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Tashirev A.,
Doctor of Technical Sciences
Pritula R.,
Tashireva A.,
Ph.D. in Biological Sciences,
Institute of Microbiology and Virology named after D.K. Zabolotnoy National Academy of Sciences of Ukraine

Identification spore-forming bacteria in associations of hydrogen-generating microorganisms

The purpose. To determine species composition of spore-forming bacteria of associations of hydrogen-generating microorganisms for assessment of an opportunity of their biotechnological application. Methods. Phonotypical and phylogenetic analysis, chemical-alytical, gas chromatography. Results. Bacillus and Clostridium predominate in structure of an probed associations. Under morphological, cultural-biochemical and molecular-genetic characteristics they identified 3 strains to species. The strain of anaerobic bacteria is identified as Clostridium butyricum VU-11. strains of aerobic bacteria - as Bacillus licheniformis BS-3 and Bacillus atrophaeus BS-1. Conclusions. Bacillic-clostridial associations are perspective for cost effective synthesis or power supply H2 from a wide spectrum of renewable biomass, including food waste.

Key words: hydrogen, associations of hydrogen-generating microorganisms, identification, Clostridium, Bacillus.

Molecular hydrogen, as an alternative energy source, has a number of technological, socio-economic and environmental benefits over fossil fuels and is its best alternative [6, 7]. Among all the methods of obtaining H2, dark fermentation (agglomeration) is considered to be the most optimal process, since it provides a technologically simple method without significant energy costs and allows the utilization of a wide variety of organic wastes [9]. Bacteria that ferment organic compounds with the formation of H2 are very common in natural (pound, cow manure, bird droppings) and technogenic (fermentation sediment methane tin, active mull aerotanks) groups [12, 14]. The application of selective factors - pasteurization or boiling - provides the selection of thermostable stabilizing bacteria that synthesize H2 [14]. So, in the past, from our soil, an association of spore-forming bacteria was isolated from the soil, which synthesized 120 liters of H2 / kg of absolutely dry mass (ASM) of potato for 7 days and reduced its mass by 17 times [1]. Later we expanded the spectrum of the studied biotopes and isolated the association of hydrogen forming microorganisms from the fermented sediment of the methane tin and active aerosol silt [3, 14]. The purpose of the research is to establish the composition of the spore bacteria of the associations of hydrogen forming microorganisms in order to assess the possibility of their biotechnological application. Materials and methods of research. Aerobic and anaerobic spore bacteria of the associations were tested. The isolation of two strains of aerobic bacteria and the study of their morphological properties was carried out in Petri dishes on medium No. 1 (Table 1) [3]. The strain of anaerobic bacteria is isolated in a thicker agar medium, using the improved Sturm method [4]. However, for the species identification, a morphological description of the colonies on the surface of the agar medium is required. For this purpose, the bacteria were cultured in Petri dishes on medium No. 2 (see Table 1) in an anaerostat in an argon atmosphere and using a reducing agent (II). To increase the biomass of anaerobes and aerobic bacteria cells for the purpose of the most extensive DNA isolation, they were cultured in glass vials of volume 3O ml (srednichishey № 3 and № 4 respectively, see Table 1). Cells were used to extract DNA in the exponential phase of growth (24-36 years of cultivation). For the anaerobic strain, the vials were sealed with elastic rubber stoppers and sealed with metal fasteners. Aerobic bacteria were cultured in tubes with cotton-gauze cork. In order to determine the type of bacteria, culture and biochemical materials, recommended in the manual on bacterial systemtics [8], were used, in the work of O.N. Revi et al. [13], as well as sequencing of 163 rRNA genes. For the amplification of the sequences of 168 rRNA genes, the primer

