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Application of fluorescent *in situ* hybridisation for monitoring methanogenic archaea in acid whey anaerobic digestion

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Abstract. Anaerobic digestion of cheese whey offers a two-fold benefit: reduction of pollution potential and biogas production. Usually effective anaerobic digestion of acid whey is hindered by low pH (~ 4.5) and low buffer capacity of the substrate. The aim of this study was to evaluate the process of acid cheese whey anaerobic digestion with respect to changes in microbial population dynamics and effective biogas production. The results showed that it is possible to obtain high methane yields (176–278 L kg^{-1} VS^{-1}) in a system with high organic loading rates (till 4.9 VS m^{-3} day) and no apparent acid inhibition on methanogenic microbial population. Moreover, *Archaea* population showed the ability to rapidly adapt and actively convert the substrate.

Key words: acidic cheese whey, biogas, fluorescent *in situ* hybridisation.

INTRODUCTION

Anaerobic digestion of energy crops, residues and wastes is of increasing interest in order to reduce greenhouse gas emissions and to facilitate sustainable development of energy supply. Production of biogas provides a versatile carrier of renewable energy, as methane can be used for replacement of fossil fuels in both heat and power generation and as a vehicle fuel (Weiland, 2010).

The worldwide production of whey by the cheese and casein industries runs into millions of tons, and yet effective utilisation of this material is not well developed (Najafpour et al., 2010). World annual production of whey is estimated to be 115 million tons and approximately 47% of the produced whey is disposed of into the environment (Leite et al., 2000). Traditionally acid whey can be used for animal feeding, however, due to high transportation costs, it is rather attractive for energy production. In dairy manufacturing plants anaerobic digestion of acidic cottage cheese whey can be introduced into the technology to reduce the energy consumption and pollution. Generally, dairy waste streams contain high concentrations of organic matters; these effluents may cause serious problems, in terms of organic load on the local municipal sewage treatment systems (Goblos et. al., 2008; Saddoud et al., 2007).

From a wastewater treatment point of view, anaerobic digestion of cheese whey offers an excellent approach. During anaerobic digestion biodegradable matter is

broken down into methane, carbon dioxide and water by microorganisms in the absence of oxygen. While anaerobic methanogenic fermentation is a multi-stage process catalysed by the coordinated activity of a wide number of individual microbial strains belonging to a number of different trophic groups, the capacity of methanogenesis is limited to members of domain Archaea, phylum Euryarchaeota which are among the most fastidious of the anaerobes. They require growth factors such as vitamins, unusual trace minerals (such as Co and Ni), fatty acids (acetate) and specific co-factors (coenzyme M) unique to methanogenic microorganisms (Montero, 2009, Nettmann, 2010). Due to the complex and sophisticated nutritional needs the identification of these organisms with traditional culture based assays is complicated or limited to the detection of only a small proportion of these organisms (Wagner et al., 1993). One of the suitable approaches in the identification and enumeration of these microorganisms has been achieved by introduction of molecular biology techniques, like denaturant gradient gel electrophoresis, Fluorescent in situ hybridisation (FISH) or cloning of 16S rDNA (Sanz & Köchling, 2007).

Raw whey acidifies rapidly, which makes it difficult to treat anaerobically (Malaspina et al., 1996; Ergüder et al., 2001; Venetsaneas et al., 2009). However, thorough control of chemical parameters during the methanogenic stage could increase biogas production rate and methane yield, with accompanying reduction in chemical oxygen demand (COD) and solid concentration (Goblos et al., 2008). Thus, the aim of this study was to evaluate the process of acid cheese whey anaerobic digestion with respect to changes in microbial population dynamics. To estimate Eubacteria and Archaea concentration dynamics during the fermentation, FISH technique which does not require cell cultivation was applied (Sanz & Köchling, 2007).

