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Microbial biodegradation as an option for waste utilization during long-term manned space missions

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Planned space flights in foreseeable future to Mars pose many important issues to be solved by nowadays science, especially the problem of organic wastes accumulated in large quantities. The flight from Earth to Mars takes about 520 days. The crew possibly could consist of 6 cosmonauts, each of them needing daily: oxygen (1 kg of liquid), water (1-2 litres), food (2-3 kg). The total weight is about 5 kg/day or 30 kg/day for the entire crew. During long-term manned missions, the wastes are prohibited to be disposed of in space. They must be recycled. It is known that specific bacteria are able to convert waste substrates into usable nutrients and minerals. The enormous challenge is: reducing the volume of wastes to generate liquid and gaseous fractions which could be used in the production of food, water and oxygen. Simultaneously, some biogas is obtained as a source of energy. We present the development of a process of biodegradation of cellulose containing substrates similar to personal hygiene materials for cosmonauts by selected non-pathogenic bacteria. Experiments were conducted in terrestrial conditions where a degree of biodegradation of 72% of cellulose containing substrates at anaerobic, mesophilic conditions was realized. The selected bacterial community was genetically identified. The most abundant species were *Bacteroides oleiciplenus*, *Clostridium butyricum*, and *Ruminiclostridium papyrosolvens*. Concentration and profile of volatile fatty acids accompanying the biodegradation process in a bioreactor were also followed.

Keywords: Cellulose containing wastes, Biodegradation, Bacterial community, Long-term manned space missions

Abbreviations: MELISSA: Micro-Ecological Life Support System Alternative; ESA: European Space Agency; AD: anaerobic digestion; ADS: anaerobic digestion sludge; DBD: degree of biodegradation; D: dilution rate; VFA: volatile fatty acids.

INTRODUCTION

Research related to Life Support Systems for long-term space flights is deepening in recent years. Due to vast operating distances and the resulting long travel times the maintenance of the crew has to be fully accomplished on board [1]. For the whole period the cosmonauts will require oxygen, water and food, as well as hygienic materials. After use they have to be recycled. An international group of experts is already working to create a closed micro-environmental ecosystem - the Micro-Ecological Life Support System Alternative (MELISSA), project of the European Space Agency (ESA) [2]. In it organic waste will be utilized by different groups of bacteria in special bioreactors. The closed system MELISSA is inspired by aquatic ecosystems and is designed as a model for studying regenerative life-support systems for long-term space missions. The split structure of the cycle and the choice of several microbial processes have been made to simplify the behavior of this artificial ecosystem. The leading elements are the production of food, water and oxygen from the organic waste of the mission. The choice of this split structure is conditioned by high reliability and safety requirements and the limited space for realization. Among the wastes that are formed in the conditions of manned space flight, a significant proportion is spent on personal hygiene. The problem of waste utilization is very acute as there is no shower on board and wipes and towels are the most common means of hygiene procedures, forming consecutively the largest waste volume. Together with this a necessary element of the closed system of manned spacecraft are considered greenhouses for the reproduction of the vegetative part of the diet of cosmonauts. Vegetable remains become another waste for recycling [3]. In addition, the waste contains products of human body secretion, which are dangerous in the sanitary-epidemiological sense.

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In the perspective of interplanetary flights based on the requirements of planetary quarantine, it is not possible to carry out the contaminated with microorganisms' debris beyond the spacecraft. On Earth, the accumulation of these residues may cause serious environmental problems. At the same time, it may serve as a significant resource, as the high organic content of vegetable residues and other cellulose containing wastes could make them a potential source for renewable energy [4].

Anaerobic digestion of organic wastes is a very attractive biotechnology also in the field of the renewable energy sources and biofuels. Anaerobic digestion is a multi-step biotechnological process with H_2 as a non-accumulating intermediate product. Recently, the interest in H_2 production through anaerobic digestion has increased [5]. Lignocellulose degradation is indeed a process with complex regulation, as has already been demonstrated for the bacterial genera *Caldicellulosiruptor, Fibrobacter* or *Ruminicoccus*. In the same time, the presence of other proteins involved in chitin degradation (chitinases and *N*-acetylglucosaminidases) and starch catabolism (such as amylases, maltases and fructofuranosidases) in the bacterial proteomes during growth in cellulose that we have observed, suggests that cellulases and hemicellulases may share their regulatory systems with other proteins [6].

