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The hydrogen production by anaerobic bacteria grown on glucose and glycerol

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Abstract

Four isolates of *Clostridium* genus were obtained from the activated sludge and one Escherichia coli isolated was found in sheep ruminal fluid. These isolates were identified by microscopic methods and by rRNA sequences. Growth, production of metabolic gases and production of organic acids were measured during the anaerobic cultivation of these isolates with glycerol as sole carbon source. It was found that these isolates from activated sludge were related to *Clostridium botulinum, C. perfringens* and *C. difficile*. One strain could not be assigned to any species but was similar to *C. botulinum,* and one ruminal bacterium was identified as *Escherichia coli.* All isolates grew on the medium with glycerol as sole carbon source with prolonged lag phase. The lag phase was shorter after adaptation of cells to glycerol. All these strains produced H_2, and CO in concentration range 10^2 μmol L^{-1}, and H_2S in concentrations lower by one order of magnitude. Kinetics of evolution of these gases was different suggesting that they are produced by independent processes. The major organic acid produced with glycerol as the carbon source was acetic acid. Butyric, formic, and propionic acids were produced in dependence on the isolated tested. Results show that metabolic gases are produced mainly in the exponential phase of growth.

Keywords: Clostridium sp., glucose, glycerol, hydrogen, carbon monoxide, hydrogen sulfide

Introduction

Hydrogen (H_2) is considered the promising clean fuel, due to its potentially higher efficiency of conversion to usable power, low or none pollutant formation and high energy density.

H_2 as fuel can be produced by biological or non-biological process. Microbial H_2 as fuel can by produced using fermentative bacteria, photosynthetic bacteria or algae. Biological H_2 production processes can be classified into three major categories: photo-oxidation of water used by algae or cyanobacteria; photodecomposition of organic compounds by photosynthetic bacteria (photo-fermentation); and dark fermentation by fermentative bacteria, that produced H_2 from organic substrates without the involvement of light (Hallenbeck and Benemann 2002).

Fermentative H_2 production has good prospects for the biotechnological applications, due to the capability to exploit variety of substrates (Nath and Das 2004, Hallenbeck and Ghosh 2009). Among bacteria used for the dark fermentation, clostridia play an important role. Their robust and diverged fermentative metabolism, easy isolation of clostridial strains, stability of cultures that includes spore formation, and presence of hydrogenases (Calusinska et al. 2010) favour clostridia as potential H_2 producers (Lin et al. 2007).

The use of complex substrates and/or waste materials for H_2 production is of great interest for the biotechnological applications. Recently, several studies appeared that confirm the suitability of *Clostridium spp.* for H_2 production on these substrates. Guo et al. (2010) reviewed papers describing H_2 production from municipal wastes, and Doi et al. (2010) described the H_2 production from apple pomace. Pattra et al. (2008) showed that the treatment by *Clostridium butylicum* of sugarcane bagasse leads to the H_2 production. Also less complex substrates, such as cellulose could be degraded by cellulolytic clostridia (Nissila et al. 2011, Geng et al. 2010). Nissila et al. (2011) showed that these could be selectively isolated from the ruminal fluid by incubation at 60 °C.

Although most studies were made with isolated strains, attempts were made to improve parameters of H_2 productions by using co-cultures. Thus, Geng et al. (2010) used the co-culture of cellulolytic and non-cellulolytic strains in order to increase cellulose hydrolysis. Similar approach adopted Sunil et al. (2009) who co-cultured *Clostridium butylicum* with *Enterococcus saccharolyticum* in order to improve hydrolysis of cellobiose, or Chang et al. (2008), who succeeded to co-culture *Bacillus thermoamylovorans* and several *Clostridium* species in order to improve hydrolytic processes and, thereby, also the H_2 production. Co-cultures of *Clostridium butyricum* and *Rhodobacter sphaeroides* was successfully tested as a model of combined phototrophic and dark fermentation process of H_2 production (Fang et al. 2006). Beckers et al. (2010) successfully used co-culture of *C. butyricum* with facultative anaerobe *Citrobacter freundii* to reliably remove oxygen contamination, which could damage physiological processes in clostridia. On the other hand, Hiligsman et al. (2011) found that the

H_2 production by 11 pure Clostridium strains and 2 facultatively anaerobic bacteria were better producers of H_2 than several sludges with bacterial consortia.

