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Production of Bio-Hydrogen Gas and Other Metabolic Gases by Anaerobic Bacteria Grown on Molasses

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Abstract: The study had aimed to characterize the production of hydrogen gases by anaerobic bacteria. One isolate was found in sheep ruminal fluid and four isolates were obtained from the activated sludge. These isolates were identified by microscopic methods and by rRNA sequences. One ruminal bacterium was identified as *Escherichia coli*, and 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*. Growth and production of the metabolic gases with molasses as sole carbon source were measured during the anaerobic cultivation by Micro-Oxymax (Columbus Instruments, Columbus, OH, U.S.A.) gas analyzer. One of the most available saccharidic waste products is molasses. The growth on molasses as carbon source was done to test the production of H_2. It was found that all tested Clostridium isolates (AK 1-4, AK 1-5, AK 1-9 and AK 1-12) and E. coli isolate (No 2- 24) had utilized molasses as carbon source monitored by production of CO_2 gas. All these strains produced H_2 gas, and CO gas in concentration range 102 μmol L^{-1}, and H_2S gas in concentrations lower by one order of magnitude. Kinetics of evolution of these gases was different suggesting that they are produced by independent processes. Results show that metabolic gases are produced mainly in the exponential phase of growth.

Keywords: Clostridium Spp., E. Coli, Molasses, Hydrogen, Metabolic Gases, Anaerobic Metabolism

1. Introduction

Hydrogen gas (H_2) is considered the promising clean fuel [1], due to its potentially higher efficiency of conversion to usable power, low or none pollutant formation and high energy density [2]. Consequently, low-cost and sufficient supply of H_2 could soon become in urgent demand [3]. Hydrogen as fuel can be produced by biological or non-biological processes. It can be produced using fermentative bacteria, photosynthetic bacteria or algae. Biological H_2 production using fermentative, photosynthetic bacteria, or algae is an environmentally friend and energy saving process [4] [5]. Therefore, it is a feasible alternative for global H_2 supply in the future [6].

The biological production of microbial H_2 as fuel can be classified cyanobacteria; photodecomposition of organic compounds by photosynthetic bacteria (photo-fermentation); and dark fermentation by fermentative bacteria [7]. Production of Fermentative H_2 has good prospects for the biotechnological applications, [8] [9]. In the dark fermentation process, different microbes can be used anaerobically to breakdown carbohydrate-rich substrates to H_2, organic acids (e.g., lactic, acetic, butyric, etc.) and alcohols [8] [10] [11]. Among bacteria which are used for the dark fermentation, clostridia play an important role. Their robust includes diverged fermentative metabolism, and easy isolation of clostridial strains, stability of cultures that includes spore formation, and presence of hydrogenases [12] favors clostridia as potential H_2 producers [13]. The suitability of Clostridium spp. for H_2 production was

confirmed by more studies [14] [15]. More waste materials were used for H_2 production in the biotechnological applications. Many literatures described production of H_2 from municipal wastes, [16] used apple pomace and [17] treated sugarcane bagasse by Clostridium butylicum. However, less complex substrates, such as cellulose could be degraded by cellulolytic clostridia [18] [19]. Several studies were made to increase the production of H_2 by using co-cultures, cellulolytic and non-cellulolytic strains, in order to increase cellulose hydrolysis [19]. Similar approach adopted [20]. Co-cultured Clostridium butylicum with Enterococcus saccharolyticum in order to improve hydrolysis of cellobiose, or [4], Succeeded to co-culture Bacillus thermoamylovorans and several Clostridium species in order to improve hydrolytic processes; also co-cultures of Clostridium butyricum and Rhodobacter sphaeroides were successfully tested as a model of combined phototrophic and dark fermentation process of H_2 production [21] [22]. Application used co-culture of C. butyricum with facultative anaerobe Citrobacter freundii on under anaerobic condition, which could damage physiological processes in clostridia. 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 [23]. On the other hand, autologous overexpression of hydrogenase in C. acetobutylicum did not augment the H_2 production [24].