MATERIALS AND METHODS

Inoculum and cheese whey

The reactor was inoculated with 1,000 ml digestate originated from a lab-scale working reactor (50 L, HRT – 50 days, substrate – granules of Medicago sativa) and supplied with acid cheese whey (Smiltenes piens, Latvia). No pre-treatment (filtration, dilution) of the substrate was performed.

The whey samples were provided from the manufacturer, collected in 5 L containers and stored at 4 °C no longer than 1 week to avoid changes in the chemical composition.

Experimental Set Up

Anaerobic digestion studies were performed in a 6.2 L glass bioreactor (EDF-5.3_1, Latvia) with a working volume of 4 L and a height to diameter ratio of 3:1. The bioreactor was equipped with novel magnetic drive placed in the upper lid. Temperature (Pt-100) and pH (Ingold, Toledo 405-DPAS SC K8S/325) in the reactor, CH4 and CO_2 concentrations in the exhaust gases (Bluesens, Germany) were measured online.

Automatic control of pH at 7.2 ± 0.2 using 12% sodium hydroxide solution was applied. NaOH solution was added to the bioreactor with peristaltic pump (Longer Pump, BT100-2J) working in on-off pumping rate mode. Temperature set point was

37.0 ± 0.2 °C. The anaerobic environment at the start was ensured by flushing with N2 until the dissolved oxygen was zero.

Chemical analyses

Standard method was applied for pH measurement (LUTRON PH-208). Total solids (TS) were determined by drying at 105 °C for eight hours. Volatile solids (VS) and ash was analysed using muffle furnace (Snol 60/300 LFN) at 550 °C for 90 minutes.

Determination of total inorganic carbon (TC) and total volatile acid (TVA) values were performed as a two-step endpoint titration using 0.1M sulphuric acid (Lahav & Morgan, 2004).

Quantification of microorganisms

During anaerobic digestion regular sampling for FISH analyses was performed. In brief, one volume of homogenised sample was fixed with three volumes of ice-cold 4% paraformaldehyde-PBS buffer (200 mM NaH_2PO_4 x H_2O, 200 mM NaH_2PO_4 x H_2O, pH 7.2) for 2 hours at 4 °C. After incubation the suspension was thrice washed with PBS buffer and applied to a glass slide. The smears were dehydrated in ethanol series (50%, 80% and 96%) for 3 minutes each. Following air-drying hybridisation buffer containing 35% formamide and 50 nM fluorescently labelled (CY3 or FITC) oligonucleotide EUB338 (5`-GCT GCC TCC CGT AGG AGT-3`) or Arch915 (5`-GTG CTC CCC CGC CAA TTC CT-3`) probe was applied to the slides and covered with cover glass. The samples were incubated in humid, sealed chambers for 3 hours at 46 °C in the dark. After hybridisation the samples were immersed in washing buffer (70 mM NaCl; 20 mM Tris-HCl, 5 mM EDTA) for 20 minutes at 48 °C. Then the samples were rinsed with ice-cold sterile distilled water and air-dried. To obtain total bacterial counts, staining with 10 μg ml^{-1} DAPI (4',6-diamidino-2-phenylindole) for 10 minutes was performed after hybridisation. Cell counts were determined with epifluorescence microscope (Leica DMLB) equipped with a 50-W power supply, mercury lamp, filter sets for DAPI (Ex: 340/380 nm; Em: > 425 nm nm), FITC (Ex: 470 ± 20 nm; Em. 515 nm) and CY3 (Ex: 535 ± 25 nm; Em. 610 ± 37 nm), and a camera (CoolSNAP Pro, Media Cybernetics, Inc, USA). For image processing Image Pro Plus 4.5.1. software (Media Cybernetic Inc., Silver Spring, MD) was used. All samples were hybridised fourfold. For statistical analyses a two-tailed T-test and Dixons Q-test was performed.