Several attempts appeared to use genetics for improving efficiency of H_2 production. Heterologous expression of clostridial hydrogenase in Escherichia coli augmented H_2 production (Subudhi and Lal, 2011). On the other hand, autologous overexpression of hydrogenase in *C. acetobutylicum* did not augment the H_2 production (Klein et al. 2010). The more extensive genetic intervention (disruption of acetate kinase and acetyl phosphotransferase) enhanced the production of butyric acid and H_2 by *Clostridium tyrobutyricum*.

Although saccharidic substrates are preferred by *Clostridium sp*., they may utilize also other substrates. Glycerol as a sole carbon source was used for its biotransformation to 1,3-propanediol (Saint-Amans et al. 2001, Gonzales-Pajuelo et al. 2006). Glycerol is nowadays an important industrial waste and it is desirable to find a way to convert is to product(s) with added value. In this paper we test four isolates of *Clostridium sp.* and one *E. coli* isolate for the production of H_2 and other metabolic gases and organic acids.

Materials and Methods

Bacterial strains

Isolation of pure bacterial culture from the sheep ruminal fluid and from the activated sludge
For pure colonies of bacteria, we have to take a sample from the liquid of the sheep stomach or from the activated sludge, and transferred it into physiological solution (supplemented with 0.02% Tween 80) and serially diluted up to 10^6 times under anaerobic conditions. After then the inoculated a physiological solution was vaccinated under anaerobic conditions on the solid RCM, which was equipped on the Petri dishes in order to obtain pure and individually colonies of anaerobic bacteria. These were propagated on the reinforced clostridial medium (RCM).

Bacteria were cultivated in anaerobic cultivation chambers in the anaerobic atmosphere formed by BBL GasPack System (Becton Dickinson) at 37 °C. All operations were performed in the Bactron I anaerobic chamber (Sheldon Laboratories).

Reinforced Clostridial Medium (RCM)
Medium was done according to Kalil et al. (2009). Its composition was following Glucose 5 gL^{-1}, Yeast extracts 13 gL^{-1}, L-Cysteine.HCl 1 gL^{-1}, agar 0.5 gL^{-1}, pH 6.8 ± 0.2. Glycerol was added instead of glucose at concentration 5 gL^{-1}.

Growth curve measurements with monosaccharides and polysaccharides as substrates

Single bacterial culture was cultivated in 5 ml media (such as RCM with glucose or other substrate) inoculated under anaerobic condition by bacterial species to have concentration of cells about $2.10^7 ml^{-1}$, and incubated under anaerobic conditions at 37 °C with rotation (IKA KS 40000 ic control) (181 rpm). Growth of bacteria was monitored by measurement of A_{550} (Biochrom colorimeter Libra 52, U.K.) at 0 hour, and after every 4th hour until stationary phase was reached.

Molecular taxonomy of bacterial isolates

Bacterial taxonomy was performed by colony PCR amplifying the 16S -23S ribosomal bacterial spacer. Fifty 50 µl PCR mix contains 1 µl 10 mM dNTP mix, 1.5 µl primer forward (B16S2 FWD 5´TTGTACACACCGCCCGTC´3.), 1.5 µl primer reverse *(B28S10 REW) (5´CCWTTCCCTCACGGTACT3´), 5 µl 10x HotMaster^{TM} Tag buffer with Mg^{2+}, 0.5 µl HotMaster^{TM} Tag DNA polymerase, and 40.5 µl DNAase - free water. DNA was provided by transferring and a very small part of bacterial colonies with toothpick. PCR was started without the adding of the DNA polymerase in the thermocycler (MasterCycler personal – Eppendorf, Germany), and after 15 minutes the DNA polymerase was added to the PCR mix. PCR products were purified and directly sequenced. Sequences were analyzed by BLAST.

Measurements of metabolic gases

Tested samples were prepared from pure bacterial cultures identified by 16S -23S ribosomal bacterial spacer DNA in 40 ml of liquid medium into 50 ml flasks with stoppers with inlet and outlet tubings and placed in Micro-Oxymax (Columbus Instruments, Columbus, OH, U.S.A.) gas analyzer equipped with sensors for O_2, CO, CO_2, H_2S, H_2 and CH_4 from which traces of O_2 were removed by flushing with pyrogallol-treated N_2. Measurement started after the system was calibrated with calibration gases. Measurements were carried out at 37 °C.

