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MICROBIOLOGIA BALKANICA 2011

7th BALKAN CONGRESS OF MICROBIOLOGY

8th CONGRESS OF SERBIAN MICROBIOLOGISTS

PROCEEDINGS

October 25-29, 2011

Continental Hotel, Belgrade, SERBIA

CIP – Katalogizacija u publikaciji
Narodna biblioteka Srbije, Beograd

Udruženje mikrobiologa Srbije, Beograd.
Knjiga radova (Elektronski izvor) – Proceedings **/** MICROBIOLOGIA BALKANICA 2011 - 7th BALKAN CONGRESS OF MICROBIOLOGY & 8th CONGRESS OF SERBIAN MICROBIOLOGISTS, 25-29. oktobar 2011; (organizator) Udruženje mikrobiologa Srbije, Udruženje medicinskih mikrobiolga Srbije; (urednici: Dragojlo Obradović, Lazar Ranin, Špiro Radulović) – Beograd
1 elektronski optički disk (CD-ROM); 12cm

Sistemski zahtevi: Nisu navedeni. Nasl. sa naslovnog ekrana. –
Radovi na engleskom jeziku.-
Tekst latinica. –
Tiraž – 600.
Abstracts. – Registar

ISBN 978-86-914897-0-01
Udruženje mikrobiologa Srbije, Beograd.

KNJIGA RADOVA / PROCEEDINGS
MICROBIOLOGIA BALKANICA 2011 - 7th BALKAN CONGRESS OF MICROBIOLOGY & 8th CONGRESS OF SERBIAN MICROBIOLOGISTS, 25-29. oktobar 2011

Izdaje / Published by:
Udruženje mikrobiologa Srbije
Nemanjina 6, 11 080 Beograd, Srbija, tel/fax: 011 2199 711, dobradovic@agrifaculty.bg.ac.rs

Za izdavača / For Publisher:
Dragojlo Obradović, predsednik Udruženja

Urednici/Editors:
Dragojlo Obradović
Lazar Ranin
Špiro Radulović

ISBN 978-86-914897-0-01

Kompjuterska obrada teksta / Computer Layout:
Jelena Zovko Belić

Tiraž / Circulation
600 primeraka / 600 copy

Umnožavanje / Copying
Megaphone d.o.o., Vladimira Rolovića 105, Beograd, Srbija

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New *Bacillus circulans* strain isolated from oil shale

Vesna Dragutinović[1], Miroslav M. Vrvić[2,3], Izabela Swiecicka[4], Olga Cvetković[2], Svetislav Tatić[5], Tanja Berić [6], Slaviša Stanković[6]

[1]Institute of Medical Chemistry, Faculty of Medicine, University of Belgrade
[2]Center of Chemistry, Institute of Chemistry, Technology and Metallurgy, University of Belgrade
[3]Faculty of Chemistry, University of Belgrade,
[4]Department of Microbiology, Institute of Biology, University of Bialystok, Poland,
[5]Institute of Pathology, Faculty of Medicine, University of Belgrade,
[6]Faculty of Biology, University of Belgrade, Serbia
Belgrade, Serbia

New strain was obtained during experiments of oil shale demineralization, which was carried out in order to get "pure" organic mater (kerogen). The application of microbiological method for demineralization of oil shale by *B. circulans* resulted in higher demineralization efficiency.

Cells of isolated strain were Gram-positive, spore-forming regular rods, 0.5 x 4 μm, with rounded ends, often in pairs. Spores were ellipsoid with terminal position. On the modified Ashby's medium, the isolates formed mucous, transparent convex colonies, with even edges and smooth surface. The strain was also catalase positive. After applying API 50CHB and 20E tests, the new strain showed a positive reaction in fermentation of ribose, glucose, fructose, N-acetil-glucosamine, arbutine, esculine, salicine, cellobiose, maltose, trehalose, glycogene, gluconate, glycerol and hydrolysis of gelatin and starch. Chemotaxonomicaly, strain is characterized by a guanine-to-cytosine DNA ratio of 43.2 mol%. The most abundant cellular fatty acids are C15:0 ai, C16:0, C16:0 ai and C17:0 ai. The BLAST sequence homology search revealed that 16S rDNA sequence of strain VD01 showed homology with 16S rRNA from *B. circulans* strains. *B. circulans* strain exhibits a unique chromosome profile as it was detected in PFGE analysis and results clearly indicated 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. The protein patterns of newly isolated strain exhibited clear bands detected within the range from 30 to 160 kDa.

On the bases of the intensive comparisons with known species of *Bacillus*, the newly isolated strain was identified as a new one of *B. circlans* VD01, which proved to be very efficient in the bacterial desilification of oil shale.

