Suitability of Organic Waste Materials for Biohydrogen Gas Production

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Abstract: *This study aimed to test the hydrogen gas (H_2) production in heterotrophic bacteria isolated from typical anaerobic habitats. To reach this goal, isolation and maintaining bacterial strains capable of producing H_2 were done. Microorganisms were isolated from sheep rumen and activated sludge and assigned to some known species by BLAST. And the genetic similarity was based on the ITS sequence. One of the isolates was Escherichia coliDH1(ME8569) which was isolated from the sheep rumen and coded by No 2-24. And the other four strains were obtained from activated sludge and identified as clostridial species. The clostridial species were Clostridium spp., Clostridium perfringens clone CVCC90. We, Clostridium difficile M68, and Clostridium botulinum E3 str. Alaska E43; were coded by AK 1-12, AK 1-9, AK 1-5, and AK 1-4, respectively. Reinforced Clostridial Medium (RCM); and Yeast extract (Y.E.) medium were used for growth and hydrogen production. The waste materials tested for hydrogen production are sunflower meal, sunflower expellers, rapeseed meal, hay, and straw. The productivity of H_2 gas with rapeseed meal was ranged 531-11250 µmol/l (AK 1-9 11250 µmol/l, AK 1-4 710 µmol/l, AK 1-5 3028 µmol/l, AK 1-12 531µmol/l); and with sunflower expeller in range 3413-18080 µmol/l (AK 1-12 18080 µmol/l, AK 1-4 5120 µmol/l, No. 2-24 3413 µmol/l) while sunflower meal between 285-8848 µmol/l (AK 1-9 8848 µmol/l, AK 1-4 7570 µmol/l, No. 2-24 285 µmol/l). E. coli and AK 1-5 did not produce hydrogen gas using rapeseed meal or sunflower meal. Hay and straw were poor substrates for H_2 production. Results indicate that the capability of yielding H_2 and the quantity of H_2 substantially depend on the carbonaceous substrates.*

Keywords: *Clostridium Spp*., *E. Coli*, Organic waste materials, Biohydrogen gas, Anaerobic Metabolism

1. Introduction

Fossil fuel is about eight percent of world energy exhaustion [1, 2], limited non-renewable energy resource, and harm the environment by emission of greenhouse gases. Because of the problems of pollution and the extinction of fossil fuels, scientists have sought to find alternative solutions to replace fossil fuels and reduce environmental pollution by using cleaner and more environmentally friendly fuel sources. Hydrogen gas (H_2) itis an extremely hopeful clean fuel for futurity, has high potential efficiency to convert to useful energy, and has minimal or no pollutants. Hydrogen is a cleaner fuel than methane and gasoline, so there are broad research interests in production and storage for long periods at acceptable costs [3]. Hydrogen gas is a non-carbon energy source and produces only water when burned. The calorific value of hydrogen gas (141.9 kJ/g) is 2.6 times (47 kJ/g) greater than natural gas and threefold (54 kJ/g) of gasoline [3]. So bio-hydrogen gas is cleaner, renewable, and sustainable energy source [4, 5, 6, 7, 8]. H_2 gas is produced biologically or non-biologically. Biological production is achieved under anaerobic or aerobic conditions. One of the biological methods is the anaerobic fermentation of biomass. The gas fermentation by anaerobic bacteria gained wide attention in several scientific, industrial, and social fields [9]. Since hydrogen gas production is yet largely dependent on fossil fuels, there is a great extent of other hydrogen production methodologies, which are considered more environmentally safe, such as photo-fermentation, dark fermentation, and photolysis [2, 10, 11, 12]. The products of anaerobic metabolism of many microorganisms show a significant role in agriculture and industry. The simplest example is the production of ethanol by yeasts; or using of *Lactobacilli* in the food manufacturing. The use of bacteria for biotechnological purposes maybe not is so widely distributed but has a necessary role in the development of biotechnology.

Among several bacterial species, *Clostridiumspecies* are important due to their ability to utilize saccharidic substrates by the butanol-acetone fermentation pathway and for H2 production. Also, the degradation of Propane-1, 2, 3-triol (glycerol) has been explored [11, 13]. Less explored is the possibility to utilize non-saccharidic (e.g., proteinaceous) substrates for biotechnological purposes.

Using waste materials or complex substrates for H_2 production is of unlimited attention for biotechnological applications. Numerous studies performed on the appropriateness of *Clostridium species* for H_2 generation by different feedstocks [14]. H_2 gas was generated from municipal wastes [15], sugarcane bagasse [16], apple pomace [17], organic waste [14], and less complex substrates like cellulose [18].