Measurement of organic acids by HPLC

Organic acids were measured by isocratic HPLC with 9 mM H_2SO_4 as mobile phase on the Shimadzu HPLC system equipped with double wavelength UV-detector (214 and 254 nm) a Watrex 300x8 mm polymer IEX column in H^+ form. Twenty µL of cultivation media obtained by centrifugation and pre-filtered through the 0.22 µm membrane was applied on the column. Chromatography was performed at 41 °C. The system was calibrated by series of standard organic acids.

Results and Discussion

Isolation and identification of microorganisms

Anaerobic microorganisms were isolated from cow and sheep ruminal content and from activated sludge and characterized as described above. The most stable and cultivatable microorganisms were isolated from the activated sludge. They mostly belonged to the genus *Clostridium* and to three species – *Clostridium botulinum*, *C. perfringens* and *C. difficile* as deduced from the ITS of 16S rRNA sequences. One strain could not be assigned to any species but was similar to *C. botulinum*. One ruminal bacterium was identified as *Escherichia coli*. Observation in microscope was in accordance with results of ITS sequences. Similarity of these isolates to deposited strains is shown from the Table 1. Isolates were anaerobically propagated on the RCM medium and kept in a refrigerator.

Table 1: Identification by molecular taxonomy of the bacterial isolates from the sheep rumen and activated sludge**.**

Number of isolate	Name of bacteria derived from the highest similarity (found in the BLAST query)	Query coverage, %	Maximal identity, %
No 2-24	*Escherichia coli DH1(ME8569)*	85	98
AK 1-4	*Clostridium botulinumE3 str. Alaska E43*	73	82
AK 1-5	*Clostridium difficile M68*	69	93
AK 1-9	*Clostridium perfringens clone CVCC90.WE*	81	99
AK 1-12	*Clostridium spp.*	14	99

AK-denoted isolates originated from the activated sludge, *E. coli* was isolated from the sheep rumen. Its fragments were amplified using primers B28S10 rew, B16S2 fwd (see Materials and Methods). Their sequences were compared with those deposited in GenBank using BLAST.

Growth characteristics with glucose or glycerol as carbon sources

The growth of clostridia isolates on glycerol as carbon source was slower than that in glucose (Fig. 1). On the other hand, *E. coli* isolate grew equally, or even better, with glycerol as carbon source. It could be noted that growth curves with clostridial isolates displayed prolonged lag phases, and did not reach stationary phase within 24 h cultivation. This indicates some adaptation of bacteria to glycerol. In order to test whether the metabolism of glycerol could be inducible, experiments with two-step cultivation was carried out. First, bacteria grew up in the media with glucose or glycerol as sole carbon sources. In the second

step, obtained suspensions were inoculated into media with the same or exchanged carbon sources. Results of these experiments are shown in the Fig. 2.

