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Original Research

Optimized Methods for Stabilization of Microbial Communities Specializing in Biodegradation of Organic Environmental Contaminants

J. Augustynowicz[1], P. Kaszycki[1], M. Kuś[1], A. Białecka[2], H. Kołoczek[1]*

[1] Department of Biochemistry, Faculty of Horticulture, University of Agriculture, Al. 29 Listopada 54; 31-425 Kraków, Poland
[2] The Dr. Jan Bóbr Centre of Microbiological Research and Autovaccines, Sławkowska 17, 31-016 Kraków, Poland

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Abstract

The aim of this study was to optimize storage conditions of a microbial community used for degradation of petroleum-derived environmental contaminants. Microorganisms were either freeze-dried or directly frozen (-20°C) in the presence of four stabilizers: trehalose, sucrose, glycerol and DMSO. It was found that preincubation with trehalose and sucrose had a positive impact on cell viability for both tested storage techniques. Disaccharide-stabilized consortia were more biodiverse than control samples (untreated with any protectants) and they retained high xenobiotic biodegradation capabilities. The effect of glycerol and DMSO was unexpectedly poor, contradicting other findings on the protective action of these compounds on monocultures. Higher cell survival was achieved only upon short-term (7 days) freezing, whereas DMSO proved to be lethal in the case of freeze-dried communities. Taking into account practical and economic reasons, the use of sucrose rather than the more expensive trehalose appears as the most efficient method for microbial consortia biostabilization during long-term storage. The experimental work provides some important data concerning the problem of elaboration of improved methods for preserving robust microbial communities to be used in environmental biotechnology practice.

Keywords: microbial consortia, biodiversity, freeze-drying, freezing, petroleum-derived xenobiotics, biodegradation

Abbreviations

ATCC – American Type Culture Collection,
DMSO – dimethylsulfoxide,
GC – gas chromatography,
IIF – intracellular ice formation,
CFU – colony forming units

*e-mail: koloczek@ogr.ar.krakow.pl

Introduction

Microorganisms capable of biodegrading petroleum-derived compounds proved efficient in a number of cleanup projects of hydrocarbon-contaminated soil and water [1-3]. Biorecultivation of polluted areas was achieved upon neutralization of environmental contaminants with specialized, multi-species microbial consortia derived from naturally existing, autochthonous bacteria and microscopic fungi [4-9]. These consortia were capable of efficient oxidation of heterogeneous xenobiotics due to synergic action of all the

component strains. Their biodiversity implied the resistance to particularly noxious and toxic agents, high adaptation potential as well as the ability to further specialize upon contact with new and unknown chemicals. Several microbial communities were developed for environmental applications in the Biochemistry Department of Agricultural University in Kraków. They were widely used for industrial wastewater treatment and bioremediation of soil affected by oil leaks from petrol stations, refinery-generated sludges, used motor and transformer oil [9-11].

Technological requirements involving storage and transportation of biocenoses prior to their use produce the need to elaborate short- and long-term methods for stabilization of cellular suspensions. Since there appears to be no single and universal storage method applicable to all microorganism species (Norm EN 12322:1999; [12]), optimal conditions should be established for each strain by separate experiments. In this paper the economic aspects, technical possibilities, and the limited access to sophisticated equipment in industrial practice were taken into consideration and two storage methods were examined: low-temperature and freeze-drying (lyophilization). Both techniques have been widely used for storage of bacterial and fungal monocultures [13-15]; however, there are no reports on stabilization of complex microbial consortia consisting of specialized strains.

Freeze-drying is based on cell drying under low pressure, following cell culture freezing. This technique, together with freezing itself, generates strong physiological stress that results mainly from the limitation of water accessibility to cellular structures (desiccation). In general, freezing first affects the extracellular solution by reducing the water pool surrounding the cell and inducing water leak out of the cell interior. This increases the protoplast tendency to shrink as well as in several other concomitant mechanical alterations that occur within cell organelles. The temperature drop below 0°C may also lead to the generation of ice crystals inside cells (*intracellular ice formation*, IIF) which is even more harmful to microorganisms. As a consequence of water leakage and IIF effect, all the cytoplasmic and cell-sap constituents become dissolved in a solution of reduced volume, resulting in the elevation of electrolyte concentration that leads to protein denaturation and enzyme inactivation. In addition, as the water accessibility drops, the van der Waals interactions between fatty acid acyl chains strengthen and thus they tend to induce phase transitions within biological membranes. This in turn reduces plasmalemma selectivity and finally impairs cellular transport [16].

In biotechnological practice there is a growing interest to use natural strategies employing endogenous cell protective mechanisms of various species that usually survive extremely adverse conditions [16-18]. Some of these organisms, upon exposure to physiological stress produce osmotically active substances that include polyhydroxylic compounds, especially disaccharides such as sucrose and trehalose.

Cryoprotectants used in industrial and medical applications comprise both naturally-produced and synthetic compounds. Three out of four protective agents tested in this study, i.e. sucrose, glycerol and synthetic dimethylsulfoxide (DMSO) have been most widely used as stabilizing factors for microbial monocultures [19]. Trehalose, in turn, was found to reveal particular cryoprotective potential with regard to bacterial suspensions and eukaryotic cultures [15, 20].

