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Changes in freshwater sediment microbial populations during fermentation of crude glycerol

Matiss Paiders [a], Vizma Nikolajeva [a,*], Galina Makarenkova [a], Liana Orola [b], Ilze Dimanta [a], Janis Kleperis [c]

[a] Faculty of Biology, University of Latvia, 1 Jelgavas Street, Riga LV-1004, Latvia
[b] Faculty of Chemistry, University of Latvia, 1 Jelgavas Street, Riga LV-1004, Latvia
[c] Institute of Solid State Physics, University of Latvia, 8 Kengaraga Street, Riga LV-1063, Latvia

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A B S T R A C T

Background: This work studied how the exposure to an unusual substrate forced a change in microbial populations during anaerobic fermentation of crude glycerol, a by-product of biodiesel production, with freshwater sediment used as an inoculum.
Results: The microbial associations almost completely (99.9%) utilized the glycerol contained in crude glycerol 6 g L^{-1} within four days, releasing gases, organic acids (acetic, butyric) and alcohols (ethanol, *n*-butanol) under anaerobic conditions. In comparison with control medium without glycerol, adding crude glycerol to the medium increased the amount of ethanol and *n*-butanol production and it was not significantly affected by incubation temperature (28 °C or 37 °C), nor incubation time (4 or 8 d), but it resulted in reduced amount of butyric acid. Higher volume of gas was produced at 37 °C despite the fact that the overall bacterial count was smaller than the one measured at 20 °C. Main microbial phyla of the inoculum were *Actinobacteria*, *Proteobacteria* and *Firmicutes.* During fermentation, significant changes were observed and *Firmicutes*, especially *Clostridium* spp., began to dominate, and the number of *Actinobacteria* and *Gammaproteobacteria* decreased accordingly. Concentration of *Archaea* decreased, especially in medium with crude glycerol. These changes were confirmed both by culturing and culture-independent (concentration of 16S rDNA) methods.
Conclusions: Crude glycerol led to the adaptation of freshwater sediment microbial populations to this substrate. Changes of microbial community were a result of a community adaptation to a new source of carbon.
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1. Introduction

With global oil reserves running out and due to environmental changes, more and more focus is made on biodiesel production, an eco-friendly, alternative fuel [1,2]. The main by-product of biodiesel production is crude glycerol, a glycerol with impurities that when stored unattended can pose a threat to the environment. During biodiesel production, the transesterification of fats and oils with an alcohol results in 100 kg of crude glycerol for every ton of biodiesel produced [3]. Therefore, efficient utilization of crude glycerol is required to make biodiesel production economically viable. A prospective direction is its use as a substrate or co-substrate for the production of various substances by microbiological fermentation. Traditionally, microbiological fermentation has been studied using microorganisms in pure cultures, however, because cheap biomass sources contain many complex compounds and impurities that make it difficult for one microorganism to utilize them efficiently, use of mixed, naturally occurring microbial associations is a viable alternative [4].

The production of 1,3-propanediol, *n*-butanol, 2,3-butanediol, citric acid, docosahexaenoic acid, eicosapentaenoic acid, polyhydroxyalkanoates, lipids and ethanol by biological processes from crude glycerol is studied. Hydrogen and methane can also be obtained biologically from crude glycerol [5,6]. These processes use different microorganisms (bacteria, fungi and microalgae) and metabolic pathways. Depending on the product to be obtained

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* Corresponding author.
E-mail address: vizma.nikolajeva@lu.lv (V. Nikolajeva).

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and the microorganism used, the culture conditions may be anaerobic, microaerobic or aerobic [7].

Many microorganisms utilize glycerol in the respiratory process, but only a small number under anaerobic conditions without the presence of external electron acceptors [8]. Glycerol metabolism via anaerobic fermentation has been observed in bacteria of the *Enterobacteriaceae* family, such as *Escherichia coli, Citrobacter freundii, Klebsiella pneumoniae* and *K. aerogenes*, as well as *Clostridium, Lactobacillus, Bacillus, Propionibacterium* and *Anaerobiospirillum* spp. [9]. Anaerobic fermentation of glycerol distinguishes between the reductive and oxidative pathways. In the reductive pathway, glycerol is first dehydrated by the coenzyme glycerol dehydratase to give 3-hydroxypropionaldehyde, which in turn is reduced by 1,3-propanediol dehydrogenase to 1,3-propanediol. In the oxidative pathway, the glycerol dehydrogenase oxidizes glycerol to dihydroxyacetone, which is further phosphorylated by dihydroxyacetone kinase. The resulting dihydroxyacetone phosphate can further undergo glycolysis to form important metabolic intermediates and end products of fermentation, such as succinic acid, propionic acid, *n*-butanol, 2,3-butanediol, lactic acid, butyric acid, acetic acid, ethanol, and formic acid as well as gases, carbon dioxide and hydrogen. Formic acid, acetic acid, H_2 and CO_2 obtained by fermentation can be directly converted into methane by the action of methanogenic archaea [6]. Acetotrophic (acetoclastic) methanogens convert acetic acid into carbon dioxide and methane. Hydrogenotrophic methanogens use hydrogen to reduce CO_2 (as well as other compounds such as formic acid) to produce methane [reviewed in [10]].

