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RESEARCH PAPER

Characterization of Acidogenesis Occurring on Rainbow Trout *(Oncorhynchus mykiss)* Sludge by Indigenous *Alcaligenes faecalis*

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Abstract For the efficient treatment of sludge accumulated on a rainbow trout farm, two novel strains were isolated that possessed the ability for acidogenic digestion. The strains were identified as *Alcaligenes faecalis* HCB2-A1 and *Alcaligenes faecalis* A2, respectively, and there was synergism between the two strains. Acidogenic digestions using the mixed culture of the two isolates were performed on various sludge mixtures under examinations of changes in major reaction parameters. Among the sludge mixtures, the most stable acidogenic digestion was observed on 1:1 mixture of primary and secondary sludge. During this acidogenesis, pH and ORP dropped to 6.5 and -274 mV within 1 day and then increased steadily. At the same time total solids, COD, and total nitrogen were reduced 58, 79.3 and 42.7%, respectively, with the COD removal rate of 13,017 mg/L/day. The C: N ratio changed from 27:1 to 10:1 as the sludge was digested, and total volatile fatty acids of 6065.3 mg/L was produced for 7 days. The results demonstrated an efficient means to treat aquaculture sludge, which is the alternative to the discharge of the sludge into the river.

Keywords: *Alcaligenes faecalis*, rainbow trout sludge, anaerobic acidification, sludge treatment

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1. Introduction

As an important export, rainbow trout (*Oncorhynchus mykiss*) is produced in many tropical and subtropical countries, the production of which is responsible for an important portion of world food security (http://www.fao.org/fishery/statistics/en). Chile is currently the largest trout producer, and other major producing countries are Norway, France, Italy, Spain, Denmark, USA, Germany, Iran, and UK. African countries also play an important role in trout production (about 1,485 kilotons per year) from inland aquaculture.

During the aquaculture of trout, sludge usually accumulates at the bottom of the sedimentation tank prior to discharge. It is composed of fish fecal matter, microbes, unutilized feed, and dead fish, among other factors. Approximately 18 ~ 30% of sludge is generated per unit of feed in rainbow trout aquaculture [1]. Due to slow degradation, the sludge has to be treated to improve the water quality of the effluent and to reduce the adverse effect of discharge on the ecosystem [2]. Many countries have adopted national legislation in recent years to intensify environmental regulations. As a result, most aquaculture farms prioritize the removal of solids from the effluent to minimize pollutant release to streams.

Anaerobic digestion using obligate anaerobes is a widely used technology in the efficient treatment of organic waste, including sludge, and is known to simultaneously generate renewable energy sources. The efficient anaerobic degradation of sludge is dependent upon the coordination of acidogenesis and methanogenesis. During the anaerobic digestion, energy release from fermentative catabolism is relatively small, and therefore a large fraction of the digested organic matter is converted into biogas. An imbalance in the rates of these two metabolic processes has largely been responsible

for the instabilities associated with anaerobic digestion. The imbalance can lead to the accumulation of intermediate acid products, which will eventually cause the inhibition of methanogenic bacteria [3]. For this reason, investigation of the acidogenic and methanogenic phases operating in two separate reactors was proposed as the best approach to achieve the process stability.

Process stability and overall degradation rates can thus be increased by separately optimizing conditions for each bacterial group [4]. Acidogenesis is responsible for the conversion of complex organics into volatile fatty acids (VFA), whose metabolic rate and production type have important effects on the whole anaerobic digestion. It was reported that hydrolysis and acid formation might be the rate-controlling steps for complex organics such as aquaculture or municipal sludge [5,6]. To achieve efficient anaerobic digestion, improvement or optimization of acidogenesis is important irrespective of the type of substrate. Recently, VFA production through acidogenesis aroused our interest, since these products have diverse applications, such as biogas and biodiesel production [7]. When compared with chemical synthesis, this alternative has additional advantages: cost-effectiveness and environmentally friendly process.

Anaerobic co-digestion approaches to optimize turnover of chemical oxygen demand (COD) in wastewater treatment under a two-phase anaerobic system have been investigated so far [8-10]. Most of these processes were based on lab-scale co-digestion, but few studies have focused on mixtures of aquaculture sludge as substrates. With some possible ecological, technological and economic benefits, these processes were usually performed on at least two different types of substrates, but occasionally on complementary substrates [11]. Despite some benefits of anaerobic co-digestion, it has not been clearly reported whether a mixture of multi-substrates has any adverse impact on co-digestion. Depending upon a synergism or an antagonism among the microbes used, incomplete or complete digestion can occur upon simultaneous digestion of dfferent types of organic wastes [12,13]. To efficiently digest multiple substrates, it is critical to obtain an optimal mixture of available substrates as well as optimal operating conditions [14,15].

The purpose of this study was to seek an on-farm and eco-friendly strategy for the safe disposal of primary and secondary sludge generated from trout farms. This is because aquaculture sludge stored in a settlement tank is often discharged into the river in most developing countries. To date, anaerobic digestion has mainly focused on fisheries processing waste, but little on sludge and wastewater produced during aquaculture. In this context, anaerobic digestion of rainbow trout sludge was suggested as an effective waste management alternative to the disposal. We first isolated potential microorganisms for acidogenic digestion and executed anaerobic acidification using the isolates. This study aimed to characterize acidogenesis occurring on an optimal mixture of rainbow trout sludge to achieve high efficiency of sludge treatment. During the acidogenesis, the formation of products and the subsequent removal of total nitrogen were also examined.