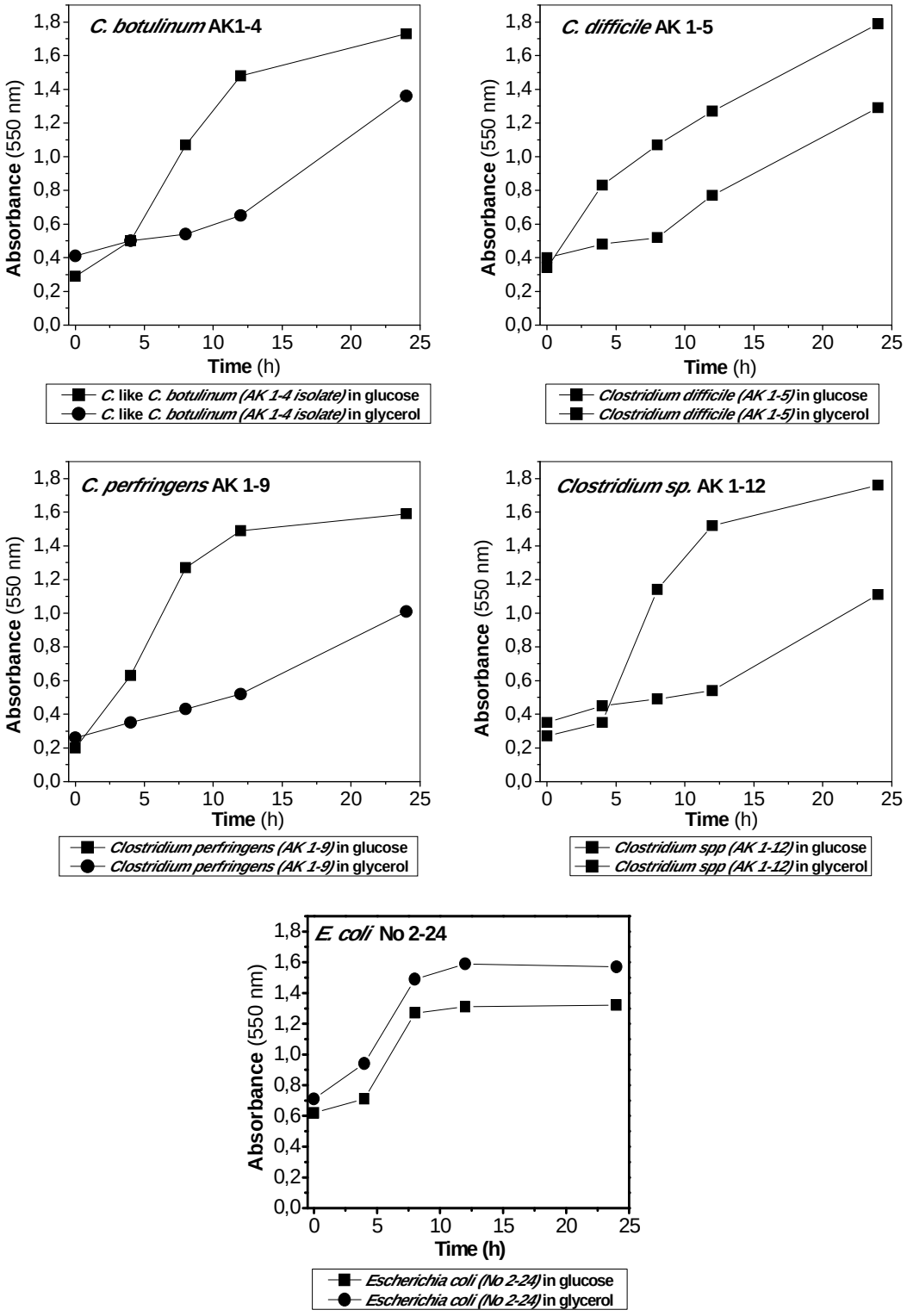

Fig. 1. Growth curves of bacterial isolates with glucose or glycerol as carbon sources. Bacterial isolates shown in the figure were anaerobically cultivated in RCM with glucose or glycerol as carbon sources as described in Material and Methods. Squares denote glucose-containing medium, circles – glycerol-containing medium.

Growth of suspension cultivated in the first step on glucose and on glycerol or glucose in the second step showed that the growth on glycerol was strongly suppressed in clostridial isolates but not in E. coli isolate, which seem to utilize glycerol without limitations (Fig. 2). Growth of suspension cultivated in the first step on glycerol and followed by the cultivation on glycerol or glucose showed significant growth without lag phase in all but AK1-12 isolate. However, densities of suspensions of glycerol-grown bacteria were significantly less than those grown in glucose-containing medium (Fig. 2). This indicates that glycerol metabolism has some adaptive features. The lowered growth yield in glycerol-containing medium could be ascribed to its lower energy content as compared with glucose.