In the case of freezing performed with slow temperature decline the use of trehalose and sucrose stimulates water leak out of the cell. This is caused by the fact that these disaccharides are unable to enter cellular interior. At the same time, however, due to the sugars' polyhydroxylic structure, they can replace water molecules and thus stabilize cell membranes by forming hydrogen bonds with proteins and phospholipid phosphate groups. They also tend to decrease the lipid phase transition temperature which enables them to retain the liquid crystal structure of bilayers [21]. In turn, the action of DMSO and glycerol is based on their penetration through plasma membranes and prevention of IIF by lowering the freezing temperature of intracellular solution [16, 19].

If the temperature drop below the freezing point is rapid enough, it may lead to a physiologically advantageous phenomenon of intracellular vitrification upon achieving a glassy matrix state of cytoplasm. A vitrified state prevents the incidents of generating ice crystallization centers due to the ceased molecular diffusion [22]. In our previous work [23] the technique of immediate freezing in liquid nitrogen (-196°C) prior to sample lyophilization was shown to preserve the relatively highest cell survival. It should be pointed out, however, that the main risk for freeze-dried cultures is caused by recrystallization during the process of unfreezing [16]. In this case, the protective potential of disaccharides may be explained analogously to the conditions of freezing with slow temperature decline.

In a paper of Augustynowicz et al. [23], the positive influence of trehalose on the survival of bacteria was shown for a multi-component biocenosis stored under various conditions. Here, the authors present a comparative study of the effect of trehalose and other protectants mentioned above, on degradation activity and biodiversity after long-term storage of specialized consortia.

Experimental Procedures

Cultivation of Microbial Community

The experimental work was performed employing bacterial biocenosis developed in the Biochemistry Department of Agricultural University, Kraków. All the constituent microorganisms were environmental, autochthonous strains, selected and isolated from soil and water sites heavily polluted with petroleum-derived hydrocarbons. A mixture of such microorganisms was then subjected to continuous selective and adaptational pressure by means of incubation with sub-lethal concentrations of organic xenobiotics. The resultant microbial community comprised bacterial species belonging to various genera of predominantly

Gram-negative strains: mainly *Pseudomonas* (constituting up to 80% of the total population), *Corynebacterium, Acinetobacter, Alcaligenes, Comamonas, Brevundimonas, Oligella, Ochrobactrum, Burkholderia, Chromobacter, Micrococcus, Moraxella, Stenotrophomonas*, and several others. Upon selection, the consortium tended to reveal unique enzymatic activities and achieved optimal metabolic potential enabling oxidation of heterogeneous organic contaminants. It proved capable of degradation of a variety of petroleum-derived chemicals such as aliphatic and aromatic hydrocarbons, polyaromatic hydrocarbons (PAH), polychlorinated biphenyls (PCBs), as well as other xenobiotics [9, 10, 24].

Bacteria were cultured at room temperature, in 300 ml Erlenmeyer flasks in a minimal liquid medium containing 23 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4 \times 7H_2O$, 7 mM KH_2PO_4, 1 mM $CaCl_2 \times 5H_2O$ (POCh, Poland), and supplemented with traces (0.025%) of casein peptone and yeast extract (BTL, Poland). As a carbon source a defined mixture of hydrocarbons was used as a selective factor for cultures to reveal the highest biodegradation potential. The mix was applied at 0.5% (v/v) final concentration and consisted of diesel-oil (Eko-diesel Plus 50 D, obtained from the Trzebinia Refinery, Trzebinia, Poland), used automotive engine oil, and lighting kerosene (1:1:1, by volume).

For all further experiments, cellular suspensions of the late-logarithmic growth phase (biomass density of 1.0-2.0 $\times 10^9$ cells/ml) were used.

Determination of Biomass Density/Cell Survival

Cell density during growth and survival tests was determined with a plating method by spreading defined volumes of appropriate culture decimal dilutions onto agar-solidified optimal media (2.5% enriched agar, BTL, Poland), incubating 3 days at 37°C and then by counting CFU (colony forming units). Prior to dilutions, in order to obtain homogeneous cell suspensions, approximately 1 ml of the original bacterial sample was sonicated in an Eppendorf tube under mild conditions for 10 min using a laboratory ultrasound washer (UN-2 Unitra/Unima, Poland).

Incubation with Stabilizers

Microbial consortia were biostabilized during storage using the following compounds (effective concentrations are given in parentheses): trehalose (0.1 M, dihydrate, ICN Biomedicals), sucrose (0.1 M, Sigma), glycerol (1.1 M, Sigma) and DMSO (1.3 M, Sigma). Prior to freezing or freeze-drying, 5 ml of bacterial suspensions were placed in 50 ml flasks and preincubated with a given compound by rotary-shaking at 160 rpm (15 min at 4°C with DMSO and 60 min at room temperature with either trehalose, sucrose, or glycerol). Both the protectant concentrations and preincubation times were chosen based upon the papers of Hubalek [19] and Augustynowicz et. al. [23].