Co-cultures often present improved performance over corresponding monocultures. The mechanisms involved may include enhanced substrate utilization, overcoming of nutritional limitations, reduction of the levels of scavengers and achieving superior overall activity, conversion and enzymatic action. In lignocellulose degradation processes, co-cultures of particular nature are superior to monocultures, as they allow division of labor in the metabolic processes that are required by the substrate. Clearly, microorganisms often lack some key metabolic pathways, which may be supplemented by others [7]

Various materials for cleaning and absorbing, including cotton towels, paper towels and napkins, "wet wipes" and gauze, are used on the International Space Station. The weekly schedule of astronauts includes cleaning the station, which involves decontamination of many surfaces with wet wipes. This leads to the accumulation of a large amount of cellulose materials, as well as other similar items such as packaging of the used provisions. To be recycled, the used textile and paper materials must be collected separately. This allows for the development of different technologies for the re-use of waste in small closed systems such as the spacecraft. Recovery of various organic wastes in space flight is an actual problem of modern astronautics and future interplanetary and other long-duration missions. Currently, organic waste is incinerated in the Earth's atmosphere during cargo vessels reentry. The maintaining of a closed ecosystem in the spaceship is considered as one of the main ways of ensuring the food and air for the crew in the long-term missions. Accordingly, ESA started to develop a high-tech bio-waste recycling system for space missions, stepping on the biological approach for recycling that included collection of data under real operating conditions. Its aim is to construct autonomous habitats in deep space, supplying astronauts with fresh air, water and food through continuous microbial recycling of organic wastes [8].

For the purpose of MELISSA the highest degree of biomass liquefaction must be achieved for more complete nutrient recovery in the following compartments. Therefore, the most challenging part is to find out the most suitable way for solid waste degradation and liquefaction [2].

The species that degrade cellulose belong mainly to the genera *Cytophaga, Cellulomonas, Cellvibrio, Bacillus, Clostridium* and *Sporocytophaga* [9]. The strictly anaerobic, thermophilic bacterium *Ruminiclostridium thermocellum* is the microorganism with the fastest documented growth rate on the recalcitrant substrate crystalline cellulose [10]. These organisms achieve a remarkable ability by forming very large extracellular multi-enzyme complexes, known as cellulosomes. Similar complexes are formed by related Clostridia (such as *Clostridium acetobutylicum, Clostridium cellulolyticum, Clostridium cellulovorans, Clostridium josui* and *Clostridium papyrosolvens*) and other anaerobic cellulose-degrading bacteria, such as *Acetivibrio cellulolyticus, Pseudobacteroides cellulosolvens* and *Ruminococcus albus* [11]. Several methods had been applied to investigate their microbial diversity: clone library of 16S rRNA genes, denaturing gradient gel electrophoresis (DGGE) analysis, fluorescence *in situ* hybridization (FISH), etc. Metagenomics is an efficient method for determining the complex microbiota structure and performing metabolic mechanism analysis. It is applied for elucidation of community structure and metabolic pathway analysis to determine the mechanism of cellulose degradation in natural consortia [12, 13]. The aim of our study was to develop a laboratory approach for bacterial degradation of cellulose containing hygiene materials similar to those used by the crews of spacecrafts accumulated during long-term space missions.

EXPERIMENTAL

Analytical methods

Cellulose concentration was estimated by a spectrophotometer using anthrone reagent according to Updegraff [14].

Volatile fatty acids concentrations were measured by a gas chromatograph "Focus GC" Thermo Scientific, equipped with a split/splitless injector, TG-WAXMS (length -30 m, ID - 0.25 mm, film - 0.25 μm) column and FID.

Released gas volume was determined using a graduated cylinder in the gas holder; concentration of methane (CH_4) - with the automatic gas analyzer "Dräger", X-am 7000, carbon dioxide (CO_2) and hydrogen (H_2)– with Gasboard gas analyser (China 3100D).

Experimental conditions

Working methane and hydrogen generating anaerobic digesters were used as a source of bacterial inocula. Laboratory bioreactors with working volumes of 1 dm^3, 2 dm^3 and 14 dm^3, at a speed of the stirrer 50 rpm were involved. The first two operated in a mesophilic mode (35-37 °C), and the third - in a thermophilic (55 °C) mode. The temperature was measured and controlled by electronic regulators, the accuracy of the regulation (in normal operation) was ± 0.5 °C. The stirring was done by constant electric motors and was about 100 rpm for all bioreactors. After feeding, each time a purge was made to ensure an anaerobic environment. Substrates used were filter paper, microcrystalline cellulose and medical gause. Whatman no. 1 filter paper was used as single rectangular pieces.