2. Materials and Methods

2.1. Sludge sample

For the experiments of acidogenic digestion under anaerobic conditions, sludge was first collected from the Ihwajeong trout farm (Sangju, Gyeongbuk Province, South Korea). The farm breeds rainbow trout using a concrete -lined recirculating aquaculture system using underground water and produces an average of 420 tons (of fish) per year. The

Table 1. Characteristics of the rainbow trout sludge collected from a trout farm

Characteristic	Primary sludge	Secondary sludge
pH	5.79	6.51
ORP (mV)	-160.4	-183.5
Total solids (%)	13.7	10.4
Volatile solids (%)	64.2	35.0
Fixed solids (%)	35.8	65.0
COD (mg/L)	127,300	70,700
Total nitrogen (mg/L)	4,270	2,930
C:N	30:1	24:1

Table 2. Chemical components of the rainbow trout sludge collected from a trout farm

Element	Primary sludge	Secondary sludge
Lipid	1.23 ± 0.04	0.16 ± 0.01
Protein	3.36 ± 0.06	1.90 ± 0.03
Carbohydrate	95.41 ± 0.10	97.94 ± 0.13
Ca (%)	1.65 ± 0.02	1.47 ± 0.02
P (%)	0.80 ± 0.02	0.62 ± 0.01
K (%)	0.02 ± 0.01	0.03 ± 0.01
Mg (%)	0.04 ± 0.01	0.05 ± 0.02
Na (%)	0.02 ± 0.01	0.03 ± 0.01
Zn (ppm)	192 ± 8	196 ± 12
Fe (ppm)	692 ± 10	890 ± 8
Mn (ppm)	102 ± 2	98 ± 5
Cd (ppm)	1.09 ± 0.08	0.67 ± 0.10
Cu (ppm)	n.d.[a]	n.d.
Hg (ppm)	n.d.	n.d.
Se (ppm)	n.d.	n.d.

[a]n.d.: not detected.

sludge was sampled from earthen ponds breeding about two-month-old trout: primary sludge was collected at a depth of 0.5 m, while secondary sludge was collected at 2 m depth. The collected sludge was sonicated for 15 min at 40 kHz and 50 W for homogeneity and then sieved to obtain particles less than 2 mm size. The characteristics and chemical components of the primary sludge and the secondary sludge were tabulated in Tables 1 and 2, respectively. The collected sludge was autoclaved for 10 min at 121°C and then preserved at -20°C prior to use in experiments.

2.2. Isolation of potential microorganisms for acidogenesis

Bacteria with potential applications to the acidogenic digestion of the main polysaccharides contained in rainbow trout sludge were isolated from soil sunk in pond bottom of a rainbow trout farm. One gram of each sludge sample was added to a sterile 250 mL flask that contained (per L): 20 g of glucose, 2 g of meat extract, 2 g of NH_4Cl, 0.5 g of K_2HPO_4, 1 g of KH_2PO_4, 0.2 g of $FeCl_2 \cdot 4H_2O$, 0.4 g of $CaCl_2 \cdot 2H_2O$, 0.3 g of $MgSO_4 \cdot 7H_2O$, 0.3 g of KCl, and 1 mL of trace element solution (pH 7.0). The trace element solution was composed of (per L): 0.05 g of H_3BO_3, 0.05 g of $ZnCl_2$, 0.03 g of $CuCl_2$, 0.05 g of $MnSO_4 \cdot H_2O$, 0.05 g of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.05 g of $AlCl_3$, 0.05 g of $CoCl_2 \cdot 6H_2O$ and 0.05 g of $NiCl_2$. The seeded flask was flushed with a sterile N_2 gas (passing through a 0.2 μm filter) for 20 min in advance, and then incubated for 3 weeks at 37°C and 160 rpm. After 3 weeks' incubation, 100 μL of culture broth taken from the flask was spread with a platinum loop onto the solid medium containing the components of the liquid culture medium, 0.1 g of bromocresol purple (as an indicator for acid production) and 1.5% agar (for solidification). The agar plate was incubated for 3 days at 37°C using air-tight gas pack (GasPak 100® System, Voigt Global Distribution Inc., USA) containing palladium pellets for removal of O_2, a chemical envelope producing H_2 and CO_2 and methylene blue as anaerobic indicator. After then a colony showing yellowish color was purely isolated. The purified isolate was obtained by repeated streaking onto the fresh agar plate. The pure strain was maintained on the agar plates sealed with parafilm, and the agar plate was stored at 4°C until use. The pure strain was transferred to a fresh agar plate every two weeks.

2.3. Tests of antagonism and synergism among isolated strains

According to the perpendicular-streak technique, as described by Alippi and Reynaldi [16], the antagonistic behavior of the screened strains was tested against each other. The agar plate on which screened strains were cross- streaked was incubated for 3 days at 37°C in the air-tight gas pack. To test synergism among the screened strains, each strain was cultivated individually, and all combined strains were cultivated in parallel. After cultivation, the production of VFA from the combined strains was compared with the sum of the VFA produced from individual cultivation of each strain.

2.4. Identification of isolates

After the potential strains for acidogenic digestion had been selected, they were primarily identified on the basis of colony morphology, Catalase test, Gram reaction of bacterial smear, and microscopic examination.

Specific identification of the isolates was performed by 16S rRNA gene sequence analysis. According to the manufacturer's instructions, DNA was extracted using an AccuPrep® Genomic DNA extraction kit (Bioneer, Korea). PCR amplification of the gene using the 158F (5'-CCAGCAGCCGCGGTAATACG-3') and 800R (5'-TAC CAGGGTATCTAATCC-3') was conducted with a PCR thermal cycler (DICE model TP600, Takara, Japan). The reaction mixture for PCR amplification contained primers (10 pmol/μL), 2.5 mM dNTPs, 10× reaction buffer, 2.5 U Taq polymerase (Takara, Japan), 1 μg DNA template and sterilized water to achieve a final volume of 50 μL. PCR was conducted under the following conditions: initial denaturation at 95°C for 5 min; 30 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec; and a final extension step at 72°C for 10 min. Phylogenetic analysis of the given sequences and their close relatives were conducted using the neighbor-joining method with 1000 bootstrap replicates using MEGA version 5 software [17].

