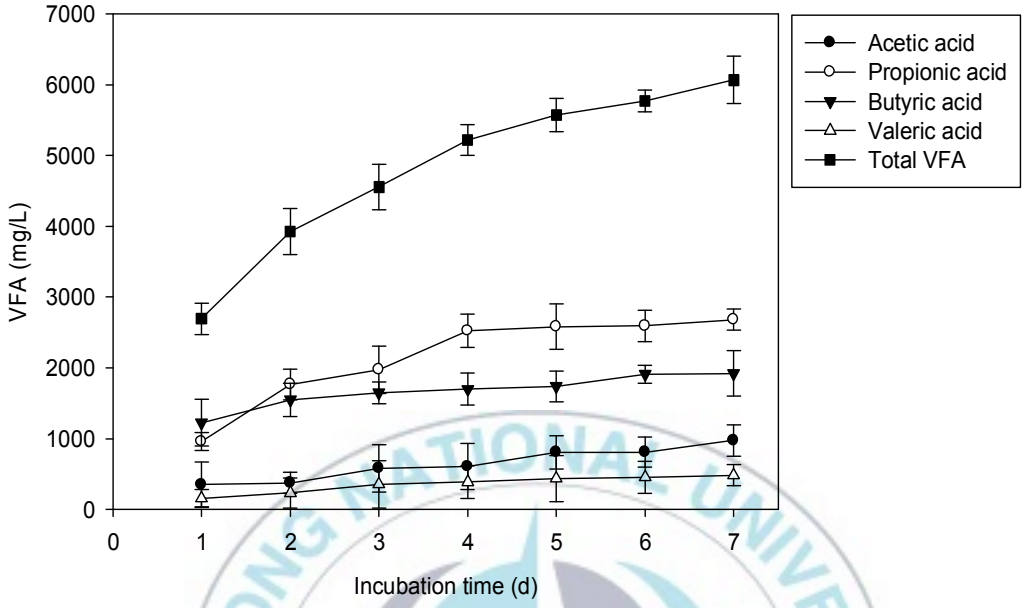


Fig. 23. Time courses of volatile fatty acids production during degradation of primary/secondary (67/33) sludge substrate using mixed culture (A1 and A2) in a 300-ml flask incubated at 37°C in a shaking incubator (160 rpm). The data are presented as the mean ± SD (n=3)

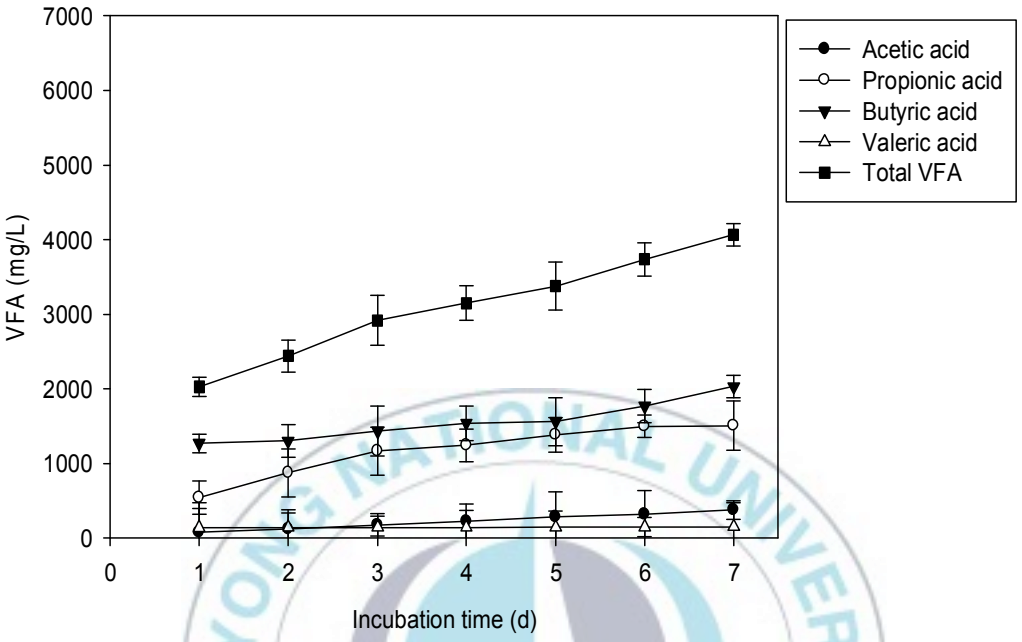


Fig. 24. Time courses of volatile fatty acids production during degradation of primary/secondary (50/50) sludge substrate using mixed culture (A1 and A2) in a 300-ml flask incubated at 37°C in a shaking incubator (160 rpm). The data are presented as the mean ± SD (n=3)

3.3.8 Effect of anaerobic digestion of rainbow trout substrate mixtures on acetic acid production

Generally the production of acetic was depressed across all the substrates with concentration ranging between 56-976 mg/L. High production levels were observed with primary/secondary (50/50) sludge substrate which showed a 64% increase during the 7-day incubation period. Primary sludge also showed high acetic acid production reaching a maximum yield of 829 mg/L after 7 days of incubation. Primary/secondary (67/33) sludge had the lowest acetic acid production although it showed an 80 % increase during the whole incubation period (Fig. 25).