Molasses are alternative carbohydrate rich substrate, and are well source of sucrose. It was used as a sole carbon source for H_2 gas evolution [25]. Some study testing molasses for hydrogen production, found that the high hydrogen yield was always associated with a high level of ethanol production [26].

The hydrogen production by the dark fermentation of molasses from sugar industry [28].

Other study used the sugar beet molasses to hydrogen and methane production as a result of two-stage anaerobic digestion [29].

Also they tested molasses for hydrogen and methane production [30].

2. Materials and Methods

2.1. Isolation of the Bacterial Strains

The sheep ruminal fluid and the activated sludge were used to isolate the bacterial culture. For pure colonies of bacteria, a sample from the ruminal fluid had been taken from the sheep stomach or from the activated sludge, and transferred into physiological solution (supplemented with 0.02% Tween 80) and serially diluted up to 10^6 times under anaerobic conditions. Then the inoculated a physiological solution was vaccinated under anaerobic conditions on the solid reinforced clostridial medium (RCM), which was equipped on the Petri dishes in order to obtain pure and individually colonies of anaerobic bacteria. The bacteria were cultivated at 37°C in anaerobic cultivation chambers in the

anaerobic atmosphere formed by BBL Gas Pack System (Becton Dickinson). All operations were performed in the Bactron I anaerobic chamber (Sheldon Laboratories).

2.2. Reinforced Clostridial Medium (RCM)

Medium composition was done as 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} [27].

2.3. Growth Curve Measurements

Single bacterial culture was cultivated in 5 ml media (RCM) and inoculated under anaerobic condition by bacterial species to have concentration of cells about $2.107ml^{-1}$, 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 A550 (Biochrom colorimeter Libra 52, U. K.) at 0 hour, and after every 4th hour until stationary phase was reached.

2.4. 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 Hot Master TM Tag buffer with Mg^{2+}, 0.5 μl Hot Master 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 (Master Cycler 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.

2.5. 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.

3. Results and Discussion

The evolution of CO_2 in tested strains was different and varied between 0.25 to 2.1% at the production maximum suggesting that not all isolates grow equally on this substrate. Differences were observed also in the production of H_2S. Only Clostridium spp. (AK 1-12) produced H_2S

concomitantly with CO_2 (Figure 1). Also, CO gas was lasted during the exponential phase and it was not evolved concomitantly with CO_2 or H_2S gas (Figure 1). The evolution of gases by AK 1-4 in exponential phase of growth was not accompanied by the large H_2 gas production although the production of CO_2 gas was extensive and temporary (Figure 2); and the evulation of CO_2 and H_2 gases of were lasted during the exponential phase of growth; but H_2 gas was consumed during the late (stationary) phase of cultivation, see (figure 2) which it is also shown that the evolution of H_2S gas is not parallel with the evolution of H_2 furthermore CO gas was formed during the exponential phase in parallel with CO_2 gas.

In Clostridium AK 1-5 and E. coli isolates (Figure 3 and 4, respectively), H_2 and CO_2 gases were produced in the exponential phase of growth and accompanied by the evolution of small amount of H_2S gas in AK 1-5 but not in E. coli (Figure 4).