2.5. Seed culture of isolates

To prepare an inoculum for acidogenic digestion experiments under an anaerobic condition, the isolated strains were cultivated. The medium used for seed culture was prepared according to that reported by Cheong and Hansen [18]. It was composed of (per L): 21.3 g of glucose, 2 g of meat extract, 2.12 g of NH_4Cl, 0.42 g of K_2HPO_4, 1 g of KH_2PO_4, 0.18 g of $FeCl_2 \cdot 4H_2O$, 0.375 g of $CaCl_2 \cdot 2H_2O$, 0.312 g of $MgSO_4 \cdot 7H_2O$, 0.25 g of KCl, 1 mL of trace element solution, 1 mL of HCl (36%), and 8 g of $NaHCO_3$ (pH 7.3). After autoclaved for 15 min at 121°C, a 250 mL flask containing the culture medium was cooled down to room temperature. Subsequently, the flask was flushed for 20 min with a sterile N_2 gas passed through a 0.2 μm filter. After 0.1 g of the purified isolates was inoculated, the flask was tightly sealed and incubated at 37°C and 160 rpm. Cells were collected when their growth reached at a late log phase, as seed culture for experiments of acidogenic digestion.

2.6. Experiment of acidogenic digestion

The experiment for acidogenic digestion was carried out on various mixtures of primary sludge and secondary sludge in a 500 mL conical flask sealed with butyl rubber stoppers (Table 3). Each sludge mixture was preliminarily homogenized, and less than 2 mm of its particle size was used after sieved. The working volume of each flask was 300 mL. The total solid included in the flask was 10% (v/v), and the flask was flushed for 20 min with a sterile N_2 gas passed through a 0.2 μm filter. After 30% (v/v) had seeded cells was inoculated, the anaerobically sealed flask was incubated at 37°C and 160 rpm. Under flashing a sterile N_2 gas, the sample was taken every 24 h from the flak during the 7 day experimental period. At each sampling time, pH was adjusted to 7.0 with few drops of 2N NaOH whenever it was below 7.0. Each experiment was carried out in triplicate.

Table 3. Various mixtures of sludge collected from a trout farm, as substrates for acidogenesis

Substrate	Mixture
1	Primary sludge only (PS)
2	Secondary sludge only (SS)
3	PS (33%) + SS (67%)
4	PS (67%) + SS (33%)
5	PS (50%) + SS (50%)

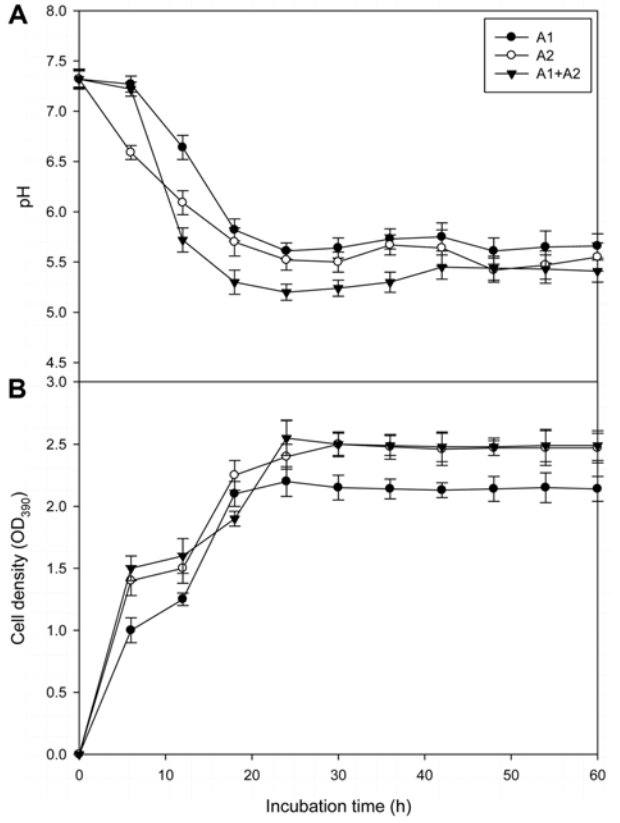

Fig. 1. Results of pH (A) and cell density (B) during the mixed culture of A1 and A2 in comparison with those during the culture of each strain individually.

2.7. Analytical methods

The pH and ORP were measured by an Istek pH/ORP meter. Total solids were measured according to the analytical method for wastewater [19]. The concentrations of chemical oxygen demand- dichromate (COD_{Cr}) and total nitrogen were measured by the spectrophotometric method using a Water-quality analyzer (Humans Co., Ltd, Korea) [20]. The concentrations of VFA including acetate, propionate, butyrate and valerate were determined by a gas chromatography (Acme-6000 series, Younglin) equipped with a cross-linked polyethylene glycol (Rtx-WAX) capillary column (30 m × 250 μm × 0.25 μm) and a FID detector. The temperatures of the injection and detector were 220 and 230°C, respectively while the oven temperature was 200°C at a speed of 15°C/min. Nitrogen (under a pressure of 30.4 kPa) was used as a carrier gas and injected at a rate of 1.0 mL/min [21].

2.8. Statistical analysis

Analysis of variance (ANOVA) was used to identify whether there were any significant differences in the acidogenic digestibility of various sludge mixtures.

3. Results

3.1. Screening of potential bacteria for acidogenesis

By repeated streaking on agar plates containing bromocresol purple, two bacterial strains were purely isolated. The two isolates demonstrated acidification ability on the simulated sludge medium and were given the names A1 and A2. From the perpendicular streak technique, it was proved that there was not any antagonistic behavior between the two strains. In addition, the culture of the mixed strains of A1 and A2 showed a bigger drop in pH due to the more active production of VFA and a better cell growth than that of the individual strain of A1 or A2 (Fig. 1).

3.2. Identification of two isolated bacteria

Based on the microscopic observation, the two isolates were very motile and in vegetative states, Catalase-negative and Gram-negative rods measuring 1.0 ~ 1.2 μm in width and 2 ~ 4 μm in length, and endospore was not formed. The colors of colonies shaping circular were light creamy (A1) and creamy (A2), respectively, and the colony size of the strain A2 had a little bigger than that of the strain A1.