271i was universal for most eubacteria. Biochemical tests for bacteria were investigated according to the methods [2]. For this anaerobic strain was cultured in test tubes with rubber stoppers, and the seeds were taken by prick or suspension, followed by purging with argon (10 min). Aerobic bacteria were cultured in tubes with cotton gauze cork. The isolation of genomic DNA and the determination of the nucleotide sequences of anaerobic strain was carried out by the employees of the Limnological Institute of the Siberian Branch of the Russian Academy of Sciences (Irkutsk) [5]. Isolation of DNA of strains of aerobic bacteria was carried out together with the staff of the Institute of Cell Biology and Genetic Engineering of the National Academy of Sciences of Ukraine (Kyiv) according to the protocols [10, 11] with modifications. Nucleotide sequences of aerobic bacteria were determined at the Institute of Molecular Biology and Genetics of the National Academy of Sciences of Ukraine. For phylogenetic analysis, the pair affinity (%) of nucleotide sequences of 16S rRNA genes of isolated strains with deposited in the GenBank database (BLAST program) was calculated. The method of joining the closest neighbors was constructed by dendrograms, where the position of the strains was determined among the near-related and typical species [5].

2. Comparative analysis of pair affinity of investigated strains with representatives of Clostridium and Bacillus in the GenBank database.%

Studies on the formation of H2 by anaerobic strain were carried out in stationary conditions in sealed vials of 120 ml volume with liquid medium No. 1. Severe anaerobes conditions were created using Propagation of the medium with argon and the reduction of iron (II) [4]. The composition of the gas synthesized during the starch fermentation was determined according to the standard method for the thermal conductivity of the catalyst on a gas chromatograph LHM-8-MD [1]. The volume of synthesized gas was measured on the syringe scale when the piston was extruded by excess gas pressure. The volume and composition of gas were determined daily. The degradation factor ("()) was calculated as rg \ 1: rg \ 2, where t1 and t2 are the initial and final ASM substrates. The duration of the fermentation (T) was determined as the time during which the gas formation took place. Research results. Morphological, culturally-biochemical and molecular-genetic characteristics were investigated to determine the species composition of strains of spore bacteria. Characteristics of anaerobic state. Colonies 1-3 mm in diameter, from round to irregular, with wavy, lopate or uneven edges; From plateaus to raised or convex; Shiny, opaque, white, smooth, with a granular or dashed interior structure; With a characteristic smell of acetic and butyric acids. Cells - straight sticks with rounded ends, mobile; Gram-positive; 0.8-1.0x2.5-3.1 μm, single, in pairs, in short or long chains. The spores oval, from the central to the subterminal, do not blow the cell. Starch hydrolyzed starch, did not form H23 during growth in meat-peptone broth (MPB), indole, ammonia, lecithinase and lipase, did not restore nitrates to nitrites; The reaction to catalase is negative. Reaction with molochku - peptonisation and formation of serum. Hydrolyzed escule. The formation of acid fermented arabinose, celibiosis, fructose, galactose, glycogen, lactose, maltose, manoz, melibiosis, raphinose, ribosomes, salicin, sucrose, trehalose, xylose and pectin. Not inhaled inositol, inulin, mannitol, rhamnose and sorbitol. The studied morphological and cultural signs allow to classify this strain to the genus Clostridium.

Fig. 1. Phylogenetic tree, where the position of the strain Clostridium sp. VU-11 among closely related and typical species of Clostridium. The scale corresponds to the 1 st substitution for 1000 p.n.

Fig. 2. A fragment of phylogenetic tree, where the position of strains BS-3 and BS-4 among the closely related and typical species of Bacillus is visible. The scale corresponds to the 1 st substitution for 1000 p.n.