In AK 1-5 isolate (Figure 3), CO gas was produced during exponential phase, and its production was not parallel with any one of produced gases. In AK 1-9 isolate (Figure 5), CO gas was produced and consumed during exponential phase and then produced again during the stationary phase of cultivation; in the Figure it is also shown that the evolution of CO gas is not parallel with the of CO_2, H_2 or H_2S.
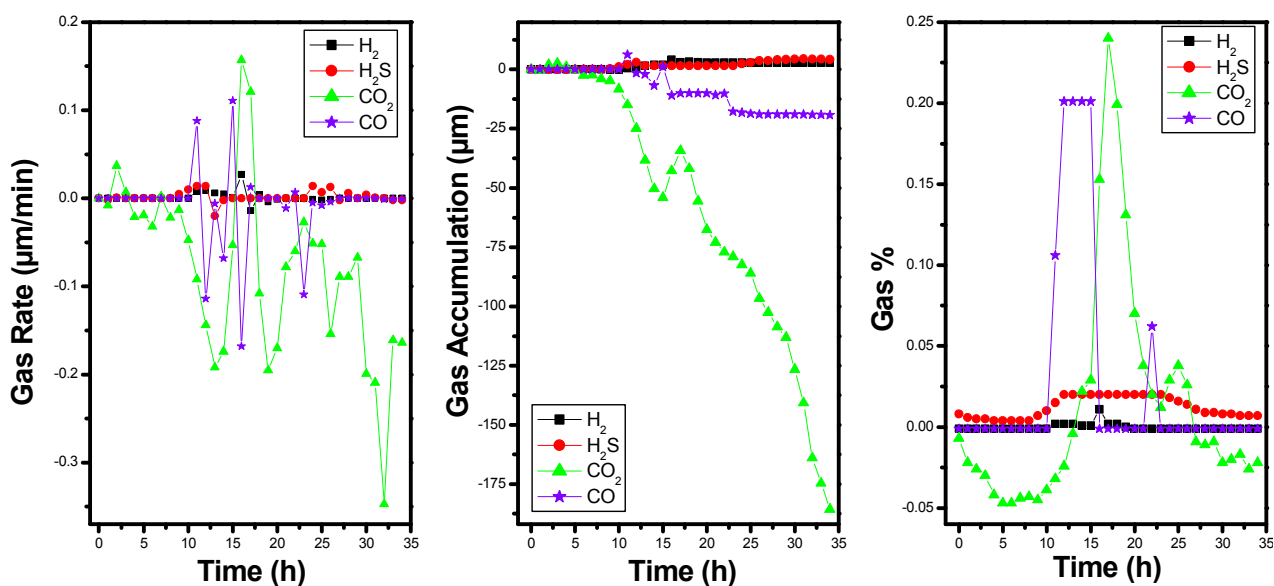

Figure 1. Evolutions of Metabolic Gases on Molasses as Substrate by Clostridium Spp. (AK 1-12 Isolate).