Production of metabolic gases

Continuous measurements of metabolic gases (O_2, CO_2, H_2, CO and H_2S) in the gas phase on the Micro-Oxymax instrument enabled us to monitor the evolution of all gases during growth of bacterial suspensions. Values of individual gases are measured successively and cyclically by this instrument and could be considered as parallel measurements. Such a measurement is illustrated by the growth of *C. difficile* AK1-5 in glycerol-containing medium, as a typical example (Fig. 3). Experiment reveals that changes in the gas content appear early, after 3-4 h of cultivation. H_2 evolution starts after 6 h cultivation and attains maximum after about 8-12 h. Prolonged cultivation leads to disappearance of H_2, probably by its utilization via reversed hydrogenase activity (Fig. 3, right panel). Changes in H_2S concentration were smaller by one order of magnitude and appear as background in this figure. Nevertheless, it seems that H_2S is produced during the cultivation of this isolate (see Fig. 4). Surprisingly, CO appeared during the cultivation in concentrations comparable to those of H_2. Concentrations of CO were elevated during the exponential phase of cultivation and their values reached the upper limit of detector after about 10 h. CO appearance was delayed by about 3 h after the appearance of H_2. The decrease of its concentration was probably caused by the cell metabolism during the last phase of cultivation. However, mechanisms involved in the CO consumption are unknown at present.

Results shown in the Fig. 3 demonstrate the decrease of the CO_2 concentrations during cultivation. This feature probably reflects the conversion of CO_2 to bicarbonate or its consumption by cells. This fact is intriguing and prevents the use of CO_2 production for as a measure of bacterial growth. Production of H_2 by *C. difficile* during growth on glucose or other saccharides was similar as to the time course but quantities of produced H_2 were different (not shown).