Microbial Consortia Storage Conditions (Stress Factors)

Immediately after incubation with a select stabilizer, microbial samples (0.5 ml specimens in Eppendorf tubes) were subjected to one of the two stress conditions (methods of storage): freezing and freeze-drying. To observe bacterial culture response to physiological stress the cell survival rate was determined and expressed as a percent of the initial cell number per ml. The survival of consortia untreated with stabilizers was always monitored in control observations (biotic controls).

Freezing was performed by placing samples in a laboratory freezer set at -20ºC. The mean gradient of temperature decline was 5ºC per min, as established with a temperature probe. After 6-month storage the samples were reanimated by rotary-shaking for 120 min (conditions given above) and then the culture cell densities were determined.

Freeze-drying was carried out using an Alpha 1-4 (Martin Christ Gefriertrocknungsan-lagen GmbH, Germany) lyophilizer. After freezing in liquid nitrogen, the samples were put into a lyophilization chamber with pressure value set at 0.35 mbar. During consecutive steps (24 h total time) the temperature of the heating shelf rose gradually to achieve approx. 20ºC of the product. Lyophilizates were stored at -20ºC for 6 months and then resuspended in 0.5 ml of water, reanimated and checked for cell survival as described above.

Microbial Culture Growth in the Presence of Xenobiotics

After storage, the re-animated 0.5 ml bacterial samples stabilized with a given compound were inoculated into 300-ml flasks containing minimal growth medium to obtain 50 ml of diluted (100-fold) microbial culture. Then, each sample was supplemented with 2% (v/v) diesel oil as a sole carbon source and rotary-shaken (160 rpm) on a laboratory shaker at room temperature for 14 days. At the beginning of the experiment, and after 3, 7, and 14 days of incubation, 2-ml specimens were collected and checked for cell density as well as subjected to gas-chromatography analysis of xenobiotic content. In order to obtain homogeneous oil emulsions, flask contents were vigorously mixed for 2 min before collection of each specimen. Apart from biostabilized samples, the appropriate biotic (bacterial consortium stored without any protectants) and abiotic (sterile water containing 2% oil) controls were always made.

Analyses of Petroleum-Derived Xenobiotic Content

The content of organic xenobiotics in biodegradation tests was monitored using a GC-17A ver. 3 (Schimadzu) gas-chromatograph equipped with a Zebron ZB-5 capillary column (Phenomenex, 30 m/Ø 0.32 mm) filled with 5% Phenyl Polysiloxane (stationary phase, 0.5 μm thickness)

and a flame-ionization detector (FID). Nitrogen served as a carrier gas and the mobile phase flow velocity, as measured at the column outlet, was set at 35 cm/s. Sample volume injected onto a column was 1 µl. Chromatographic runs were carried out in a splitless mode and the temperature gradient was preset as follows: feeder at 250ºC, detector at 330ºC, column at 40ºC for 1 min, then the increase up to 330ºC (10ºC/ min).

Xenobiotics were extracted from aqueous suspensions with n-hexane (Sigma) with a two-step procedure. A 1-ml sample was treated with extraction solvent (1:1 v/v) and vigorously mixed (micro-shaker Techno Kartell TK 3S, Poland) for 1 min. Then, 0.8 ml of the resultant upper hydrocarbon phase was collected and the remaining volume was treated with hexane and extracted again. Both collected hydrophobic-phase fractions were pooled and subjected to GC analysis.

Determination of Species Content of Microbial Biocenosis

Freeze-dried microbial communities, stabilized with sucrose or trehalose and stored for 6 months were examined together with the biotic control. The samples were then reanimated (see above) and incubated with oil-derived xenobiotics for 3 days. The identification of species and determination of cell population density were carried out by plating appropriate decimal dilutions onto the following solid media (all from bioMerieux): optimal tryptic soy agar (TSA) supplemented with 5% sheep blood as well as the selective CPS ID3 (revealing both Gram-positive and Gram-negative bacteria), Cetrimide Agar (specific to *Pseudomonas*) and Candida ID2 (specific to yeast). The biological material was incubated under aerobic conditions at 35°C for 72 h and the formed bacterial colonies were examined. Since all the identified microorganisms were Gram-negative rods, each colony that revealed distinct morphological appearance was selected and a bioMerieux ID 32 GN biochemical kit was used to identify the genus and species. The kit is a standardized, semi-automatic system of high reliability (above 90%) and reproducibility, enabling identification of 50 bacterial genera (114 strains). To improve the test accuracy, up to 3 independent passages of each isolated microorganism were subjected to evaluation. The frequency of individual isolates in the original community was also determined.

All the chemicals used were of analytical grade. Experimental results represent mean values of at least three independent physiological runs, each performed in four measurement series.

