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Biogas and hydrogen production from glycerol by *Enterobacter aerogenes* and anaerobic microbial communities

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Abstract. Biological hydrogen production by anaerobic fermentation of widely available renewable resources is a promising and advantageous area. Using microbiological hydrogen production from crude glycerol biodiesel-derived waste was utilized by obtaining renewable energy carrier. The purpose of this research was to study biogas and hydrogen production by *Enterobacter aerogenes* MSCL 758 and by natural microbial communities. Growth medium was supplemented with analytical grade, technical grade or crude glycerol. Inoculants from old municipal landfill, manure and lake sludge were also used. Biogas production was analyzed using Automatic Methane Potential Test System II. Part of the experiments were carried out in serum bottles and evolved gases were tested using mass-spectrometry. Fluorescence *in situ* hybridization was used for bacterial population dynamic determination. Optimal concentration for crude glycerol was found to be six grams per liter. Amount of hydrogen was significantly higher and amount of nitrogen gas was lower in case of analytical grade glycerol usage in comparison to crude glycerol fermentation. *E. aerogenes* acted in synergy with landfill substrate and manure in biogas production from technical grade and analytical grade glycerol. It was not the case for crude glycerol usage. Addition of *E. aerogenes* increased overall amount of produced hydrogen. Obtained results showed potential of *E. aerogenes* for use in bioaugmentation purposes for fermentation of glycerol. Lake sludge inoculum contained microorganisms necessary for the production of hydrogen as well as biogas from glycerol. Clostridia and Gammaproteobacteria were predominant in the inoculum. Cultivable bacteria *Bacillus licheniformis, Burkholderia cepacia*, *Hafnia alvei* and unidentified *Clostridium* species were found to be predominant after six days of fermentation.

Key words: bacteria, bioaugmentation, fermentation, inoculum.

INTRODUCTION

Biological hydrogen production by bacterial anaerobic fermentation of widely available renewable resources is a promising and advantageous area. By microbiological hydrogen production from crude glycerol not only renewable energy carrier is obtained, but it is done by utilizing biodiesel-derived waste (Yazdani & Gonzalez, 2007).

There are two ways of glycerol metabolism during anaerobic fermentation – oxidative and reductive (Biebl et al., 1999). In the reducing pathway, glycerol or 1,2,3-

propanetriol is converted to 1,3–propanediol. In the oxidative pathway, glycerol is firstly converted to dihydroxyacetone, then dihydroxyacetone is phosphorylated and the phosphorylated product is metabolized through glycolysis. Pyruvate may be further converted to various end-products depending on the microorganism (Sarma et al., 2012). Fermentation products such as 1,3-propanediol, butanol, formic acid, propionic acid, succinic acid and ethanol as well as gases H_2 and CO_2 are produced (Yazdani & Gonzalez, 2007; Viana et al., 2012). Various bacterial strains are considered promising for glycerol utilization because of possibility to ferment crude glycerol, and H_2 is one of the end-products of this process (Ito et al., 2005). Hydrogen production comparing to production of 1,3-propanediol is more valuable, hydrogen has higher energy content (142.9 kJ g^{-1}) and it results in higher yield and productivity (Sarma et al., 2012). Ability to metabolise glycerol is possible for following genera of anaerobic and facultative anaerobic bacteria: *Klebsiella, Citrobacter, Enterobacter, Clostridium, Lactobacillus, Bacillus, Propionibacterium* and *Anaerobiospirillum* (Yazdani & Gonzalez, 2007; Markov et al., 2011). Most productive microorganisms that grow anaerobically on glycerol as the sole carbon and energy source are *Citrobacter freundii, Clostridium butyricum, C. pasteurianum, Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Pantoea agglomerans*, *Rhodopseudomonas palustris* and *Thermotoga neapolitana* (Ito et al., 2005; Sarma et al., 2012). Facultative anaerobe *Enterobacter aerogenes* and anaerobe *Clostridium* species are the most commonly used (Mangayil et al., 2012). *E. aerogenes* is a mesophile and has a temperature optimum of 30–37 °C (reviewed in Fanning et al., 2016). Advantage of *E. aerogenes* in comparison with clostridia is uninhibited H_2 production by high H_2 pressures (Tanisho et al., 1987). However, improved yield of H_2 has been reported after removal of CO_2 because high CO_2 concentration can favour the production of fumarate or succinate, which contributes to consumption of electrons, and therefore decreases hydrogen production (Tanisho et al., 1998). Recently, Patil et al. (2016) discovered a perspective bacterium *Anaerobium acetethylicum*, representing a new genus within the order *Clostridiales*, which degrades glycerol via glyceraldehyde-3-phosphate and produces mainly ethanol and hydrogen and therefore is a future candidate for bioethanol and biohydrogen production.

