BIOLOGICAL SCIENCES

HYDROGEN PRODUCTION FROM CELLULOSIC MATERIALS BY NATURAL MICROBIAL ASSOCIATION FROM SOIL ENRICHED BY *CLOSTRIDIUM* AND *BACILLUS* MICROORGANISMS

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Abstract
Study of hydrogen production from renewable raw materials using biological methods is relevant because it does not have adverse effects on the environment. The aim of this work is to study hydrogen yield by natural microbial associations enriched by microorganisms of *Clostridium* and *Bacillus* genera. Natural microbial associations from soil and from a lake have been isolated; their ability to ferment cellulose with molecular hydrogen evolution has been investigated. Natural microbial association obtained from soil proved to be the most effective as it is dominated by 2 microorganism genera – *Clostridium* and *Bacillus*. It has been shown that additional enrichment of natural microbial associations by the microorganisms of *Clostridium* and *Bacillus* genera with a ratio 1:2.5 increases hydrogen yield by 48% and hydrogen content reaches 87.5% in the resulting biogas. This approach enables a reduction in substrate processing time.
Keywords: natural microbial association, hydrogen, cellulose, agricultural waste, *Clostridium, Bacillus.*

Introduction. Progressing shortage of fossil fuels and environmental pollution by their production waste requires searching for renewable environmentally friendly energy sources. More than 80% of energy is currently obtained from fossil fuels, which leads to gradual climate change, global warming, rapid depletion of natural resources [7]. Therefore, almost all countries are looking for alternative, renewable energy sources, such as biofuels, which can be obtained from renewable raw materials.

Existing methods of hydrogen production are power-consuming processes; all technologies, except for water electrolysis and biomass gasification, require fossil fuels as the source of hydrogen [13].

Promising is the use of renewable raw materials, such as agricultural waste, as a substrate for hydrogen production. This way, the yield of hydrogen from crushed stalks and cobs of corn under mesophilic conditions is 16.1 –20.4 mmol H_2/kg [3].

According to the sources, high hydrogen yield during cellulose fibers decomposition was achieved by the pure culture of microorganisms *Clostridium thermocellum* under thermophilic conditions and was 11.2 mmol H_2 per kg of substrate [4]. However, most of the current research is focused on the use of microbial associations and co-cultivation systems, which increases yield and rate of hydrogen production compared to the pure culture. According to the sources, hydrogen yield in case of an isolated strain use was 0.96 – 1.07 mmol H_2 per g of cellulose [6] and in case of a microbial association use – 1.6 – 2.18 mmol H_2 per g of cellulose [2]. Other studies have shown that fermenting cellulose substrates by an association dominated by microorganisms *Thermoanaerobacterium thermosaccharolyticum* and *Clostridium* under thermophilic conditions gives hydrogen yield of 7.22 mmol H_2/g of carboxymethylcellulose at a concentration of 0.25 g/dm^3 [9]. Use of a microbial association of the genera *Enterococcus* and *Clostridium* for the fermentation of wheat straw in a two-stage process allowed to obtain 79.5 cm^3 of H_2 per g of substrate [12]. Fermentation of cellulose-containing raw materials by the natural microbial association under mesophilic conditions gives hydrogen yield of 61.3 cm^3 H_2/g of substrate with cellulase activity of 0.19 mmol/min·ml [11].

Use of associations of different species and genera of microorganisms has a number of advantages:

- wide range of different substrates use;
- log phase of growth delay reduction;
- resistance to external fluctuations of waste composition and system stability;
- higher hydrogen yield [8].

This paper identifies promising microbial associations for hydrogen production and investigates the effectiveness of the process in case of enriching the natural microbial association by hydrogen-producing microorganisms of *Clostridium* and *Bacillus* genera.

The objective was to study hydrogen yield with the natural microbial association enriched by the microorganism of *Clostridium* and *Bacillus* genera. The research was split into the following tasks:

- Identify the most effective natural microbial association of cellulose destructors and molecular hydrogen producers;
- Identify the dominant microorganism genera in the association;
- Study the fermentation process and the resulting gas mixture composition in case of the natural soil microbial association enriched by the dominant microorganisms isolated from it.