According to the partial 16S rRNA gene sequence analysis, the species-specific identification of the rod-shaped isolates was performed. The 1539 bp- (A1) and 1691 (A2) bp-sized fragments of the 16S rRNA genes of the two isolate were

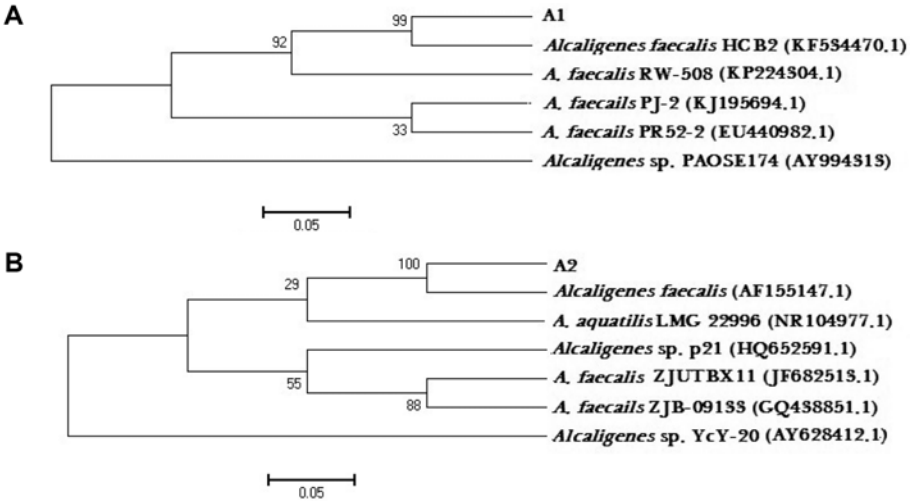

Fig. 2. Phylogenetic tree based on the partial 16S rRNA gene sequences of the strain A1 (A), the strain A2 (B) and other related *Alcaligenes* species. Bootstrap values (expressed as percentages of 1,000 replicates) > 50% are given at branching points of interest. The scale bar indicates 5% of sequence variation.

amplified and sequenced, respectively. Homology searches revealed that the strain A1 was closely related to *Alcaligenes faecalis* HCB2 (KF534470.1; 99% similarity), *A. faecalis* RW-508 (KP224304.1; 99% similarity), *A. faecalis* PR52-2 (EU440982.1; 98% similarity), *A. faecalis* PJ-2 (KJ195694.1; 98% similarity), and *Alcaligenes* sp. PAOSE174 (AY994313.1; 98% similarity). The strain A2 was closely related to *A. faecalis* (AF155147.1; 100% similarity), *A. aquatilis* LMG22996 (NR1049771; 99% similarity), *Alcaligenes* sp. p21 (HQ652591.1; 99% similarity), *A. faecalis* ZJUTBX11 (JF682513.1; 99% similarity), *A. faecalis* ZJB-09133 (GQ438851.1; 98% similarity), and *Alcaligenes* sp. YcX-20 (AY628412.1; 99% similarity). The relation between each isolated strain and other related strains were closely shown in the phylogenetic tree based on the partial 16S rRNA gene (Fig. 2). As a result, the isolated two strains were designated as *Alcaligenes faecalis* HCB2-A1 and *Alcaligenes faecalis* A2, respectively.

3.3. Changes in pH and ORP during acidogenesis on sludge mixtures

Acidogenic digestion using two isolated strains was carried out on various sludge mixtures. Based on the result of the preliminary experiment, two strains were combined 1:1, at which the highest production of VFA was obtained with the highest content of acetic acid. Due to the production of VFA, pH starting at 7.3 dropped rapidly to 6.4 ~ 6.6 within 1 day in all the digestions on various sludge mixtures (Fig. 3A). After then, it increased steadily. Likewise pH, ORP dropped drastically to below -250 mV within 1 day for all the sludge mixtures when acidogenic digestion proceeded. After then, it increased steadily during the rest of the incubation period. The acidogenic digestion performed

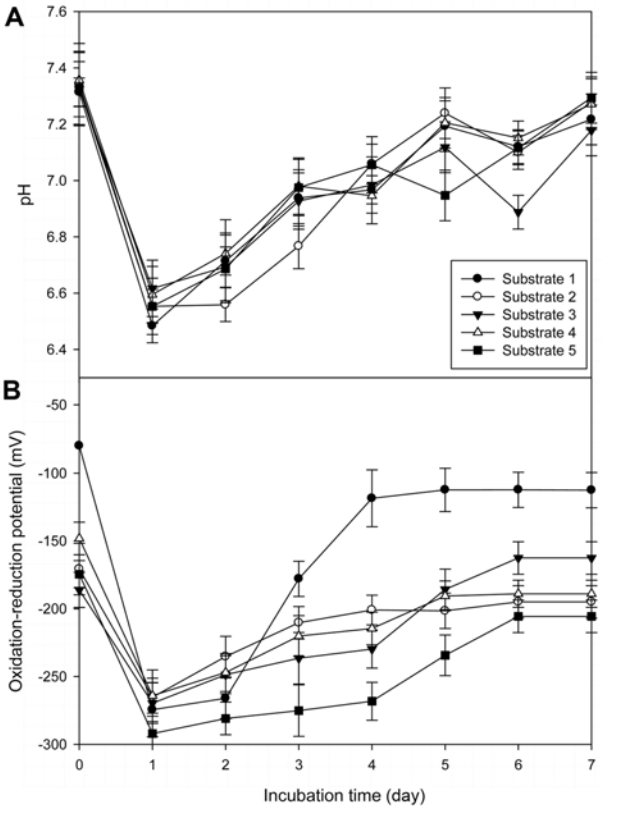

Fig. 3. Time courses of changes in pH (A) and ORP (B) on various sludge mixtures during the acidogenic digestions by mixed isolates in a 300 mL flask incubated at 37°C and 160 rpm. The data are presented as the mean ± SD (n = 3).

on the Substrate 5 showed the lowest ORP value (-274 mV) during the 7 day incubation, accompanying with the lowest recovery after 1 day (Fig. 3B). With the highest recovery after 1 day, however, ORP increased to -112 mV when the