Results

Preliminary studies of bacterial consortium survival proved that the original biocenosis remained stable when stored for 30 days at +4ºC with no exogenous protective factors and no active aeration. Under such conditions the

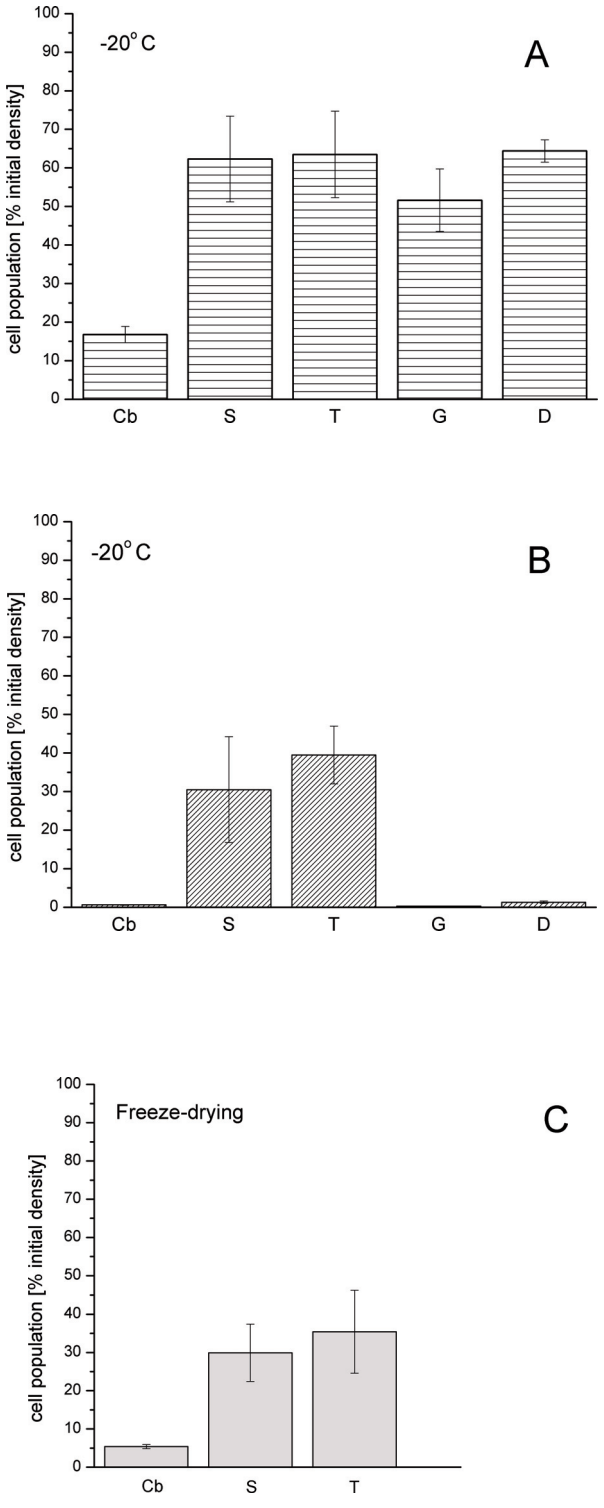

Fig. 1. Influence of storage conditions on cell survival of bacterial consortia stabilized with 0.1 M sucrose (S), 0.1 M trehalose (T), 1.1 M glycerol (G) and 1.3 M DMSO (D). (A) short-term (7 days) and (B) long-term (6 months) freezing at -20ºC , (C) long-term (6 months) storage after freeze-drying. (Cb) biotic control (lack of any protectant). In the diagram, relative mean (n=7) values of cell number per ml of culture are given and expressed as [%] of the initial microorganism population density (1.5×10^9 cells/ml).

cell population density was kept above 10^9 per ml and full physiological activity of consortia was achieved immediately on aerating and warming the suspension to room temperature (data not shown). However, a long-term (6 months) storage at +4ºC led to the total loss of the cell, which indicated a need for studies on optimizing culture freezing and lyophilization conditions.

Microorganism Survival under Freezing Conditions

As a consequence of freezing the microorganism suspensions and their subsequent storage at -20ºC for 7 days, 17% cell viability was established as compared to the original community. In this case, all the protective compounds used, i.e. sucrose, trehalose, glycerol and DMSO, had a positive impact on bacterial survival and led to the 3-4-fold increase of cell number, so that the value of 50-65% of the initial cell population was obtained (Fig. 1A).

Disaccharides proved to be highly effective stabilizing factors also in the case of long-term (6 months) consortia storage (Fig. 1B). For sucrose and trehalose, approximately 30% and 40% of the original cell viability was preserved, respectively. This means that the living cell number was up to 50 times higher as compared with the control experiment (non-stabilized biocenoses). At the same time, a relatively small positive effect of DMSO was observed (Fig. 1B bar D) whereas astonishingly, the presence of glycerol appeared to be detrimental (Fig. 1B bar G).