Material and methods. Soil samples and silt from a lake were used to obtain the microbial association of cellulose destructors and molecular hydrogen

producers. Weighing of soil samples was performed using technical scales T-200.

To prepare the inoculum, 5 g of soil was added to 250 cm^3 of distilled water. To inactivate the methanogens, the soil suspension was kept in a water bath at 90°C for one hour. The ratio of inoculum to medium was 1:5.

Cultivation was performed in 300 cm^3 vials filled by 70% with inoculum, water and a specific substrate, which were sealed with a rubber stopper and a screw clamp. The process was performed under anaerobic mesophilic conditions at 30-35°C, in a dry air thermostat TC-80M (MEDLABORTEKHNIKA, Odesa, Ukraine), in a batch mode. The degree of anaerobicity of the medium was monitored by the color change of resazurin (CP) solution (0.15 g/dm^3), which was added in an amount of 1 cm^3/dm^3.

Filter paper (white tape) was used for control as a source of cellulose. To prevent nutrient deficiency in the control experiment, the following was added into a vial containing 200 cm^3 of tap water: 0.2 g of KH_2PO_4; 0.2 g NH_4NO_3; 0.1 g $MgSO_4 \cdot 7H2O$; 0.01 g $CaCO_3$ [5].

For substrate a mixture of corn and sunflower waste was used in a ratio of 1:1. It was pre-grinded to sizes of 3-5 mm and pre-treated with 20% alkali solution: the grinded substrate was placed into a beaker, poured with 100 cm^3 of 20% NaOH solution and left for 3 hours at room temperature with periodic stirring. The solution was drained and the solid residue was washed with distilled water until achieving neutral pH.

The composition of the gas synthesized in the process of microbial destruction was determined by the standard methods of gas chromatography [1] using a gas chromatograph LHM-5MD (EXPERIMENTAL FACTORY CHROMATOGRAPH, Moscow, Russia). The temperature of the columns, the evaporator and the detector - 50°C. Carrier gas - argon, gas flow rate - 30 cm^3/min. The volume percentage of H_2, CO_2, N_2 and O_2 in the gas mixture was calculated based on the calibration data. Coefficients for calculation: $K(H_2)$ = 0.00142, $K(N_2)$ = 0.0065, $K(O_2)$ = 0.005, $K(CO_2)$ = 0.029, $K(CH_4)$ = 0.0026.

For selective isolation of the association, that effectively decomposes cellulose with the release of molecular hydrogen, chemically defined liquid Omelianski growth medium was used with the following composition, g: $(NH_4)_3PO_4$ - 1,0; K_2HPO_4 - 1.0; $MgSO_4$ - 0.5; NaCl - 0.1; $CaCO_3$ - 2.0; $FeSO_4$ - 2 drops of 1% solution; peptone - 0.6; distilled water - 1000 cm^3.

To isolate axenic colonies of anaerobic microorganisms, a standard streaking process was used [10] in petri dishes in an anaerostat. Medium composition, g/cm^3: K_2HPO_4 - 30; KH_2PO_4 - 2; $MgSO_4$ -1; NH4Cl - 1; $CaCO_3$ - 0.1; $FeCl_2$ - 0.4; agar - 15; microcrystalline cellulose powder - 10. The medium was sterilized by autoclaving for 20 minutes at 50.65 kPa, 121°C. After sterilization, 2 drops of indicator (resazurin (CP)) were added to visually control the environment's redox potential. 15 cm^3 of agar medium was added to petri dishes and spreaded by a microbiological loop using streaking technique. After loading the dishes into a container, 30-50 g of calcined (1h at 100°C) granulated (d = 2–4 mm) silica gel (CP) to avoid condensation. The air was replaced with argon by a 3-fold cycle of "evacuation-filling with argon". One cycle consists of evacuation at a pressure of 202.6 kPa for 5 minutes and then filling the anaerostat with argon to balance the atmospheric pressure. Inert gas argon (DSTU 10157-79, first grade) containing O_2 at a concentration not exceeding 0.002% was used in the work.

Cell morphology was studied by light microscopy using an XSP-139TP microscope (ULAB SCIENTIFIC INSTRUMENTS CO. LTD, Jiangsu, China) with a magnification of 1000x. Gram staining was performed according to the common method [5].