Among the impurities (methanol, salts of potassium, and sodium, heavy metals etc.) in crude glycerol, long chain fatty acids, chlorides, and sulfates can have a serious effect on the metabolism of microorganisms and therefore, it is necessary to dilute it. In the presence of sulfates, in turn, sulfate-reducing bacteria compete with acetotrophic methanogenic archaea and consume acetic acid, releasing H_2S, which at sufficient concentration can inhibit anaerobic microorganisms [6]. In comparison with pure cultures, the specific advantages of using mixed consortia are: (1) the possibility of utilizing cheaper mixed substrates, (2) combination of enzymatic systems of different microorganisms and synergy, which may allow more efficient utilization of substrate with a narrower spectrum of products obtained, (3) there is no need for sterile conditions to reduce production costs [4].

Natural microbial communities usually consist of several hundred species with various metabolic activities and life-styles. Although conventional cultivation methods are used for microbial composition studies in freshwater sediment, it is believed that only 0.25% of the cells present in sediment samples can be cultured [11]. Therefore, it is not possible to determine the full diversity of microorganisms by cultivation alone and molecular methods such as 16S rDNA analysis should be used [12,13]. Phylogenetic analyses provided by Tamaki et al. [11], Shao et al. [14] and Zhang et al. [15] showed that *Proteobacteria* was the most abundant and largest phylum in the like sediments. In freshwater sediments, bacterial production rate is closely linked to substrate supply and temperature [16]. The sources of organic matter to sediments can be qualitatively different. Complex structural polysaccharides and phenolic polymers may comprise a greater fraction of the organic input to freshwater systems [17]. Orland et al. [18] highlighted that future changes to both sediments and lake waters can modify how sediment microbial communities develop with consequences for important ecosystem functions like carbon cycling. In laboratory experiments, adaptation times ranged from days to weeks, depending on the type of inoculum, molecules being assessed and of the experimental conditions [reviewed in [19]].

The aim of this study was to investigate how the exposure to an unusual substrate forced a change in the composition of microorganisms during the anaerobic fermentation of crude glycerol using inoculum from freshwater sediment.

2. Materials and methods

2.1. Fermentation experiments

A sample of freshwater sediment collected from a quarry in Dalbe (Latvia) five meters from the shore was used as an inoculum. The sediment in the amount of 0.5 mL was inoculated into 100 mL volume glass serum bottles (Supelco, USA) containing 70 mL of sterile medium [tryptone 1.0 g L^{-1}, cysteine hydrochloride monohydrate 0.5 g L^{-1}, with or without crude glycerol (Bio-Venta Ltd., Latvia) 6.0 g L^{-1}]. The serum bottles were hermetically sealed with a sterile rubber stopper (Gotlands Gummifabrik, Sweden) and aluminum cap, and flushed with inert gas–argon gas (99.99% purity) (for approximately one and a half minutes) to create anaerobic environment. A portion of the samples was incubated in an incubator at 37 °C and the other part at room temperature (20 °C) for 4 or 8 d. The experiment was performed in duplicates.

After incubation, the volume of gas released during fermentation was determined by piercing the rubber stopper of the serum bottle with a 50 mL disposable gas-tight syringe. In case of an excess pressure in the bottle, the gas flowed into a syringe where the volume of gas could be determinate.

2.2. Biochemical analyses

The pH of the media before and after incubation was determined using a pH meter AD1405 (Adrona, Latvia). After incubation, approximately 20 mL of medium from each sample were frozen and stored at −20°C until DNA extraction and chromatographic measurements could be performed.

Glycerol content was determined with a HPLC Agilent 1290 Infinity (Agilent Technologies, USA) coupled to an Agilent 6230 TOF LC/MS mass spectrometer using an Atlantis dC18 (Waters, USA) column (column length 150 mm, internal diameter 2.1 mm) in gradient mode. The mobile phases consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. Analytical conditions: mobile phase rate 0.3 mL min^{-1}, column temperature 30°C, sample volume to be injected 1 μL, positive electrospray, detection range *m/z* 50–1000, drying gas temperature 325°C and flow rate 10 mL min^{-1}, fragmentation voltage 90 V. For chromatography analyses, 2 mL of the medium were centrifuged to remove bacterial and sediment deposit. Supernatant was used for analysis and diluted 1:2 with acetonitrile. Each sample was injected into the chromatographic system three times. Standard glycerol solutions in acetonitrile/water (1:1) were prepared and a calibration graph was constructed to determine the glycerol content. MassHunter Qualitative Analysis software was used for data processing.

