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PREPARATION OF SEDIMENTARY ORGANIC MATTER CONCENTRATES BY MICROBIOLOGICAL METHODS

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Investigation on combined bacterial demineralization of sediments i.e., removal of silicates (desilicification) by *Bacillus circulans* and removal of pyrite (depyritization) by *Acidithiobacillus ferrooxidans* are reviewed in this paper. These methods represent a new approach in solving one of important organic geochemical problems, isolation of native organic matter (kerogen) concentrate from sediments. Using the oil shale from Aleksinac as substrate, the most important aspect of bacterial demineralization was examined: the question of whether or to what extent do the applied bacteria affect the composition and structure of kerogen. High efficiency of *Acidithiobacillus ferrooxidans* was observed in depyritization experiments (*ca* 91%). On the other hand, in 30-day-leaching period noticeable elimination of silicates by *Bacillus circulans* (*ca* 40%) was achieved. For detailed analyses prior to and after bacterial demineralization, besides standard methods (such as i.r. spectroscopy, elemental microanalysis, micropetrographic analysis), modern analytical

instrumental methods were also used (e.g., P-GC and P-GC-MS). The observed results suggested that during these processes the sedimentary organic matter remained native.Thus, the advantage of bacterial *vs.* chemical demineralization process was demonstrated.

Key words: Aleksinac oil shale, organic matter concentrate, kerogen concentrate, depyritization, *Acidithiobacillus ferrooxidans*, desilicification, *Bacillus circulans*

INTRODUCTION

Investigation of chemical composition and structure of kerogen generally require preliminary isolation of unchanged organic matter. Isolation of native kerogen is a difficult task, partly because of the fact that kerogen in sediments is intimately mixed with large amounts of diverse minerals, such as carbonates, silicates and pyrite. The carbonates and silicates are removed by treatment of the sediment with dilute acids and mixtures of concentrated hydrochloric and hydrofluoric acids, respectively. Pyrite may also be removed by chemical treatment of the substrates. However, chemical reagents used for this purpose were shown to attack the kerogen as well, modifying it to a certain degree (BERGER, 1963; EGLINTON *et al.*, 1969). Different chemical reagents have been proposed for the removal of pyrite, including nitric acid (EGLINTON *et al.*, 1969; HIMUS *et al.*,1949; DANCY *et al.*,1950; LAWLOR *et al.*,1963), nascent hydrogen produced from mixture of zinc and hydrochloric acid (DANCY *et al.*,1950; McIVER), lithium aluminum hydride (LAWLOR *et al.*,1963; YEN *et al.*,1976), sodium borhydride (LAWLOR *et al.*,1963; YEN *et al.*,1976) and iron (III) salts in acidic medium (VITOROVIĆ *et al.*,1980). All these reagents were shown to affect the composition and structure of kerogen. Therefore, elimination of pyrite represents one of major problems in the native kerogen isolation procedures (DURAND *et al.*, 1980; VRVIĆ *et al.*,1983).

To solve the problem of pyrite elimination, but bearing in mind the need of preserving the kerogen unchanged (VRVIĆ *et al.*,1983; VRVIĆ *et al.*,1988), depyritization of ancient sediments by chemolithoautotrophic bacterium, *Acidithiobacillus ferrooxidans* (*A. ferrooxidans*), was proposed. The efficiency of the process was always found to be high. It depended on the nature of the substrate, the ratio of solid to liquid phases, as well as other experimental conditions. Investigation of the composition and structure of Aleksinac oil shale organic matter concentrate showed that by bacterial depyritization its composition and structure remained unchanged (CVETKOVIĆ *et al.*, 1993).