To increase the yield of hydrogen, the natural association from soil was enriched with microorganisms of *Bacillus* and *Clostridium* genera isolated from the same association. To achieve that, microorganisms of *Clostridium* genus were re-inoculated from the petri dishes in the anaerostat to a liquid Omelianski growth medium, and microorganisms of *Bacillus* genus were re-inoculated onto nutrient broth and cultivated to increase biomass.

Results. During the cultivation of natural associations from soil and from a lake using filter paper as the only source of carbon, the gas-phase composition changed over time. The vials were sealed with air to inhibit the development of methanogenic microorganisms for which oxygen is toxic. Because of that, initially the nitrogen content was the same as in the air; over time its percentage concentration in the gas-phase decreased, due to the increase of other gases (H_2 and CO_2). The amount of oxygen over time decreased to complete absence in the association from soil. The amount of carbon dioxide and hydrogen increased during the cultivation process. The hydrogen yield after 6 days is 2.5 times higher when using the association from soil than when using the association from a lake (Table 1).

Table 1

Change in the qualitative and quantitative composition of the gas mixture during cultivation

Day	Parameter	Inoculum from a lake	Inoculum from soil
3	H_2, %	0,5	0
	N_2,%	75	77,9
	CO_2, %	20	16
	O_2, %	4,5	6,1
4	H_2, %	2,7	2,4
	N_2,%	72,8	75,7
	CO_2, %	21	18,9
	O_2, %	3,5	3
5	H_2, %	4,5	13,5
	N_2,%	71	65
	CO_2, %	21,1	21,5
	O_2, %	3,4	0
6	H_2, %	14,5	34,7
	N_2,%	61	42
	CO_2, %	23,4	23,3
	O_2, %	1,1	0

As Table 1 shows, for hydrogen production the most effective was the microbial association from soil (*Bacillus*, *Clostridium*, *Streptobacillus*, *Diplobacillus*, dominated by *Bacillus* and *Clostridium* genera). The rate of cellulose decomposition was 3 times higher when using the inoculum from soil compared to the inoculum from a lake (*Bacillus*, *Clostridium*, *Streptobacillus*, *Diplobacillus*, *Desulfotomacullum*, *Methanosarcina* dominated by *Diplobacillus* and *Streptobacillus* genera): 0.6 and 0.2 mg/h, respectively.

As the graph on (Fig. 1) shows, the association from a lake adapted faster and began to produce hydrogen, but the hydrogen yield was insignificant (max = 14.5%). The association from soil had a longer period of adaptation, but after 4 days it started actively producing hydrogen, the yield (max = 34.7%) was 2.5 times higher than in the association from a lake.

After 6 days, hydrogen yield decreased.