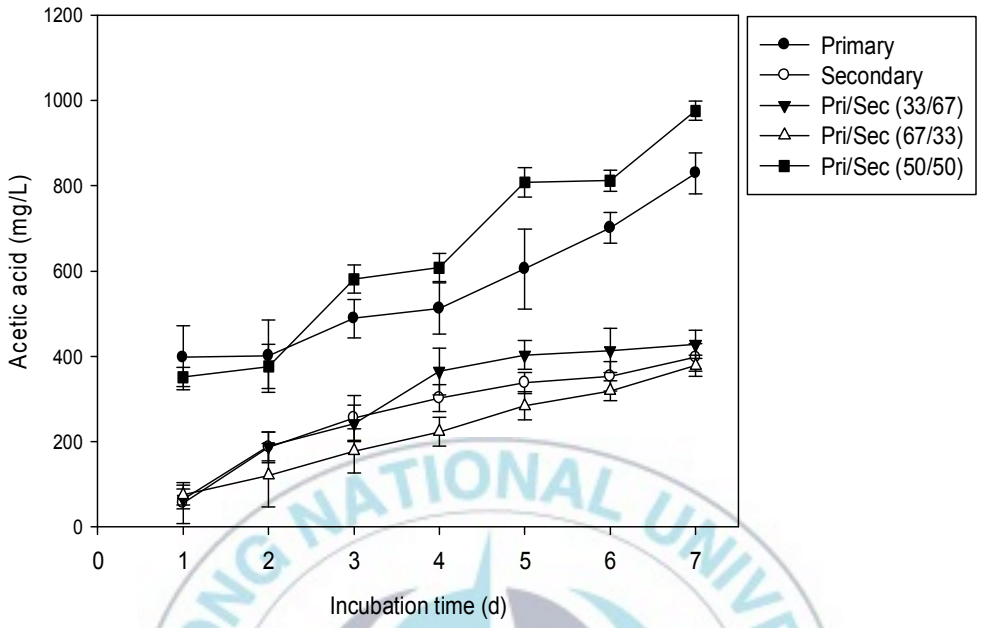


Fig. 25. Time courses of acetic acid production during degradation of rainbow trout sludge substrates using mixed culture (A1 and A2) in a 300-ml flask incubated at 37°C in a shaking incubator (160 rpm). The data are presented as the mean ± SD (n=3)

3.3.9 Effect of anaerobic digestion of rainbow trout substrate mixtures on total volatile fatty acids (VFA) production

There was a notable increase in total VFA in all the sludge substrates during the incubation period. On average primary sludge and primary/secondary (50/50) VFA production rates and concentrations were almost similar. On the other hand secondary only, primary/secondary (33/67) and primary/secondary (67/33) also behaved almost similarly in terms of VFA production rates and concentrations at the end of the incubation period. The highest VFA production was achieved in primary sludge only which reached 6119.40 mg/L while primary/secondary (33/67) sludge achieved 6065.32. The lowest VFA production was observed in secondary sludge only which only managed to produce a maximum of 3888 mg/L throughout the incubation period (Fig 26).

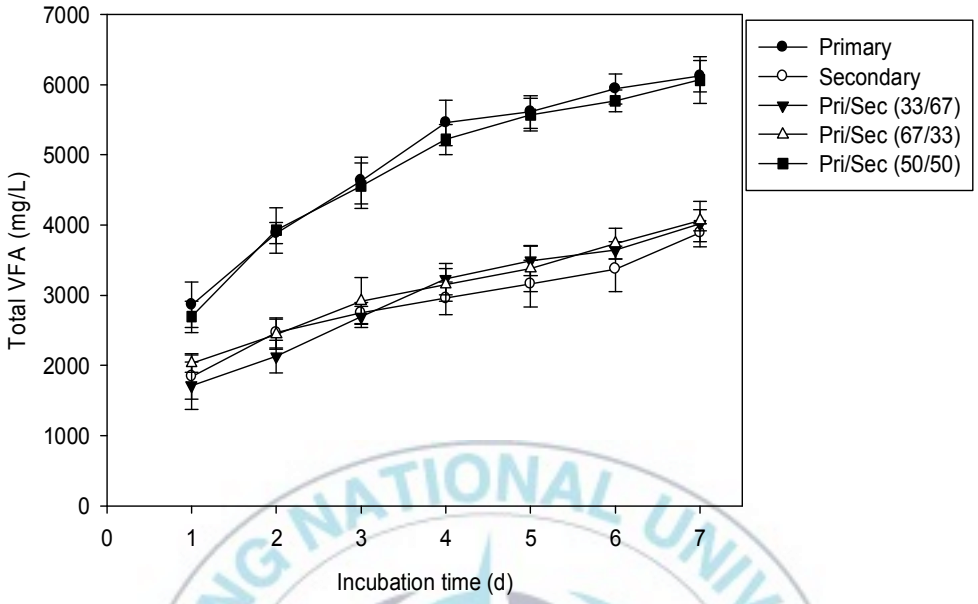


Fig. 26. Time courses of total volatile fatty acids production during degradation of rainbow trout sludge substrates using mixed culture (A1 and A2) in a 300-ml flask incubated at 37°C in a shaking incubator (160 rpm). The data are presented as the mean ± SD (n=3)

4. Discussion

4.1. Identification and characterization of useful sludge degrading isolates

Although A1 and A2 were most closely aligned to *Alcaligenes faecalis* with 99% and 98% similarity respectively, their colony morphology and acidogenic abilities were different. After same incubation period colonies for A2 were larger than for A1. In addition A2 showed a higher acidogenic activity by a larger drop in pH and higher production of volatile fatty acids.

4.2. Acidogenic ability of screened strains

Among the selected strains A2 displayed the highest volatile fatty acid production ability and hence the lowest pH values. The lowest pH and the highest VFA production were achieved within 24 hours. This shows that most of the acidogenic activity was done within 24 hours. In addition even the cell growth stabilized after 24 hours, this might be because of the depletion of the substrates in medium. When the strains were incubated together there was a lower pH and a higher VFA production achieved. This shows a comensal interaction in which the two acidogens feed on and

efficiently digest the substrates. The use of the two acidogens added an advantage to the acidogenic process making it more resistant to shock loading. Analysis of variance showed a significant difference between single bacteria and the co-mixture with $p < 0.05$. Generally the presence of VFAs leads to a pH drop in the digester and VFA toxicity is higher at a pH below 7 (Hwang *et al.,* 2007). The pH dropped to below 6 thus the possibility of VFA toxicity. This was also shown by the retardation in cell growth and biochemical activity shown by increase in the oxidation-reduction potential.