To clarify the taxonomic status of Clostridium sp. VU-11 we used sequences of 16S rRNA genes. Comparative analysis of couples affinity shows that Clostridium butyricum is the most closely related species for the investigated strain - 98.6% affinity (Table 2). Additionally, the phylogenetic position was confirmed by the construction of the dendrogram. Thus, the strain VU-11 has a high similarity to C. butyricum VPI3266 and forms a joint cluster with it on the dendrogram (Figure 1). Thus, the strain VU-11 is identified as S. butyricum. Characteristics of aerobic strains. Collisions of the strain BS-3 of irregular shape, d = 1-4 mm, with a wavy edge, raised, white, matte, viscous (stretching behind the loop). Columns of the BS-4 strain of irregular shape, d> 5-10 mm, with an uneven edge, loose, white or translucent, matte or shiny, viscous. Colonies of the strains have a distinctive sharp smell. Strain cells are gram-positive; Straight sticks with rounded ends; Moving; Located singly or in diplobaciles or chains of 3-5 cells. Disputes - central, elliptical, do not blow up the cell. The size of the BS-3 strain cells is 0.8-0.9x1.3-2.4 μm, the BS-4 strain is 0, 9-1.0x2.0-2.1 μm. Both strains hydrolyzed starch; Were capable of anaerobic growth in meat-peptone agar (MPA); Fermented glucose with the formation of acid, but not acetone; Catalase-positive; Nitrates were restored but did not form N2; Hydrolysed casein, gelatin and esculin; Grow in BCH for 7% NaCl and used cyflat. In addition, the BS-3 strain of oxidazospositive was grown in BCH at 50-55 ° C. Oxidized with the formation of acid arabinose, celibiosis, fructose, galactose, maltose, mannitol, mannose, ribose, sucrose, trehalose and pectin. Did not use glycogen, inulin, melibiose, raffinose, rhamnose and xylose; Weak acid formation was detected by using inositol, lactose, salicin and sorghum. The BS-4 strain is oxidazonegative and did not grow in BCH for 50-55 ° C. Oxidized with the formation of acid only fructose, maltose, sucrose and pectin. Weak acid formation was detected with the use of celibiosis, maniosis, salicin, sorbitol and trehalose; Did not use the rest of the sugar. The studied morphological and cultural features allow to classify these strains to the genus Bacillus. A comparative analysis of the pair affinity shows that the most closely related species for BS-3 strain - V. licheniformis (98.93%) (see Table 2). For BS-4 strain Fig. 3. Dynamics of formation of H2 during fermentation of starch C. Liuyugisit VU-11 received ambiguous result. The filo-genetic position of the strains was clarified during the construction of the dendrogram (Fig. 2). Consequently, the strain of WB-C was identified as V. Isheni-gtig, and strain VZ-4 as B. aioriaeiz. The formation of H2 by the anaerobic strain VU-11 has been confirmed at the stage of its isolation in the stratum of an agar medium with starch (H2> 6%) [4], as well as during cultivation in an anesthetic (H2 = 10-17%). Therefore, it was expedient to investigate the formation of H2 by the already identified S. pneumophilic strain VU-11 during the fermentation of the model starch substrate (0.7 g) in liquid medium No. 1 (Fig. 3). The average maximum concentration of H2 was 31 ± 4.5% on the 2nd day. For 6 ± 0,9 days the strain synthesized 110 ± 19,8 liters of H2 in terms of 1 kg of ACM starch and reduced its mass in 7,9 ± 2,7 times. Thus, based on the species composition and properties of the spore bacteria of the associations of hydrogen-forming microorganisms for the extraction of H2 from the waste, the following advantages of bacillary-clostridial associations can be distinguished: • bacteria are not pathogenic; Are prototrophic, that is, they do not require additional value compounds (vitamins, trace elements, etc.); Have a high rate of metabolism, which ensures effective synthesis of H2; • with the formation of H2, they can dispose of a wide range of simple and complex sugars that are components of food waste; • bacteria are mesophilic (25-35 ° C), that is, they do not require high energy consumption (support G) in the technological process. It has also been established that aerobic spore-forming bacteria of associations during growth decrease the redox potential [3], which is obviously the key to creating optimal conditions for the growth of precisely anaerobic H2-forming clonidiosts during the fermentation of waste by associations .

Conclusions

The phenotypic and phylogenetic analyzes methods have established the specific composition of spore-forming bacteria of the associations of hydrogen-digestive microorganisms. The isolated strain of anaerobic bacteria is identified as SiohigisPity Liuyugisit VU-11, 2 aerobic states - as Vasyliv Ishenii VB-Z and VasyNig AioryAbeiz V8-4. During the fermentation of a modal substrate of starch in a liquid medium C. Liuyhusiit VU-11 for 6 ± 0,9 days synthesizes 110 ± 19,8 l N / kg of ASM substrate and reduces its mass in 7,9 ± 2,7 times. Based on the species composition of spore bacteria and their properties, it has been established that bacillary-clostridial associations are promising for cost-effective synthesis of energy carrier H2 from a wide range of renewable biomass, in particular, food waste.

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