Anaerobic cultivation techniques and appliances were used. The bacterial inoculum from the laboratory bioreactors was cultured at 37 °C and 55 °C, respectively, under anaerobic conditions created in anaerobic jars of anaerobic gas-generating gasPakTM EZ sachets for anaerobic container system (Becton Dickinson, 260678). The anaerobic conditions of the environment were proved by indicators (Merck, 1.15112.0001). Process duration was 7-30 days.

Cultivation media

For 1 L of 520 DSMZ medium preparation the following components were required: 1.30 g of $(NH_4)\ 2SO_4$, 1.50 g of KH_2PO_4, 2.90 g of $K_2HPO_4 \times 3\ H_2O$, 1.25 ml of $FeSO_4 \times 7\ H_2O$ solution (0.1% w/v in 0.1 N H_2SO_4), 1.00 ml of a solution of (0.50 g of Na-resazurin solution (0.1% w/v), 0.20 g of $MgCl2 \times 6H_2O$, 75.00 mg of $CaCl_2 \times 2\ H_2O$, 6.00 g of cellobiose (the composition is presented below), 2.00 g of yeast extract, 1.50 g of Na_2CO_3 and 0.50 g of L-cysteine-HCl × H_2O.

For 990 ml of SL-10 trace elements: 10.00 ml of HCl (25%, 7.7 M), 1.50 g of $FeCl_2 \times 4\ H_2O$, 70.00 mg of $ZnCl_2$, 100.00 mg of $MnCl_2 \times 4\ H_2O$, 6.00 mg of H_3BO_3, 190.00 mg of $CoCl_2 \times 6\ H_2O$, 2.00 mg of $CuCl_2 \times 2\ H_2O$, 24.00 mg of $NiCl_2 \times 6\ H_2O$ and 36.00 mg of $Na_2MoO_4 \times 2\ H_2O$.

For 1 L of 122 DSMZ medium, the following components were required: 1.30 g of $(NH_4)_2SO_4$, 2.60 g of $MgCl_2 \times 6\ H_2O$, 1.43 g of KH_2PO_4, 5.50 g of K_2HPO_4, 0.13 g of $CaCl_2 \times 2\ H_2O$, 6.00 g of Na_2-β-glycerol phosphate (0.1% w/v in 0.1 N H_2SO_4), 0.25 g of reduced L-glutathione, 4.50 g of yeast extract, 0.50 ml of Na-resazurin solution (0.1% w/v) and 5.00 g of of cellobiose. Cultivation temperature was 37°C and 55°C.

Metagenome sequencing

Genomic DNA was extracted using a GeneJET genomic DNA purification kit (Thermo Fisher Scientific). DNA concentration and quality were measured using QB 3000 spectrophotometer. Illumina 16S library construction was performed using 16S rRNA gene specific primers, followed by MiSeq 2×300 bp sequencing, and FastQC quality control (Macrogen Inc., South Korea). The assembly results showed that quality-filtered data contained 43 056 268 total bases and 96 208 read counts. The percentage of Q20 quality reads was 97.03%.

Statistical analysis

The data are average from three independent experiments. Bars – ± SD (standard deviation). The values are statistically significant ($p<0.05$).

RESULTS AND DISCUSSION

Biodegradation of cellulose containing substrates by anaerobic microbial consortia

With the purpose of isolating cellulolytic microorganisms, batch anaerobic processes were carried out. Inoculum was taken from a working bioreactor containing powdered/microcrystalline cellulose (15 g/l) at a mesophilic regime. Bacterial communities were taken from the mesophilic and thermophilic bioreactors containing the same substrates but operating under different temperature regimes. Experiments at a mesophilic regime started with substrates: Whatman cellulose and filter paper. At the 15th day of the process partial degradation was registered (Figure 1 B), while after 20 days all pieces of filter paper disappeared (Figure 1C).

Fig. 1. Microbial degradation of filter paper pieces: at the beginning of the process (A), after 15 days (B) and after 20 days (C).

Biodegradation of cellulose containing components similar in composition to waste hygiene materials used by space crews was estimated. Lignocellulosic biomass possesses an inherent complexity and heterogeneity, so the efficient biodegradation requires activities of different types of hydrolytic enzymes and involvement of complex microbial communities that can work efficiently and synergetically. The generation of a complex microbial consortium is a promising approach for the efficient biomass decomposition [15].