Organic acids and alcohols were measured using a HPLC Agilent 1100 and a Shodex Asahipak SH1011 (Showa Denko, Japan) column (column length 300 mm, internal diameter 8.0 mm) and SH-G pre-column (length 50 mm, internal diameter 6.0 mm) at 50°C with a mobile phase of 0.01 N H_2SO_4 by R. Scherbak at the Institute of Microbiology and Biotechnology of the University of Latvia. The mobile phase rate was 0.6 mL min^{-1}. The volume of sample to be injected was 5 μL. A differential refractometric detector RID G1362 was used to determine ethanol. Organic acids were determined using a diode array detector DAD G1315B at 210 nm. Alcohols were determined using an Agilent 6890N capillary gas chromatograph with a flame ionization detector.

2.3. Cultivation and identification of microorganisms

Number of heterotrophic bacterial colony-forming units (CFU) per mL of medium was determined by preparing a series of five dilutions (1:10) and plating 100 μL of each dilution on Petri plates with R2A agar (Sifin Diagnostics, Germany) in duplicate. Incubation was performed at 20 ± 2°C. The first replicate was cultured for 4 d under aerobic conditions and the second replicate was cultured for 10 d under anaerobic conditions (BD GasPak EZ, Becton, Dickinson and Company, USA). To determine the presence of fungi, 100 μL of the original medium and the first dilution series were plated on Malt extract agar (Biolife, Italy) which was incubated 4 d under aerobic conditions.

After determining the number of CFU, different morphotypes were selected from each sample and 1–3 times re-inoculated in new medium to obtain pure cultures. Microscopy revealed the morphology of bacteria, as well as Gram reaction, catalase assay, indole assay, and oxidase assay were performed. Pure cultures were identified using BD BBL™ Crystal™ Identification Systems: Anaerobe ID Kit, Enteric/Nonfermenter ID Kit, and Gram-Positive ID Kit (Becton, Dickinson and Company, USA).

2.4. Extraction of DNA, and qPCR

Total DNA extraction from the samples was performed with the PowerSoil DNA Isolation Kit (MO BIO Laboratories, USA). Concentration and purity of DNA were determined with NanoDrop 2000c (Thermo Fisher Scientific, USA). $A_{260/280}$ ranged from 1.71 to 2.18. Real-time quantitative PCR was performed with bacterial group-specific pairs of 16S rDNA primers (Sigma-Aldrich) using Applied Biosystems 7300 qPCR (Thermo Fisher Scientific, USA). The primers and their sequences are shown in Table 1. The obtained data were analyzed with the 7300 System SDS program.

The qPCR mixture in volume of 25 μL contained Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific) with primer (0.2 μM) and 1 μL (5–10 ng) of DNA. Thermal cycles consisted of a 10 min initial denaturation at 95°C followed by 40 cycles of denaturation at 95°C for 30 s, hybridization at 60°C for 30 s and extension at 72°C for 60 s. All reactions were performed in triplicate.

The following strains were used to prepare standard curves: *Pantoea agglomerans* MSCL 652 for *Gammaproteobacteria*, *Bacillus subtilis* MSCL 897 for *Firmicutes*, *Rhodococcus equi* MSCL 762 for *Actinobacteria* and *Methanosaeta harundinacea* MSCL 1074 for *Archaea*. To confirm the specificity of amplified PCR products, all qPCR reactions were followed by melting curve analysis and agarose gel electrophoresis.

2.5. Statistical analysis

The R program version 3.5.3 was used for statistical processing of data. Statistical significance was defined as $P < 0.05$. Arithmetic means were compared using the *t*. test function to compare related and independent sample sets.

Table 1
Primers used in this study.

Primer	Specificity	Sequence (5′ → 3′)	Reference
Actino235	*Actinobacteria*	CGCGGCCTATCAGCTTGTTG	[36]
Eub518R		ATTACCGCGGCTGCTGG	[37]
Arch967F	*Archaea*	AATTGGCGGGGGAGCAC	[38]
Arch1060R		GGCCATGCACCWCCTCTC	
Lgc353	*Firmicutes*	GCAGTAGGGAATCTTCCG	[39]
Eub518R		ATTACCGCGGCTGCTGG	[37]
Gamma877F	*Gamma-*	GCTAACGCATTAAGTRYCCCG	[40]
Gamma1066R	*proteobacteria*	GCCATGCRGCACCTGTCT	

3. Results and discussion

3.1. Volume of gas produced during fermentation

When determining the volume of gas after 4 and 8 d of fermentation, the highest volume of gas produced was found in a sample cultured at 37°C with crude glycerol after 4 d of incubation with a mean value of 44.3 mL and a maximum result of 53.0 mL gas per 70 mL medium (Fig. 1). Addition of crude glycerol increased the volume of gas produced in the sample by an average of 23.8 mL in comparison with the control without glycerol ($P = 2.37 \times 10^{-5}$). In the control samples without sediment inoculum gas was not produced.