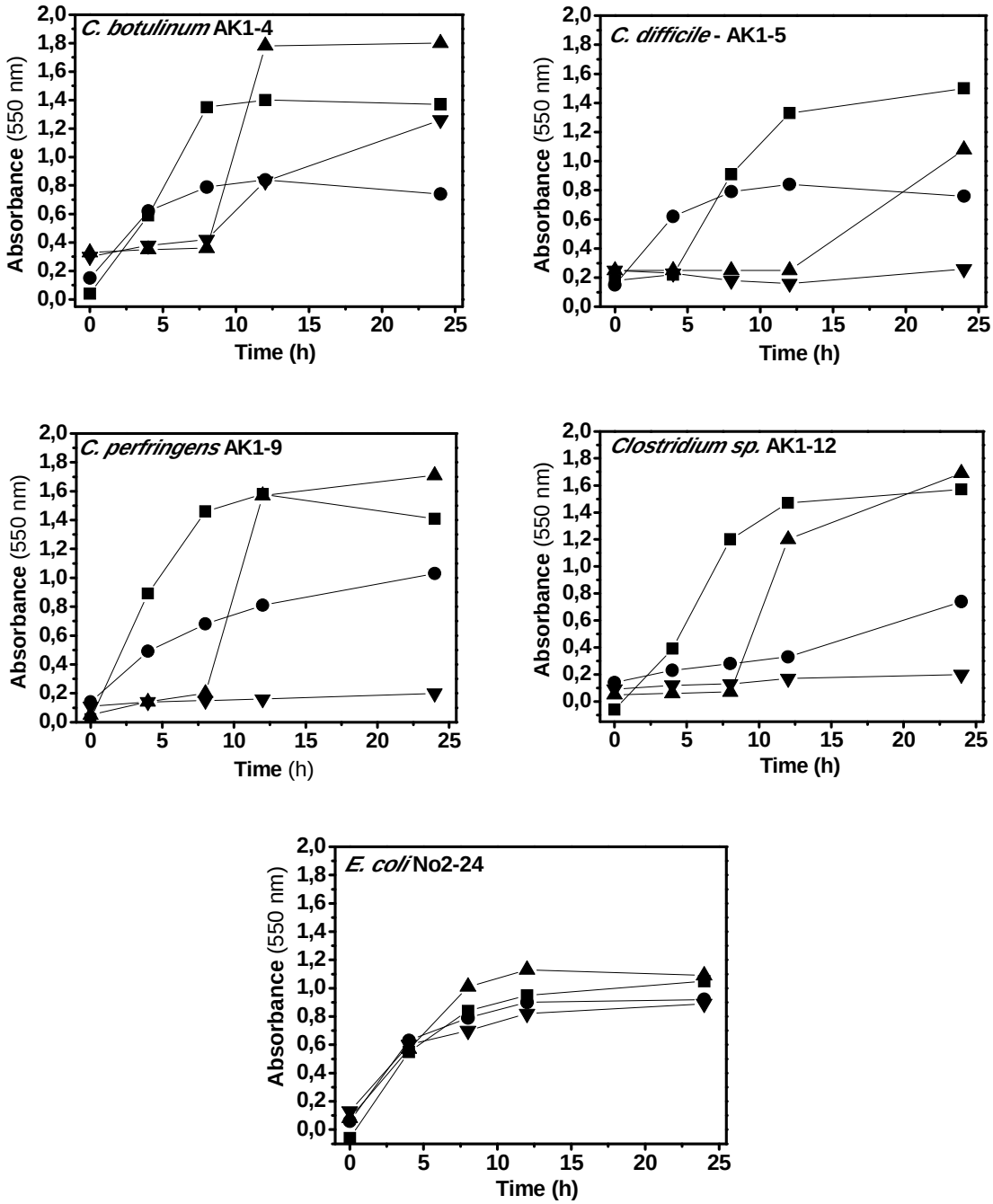

Fig. 2. Growth of bacterial isolates on glucose and glycerol as carbon sources.
Bacterial isolates shown in the figure were cultivated on either glucose or glycerol and the 24 h culture was used as inoculum for the fresh medium with the same carbon source or with the exchanged carbon source. Growth curves were measured spectrophotometrically for 24 h. Orders of carbon sources were following for each isolate: —■— from glycerol to glucose, —●— from glycerol to glycerol, —▲—from glucose to glucose, —▼— from glucose to glycerol.

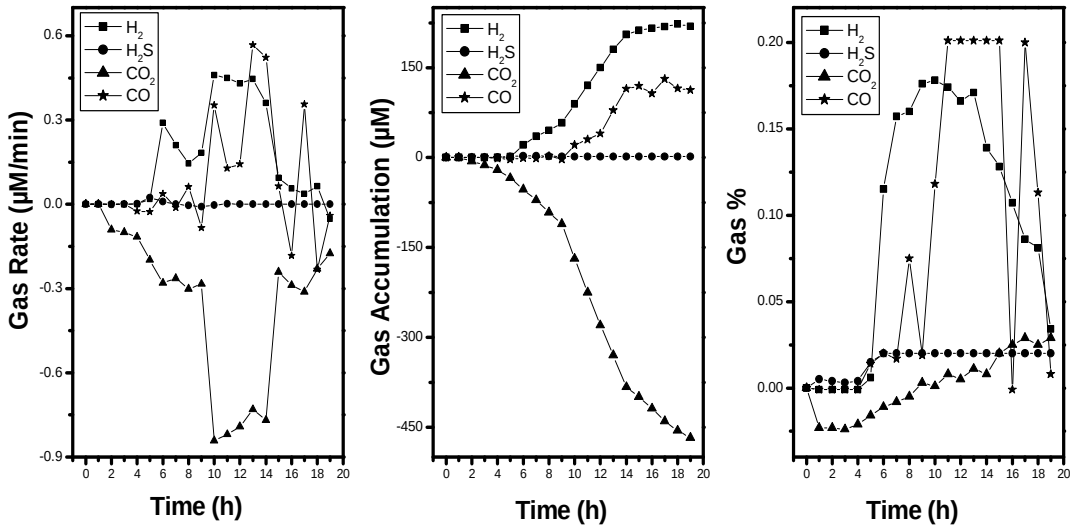

Fig. 3. Evolution of metabolic gases during growth of *Clostridium difficile (AK 1-5 isolate)* on glycerol as sole carbon source.
Graphs were plotted from data produced by gas analyzer for CO, H_2, CO_2 and H_2S. They illustrate three possible outputs of instrument from left to right: Rate of gas changes, gas accumulation and immediate gas concentration in %. Values of parameters for oxygen are not shown. All parameters are plotted in the linear scale.

Hydrogen production on various substrates

Kinetics of the evolution of metabolic gases indicates that hydrogen is produced mainly during the exponential phase of growth. The evolution, even the concentration of hydrogen in atmosphere over the suspension, decreases during the cultivation suggesting the hydrogen consumption during the late (stationary) phase of cultivation. It is also shown that the evolution of H_2S is not parallel with the evolution of hydrogen. The evolution of CO_2 exceeds by order of magnitude the evolution of H_2.