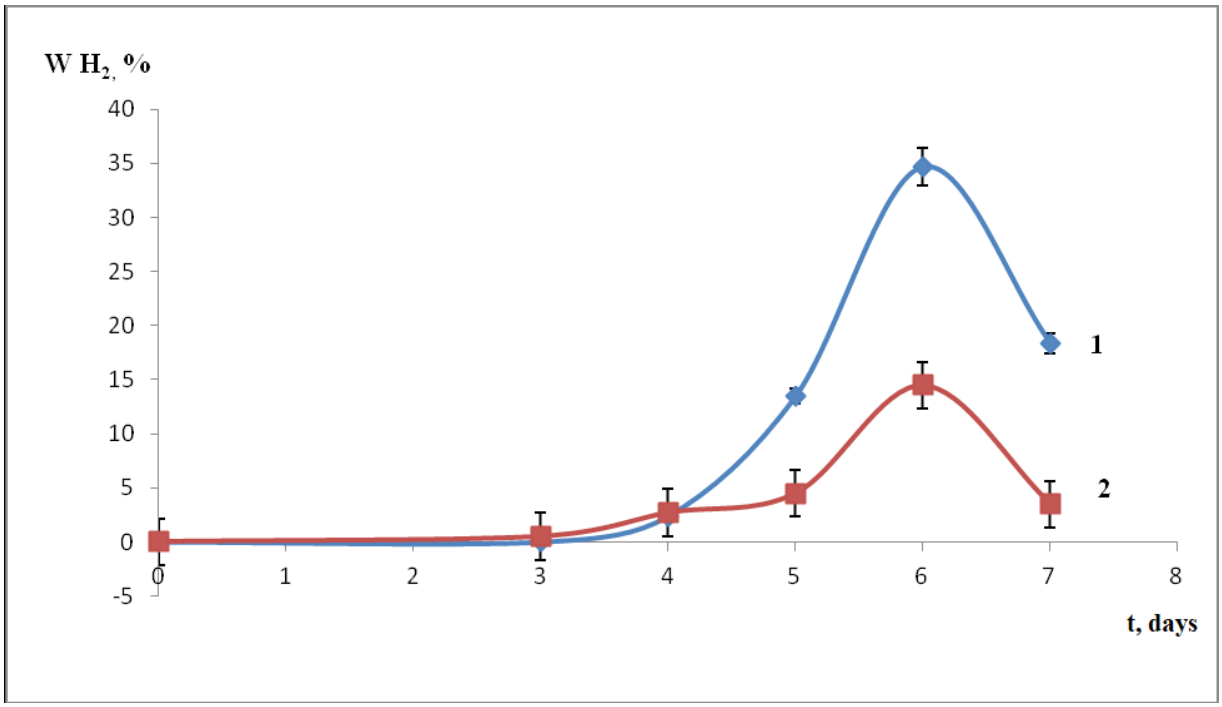

Figure 1. Change in hydrogen yield (W) depending on the cultivation period (t) of different microbial associations: 1 - from soil, 2 - from a lake.

Microscopic examination of the association from a lake was dominated by bacteria of *Bacillus* genus with a large number of spores. The association from soil was dominated by bacteria of *Clostridium* genus; bacteria of *Bacillus* genus were also present but in smaller numbers.

By repeated re-inoculation (to prevent contamination by other microorganism species) of the obtained microbial association in an anaerostat, axenic colonies of anaerobic microorganisms of *Clostridium* genus were isolated.

Axenic colonies of aerobic microorganisms of *Bacillus* genus were obtained by repeated re-inoculation of the obtained association on a solid growth medium with oxygen presence (Fig. 2).

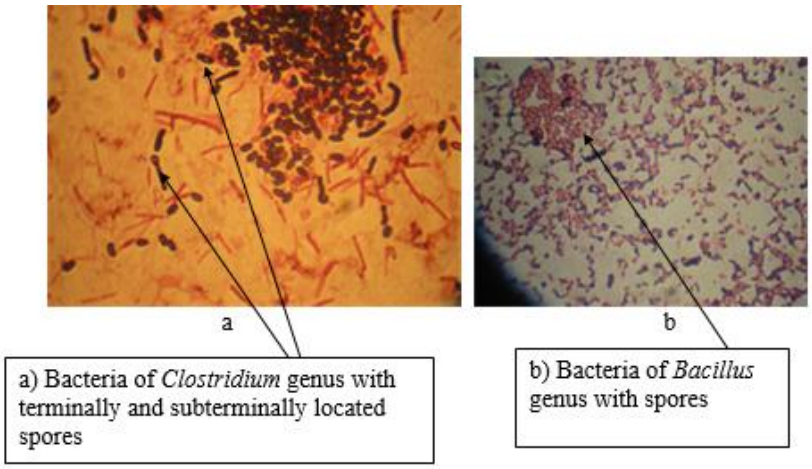

Figure 2. Smear from petri dishes: a - from anaerostat, b - from incubated in aerobic conditions.

Finally, different types of enrichment were explored. Table 2 shows gas mixture contents depending on the microorganism types used for the association enrichment.

Table 2

Gas mixture composition during the fermentation of sunflower waste enriched by the natural microbial association dominated by *Clostridium* and *Bacillus* genera