Statistically significant ($P = 0.0433$) higher volume of gas was detected in the samples cultured at 37°C compared to samples cultured at 20°C. Temperature differences changed the volume of gas by an average of 6.1 mL. The duration of cultivation did not have a statistically significant ($P = 0.3278$) effect on the amount of gas released. This could be explained firstly, with the large differences between the results in both replicates of the samples, this is likely due to the lack of homogeneity in the sediment inoculum, and secondly, with the activity of hydrogenotrophic methanogenic archaea or homoacetogenic bacteria. Homoacetogenic bacteria have the ability to use hydrogen as an electron donor for CO_2 reduction to yield acetate [20] and include members of the *Clostridia* class such as *Clostridium ljungdahlii* and *C. autoethanogenum* [21]. Although this work did not analyze the composition of the gas released by fermentation, it is reasonable to assume that mainly CO_2 and H_2 were produced. Our previous experiments with fermentation of analytical glycerol with sediment inoculum showed that after two days incubation at 37 °C, the gas phase of the medium contained 27.7% CO_2 and 16.2% H_2. Small amounts of H_2S were also detected in the media, as well as the presence of methane in some samples.

3.2. pH

A decrease of pH of the media was observed in all samples during fermentation. In the case of medium containing crude glycerol this decrease was statistically more pronounced ($P = 1.65 \times 10^{-6}$) compared to medium without crude glycerol 5.29 ± 0.07 and 6.13 ± 0.11, respectively. No statistically significant ($P = 0.2389$) effect was observed for the incubation temperature. The more pronounced decrease in the pH of the crude glycerol-containing medium can be explained by the formation of organic acids. Investigating the fermentation of *Klebsiella (Enterobacter) aerogenes* at various concentrations of crude glycerol, Paiders et al. [22] found that as the initial concentration of crude glycerol increased, the pH of the medium decreased during incubation.

3.3. Concentration of glycerol and metabolites in growth media

In the non-incubated crude glycerol samples and in the sediment inoculum-free crude glycerol samples after incubation, the liquid chromatographic results showed an average glycerol concentration of 4.89 ± 0.41 g L^{-1} (Table 2), indicating approximately 81.5% glycerol content in the crude glycerol used in the study. After 4 and 8 d fermentation, >100-fold glycerol utilization was observed in all samples. There was only 0.019–0.037 mg L^{-1} left. No significant effect on glycerol utilization was observed for either incubation time or incubation temperature.

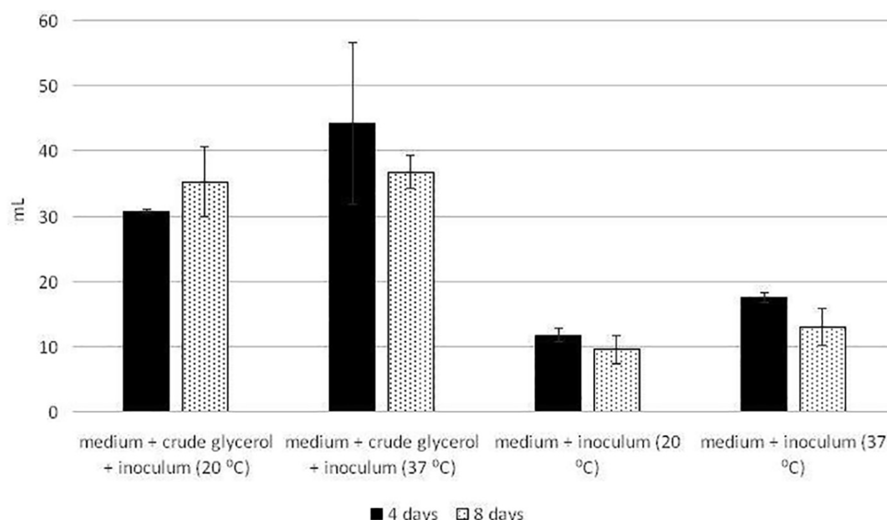

Fig. 1. Gas volume (mL) after 4 and 8 d of cultivation at 20 °C and 37 °C per 70 mL of medium.

Table 2
Concentration of glycerol and metabolites in growth media at the beginning and after 4 and 8 d of cultivation at 20 °C and 37 °C. N – not detected.

