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ORIGINAL PAPER

Correlation of microbial mass with ATP and DNA concentrations in acidogenesis of whey permeate

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Abstract In this paper, we examine variations in the contents of ATP and DNA per unit microbial mass in an acidogenesis of whey permeate. We also introduce a novel approach to estimate microbial mass by measuring ATP and DNA when the ratios of ATP and DNA to microbial mass vary. Acidogenic experiments were performed at 35°C and pH 6.0 in batch mode. The amounts of ATP and DNA per unit microbial mass were not consistent during the incubation except during the post-decay phase. Especially within the exponential phase, each showed a 10-fold difference between maximal and minimal values. In this case, the conventional method which converts ATP or DNA concentration into microbial mass using a fixed conversion factor can give inaccurate results. While the constant ratios of 0.74 mg ATP/g VSS and 1.96 mg DNA/g VSS were determined for the post-decay phase, the ATP and DNA concentrations showed strong linear relationships with the microbial mass ($r^2 = 0.99$) within the ranges of 0.039–1.078 mg ATP/l and 0.075–2.080 mg DNA/l, respectively. The linear regression equations are as follows: (1) microbial mass concentration (mg/l) = 478.5 × ATP concentration (mg/l) + 293.5, (2) microbial mass concentration (mg/l) = 257.2 × DNA concentration (mg/l) + 250.4. Therefore, changes in the mass of the acidogenic population should be monitored by the combined use of the regression equations obtained in the exponential phase and the constant ratios determined in the post-decay phase. This procedure should be widely applicable to the acidogenesis of dairy processing wastewaters, especially of a highly suspended organic wastewater such as whey.

Keywords Acidogenesis · ATP · Dairy processing wastewater · DNA · Microbial mass · Whey permeate

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Introduction

Whey is a by-product of cheese production obtained from the separation of butter fat and casein as curd from milk. In the cheese-making industry, about 90% of the whole milk processed into cheese is discharged as whey. The organic matter in whey causes a chemical oxygen demand (COD) in the range of 40–75 g/l, an indication of high pollution potential (Rajeshwari et al. 2000; Lee et al. 2003). Biological systems, either aerobic or anaerobic, can be used in the treatment of whey. However, anaerobic digestion offers several advantages over aerobic process, especially in the treatment of high strength organic wastewaters such as whey: the low energy requirement for process operation, low sludge production rate, and production of

methane, a readily available energy source (Speece 1996; Yu et al. 2002).

Anaerobic digestion is a metabolic series composed of three stages: hydrolysis, acidogenesis, and methanogenesis. These reactions are carried out by various symbiotic microorganisms, which are broadly divided into two groups: acidogens and methanogens (McCarty and Smith 1986; Speece 1996). The former is the bacterial group which hydrolyzes and ferments organic materials in the wastewater and produces various organic acids and some alcohols, and the latter is the archaeal group which produces methane utilizing the acidogenic products. Conventional wisdom suggests that an overall process enhancement must be based on an understanding of the behavior of acidogens since they play a primary role in producing short-chain organic acids, major substrates for methanogens (Yang et al. 2003).

In almost all biological processes, accurate estimation of microbial mass is essential for the biokinetic modeling which provides fundamental information for the design and control of biological processes (Robertson et al. 1998). The most widely used method of quantifying microorganisms in biological wastewater treatment is the volatile suspended solids (VSS) test (APHA-AWWA-WEF 2005). The VSS method measures the dry weight of microorganisms per unit volume of reaction fluid, which is readily obtained by separating, drying, and weighing the microorganisms from the fluid. This method has high accuracy only when the wastewater is totally soluble. Anaerobic processes, however, are frequently used to manage highly suspended organic wastewaters, including agricultural, domestic and food processing wastewaters. Acidogens play a key role in converting the suspended organic materials to various organic acids, hydrogen, and carbon dioxide, major substrates for methanogens. In this situation, the VSS test can misestimate acidogenic microbial mass because the test can not differentiate between microorganisms and suspended organic particles in the wastewater (Yu et al. 2002). Therefore, ways of measuring acidogenic microbial mass other than the VSS test are essential for better comprehension of the acidogenic phase in anaerobic processes.