In order to eliminate silicates from the Aleksinac oil shale organic matter concentrate, also bearing in mind that unchanged organic matter concentrate should be obtained, several bacterial desilicification experiments were

carried out by chemoorganoheterotrophic bacterium *Bacillus circulans*-Jordan (*B. c.*-Jordan) (CVETKOVIĆ *et al.*, 2001; DRAGUTINOVIĆ *et al.*,1997; VRVIĆ *et al.*, 1989). These experiments indicated that siliceous bacteria may have potential as an alternative, biochemical agent for the isolation of native kerogen, and justified further efforts towards continued evaluation of this advantageous process.

The best desilicification results were obtained by using a zymogenous strain of *B. c.* (VRVIĆ *et al.*, 1989) wich was found to be much more efficient compared to *B.c.* Jordan. A new strain, identified as *B. c.* CHBC-1, was isolated from oil shale on modified Ashby's medium and identified by morphological and taxonomic methods. The desilicification efficiency of the zymogenous strain was about 70 % (unpublished results).

MATERIAL AND METHOD

Oil shale sample

The oil shale samples originated from the Aleksinac oil shale deposit (Serbia), an Oligocene-Miocene lacustrine sediment.

The sample for depyritization experiments was a composite consisting of proportional quantities of five samples from the 476.10 to 491.60 m interval of core 19. The composite was homogenized and powdered to -63 μm in a ball mill.

The substrate used in all experiments was a HCl-kerogen concentarte obtained by treatment of the composite with 0.2M hydrochloric acid for 4h on a steam bath. The residue was rinsed with distilled water and dried at 80°C to constant weight. The HCl-concentrate was finally Soxhlet extracted with an azeotrope mixture of benzene and methanol for 93h to remove the bitumen. The dry HCl- kerogen concentrate (KC) represented 78.5% of initial dry composite.

The desilicification experiments were carried out with a composite of proportional quantities of the shale from the 365.1-390.1 m interval of core BS-14. The composite was homogenized and powdered to <100μm. A concentrate obtained from the composite by treatment with dilute hydrochloric acid served as a reference sample. Standard analytical methods were used for the characterization of the organic matter in the reference and bacterially treated samples, as well as for the analysis of the ash, which served as a basis for evaluating the desilicification efficiency.

Bacterial depyritization

Bacterial depyritization was carried out by *A. ferrooxidans*, isolated from the coal dump at the Aleksinac brown coal mine (VRVIĆ *et al.*, 1983). The *A. ferrooxidans* culture was activated by successive reseeding, repeated several times, on a fresh 9K medium (SILVERMAN *et al.*,1959), and 6-day incubation at 28°C and pH 2.5.

The experiments were carried out in sterile 500 cm^3 Erlenmeyer flasks. The flasks containing 6.00 g of HCl-kerogen concentrate, closed with microbiological stoppers, were once more sterilized by 1h heating at 80°C. Sterile leaching solution was then added. The solid/liquid phase ratio was adjusted to 1:12. Finally, the same volume of leaching solution was added, containing the *A. ferrooxidans* biomass. Five Erlenmeyer flasks, prepared in identical way, were placed on a horizontal shaker (New Brunswick Scientific Co., Model No B-82, 200 oscillations per minute) in a thermostated room at 28°C.

Bacterial desilicification

An activated culture of *B. c.*Jordan, obtained from the Center of Microbiology, Sofia, Bulgaria, adapted to modified Ashby's medium, was used as the inoculum in the desilicification experiments, which were run in a rotary shaker for 30 days at 35°C, involving reseeding every third day. After 30 days the substrate was finally separated by centrifugation and treated with dilute HCl, rinsed with distilled water and dried at 80°C.

Analytical methods

The following methods and instruments were used for the analyses of the composite samples, concentrates and demineralized products of (depyritization and desilicification).

pH. Radiometer, Copenhagen, type PHM 26, with a combined electrode GK 2401 B from the same manufacturer.

Number of microorganisms. The McCredy's method of most probable number (MPN) (FISHER *et al.*, 1975).

Elemental organic analysis (C, H, N and S) was carried out by standard microanalytical methods.