The experiments continued by biodegradation of medical sterile gauze at anaerobic mesophilic conditions for 20 days (Fig. 2). Gauze, used by cosmonauts as sanitary napkins, consists of natural cellulose fibers (70-100%) and inclusions of synthetic polymeric materials (up to 30%).

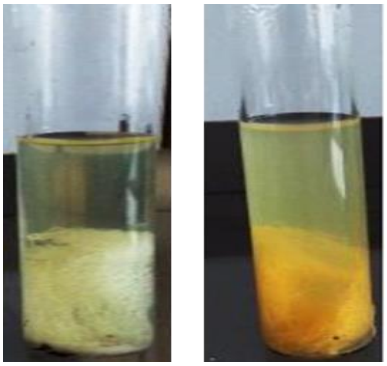

Fig. 2. Biodegradation of gauze at anaerobic mesophilic conditions for 10 days

Biodegradation of gauze at anaerobic mesophilic conditions was realized also at thermophilic conditions. The comparison did not reveal a significant difference in the residual cellulose between the 14th and 21st day at the two different temperatures. The mesophilic bacterial community after 14 days degraded cellulose to a greater extent (about 59%) compared to the thermophilic (about 35%), so we focused our attention on mesophilic processes. But for space flight conditions, maintaining a high temperature requires energy consumption and is not life-supporting for astronauts in long flights as it is one of the limiting factors. And our results showed the opposite. It is known that thermophilic cellulolytic bacteria of the genus Clostridium are the most promising agents capable to destroy cellulose with various degrees of ordering. But for the conditions of a space flight, such energy consuming process is not applicable.

Single colonies were isolated (using Mueller Hinton agar and TSA) at the end of the biodegradation process. They showed cellulolytic activity. Presence of spore-forming rod-shaped clostridial forms was established, following the light microscopic observations (Figure 3). From the photodocumentation, the presence of Gram - (-) spore-forming rod-shaped (short and long) and Gram - (+) clostridial typical forms is observed. Some of the cells are in the process of division, so they look bipolar.

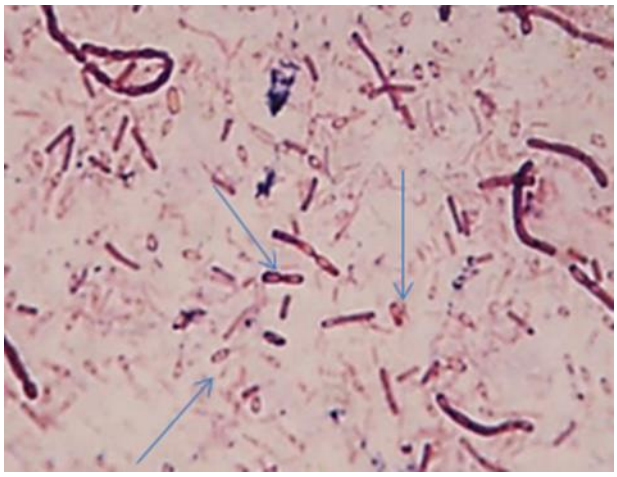

Fig. 3. Light microscopy image of the bacterial consortium realizing biodegradation of cellulose.

Stock cultures were prepared and preserved at -20ºC with a cryoprotector for further use.

Next experiments were conducted for degradation of cellulose containing wastes using the selected microorganisms.

Cellulose degradation, gas formation and volatile fatty acids accumulation

The dynamics of the degradation of a model substrate (filter paper) by the selected mesophilic bacterial community showed most intense degradation during the first 17 days of the process. The correlation between substrate degradation and biogas accumulation is presented on Figure 4.