Glycerol may be used in biogas production to improve both hydrogen and methane yield. Crude glycerol has been added to the anaerobic fermentation as a co-substrate (Yang et al., 2012). Fountoulakis & Manios (2009) reported increase of methane yield 2.6 times after addition of 1% of crude glycerol to fermentation medium. Bruna et al. (2010) have reported production of hydrogen from crude glycerol with anaerobic sludge. Fermentation of crude glycerol has a problem in comparison with analytical grade and technical grade glycerol because different impurities are known to inhibit growth of microorganisms (reviewed in Sarma et al., 2012). Despite the variations in the proportion of components, samples of crude glycerol were shown to contain glycerol, soap, methanol, fatty acid methyl esters, water, glycerides, free fatty acids, and ash (Hu et al., 2012). Glycerol content varies from 40% to 90% (Mane & Rode, 2013). For example, Ito et al. (2005) used crude glycerol with 41% glycerol content, Mangayil et al. (2012) with 45%, Priscilla et al. (2009) with 70% but Jitrwung & Yargeau (2015) with 81% glycerol content. Application of mixed microbial cultures has been proposed as strategy for improved hydrogen production (Sarma et al., 2012). Yield of 0.86 mol H_2 mol^{-1} glycerol was reported by Jitrwung & Yargeau (2015) and 6 moles H_2 mol^{-1} glycerol

(75% of theoretical, 8 moles H_2 mol^{-1} glycerol) was reported by Sabourin-Provost & Hallenbeck (2009).

The aim of this study was to investigate possibilities of Enterobacter aerogenes to produce biogas and hydrogen from crude, technical grade and analytical grade glycerol and to evaluate mixed natural microbial populations as potential inoculants for hydrogen production from glycerol.

MATERIALS AND METHODS

Fermentation experiments

This study focused on biogas and hydrogen production by pure culture of *Enterobacter aerogenes* MSCL 758 and by mixed anaerobic microbial communities in batch experiments. Natural microbial inoculants from old municipal landfill substrate, manure and lake sludge were used in different experiments and they were added to sterile growth media. Ten grams of landfill substrate and manure were suspended in 90 mL of sterile water and activated 18 h at temperature of 20 °C. After, 1 mL of the suspension as an inoculant was added to 400 mL of culture medium in 600-mL reactors or for the serum bottle experiments – 88 μl to 35 mL of culture medium. One mL of lake sludge was added to 35 mL culture medium in serum bottles. Cultivation was carried out triplicate with each sample in sterile microbiological growth medium consisted from tryptone (Fluka Biochem, Switzerland) 1.0 g L^{-1}, yeast extract (SIFIN, Germany) 2.5 g L^{-1} and cysteine hydrochloride (Sigma-Aldrich, Germany) 0.5 g L^{-1}, pH 7.5. Growth medium was supplemented with glycerol source 3–12 g L^{-1} (w/v): crude glycerol with glycerol content 40%, technical grade glycerol with glycerol content 82% (Balteko Ltd., Latvia) or analytical grade glycerol (Stanlab, Poland).

Part of the anaerobic cultivations were in 600-mL reactors with 400-mL working volume according to the instruction of the Automatic Methane Potential Test System (AMPTS) II kindly provided by Bioprocess Control AB, Sweden (Strömberg et al., 2014). Data were recorded by AMPTS II software. In experiments with *Enterobacter aerogenes*, media were supplemented with $NaHCO_3$ 5.5 g L^{-1} and solution of microelements (Angelidaki et al., 2009) 10 mL L^{-1}. Initial pH value was adjusted to 7.5 with solution of HCl. Reactors were flushed with argon gas (AGA Ltd., Latvia) to remove oxygen. Other part of experiments were performed in 50 mL serum bottles (Supelco Analytical, USA) with a working volume of 35 mL. Bottles were sealed with butyl rubber stoppers (Gotlands Gummifabrik, Sweden) and aluminium crimps (Supelco Analytical, USA), and flushed with argon. Cultivation time differed between experiments from 24 h to six days. Cultivation was maintained at temperature of 37 °C and 20 °C.

Analytical methods

Gas production was analyzed in two ways. Automatic system AMPTS II was used for detection of biogas volume. Qualitative analysis of evolved gases from the headspace of bottles in amount of 10 cm^3 were collected by syringe from the test system and injected into the RGA Pro-100 mass spectrometer with input node (HyEnergy, Setaram, France). Data were assessed by SR Residual Gas Analyzer with RGA 3.0 software.