4.3. Kinetics of the rainbow trout sludge substrate biodegradation

The kinetics of bidegradation was studied in terms of conversion efficiency of rainbow trout sludge substrate to volatile fatty acids, pH level, oxidation reduction potential, and carbon and nitrogen mineralization during 7 days of incubation. During biodegradation of RTSS in 300 ml conical flasks there was an observed drop in oxidation reduction potential across all substrates. The biggest drop in ORP occurred within 24 h of incubation. This shows high biochemical activity in the substrates with the highest cell activity

observed in 50PS:50SS. The pH was adjusted every day however there was a decrease in the pH drop from the adjusted level with increase in the incubation time. This shows retardation in the biodegradation which might be due to decrease in substrate concentration of VFA toxicity. The oxidation reduction potential remained low although retarding showing that all the reactors were quite stable and able to withstand occasional disturbances such as fluctuation in temperature and mixing during sampling and pH adjusting. The adjustment of pH allowed the maintenance of a longer interval of observed high VFA production and COD removal.

In anaerobic digestion the composition of the starting material is important in the sense that there is a need for a suitable ration between carbon and nitrogen. Furthermore, main intermediates in the conversion are volatile fatty acids. If a high concentration of VFA is formed, pH will be reduced and that can reach levels when the acidogenic bacteria are severely inhibited and even may die. Therefore it is important to have buffering capacity in the system i.e. products or interventions that will counteract the effects of the VFA. It is known that carbohydrate-rich substrates are good producers of VFA and that protein rich substrates yield a good buffering capacity.

Therefore it seems realistic to investigate the biomass wastes when setting up an anaerobic digestion process (Foresti, 2001)

4.3.1. Carbon and Nitrogen mineralization

Anaerobic digestion is commonly used in wastewater sludge treatment. However low biodegrability of sludge remain an issue in anaerobic degradation. Primary sludge was relatively young (SRT 1 month) which means it contained a high proportion of biodegradable organic matter compared to secondary sludge (1-9 months) where nutrient removal was at an advanced stage. Sludge flocs contain a high amount of free or bounded water that is attached to the sludge floc structure extracellular polymeric substances (EPS) by electrostatic interactions and hydrogen bonds. Autoclaving had a direct effect on the bounded water, since they destabilize the floc structure, breaking hydrogen bonds between hydroxyl groups of EPS polymers and water molecules and electrostatic interactions between water molecules and induced dipoles of other functional groups in the EPS structure. This led to the release of bounded water increasing the degradability of the substrates. Organic sludge undergoes considerable changes in its physical and biological properties once it is generated. Factors

such as sludge pH, salinity, mineral composition, temperature, loading rate, hydraulic retention time (HRT), carbon to nitrogen ratio and volatile fatty acid content influence the digestibility of the sludge. Results show that the RTSS were steadily digested throughout the digestion period. With the highest reduction in total solids % and COD removal being observed in primary sludge which contained high proportion of biodegradable material. The 50PS:50SS mixture also gave low resultant COD and total solids % maybe because of a balance brought by mixing a substrate with a high buffering capacity (PS) and one which is carbon rich (SS). The reductions in total solids compare well with the solids reductions which have been reported by other researchers examining digestion. For example with activated sludge; Parking and Owen (1986) reported 20-50% total solids reduction while Callaghan *et al.,* (1999) reported 45-80 % TS reduction. Results show that anaerobic digestion is an attractive approach for aquaculture sludge management. It allows addressing many of the problems associated with the traditionally used management methods, such as municipal waste treatment systems and discharge into receiving water bodies. Analysis of variance showed a significant difference the different mixtures for C: N, Total N and COD with $p < 0.05$. The results support

findings by Reed *et al.,* (1995), who reported that the anaerobic digestion of RAS sludge can significantly reduce its volume to more than 90% due to its high digestion efficiencies, consequently lowering sludge transport and external treatment costs which are major factors in the feasibility of most aquaculture operations. In addition Cakir and Stenstorm, (2005), also reported 51-96% anaerobic treatment efficiency. Moreover the polluting strength of the treated sludge is significantly lower than that of raw sludge as shown by the resulting lower total N and C: N ratios in the RTSS. The C:N ratio of the co-digested mixtures which ranged between 20-28 was within the C:N ratios required for stable biological conversions reported by others on anaerobic digestion of organic wastes. Kayhanian and Hardy (1994) reported C:N ratios between 25 and 30 as being optimal. However, some investigators such as Gunaseelan (1995) suggested C: N of 11 being satisfactory for anaerobic digestion. Many of the trout farms are small and isolated therefore in practical terms, in the long term the amounts of organic waste generated at any particular site may not be sufficient to make digestion cost-effective. However the establishment of a centralized facility among several integrated operations on the farms would deal with such a situation.

4.3.2. Volatile fatty acids production

High concentrations of long-chain fatty acids originating from the fish feed is a significant factor contributing to inhibition of sludge digestion (Eikebroke, 2006). Dilution of the sludge with distilled water helped overcome these problems. The dilution with distilled water also might have reduced the sodium concentration (and problem of those of other salt ions) which was reported by Gebauer (2004) to be inhibiting to anaerobic digestion of brackish aquaculture sludge. In a well operating system organic acids such as propionic acid, butyric acid are mostly converted to acetic acid and H_2 gas. The production of acetic acid was repressed in this experiment because of the pH buffering which was done every 24 hours this helped to enrich the acidogenic bacteria. Analysis of variance showed a significant difference the different mixtures for total VFA and the different fatty acids with $p < 0.05$. The dominant liquid phase metabolites waste was butyric acid with concentrations surpassing 1000 mg/L. The response of this digestive system to increased C:N ratio was demonstrated by butyric acid which increased in concentration with increase in nitrogen mineralization which was the same trend observed by Cooney *et al.,* 2007. The different