Quantitative IR specta. KBr pellet (1:100). 0.2% KSCN internal standard (λmax 2065 cm^{-1}), IR spectromer Perkin-Elmer Model 457, range 4000-400cm-1. Quantitative estimation of the participation of individual bands was based on the heights of these bands determined relative to the base lines of the corresponding areas.

Micropetrographic investigations, (petrographic composition, huminite reflectance, fluorescence). Reflected light, normal or fluorescent. Polished blocks in epoxy resin. Leitz-Knott photomicroscope MPV. Objective 40:1 or 25:1, oil immersion, ocular GF 25X, measuring blend 0.5nm, monochromatic light 546nm. Qualitative and quantitative analysis of macerals: point counting method (Point Counter) with 500 points. Optical properties of macerals of the huminite group: the method of reflectance measurements on coaly type grains.

Pyrolysis-gas chromatography. The depyritization samples were analyzed by pyrolysis-gas chromatography (P-GC) using a CDS Model 120

Pyroprobe and a Varian 3700 gas chromatograph. Pyrolysis was carried out at 800°C for 20s on a ribbon filament. Separation was accomplished with a 60m (0.25 μm film thickness) DB-1 column. Oven conditions were 350°C (held 15 min). Injection temperature was 290°C.

Pyrolysis-gas chromatography-mass spectrometry (P-GC-MS) was completed using a CDS Model 122 Pyroprobe interfaced to a Hewlett Packard 5890A gas chromatograph, which was in turn interfaced to a VG 70-250SE mass spectrometer, operated at 500 resolution. Pyrolysis was conducted on a ribbon probe for 5 s at 800°C. Quantitative pyrolysis-gas chromatographic analyses of depyritization samples were performed with an internal standard (poly-*tert*-butylstyrene) eluted as third peak in a triplet at a retention time of approx. 30 min. The identification was based on: m/z 253.29 for C_{20+} *n*-alkanes, m/z 217.19 for steranes, and m/z 191.18 for pentacyclic triterpanes. However, all biomarker identifications were tentative as they have not been checked thoroughly by full scan GC-MS experiments. Nevertheless, the compound distributions (regardless of identity) for the original and depyritized kerogens were very similar. Further GC and MS details are described by (LARTER *et al.*, 1985) and (CURIALE *et al.*, 1991).

Pyrolysis-gas chromatography-mass spectrometry (P-GC-MS) of desilicification samples was conducted using a high performance flow programmable thermal extraction-pyrolysis injector mounted on a Hewlett-Packard quadrupole mass spectrometer (volatile organics were purged at 320°C for 5 min; pyrolysis was carried out by heating the sample from 100°C/min to 600°C, the pyrolysis products were cryogenically focused before separation with a low bleed capillary column, ionization and detection in the mass spectrometer).

RESULTS AND DISCUSSION

Bacterial depyritization

The best approach in answering the question whether the kerogen is changed during bacterial depyritization of an oil shale with *A. ferrooxidans* is to compare the properties of the organic matter in the substrate before bacterial treatment with the properties of the depyritization product.

The initial substrate, the HCl-kerogen concentrate (KC_0) of the oil shale from Aleksinac, contained 41.9% organic matter and 13.7% pyrite (calculated on the basis of sulphur content). This substrate was treated with *A. ferrooxidans* for 2, 4, 6 and 8 days. The number of microorganisms was constant during the 8 days (10^{11} cells/cm^3). The maximal removal of pyrite in 8 days was 74.3%, relative to the initial pyrite in the kerogen concentrate (Table 1).

The elemental composition of the organic substance in the original substrate and the substrates in the course of the depyritization process are given in Table 1.

It is not reasonable to expect any conclusion based on direct correlation of the absolute values observed for C, H and N in the organic substance of the initial and treated substrates, because all substrates contained *ca* 50% mineral matter which may have influenced the reliability of the ultimate analysis (DURAND 1980). Hence, atomic H/C and O/C ratios, IR, parameters which generally are used for the characterization and classification of kerogens were used only for correlation purposes (van KREVELEN 1961). Approximately the same, or similar, atomic H/C and O/C ratios calculated for the initial substrate and the treated samples (Table 1), suggested that kerogen elemental composition did not change substantially during the bacterial depyritization of this oil shale.