Microorganism Survival in Freeze-Dried Consortia

Lyophilization of biocenoses bears double stress conditions that result from freezing and dehydration. When the cells were reanimated immediately after freeze-drying of microorganism suspensions (no storage, data not shown), the number of CFU detected on plates decreased dramatically to approximately 15% of the initial value. The addition of trehalose and sucrose substantially suppressed this negative effect and led to cell viability 4-times higher than in control. Glycerol and DMSO pretreatment had no protective result: the presence of the former agent decreased the viability of cell population below the control level, whereas DMSO proved to be strongly toxic and reduced the survival over four orders of magnitude. Due to these and earlier (Fig. 1B) observations, both compounds were excluded from further studies on optimizing microbial storage conditions.

In the case of lyophilized consortia stored at -20ºC for 6 months, the application of disaccharides proved again their significant protective potential that led to an approximate 5-fold increase in cell survival relative to control. As seen in Fig. 1C, the administration of sucrose or trehalose enabled us to preserve 30% and 35% of cells, respectively, as compared to the original population density (microbial consortium prior to freeze-drying).

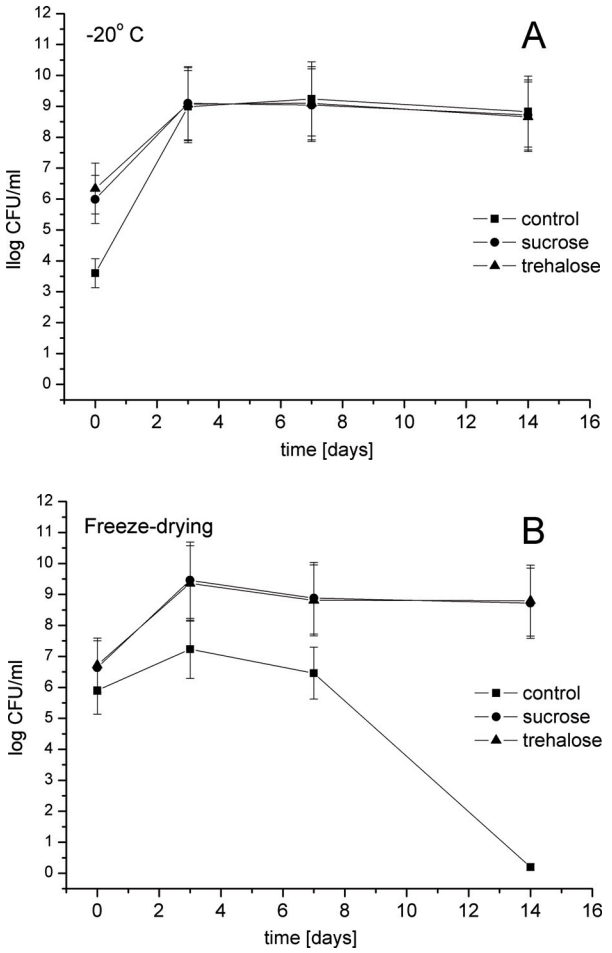

Fig. 2. Microorganism population dynamics upon incubation of bacterial consortia with 2% (v/v) diesel oil, after long-term (6 months) storage of frozen (A) and freeze-dried (B) samples. The initial biocenosis cell density was (1.3×10^9 cells/ml). (■) control experiment (lack of protectant), stabilization with sucrose (●) and trehalose (▲).

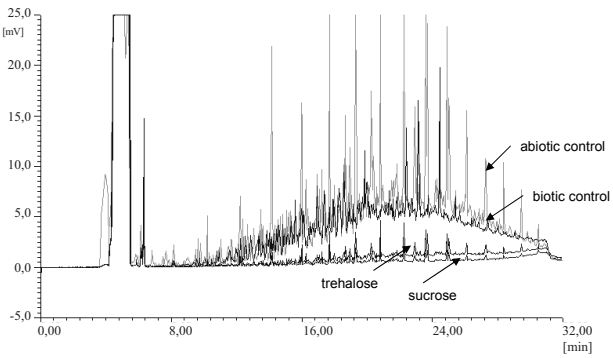

Fig. 3. Gas chromatography profiles obtained for organic extracts of 3-day microbial cultures in the presence of 2% diesel oil. Microorganisms were reanimated after 6-month storage of lyophilized samples. GC chromatogram descriptions: "sucrose" and "trehalose" – protective compounds used; "biotic control" – non-stabilized biocenosis, "abiotic control" – blank experiment (2% oil suspension in water).

Metabolic Activity of Biostabilized Microbial Communities

After 6-month storage, consortia stabilized with sucrose or trehalose and subjected either to direct freezing or to freeze-drying, were cultured in the presence of 2% diesel oil that served as a source of carbon. The most dynamic growth was observed for the first 3 days of incubation, when the cell number reached the level of initial biomass density (Fig. 2). Biomass growth upon assimilating the protectants themselves could be excluded since these compounds were present at very low concentrations due to a 100-fold dilution of bacterial suspensions prior to experiments.

Note that in the control experiment (lyophilization with no protective substances) the cell population growth was observed only within the first phase (3 days) of incubation with xenobiotics (Fig. 2B). Then, the cell number dramatically decreased to reach zero value upon 14 days of incubation, which suggests substantially lower metabolic potential of the non-stabilized biocenosis (see: Discussion).