Medium with		Temperature (°C)	Days	Concentration, g L^{-1}				
Crude glycerol	Inoculum			Glycerol	Acetic acid	Butyric acid	Ethanol	*n*-butanol
+	–	20	0	4.950 ± 0.778	0.009	<0.001	0.153	0.002
+	+	20	0	4.725 ± 0.474	0.008	<0.001	0.141	0.002
–	–	20	0	N	0.002	<0.001	0.150	0.003
–	+	20	0	0.003	0.002	<0.001	0.150	0.003
+	+	20	4	0.029 ± 0.003	0.120 ± 0.149	0.029 ± 0.010	1.512 ± 0.012	0.012 ± 0.003
+	+	37	4	0.029 ± 0.004	0.542 ± 0.206	0.039 ± 0.023	1.167 ± 0.753	0.012 ± 0.005
–	+	20	4	0.019 ± 0.001	0.522 ± 0.409	0.122 ± 0.039	0.396 ± 0.003	0.004
–	+	37	4	0.031 ± 0.013	0.703 ± 0.232	0.084 ± 0.045	0.336 ± 0.024	0.003
–	–	37	4	N	0.006 ± 0.007	0.012 ± 0.007	0.148 ± 0.011	0.003
+	–	37	4	5.165 ± 0.191	0.015 ± 0.006	0.013 ± 0.011	0.150 ± 0.008	0.003
+	+	20	8	N	0.341 ± 0.462	0.059 ± 0.046	1.496 ± 0.089	0.009 ± 0.008
+	+	37	8	0.037 ± 0.010	0.859 ± 0.011	0.105 ± 0.018	1.435 ± 0.366	0.011 ± 0.005
–	+	20	8	0.026 ± 0.007	1.015 ± 0.013	0.174 ± 0.059	0.479 ± 0.006	0.003 ± 0.001
–	+	37	8	0.027 ± 0.004	0.990 ± 0.019	0.155 ± 0.045	0.412 ± 0.041	0.003
–	–	37	8	N	0.002 ± 0.003	0.018 ± 0.013	0.149 ± 0.013	0.003
+	–	37	8	4.735 ± 0.191	0.016 ± 0.005	0.021 ± 0.014	0.166 ± 0.011	0.003

Of all the organic compounds detected, the highest concentration increase was observed for ethanol with a maximum mean concentration of 1.512 g L^{-1} in the crude glycerol sample incubated for 4 d at 20°C. The presence of crude glycerol in the medium resulted in a statistically significant (P = 5.21 × 10^{-5}) increase in ethanol production (0.997 g L^{-1} on average). The incubation time and temperature selected did not have a statistically significant effect on ethanol concentration. The highest ethanol yield was calculated after incubation at 20°C for 4 d, 0.23 g of ethanol produced per gram of glycerol utilized, which is among the ethanol yields reported in the literature. For example, *Klebsiella aerogenes* gave an ethanol yield of 0.43 g g^{-1} in the fermentation of crude glycerol with parallel hydrogen production [23]. Metsoviti et al. [24] obtained an ethanol yield of 0.33 g g^{-1} in the fermentation of crude glycerol with *Klebsiella oxytoca*, in parallel with the production of 1,3-propanediol (0.28 g g^{-1}) and 2,3-butanediol (0.08 g g^{-1}). Unfortunately, the concentration of 1,3-propanediol and 2,3-butanediol was not determined in our study, which did not allow us to get a complete picture of the metabolic processes. The obtained results showed that sediment bacteria are able to metabolize an unusual substrate for their habitat – glycerol – in 4 d.

Crude glycerol-containing samples also showed an increase in *n*-butanol, showing a maximum concentration of 0.012 g L^{-1} after 4 d of incubation. However, temperature and incubation time did not have a statistically significant effect on *n*-butanol concentration. The addition of crude glycerol to the basal medium had a statistically significant (P = 0.0025) effect, increasing the *n*-butanol concentration by an average of 0.008 g L^{-1}. In some cases, a slight increase in the concentration of isobutanol (0.1–0.7 mg L^{-1}) after the fermentation process was also detected.

The main organic acids detected were acetic and butyric acid (Table 2), but formic acid was detected in small quantities in separate replicates of the samples. Concentration of lactic acid was <0.001 g L^{-1} in all cases. Acetic acid showed statistically significant (P = 0.0438) elevated concentrations in crude glycerol-free samples after fermentation compared to crude glycerol-containing samples. The maximum detected acetic acid concentration was 1.025 g L^{-1} in the crude glycerol-free sample, which was incubated for 8 d at 20°C. Increasing the incubation period from 4 to 8 d resulted in a significant (P = 0.0080) increase in acetic acid concentration by an average of 0.33 g L^{-1}, without suggesting acetotrophic methanogenic activity but not excluding the activity of acetogenic

bacteria. Increasing the temperature from 20°C to 37°C also showed a positive significant (P = 0.0352) effect, increasing the acetic acid concentration by an average of 0.27 g L^{-1}.

Butyric acid also showed significant (P = 0.0023) higher concentrations in crude glycerol-free samples, reaching a maximum concentration of 0.22 g L^{-1}. A positive significant effect (P = 0.0096) on increasing the incubation time from 4 to 8 d by increasing the concentration of butyric acid by 0.06 g L^{-1} was also found. Incubation temperature did not have a statistically significant effect on butyric acid concentration.