Table 1. Ultimate analysis of the organic substance in initial and depyritization concentrates

Organic matter elemental composition (ash free basis)					H/C ratio	O/C Ratio	Depyritization efficiency (% of eliminated pyrite relative to initial pyrite)	
Substrate	%C	%H	%N	%(O+Sorg) (by difference)			Based on residual sulphur	Based on residual iron
KC	62.26	7.99	1.95	27.80	1.54	0.33		
KC_0	62.81	8.87	2.02	26.30	1.69	0.31	2.3	9.2
KC_2	62.81	8.80	2.43	19.96	1.53	0.22	19.8	34.7
KC_4	66.60	8.67	2.03	22.70	1.56	0.26	49.5	75.1
KC_6	66.95	8.51	2.20	22.34	1.53	0.25	65.5	87.5
KC_8	67.89	9.12	2.13	20.86	1.61	0.23	74.3	91.1

Infrared spectra were taken for the initial substrate and all inoculated and control test samples. Figure 1 (a,b) shows, as example, only two most relevant spectra, one of the original HCl-kerogen concentrate, and the other of the final demineralized sample, i.e. the substrate treated with *A. ferrooxidans* for 8 days. The IR spectra of these substrates were rather complex and therefore their direct correlation was not informative. According to literature data (DURAND 1980; BELLAMY 1954; ESPITALIE *et al.*, 1973), a few absorption bands are representative of kerogen structure and hence suitable as correlation parameters. For example, 2920cm^{-1}, 2850cm^{-1} and 1475 cm^{-1} bands are representative for aliphatic structures, and 1720cm^{-1} and 1710cm^{-1} for carbonyl groups.

Quantitative estimation of the participation of individual structural units in the organic matter concentrate was based on heights of the corresponding bands. Since eventual changes in the organic matter during the depyritization process might be expected mainly in the most sensitive oxygen functional groups, ratios of the quantitative estimates of individual structural units (e.g., the >C=O/≡CH ratio) were taken as a basis for the evaluation of structural changes caused by the bacterial depyritization. The values of these ratios suggested that

the corresponding oxygen functional group in the concentrate organic matter was stable during the 8 days of bacterial treatment.

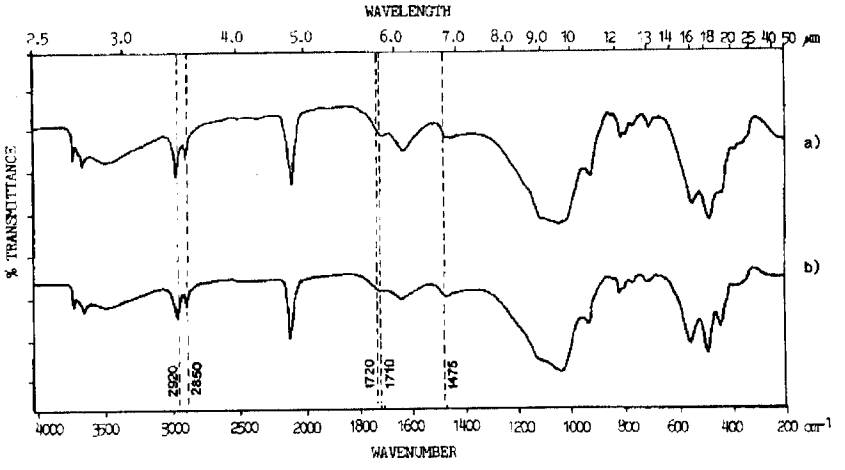

Fig. 1. Infrared spectra of the initial concentrate (a), and the substrate treated with *Acidithiobacillus ferrooxidans* for 8 days (b)