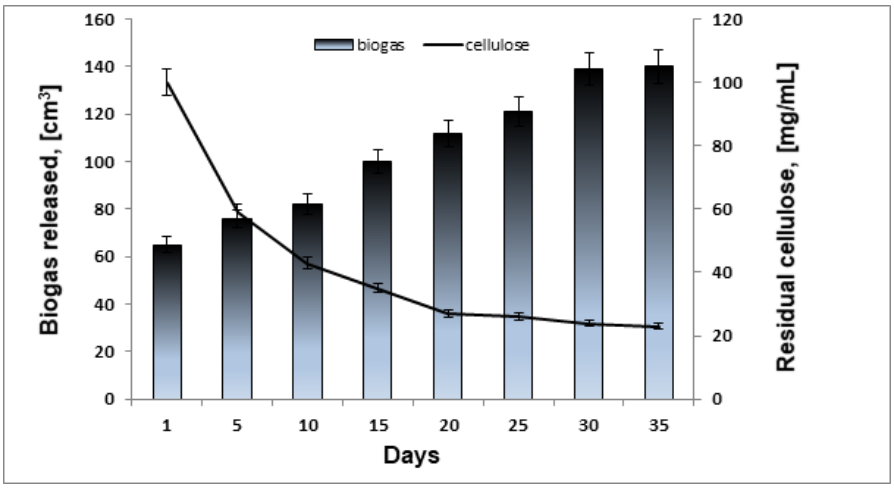

Fig. 4. Dynamics of cellulose biodegradation in relation to biogas released.

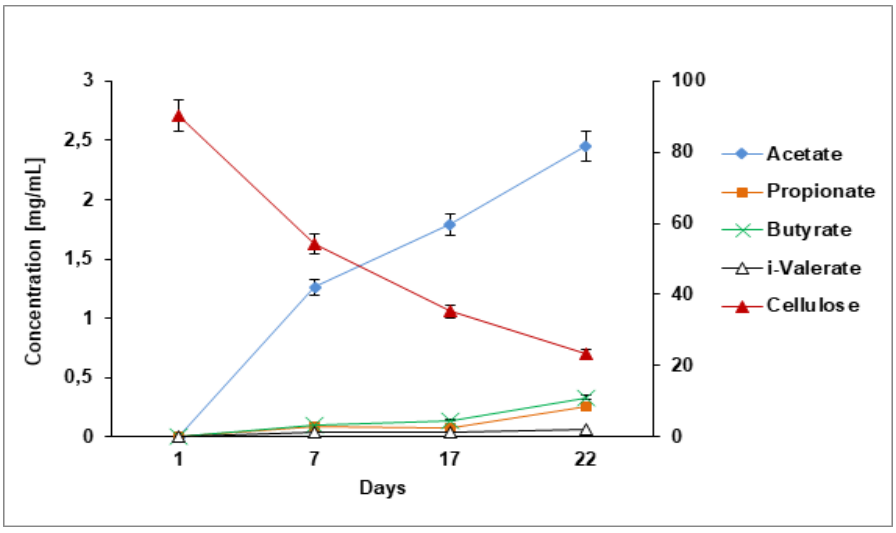

Fig. 5. Profile of fatty acids at the end of the process of biodegradation.

The degree of cellulose biodegradation was determined to be 72% at 37 ºC. The process was accompanied by biogas release, in which volume the percent of hydrogen was calculated to be up to 45%. These results are comparable with those obtained by Chu *et al*. [16] and Lay [17], which might show that the degradation process is paired with hydrogen production. In that case we could suggest that the cellulose degradation was realized by mixed microbial consortia *via* mixed acid fermentation. Hydrogen is known as a clean energy resource and is one of the most important elements. The biological production of hydrogen by using wastes and other biomass as raw materials has been attracting attention as an environmentally friendly process that does not consume fossil fuels. While conversion of biomass resources to hydrogen gas by fermentation has been extensively studied, most studies have been carried out with pure cultures of the isolated strains [18].

Profiles of volatile fatty acids - a major metabolic product in anaerobic biodegradation were investigated. Volatile fatty acids can serve as substrates for the next step in a closed micro-environmental life support system, such as MELISSA. The concentration of VFA was followed during the fermentative process. The biodegradation of the cellulosic material lead to accumulation in the medium mainly of acetate, followed by butyrate and propionate in the process with inoculum from a mesophilic hydrogenic reactor and substrate filter paper (Figure 5).

This result is also obtained from other authors [16, 19, 20]. According to Zang *et al*. [19], the concentration of acetate increased as pH dropped below 6.5 and decreased as pH and butyrate increased. This result suggests that the variation in pH led to changes in the distribution of fermentative products. Hydrogen production and decrease in pH were accompanied by the formation of VFA and ethanol throughout the cellulose-consuming

fermentation. In our experiments the total concentration of VFAs was 3 mg/ml, acetate was 80% among other VFA detected.

Metagenomics

We continued with identification by molecular-biological methods the bacterial population which can degrade cellulose from different sources as filter paper and other hygienic materials.