RESULTS AND DISCUSSION

Numerous studies have addressed the use of cheese whey for anaerobic treatment (Yan et al., 1989; García et al., 1991; Patel et al., 1995; Ghaly et al, 2008) however, data on acid cheese whey treatment without dilution with respect to microbial population dynamics is limited. Since raw whey has low bicarbonate alkalinity (2,500 mg L^{-1} as CaCO3) and high COD (up to 70 g L^{-1}) concentration, its anaerobic treatment is complicated (Malsapina et.al, 1996; Kavacik & Topaloglu, 2010). The main components of cheese whey are lactose (44–52 g l^{-1}), protein (6.1–6.6 g L^{-1}), fat (0.2–0.3 g L^{-1}) and minerals (5–7.9 g L^{-1}) (Fox et al., 2000). The analyses on chemical composition of acid cheese whey and inoculum showed very low pH values for the

whey (Table 1.) but contained high amounts of total solids (TS) – 5.58% and volatile solids (VS) – 4.24%.

Table 1. Chemical composition of inoculum and whey

Parameter	Inoculum	Whey
pH (20 °C)	7.92	4.54
Total solids (% w w^{-1})	1.89	5.58
Volatile solids (% w w^{-1})	1.34	4.24
$COD_{unfiltred}$ (g O_2 L^{-1})	-	78.30
$COD_{filtred}$ (g O_2 L^{-1})	-	31.50
Ash (% w w^{-1})	0.59	0.62
Protein (% w w^{-1})	-	0.30
NPN (% w w^{-1})	-	0.20
Lactose (% w w^{-1})	-	4.85
Fat (% w w^{-1})	-	0.05

The results showed that the main component of whey is lactose, which can be easily degraded into acid products – mostly to acetate and propionate. The whey contained low concentrations of fat and proteins. Fresh whey had COD – 78.30 g O_2 L^{-1}, so it could be considered as a strong waste (Ghaly, 2000). The removal of fats and proteins decreased the COD value by 59.8%. Since whey has a typical COD value of 60.0–100.0 g O_2 L^{-1}, anaerobic digestion is the only viable biological method for treating this waste (Gelegenis et al., 2007).

The ability to neutralise organic acids and maintain constant pH in an anaerobic digester is determined by total alkalinity in the form of total inorganic carbon. A steady level of the total inorganic carbon in the digester was achieved on the third day and ranged from 10.25 to 10.94 g kg^{-1} (Fig.1). For an operating digester, total alkalinity of 2.5–8.2 g L^{-1} as CaCO3 is recommended (Fox et al., 1992; Ghaly et. al, 2000). The average total alkalinity values in this study were higher than reported previously and recommended, however, there was no apparent sign of inhibition.

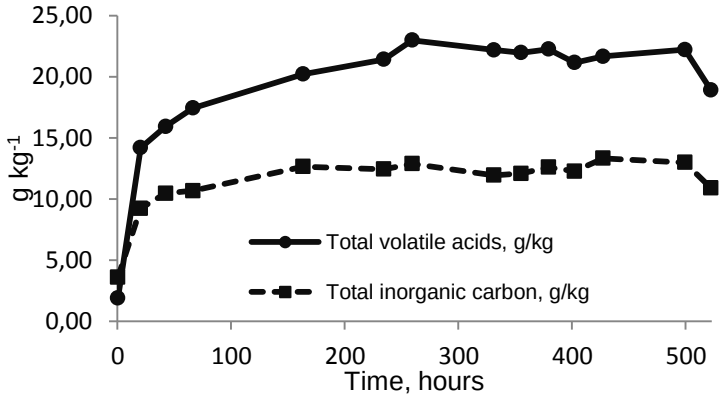

Figure 1. Total volatile acids and total inorganic carbon from lab-scale reactor processing acid whey.