The pH values were measured using a pH meter AD 1405 (Adrona Ltd., Latvia).

Microbiological analyses

The amount of culturable microorganisms was determined by serial dilution of broth or inoculant samples by obtained dilution spreading on agar plates and counting of colony-forming units (CFU) after incubation. Serial dilutions were plated in duplicate on the R2A (Becton & Dickinson, France). For each spread plate 0.1 mL of each dilution was used. R2A plates were incubated at 37 °C or 22 °C for 2–7 days aerobically or for seven days anaerobically (GasPak Anaerobe Pouch, Becton & Dickinson, USA), results were expressed as CFU per mL. Based on colony and cell morphology, predominant cultures (morphotypes) were isolated from the highest dilutions and purified using streaking method. Isolated cultures were identified biochemically with BBL® Crystal™ Gram-Positive ID kit, Enteric/Nonfermenter ID kit and Anaerobe ID kit (Becton & Dickinson, USA). Genera of the isolated fungi were identified using macroscopic and microscopic appearance and keys (Kiffer & Morelet, 2000).

Fluorescence *in situ* hybridization (FISH) and epifluorescence microscopy were used for determination of bacterial population dynamics. Basic method described by Pernthaler et al. (2001) and Fuchs et al. (2007) was used. Samples were air-dried, fixed in 4% para-formaldehyde for 1.5–2 hours. After drying, samples were dehydrated in ethanol (50, 80, and 96%). The 16S rRNA-targeted oligonucleotide probes were purchased from Eurofins MWG Operon (Germany). Probes LGC353B (Felske et al., 1998), GAM42a (Manz et al., 1992) and Chis150 (Franks et al., 1998) were marked at their 5'-end with Cy3, but ARC915 (Stahl & Amann, 1991) was marked with RGR. Epifluorescent microscope DM 2000 (Leica Microsystems, Germany) was used for examination of hybridization results.

Statistical analysis

Statistical analysis was performed using R version 3.2.3. One-way analysis of variance (ANOVA) was used to determine statistical significance ($P < 0.05$) of differences between experimental groups. The strength of association between two groups was determined by calculation of Pearson correlation coefficient.

RESULTS AND DISCUSSION

Experiments with *Enterobacter aerogenes*

Some authors have shown that hydrogen production was not affected by the impurities (Mangayil et al., 2012) but other experiments demonstrated that optimal concentration of crude glycerol as a co-substrate is, for example, 1 g L^{-1} (Fountoulakis et al., 2010) or 3–6% (Amon et al., 2004). Methanol can be removed by evaporation through autoclaving (Denver et al., 2008) as it also could be the case for this study. Optimal concentration of crude glycerol obtained from local producer Balteko Ltd. was found to be six grams per liter (Fig. 1). Although concentration of six g L^{-1} gave the highest volume of hydrogen and total gas volume, there was no significant difference between concentrations of three, six and 12 g L^{-1} ($P > 0.05$). A strong positive correlation was found between evolved gas volume and volumetric fraction of hydrogen ($r = 0.8240$, $P = 0.0005$) after 24 h of fermentation at 37 °C.

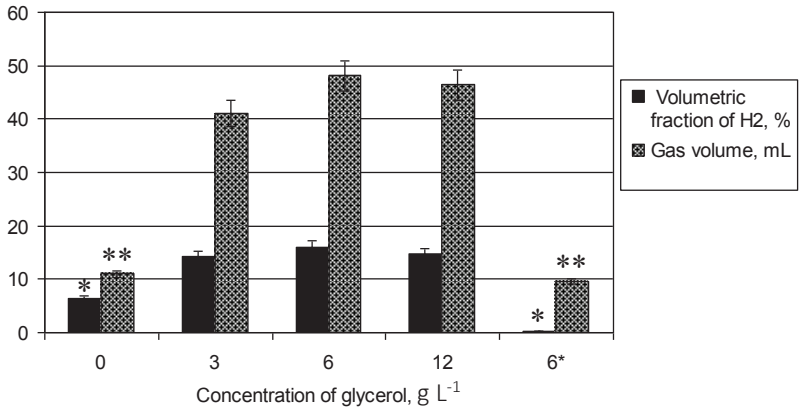

Figure 1. The volumetric fraction of hydrogen produced by *E. aerogenes* in the gas phase of growth medium without crude glycerol (0), with crude glycerol (3, 6 and 12 g L^{-1}) and crude glycerol in water (6*; 6 g L^{-1}) after 24 h of incubation at 37 °C. * – volumetric fraction of H_2 significantly different ($P < 0.05$) from other variants; ** – gas volume significantly different ($P < 0.05$) from other variants.