To investigate the microbial diversity involved in the production of hydrogen from cellulose, a metagenome profiling was performed (Figure 6).

Our results showed that cellulose degradation is most probably due to the presence of members of genera *Clostridium* (DNA copies 5480), *Bacteroides* (DNA copies 4263), and *Ruminiclostridium* (DNA copies 2590). Among them, the most spread species are *Clostridium butyricum*, *Bacteroides oleiciplenus* and *Ruminiclostridium papyrosolvens*. About ten times less in the sample were the representatives of the genera *Dendrosporobacter*, *Oscillibacter* and *Caproiciproducens* with the most abundant species *O. ruminantium*, *Capr. galactitolivorans* and *D. quercicolusi.*

A number of structurally stable multispecies consortia with high cellulose-degrading activity were obtained by successive culture enrichments using agricultural biomass as the sole carbon source under meso- and thermophilic conditions. These symbiotic consortia can efficiently degrade various cellulosic materials. Ruminal methanogenic communities were composed of hydrogenotrophic methanogens dominated by the order *Methanobacteriales* regardless of the host species. The methanogenic communities changed significantly during the enrichment procedure, but still the strict hydrogenotrophic *Methanobacteriales* and *Methanomicrobiales* were the predominant orders in the enrichment cultures [21].

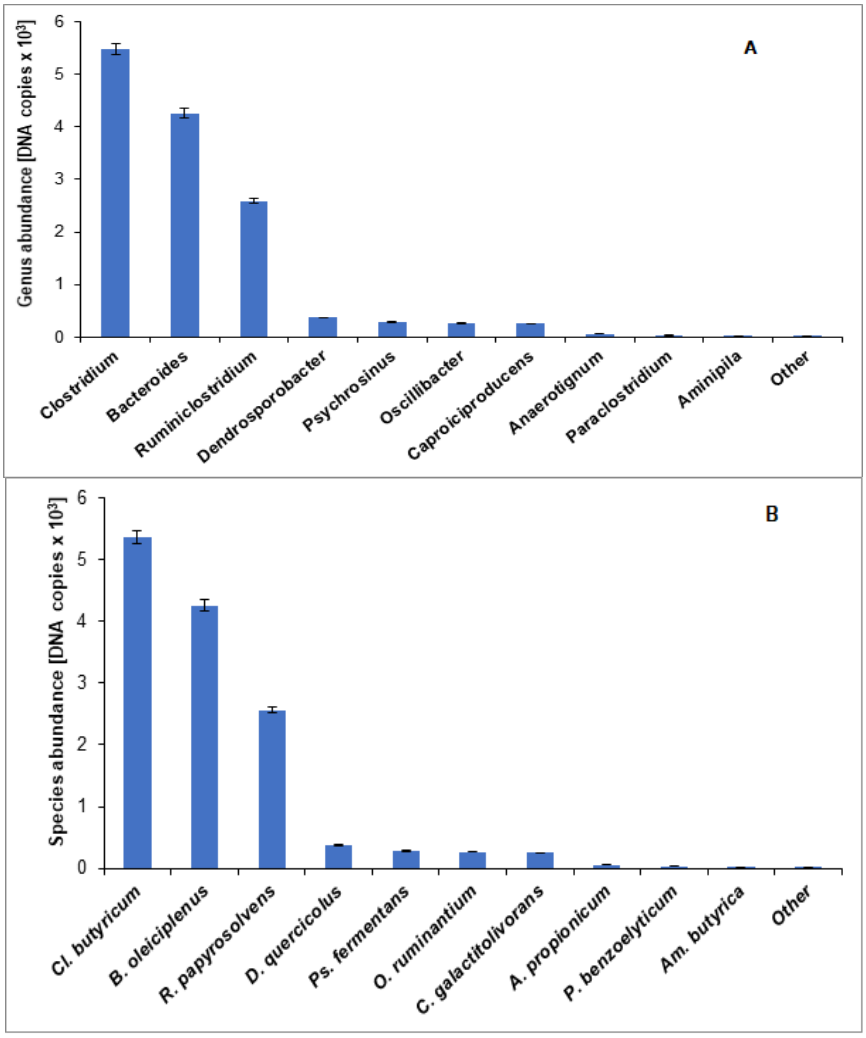

Fig. 6. Genus (A) and species (B) abundance in the investigated microbial consortium.