Those *Clostridial* species (*Clostridium spp., Clostridium perfringens, Clostridium difficile, and Clostridium botulinum*) and E. coli spp produced H2 production by using saccharide substrate [11, 19], glycerol [11], milk and blood [19], and molasses [20].

2. H_2 Production by Fermentation in Bacteria

In fermentation, carbohydrates such as starch are converted to monosaccharides, subsequently to pyruvate via glycolysis, and pyruvate to H_2 and organic acids (methanoic, ethanoic, butanoic acid) at the end step. Bacteria grown on organic substances degraded them to provide metabolic energy and building blocks for growth.

Fermentative bacteria, such as *Clostridia* and enteric bacteria [12, 14], use the fermentation process to extract H_2 from carbohydrates (starch, glucose).

The products of the fermentation of glucose are acetic acid (eq. 1) and butyric acids (eq. 2) beside the H_2 gas.

$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CH_3COOH + 2CO_2$ (1)

$C_6H_{12}O_6 \rightarrow 2H_2 + CH_3CH_2CH_2COOH + 2CO_2$ (2)

One mole of glucose will yield 2mol of H_2 by facultative anaerobic bacteria and 4mol by strictly anaerobic bacteria.

3. Material and Methods

3.1- Isolation and characterization of pure bacterial species

As described previously [11, 20], the bacterial strains were isolated from the activated sludge and the sheep rumen fluid. The tested bacteria propagated on RCM. Cultivation was carried out under an anaerobic atmosphere (anaerobic cultivation chambers). Free oxygen atmosphere was achieved using of BBL GasPack System (Becton Dickinson), and the cultivation was done in the Bactron 1 anaerobic chamber (Sheldon Laboratories) [11, 19, 20]. And the characterization was carried out by sequencing ITS fragments as described previously [20].

3-2. Molecular taxonomy

The molecular taxonomies of the bacterial isolate were achieved by colony PCR amplifying the 16S-23S ribosomal bacterial spacer. As described by R. Jame et al. [11, 20] the PCR mixture contains a 50 µl PCR mix contains 40.5 µl DNAase-free water, 1 µl 10 mMdNTP mix, 1.5 µl primer reverse *(B28S10 REW) (5´CCWTTCCCTCACGGTACT´3), 1.5 µl primer forward (B16S2 FWD 5´TTGTACACACCGCCCGTC´3), 5 µl 10x HotMaster™ Tag buffer with Mg^{2+}, 0.5 µl, and HotMaster™ Tag. The PCR by a thermocycler was started with a little fraction of bacterial colonies (MasterCycler personal – Eppendorf, Germany), and the DNA polymerase was added to the PCR mixture after 15 minutes. Then the purification and sequencing of PCR products were carried out before analysis by the BLAST.

3-3. Reinforced clostridial medium (RCM)

The composting of the growth medium was prepared based on the Kalil et al. method [21]. But in this study, other substrates than glucose were added, such as rapeseed meals and sunflower expeller. The RCM contented a 40g/l of the tested substrate (rapeseed meals or sunflower expeller), Yeast extracts 13g/l, agar 0.5 g/l, L-Cysteine.HCl 1g/l, and pH 6.8±0.2.

3-4.Yeast extract (Y.E.) with waste material

The cultivation medium composition was the following: yeast extract 1g/l, waste material: 40g/l(sunflower meals, rapeseed meals, or sunflower expeller) or¾ volume of the used bottle (straw or hay), and pH 7.

3-5. Metabolic gases measurements

Specific flasks (50 ml volume) with stoppers equipped with outlet and inlet tubings; were used for the cultivation of 40 ml of the liquid medium of the pure bacterial cultures. Flasks were connected to the gas analyzer (Micro-Oxymax) (Columbus Instruments Columbus, OH, U.S.A.); the gas analyzer equipped with O_2, CO, CO_2, H_2S, H_2, and CH4 sensors. The system was flushed with pyrogallol-treated N2 to remove any traces of O_2 gas. Measuring the metabolic gases was executed at 37˚C.

4. Results

4.1. Growth with carbon sources

CO_2 was expected to be the major metabolic gas. The measurement of CO_2 production could be regarded as a measure of metabolic activity. The

evolution of CO_2 by *E. coli* and *Clostridium spp.* could monitor the growth of cells.

4.1.1.Growth with sunflower expeller and sunflower meal

Sunflower expeller and sunflower meal are abundant waste materials from the edible oil industry.