Although pH effects on the growth of *E. aerogenes* was not studied, as well as pH was not affected during experiments, the pH value was measured at the beginning and after 24 h of cultivation (Fig. 2). Increase of initial pH value was observed together with increase of initial concentration of crude glycerol, especially in the case of glycerol solution in water instead of solution in the growth medium. Apparently, the medium had buffering properties. The broth became more acidic during incubation in all variants, up to pH 4.5 (with glycerol 12 g L^{-1}) and together with increase of glycerol concentration. Tanisho et al. (1989) showed that the most suitable pH for cell mass productivity of *E. aerogenes* is around pH 7.0, and as pH decreases below pH 7.0, the cell weight measured after cultivation also decreases. However, *E. aerogenes* is able to multiply in pH range 4.4–9.0 (reviewed in Fanning et al., 2016). Decrease of pH value during incubation is due to the formation of metabolism products such as succinic acid and propionic acid (Clomburg & Gonzalez, 2013). Certainly, results with values of pH below 5 are too low for good growth and productivity and pH should be corrected and maintained near optimal level. Tanisho et al. (1989) showed that the evolution rate and the yield of hydrogen have maximum values at a pH of approximately 5.8 but Lin & Lay (2004) reported maximum output occurred at pH values between 6 and 7.

Landfill substrate and manure as inoculants with or without inoculation of *E. aerogenes* were used to compare analytical grade, technical grade and crude glycerol as a co-substrate for gas production (Fig. 3). Cumulative gas volume was calculated in AMPTS II after four days of incubation. *E. aerogenes* together with landfill substrate and manure gave 55%, 42% and 8% higher gas volume than landfill substrate and manure without bacterium correspondingly from analytical grade, technical grade and crude glycerol. Parallel experiments with measurements of gas volume and composition were performed in the serum bottles (Fig. 4) because AMPTS II can only distinguish between CO_2, which is captured in the CO_2 trap, and 'not CO_2', which is assumed to be CH_4. The system is not capable of differentiating between CH_4, N_2, O_2 or other gases (Strömberg et al., 2014) including argon used for initial flushing of reactors. Obtained

results showed that methane was not produced in this experiment, but AMPTS II records reflected the volume of argon, hydrogen and nitrogen. Gas volume obtained from technical grade glycerol in AMPTS II did not significantly differ ($P > 0.05$) from gas volume obtained from analytical grade glycerol with one exception. Significantly higher gas volume was obtained from analytical grade glycerol than from technical grade glycerol in variant with *E. aerogenes* together with inoculants obtained from landfill substrate and manure (Fig. 3). Increase was based on the presence and action of *E. aerogenes*. Landfill substrate and manure inoculum acted in synergy with *E. aerogenes* in fermentation of technical grade and analytical grade glycerol. Synergy was not observed in case with crude glycerol ($P > 0.05$).

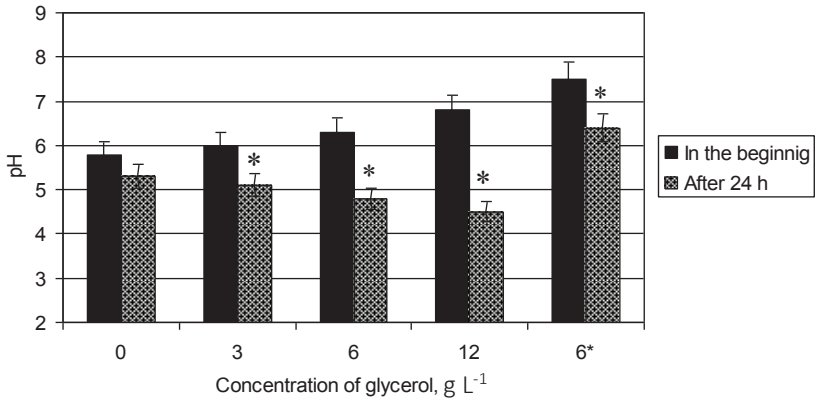

Figure 2. Values of pH of *E. aerogenes* growth media without crude glycerol (0), with crude glycerol (3, 6 and 12 g L^{-1}) and crude glycerol in water (6*; 6 g L^{-1}) in the beginning of the experiment and after 24 h of cultivation at 37 °C. * – significant difference ($P < 0.05$) between beginning and 24 h of incubation.