The purpose of micropetrographic examination of the substrates prior to, during (2, 4, 6 and 8 days), and after *A. ferrooxidans* bacterial depyritization was not to follow the efficiency of pyrite elimination, but rather to try to detect, by this optical method, any change of the kerogen. Therefore, in addition to general composition (amorphous and coaly type kerogen and pyrite), huminite reflectance and fluorescence intensity were also determined, in an effort to monitor any change of kerogen as defined by these properties. The results of micropetrographic examinations are shown in Table 2. The depyritization efficiency in 8 days, according to the micropetrographic analysis, was 71.4%. Bearing in mind the limits of experimental error with this method, the ratios of amorphous *vs* coaly type kerogen suggested that the maceral distribution of the kerogen in the various substrates has not been noticeably different.

Pyrolysis-gas chromatographic (P-GC) analysis was performed of the initial concentrate organic matter (Fig. 2(a)) and the substrate bacterialy treated for 8 days (Fig. 2 (b)). The ratios of compound types within the samples were taken as a basis for detecting structural changes. The homologous series of *n*-alkanes and *n*-alkenes in the chromatograms of the initial and depyritized substrates were determined according to the location of the coinjected standard (near the C_{12} *n*-alkane) of *ca* 30 min retention time and the location of prist-1-ene. The CPI, the ratio of higher *vs* lower alkanes, and the alkanes and the

alkanes/alkene ratio were determined for C_8-C_{24} identified range. The corresponding calculated values presented in Table 3 did not suggest any change in the kerogen structure as a result of the depyritization process.

Table 2. Micropetrographic examination of organic matter in initial and depyritized concentrates

Substrate	Composition (normalized to 100%)			Huminite reflectance % R_0	Fluorescence intensity*	Amorphous/coaly type kerogen ratio
	Amorphous kerogen (alginite B+liptinite) vol %	Coaly type kerogen vol %	Pyrite vol %			
KC	72.00	14.00	14.00	0.41±0.05	+++	5.1
KC_0	70.00	11.00	19.00	0.38±0.05	+++	6.4
KC_2	71.50	13.50	15.00	0.40±0.04	+++	5.3
KC_4	74.00	14.50	11.50	0.40±0.04	+++	5.1
KC_8	81.00	15.00	4.00	0.38±0.04	+++	5.4

*The intensity of fluorescence was graded according to an internal scale (+ to +++)

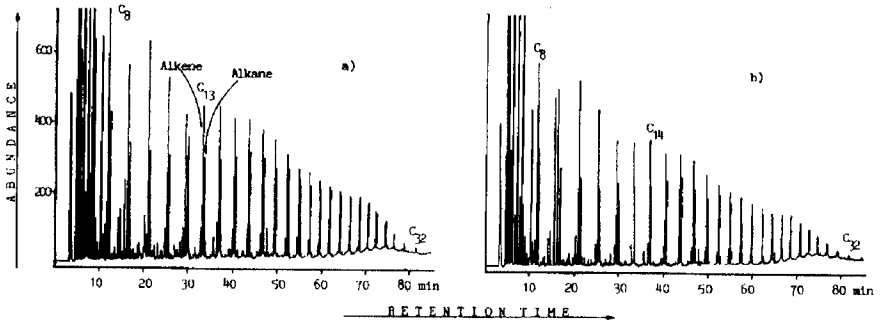

Fig. 2. P-GC chromatograms of the initial concentrate (a) and the substrate treated by *Acidithiobacillus ferrooxidans* for 8 days (b)

Table 3. Ratios (based on peak heights) calculated on the basis of P-GC or P-GC-MS analyses of the initial concentrate and the substrates, or pyrolyzates, respectively

Parameter	Samples	
	KC	KC_8
CPI for the C_8-C_{24} range	1.00	1.00
C_8-C_{19} *n*-alkanes/ C_{20}-C_{24} *n*-alkanes	3.68	3.84
n-alkanes/ *n*-alkenes	0.69	0.67
C_{27}-5α(H).20R: C_{28}-5α(H).20R: C_{29}-5α(H).20R	2:1:3	2:1:3

P-CG-MS analyses of the same two substrates (KC and KC_8) were also carried out. Pairs of corresponding mass chromatograms: m/z 253.29 (C_{20+} *n*-alkanes), m/z 217.19 (steranes) and m/z 191.18 (terpanes) are shown in Figs. 3-5 (a,b), respectively.