The comparison of H_2 production by tested isolates with glycerol as a carbon source is summarized in the Table 2. These data are compared with those obtained using glucose as a sole carbon source. Results show that there are substantial differences between individual isolates with both substrates. In addition, results show that the yield of H_2 is lower with glycerol as substrate also varies between individual isolates. This indicates that the putative utilization of clostridia or enterobacteria for H_2 production may need a strong selection of suitable isolate(s).

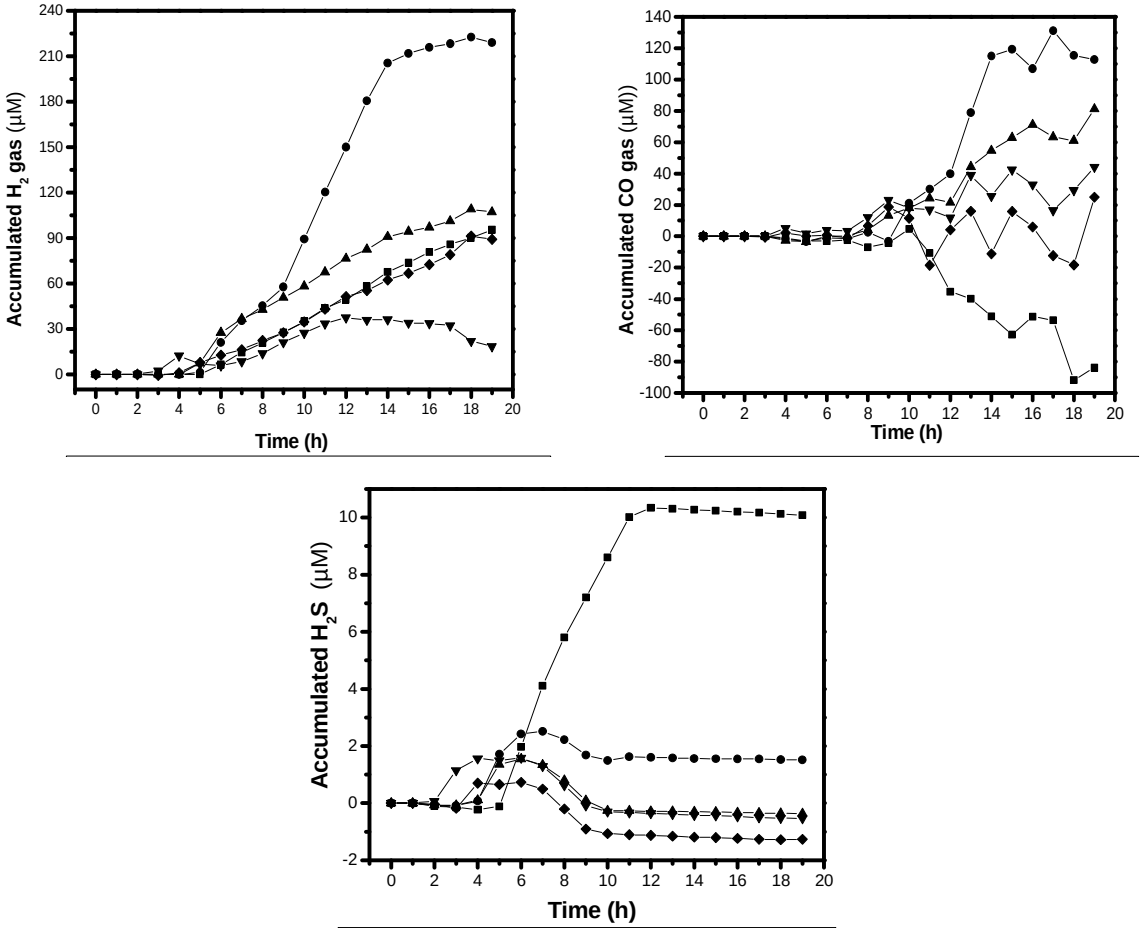

Fig. 4. Glycerol as substrate for production of: (left panel) H_2; (right panel) CO and (bottom panel) H_2S by *Clostridium* and *E. coli* isolates in RCM.
Accumulated H_2, CO and H_2S values read out from Micro-Oxymax were plotted against time of cultivation for all isolates as follows: ■ – C. botulinum AK1-4, ● – C. difficile AK1-5, ▲ – C. perfringens AK1-9 strain, ▼ – Clostridium sp. AK1-12 strain, ♦ - E. coli No2-24 strain.