The presented observations correlate with the studies of xenobiotic degradation with microbial consortia tested after freeze-drying. Exemplary GC profiles of organic extracts obtained from microbial samples are shown in Fig. 3. Disaccharide-stabilized biocenoses revealed substantially elevated biodegradation potential (about 10-fold) relative to control, and the removal of almost all the xenobiotic pool was completed within 14 days (Table 1).

Total contents of oil-derived organic substances, extracted from microbial cultures were expressed as areas under respective chromatograms. They are compared in Fig. 4 and Table 1, for consortia stored by means of freezing (Fig. 4A) and freeze-drying (Fig. 4B). It should be noted that for the latter storage method the protective effect of sugar stabilizers was significantly higher (Figs 3, 4B, Table 1).

The concentration of organic compounds in the blank test (abiotic control, i.e. aqueous 2% oil suspension) varied insignificantly throughout a 14-day experiment (data not shown). Thus, the error resulting from evaporation of low-boiling hydrocarbons upon prolonged rotary-shaking of the samples could be neglected.

Effect of Protectants on Consortia Biodiversity

Microbiological analyses were carried out to determine the influence of sucrose and trehalose on species content and cell number in cultures incubated for 3 days in the presence of 2% diesel oil. Lyophilized consortia were selected for evaluation since this method of storage led to the most pronounced differences in viability and metabolic activity in biostabilized cultures relative to control (see above).

Preincubation with either of the two disaccharides resulted in preserving five bacterial species, whereas only two were determined in a control experiment. Detailed identification of the strains isolated after reanimation of freeze-dried suspensions (Table 2) revealed differences in species content in samples administered with sucrose and trehalose. However, all the identified strains were Gram-negative bacteria, and two species: *Pseudomonas putida* and *Stenotrophomonas maltophilia*, were present in all samples, including control. Total cell densities in bioprotected samples were significantly higher than in control, which is in accord with earlier observations (Fig. 2B).

Table 1. Biodegradation efficiency of organic compounds obtained with bacterial consortia stored for 6 months at -20°C after direct freezing and freeze-drying, and then cultivated for 14 days in the presence of 2% diesel oil. The content of xenobiotics was calculated as areas under chromatograms with standard deviations not greater than 11% of the original values. Abiotic control – blank experiment (2% oil suspension in water); biotic control – non-stabilized biocenosis; sucrose and trehalose – protective compounds used.

Sample	Xenobiotic content [a.u.]	
	freezing	**freeze-drying**
abiotic control	5837	5837
biotic control	417	773
sucrose	208	72
trehalose	296	123

Table 2. Bacterial species and cell population determined in microbial consortia cultured for 3 days in the presence of 2% (v/v) diesel oil after 6-month storage of freeze-dried samples. Sucrose or trehalose was applied as a stabilizing agent; control – lack of any protectant used. The initial biocenosis cell density before lyophilization was 1.5×10^9 CFU/ml.

Bacterial species	**Number of bacterial cells per ml** [CFU/ml]		
	Stabilizing agent		**Control**
	Sucrose	**Trehalose**	
Pseudomonas putida	2.0×10^9	5.0×10^8	5.0×10^8
Stenotrophomonas maltophilia	2.0×10^8	2.0×10^8	3.2×10^8
Providencia rettgeri	1.1×10^8	–	–
Alcaligenes xylosoxidans	4.0×10^5	–	–
Alcaligenes denitrificans	3.0×10^6	–	–
Pseudomonas aeruginosa	–	2.0×10^5	–
Ochrobactrum anthropi	–	5.0×10^8	–
Morganella morganii	–	1.0×10^8	–
Total [CFU/ml]	2.3×10^9	1.3×10^9	8.2×10^8