mixtures basically increased C:N ratio which generally destabilized the digestion process in mixtures with high C:N ratio and the microorganism activity was stabilized at lower C:N ratio of 20. Propionic acid and butyric acid are the most important syntrophic intermediates. In anaerobic syntrophic interactions their production and subsequent degradation is regarded as the rate limiting step because of thermodynamic interactions (de Bok *et al.,* 2004). The results from this study show that acid concentration increased as RTSS was incubated. This strongly implied that the acidogens were able to steadily digest the RTSS. The best digestion response was again observed in primary sludge and 50PS:50SS sludge substrates. Because of the pH buffering which was performed on the digestion process the VFA concentrations were kept low thus preventing toxicity and stabilizing the digestion process. It has been shown earlier by others examining anaerobic digestion that increase in propionic acid ratio greater than 1.4 and a build up of acetic acid and butyratic acid above 200 mM as well as 100 mM of propionic acid can cause process inhibition and ultimate digester failure (Hill *et al.,* 1987, Ahring *et al.,* 1995). Anaerobic digestion of aquaculture sludge is a fairly new concept because in the traditional methods of aquaculture in ponds, flow-through systems or net pens-sludge is not

collected . Anaerobic sludge digestion from freshwater RAS was first reported in the 1990's with little success (Kugelman and van Gorder (1991), Lanari and Franci (1998)). Based on those studies the authors suggested a continuously stirred treatment reactor system operating under mesophilic conditions with diluted wastewater (to overcome the inhibition of ammonia) for aquaculture sludge digestion. Therefore the results from this study are quite promising as a cost effective and efficient treatment of rainbow trout sludge as the different mixtures had a significant effect on the product distribution.

4.4. Characteristics and reutilization of rainbow trout sludge substrate after biodegradation

In recent years, the aquaculture industry has had to develop various management strategies to reduce the environmental impacts of aquaculture manure waste to meet legislative and regulatory requirements for effluent control. Regulatory agencies usually restrict the final effluent concentrations of suspended solids, total phosphorus and total nitrogen that may be released into a receiving watershed (Iversen, 1995). Although significant progress has been made in reducing the overall quantity of wastes produced from fish farms through development of improved diets and feed delivery systems (Iversen, 1995; Thorpe and Cho, 1995), significant amounts of both soluble and settleable materials remain that must be removed from the effluent stream. Settled solids (primarily manure and uneaten feed) from aquaculture operations are commonly disposed of by applying them to farmlands as a fertilizer supplement (Westerman *et al.,* 1993).

Land disposal of livestock manures, including that from fish, requires proper management to fully recover the fertilizer value of the manure, as well as to

prevent environmental impacts through unwanted loss of nutrients in the run-off into surrounding watersheds (Sweeten, 1992). To evaluate the potential of aquaculture solid waste for use as a fertilizer, the aquaculture industry and regulatory agencies require analytical data regarding the concentrations of various plant nutrients and trace metals found in this waste. Therefore, this study adds to the small database of information relating to the nutrient composition of aquaculture waste from a rainbow trout farm. The chemical characteristics of interest when livestock manure is used as a soil fertilizer include the concentrations of plant macronutrients and micronutrients, as well as certain toxic substances, such as heavy metals (Barrington, 1991). Fish manures tend to be highly variable in their chemical content, which is also the case with other animal manures (Fulhage, 1992; Olson, 1992; Smith, 1992; Westerman *et al,*. 1993). The physical and chemical composition of fish manure is influenced by several factors, including the type of rearing tank or pond, species and size of fish, feed and feeding systems, water flow dynamics, manure handling procedures, and storage time and environment (Mudrak, 1981; Muir, 1982; Olson, 1992; Westerman *et al.,* 1993). The rainbow trout farm used in this study produced about 420 tonnes per year using underground water in a concrete lined

recirculating aquaculture system. The farm has been operating for ten years while the waste sampled from earthen ponds was between 1-12 months old. From the analysis of the results, it was observed that the concentrations are comparable with what was observed from previous research as shown in Table 8. Despite the farm being in operation for many years there is notable absence of heavy metals i.e. Cu, Hg and Se. Since underground water is being used in the RAS on the farm there is little or no contamination of the water used in the aquaculture system. The effluent sludge had similar levels of N, P, Ca, and Mg, and lower levels of K compared to a study by Naylor *et al.,* (1999) on beef, dairy cattle, poultry and swine manure. Long residence time in settling basins, ponds, or storage tanks lowers the nutrient content of fish manure (Olson, 1992). Chemical and microbial decomposition followed by leaching of the solids contributes to the portion of the total N and P that is in soluble form (Butz and Vens-Cappell, 1982; Parjala *et al.,* 1984). Effluent sludge that had accumulated in settling basins for 1-9 months (secondary sludge) had lower levels of N, P, and Ca but higher levels of K, Na, and Mg than fresh primary sludge (i.e., one month old; Table 8).

Table 8. Chemical composition of sludge from rainbow trout farm (this study) compared with values reported in previous studies (dry-weight basis). Data are presented as ranges or means.

Element	This study		Westerman *et al* (1993)		Naylor *et al.* (1999)[e]	Olson (1992)[f]
	Primary[a]	Secondary[b]	PS[c]	SS[d]		
Lipid	1.23 ± 0.000	0.16 ± 0.000	---	---	---	---
Protein	3.36 ± 0.020	1.90 ± 0.000	---	---	---	---
Carbohydrate	95.41 ± 0.000	97.94 ± 0.000	---	---	---	---
Ca (%)	1.65 ± 0.023	1.47 ± 0.018	1.18-4.43	0.34-2.70	6.99 ± 2.71	---
P (%)	0.80 ± 0.006	0.62 ± 0.006	0.88-6.60	0.35-1.85	2.54 ± 1.20	1.34-3.51
K (%)	0.02 ± 0.000	0.03 ± 0.001	0.05-0.96	0.29-0.88	0.10 ± 0.05	0.29-0.43
Mg (%)	0.04 ± 0.000	0.05 ± 0.001	0.18-0.44	0.35-0.60	0.53 ± 0.59	---
Na (%)	0.02 ±0.002	0.03 ± 0.001	0.023-0.351	0.035-0.052	---	---

