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Characterisation of New *Bacillus circulans* Strain Isolated from Oil Shale

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Summary

A new strain of *Bacillus* sp. was obtained during experiments of oil shale demineralization, which were carried out in order to get 'pure' organic matter (kerogen). The demineralization efficiency of newly isolated strain was found to be substantially higher in comparison with that of *Bacillus circulans* Jordan 1890. On the basis of the biochemical characteristics, protein patterns and fatty acid composition, as well as the whole genome profile and 16S rDNA sequencing, the new strain was identified as *Bacillus circulans* VD01.

Key words: Bacillus circulans, oil shale, kerogen, siliceous bacteria

Introduction

Kerogen is a mixture of organic compounds. It represents an insoluble component of oil shale, a compact sedimentary rock of homogenous fine-grained composition. The preparation of pure and unaltered kerogen concentrate is of geochemical interest (*1*). Investigations of the kerogen chemical composition and structures generally require preliminary isolation of natural organic material. The isolation of native kerogen is a difficult task due to its complex nature and insolubility, and the fact that in sediments kerogen is mixed with large amounts of diverse minerals, such as carbonates, silicates and pyrite. Microbial depyritization by the action of chemolithoautotrophic bacterium *Acidithiobacillus ferrooxidans* was found to be quite efficient (*2*). Siliceous bacterium *Bacillus circulans* was also proposed for bacterial desilicification of the oil shale (*3*).

B. circulans is a typical chemoorganoheterotrophic bacterium using mono-, di- and polysaccharides and polyhydroxylic alcohols as sources of carbon, energy and electrons. The efficiency in disintegrating silicates and aluminosilicates is not equal in all strains of siliceous bacteria, and it varies depending on the nature of silicates and the metabolic products of bacteria, primarily organic acids and the mucous polysaccharide capsules. The activity of *B. circulans* is attributed to its metabolites and their specific reactions such as acidolysis, alkalysis and complexolysis (*4*).

The aim of this study is to identify newly isolated strain of bacteria from Aleksinac oil shale (Serbia) that exhibited very high demineralization efficiency.

Materials and Methods

An unidentified strain was obtained from Aleksinac oil shale and adapted to grow on modified Ashby's me-

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dium (*3*), incubated at 30 °C for 15 days. The strain was activated by successive seeding and appropriate tests for identification of *Bacillus* genus were performed (*5*). Activated culture of *B. circulans* Jordan 1890, received from the Center of Microbiology, Sofia, Bulgaria, and adapted to modified Ashby's medium, was used for desilicification.

Phenotypic analysis

The morphology of cells was examined by Gram staining and the presence of spores was determined using Ziehl-Neelsen stain as previously described (*5*). In brief, culture grown in modified Ashby's medium was subjected to high temperature (80 °C) for 10 min and plated on solid modified Ashby's medium. After overnight incubation at 37 °C, staining procedure was performed.

Biochemical characteristics of the new strain

Bacilli, fulfilling the morphology and general biochemical characteristics of genus *Bacillus*, were assayed using commercial kits API 20E and 50CHB (API System, bioMérieux, Marcy l'Etoile, France) as described by Berkeley *et al.* (*6*). The strain was identified by analysing the test results (obtained according to the manufacturer's protocol) with API Lab Plus software and named *B. circulans* VD01.

DNA analysis

For DNA extraction, cells were grown for 24 h in nutrient medium (*7*) on a rotary shaker at 200 rpm and 30 °C. DNA was extracted and purified by the conventional method (*8*). The purity and quality of each DNA preparation were determined by monitoring the ratio of absorbance at 260 nm to that at 280 nm (1.8 to 2.1) and the ratio of absorbance at 260 nm to that at 230 nm (1.8 to 2.1). The DNA samples were thermally denaturated by setting the temperature from 60 to 100 °C; at a rate of 1 °C per minute. The standard sample contained calf thymus DNA (SERVA GmbH, Heidelberg, Germany). The changes in absorbance were measured at 260 nm. All determinations were done by absorbance spectrophotometer Gilford® G-250 (Gilford Instrument Laboratories Inc., Oberlin, OH, USA). The guanine-plus-cytosine (G+C) composition of the samples was calculated as previously described (*9*).