Graphs were plotted from data produced by gas analyzer for O_2, 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%. Parameters in the linear scale, O_2 values omitted.
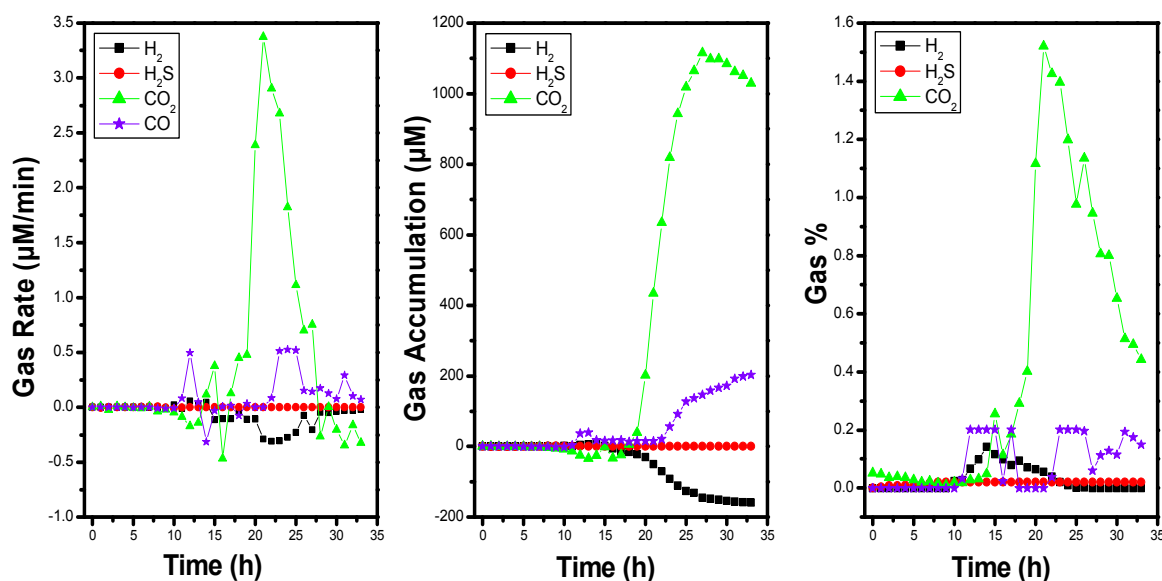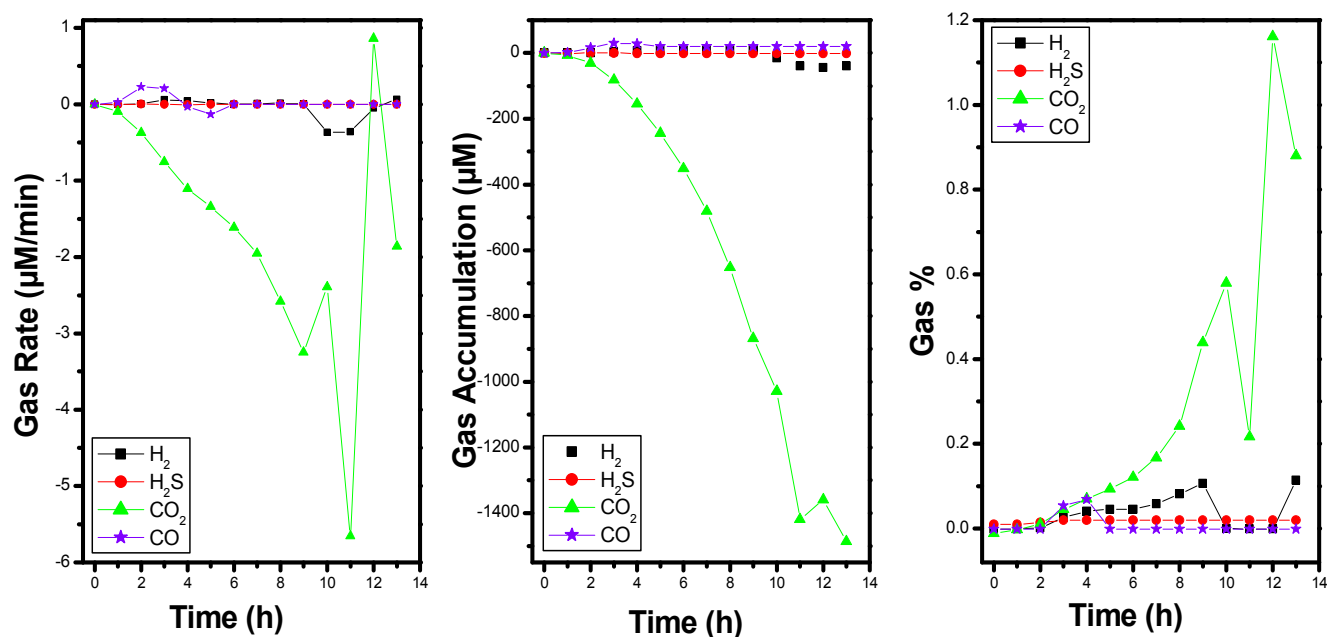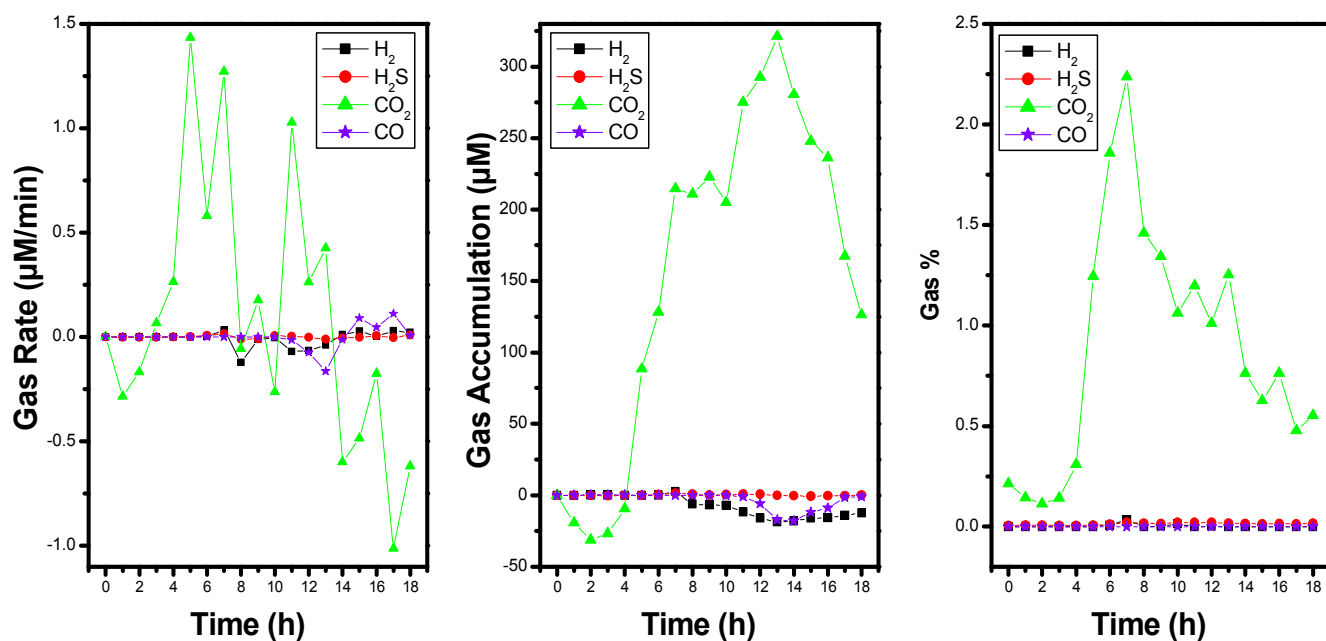