3.4. Number of cultivated microorganisms

Initially, immediately after the addition of the sediment inoculum, the medium contained 9950 aerobically growing bacterial CFU mL^{-1} (Fig. 2a). The maximum number of aerobic microbial CFU was 5.7×10^6 CFU mL^{-1} of the medium without crude glycerol after 4 d incubation at 20°C. Addition of crude glycerol had a significant (P = 0.0447) negative effect on number of aerobic bacteria. Increasing incubation temperature from 20°C to 37°C also significantly (P = 0.0090) reduced number of aerobic bacterial CFU. This indicates that the anaerobic environment containing crude glycerol, especially at elevated temperatures, is unfavorable to aerobically growing bacteria.

The medium contained on average 5600 anaerobically growing bacterial CFU mL^{-1} at the beginning (Fig. 2b). After incubation, an increase in CFU number was observed in all inoculum-containing samples with a maximum value of 3.0×10^6 CFU mL^{-1} in the crude glycerol-containing sample incubated for 4 d at 20°C. No statistically significant (P = 0.5433) effect on the anaerobic bacterial CFU was observed with the addition of crude glycerol. Increasing the temperature from 20°C to 37°C showed a negative significant (P = 0.0074) effect, reducing the number of CFU per mL medium by an average of 1.3×10^6. An increase in incubation time from 4 to 8 d did not significantly affect the CFU number of anaerobically growing microorganisms in crude glycerol-containing medium, whereas it increased in crude glycerol-free medium ($P < 0.05$). The results indicate a loss of activity of anaerobic bacteria between 4 and 8 d due to unfavorable conditions in media containing crude glycerol. In a contrast, crude glycerol-free medium still maintained favourable conditions for their growth. Although literature data indicate a more efficient utilization of crude glycerol at 37°C [25] and also in our study the highest volume of gas produced at 37°C, both anaerobically and aerobically growing bacteria showed higher number of CFU at 20°C than at 37°C.

The inoculated medium contained on average 380 fungal (filamentous fungus *Acremonium* sp.) CFU mL^{-1} at the beginning of incubation. There were no fungi detected after incubation because aerobic fungi lose viability during anaerobic cultivation [26].

3.5. Identified bacterial species

The dominant cultivated bacterial species were isolated from the solid media and identified by biochemical properties. Representatives of *Actinobacteria*, *Firmicutes* and *Proteobacteria* phyla were identified for aerobically cultured microorganisms (Table 3). *Firmicutes* were isolated from all samples and all of the isolated aerobic *Firmicutes* belonged to the *Bacilli* class. These included the facultative anaerobes *Bacillus licheniformis* and *Enterococcus* spp., as well as *Brevibacillus brevis*, which is classified as a mandatory aerobic bacterium [27], but maintained viability under the experimental anaerobic conditions.

B. licheniformis and *B. brevis* were found in all samples incubated at 37°C, but only in one sample incubated at 20°C. This trend is explained by choosing preferable growth temperatures for both bacteria close to 37°C. The majority of the strains of *B. brevis* did not grow at 20°C. The minimum temperature for *B. licheniformis* cultivation is 15°C, whereas for the *Enterococcus* genus it is approximately 10°C [28].

Among the aerobic cultured microorganisms, three members of the *Gammaproteobacteria* class, facultative anaerobes, were also identified [28], i.e. *Aeromonas hydrophila*, *A. veronii* and *Pantoea agglomerans*. Możejko-Ciesielska and Pokoj [29] isolated *Aeromonas* strains, which were genetically similar to *A. hydrophila* and capable of synthesizing polyhydroxyalkanoates in crude glycerol fermentation, from the activated sludge of sewage treatment plant.

Representatives of *Actinobacteria* and *Firmicutes* phyla were identified from microorganisms cultured under anaerobic conditions (Table 3). All these species are strictly anaerobic [29,30]. All anaerobic *Firmicutes* belonged to the *Clostridia* class. Bacteria of the genus *Clostridium* were found in all of the samples. *Clostridium* is one of the most widely studied genera in the anaerobic fermentation of crude glycerol with the potential to produce 1,3-propanediol and butanol specifically [6,31]. From the species isolated in our study, *Clostridium beijerinckii* and *C. butyricum* as 1,3-propanediol producers is of great interest in the fermentation of crude glycerol [32,33].

3.6. Analysis of microbial populations by qPCR

The molecular analysis revealed that diverse bacterial populations were in the medium before incubation, coming from the sediment inoculum. The highest concentration of DNA was found for *Actinobacteria* (4.56 ng mL^{-1}), followed by *Gammaproteobacteria* (3.84 ng mL^{-1}) and *Firmicutes* (2.23 ng mL^{-1}) (Fig. 3).