Growth utilizing sunflower meal supplemented with 0.1% yeast extract was detected by *Clostridium* isolates (AK 1-4 and AK 1-9) and *E. coli* isolate (No 2-24). Also, the growth was observed by *Clostridium* isolates (AK 1-4 and AK 1-12) that utilized sunflower expeller in RCM; and *E. coli* isolate that utilized sunflower expeller supplemented with 0.1% yeast extract. Under these conditions, growth started later than that observed with saccharidic substrates. It could be inferred from the CO_2 evolution that the growth was quite poor under these conditions.

4.1.1.1.Evolution of hydrogen gas with sunflower meal and sunflower expeller

The productivity of H_2 gas with sunflower expeller is in the range 3413-18080 µmol/l; AK 1-12 18080 µmol/l, AK 1-4 5120 µmol/l, and No. 2-24 3413 µmol/l. While the productivity by sunflower meal is between 285-8848 µmol/l; AK 1-9 8848 µmol/l, AK 1-4 7570 µmol/l, and No. 2-24 285 µmol/l. And no hydrogen gas was detected by AK 1-5 by using sunflower meal. The data for H_2 production on sunflower meal and sunflower expeller was scheduled in table no.1.

Table 1: The maximum H_2gas productivity on sunflower by *E. coli* and *Clostridium*strains

Maximal H_2 (µmol/l) with sunflower meal or expeller	**Bacterial strain**				
	***Escherichia coli DH1(ME8569)* (No 2-24)**	***Clostridium spp* (AK 1-12)**	***Clostridium perfringens clone CVCC90.WE* (AK 1-9)**	***Clostridium difficileM68* (AK 1-5)**	***Clostridium E3 str. Alaska E43* (AK 1-4)**
H_2 (µmol/l) from Y.E. with sunflower meal	285	_	8848	X	7570
H_2 (µmol/l) from RCM with sunflower expeller	3413 in Y.E.	18080	_	_	5120

– ≡ Don't tested, X ≡ H_2 not detected, AK ≡ Activated sludge, No 2 ≡ Sheep 2

4.1.2. Growth with rapeseed meal

Growth on rapeseed meal was tested by *E. coli* and *Clostridium* isolates (AK 1-5, AK 1-9, and AK 1-12) in a medium containing yeast extract (0.1 %); and by *Clostridium* isolates AK 1-4 in RCM medium.

All tested *Clostridium* isolates were grown on rapeseed meals. With this substrate, some delay in the onset of CO_2 production (except AK 1-12 isolate).

4.1.2.1. Evolution of hydrogen gas with rapeseed meal

Accumulated H2 was detected in the four *Clostridium* strains (AK 1-4, AK 1-5, AK 1-9, and AK 1-12) but not in the *E. coli* strain (No 2-24). The 710 µmol/l was detected by *Clostridium* isolate AK 1-4 grown on RCM medium with rapeseed meal; and 11250 µmol/l, 3028 µmol/l, and 531.2 µmol/l were detected by *Clostridium* stains AK 1-9, AK 1-5, and AK 1-12 respectively, in the medium supplemented with 0.1 % yeast extract (Table 2). Thus, there are dramatic differences in utilizing this substrate and H_2 production between strains used.

Table 2: The maximum H_2 gas productivity on rapeseed meal by *E. coli* and *Clostridium* strains

Maximal H_2 (µmol/l) obtained with rapeseed meal	**Bacterial strain**				
	***Escherichia coli DH1(ME8569)* (No 2-24)**	***Clostridium spp* (AK 1-12)**	***Clostridium perfringens clone CVCC90.WE* (AK 1-9)**	***Clostridium difficileM68* (AK 1-5)**	***Clostridium E3 str. Alaska E43* (AK 1-4)**
H_2 (µmol/l) from Y.E. with rapeseed meal	X	531.2	11250	3028	710In RCM

– ≡ Don't tested, X ≡ H_2 not detected, AK ≡ Activated sludge, No 2 ≡ Sheep 2

4.1.3. Growth and evolution of hydrogen gas with straw and hay

Straw and hay were used as substrates for three isolates, two *Clostridium* isolates (*Clostridium* isolate AK 1-4, *Clostridium* isolate AK 1-12), and *E. coli* isolate (No 2-24). Tested isolates failed to produce the H_2. Neither CO_2 production nor a significant H_2 production was observed. Therefore, corresponding data are not listed here.

5. Discussion

5.1. Isolation and characterization of bacterial strains

The results of the isolation and the ITS sequences assigned most bacteria to known genera, which are facultative or anaerobic bacteria. Unfortunately, only one isolate of enterobacteria (sheep rumen) was found to be *E. coli DH1 (ME8569)*, with a Maximum identity of 98 %. And the other four strains were obtained from activated sludge and identified as clostridial species. Those species are *Clostridiumspp.* (Maximal identity 82 %), *Clostridium perfringens clone CVCC90 WE* (Maximal identity 99 %), *Clostridium difficile M68* (Maximal identity 93 %), and *Clostridium botulinum E3 str. Alaska E43* (Maximal identity 82 %); which are coded by AK 1-12, AK 1-9, AK 1-5, and AK 1-4 respectively [19].