Figure 2. Evolutions of Metabolic Gases on Molasses as Substrate by Clostridium (AK 1-4 Isolate).

Graphs were plotted from data produced by gas analyzer for O_2, 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%. Parameters in the linear scale, O_2 values omitted.

Figure 3. *Evolutions of Metabolic Gases on Molasses as Substrate by Clostridium Difficile (AK 1-5 Isolate).*

Graphs were plotted from data produced by gas analyzer for O_2, 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%. Parameters in the linear scale, O_2 values omitted.

Figure 4. *Evolutions of Metabolic Gases on Molasses as Substrate by E. Coli (No 2-24 Isolate).*

Graphs were plotted from data produced by gas analyzer for O_2, 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%. Parameters in the linear scale, O_2 values omitted.

Figure 5. *Evolutions of Metabolic Gases on Molasses as Substrate by Clostridium Perfringens (AK 1-9 Isolate).*

Graphs were plotted from data produced by gas analyzer for O_2, 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% Parameters in the linear scale, O_2 values omitted.
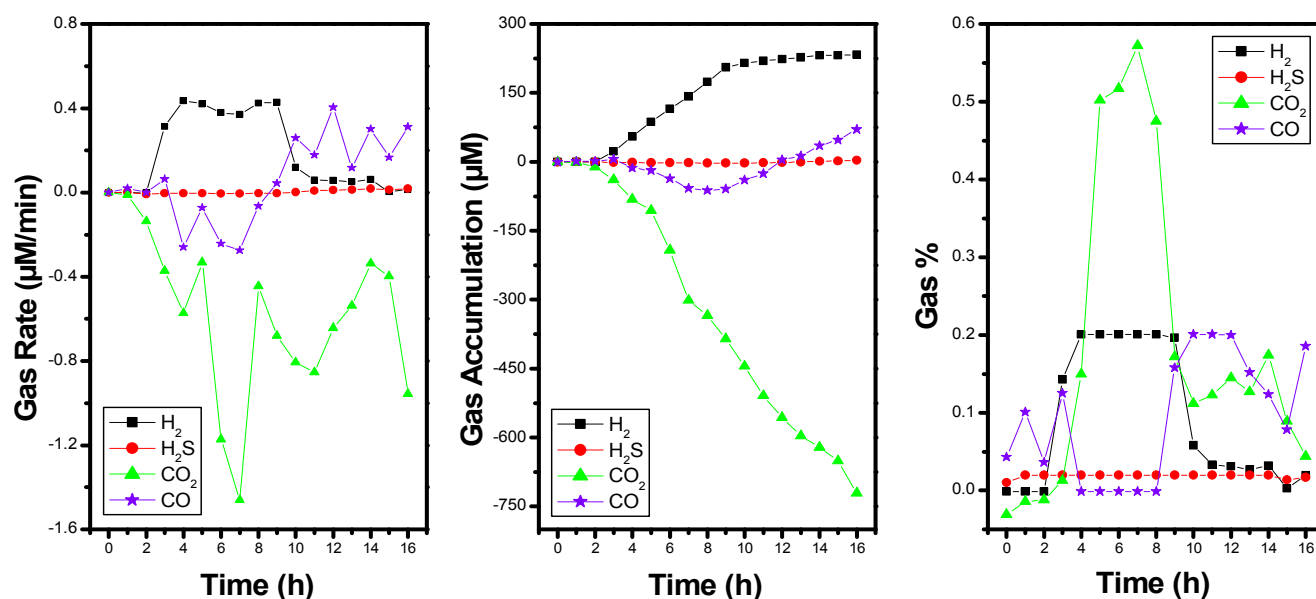
The maximum amount of produced H_2 upon utilizing molasses as carbon source was observed by *Clostridium perfringens* (AK1-9 isolate) (4615µmol/l) Figure 6A, Table 1). Other isolates produced up to 100 times lower concentrations of H_2 (Figure 6A, Table 1). This suggests that molasses is less universal substrate for H_2 production. The maximum amount of accumulated CO using molasses as carbon source was detected in *Clostridium botulinum*-like strain (AK 1-4 isolate). In *Clostridium perfringens* (AK 1-9

isolate) the CO gas evolution and/or consumption was inverse to that of H_2 (Figure 6A, B). Also, smaller amount of CO but higher than of H_2 was detected in *Clostridium difficile* (AK 1-5 isolate).

