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ZYMOGENIC *Bacillus* sp. REVEALED IN ALEKSINAC (SERBIA) OIL SHALE

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Abstract. The demineralisation experiments are aimed at preparing native sedimentary organic matter concentrates, consisting mainly of insoluble kerogen. Initial desilicification experiments of three Aleksinac (Serbia) oil shale samples were carried out with the help of *Bacillus circulans* J o r d a n. The process was followed by pH measurement and determination of the number of microorganisms. Demineralisation efficiency was evaluated based on the ratio of SiO_2, Al_2O_3 and Fe_2O_3 contents in the substrates before and after the experiments. Surprisingly, in all experiments much better desilicification was observed in control tests compared to inoculated substrates. Discovery of a zymogenic culture in all three oil shale samples explained the apparent anomaly. Based on physiological, biochemical and taxonomic characteristics, the isolated zymogenic culture was identified as *Bacillus* sp.

Keywords: oil shale, bacterial desilicification, *Bacillus* sp.

AIMS AND BACKGROUND

Isolation of native sedimentary organic matter, consisting mainly of insoluble kerogen, is of organic-geochemical interest. Physical[1,2] and chemical[3–5] methods were often used for this purpose. However, these demineralisation experiments showed the structure of the native organic matter to have been changed[5]. For that reason bacterial demineralisation of sediments is investigated aimed at isolating unchanged, native sedimentary organic matter. Thus, bacterial depyritisation of Aleksinac oil shale by *Acidithiobacillus ferrooxidans* was shown to be quite efficient (ca. 91% pyrite removed)[6], while the organic substance seemed to have remained unchanged. Since in addition to pyrite a more complete demineralisation required removal of aluminosilicates, bacterial desilicification was attempted by using the *Bacillus circulans.*

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EXPERIMENTAL

Three samples of Aleksinac (Serbia) oil shale, an Oligocene–Miocene lacustrine sediment, were used as substrates in the first bacterial desilicification experiments. One of the substrates (1) represented a composite of several borehole samples, and the other two (2 and 3) were individual samples originating from two different deep boreholes. Pulverisied samples (< 100 μm) were characterised by the amount of ash (850°C) and ash analysis, as well as the amount of organic matter. In all three samples just a minor part of the native organic matter was soluble in toluene/methanol mixture (Table 1).

Table 1. Characterisation of Aleksinac oil shale samples

Sample	Organic matter (by difference) (%)	Bitumen (soluble organic matter) (%)	Ash (%)	SiO_2 (%)	Al_2O_3 (%)	Fe_2O_3 (%)
1	28.9	1.0	71,1	39.9	5.2	7.1
2	42.9	2.2	57.1	23.9	8.1	4.5
3	23.9	1.3	76.1	26.1	6.2	3.8

Desilicification experiments were carried out with a hemoorganoheterotrophic culture *Bacillus circulans* J o r d a n[7] (*B. circulans*) obtained from the Center of Mineral Biotechnology in Sofia (Bulgaria). It was adapted on a modified Ashby medium[8] and activated by successive reseeding on a fresh modified Ashby medium. The experiments were carried out in sterile 500-cm^3 hollow Erlenmeyer flasks, on a rotation shaker LAB TH (A. Kuchner), 200 rpm at 30°C. The solid/liquid ratio was 1:50 (w/v). The samples (except in control tests) were inoculated by a 1:10 (v/v) suspension of *B. circulans*. Every third day, fresh modified Ashby medium was added to all Erlenmeyer flasks, and a fresh biomass only to inoculated samples. Demineralisation was controlled by every third day pH measurement of the suspension before adding the fresh medium (Radiometer Copenhagen, type PHM 26, combined electrode GK 2401 B), determination of the number of microorganisms in the suspension (by the method of serial dilution at potato agar[9]), and determination of the amount of ash and organic matter (by difference) in the substrates at the end of demineralisation. After 21 days of shaking the samples were treated by hydrochloric acid (1:1, v/v) and rinsed with distilled water, dried to constant mass at 105°C, and finally analysed in the same way as original samples.

The sources of carbon and energy were tested by using D-glucose, D-mannitol, D-xylose, L-arabinose, and starch. DNA determinations were based on the ratio of 260 and 280 nm (1.8–2.1) and 260 and 230 nm (1.8–2.1) absorbances (spectrophotometer Gilford G-250) (Ref. 10). DNA was isolated by a conventional method[11] and denatured by heating in the interval of 60 to 100°C (1°C per min). Calf thymus (SERVA) DNA standard was used as control. An 87°C denaturation

temperature was observed. The content of guanine plus cytosine (G+C) was calculated by using a corresponding equation[12].

RESULTS AND DISCUSSION

Unexpected inverse ratios of demineralisation efficiencies were observed between inoculated and control tests. Namely, demineralisation efficiencies in control tests were found to be significantly higher than in inoculated tests. Therefore, identical experiments were repeated 5 times. In all series, substantially higher bacterial efficiency in control tests was confirmed. Average demineralisation efficiencies observed in the 5 consecutive series of experiments in inoculated and control tests are shown in Fig. 1.