The concentrations of cellular components, such as protein, deoxyribonucleic acid (DNA), nicotinamide adenine dinucleotide (NADH), and adenosine triphosphate (ATP) in a cell culture have been used to estimate microbial concentration without measuring the dry weight (Lee et al. 2006). Among them, ATP and DNA assays have been considered to be the most practical for estimating microbial levels in suspended organic wastewaters and widely applied to biological wastewater treatment processes (Jorgensen et al. 1992; Liebeskind and Dohmann 1994; Hwang and Hansen 1998; Yu et al. 2002). Both ATP and DNA are ubiquitously present in living cells. ATP is a central bioenergy compound that is required in metabolic reactions, and is rapidly degraded after cell death. DNA is a fundamental genetic constituent of all microorganisms and is highly correlated with microbial proliferation.

ATP and DNA can also be conveniently extracted and quantified from a wastewater sample due to the recent development of commercial assay systems (Obata et al. 2001; Ishida et al. 2002). Although ATP and DNA concentrations can be directly used to represent the microbial concentration, the VSS concentration calculated from the measured ATP and/or DNA content has been widely reported in the literature primarily for comparison with relevant research works. Constant conversion factors are typically used for the VSS calculation regardless of microbial growth phases (Stoeck et al. 2000; Eiland et al. 2001; Yu et al. 2002). However, it must be considered that the cell size and the contents of intracellular materials may change with microbial growth (Akerlund et al. 1995; Hjort and Bernander 1999; Shuler and Kargi 2002). The ratios of ATP and DNA to microbial mass, in this case, are not likely to be consistent and care must be taken in calculating VSS concentrations using ATP and DNA content. Therefore, this research was conducted to examine the changes in the contents of ATP and DNA per unit microbial mass in acidogenesis of whey permeate, and to introduce a novel approach to estimate the VSS concentration when the ratios of ATP and DNA to microbial mass vary with growth phases.

Materials and methods

Acidogen inoculum system

Anaerobic seed sludge from a local municipal wastewater treatment plant (Pohang, Korea) was cultivated in a lab-scale completely stirred tank reactor (CSTR)

with a working volume of 1.5 l to enrich acidogens. The acidogen inoculum system was operated with dilute whey wastewater of 20.0 g COD/l at 0.5 days hydraulic retention time (HRT). Temperature was held at 35°C and the pH was maintained at 6.0 with 3.0 N NaOH. The absence or repression of methanogenic activity in the inoculum system was verified by the lack of methane production. Steady state effluent from the system was used as seed culture for subsequent acidogenesis experiments.

Wastewater and batch acidogenesis

Whey is highly organic and contains most of the essential nutrients for microbial growth (Hwang and Hansen 1998), but suspended organic materials in whey, mostly insoluble protein, can cause an error in measuring microbial mass using the VSS test. Ultrafiltered whey, whey permeate, contains most of the soluble organics in whey and still has a high pollution potential close to that of whey (Hwang and Hansen 1992; Rajeshwari et al. 2000). In this study, therefore, whey permeate was used as a substrate to minimize the confounding effect due to presence of the suspended organic materials in measuring VSS concentration as microbial mass.

Dried whey permeate powder was obtained from Gossner's cheese plant (Logan, Utah, USA) and dissolved in distilled water to give 10.0 g COD/l. The experiment was done in batch mode with a working volume of 5 l. Temperature was held at 35°C and the pH was maintained at 6.0 with 3.0 N NaOH. The initial seeding ratio was 1% (v/v).