Initially, an organic loading rate (OLR) of 2.8 kg VS m^{-3} day was maintained (Fig. 2). Then it was slowly increased to 6.8 kg VS m^{-3} day. The increase in ORL resulted in elevated total volatile acids concentration (Fig. 1) which can be explained by rapid conversion of whey lactose to volatile fatty acids by acidogenic microorganisms (Ghaly, 2000). The resultant elevated acid concentrations can act as inhibitors for methanogens which have lower growth rates than acetogens (Goblos et. al., 2008). To overcome the increase in acid inhibition, ORL concentration was decreased till 2.8 VS m^{-3} day. Only at a final stage a steady state with simultaneous ORL of 6.2 kg VS m^{-3} day was achieved.

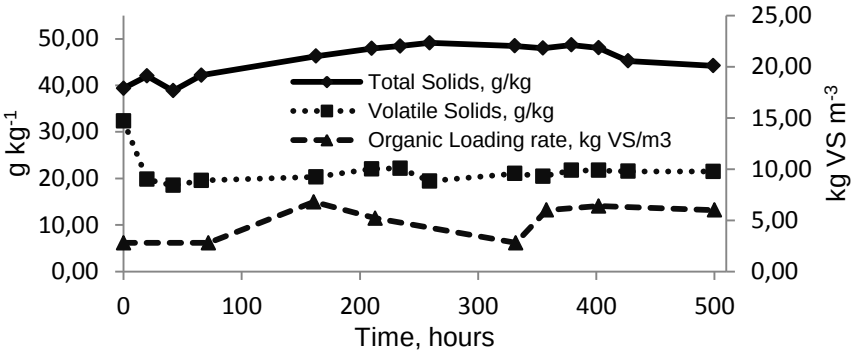

Figure 2. Daily total solids and volatile solids from lab-scale reactor processing acid whey, and organic loading rate.

The average biogas yield during the study was 348.74 L kg^{-1} VS (0.56 L L^{-1}reactor d^{-1}) where methane yielded around 50% (Fig 3.). The methane concentration decreased every two days when the digestate was poured off to maintain a constant working volume in the reactor. The obtained methane yields were between 176–278 L kg^{-1} VS. The highest methane content of 63.2% was reached when OLR was increased from 2.8 till 6.0 kg VS m^{-3}. However, higher OLR (6.4 kg VS m^{-3}) did not lead to higher methane yields which could be explained with potential ammonia or long chain fatty acids inhibition (Pitk et al., 2012).

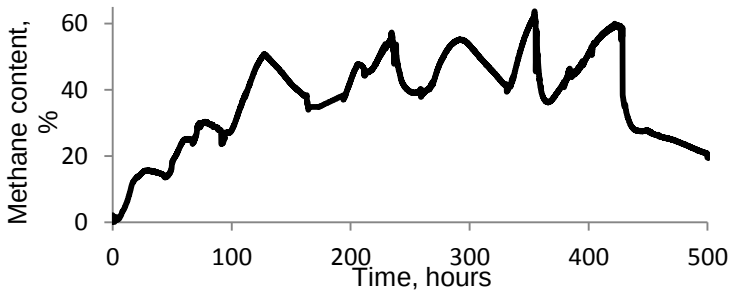

Figure 3. Methane content in the biogas formed in lab-scale reactor processing acid whey.

In situ hybridisation analyses showed that initially in the inoculum *Archaea* constituted almost 45% of all microorganisms and represented more than 5*109 cells

per ml of the inoculum. After the addition of the inoculum to the whey substrate, a significant increase ($p < 0.05$) in *Archaea* concentration was observed already after 20 hours of supply and reached more than 1.3*1010 cells/ml, indicating an active growth. Similarly, an increase in *Eubacteria* counts was observed, however, this did not account for a significant increase ($p > 0.05$). The comparison of cell concentration with respect to ORL, did not show such a rapid increase in cell counts, including *Archaea* (Fig. 5).