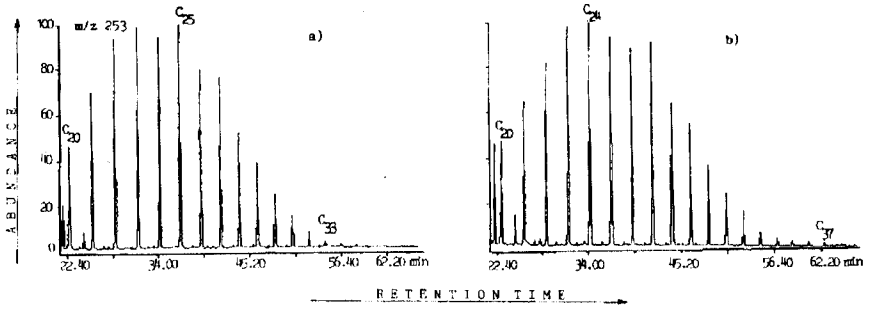

Fig. 3. P-GC-MS m/z 253.29 (C_{20+} *n*-alkanes) mass chromatograms of the initial concentrate (a), and the substrate treated by *A. ferrooxidas* for 8 days (b)

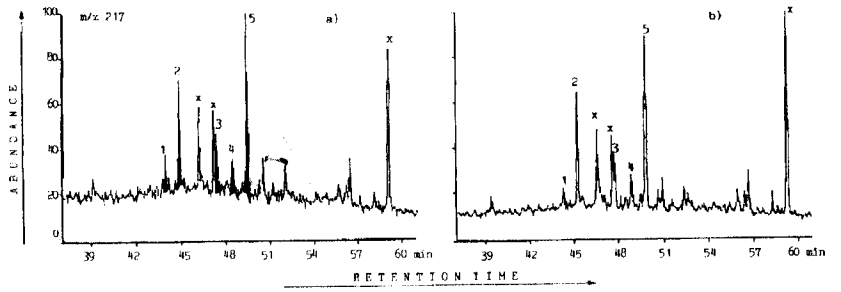

Fig. 4. P-GC-MS m/z 217.19 (steranes) mass chromatograms of the initial concentrate (a), and the substrate treated by *A. ferrooxidans* for 8 days (b)

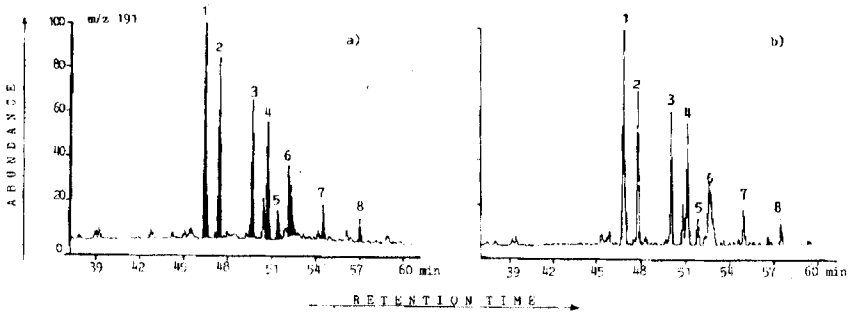

Fig. 5. P-GC-MS m/z 191.18 (pentacyclic triterpanes) mass chromatograms of the initial concentrate (a), and the substrate treated by *A. ferrooxidans* for 8 days (b)

The m/z 253.29 chromatograms (Fig. 3(a,b)) were very similar. From the sterane (m/z 217.19) chromatograms (Fig. 4(a,b)), the ratios of identified C_{27}, C_{28} and C_{29} stereoisomers were shown to remain unchanged during bacterial depyritization (KC and KC_8, Table 3).