5.2. The total production of hydrogen gas by several organic waste materials

Production of hydrogen gas by using complex lignocellulosic carbon substrates was tested by *Clostrodial spp. and E. coli spp.*; these isolates were tested for H_2 production by using: saccharide substrate [11, 19], glycerol [11], milk and blood [19], and molasses [20]. *E. coli* was used for H_2 production for several decades [22].While the hydrogen gas was not produced by the straw or the hay when were used as growth substrates.

Sunflower meal, sunflower expeller, and rapeseed meal were found to be good substrates for the growth of clostridial isolate except AK 1-5 by using sunflower meal and *E. coli* by using rapeseed meal.

By growth on the sunflower expeller, AK 1-12 is the best hydrogen producer of all tested isolates (Ak 1-12, AK 1-4, and No. 2-24), table 1. by using sunflower meal, AK 1-9 and AK 1-4 isolates produced H_2 in good amount; and AK 1-9 was found to be the best producer of all tested isolates (Ak 1-9, AK 1-4, AK 1-5, and No. 2-24), see table 1.

AK 1-4 and AK 1-9 isolates are good producers with monosaccharides (glucose) and disaccharides (lactose and Cellobiose) [11] but failed to grow and produce H_2 on starch and cellulose [11].

By using rapeseed meal the AK 1-5 is the best hydrogen producer of all tested strains (Ak1-12, AK 1-4, AK 1-9, AK 1-5, No 2-24). The productivity of hydrogen gas by *E. coli*isolate (No 2-24) was better by using sunflower expeller (3413 µmol/l), traces by using sunflower meal (285 µmol/l); while no hydrogen was produced by using rapeseed meal, see table 2. Therefore, the good performance in the utilization of sunflower and rapeseed meals is enigmatic. The activities of hydrolytic enzymes participating in the hydrolysis of hemicelluloses should be determined later. Production of H_2 by tested isolates was strongly dependent on the carbon sources.

The evaluation and production of the H_2 gas were carried out using waste materials such as straw, hay, and food oil industry substrates (sunflower meal, sunflower expeller, and rapeseed meal).

The major metabolic gas produced by most of the microorganisms was CO_2. Mostly, its production could be theoretically considered as a measure of growth. Concentrations of H_2S during cultivations were much lower than those of all other gases and did not exceed 0.025 %. None of the substrates used as carbon sources was stimulating the production of H_2S selectively. It could be deduced that the process of sulfate respiration does not occur in the isolates tested. A low evolution of H_2S may be of advantage for the production of H_2 as H_2S is contaminant which has to be removed from the H_2.

6. Conclusion

This study aimed to characterize the productivity of H_2 gas by a test isolated from typical anaerobic habitats and cultivated on defined and complex substrates. The results obtained could be summarized as follows:

Anaerobic bacteria were isolated from sheep rumen and the activated sludge. These bacteria were characterized by 16S rRNA sequences and by tolerance to oxygen. The prevalent number of bacterial isolates was similar to known bacterial species.

Five isolates were used in this study. One of the isolates is *Escherichia coli DH1(ME8569)* which is isolated from the sheep rumen, and the other four strains were obtained from activated sludge and identified as clostridial species. These species are

Clostridium spp.; Clostridium perfringens clone CVCC90 WE, Clostridium difficile M68, and *Clostridium botulinum E3 str. Alaska E43.*

These isolates were cultivated under anaerobic atmosphere with RCM or Y.E. by adding sunflower, rapeseed meal, sunflower expeller, hay, or straw.

The measuring of metabolic gases (O_2, CO_2, CO, H_2, H_2S, and CH_4) was carried out simultaneously by the gas analyzer (Micro-Oxymax). And found that all isolates produce CO_2, CO, H_2, and H_2S depending on the carbon source.

Hay and straw were poor substrates for H_2 production. On the other hand, sunflower meal, sunflower expellers, and rapeseed meal were ideal substrates for the H_2 production in AK 1-4 and AK 1-9 isolates.

The kinetics of H_2 evolution shows that it was created through the exponential phase of growth. In some experiments, it is consumed in the aged culture of bacteria.

Results indicate that the capability of yielding H_2 and the quantity of H_2 substantially depend on the carbonaceous substrates.

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