Analytical methods

A gas chromatograph (6890 Plus, Agilent, Palo Alto, CA, USA) equipped with an Innowax capillary column (Agilent) and a flame ionization detector was used to quantify volatile fatty acids (VFAs) and ethanol. Helium was used as a carrier gas at a flow rate of 2.5 ml/min. An ion chromatograph (Personal 790 IC, Metrohm, Switzerland) equipped with a PRP-X300 ion exclusion column (Hamilton, Reno, NV, USA) was used to quantify lactate. Perchloric acid solution of 1.5 mM was used as an eluent at a flow rate of 1.0 ml/min. COD was measured by the closed reflux colorimetric method and solids were determined according to the procedures in Standard Methods (APHA-AWWA-WEF 2005). Total carbohydrate was analyzed by the phenol-sulfuric acid method (Dubois et al. 1956). The concentrations of ammonia, organic nitrogen and protein were measured according to the Kjeldahl method (Zapsalis and Beck 1986; APHA-AWWA-WEF 2005). Lactose concentration was measured enzymatically using a commercial kit (Lactose/D-Galactose test kit, Boehringer Mannheim, Mannheim, Germany). All experimental analyses were performed in duplicate.

ATP and DNA assays

ATP was extracted using a modification of the boiling water method (Lee et al. 2006). One milliliter of the wastewater sample was centrifuged at 16,000 g for 5 min. The supernatant was decanted and 1 ml of boiling deionized water (DW) was added. The mixture was further heated in a boiling water bath for 5 min. The extract was then filtered through a 0.45 μm Acrodisc syringe filter (Pall Corporation, Ann Arbor, MI, USA) to remove suspended particles. ATP concentration in the filtrate was measured using the firefly bioluminescent reaction (Nelson 1991). Twenty microliters of the filtrate was mixed with 100 μl of luciferin-luciferase reagent solution, and a microplate luminometer (Luminoskan Ascent, Labsystems, Helsinky, Finland) was used to measure bioluminescence intensity. The assay solution was prepared using a commercial kit (ENLITEN ATP Assay System, Promega). All ATP extractions were performed in duplicate.

DNA was extracted using a fully automated nucleic acid extractor (Magtration System 6GC, PSS Co., Chiba, Japan) employing magnetic bead technology. An automated DNA extraction is known to extract DNA with high purity and reproducibility (Obata et al. 2001). One milliliter of the wastewater sample was centrifuged at 16,000 g for 5 min and the supernatant was decanted. The pellet was washed with 500 μl of deionized and distilled water (DDW) and was centrifuged again in the same manner in order to ensure a maximal removal of residual medium. The supernatant was carefully removed, and the pellet was resuspended in 100 μl of DDW. Genomic DNA was immediately extracted from the suspension using the automated nucleic acid extractor with a Magtration Genomic DNA Purification Kit (PSS Co., Chiba, Japan). Purified DNA was eluted

with 100 µl of Tris–Cl buffer (pH 8.0). The concentration of extracted DNA was measured using a fluorometer (TD-700, Turner Designs, Sunnyvale, CA, USA) with PicoGreen dsDNA Quantification Reagent (Molecular Probes, Eugene, OR, USA). All DNA extractions were performed in duplicate.

Results and discussion

Acidogenic reactor performance

Soluble carbohydrate content in the dilute whey permeate used in this study was 7.8 g glucose equivalent/l (data not shown) corresponding to a theoretical oxygen demand of 8.4 g/l (i.e., 7.8 g glucose/l × 1.07 g O_2/g glucose). This indicates that carbohydrate was the major soluble organic substance composing 93.8% of soluble COD (SCOD) in the wastewater (data not shown).