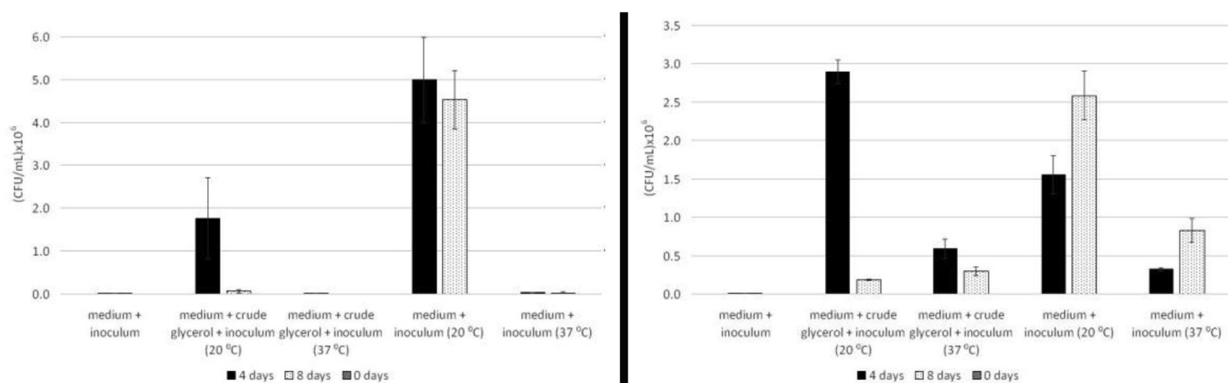

Fig. 2. Number of CFU of aerobically (a) and anaerobically (b) growing bacteria per mL of growth medium at the beginning and after 4 and 8 d of cultivation at 20°C and 37°C.

Table 3
Isolated and identified predominant aerobically and anaerobically (species marked with *) growing bacteria.

Phylum	Species	Sample								
		Without crude glycerol					With crude glycerol			
		0 days	4 days		8 days		4 days		8 days	
		20°C	20°C	37°C	20°C	37°C	20°C	37°C	20°C	37°C
Actinobacteria, number of species		1			1	1		3	1	
	*Actinomyces israelii**							+		
	*Collinsella aerofaciens**				+					
	Corynebacterium propinquum								+	
	Leifsonia aquatica					+				
	Rhodococcus hoagii							+		
	Rothia dentocariosa							+		
	Trueperella pyogenes	+								
Firmicutes, number of species		4	3	7	2	4	4	6	6	6
	*Anaerococcus tetradius**								+	
	Bacillus licheniformis	+		+		+		+	+	+
	Brevibacillus brevis	+		+		+		+		+
	*Clostridium beijerinckii**							+		+
	*Clostridium butyricum**			+			+	+	+	+
	*Clostridium clostridioforme**			+						
	Clostridium sp.*	+	+	+	+	+	+	+	+	+
	Enterococcus faecalis							+		
	Enterococcus faecium		+	+			+		+	
	Enterococcus raffinosus				+					
	*Eubacterium limosum**			+						
	*Hathewaya histolytica**									+
	Peptostreptococcus sp.*		+				+			
	*Tissierella praeacuta**	+				+			+	+
Proteobacteria, number of species		1	1		1		1			
	Aeromonas hydrophila	+					+			
	Aeromonas veronii		+				+			
	Pantoea agglomerans				+					

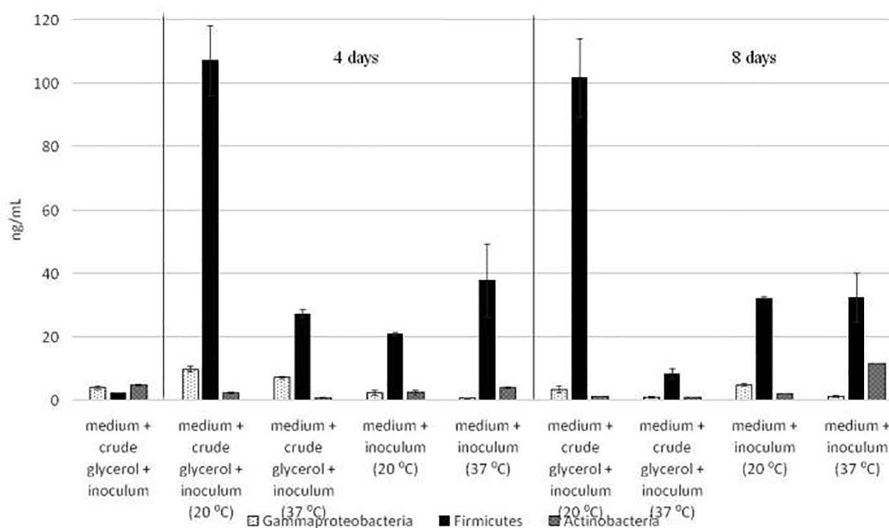

Fig. 3. *Gammaproteobacteria*, *Firmicutes* and *Actinobacteria* DNA concentrations (ng mL^{-1}) in growth media without incubation (medium + crude glycerol + inoculum) and after incubation at 20°C or 37°C for 4 or 8 d with or without crude glycerol.