The distribution of pentacyclic triterpanes observed in the m/z 191.18 chromatograms (Fig. 5 (a,b)), and the comparision of the calculated ratios of individual members, is shown in Table 3. These results also suggested that the organic matter did not suffer noticeable change during the *A. ferrooxidans* bacterial depyritization treatment.

Bacterial desilicification

To determine whether the composition and structure of the sedimentary organic matter had changed during bacterial desilicification by *B. c.*, the properties of the organic material in the reference sample were compared with the properties of the same material in the bacterially treated sample.

Desilicification experiments, which consisted of leaching for 30-days with *B. c.* Jordan in the modified Ashby's medium, were repeated four times. In all of these experiments, a progressive decrease in the pH was observed, indicating that organic acids, responsible for the desilicification (KRUMBEIN 1983), were constantly being produced. In spite of this, the leaching effect, expressed *via* the sum of SiO_2, Al_2O_3 and Fe_2O_3 relative to the contents of the same oxides in the reference sample, was not 100%. This fact suggested that the leaching effect depended on the type of mineral components in the shale sample, as well as on the relatively low desilicification efficiency of this particular *B. c.* strain. It was considered that a comparison of the composition and structure of the organic matter prior to and after 30-days exposure to *B. c.* is a sound basis for checking the kerogen stability during *B. c.* desilicification. The product resulting from the most efficient desilicification experiment (elimination of 24.3% of SiO_2 + Al_2O_3 + Fe_2O_3 relative to reference sample) was used for further examination.

The main motive of demineralizing the sediment was to remove most of the mineral matter interfering with the organic matter analysis. As the desilicification efficiency of the used *B. c.* Jordan strain was still rather low (~25%), and the relatively high remaining mineral matter content implied that elemental analysis may not be the best method for checking the stability of the organic matter during bacterial desilicification. This fact must be kept in mind when elemental analysis results are evaluated.

Atomic H/C ratios of the organic matter prior to and after 30-day exposure to *B. c.* (Table 4) differed only slightly, suggesting that the composition of the organic matter during bacterial desilicification remained stable with respect to this ratio. In contrast, the O+Sorg/C ratio of the organic matter of the

bacterially treated sample was somewhat higher. These results suggested that further investigation of the real source of the observed differences is needed, as well as further checks of the reliability of elemental analysis of samples containing high concentrations of mineral components (DURAND 1980).

The stability of the sedimentary organic matter during bacterial desilicification treatment was also monitored by infrared spectroscopy. The spectra of the substrates were complex, and direct correlation inconclusive. The ratios of quantitive estimates of the absorption bands of certain functional groups, representative of specific structures of sedimentary organic matter, were taken as the basis for the evaluation of structural changes caused by bacterial desilicification (Table 4). In summary, the IR data did suggest dramatic structural changes resulting from the 30-day bacterial treatment.

Table 4. Composition and intensity ratios of certain functional group bands in the IR-spectra of the reference and bacterially treated samples

	Reference sample	Bacterially treated sample
Atomic ratios		
H/C	1.79	1.75
O+Sorg/C	0.18	0.26
Ash %	53.80	43.40
Content of ash components, relative to the reference sample, %		
Al_2O_3	7.80	6.20
SiO_2	38.60	29.00
Fe_2O_3	4.80	3.60
IR-band intensity ratios of selected functional groups		
CH/>C=O	2.19	2.25
CH/OH	3.34	3.60
>C=O/OH	1.53	1.60