Molasses as a waste product of sugar production was expected to be a good carbon source for bacteria. However, disappointing that bacteria did not grow well on molasses, with poor evolution of CO_2 (Figure 1-5). H_2 was evolved only with one isolate (AK 1-9). The reason of this failure did not analyze. Probably, high concentrations of Ca^{2+} and the presence of many other carbon compounds besides sucrose disqualified molasses as good substrate for bacteria. Also the absence of ß-fructosidase activity in other isolates of bacteria could be the reason for failing to grow on the molasses.
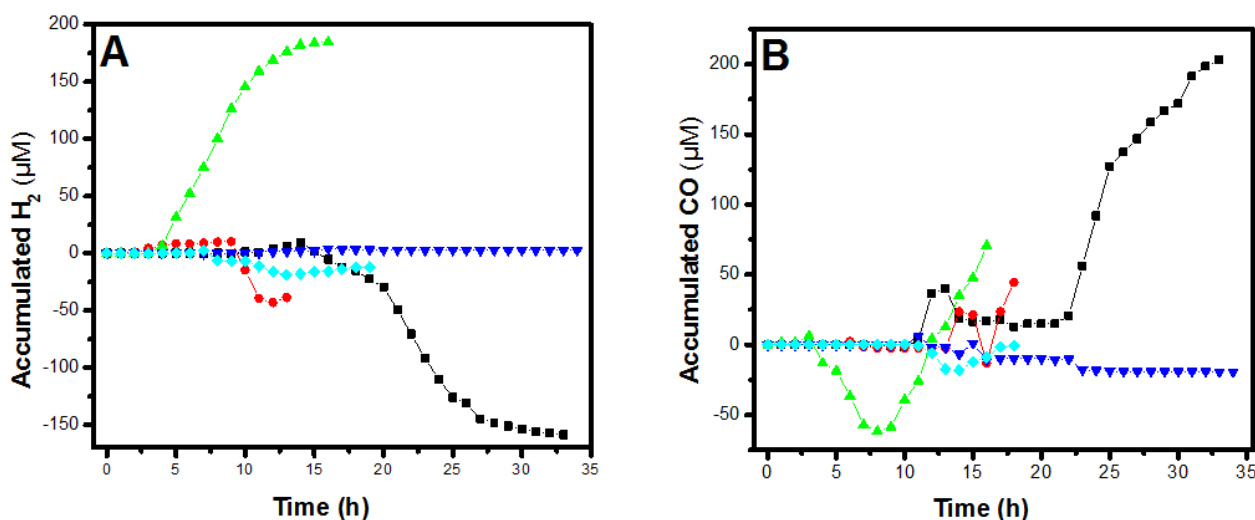

Figure 6. *Molasses as Substrate for Production of: (A) H_2 Gas; (B) CO gas by Clostridium and E. Coli Isolates in RCM.*

—■— *C. botulinum* like strain (AK 1-4 isolate), —●— *C. difficile* (AK 1-5 isolate), —▲— *C. Perfringens* (AK 1-9 isolate), —▼— *Clostridium spp.* (AK 1-12 isolate), —◆— *E. coli* (No 2-2-4 isolate).

Accumulated H_2 production read out from Micro-Oxymax

***Table 1.** H_2 Production on Molasses by Clostridium Isolates and by E. coli (No 2-24 Isolate).*

Maximal H_2 concentration (µmol/l) obtained upon cultivation with molasses	Bacterial species				
	Clostridium perfringens (AK 1-9 isolate)	*Clostridium difficile* (AK 1-5 isolate)	*Clostridium spp* (AK 1-12 isolate)	**Clostridium* (AK 1-4 isolate)	*Escherichia coli* (No. 2-24 isolate)
H_2 (µmol/l) from RCM with Mol.	4615	252.5	75	227	60

*Similar to *Clostridium botulinum* strain, Mol. ≡ Molasses, AK ≡ Activated sludge, No 2 ≡ Sheep 2.
Data from the Figure 5A were re-calculated for 1 l medium.

4. Conclusion

Five isolates were used to study the production of metabolic gases. Four isolates were from activated sludge and all belonged to the genus *Clostridium*: *C. perfringens* (AK 1-9 isolate), *C. difficile* (AK 1-5 isolate); one strain could be assigned to *Clostridium spp.* (AK 1-12 isolate); and one strain similar to *C. botulinum* (AK 1-4 isolate). The fifth was a rumen bacterium, which was identified as *Escherichia coli* (No 2- 24 isolate). These isolates were cultivated anaerobically with molasses as complex carbon sources. Metabolic gases (O_2, CO_2, CO, H_2, H_2S and CH_4) were measured simultaneously by means of Micro-Oxymax gas analyzer with optical detection. It was found that all isolates produce CO_2, CO, H_2, and H_2S dependent on the carbon source. Molasses was utilized only by AK 1-9 isolate.

Results suggest that the ability of tested bacteria to produce H_2 is universal. However, quantity of produced H_2 strongly depends on the carbon source. Comparative study of five isolated suggested that several aspects of utilization of carbon sources is highly variable between individual isolates. Thus, successful strain for the biotechnological use should be selected or modified in order to achieve optimal properties for the utilization of complex systems.

Acknowledgements

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