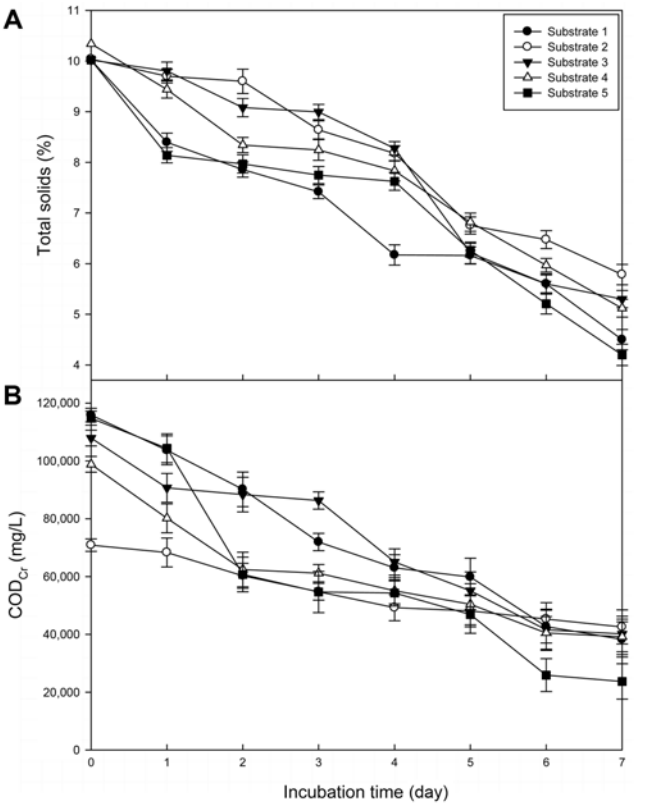

Fig. 4. Time courses of changes in total solids (A) and COD_{Cr} (B) on various sludge mixtures during the acidogenic digestions by mixed isolates in a 300 mL flask incubated at 37°C and 160 rpm. The data are presented as the mean ± SD (n = 3).

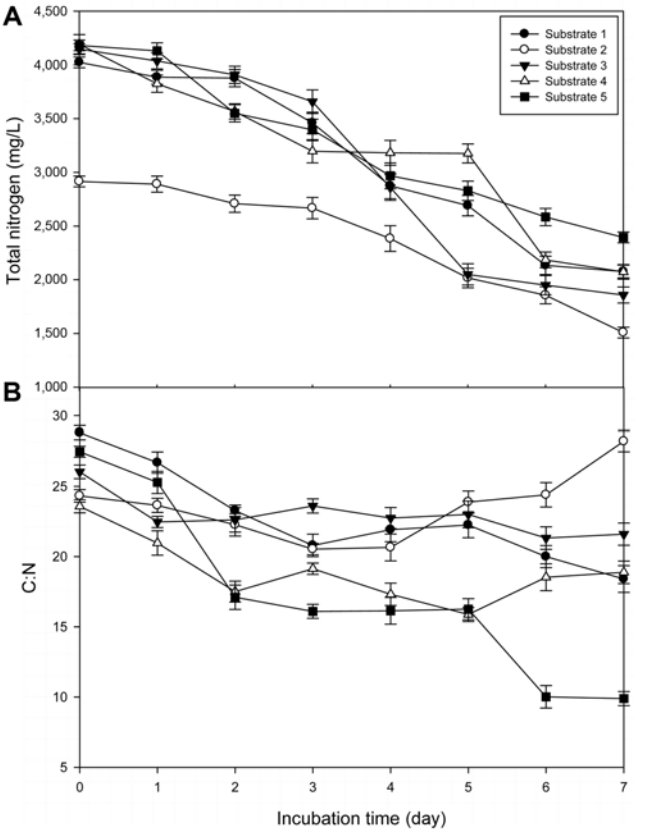

Fig. 5. Time courses of changes in total nitrogen (A) and C:N ratio (B) on various sludge mixtures during the acidogenic digestions by mixed isolates in a 300 mL flask incubated at 37°C and 160 rpm. The data are presented as the mean ± SD (n = 3).

Substrate 1 was used in the acidogenic digestion.

3.4. Changes in total solids and COD during acidogenesis on sludge mixtures

Due to the digestion by the two isolated bacteria, there was a gradual decrease in total solids for all the sludge mixtures throughout the 7 day incubation. The most significant change in total solids was observed on the Substrate 5 with 58% reduction (Fig. 4A). The half reduction of the total solids in the digestion on the Substrate 5 was completed within 4 days. The reductions in total solids were found to be 55, 42, 47, and 50% for the Substrate 1, the Substrate 2, the Substrate 3 and the Substrate 4, respectively. Likewise total solids, COD was decreased by the microbial activity for all the sludge mixtures throughout the 7 day incubation (Fig. 4B). The digestion on the Substrate 2 revealed the lowest reduction (39.8%) in COD, while that on the Substrate 5 had the highest reduction (79.3%) in COD with the highest removal rate (13,017 mg/L/day). The digestions on the other sludge mixtures revealed 67.0, 62.8, and 60.4% of reductions in COD for the Substrate 1, the Substrate 3 and the Substrate 4, respectively.

3.5. Changes in total nitrogen and C:N ratio during acidogenesis on sludge mixtures

From the microbial consumption of nitrogen for the digestion on the sludge mixture, its concentration was decreased throughout the 7 day incubation (Fig. 5A). The highest (55.2%) and the lowest (42.7%) reductions in total nitrogen were obtained from the digestions on the Substrate 3 and the Substrate 5, respectively. Meanwhile, reductions in total nitrogen were found to be 48.4, 48.2, and 50.5% for the digestions on the Substrate 1, the Substrate 2 and the Substrate 4, respectively. During the digestion on each sludge substrate, the change in C:N (COD to total nitrogen) ratio was evaluated (Fig. 5B). The biggest change from 27:1 to 10:1 in C: N ratio was obtained from the digestion on the Substrate 5. On the contrary, the smallest change was obtained from the digestion on the Substrate 2.