Table 2. Hydrogen production by clostridia isolates and Escherichia coli on glycerol as carbon source.

Maximal H_2 concentration (µmol/l) obtained upon cultivation	Bacterial species				
	Clostridium perfringens (AK1-9)	*Clostridium difficile* (AK1-5)	*Clostridium sp.* (AK1-12)	*Clostridium botulinum.* (AK1-4)	*Escherichia coli* (No.2-24)
H_2 (µmol/l) from RCM with Glc	9378	7433	4070	5018	9029
H_2 (µmol/l) from RCM with glycerol	2726	5566	938.3	2384	2282

Table 3: Produced organic acids RCM medium with glucose or glycerol as sole carbon sources by bacterial isolates AK 1-4, AK 1-5, AK 1-9, AK 1-12 and No 2-24.

Concentration of acids (mmol/l) in medium normalized to 1 A_{550} of suspension with glucose/glycerol as carbon sources	**Tested microorganism**				
	Escherichia coli (No.2--24 isolate)	***Clostridium sp. (AK1-12 isolate)***	***Clostridium perfringens (AK1-9 isolate)***	***Clostridium difficile (AK1-5 isolate)***	***Clostridium botulinum (AK1-4 isolate)***
Formic acid	N.D./4.47	2.13/2.65	1.19/1.07	0.30/N.D.	1.55/1.66
Malic acid	0.17/0.98	0.85/0.57	0.97/1.10	0.04/0.14	0.17/0.31
Citric acid	0.01/N.D.	0.85/0.60	0.03/0.25	N.D./N.D.	N.D./0.03
α-ketoglutaric acid	1.03/0.44	0.05/N.D.	N.D./N.D.	0.02/N.D.	0.05/0.03
Acetic acid	7.71/4.56	4.21/6.67	3.10/3.48	6.59/5.90	3.81/0.69
propionic acid	3.77/1.22	4.21/1.31	0.69/0.19	N.D./1.20	0.89/0.51
Butyric acid	N.D./N.D.	2.69/N.D.	1.14/2.04	N.D./N.D.	2.60/3.08
Fumaric acid	0.14/0.08	N.D./N.D.	N.D./N.D.	0.11/N.D.	N.D./N.D.
Succinic acid	N.D./N.D.	N.D./N.D.	N.D./N.D.	N.D./N.D.	N.D./N.D.

N.D. – not detected

The production of CO is a process, which was present in all isolates with variable intensities and one could speculate that all clostridia may exhibit this metabolic feature. One should note that the production of H_2 is stable but the production of CO is fluctuating (Fig. 4). This indicates some uncertainty either in the measurements, or in the character of metabolism (e.g. oscillations of CO concentration). These possibilities and the role of CO in clostridial metabolism should be analyzed in the future. Nevertheless, comparison of the evolution of CO and H_2 reveals that there is some loose relationship between production of H_2 and CO in some isolates (e.g. AK1-4 and AK1-9) but not between all tested isolates. The production of H_2S was pronounced in AK1-4 only. Other isolates exhibited a temporary accumulation of H_2S only (Fig. 4).

Production of organic acids

Organic acids were excreted by tested strains cultivated with glycerol as sole carbon source in RCM medium. The major excreted acid was acetic acid except AK1-4 isolate, which produced formic and butyric acids instead. Succinic acid was not detected in any cultivation medium of the tested *Clostridium* isolates or *E.coli* isolate (Table 3). *Clostridium perfringens* (AK1-9 isolate) also produced butyric acid. Other acids were produced only in trace quantities as detected by our method.

These experiments showed that the measurement of metabolic gases is the useful method of monitoring bacterial fermentation, which could not only measure the production of H_2 but also to reveal processes unknown so far, such as CO production. Experiments also

showed that *Clostridium sp*. utilize glycerol and convert it to H_2 and organic acids strongly dependent on the bacterial isolate. Thus, there is a possibility to use glycerol as a substrate for H_2 production, although, probably, optimization of bacterial strains, or their genetic modification, will be desired.

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