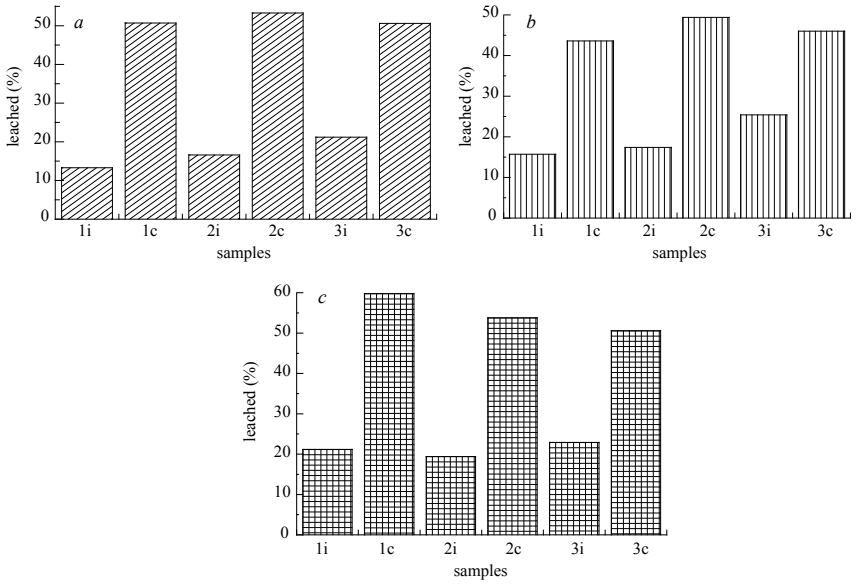

Fig. 1. Leaching (%) of silica (*a*), alumina (*b*) and iron (*c*) from inoculated (1i, 2i, 3i) and control (1c, 2c, 3c) tests

In inoculated tests the pH gradually changed from 7 ± 0.2 at the beginning to 5 ± 0.2 after 21 days of demineralisation process, while in control tests, in almost all cases, already after 12 days it decreased to pH 2.5 ± 0.2. The number of microorganisms in inoculated tests during the 21 days of experimenting was in the range of E-8 – E-9 cells/cm^3, which represented a good basis for a successful desilicification. On the other hand, the number of microorganisms in control tests was also high, E-9 cells/cm^3, indicating the presence of an unknown ‘biological agent’, activated under the applied experimental conditions.

Explanation of the observed unexpected demineralisation in control tests imposed itself as a research priority at that moment. Higher demineralisation efficiency in control tests required to be explained from microbiological point of view. Of course, it was presumed that oil shale samples contained a zymogenic culture. To check this presumption the supposed zymogenic culture had to be isolated from the control tests and identified by determining its physiological and morphological characteristics. Based on the observed denaturation temperature, the calculated content of G+C pairs was found to be 43.2 mol. %. In several cases the mol. % values of G+C pairs were higher compared to *B. circulans* reference values, but similar discrepancies were shown to be possible[13]. The morphology of cells grown on the modified Ashby medium was analysed microscopically. Rod-shaped cells of 0.5 × 4 μm dimensions with rounded ends, most often in pairs, and gram-positive spores of ellipsoid shape and terminal position were isolated from demineralisation control tests. The culture isolated on the modified Ashby medium formed mucous, transparent, convex colonies of smooth edges and surfaces. The observed physiological and biochemical characteristics of the zymogenic culture isolated from the Aleksinac oil shale are shown in Table 2.

Table 2. Physiological and biochemical characteristics of the zymogenic culture

Characteristics	Strain
Catalase	+
Anaerobic growth	–
The Voges–Proskauer test	–
pH in V-P broth < 6	v
Acid from: D-glucose	+
L-arabinose	+
D-xylose	+
D-mannitol	+
Gas from glucose	–
Hydrolysis of casein	–
gelatin	+
starch	+
Nitrate reduced to nitrite	+
Growth at 40ºC	+
50ºC	–
60ºC	–
Growth in 7% NaCl	v

+ Positive; – negative; v – variable.

CONCLUSIONS

Based on physiological and morphological characteristics[14,15], the revealed zymogenic culture was unambiguously shown to belong to *Bacillus* sp. In inoculated oil shale substrates the nonactivated zymogenic culture could not demonstrate the high desilicification efficiency shown in control tests, presumably because it was not able to compete with the adapted and activated *Bacillus circulans* J o r d a n, added in the form of fresh biomass every third day to inoculated samples. Thus, in the presence of *B. circulans* it remained nonactivated. Its higher desilicification efficiency demonstrated in control tests was most probably resulting from a more intensive biosynthesis of organic acids[16,17], i.e., in the absence of *B. circulans*. Its desilicification efficiency suggests its belonging to *Bacillus* sp. However, such a presumption requires further microbiological investigation.

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