Amplification of 16S rDNA by PCR and sequence determination

In order to obtain 16S rDNA sequences, polymerase chain reaction (PCR) was carried out using primers $P1_{16S}$ (5'-GAATCTTCCACAATGGACG-3') and $P2_{16S}$ (5'-TGACGGGCGGTGTGTACAAG-3') according to the following protocol: 94 °C for 2 min, 30 cycles at 94 °C for 30 s, 50 °C for 30 s, 68 °C for 30 s and 72 °C for 10 min. The purified PCR fragments were sequenced with both primers and compared to 16S rRNA gene sequences in the public database using BLAST (NCBI, Bethesda, MD, USA).

PFGE of genomic DNA

The low melting point agarose gel (Sigma-Aldrich, St. Louis, MO, USA) embedded chromosomal DNA of the newly isolated strain was prepared from 6-hour-old cultures according to Gaviria Rivera and Priest (*10*). The DNA was digested with 30 U of *Not*I (MBI Fermentas, Vilnius, Lithuania) or 25 U of *Asc*I (New England BioLabs GmbH, Frankfurt, Germany) for 4 h. Pulsed field gel electrophoresis (PFGE) was performed using the CHEF-DR II System (Bio-Rad Laboratories, Hercules, CA, USA) at 14 °C and 5.3 V/cm, using 1.0 % agarose gel (Sigma-Aldrich) in 0.5×TBE (100 mM Tris, 100 mM borate, 200 μM EDTA). For either *Not*I or *Asc*I digestion, the electrophoresis was carried out for 22 h with pulse from 10 to 130 s. After staining with ethidium bromide solution (1 $\mu g/cm^3$), the gels were photographed under UV light using the Gel Doc 2000 System (Bio-Rad Laboratories) and analysed with the Quantity One v. 4.1.1 program (Bio-Rad). The total molecular mass of the genome was determined by adding the fragments produced by restriction enzymes, the sizes of which were determined by comparison to yeast marker, as described by Carlson *et al*. (*11*).

SDS-PAGE of whole cell proteins

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Costas (*12*). Electrophoresis was performed at the constant current of 20 mA, using a 10 % separating gel (pH= 8.8) and a 5 % stacking gel (pH=6.8). The mini gel electrophoresis equipment SE260 was purchased from Hoefer Scientific Instruments (San Francisco, CA, USA). Molecular mass standard (M_r=15–250 kDa) was purchased from GE Healthcare (Little Chalfont, UK).

Determination of fatty acid composition

The lyophilized cell fatty acids (composition of 100 mg in 5 mL of NaOH-CH_3OH) were heated for 15 min at 100 °C. The saponified material was cooled and acidified to pH=2. Boron trifluoride-methanol (BF_3–CH_3OH) reagent was added to 4 mL of saponified material, and the mixture was reheated at 100 °C for 5 min. The content of the tube was added to 10 cm^3 of saturated sodium chloride solution and the methyl esters were extracted twice with an equal volume of 1:4 trichlormethane/hexane (*13*).

Determination of fatty acid composition of bacterial biomass was performed on Agilent 6890 chromatograph with Agilent 5973 mass detector (Agilent Technologies, Santa Clara, CA, USA).

Bacterial desilicification

The newly isolated strain of *B. circulans* was activated by growing on a modified Ashby's medium. The experiments were carried out in the 500-cm^3 Erlenmeyer flask with a solid/liquid medium ratio of 1:50 (by mass per volume), on a rotary shaker at 200 rpm on 30 °C for 30 days. Every third day fresh medium was added to the substrate. Several parameters, such as pH, the number of microorganisams as well as the amount of residual silicon dioxide, aluminium oxide and ferric oxide in the substrate ash were monitored throughout the experiment. The number of microorganisms was determined by the method of serial dilutions on a potato dextrose agar plate (pH=7).