Zn (ppm)	200 ± 0.000	200 ± 0.000	130-590	160-500	604.9 ±
Fe (ppm)	700 ± 10.00	900 ± 20.00	---	---	1,942 ±
Mn (ppm)	100 ± 0.000	100 ± 0.000	---	---	487.8 ±
Cd (ppm)	1.09 ± 0.077	0.67 ± 0.102	---	---	1.13 ±
Cu (ppm)	n.d.	n.d.	0	0-60	33.4 ±
Hg (ppm)	n.d.	n.d.	---	---	0.05 ±
Se (ppm)	n.d.	n.d.	---	---	0.50 ±

[a]Trout effluent sludge from settling ponds, less than one month old

[b]Trout effluent sludge from settling ponds, 1-12 months old

[c]Trout manure samples from raceway settling sections, less than two weeks old

[d]Trout manure samples from settling basins, 1-9 months old

[e]Trout settleable solid wastes from gravitational settling units, 1-12 months old

[f]Trout manure from three fish farms using concrete settling basins, unknown age

The results from this study indicate that sludge from aquaculture waste is similar in its chemical composition to other livestock manures, and thus, it should be able to be stored and disposed of using similar codes of practice that are widely in use in terrestrial agriculture as an agricultural fertilizer with little or no phytotoxicity problems. Further laboratory or field tests are however necessary to test the availability of the nutrients for a positive biofertilization capacity. Land application of manure should follow a nutrient management plan that includes an annual nitrogen and phosphorus balance determination (Fulhage, 1992; Sweeten, 1992). A nutrient management plan takes into account the nutrients present in the soil, nutrients in the manure after storage or treatment, nutrient availability to plants, crop uptake as a function of realistic yield goals and potential for leaching and run-off following application (Fulhage, 1992; Sweeten, 1992). While the data from this study is useful to demonstrate the utility of fish effluent sludge as an agricultural fertilizer, sludge N/P/K analysis before application is still essential for proper nutrient management because of the high variability in nutrient composition (Barrington, 1991; Fulhage, 1992; Smith, 1992; Sweeten, 1992; Westerman *et al.,* 1993). In addition, it is presumed that these data will also help regulatory agencies and farmers

make sensible and pragmatic decisions concerning the appropriate and safe disposal of effluent sludge collected from land-based trout culture facilities.

5. Conclusions

Two strains isolated from pond-bottom soil and identified as *Alcaligenes faecalis strain HCB2- A1* and *Alcaligenes faecalis A2* were determined to be useful sludge degrading strains. Strain A2 exhibited the highest acidogenic ability compared to A1 however the acidogenic ability of both strains were improved when cultured together producing a maximum of 6632 mg/L total volatile fatty acids. A gas chromatographic analysis done on the culture supernatant revealed that propionic and butyric acid were the dominant volatile fatty acids. The fermentation of rainbow trout sludge substrate revealed a steadily digesting system with total solids for the substrates being reduced by between 42-58%. With 50PS:50SS mixture exhibiting the greatest reduction in total solids percentage. Chemical oxygen demand removal was also revealed in all substrates with removal efficiency in all the substrates ranging between 40-80% with 50PS:50SS having the highest (80%). This was also shown with the gradual decline in total nitrogen % and C:N ratio with digestion stabilizing around C:N ratio of 20:1. There was a significant difference in all parameters measured therefore the null

hypothesis that co-mixture of primary and secondary sludge does not enhance acidogenic potential can be reject since there is sufficient evidence from the data.

Anaerobic degradation process stability and overall degradation rates can thus be increased by separately optimizing conditions for each bacterial group. However, product formation by a mixed acidogenic population is a very complex process and is greatly influenced by many factors. These factors include wastewater specificity, reactor configuration, hydraulic retention time (HRT), influent organic concentration, organic loading rate, pH, and temperature, oxidation–reduction potential and nutritional requirements. Proteins are degraded slower than carbohydrates under acidogenic conditions thus mixing primary and secondary sludge substrates helped to improve the digestion efficiency. The determination of COD has shown that it can be used to evaluate the efficiency of acidogenesis with results clearly indicating that the COD removal in mixed substrates (50/50) was higher than that in unmixed substrates thus PS/SS (50/50) had a better capacity to respond to the impact of organic loading. Acetate, propionate and butyrate were found to be the three main acidogenic products in this study, in agreement with previous reports from other researchers who

observed that alcohols, valerate and other organic acids produced during sludge fermentation could be negligible. Therefore, it was assumed that the PS/SS (50/50) mixed effluent containing a smaller portion of propionate was favorable for the subsequent anaerobic digestion. Apart from enhancing anaerobic sludge treatment with regard to providing more favorable conditions for digestion, mixing of the substrates was possibly meaningful for sludge pretreatment, and even for sludge digestion for the production of acetate. If sludge is allowed to mix to a certain level, not only could the biodegradability be improved, but also COD removal could be promoted to reduce the organic loading in the subsequent treatment. On the other hand, acceleration of hydrolysis and optimization of the fermentation by mixing substrates were likely to provide inspiration to enhance the anaerobic digestion of excess sludge. As is well known, hydrolysis/fermentation is the rate-controlling step in sludge digestion, the mixing of the substrates was helpful to improve anaerobic acidogenesis and be used as a pretreatment method to create a favorable feeding condition for subsequent treatment.

The data from this research is not only helpful in establishing stabilized conditions for a full anaerobic digestion process for rainbow trout effluent sludge but it also help regulatory agencies and farmers make sensible and

pragmatic decisions concerning the appropriate and safe disposal of effluent sludge collected from land-based trout culture facilities.