The majority of the *Actinobacteria* are free-living organisms that are widely distributed in both terrestrial and aquatic ecosystems. Most *Actinobacteria* are aerobic, but there are exceptions [reviewed in [34]]. After fermentation, a decrease in *Actinobacteria* DNA concentration was observed in all samples except for crude glycerol-free medium after 8 d fermentation at 37°C, where the concentration increased 2.5-fold and showed a statistically significant ($P = 0.0002$) difference. Overall, the presence of crude glycerol in the media had a negative effect ($P < 0.05$) on the DNA concentration of *Actinobacteria*. These bacteria were unable to adapt to the use of crude glycerol, at least during the experiment.

During incubation, an increase in the concentration of *Gammaproteobacteria* DNA was detected in two samples – crude glycerol-containing medium after 4 d incubation at 20°C ($P = 0.0029$) and in the same 37°C incubated sample ($P = 0.0176$) (Fig. 3). A significant ($P = 6.12 \times 10^{-6}$) incubation temperature effect on *Gammaproteobacteria* DNA concentration was found, with lower results at 37°C than at 20°C. In crude glycerol-containing media, DNA concentration decreased ($P = 5.58 \times 10^{-7}$) with an increase in incubation time from 4 to 8 d, whereas in crude glycerol-free media an increase in concentration ($P = 0.0285$) was observed. The results indicate that crude glycerol-containing

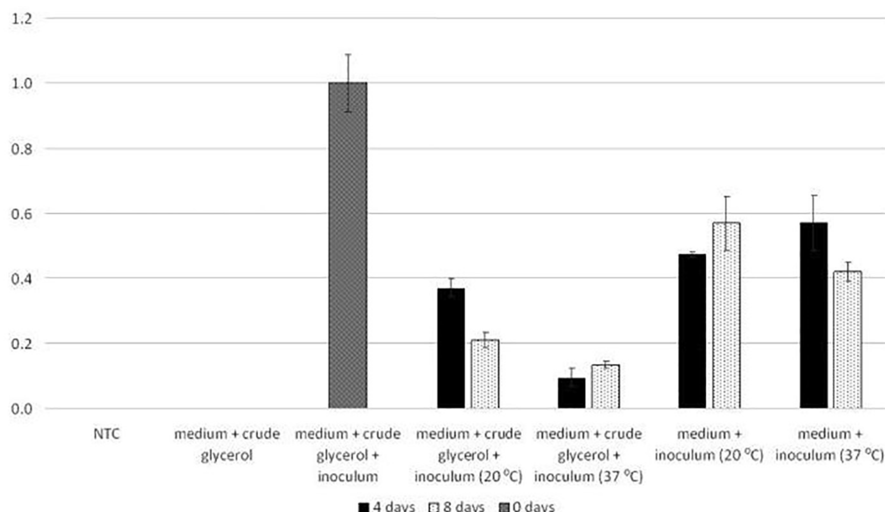

Fig. 4. Concentration of *Archaea* DNA (ng mL^{-1}) in growth media. NTC – qPCR no template control.

media initially provided favourable conditions for the growth of *Gammaproteobacteria*, which worsened after a few days of incubation. In crude glycerol-free media, *Gammaproteobacteria* continued to multiply even after 4 d of fermentation.

After fermentation, *Firmicutes* showed significantly higher results than *Actinobacteria* ($P = 3.65 \times 10^{-5}$) and *Gammaproteobacteria* ($P = 2.97 \times 10^{-5}$). *Firmicutes* had an average DNA concentration of 25.5 times that of *Actinobacteria* and 30.6 times that of *Gammaproteobacteria*. There was no significant difference between *Actinobacteria* and *Gammaproteobacteria* DNA concentrations after fermentation ($P = 0.7568$). These results are consistent with microorganism cultivation experiments, which were also dominated by *Firmicutes*. The ability to utilize glycerol under anaerobic conditions also has been observed primarily in *Gammaproteobacteria*, *Firmicutes* and *Actinobacteria* [8]. The highest concentration of *Firmicutes* DNA was in crude glycerol-containing medium incubated at 20°C, which was significantly higher ($P < 0.05$) than in the medium incubated at 37°C.

During the fermentation, the *Archaea* DNA concentration decreased in all samples ($P = 0.0001$) (Fig. 4). The effect of crude glycerol was also determined with lower concentrations of DNA in samples containing crude glycerol ($P = 1.96 \times 10^{-5}$). Overall, the *Archaea* DNA results obtained do not indicate the presence of methanogenesis in the culture media.

Lower concentrations of DNA were observed in the crude glycerol-containing medium for all groups of microorganisms studied at 37°C compared to the one maintained at 20°C, which is consistent with the results of the CFU number. However, as the major hydrogen producers belong to *Firmicutes* and *Gammaproteobacteria* [35] and the gas volume was highest at 37°C, the qPCR results also indirectly suggest a possible bacterial-unfavorable condition before reaching the 4-d fermentation time of crude glycerol at 37°C. Thus, crude glycerol led to the adaptation of sediment microbial populations to this substrate. Changes of microbial community were a result of a community adaptation to a new source of carbon.

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Conflict of interest

All authors declare that they have no conflict of interest.

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