3.6. Change in total VFA during acidogenesis on sludge mixtures

As acidogenic digestion proceeded, there was a notable increase in VFA production from all the sludge mixtures

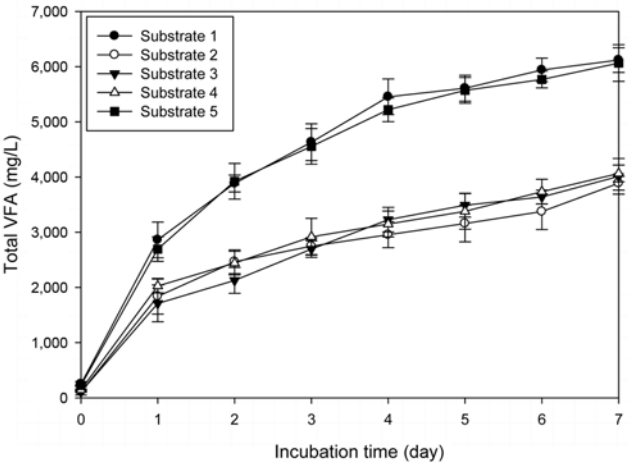

Fig. 6. Time courses of changes in total VFA on various sludge mixtures during the acidogenic digestions by mixed isolates in a 300 mL flask incubated at 37°C and 160 rpm. The data are presented as the mean ± SD (n = 3).

(Fig. 6). The 7 day-VFA production by the digestion was highest (6119.4 mg/L) on the Substrate 1, with steady accumulation. The VFA production by the digestion on the Substrate 5 (6065.3 mg/L) was comparable to that on the Substrate 1. Compare with the above, the VFA productions by the digestions on the Substrate 2, the Substrate 3, and the Substrate 4 were much lower (about 3,890 mg/L). The produced VFAs were mainly propionate and butyrate for all the sludge mixtures (Table 4). The highest concentration (2682.0 mg/L) of propionate was obtained from the digestion on the Substrate 5, while that (2136.0 mg/L) of butyrate was obtained from the digestion on the Substrate 1.

4. Discussion

Among the six strains isolated from the sludge accumulated in rainbow trout farm, only two strains showed the ability of acidogenic digestion. These indigenous strains were identified as *A. faecalis* HCB2-A1 and *A. faecalis* A2, respectively. The strain A2 showed higher cellular growth and higher VFA production than strain A1. On the other hand, the strain A1 showed better production of acetic acid than the strain A2. There was no antagonism between the two strains, and drop in pH was more significant in the mixed culture of the two strains, compared with that in the individual culture of each strain. This indicates there was some synergism between the two strains, which was good for acidogenic digestion. In the anaerobic decomposition of organic materials, heterotrophs are mostly used [22]. This type of biological treatment generally relies not solely on a single strain, but on a microbial consortium [23]. For this reason, a group of microbes including *Actinomycetes*, *Thermomonospora*, *Ralstonia* and *Shewanella* was reported to be used for the production of VFA from the degradation of food waste [24]. Meanwhile, *Alcaligenes* species were reported to involve into the acidification process of pig manures [25], and among these species, *A. faecalis* was often found in sludge anaerobic acidification process [26]. As a result, the two isolates were eligible for acidogenic digestion.

Although anaerobic digestion is commonly used in wastewater sludge treatment, biologically low degradability of sludge becomes an issue. Once organic sludge is generated, it undergoes considerable changes in physical and biological properties. Therefore, the digestibility of the sludge is influenced by its pH, temperature, salinity, mineral composition, loading rate, hydraulic retention time, carbon to nitrogen ratio and VFA content. Primary sludge used in this study was relatively young (about 1 month of retention time). Thus, it contained a high proportion of biodegradable organic matter, compared with secondary sludge. Sludge flocs contain a high amount of free or bounded water, and extracellular polymeric substances play important roles in sludge floc structure [27]. Since water entrapped in sludge flocs could be released by the floc destruction, all the sludge mixtures were preliminarily autoclaved in this study to enhance degradability of sludge.

For acidogenic digestions by the two isolated microbes on various sludge mixtures, changes in major parameters including pH, ORP, total solids, COD, total nitrogen and production of VFA were examined during 7 day incubation. Within 1 day incubation, pH dropped sharply in all the digestions on various sludge mixtures, accompanying with a big drop in ORP. This phenomenon happened because VFA was produced from the active degradation of sludge by the two isolates. It was reported that pH had a crucial

Table 4. Composition of VFA produced from anaerobic digestions on various mixtures of sludge after 7 day incubation

Substrate	VFA (mg/L)				
	Acetate	Propionate	Butyrate	Valerate	Total
1	829.7	2,512.0	2,136.0	641.7	6,119.4
2	397.5	1,330.2	1,781.2	378.7	3,887.6
3	428.1	1,490.1	1,735.3	359.0	4,012.5
4	378.2	1,506.2	2,030.8	150.0	4,065.2
5	976.0	2,682.0	1,922.8	484.5	6,065.3

influence on both the performance of acidogenesis and the formation of intermediate products, implying the importance of pH control [28]. In this study, pH was adjusted to 7.0 three times at day 1, 2, and 3. Due to the pH adjustment, the inhibition of VFA on the cell activity of two isolates could be reduced, resulting in the continuous production of VFA from the sludge.

As the sludge was degraded, the concentrations of solids, COD and nitrogen were decreased. The highest reductions in total solids (58%) and COD (79.3%) were observed from the digestion on 1:1 mixture of primary and secondary sludge. This indicates that the balance brought by mixing of the two different sludge characters was important for better acidogenic digestion. The degree of reduction in total solids obtained from this study was comparable, compared with those reported by other researchers: 20 ~ 50% [29] and 45 ~ 80% [8] in digestions of activated sludge, respectively. In addition, Reed *et al.* [30] reported that the volume of return activated sludge could be reduced by 90% by the anaerobic digestion. Cakir and Stenstorm [31] also reported treatment efficiency of 51 ~ 96% *via* anaerobic sludge digestion. Consequently, anaerobic digestion could lower sludge transport and external treatment costs that are major factors for the feasibility of most aquaculture operations. Moreover, the pollution strength of the treated sludge was significantly lower than that of raw sludge due to the reduction in total N. The C:N ratios of the sludge mixtures used in this study were in a range of 24 ~ 30. For stable anaerobic digestion of organic wastes, C:N ratios between 25 and 30 were reported to be optimal for high-solids anaerobic digestion of municipal solid waste [32]. However, C: N of 11 was also suggested for satisfactory anaerobic digestion of organic waste [33]. It may be dependent on the property of a substrate, microorganisms used and reaction conditions.