Anaerobic wastewater treatment offers improved energy conservation with potential reduction in greenhouse gas emissions. During the last 200 years atmospheric concentration of greenhouse gases, CO_2, CH_4 and N_2O have increased due to anthropogenic activities such as production and use of fossil fuels and other agricultural and industrial activities. Another advantage of integrating an AD unit into the RAS is its smaller environmental footprint compared to traditional systems, specifically to WSPs that require large areas of land (sometimes with high agricultural value) and to discharge into receiving water bodies that pollutes local environment. The localized treatment of aquaculture sludge in anaerobic digesters is a preferable alternative. Some farmers use the water for irrigation and sludge for land spread, however in the case of brackish/marine water this might lead to soil and groundwater salinization. The results from this study show that the use of primary sludge only or in equal proportion with secondary sludge are the most promising substrates for anaerobic digestion of rainbow trout sludge. However these results need to be put into a proper context by comparison with other digestion systems. In addition the

resultant full digestion to biogas need to be investigated and scaling up to a bigger reactor is required.

As the AD of aquaculture sludge is a fairly new concept, information is still lacking and further research is required. Aside from further optimization of the current systems, the research community should be looking at ways to further reduce the sludge mass as well as improve on the “benefits” from the sludge treatment, such as methane production or nitrogen removal. The stabilized sludge characteristics need to be identified and tested and its potential benefit should be assessed. Issues such as potential accumulation of heavy metals, the presence of chemicals, pathogens and odors, and soil salinization should be addressed, as well as the potential availability of beneficial compounds such as nitrogen and phosphorus. This study however demonstrates that with mixing of substrates there is little or no problems of heavy metal toxicity. It is generally considered that digestate from anaerobic digestion are not generally suitable for putting directly onto land. They are too wet, contain a notable amount of volatile fatty acids which are somewhat phytotoxic and, if digestion has not occurred within the thermophilic range of temperatures are not hygienized. However in a pH controlled digestion process as used in this study demonstrate that problems

of phytotoxicity can be avoided. If pH is not controlled the VFA concentration is elevated thus there develop a need for post treatment after anaerobic digestion to obtain a high quality finished product. The future of anaerobic digestion should be sought in the context of an overall sustainable waste-management perspective in comparison with aerobic treatment which produces large and uncontrolled emissions of volatile compounds such as ketones, aldehydes, ammonia and methane. Biogas collection also prevents the production of undesired odors associated with sludge stabilization in open ponds. For sustainable energy development in the developing world, low cost “low tech” renewable energy systems for rural areas, peri-urban or isolated aquaculture farms like anaerobic digestion used in this study have high potential for application.

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References

Ahring B. K., Sandberg M. and Angelidaki I. 1995. Volatile acids as indicators of process imbalance in anaerobic digesters. Appl. Microbiol. Biot. 43: 559-565

APHA (American Public Health Association), 1998. In: Clesceri, Greenberg, Trussell (Eds.), Standard Methods for the Examination of Water and Wastewater, 20th ed. Washington, DC.

Alippi, A. M. and Reynaldi, F. J. 2006. Inhibition of the growth of Paenibacillus larvae, the causal agent of American foulbrood of honeybees, by selected strains of aerobic spore forming bacteria isolated from apiarian sources. J. Invert. Pathol., 91: 141-146.

Alvarez J. A, Otero L and Lema J. M. 2009. A methodology for optimizing feed composition for anaerobic co-digestion of agro-industrial wastes. Bioresour. Technol., 101: 1153-1158.

Alvarez R. and Lidén G. 2008. Semi-continuous co-digestion of solid slaughterhouse waste, manure, and fruit and vegetable waste. Renew. Energ., 33(4): 726-734.

Alvarez J., Tilche A. and Cecchi F. (Eds), Proceedings of the Second International Symposium on Anaerobic Digestion of Solid Wastes, Barcelona, Vol 2. Grafiques 92, 15-18 June, pp 274-277.

Axler R. P. 1997. Characteristics of effluent and sludge from two commercial rainbow trout farms in Minnesota. Prog. Fish-Cult., 59:161-172.

Barrington, S. E 1991. Characteristics of livestock manures. Pages 19-35 in D. A. Leger, N. K. Patni, and S. K. Ho, editors. Proceedings of the national workshop on land application of animal manure. Canadian Agricultural Research Council, Ottawa.

Bergheim A, Tyvold T and Seymour E. A. 1991. Effluent loadings and sludge removal from landbased salmon farming tanks. In: Depauw, N., Joyce, J. (Eds.), Aquaculture and the Environment: Short Communications

and Abstracts, Number 14. European Aquaculture Society, Bredene, Belgium, Page 27.

Bergheim A, Sanni S, Indrevik G and Hølland P. 1993. Sludge removal from salmonid tank effluent using rotating microsieves. Aquacult. Engineer., 12: 97–109.

Bergheim A. and Brinker A. 2003. Effluent treatment for flow-through systems and European environmental regulations. Aquacult. Engineer., 27:61-77.

Beveridge M C M, Phillips M J and Clarke R. M. 1991. Quantitative and qualitative assessment of wastes from aquatic animal production. In: Brune D E., Tomasso J.R. (Eds.), Aquaculture and water quality. World Aquaculture Society, Baton Rouge, Louisiana, pp. 506-533.

Boardman G D, Maillard V, Nyland J, Flick G J and G. S. Libey. 1998. The characterization, treatment and improvement of aquacultural effluents. Departments of Civil and Environmental Engineering, Food Science and

Technology, and Fisheries and Wildlife Sciences. Virginia Polytechnic Institute and State University. Blacksburg, VA. 123 pp.

Boyd C E, Gross A and Rowan M. 1998. Laboratory study of sedimentation for improving quality of pond effluents. J. Appl. Aquacult., 8: 39–48.

Butz, L, and B. Vens-Cappell. 1982. Organic load from the metabolic products of rainbow trout fed with dry food. Pages 73-82 *in* J. S. Alabaster, editor. Report of the EIFAC workshop on fish-farm effluents. EIFAC (European Inland Fisheries Advisory Commission) Technical Paper 41.