NEW *BACILLUS CIRCULANS* STRAIN ISOLATED FROM OIL SHALE

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[1]Faculty of Medicine, University of Belgrade, Serbia, [2]Center of Chemistry, IChTM, University of Belgrade, Serbia, [3]Faculty of Chemistry, University of Belgrade, Serbia, [4]Institute of Biology, University of Bialystok, Poland, [5]Faculty of Biology, University of Belgrade, Serbia

INTRODUCTION

B. circulans is a typical chemoorganoheterotrophic bacterium using mono-, di- and polysaccharides and polyhydroxylic alcohols as source 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 bacterial metabolic products, 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.

Kerogen is a mixture of organic compounds. It represents an insoluble component of oil shale, a compact sedimentary rock of homogenous fine-composition. Preparation of pure and unaltered kerogen concentrate was always of geochemical interest. Investigations of the kerogen chemical composition and structures generally requires preliminary isolation of unchanged 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 intimately with large amount of diverse minerals, such as carbonates, silicates and pyrite.Siliceous bacterium *B. circulans* was also proposed for bacterial desilicification of the oil shale.

RESULTS

Spores were ellipsoid with terminal position. On the modified Ashby's medium, the colonies formed mucoid, transparent convex colonies, with even edges and smooth surface. Since the strain was also catalase positive, it was clear that the newly isolated strain belongs to genus *Bacillus*.

After applying API 50CHB and 20E tests, the new strain showed a positive reaction in fermentation of ribose, glucose, fructose, N-acetil-glucosamine, arbutine, esculine, salicine, cellobiose, maltose, trehalose, glycogene, gluconate, glycerol and hydrolysis of gelatin and starch. According to the biochemical features, newly isolated strain was very similar to *B. circulans*.

Chemotaxonomicaly, strain is characterized by a guanine-to-cytosine DNA ratio of 43.2 mol%. Hence the range of mol % G + C in *Bacillus* strains is from about 33 to 65%, although strains of most species cluster between 40-50%, we have good correspondence here. Percentage calculated was somewhat higher than the reference value for *B. circulans*, but variations are possible and they show a wide range .

The BLAST sequence homology search revealed that 16S rDNA sequence of strain VD01 showed homology with 16S rRNA from *B. circulans* strains.

The most abundant cellular fatty acids are C15:0 ai, C16:0, C16:0 ai and C17:0 ai and collectively account for up to 85% of the total fatty acid content. (Table 1).

Fatty acid	Fatty acid content (% of total fatty acids)	Fatty acid content (% of total fatty acids)
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

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

CONCLUSION

On the bases of the intensive comparisons with known species of *Bacillus*, the newly isolated strain was identified as a new one of *B. circulans* VD01, which proved to be very efficient in the bacterial desilicification of oil shale.

MATERIAL AND METHODS

Biochemical characteristics

Bacilli, fulfilling the morphology and general biochemical characteristics of genus *Bacillus*, were assayed in commercial kits API 20E and 50CHB as described by Berkeley *et al.* Strain was identified by analyzing the test results (scored according to manufacturer's protocol) with API Lab Plus PC version software.

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') and according to the protocol as follows: 94ºC for 2 min, 30 cycles at 94 ºC for 30 s, 50 ºC for 30 s, 68 ºC for 30 s and 72 ºC 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.

PFGE of genomic DNA

The low melting point agarose (Sigma Chemical Co, St. Louis, USA) embedded chromosomal DNA of the newly isolated strain was prepared from 6 h cultures according to Gaviria Rivera and Priest . The DNA was digested with 30U of *Not*I (MBI Fermentas, Vinius, Lithuenia) or 25U of *Asc*I (New England BioLabs GmbH, Frankfurt, Germany) for 4 h. PFGE was performed using the CHEF-DR II System (Bio-Rad Laboratories, Hercules, USA) at 14 °C and 5.3 V/cm, using 1.0% agarose (Sigma) gel in 0.5xTBE (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 µg ml^{-1}), the gels were photographed in U.V. light using the Gel Doc 2000 System (Bio-Rad Laboratories, Hercules, USA) and analysed with the Quantity One PC version 4.1.1 program (Bio-Rad). The total molecular weight 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. (*1*).

SDS-PAGE of whole cell proteins

The analysis was performed as described by Costas (*2*). 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). Molar mass standard (15-250 kDa) was purchased from Amersham (Bioscience).

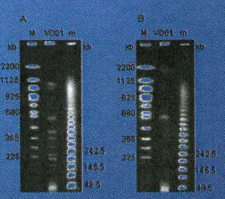
Determination of fatty-acid composition (FAME)

The lyophilizied cell fatty acid composition of (100 mg in 5 ml of NaOH-CH_3OH) was 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 in portion of saponified material (4 ml), and the mixture was re-heated for 5 min at 100°C. The content of the tube was added to 10 ml of saturated sodium chloride solution and the methyl esters were extracted twice with an equal volume of 1:4 trichlormethan : hexane.

Determination of fatty-acid composition of bacterial biomass was performed on Agilent 6890 chromatograph with Agilent 5973 mass detector (MSD).

*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

PFGE of genomic DNA



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