Figure 1 represents the degradation of soluble carbohydrate along with the accumulation of acidogenic products. All the concentrations were expressed as COD equivalent for convenient comparison. A total of 98.5% of the initial soluble carbohydrate was degraded during the 32 h of incubation, and the major portion, 95.7%, was utilized during the first 22 h period. The total amount of acidogenic products increased up to 6,599 ± 110 mg COD/l at 22 h, and remained relatively constant thereafter. For this experiment, lactate, ethanol, and short chain VFAs (C_2–C_6) including acetate, propionate, butyrate, valerate, and caproate were detected. These are the normal products of carbohydrate fermentation (Kissalita et al. 1989; Yang and Tang 1991). The main products formed in order of concentration were lactate, ethanol and acetate, of which maximal concentrations were 3,407 ± 70, 2,339 ± 23 and 900 ± 8 mg COD/l, respectively. Among the minor products, only valerate showed a concentration of higher than 100 mg COD/l, with a maximum of 164 ± 14 mg COD/l at 12 h. Propionate, butyrate, and caproate, reached only to 32.5 ± 0.6, 74.3 ± 1.9, and 31.1 ± 0.3 mg COD/l, respectively. Product composition in acidogenesis of dairy processing wastewater largely depends on the source of inoculum and the operating conditions of processes (Kissalita et al. 1989; Foernier et al. 1993). If removal or consumption of hydrogen is limiting, acidogens have to avoid the reduction of protons with electrons generated by catabolism. This metabolic shift increases the production of more reduced compounds such as lactate and ethanol (Foernier et al. 1993). Accordingly, our acidogenic system probably was operating under hydrogen accumulating conditions, with relatively low activity of hydrogen consuming populations.

Figure 2 shows the variations in VSS, ATP and DNA concentrations during the incubation. Growth phases were determined based on the profile of VSS content. The lag, exponential, decay, and post-decay phases were assumed to be the 0–6 h, 6–22 h, 22–24 h, and 24–32 h incubation periods, respectively. The

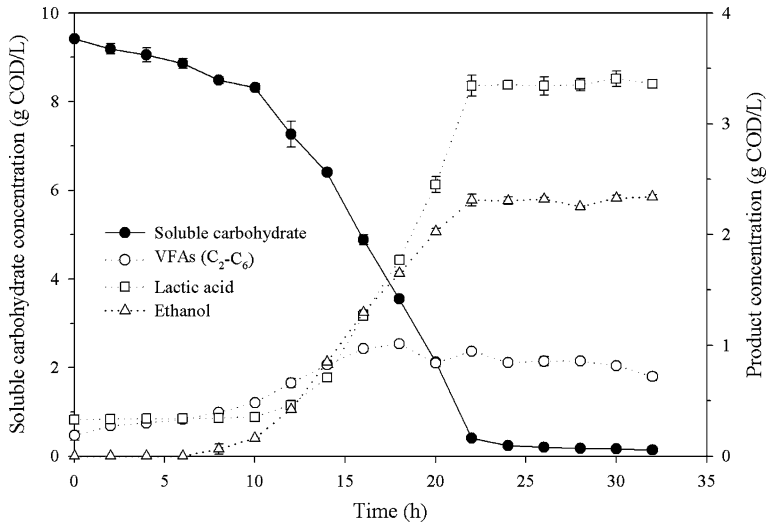

Fig. 1 Degradation of soluble carbohydrate and accumulation of acidogenic products