At the end of the leaching period the samples were treated with distilled water and hydrochloric acid (1:1,

by mass per volume). The solid residue was used to prepare the ash (800 °C) by the analysis of Si, Al and Fe by spectrophotometry on a Varian 475 atomic apsorption spectrophotometer (Varian Inc., Agilent Technologies) (*2*).

Results and Discussion

The application of microbiological method for demineralization of Aleksinac oil shale (Serbia) by *B. circulans* Jordan 1890 resulted in higher demineralization range in the control than in the inoculated samples. The results of demineralization were checked by multiple repetition and finally newly isolated strain was obtained from the control test substrates.

The cells of the isolated strain were Gram-positive, spore-forming regular rods, 0.5×4 μm, with rounded ends, often in pairs. Determination of cell length and width was done in repetitive independent measurements, variation of values being less than 5 %. The obtained results for cell length and width were in accordance with the average values for the *B. circulans* species.

Spores were ellipsoidal, located at a terminal position. On the modified Ashby's medium, the isolates formed mucous, transparent convex colonies, with even edges and smooth surface. Since the strain was also catalase positive, it was clear that the newly isolated strain belonged to genus *Bacillus*.

After applying API 50CHB and 20E tests (bioMérieux), the new strain showed a positive reaction in fermentation of ribose, glucose, fructose, N-acetyl glucosamine, arbutine, esculine, salicine, cellobiose, maltose, trehalose, glycogene, gluconate, glycerol and hydrolysis of gelatin and starch. According to the biochemical features, the newly isolated strain was very similar to *B. circulans.* The BLAST sequence homology search revealed that 16S rDNA sequence of strain VD01 showed homology with 16S rRNA from *B. circulans* strains.

Chemotaxonomically, the strain is characterized by a G+C DNA molar ratio of 43.2 %. There is good correspondence here with the molar ratio of G+C (%) in *Bacillus* strains, which ranges from about 33 to 65 % (*14*). The percentage was somewhat higher than the reference value for *B. circulans*, but variations are possible and they show a wide range (*15*). The most abundant cellular fatty acids are *ai*C15:0, C16:0, *ai*C16:0, and *ai*C17:0 and collectively account for up to 85 % of the total fatty acid content (Table 1; *16*).

Table 1. Fatty acid composition of *Bacillus circulans* VD01 strain

Fatty acid	$(m(\text{fatty acid})/\Sigma m(\text{fatty acid}))$ %	$m(\text{fatty acid})/\Sigma m(\text{fatty acid})$*
C_{14}	2.6	5
iC_{14}	2.0	4
iC_{15}	35.7	20
aC_{15}	17.0	30
C_{16}	23.6	21
iC_{16}	4.6	6
iC_{17}	9.4	7
aC_{17}	5.1	7

*Fatty acid composition according to O´Leary and Wilkinson (*16*)

B. circulans VD01 strain exhibits a unique chromosomal profile as it was detected with PFGE analysis (Fig. 1). The *Asc*I restriction yielded pattern of three bands ranging from 580 to 2300 kb, while the *Not*I digest produced nine bands, between 180 and 1300 kb (Table 2). The total genome size of the studied *B. circulans* was calculated to be 4565 kb (*Not*I restriction) or 4580 kb (*Asc*I restriction). The present results clearly indicate that both *Acs*I and *Not*I treatments are well adapted to PFGE of *B. circulans*, because they generated DNA fragments well resolved for visual comparison. This method has been well exploited for the investigation of Gram-negative (*17*), as well as Gram-positive bacteria (*10,18*). However, to the best of our knowledge, this has been the first report in which PFGE was used for *B. circulans* genome description.