Day	Parameter	Natural association	Natural association enriched by *Clostridium*	Natural association enriched by *Bacillus*	Natural association enriched by *Clostridium* and *Bacillus 1;1*	Natural association enriched by *Clostridium* and *Bacillus 2,5;1*	Natural association enriched by *Clostridium* and *Bacillus 1;2,5*
0	H_2, %	0	0	0	0	0	0
	N_2,%	49,5	44,4	49,1	45,5	46,2	47,1
	O_2, %	10,75	9,4	1,5	6,5	8,5	2,1
1	H_2, %	3,5	4,2	8,3	5,5	6,1	10,5
	N_2,%	45,5	42,3	40,6	41,3	38,7	37,5
	O_2, %	6,8	5,1	0	1,1	1,8	0
2	H_2, %	16,5	17	47,8	23,1	68	87
	N_2,%	42,1	40	38,2	40	32	14
	O_2, %	2,3	0	0	0	0	0
3	H_2, %	38,7	46,5	27	51,5	55	70
	N_2,%	35,1	33,1	30,1	31	30	14
	O_2, %	0	0	0	0	0	0
4	H_2, %	19	21	15	23,5	25	30,1
	N_2,%	35	32	30	30	27	13
	O_2, %	0	0	0	0	0	0

As Table 2 shows, enrichment of a natural microbial association by *Clostridium* only or by *Bacillus* only lead to a decrease in hydrogen yield compared to the control measurement of cellulose-containing raw materials fermentation. Instead, when both *Clostridium* and *Bacillus* genera were added into the natural microbial association, a significant increase in hydrogen yield was observed compared with the control (Fig. 3). The highest hydrogen yield was observed when the association was enriched with *Clostridium* and *Bacillus*, ration 1;2,5.

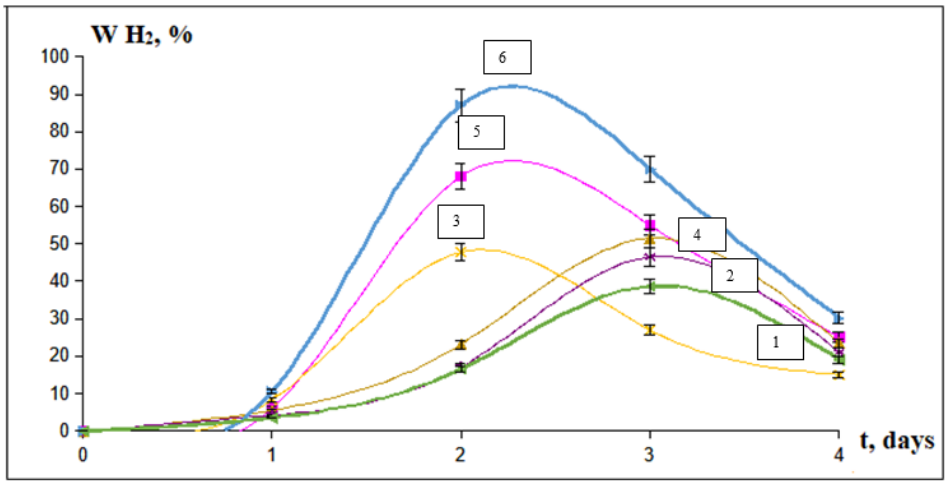

Figure 3. Change of the hydrogen content in biogas (W) during the fermentation of sunflower waste: 1 - natural association, 2 - association enriched with Clostridium, 3 - association enriched with Bacillus, 4 - association enriched with Clostridium and Bacillus, 1;1, 5 - association enriched with Clostridium and Bacillus, 2,5;1, 6 - association enriched with Clostridium and Bacillus, 1;2,5.

Discussion. During the cultivation gradual decrease of the amount of oxygen in the association from soil was due to its consumption by microorganisms of *Bacillus* genus. The amount of carbon dioxide and hydrogen increased during the cultivation process, as they are the final metabolites.

As the results show, the association from soil was 2.5 times more effective than the association from a lake. This was due to the fact that the association from soil was dominated by microorganisms of the two genera: *Clostridium* and *Bacillus*. Microorganisms of *Bacillus* genus are facultative aerobic bacteria that initially use oxygen dissolved in the medium for respiration, and in the absence of oxygen in the environment carry out anaerobic fermentation of the mixed type, releasing hydrogen from cellulose-containing raw materials. Thus, they create the necessary conditions for obligate anaerobic microorganisms of *Clostridium* genus, which in turn emit hydrogen in the process of butyric acid fermentation, which leads to an increase in hydrogen yield as Figure 1 shows.