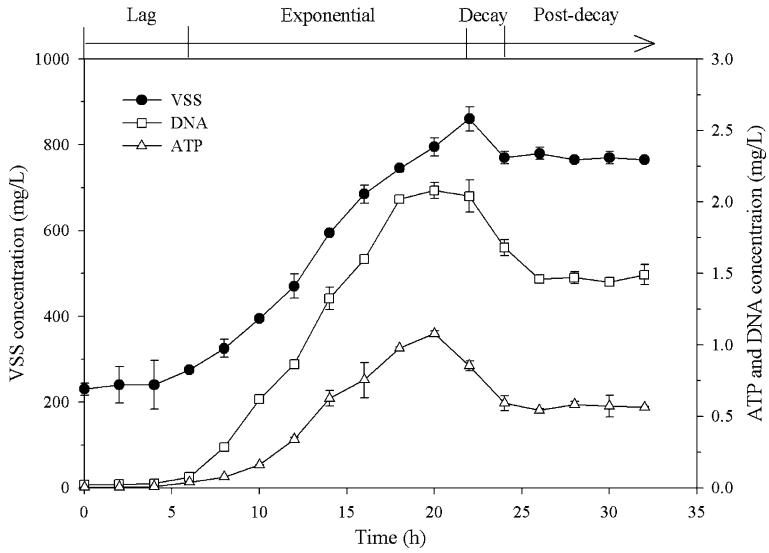

Fig. 2 Variations in VSS, ATP, and DNA concentrations. Growth phases were determined based on the profile of VSS content

stationary phase was not found with the 2-h sampling schedule. VSS concentration increased up to 860 mg/l during the first 22 h period. On the other hand, the ATP and DNA concentrations reached their maximum values of 1.08 ± 0.02 and 2.08 ± 0.06 mg/l, respectively, at 20 h incubation. This implied that the changes in metabolic and genetic activities occurred prior to the change in microbial mass. It has been reported that nucleic acid reaches its maximal concentration and starts to decrease about one doubling time (T_d) before protein which constitutes the major part of cell dry weight (Shuler and Kargi 2002). Doubling time of acidogens utilizing whey or lactose at mesophilic temperature ranges from 1.7 to 1.9 h (Kissalita et al. 1989; Yu et al. 2002), which is close to the sampling interval of 2 h in this study. These findings support our observation that ATP and DNA reached their maximal concentration one sampling interval (i.e., 2 h) before VSS.

Variations in the contents of ATP and DNA

Figure 3 represents the variations in the ratios of ATP and DNA to VSS during microbial growth. It is evident that the contents of ATP and DNA per unit microbial mass were not consistent. The ATP to VSS ratio increased up to 1.36 ± 0.04 mg/g at 20 h, while the DNA to VSS ratio had a maximum of 2.71 ± 0.05 mg/g at 18 h. Each ratio then gradually decreased to reach a plateau. Within the exponential phase, the maximal value of the ATP to VSS ratio was 9.6 times the minimum, and the maximum of the DNA to VSS ratio was 9.9 times its minimum. This indicates that the acidogenic culture was not in a balanced growth even in the exponential phase. It corresponds to the observation of the imbalance between DNA and cell mass in an exponentially growing *Escherichia coli* culture (Akerlund et al. 1995). The imbalanced growth would be due to the changes in unit cell mass and cellular composition during microbial growth (Akerlund et al. 1995; Shuler and Kargi 2002). Cell size and morphology are known to be a function of microbial growth rate rather than life cycle (Shuler and Tsuchiya 1975). Active cells in exponential growth continue to divide at the same rate, but the amount of material incorporated into the cell decreases continuously with time in a batch culture. In this situation, the cell controls its surface to volume ratio to maintain sufficient transport of materials into the cell, resulting in a decrease in cell size. And, in order to maintain the proper size, the synthesis of non-essential cellular components, such as storage materials, is limited or ceased, resulting in changes in cell composition (Shuler and Tsuchiya 1975).

The ratios of ATP and DNA to VSS reached constants of 0.74 ± 0.03 mg/g and 1.96 ± 0.13 mg/g, respectively, in the post-decay phase (i.e., after 24 h incubation). The residual concentration of soluble carbohydrate in that period was less than