From the cellulose degrading bacteria, *Bacteroides oleiciplenus* is a representative of a group of microbes that constitute the most abundant members of the intestinal microflora of mammals. This organism produces many extracellular enzymes which assist in the breakdown of complex plant polysaccharides such as cellulose and hemicellulose and host-derived polysaccharides such as mucopolysaccharides. *Clostridium aciditolerans* is an obligately anaerobic, spore-forming, moderately acid-tolerant bacterium. The strain JW/YJL-B3T utilized beef extract, casamino acids, peptone, tryptone, arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, raffinose, ribose, sucrose, xylose, pyruvate, glutamate and inulin as a carbon and energy source. There were no indications of growth under aerobic or autotrophic conditions. The isolate produced acetate, butyrate and ethanol as fermentation end products from glucose [22].

The species *Ruminiclostridium papyrosolvens*, according to Dassa *et al.* [23], is able to degrade cellulose. *C. butyricum, C. saccharolyticum, C. galactitolivorans* and *D. quercicolus* are bacterial species which produce hydrogen from cellulose sources. *C. butyricum is a* producer of hydrogen from molasses [24], while the major products of *D. quercicolus* grown in a medium with yeast extract, peptone and fructose were acetate, propionate, propanol, and hydrogen [25]. The strict anaerobe *C. galactitolivorans*, Gram-positive, non-spore-forming, rod-shaped bacterial strain was isolated from an anaerobic digestion reactor during a study of bacteria utilizing galactitol as a carbon source [26].

Both *Bacteroides* and *Clostridium* are anaerobic rod-shaped bacteria with indispensable role in cellulose degradation in living nature. *Bacteroides oleiciplenus* is known to produce extracellular enzymes which assist in the breakdown of complex plant polysaccharides such as cellulose and hemicellulose [27]. *C. butyricum* also has high capacity for cellulose degradation [28]; *Ruminiclostridium cellulolyticum* is anaerobic bacterium that depolymerizes cellulose and related plant cell wall polysaccharides *via* production of large extracellular multi-enzyme complexes termed cellulosomes [29].

Depending on the nature of the sample, distinct cellulose-degrading microbial communities could be found. By Wang *et al*. [30], a cellulolytic microbial community capable to degrade cotton and paper contains *Bacillus thermoamylovorans, Paenibacillus barengoltzii, Proteobacterium, Pseudoxanthomonas taiwanensis, Rhizobiaceae, Beta proteobacterium, Petrobacter succinimandens, Tepidiphilus margaritifer*. By omics-based research other authors found a community with cellulose activity contacting *Clostridium sporogenes, Clostridium thermosuccinogenes, Clostridium thermocellum, Clostridium straminisolvens, Brevibacillus borstelensis, Cellulosilyticum lentocellum* [31]. Metagenome investigations of biodiversity in an anaerobic digester for biogas production by Sun *et al*. [32] showed that ubiquitous genera involved in the cellulose hydrolysis step include *Clostridium*, *Bacteroides*, *Succinivibrio*, *Prevotella* and *Ruminococcus*. *Fibrobacter*, formerly grouped to *Bacteroides*, as well as *Ruminococcus* and uncultured bacteria have also been suggested to play important roles in cellulose hydrolysis in the rumen; *Firmicutes* and *Bacteroidetes* were suggested to be important in a hydrolytic/acidogenic digester fed with dried hay and straw [32].

Illumina high-throughput sequencing was used to explore the microbial communities and functions in anaerobic digestion sludge (ADS) from two wastewater treatment plants based on a metagenomic view. Taxonomic analysis indicated that *Proteobacteria* (9.52–13.50 %), *Bacteroidetes* (7.18 %–10.65 %) and *Firmicutes* (7.53 %–9.46 %) were the most abundant phyla in the ADS [33].

A series of recent studies had indicated that enriched microbial communities, obtained from environmental samples through selective processes, can effectively contribute to lignocellulose degradation, combining ecological theory and enrichment principles to develop effective lignocellulose-degrading minimal active microbial consortia [34]. Using them with optimal substrate supply, appropriate mixing, maintaining pH at the optimum level and removing the resulting fermentation products can significantly improve the degree and rate of degradation of the substrates used.

CONCLUSIONS

Microbial degradation of cellulose containing substrates similar in composition to the hygiene materials used by space crews was realized by a selected genetically identified bacterial community. An effective anaerobic biodegradation process was accomplished applicable to laboratory biotechnology.

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