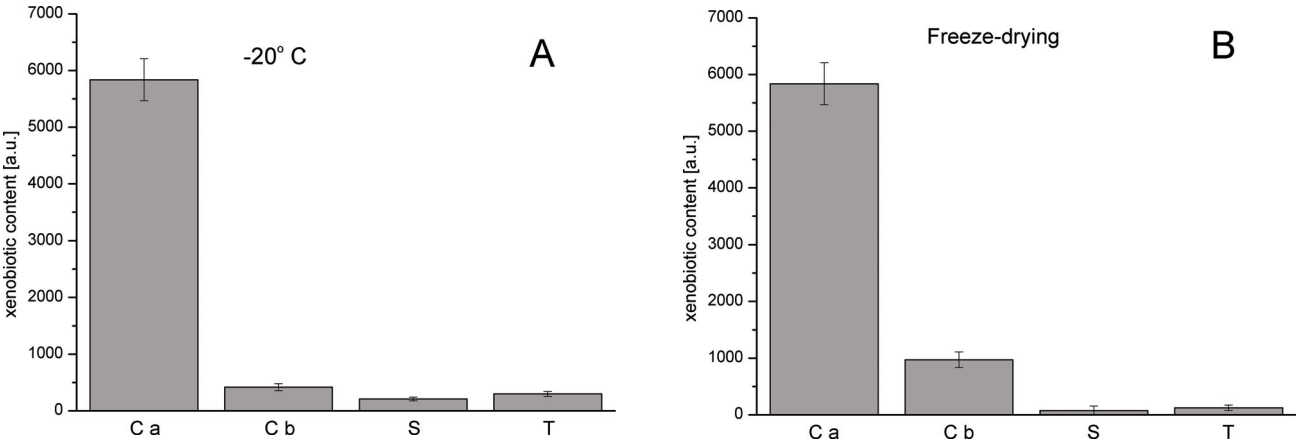

Fig. 4. Biodegradation efficiency of organic compounds obtained with bacterial consortia stored for 6 months at -20°C after direct freezing (A) and freeze-drying (B), and then cultivated for 7 days in the presence of 2% diesel oil. Bars represent the total content of organic fraction, expressed as areas under respective chromatograms (cf. Fig. 3). (Ca) abiotic and (Cb) biotic controls; (S) sucrose and (T) trehalose stabilization.

Discussion

Freezing and lyophilization are common methods of long-term preservation of biological material [25, 26]. In particular, the latter technique is the most convenient one for microbial biomass storage and has been widely applied to various valuable strains used in industrial biotechnology [15]. The main advantages of freeze-drying originates from substantial reduction of initial cell suspension volumes and from conditions that enable preserving high culture viability for a relatively long time. The above attributes proved to be especially suitable for transport and management of strain culture collections [27, 28]. Although the sample preparation procedures as well as technical requirements for lyophilization must comply with precisely defined laboratory conditions, the storage of lyophilizates is relatively simple, and typically it requires that ampoules be kept in a cool place (at 3-8°C) [29]. All the methods of freeze-drying elaborated upon until now have dealt with microorganism monocultures [19, 27, 28]. To our knowledge, detailed procedures concerning storage of complex bacterial consortia have not yet been optimized and they still require thorough studies.

Recently, the technique of cell deep freezing (below -70°C) is being developed [12, 29, 30]. The conditions for low-temperature preservation of a multi-component community were optimized in our previous work [23], which proved trehalose to be effective as a cryoprotecting agent. However, it should be stressed that the methods employing cryogenic temperatures are applicable to small culture volumes and they require access to a modern and well-equipped laboratory. Thus, for practical reasons, it appears that for industrial-scale volumes (the order of liters) the most suitable and economically reasonable method for microbial culture storage is freezing at -20°C.

Cell freezing and lyophilization negatively affected non-stabilized biocenoses in all the studied experimental models. Stress conditions generated by the two techniques were different: for freezing, a slow temperature decrease of approximately 5ºC per min. was observed, and for freeze-drying, a rapid temperature drop and dehydration resulted from the immersion in liquid nitrogen and subsequent sublimation under low pressure during the lyophilization process.

The efforts to optimize microbial consortia storage conditions were aimed at minimizing the negative effects of the observed stress factors. It is known that microorganism tolerance to physiological stress is different for different species [12, 13, 30]. Hence, the elaboration of one and universal method of bioconservation of mixed-culture communities is difficult. In order to keep the high metabolic potential of specialized biocenoses, both maximum cell viability and species biodiversity should be preserved. A promising approach to deal with the above problems is to biostabilize microbial consortia using chemical protective factors prior to storage.

The bacterial communities used in this work retained high survival rates when kept at +4ºC for 4 weeks in the absence of any protecting agent and with no active aeration. The observed stability gives evidence for strong adaptive potential to the imposed stress conditions and could be explained by a cell's ability to produce endogenous protective factors. On the other hand, it is known [12, 30] (and our research, not shown) that the viability of bacterial monocultures stored at +4ºC decreased rapidly over time. In the case of the consortia, the drop of cell survival was observed only when the storage periods exceeded 4 weeks and within 6 months the bacterial population died out completely. For these as well as practical reasons, cooling of the microbial suspension to +4ºC is the suggested method for consortia storage under 4 weeks.

The negative effects of both cell freezing and freeze-drying were suppressed by the application of sucrose or trehalose. Possibly, the use of protective compounds made it possible to preserve the integrity of membrane proteins and lipids present on the outer side of plasmalemma (see: Introduction). Cell viability levels in biostabilized cultures reached 30%-40% of the initial bacterial population density,

whereas in control tests the observed survival rate never exceeded 6%. This is a satisfactory result and can be compared with the literature data concerning bacterial and fungal monocultures [13, 31]. Moreover, preincubation with any of the protecting sugars enabled the consortia to keep high metabolic activity. This physiological potential was exhibited by means of culture growth on petroleum products (2% diesel oil) and was proved by direct GC measurements of hydrocarbon biodegradation.

It should be mentioned here that, unlike the freeze-drying conditions, consortia stored at -20ºC with no added protectants (biotic control, Fig. 2A) retained potential for growth in the presence of xenobiotics, although the capability for degradation of hydrocarbons was lower (Table 1). Since several potent strains might be lost upon long-term storage and to reduce such risk, the use of sucrose or trehalose is recommended also for cell freezing.