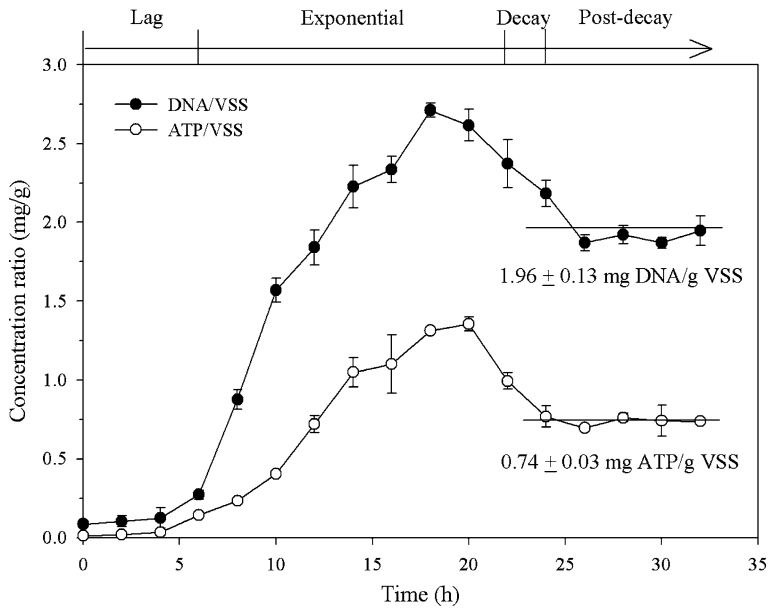

Fig. 3 Variations in the ratios of ATP and DNA to VSS. Growth phases were determined based on the profile of VSS content

235.7 ± 5.3 mg COD/l corresponding to 97.5% removal efficiency (Fig. 1). This could be because net microbial growth, and net synthesis of genetic and energy compounds for proliferation cease after the end of exponential growth (Shuler and Kargi 2002). Therefore, it would be possible to use the constant ratios as conversion factors to convert measured ATP and DNA concentrations into microbial mass in similar situations.

Correlation of ATP and DNA to microbial mass

Conventional indirect microbial quantification converts the measured concentrations of ATP and DNA into microbial mass using a fixed conversion factor (Stoeck et al. 2000; Eiland et al. 2001; Yu et al. 2002). This is based on the assumption that the ratios of ATP and DNA to microbial mass are consistent. However, our experimental data showed that those ratios varied continuously during microbial growth (Fig. 3). Therefore, a method to compensate the variations in the ratios of ATP and DNA to VSS is required for more accurate estimation of microbial mass.

The concentrations of ATP and DNA were plotted against the VSS concentration. Each plot was highly linear with r^2 of 0.99 within the period (from 6 to 20 h) in the exponential phase (Fig. 4). All terms of the linear regression models were significant at the 0.1% α-level. These indicate the adequacy of the models (Eqs. 1, 2), which meant the linear regression equations could estimate the microbial concentrations from the concentrations of ATP and DNA.

$$\text{Microbial concentration (mg/l)} = 478.5 \times \text{ATP concentration (mg/l)} + 293.5 \quad (1)$$

$$\text{Microbial concentration (mg/l)} = 257.2 \times \text{DNA concentration (mg/l)} + 250.4 \quad (2)$$

The applicable ranges of Eqs. 1 and 2 were 0.04–1.08 mg/l in ATP concentration and 0.08–2.08 mg/l in DNA concentration, respectively.

Changes in the mass of the acidogenic population can be monitored by the combined use of the regression equations obtained in the exponential phase and the conversion factors determined in the post-decay phase. To our knowledge, this is the first report on the variations in the contents of ATP and DNA per unit microbial mass in a mixed culture acidogenesis of dairy processing wastewater. Quantification errors caused by the variations in the contents of ATP and DNA per unit microbial mass can be minimized by using the proposed method. It is suggested that variations in the ratio of ATP or DNA to microbial mass should be considered in the indirect quantification of microbial mass.