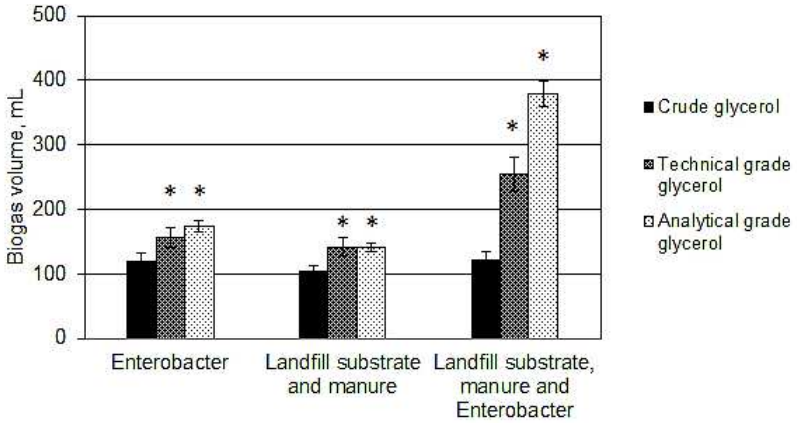

Figure 3. Biogas volume detected in AMPTS II after four days of incubation of growth medium containing glycerol source (10 g L^{-1}) and landfill substrate and manure with or without inoculation of *E. aerogenes* depending on the substrate and type of glycerol. Glycerol concentration: crude glycerol – 40%; technical grade glycerol – 82%; analytical grade glycerol – 99.5%. * – significant difference ($P < 0.05$) between variant with crude glycerol in the same substrate and inoculant group.

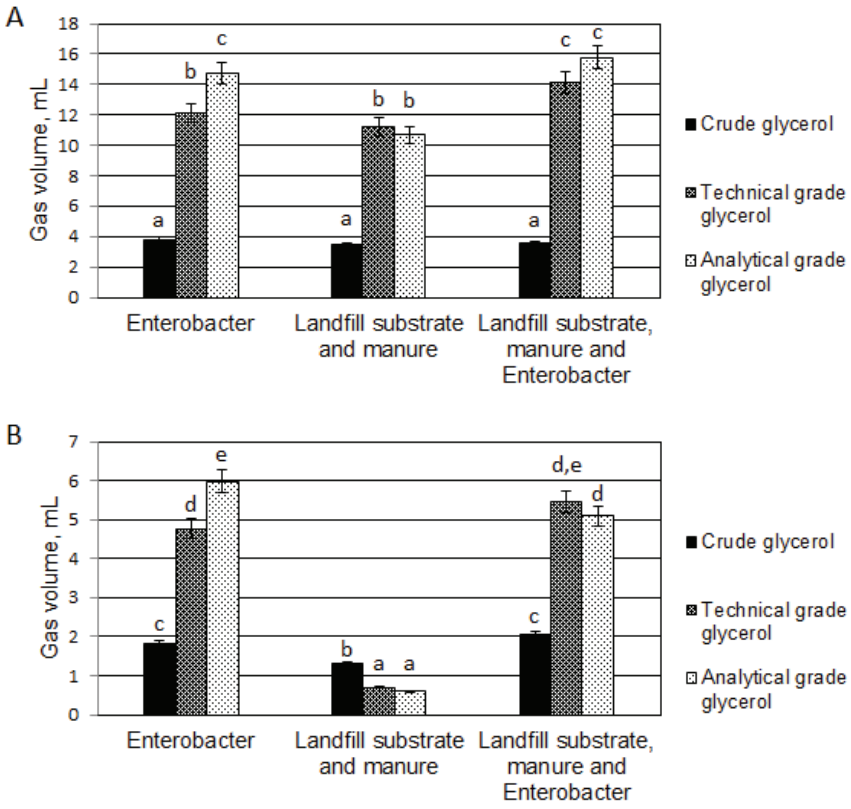

Figure 4. Carbon dioxide (A) and hydrogen (B) gas volume produced in serum bottles parallel with AMPTS II experiment after four days of incubation of growth medium containing glycerol source (10 g L^{-1}) and inoculum of landfill substrate and manure with or without *E. aerogenes* depending on the substrate and type of added glycerol. Glycerol concentration: crude glycerol – 40%; technical grade glycerol – 82%; analytical grade glycerol – 99.5%. Means sharing the same letter are not significantly different ($P > 0.05$) from each other in the corresponding part A or B.