Cakir, F.Y. and Stenstrom M.K., 2005. Greenhouse gas production: a comparison between aerobic and anaerobic wastewater treatment technology. Water Res., 39: 4197–4203.

Callaghan F. J., Wase D. A. J., Thayanithy K and Forster C. K. 1999. Co-digestion of waste organic solids: batch studies. Bioresour. Tech. 67: 117-122

Callaghan F. J, Wase D. A. J, Thayanithy K and Forster C. F. 2002. Continuous co-digestion of cattle slurry with fruit and vegetable wastes and chicken manure. Biomass Bioenerg., 22(1): 71-77.

Cheong D. and Hansen C. L. 2006. Acidogenesis characteristics of natural, mixed anaerobes converting carbohydrate-rich synthetic wastewater to hydrogen. Process Biochem., 41: 1736–1745

Chile, Environmental Regulation No. 320, Article 4. 2001

Cooney M., Maynard N., Cannizzaro C. and Benemann J. 2007. Two phase anaerobic digestion for production of hydrogen-methane mixtures. Biores. Tech. 98: 2641-2651.

Cripps S. J. 1992. Characterization of an aquaculture effluent based on water quality and particle size distribution data. In: Proceedings of Aquaculture ’92 Conference, Orlando, FL.

Cripps S. J. 1995. Serial particle size fractionation and characterization of an aquacultural effluent. Aquacult., 133: 323-339.

Cripps S. J and Bergheim A. 2000. Solids management and removal for intensive land-based aquaculture production systems. Aquacult. Engineer., 22: 33-56.

De Bok F. A. M., Plugge C., Stams A. J. M., 2004. Interspecies electron transfer in methanogenic propionate degrading consortia. Water Re., 38: 1368-1375

Fantozzi F and Buratti C. 2009. Biogas production from different substrates in an experimental Continuously Stirred Tank Reactor anaerobic digester. Bioresour. Technol., 100: 5783–5789.

Flimlin G, Sigiura S and Ferraris R. P. 2003. Examining phosphorus in effluents from rainbow trout (*Oncorhynchus mykiss*) aquaculture. Rutgers Cooperative Extension Bulletin. New Jersey Agricultural Experiment Station. Rutgers State University of New Jersey.

Fornshell, G. 2001. Settling Bain Design. Western Regional Aquaculture Center. University of Washington, School of Fisheries. Pub. No. 106.

Foy, R. H., Rosell, R., 1991. Fractionation of phosphorus and nitrogen loadings from a Northern Ireland fish farm. Aquacult., 96: 31-42.

Foresti E. 2001. Perspective on anaerobic treatment in developing countries. Water Sci. Technol. 41: 141-148

Fricke K, Santen H, Wallmann R, Huttner A and Dichtl N. 2007. Operating problems in anaerobic digestion plants resulting from nitrogen in Municipal Solid Waste. Waste Manage., 27: 30–43.

Fulhage, C. 1992. Nutrient management in livestock waste systems. Pages 110-115 *in* Blake, J., Donald J, and Magette W, editors. 1992. National livestock, poultry and aquaculture waste management, Proceedings of the national workshop. American Soc. of Agric. Eng., St. Joseph, Michigan.

Gatlin D and Hardy R. 2002. Manipulations of diets and feeding to reduce losses of nutrients in intensive aquaculture. In: Tomasso, J.R. (Eds.), Aquaculture and the Environment in the United States. U.S. Aquaculture Society, Baton Rouge, Louisiana, pp. 155-166.

Gebauer, R., 2004. Mesophilic anaerobic treatment of sludge from saline fish farm effluents with biogas production. Bioresour. Technol., 93: 155–167.

Gunaseelan V. G., 1995. Effects of inoculums/substrate ratio and pretreatments of methane yield from pantenium. Biomass Bioenerg. 8: 39-44

Heinen J. M, Hankins J. A and Adler P.R. 1996. Water quality and waste production in a recirculating trout-culture system with feeding of a higher-energy or lower-energy diet. Aquacult. Res., 27: 699-710.

Hill D. T., Cobb S. A., and Bolte J. P. 1987. Using volatile fatty acids relationships to predict anaerobic digester failure. Trans. ASAE 30: 496-501

Hwang M. H., Jang N. J., Hyum S. H. and Kim I. S. 2004. Anaerobic bihydrogen production from ethanol fermentation: the role of pH. J. Biotech. 111: 297-309.

(IDEQ) Idaho Department of Environmental Quality. 1998. Idaho Waste Management Guidelines for Aquaculture Operations. Idaho Department of Health and Welfare, Division of Environmental Quality, Twin Falls, ID.

Ike M, Inoue D, Miyano T, Liu T. T, Sei K, Soda S and Kadoshin S. 2010. Microbial population dynamics during startup of a full-scale anaerobic digester treating industrial food waste in Kyoto eco- energy project. Bioresour. Technol., 101: 3952–3957.

ISO (International Organization for Standardization), 2002. Water quality determination of the chemical oxygen demand index (ST-COD) - small-scale sealed-tube method, ISO 5705:2002(E). International Organization for Standardization, Geneva. 18 pp.

Iversen, T. M. 1995. Fish farming in Denmark: environmental impact of regulative legislation. Water Sci. and Tech., 31:73-84.

Kayhanian M. and Hardy S. 1994. The impact of four design parameters on the performance of high-solids anaerobic digestion of municipal solid waste for fuel gas production. Environ. Technol., 15: 557-567

Kelley L. A, Bergheim A and Stellwagen J. 1997. Particle size distribution from freshwater fish farms. Aquacult. Intern., Vol. 5, No. 1, pp. 65-78.

Kim J. K, Nhat L, Chun Y. N and Kim S.W. 2008. Hydrogen production condition from food waste by dark fermentation with *Clostridium beijerinckii* KCTC 1785. Biotechnol. Bioprocess Eng., 13: 499–504.