The composition of the substrate is one of the influential factors for a suitable acidogenic digestion, and it may affect the formation of intermediate products. It was reported that carbohydrate-rich substrates were good producers of VFA and protein-rich substrates yielded a good buffering capacity [34]. In this study, 1:1 mixture of primary and secondary sludge was found to be the best substrate for acidification by the two isolates. From all the above results, anaerobic digestion appeared to be an attractive approach for the management of aquaculture sludge. As a result, this biological treatment would allow addressing problems associated with the traditionally used management methods, especially discharge into receiving water body.

In the sludge digestion, a high concentration of long-chain fatty acids originating from the fish feed can be an inhibitory factor [35]. This inhibitory effect can be more significant if the feed is not ingested by fish and thus accumulates into sludge floc. In this study, the dominantly produced VFAs on sludge mixtures were propionate and butyrate. Although these two organic acids are converted mostly to acetate and H_2 gas, acetate was not actively produced. It was reported that metabolic pathways involving acetic and butyrate production appeared to be favored at pH 4.5 ~ 6.0, while neutral or higher pH might promote propionate production [36]. In this study, pH was adjusted three times for a better production of VFA from the sludge mixture, and accordingly, the production of VFA increased as sludge was degraded. Probably, low production of acetate resulted from the pH adjustment in spite of a relatively high VFA production. Moreover, it was reported in anaerobic digestion that more than 200 mM of acetate and butyrate or 100 mM of propionate could cause process inhibition, resulting in ultimate digestion failure [37,38]. This strongly implies that the acidogenic digestion by the isolates was stable without process inhibition in this study. Propionate and butyrate are the most important syntrophic intermediates. This is because the subsequent degradation after their production is regarded as a rate limiting step due to thermodynamic interaction [39]. In addition, degradation of propionate is a central key to improving the performance of anaerobic digestion system, since propionate is generally accumulated in anaerobic digesters. To cause not any inhibition in the biodegradation of propionate, many factors have to be satisfactorily controlled, such as pH, temperature, VFA, the partial pressure of H_2, and toxins [40].

Anaerobic sludge digestion from freshwater recirculating aquaculture system was first reported in the 1990's with little success [41,42]. After then, a continuously stirred treatment reactor system operating under mesophilic conditions with diluted wastewater has been suggested to overcome the inhibition of ammonia. Besides, disposal of aquaculture sludge into wastewater treatment systems is often prohibited. This is because it usually involves high volumes with high organic matter content and/or salts that might interfere with the treatment of municipal sludge [43]. The worse than this is a discharge of sludge into the water receiving bodies. Recently, the treatment of aquaculture sludge in waste stabilization ponds has been reported to be a preferable alternative, with 17 ~ 30% conversion of influent organic carbon into methane [44]. Yet, the utilization of aquaculture sludge remains to be a fairly new concept. Therefore, the result obtained from this study is meaningful to provide an efficient treatment means for aquaculture sludge mixtures.