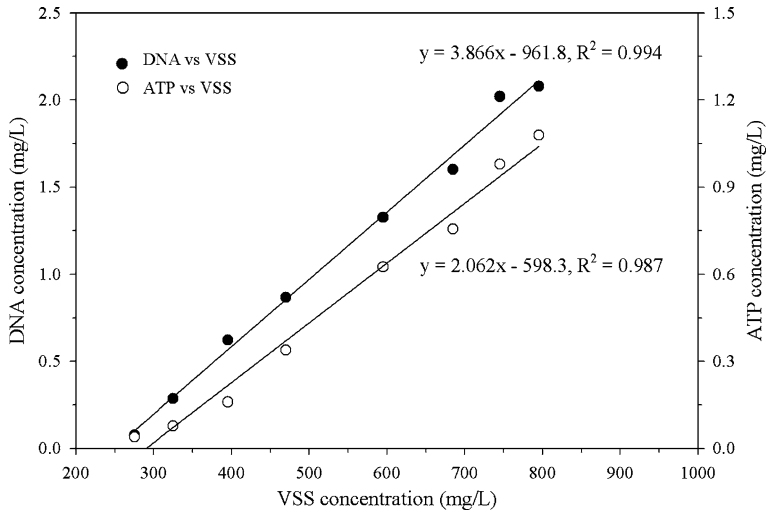

Fig. 4 ATP and DNA concentrations versus VSS concentration. Each equation was obtained by linear regression through the data points

A summary of the characteristics of dairy processing wastewaters obtained from literature is given in Table 1. These wastewaters commonly contain high VSS of up to 21.8 g/l. Such a high amount of substrate VSS can cause errors in the quantification of microbial mass using the VSS test in anaerobic treatment of dairy processing wastewater. The proposed method would offer a practical solution to quantify acidogenic populations utilizing such wastewaters with improved reliability, especially during pre-acidification of two-phase anaerobic digestion. The two-phase process is particularly suitable for treatment of highly suspended organic wastewaters, such as dairy processing wastewaters (Demirel et al. 2005). The performance of an acidogenic reactor is of paramount importance in two-phase anaerobic digestion of wastewater, since the acidogenic reactor should provide the most appropriate substrate for the subsequent methanogenic reactor. Consequently, the improved method introduced in this study could be helpful to understand acidogenesis and enhance the performance of a two-phase anaerobic digestion process treating dairy processing wastewater.

Conclusions

Improved methods to estimate the microbial mass by measuring ATP and DNA when the ratios of ATP and DNA to microbial mass vary were presented. Our experimental data showed that the ratios were not consistent during microbial growth. Each ratio showed about 10-fold difference between its maximal and minimal values in the exponential phase, and maintained a constant level in the post-decay phase. The constant ratios, 0.74 mg ATP/g VSS and 1.96 mg DNA/g VSS, can be used as the conversion factors for estimation of acidogenic microbial mass in similar situations. On the other hand, the ATP and DNA concentrations were linearly related to the microbial mass during their exponential increases. The linear regression models derived were highly

Table 1 Characteristics of dairy-processing wastewaters from full-scale operations

Wastewater	COD (g/l)	pH	Suspended solids (mg/l)	Volatile suspended solids (mg/l)	Total solids (mg/l)	Reference
Milk-processing	4.4	8.6	870	485	3,300	(Demirel and Yenigun 2004)
Yoghurt-processing	1.2–9.2	5.8–11.4	340–1,730	255-830	2,705–3,715	(Demirel and Yenigun 2006)
Whey	74.8	4.9	22,400	21,750	67,070	(Ghaly et al. 2000)
Cheese-processing	63.1	3.4	12,500	12,100	53,000	(Hwang and Hansen 1998)
Creamery	2.0–6.0	8–11	350–1,000	330–940		(Kasapagil et al. 1994)
Cheese-processing	1.0–7.5	5.5–9.5	500-2,500			(Monoy et al. 1995)
Whey	61.0		1,780	1,560		(van den Berg and Kennedy 1992)

adequate (r^2 = 0.99) and significant at the 0.1% α-level in the ranges of 0.039–1.078 mg ATP/l and 0.075–2.080 mg DNA/l, respectively. The regression equations and the conversion factors determined in the post-decay phase should be widely applicable to estimating acidogenic microbial mass in acidogenesis of dairy processing wastewater.

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