Kugelman I.J. and van Gorder S. 1991. Water and energy recycling in closed aquaculture systems. Engineering Aspects of Intensive Aquaculture. Proc. Aquaculture Symposium, Cornell University, April 4–6, 1991. : Northeast Regional Agricultural Engineering Service (NRAES)-49. New York, Ithaca, pp. 80–87.

Lanari D. and Franci C., 1998. Biogas production from solid wastes removed from fish farm effluents. Aquat. Living Resour. 11, 289–295.

Lee J, Song J and Hwang S. 2009a. Effects of acid pre-treatment on bio hydrogen production and microbial communities during dark fermentation. Bioresour. Technol. 100: 1491–1493.

Malta-Alvarez J, Mace S and Llabres P. 2004. Anaerobic digestion of organic solid wastes, an overview of research achievements and perspectives. Bioresour. Technol., 74(1): 3-16.

Muir, J. E 1982. Economic aspects of waste treatment in fish culture. Pages 123-135 *in* J. S. Alabaster, editor. Report of the EIFAC workshop on fish-farm effluents. EIFAC (European Inland Fisheries Advisory Commission) Technical Paper 41.

Natella M, Yossi T and Amit G. 2010. Anaerobic digestion of sludge from intensive recirculating aquaculture systems. Aquacult., 306

Naylor S. J, Mocca R. D and Durant G. M. 1999. The Chemical Composition of Settleable Solid Fish Waste (Manure) from Commercial Rainbow Trout Farms in Ontario, Canada. North American J. Aquacult., 61:21-26.

Olson, G. L. 1992. The use of trout manure as a fertilizer for Idaho crops. Pages 198-205 *in* Blake, J., Donald J, and Magette W, editors. 1992. National livestock, poultry and aquaculture waste management, Proceedings of the national workshop. American Soc. of Agric. Eng., St. Joseph, Michigan.

Parjala, E., A. Tamminen, and O. V. Lingqvist. 1984. Pollution loadings from Finnish inland fish-farms: a case study. Aqua Fennica 14:205-214.

Parkin G. F. and Owen W. F. 1986. Fundamentals of anaerobic digestion of wastewater sludges. J. Environ. Eng. 112: 867-920

Qigui Niu, Toshimasa Hojo, Wei Qiao, Hong Qiang, Yu-You Li (2014), Characterization of methanogenesis, acidogenesis and hydrolysis in thermophilic methane fermentation of chicken manure. Chemical Engineering Journal 244 587–596

Reed, S.C., Crites, R.W. and Middlebrooks, E.J. 1995. Natural Systems for Waste Management and Treatment. McGraw-Hill, New York.

Salam B, Islam M and Rahman M. T. 2009. Biogas from Anaerobic Digestion of Fish Waste. International Conference on Mechanical Engineering (ICME2009), 26-28 December 2009, Dhaka, Bangladesh.

Schwartz M. R and Boyd C. E. 1994. Channel catfish pond effluents. Prog. Fish-Cult., 56:367-377.

Smith J. J. 1992. Our responsibility in animal waste management. Pages 121-127 *in* Blake, J., J. Donald, and W. Magette, editors. 1992. National livestock, poultry and aquaculture waste management, Proceedings of the

national workshop. American Society of Agricultural Engineers, St. Joseph, Michigan.

Stewart N. T, Boardman G. D and Helfrich L. A. 2006. Characterization of nutrient leaching rates from settled rainbow trout (*Oncorhynchus mykiss*) sludge. Aquacult. Eng., 35:191–198

Summerfelt S. T., 1999. Waste handling systems. In: Wheaton, F.W., (Vol. Ed.), CIGR (International Commission of Agricultural Engineering) handbook of agricultural engineering, Vol. II. Part I. Aquacult. Eng. American Society of Agric. Eng. St. Joseph, Michigan, pp. 309-350.

Summerfelt S. T. and Wade E. M. 1997. Recent advances in water treatment processes to intensify fish-production in large recirculating systems. In: Tomasso M. (Ed.), Advances in Aquacultural Engineering (Proceedings). Northeast Regional Agricultural Engineering Service, Ithaca, NY, pp. 575-588.

Sweeten, J. M. 1992. Livestock and poultry waste management: a national overview. Pages 4-15 *in* Blake, J., J. Donald, and W. Magette, editors. 1992. National livestock, poultry and aquaculture waste management, Proceedings of the national workshop. American Society of Agricultural Engineers, St. Joseph, Michigan.

Thirumurugan P and Gopalakrishnan A N. 2012. Effect of substrate to inoculum ratio (S/I) on liquefaction and biomethanation of fish market waste. J. Sci. Ind. Res., 71: 699-702.

Thorpe, J. E., and C. Y. Cho. 1995. Minimizing waste through bioenergetically and behaviourally based feeding strategies. Water Sci. and Tech., 31:29-40.

Trzcinski A. P. and Stuckey D.C. 2010. Treatment of municipal solid waste leachate using a submerged anaerobic membrane bioreactor at mesophilic and psychrophilic temperatures: analysis of recalcitrants in the permeate using GC-MS. Water Res., 44: 671–680.

VWRC (Virginia Water Resource Center). 2002. Benthic TMDL reports for six impaired stream segments in the Potomac-Shenandoah and James River basins. Submitted to Virginia Departments of Environmental Quality and Conservation and Recreation. Richmond, VA.

Wang S. L, Liu C. P and Liang T. W. 2012. Fermented and enzymatic production of chitin/chitosan oligosaccharides by extracellular chitinases from *Bacillus cereus* TKU027. Carbohydrate Polymers., 90: 1305-1313.

Westerman, P W., J. M. Hinshaw, and J. C. Barker. 1993. Trout manure characterization and nitrogen mineralization rate. Pages 35-43 *in* Wang J. K., editor. Techniques for modern aquaculture. American Society of Agricultural Engineers, St. Joseph, Michigan.

Willett I. R and Jakobsen P. 1986. Fertilizing properties of trout farm waste. Australia, Elsevier, Agric. Wastes, Volume 17, Issue 1, Pages 7–13.