Amount of hydrogen also was significantly higher in the case of analytical grade glycerol usage in comparison with crude glycerol fermentation. Nitrogen gas in amount of 0.4–0.7% in the headspace was detected only in variants with crude glycerol. Landfill substrate and manure gave small quantity of hydrogen, not exceeding 1.3 mL per one serum bottle. Additional inoculation with *E. aerogenes* increased the amount of hydrogen 1.6, 7.8 and 8.6 times in variants with crude glycerol, technical grade glycerol and analytical grade glycerol respectively (Fig. 4B). However, synergy was not found. These volumes were not significantly higher than without landfill substrate and manure, i.e. they were similar to variant with *E. aerogenes* alone. That means that landfill substrate and manure did not contain enough number of microorganisms appropriate for hydrogen production at least in these conditions. Unfortunately, no information about chemical composition of landfill substrate and manure was obtained. It is known that hydrogen production is sensitive to both various inhibitors and the ratios of C : N and C : P and, for example, low nitrogen concentrations also require low phosphorous

contents for high productivity (Argun et al., 2008). In the same time, *E. aerogenes* increased the amount of produced carbon dioxide 1.3 and 1.5 times in variants with technical grade and analytical grade glycerol (Fig. 4A). Amount of CO_2 increased 1.1 times in comparison with *E. aerogenes* alone (statistically significant only in the case of technical grade glycerol). There was a strong correlation ($r = 0.8235$, $P = 0.0064$) between gas volume and volumetric fraction of hydrogen as well as between gas volume and volumetric fraction of carbon dioxide ($r = 0.9762$, $P = 0.00001$).

Acs et al. (2015) carried out bioaugmentation of biogas production by hydrogen-producing *Enterobacter cloacae*. This lead to the altered microbial community and increased biogas production, and authors proposed development of syntrophic relationships between polymer-degrading and H_2-producing Clostridia and *E. cloacae*. Results of this study showed potential of *E. aerogenes* for use in bioaugmentation purposes particularly for fermentation of glycerol.

Experiments with mixed natural microbial communities

Lake sludge inoculum was used in crude glycerol fermentation experiments in the serum bottles with mixed natural microbial communities during six days at temperature of 20 °C and 37 °C. Carbon dioxide was the most produced gas at both temperatures, especially at 37 °C. The highest volume of hydrogen was found after two days of incubation at 37 °C (Fig. 5). Volume of hydrogen was 1.6 times lower at 20 °C than at 37 °C. Methane was detected in one case, i.e. it occurred in amount of 0.45 mL after six days at 37 °C simultaneously with decrease of volume of hydrogen. This suggests that hydrogenotrophic methanogens had begun operating. Homoacetogenesis and aceticlastic methanogenesis are more pronounced at lower temperatures (reviewed in Schink & Stams, 2013). FISH analyses confirmed increased amount of Archaea in this case (Fig. 6). Cultivation of Archaea was not attempted, however, results show that number of aerobically growing bacteria remained higher than number of anaerobically growing bacteria during all experiment regardless of the temperature with exception of two days at 20 °C (Fig. 7).

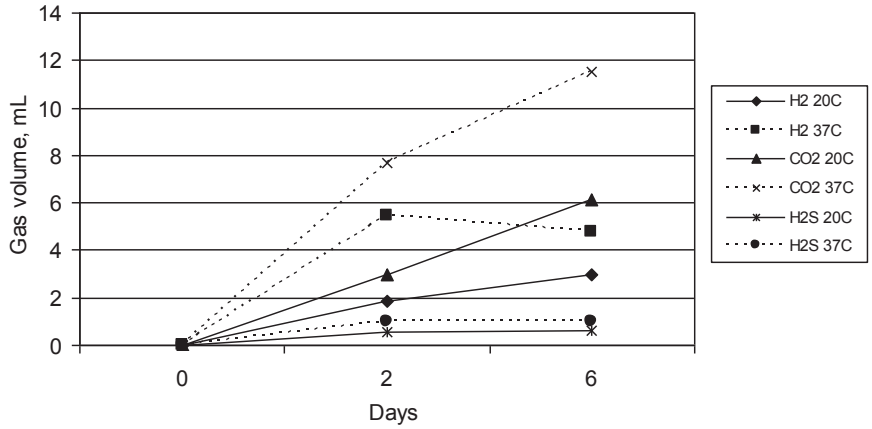

Figure 5. Cumulative volume of gases produced during anaerobic incubation of medium containing lake sludge inoculum and analytical grade glycerol 10 g L^{-1} at temperature of 20 °C or 37 °C.