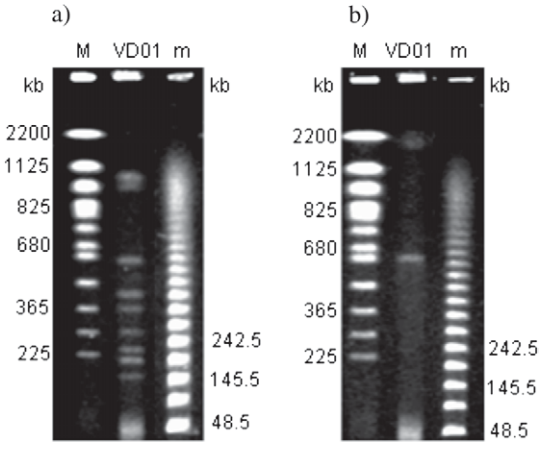

Fig. 1. PFGE patterns of *Bacillus circulans* VD01 genomic DNA digested with: a) *Not*I and b) *Asc*I. Line M=yeast chromosome PFGE marker (Bio-Rad), line m=λ DNA concatemer marker (Sigma-Aldrich), kb=kilobases

Table 2. *Bacillus circulans* VD01 genome size

*Asc*I digestion band size/kb	*Not*I digestion band size/kb
580	180
1700	210
2300	240
	280
	365
	430
	580
	980
	1300
Σ=4580 kb	Σ=4565 kb

The protein patterns of the newly isolated strain exhibited clear bands detected within the range from 30 to 160 kDa.

With the introduction of modern taxonomic techniques such as numerical phenetics, DNA base composition determinations, DNA reassociation experiments or DNA restriction patterns allowing for the sequence ho-

mology between strains to be estimated, it has become apparent that strains of genus *Bacillus* are more heterogeneous than hitherto suspected. Considerable genetic diversity among species suggests that the genus should perhaps be split into several, more homogenous taxa. The bacteria have been classified in six large groups or aggregates of clusters, which in many ways equate with genera.

B. circulans belongs to group I together with *B. polymyxa* as a reference organism and other species such as *B. alvei* and *B. macerans*, which all produce oval spores that distend the mother cell. These bacteria are facultative anaerobes that ferment a variety of sugars and have reasonably fastidious growth requirements in vitamins and amino acids. They secrete numerous extracellular carbohydrases such as amylases and β-glucanases including cellulases, pectinases and pullulanases.

Bacterial desilicification

A 30-day metal leaching by new *B. circulans* VD01 strain, expressed in percentage, relative to initial amounts of individual mineral constituents in the carbonate-free substrate (samples were treated by hydrochloric acid solution) is shown in Fig. 2. In inoculated tests the number of bacteria during 30 days was in the order of 10^{-8} to 10^{-9} cells/cm^3 and pH value decreased from initial pH=7 to pH=2.5 as a result of anabolic intermediate production of organic acids.

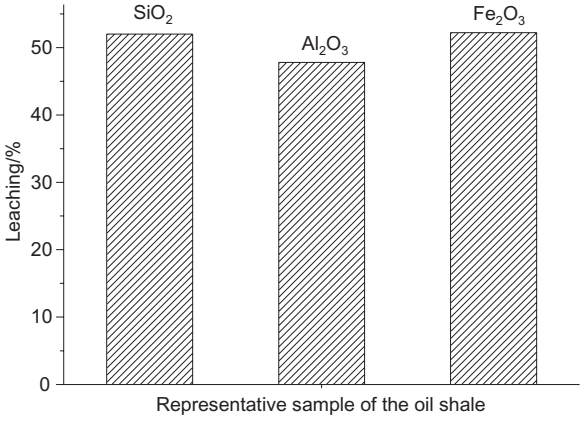

Fig. 2. Percentage of leaching of silicon oxide (SiO_2), aluminium oxide (Al_2O_3) and iron(III) oxide (Fe_2O_3) of representative sample of the oil shale

Bacterial desilicification processes demonstrated obvious advantages as compared to chemical methods applied for the same purpose. This is due to the fact that bacterial desilicification of the organic matter leaves the substrate unchanged. The microorganisms do not use the organic matter of the substrate as a source of carbon. Therefore, it should be of interest to continue with the investigation of this strain in terms of optimizing the conditions of cultivation, inoculation of sediments, *etc.*

Conclusion

On the basis of the intensive comparisons with known species of *Bacillus*, the newly isolated strain was identified as a new *B. circulans* strain VD01, which proved to be very efficient in the bacterial desilicification of oil shale. The oil shale demineralization using bacteria on a laboratory scale is an introductory step in biotechnology/biobenefication of this raw material as an alternative source of energy.

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