In contrast to the effect of disaccharides, DMSO and glycerol had no stabilizing action on long-term stored microorganisms, and in several cases they revealed an inhibitory or even toxic effect. The observed inapplicability of these substances for protecting complex consortia is quite an unexpected result especially when one considers their reported positive influence on microorganism monocultures: both DMSO and glycerol were shown to be highly-efficient stabilizers for bacteria, microscopic fungi, viruses, algae and protozoans [19]. Furthermore, glycerol is listed in ATCC standards and recommendations as an agent particularly applicable for preparing protective solutions for microbial suspensions [32]. In order to explain the above discrepancies, a complex mechanism of action of both DMSO and glycerol should be considered, which might lead to the final lack of any positive effect on mixed consortia. As these low-molecular chemicals are easy membrane-penetrating agents, they can promote vitrification of cytoplasm upon a rapid temperature drop; however, they may not be able to stabilize the formed glassy-matrix, as shown by Wolf and Bryant [33] and Rall and Fahy [34].

Microbiological analyses (Table 2) revealed that lyophilizates stored after preincubation with trehalose or sucrose were much more biologically diverse (five bacterial species determined) in comparison with the control experiment (two species). Both studied protectants preserved higher cell survival, but each of them lead to different species content of the resultant biocenoses. Note that two strains, namely *Pseudomonas putida* and *Stenotrophomonas maltophilia*, were present in all samples, including the control. Thus, it appears that although the presence of these two species is important for effective xenobiotic biodegradation, the other strains are also necessary to develop full enzymatic potential of bioremediation.

The original microbial community used in the study consisted of autochthons obtained from contaminated sites, which were capable of biodegradation of organic pollutants. It is well documented that for bioremediation purposes the development of mixed microbial consortia should be based on indigenous microflora rather than on commercially available, wild type collection strains [1-3, 5-11, 24, 35, 37, 38]. The autochthonous microorganisms are better fitted to the presence of xenobiotics and they often reveal rare metabolic pathways enabling efficient biodegradation. At the beginning of the experiment the community consisted of 39 species, mostly Gram-negative bacteria with dominating genus *Pseudomonas*, as determined in previous studies [11, 24]. The number of species, identified in cryoprotected and then reanimated consortia, was reduced when compared to the initial biocenosis. The observed decrease resulted from spontaneous mechanisms of bacterial communities selection and adaptation which was a typical phenomenon induced by the presence of environmental contaminants [35].

It should be stressed here, that the culture-dependent method employed for microbiological qualitative and quantitative evaluation, although simple and practical, is only partially accurate and reliable. This significant problem is created mainly by the presence of the non-cultivable strains under laboratory conditions [36]. Thus, our results might be further supported by other, culture-independent determinations such as 16S rRNA phylogeny evaluation, in order to better describe the microbial consortia. However, as discussed by Lovley [37], in bioremediation studies the methods applying genomic molecular analyses should be regarded as complementary rather than alternative to conventional microbiological techniques.

All the collected data regarding construction of microbial consortia used to biodegrade environmental contaminants confirm our idea of synergic effects revealed by biodiverse biocenoses. The metabolic activity of microorganisms cooperating in a community was always substantially higher than in monocultures [9-11, 24].

When the 6-month-stored bacterial lyophilizate control was grown in the presence of petroleum-derived compounds, a dramatic drop of cell numbers was observed within the second stage of incubation (7-14 days, Fig. 2). The cell population decrease may have been caused by two mechanisms. First, it could result from the generation of secondary, toxic metabolites upon degradation of heavy hydrocarbon fractions [38]. In such a case, the relatively more diverse consortia stabilized with protectants possibly kept the biochemical potential to neutralize toxic intermediates. Second, a heterogeneous hydrocarbon content of diesel oil might contain fractions of different metabolic accessibilities. Thus, it seems that the non-stabilized control cultures have not been able to assimilate the less-accessible carbon fraction and they died out earlier than the more complex, protectant-treated consortia that were able to produce a richer pool of enzymes.

In summary, the ability to maintain higher biodiversity in microbial samples stabilized by protective sugars – sucrose or trehalose – appears to serve as a key condition for acquiring high potential for degradation of hydrocarbons. This is because multi-species consortia become capable of bioremediation of complex xenobiotic mixtures due to complementation of specialized metabolic pathways of individual strains, which finally produces the effect of synergy.

Based on the obtained results it can be inferred that both freeze-drying and freezing at –20ºC can be applied as efficient techniques for long-term storage of complex microbial communities, provided that microorganisms have been properly biostabilized to retain high survival rates and biodiversity. Direct freezing should be used in the case of large cell suspension volumes and lyophilization appears to be particularly suitable for preserving inocula of specialized bacterial formulations. For the most efficient bioconservation, a supplementation of cell cultures with 100 mM sucrose as a protecting agent is recommended. Although the application of trehalose brings a similar positive effect, the high cost of the latter disaccharide makes its use for the industrial scale economically less reasonable.

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