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References

1. Heinen, J. M., J. A. Hankins, and P. R. Adler (1996) Water quality and waste production in a recirculating trout-culture system with the feeding of a higher-energy or lower-energy diet. *Aquac. Res*. 27: 699-710.
2. Wang, S. L., C. P. Liu, and T. W. Liang (2012) Fermented and enzymatic production of chitin/chitosan oligosaccharides by extracellular chitinases from *Bacillus cereus* TKU027. *Carbohyd. Polym*. 90: 1305-1313.
3. Liu, Y., Y. Zhang, X. Quan, Y. Li, Z. Zhao, X. Meng, and S. Chen (2012) Optimization of anaerobic acidogenesis by adding Fe powder to enhance anaerobic wastewater treatment. *Chem. Eng. J.* 192: 179-185.
4. Dinopoulou, G., T. Rudd, and J. N. Lester (1988) Anaerobic acidogenesis of a complex wastewater: 1. The influence of operational parameters on reactor performance. *Biotechnol. Bioeng*. 31: 958-968.
5. Miron, Y., J. Zeeman, B. van Lier, and G. Lettinga (2000) The role of sludge retention time in the hydrolysis and acidification of lipids, carbohydrates and proteins during digestion of primary sludge in CSTR systems. *Water Res.* 34: 1705-1713.
6. Speece, R. E. (1983) Anaerobic biotechnology for industrial wastewater treatment. *Environ. Sci. Technol*. 17: A416-A427.
7. Fontanille, P., V. Kumar, G. Christophe, R. Nouaille, and C. Larroche (2012) Bioconversion of volatile fatty acids into lipids by the oleaginous yeast *Yarrowia lipolytica*. *Bioresour. Technol*. 114: 443-449.
8. Callaghan, F. Jj, D. A. J. Wase, K. Thayanithy, and C. F. Forster (1999) Co-digestion of waste organic solids: Batch studies. *Bioresour. Technol*. 67: 117-122.
9. Kabouris, J. C., U. Tezel, S. G. Pavlostathis, M. Engelmann, J. Dulaney, R. A. Gillette, and A. C. Todd (2009) Methane recovery from the anaerobic co-digestion of municipal sludge and FOG. *Bioresour. Technol*. 100: 3701-3705.
10. Kassuwi, S. A. A., A. M. Mshandete, and A. K. Kivaisi (2012) Anaerobic co-digestion of biological pretreated Nile perch solid waste with a vegetable fraction of the market solid waste. *ARPN J. Agri. Biol. Sci*. 7: 1016-1031.
11. Alvarez, R. and G. Lidén (2008) Semi-continuous co-digestion of solid slaughterhouse waste, manure, and fruit and vegetable waste. *Renew. Energy* 33: 726-734.
12. Callaghan, F. J., D. A. J. Wase, K. Thayanithy, and C. F. Forster (2002) Continuous co-digestion of cattle slurry with fruit and vegetable wastes and chicken manure. *Biomass Bioenerg*. 22: 71-77.
13. Malta-Alvarez, J., S. Mace, and P. Llabres (2004) Anaerobic digestion of organic solid wastes, an overview of research achievements and perspectives. *Bioresour. Technol*. 74: 3-16.
14. Alvarez, J. A., L. Otero, and J. M. Lema (2009) A methodology for optimizing feed composition for anaerobic co-digestion of agro-industrial wastes. *Bioresour. Technol*. 101: 1153-1158.
15. Thirumurugan, P. and A. N. Gopalakrishnan (2012) Effect of the substrate to inoculum ratio (S/I) on liquefaction and biomethanation of fish market waste. *J. Sci. Ind. Res*. 71: 699-702.
16. Alippi, A. M. and F. J. Reynaldi (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.
17. Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol*. 28: 2731-2739.
18. Cheong, D. and C. L. Hansen (2006) Acidogenesis characteristics of natural, mixed anaerobes converting carbohydrate-rich synthetic wastewater to hydrogen. *Proc. Biochem*. 41: 1736-1745.
19. APHA (1998) *Standard methods for the examination of water and wastewater*. 20th ed., American Public Health Association, Washington DC, USA.
20. ISO (2002) *ISO 15705:2002(en), Water quality determination of the chemical oxygen demand index (ST-COD) - small-scale sealed-tube method*. International Organization for Standardization, Geneva 18.
21. Niu, Q., T. Hojo, W. Qiao, H. Qiang, and Y. –Y. Liu (2014) Characterization of methanogenesis, acidogenesis and hydrolysis in thermophilic methane fermentation of chicken manure. *Chem. Eng. J*. 244: 587-596.
22. Fricke, K., H. Santen, R. Wallmann, A. Huttner, and N. Diehtl (2007) Operating problems in anaerobic digestion plants resulting from nitrogen in municipal solid waste. *Waste Manage*. 27: 30-43.
23. Fantozzi, F. and C. Buratti (2009) Biogas production from different substrates in an experimental continuously stirred tank reactor anaerobic digester. *Bioresour. Technol*. 100: 5783-5789.
24. Ike, M., D. Inoue, T. Miyano, T. T. Liu, K. Sei, S. Soda, and S. Kadoshin (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.
25. Zhang, D., X. Yuan, P. Guo, Y. Suo, X. Wang, W. Wang, and Z. Cui (2011) Microbial population dynamics and changes in main nutrients during the acidification process of pig manures. *J. Environ. Sci*. 23: 497-505.
26. Liu, Y., L. Xin, X. Kang, Y. Yuan, M. Jiao, J. Zhan, and M. Du (2015) Effect of extracellular polymeric substances disintegration by ultrasonic pretreatment on waste activated sludge acidification. *Int. Biodeter. Biodegrad*. 102: 131-136.
27. Nguyen, M. T., N. H. M. Yasin, T. Miyazaki, and T. Maeda (2014) Enhancement of sludge reduction and methane production by removing extracellular polymeric substances from waste activated sludge. *Chemosphere* 117: 552-558.
28. Dareioti, M. A., A. I. Vavouraki, and M. Kornaros (2014) Effect of pH on the anaerobic acidogenesis of agroindustrial wastewaters for maximization of bio-hydrogen production: A lab-scale evaluation using batch tests. *Bioresour. Technol*. 67: 218-227.
29. Parkin, G. F. and W. F. Owen (1986) Fundamentals of anaerobic digestion of wastewater sludges. *J. Environ. Eng*. 112: 867-920.
30. Reed, S. C., R. W. Crites, and E. J. Middlebrooks (1995) *Natural systems for waste management and treatment*. McGraw-Hill, NY, USA.
31. Cakir, F. Y. and M. K. Stenstrom (2005) Greenhouse gas production: A comparison between aerobic and anaerobic wastewater treatment technology. *Water Res*. 39: 4197-4203.
32. Kayhanian, M. and S. Hardy (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.
33. Gunaseelan, V. G. (1995) Effects of inoculums/substrate ratio and pretreatments of methane yield from Parthenium. *Biomass Bioenerg*. 8: 39-44.
34. Foresti, E. (2001) Perspective on anaerobic treatment in developing countries. *Water Sci. Technol*. 44: 141-148.

35. Gebauer, R. (2004) Mesophilic anaerobic treatment of sludge from saline fish farm effluents with biogas production. *Bioresour. Technol*. 93: 155-167.
36. Guo, X. M., E. Trably, E. Latrille, H. Carrre, and J. P. Steyer (2010) Hydrogen production from agricultural waste by dark fermentation: A review. *Int. J. Hydrogen Energy* 35: 10660-10673.
37. Hill, D. T., S. A. Cobb, and J. P. Bolte (1987) Using volatile fatty acids relationships to predict anaerobic digester failure. *Transactions of the ASABE* 30: 496-501.
38. Ahring, B. K., M. Sandberg, and I. Angelidaki (1995) Volatile acids as indicators of process imbalance in anaerobic digesters. *Appl. Microbiol. Biotechnol*. 43: 559-565.
39. de Bok, F. A. M., C. Plugge, and A. J. M. Stams (2004) Interspecies electron transfer in methanogenic propionate-degrading consortia. *Water Res.* 38: 1368-1375.
40. Li, J., Q. Ban, L. Zhang, and A. K. Jha (2012) Syntrophic propionate degradation in anaerobic digestion: A review. *Int. J. Agric. Biol*. 14: 843-850.
41. Kugelman, I. J. and S. van Gorder (1991) Water and energy recycling in closed aquaculture systems. *Proceedings from the Aquaculture Symposium, Cornell University*. April 4-6, Ithaca, NY, USA.
42. Lanari, D. and C. Franci (1998) Biogas production from solid wastes removed from fish farm effluents. *Aquat. Living Resour*. 11: 289-295.
43. Mirzoyan, N., Y. Tal, and A. Gross (2010) Anaerobic digestion of sludge from intensive recirculating aquaculture systems: Review. *Aquacult.* 306: 1-6.
44. Green, F. B., L. Bernstone, T. J. Lundquist, J. Muir, R. B. Tresan, and W. J. Oswald (1995) Methane fermentation, submerged gas collection, and the fate of carbon in advanced integrated wastewater pond systems. *Water Sci. Technol*. 31: 55-65.