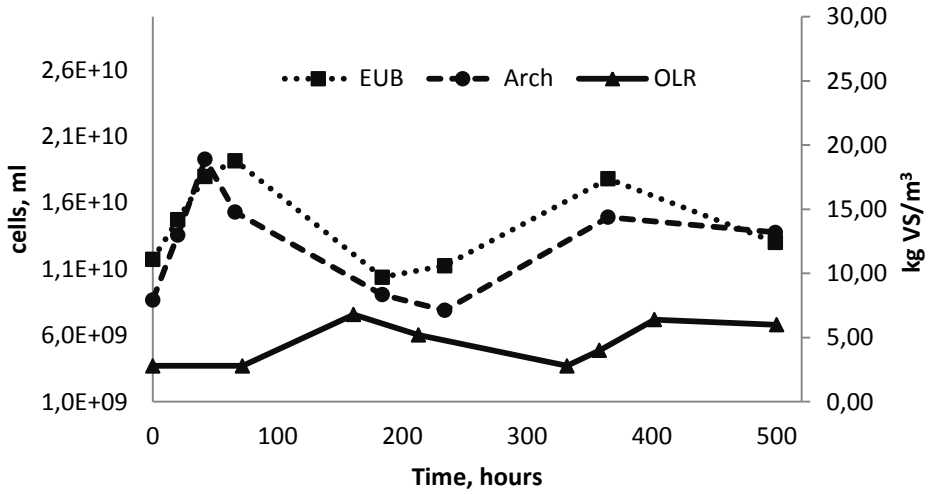

Figure 4. Amount of *Archaea* (Arch) and *Eubacteria* (EUB) in the reactor during anaerobic treatment with various OLR.

The highest *Archaea* proportion was observed after 45 hours of fermentation when simultaneously one of the highest methane concentrations were recorded (55.9%). Rapid increase in OLR (to 6.8 VS m^{-3} day) after 80 hours of fermentation, decreased cell concentration, but when OLR was decreased to 4.9 VS m^{-3} day *Archaea* amount increased.

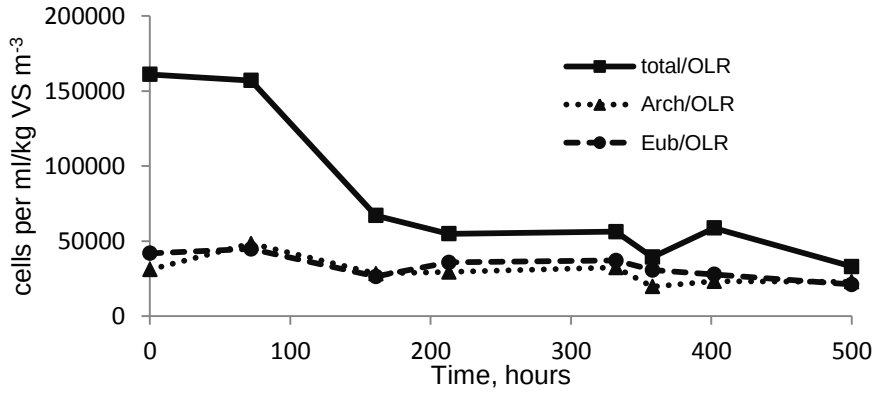

Figure 5. Changes in total microbial, *Eubacteria* and *Archaea* concentration with respect to ORL.

CONCLUSIONS

The study showed that anaerobic digestion in mesophilic and pH-controlled (7.2 ± 0.2) conditions is a potential technology for treatment of undiluted acid whey. OLR did not show inhibitory properties on methanogenic *Archaea* to 4.9 VS m^{-3} day ORL. The highest methane content of 63.2% was obtained when OLR was increased from 2.8 to 6.0 kg VS m^{-3}. On average 42.5% methane yields were obtained in the biogas produced. Relatively low fluctuations in microbial counts still showed that elevated *Archaea* counts are observed at the same time when biogas yields tend to increase.

Thus, anaerobic digestion of acid whey without manure can be used as a sustainable and attractive technology to convert dairy waste to energy.

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