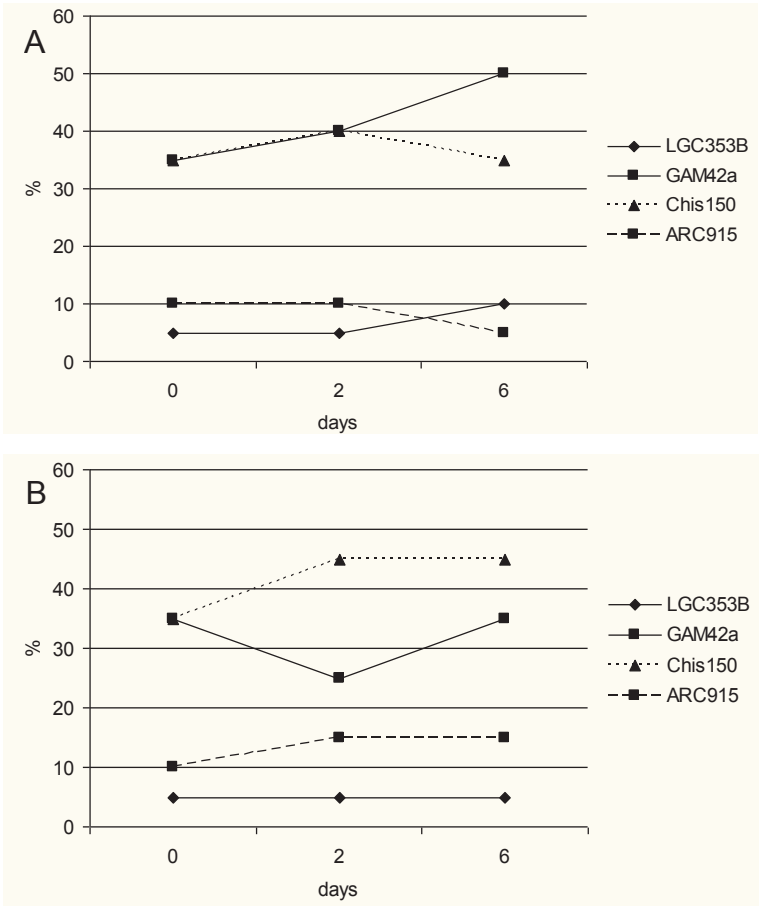

Figure 6. Bacterial groups determined by FISH in fermentation liquid in the beginning and after two and six days of anaerobic incubation at 20 °C (A) and 37 °C (B). Results are expressed as a percentage of all observed cells and been rounded to 5%. LGC353B – probe for *Bacillus* spp.; GAM42a – probe for Gamma-Proteobacteria; Chis150 – probe for *Clostridium* clusters I and II; ARC915– probe for Archaea.

FISH analyses indicated presence of all four studied groups: *Bacillus* spp., Gamma-Proteobacteria, *Clostridium* clusters I and II, and Archaea (Fig. 6). Clostridia and Gammaproteobacteria were predominant in the lake sludge inoculum. Several species were isolated: *Clostridium butyricum* and *C. hastiforme* from class Clostridia, and *Pantoea agglomerans* and *Serratia plymuthica* from class Gammaproteobacteria. Amount of Bacilli and Archaea did not exceed 10% during the experiment (Table 1). However, *Bacillus licheniformis* was isolated and found to be between the predominant after two and six days of incubation at both temperatures. *B. licheniformis* is a widespread facultatively anaerobic bacterium, which reduces nitrate to nitrite and utilizes glycerol (reviewed in Logan & De Vos, 2015). Already Kalia et al. (1994) and Porwal et al. (2008) isolated hydrogen-producing *B. licheniformis* strains from cattle dung and Porwal et al. (2008) also from contaminated food. This facultative anaerobic bacterium produced 0.5 ml H_2 mol^{-1} glucose (Kumar et al., 1995). Unidentified

Clostridium species were recovered after six days of incubation regardless of temperature. Microbial metagenome analyses of biogas reactors usually detect a large number of reads with unidentified microbial origin, indicating that anaerobic degradation process may also be conducted by up to now unknown species (Rademacher et al., 2012).