The difference between the 2 associations was that during the cultivation in the association from soil oxygen content was gradually reduced to its complete disappearance on the 5th day of cultivation, whereas in the association from a lake oxygen remained present until the end of the cultivation and as a result anaerobic microorganisms that were main hydrogen producers couldn't effectively develop.

The decrease of hydrogen yield after 6 days is because of the inhibition by the end products of metabolism.

During the fermentation of organic waste under anaerobic conditions, two processes are possible - formation of hydrogen or methane. Since the process of methane formation reduces the yield of hydrogen, a necessary condition for the creation of hydrogen production technology is the elimination of methanogenesis. The advantage of the microbial association based on *Clostridium* and *Bacillus* genera is that residual oxygen in the environment leads to inhibition of methane-forming microorganisms, while aerobic bacteria use dissolved oxygen, creating the necessary conditions for subsequent growth of anaerobic clostridia.

Enrichment by one microorganism genus (*Clostridium* only or *Bacillus* only) proved to be ineffective. This can be explained by the dominance of one microorganism species, trophic bonds destruction in the association and, as a result, leads to a decrease in the efficiency of complex substrates fermentation. Significant increase in hydrogen yield in case of enrichment by both genera is due to the fact that bacteria of *Bacillus* genus emit hydrogen and create the necessary conditions for an anaerobic process of cellulose fermentation with the participation of microorganisms of *Clostridium* genus. The enrichment was most effective when the ratio of microorganisms of *Clostridium* genus to microorganisms of *Bacillus* genus was 1:2.5.

The graph on (Fig. 3) shows that addition of microorganisms of *Clostridium* and *Bacillus* genera reduced the adaptation time and increased the rate of substrate utilization, which leads to the process time reduction.

Conclusions

1. Hydrogen yield during fermentation depends on the species composition of the association. To obtain hydrogen from cellulose-containing raw materials, the microbial association from soil, where the microorganisms of two genera (*Clostridium* and *Bacillus*) are dominant, is more effective.

2. It was proved that enrichment of the natural association by microorganisms of *Clostridium* and *Bacillus* genera at a ratio of 1:2.5 increases hydrogen content in biogas to 87%.

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СРАВНИТЕЛЬНАЯ МОРФОФУНКЦИОНАЛЬНАЯ ХАРАКТЕРИСТИКА ЩИТОВИДНОЙ ЖЕЛЕЗЫ БЕЛОЙ ЛАБОРАТОРНОЙ КРЫСЫ, КРОЛИКА И СОБАКИ

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COMPARATIVE MORPHOLOGICAL AND FUNCTIONAL CHARACTERISTICS OF THE THYROID GLAND OF THE WHITE LABORATORY RAT, RABBIT, DOG

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Аннотация

В статье рассматриваются сравнительная анатомия щитовидной железы белой лабораторной крысы, кролика, собаки. Дана морфологическая характеристика щитовидной железы. Подробно даны результаты исследования щитовидной железы на макро-микроскопическом уровне. Выявлены наиболее значимые морфологические показатели щитовидной железы у исследуемых животных.

Abstract

The article deals with the comparative anatomy of the thyroid gland of the white laboratory rat, rabbit, dog. Given the morphological characteristics of the thyroid gland. The results of the study of the thyroid gland at the macro-microscopic level are given in detail. The most significant morphological parameters of the thyroid gland in the studied animals were revealed.

Ключевые слова: морфология, анатомия, щитовидная железа, эндокринология, гормон, грудная полость, крыса, кролик, собака, доли, эпителий, кровеносные сосуды.

Keywords: morphology, anatomy, thyroid, endocrinology, hormone, chest cavity, rat chest, rabbit, dog, share, epithelium, blood vessels.

Совокупность эндокринных желез образует эндокринную систему, в которой можно выделить несколько составляющих частей. Эндокринные железы выделяют гормоны неспосредствен , но в кровь, межклеточную жидкость, лимфу. Эндокринные железы подразделяются на группы. По морфологической связи с центральный нервной системы, делятся на центральные (гипоталамус, гипофиз, эпифиз) и периферические –щитовидая, половые, надпочечных и т.д. [1,2,3].

Щитовидная железа образуется из эктодермального эпителия непарного срединного выроста вентральной стенки передней кишки. Эпителиалные клетки формируют сложную систему тяжей. Из мезенхимы развивается соединительная ткань,