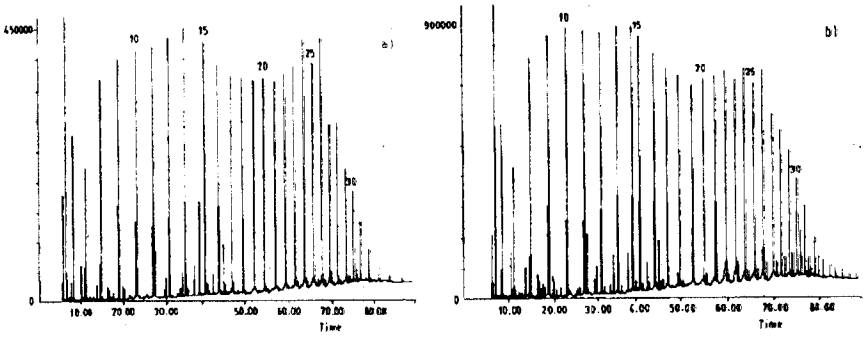

Fig. 6. P-GC chromatograms of the initial concentrate (a) and the substrate treated by *Bacillus circulans* for 8 days (b)

P-GC and P-GC-MS analyses were also carried out for the samples set. The total ion chromatogram of the bacterially treated sample was not significantly different from that of the untreated sample, with respect to the straight-chain hydrocarbon components (*n*-alkanes). Moreover, the pyrogram of the bacterially treated sample showed a bimodal distribution in the aliphatic hydrocarbon homologous series (n-C_7-C_{37}), as did the pyrogram of the reference sample. In addition, P-GC-MS analyses for the same sample pair did not show substantial changes resulting from bacterial treatment.

The pyrolyzates were very similar, and only minor variations were observed in the relative peak intensities. All major components were present in both pyrolyzates.

CONCLUSION

The experiments of bacterial demineralization, i.e., depyritization by *A. ferrooxidans* and desilicification by *B. c.*, carried out with Aleksinac oil shale as substrate, showed high efficiency in removal of pyrite (*ca* 91%) and silicates (*ca* 40%). Evidence of stability of sedimentary organic matter during bacterial demineralization was obtained by modern instrumental methods. Based on combined experimental approaches, the organic matter from Aleksinac oil shale seemed to remain unchanged during bacterial demineralization, thus demonstrating the advantages of bacterial *vs* chemical demineralization processes. Considerably greater efficiency in removal of silicates was achieved by zymogenous CHBS-1. However, the stability of the composition and structure of organic matter concentrates in this case is still to be examined.

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PRIPREMANJE KONCENTRATA ORGANSKE SUPSTANCE PRIMENOM MIKROBIOLOŠKIH METODA

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Izvod

U ovom radu su prikazani rezultati proučavanja bakterijske demineralizacije, izdvajanja silikata (desilicifikacije) pomoću *B.c.* i izdvajanja pirita (depiritizacija) pomoću *A. ferrooxidan.* Predložene metode su novi pristup rešavanju jednog od veoma važnih geohemijskih problema, izolovanja organske supstance (kerogena) iz sedimenata. Upotrebom uljnog škriljca iz Aleksinca, kao model supstrata, proučavana su dva najvažnija aspekta bakterijske demineralizacije: da li i kom stepenu deluje upotrebljena bakterija na sastav i strukturu kerogena. U eksperimentima depiritizacije pomoću *A. ferrooxidans* postignuta je visoka efikasnost (91%) udaljavanja pirita. Znatna eliminacija silikata (40%) ustanovljena je posle 30-dnevnog perioda luženja silikata pomoću *B.c.* Jordan. Za detaljnu analizu pre i posle bakterijske demineralizacije, pored standardnih uobičajenih metoda (kao npr. IR spektroskopija, elementarna mikroanaliza, mikropetrografska analiza) primenjene su i druge analitičke metode (kao npr. P-GC, P-GC-MS). Dobijeni rezultati su pokazali da organska materija sedimenata ostaje nativna tokom ovih procesa. Ovim rezultatima je pokazana prednost bakterijske u odnosu na hemijsku demineralizaciju.

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