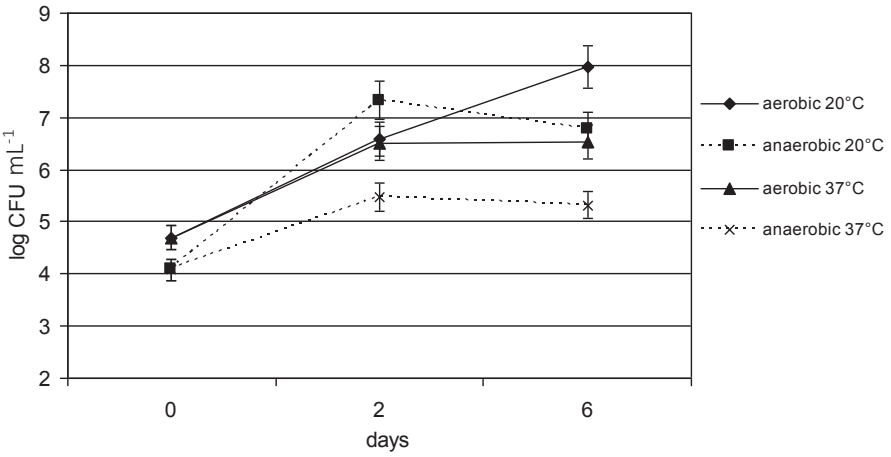

Figure 7. Amount of CFU of aerobically and anaerobically growing bacteria during anaerobic incubation of medium containing lake sludge inoculum and analytical grade glycerol 10 g L^{-1} at temperature of 20 °C or 37 °C.

Enterococci and *Enterobacteriaceae* were the most identified microorganisms which persist after anaerobic digestion of cattle manure (Resende et al., 2014). *Burkholderia cepacia* was found between predominant species in the sludge inoculum and in the fermentation broth after six days of anaerobic incubation at both temperatures (Table 1). Also Resende et al. (2014) identified *B. cepacia* in 8.87% of samples. *B. cepacia* is commonly found in natural materials, particularly soil and it is extremely versatile from the metabolic standpoint (reviewed in Palleroni, 2015). It is a strict aerobe and does not perform nitrate reduction. However, Hutchison et al. (1998) demonstrated that without visible growth during anaerobic incubation, viable *B. cepacia* could be cultured after seven days.

Lake sludge inoculum contained a little amount of fungi. There was *Penicillium* sp. about 1 CFU mL^{-1} (Table 1). Fungi were not detected after two and six days of incubation. Also loss of viability of fungus *Trichoderma asperellum* during anaerobic incubation was previously observed (Nikolajeva et al., 2015). Other fungi belonging to order *Neocallimastigales,* which are anaerobic and are usually found in the digestive tracts of herbivorous animals are recognized as promising candidates for improvement of degradation of fibrous material in fermentation reactors (Dollhofer et al., 2015).

Table 1. Predominant species and class of microorganisms isolated from fermentation liquids and identified in the beginning (0 days) and after two and six days of anaerobic incubation at 20 °C and 37 °C

Species	Class	0 days	2 days 20 °C	2 days 37 °C	6 days 20 °C	6 days 37 °C
Agrobacterium sp.	Alphaproteobacteria	X				
Bacillus cereus	Bacilli	X				
Bacillus licheniformis	Bacilli	X	X	X	X	X
Bacillus sp.	Bacilli			X		
Burkholderia cepacia	Betaproteobacteria	X		X	X	X
Clostridium butyricum	Clostridia	X	X	X		
Clostridium hastiforme	Clostridia	X				
Clostridium spp.	Clostridia				X	X
Hafnia alvei	Gammaproteobacteria				X	X
Pantoea agglomerans	Gammaproteobacteria	X				
Penicillium sp.	Eurotiomycetes	X				
Serratia fonticola	Gammaproteobacteria		X			
Serratia plymuthica	Gammaproteobacteria	X				
Sphingobacterium multivorum	Sphingobacteria	X				
Sphingomonas paucimobilis	Alphaproteobacteria	X				

CONCLUSIONS

Microbiological production of biogas and hydrogen by anaerobic fermentation of glycerol requires further investigation. *Enterobacter aerogenes* is a facultatively anaerobic bacterium that can be used for glycerol fermentation purposes.

In lab-scale experiments *E. aerogenes* produced hydrogen from all types of glycerol source. *E. aerogenes* acted in synergy with landfill substrate and manure inoculum for biogas production from technical grade and analytical grade glycerol. Synergy was not observed in the case of crude glycerol.

Lake sludge inoculum unlike landfill substrate and manure inoculum supported hydrogen production from glycerol. Inoculated mixed microbial populations from lake sludge changed during fermentation. Increase of proportion of Clostridia and Archaea was observed at 37 °C while increase of Gammproteobacteria and *Bacillus* spp. was observed at 20 °C, this temperature was too low for successful fermentation process.

Additional studies are necessary to confirm the potential of *E. aerogenes* and lake sludge inoculum for use in bioaugmentation purposes for mesophilic fermentation of glycerol as a co-substrate together with common feedstocks.

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