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Microbial degradation of bitumen

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Summary. A blown bitumen Mexphalte R 90/40 with a high content of saturated hydrocarbons was degraded by several microorganisms to the same extent. In batch cultures of *Saccharomycopsis lipolytica*, maximal biodegradation was estimated to be about 9% w/w, $3.2 \cdot 10^{-3}$ g/cm^2 and $3.1 \cdot 10^{-3}$ cm of degraded bitumen. The Mexphalte R 90/40 degradation rate was closely coupled to biofilm formation. The microbial activity concerned predominantly the oxidation of saturated hydrocarbons. A direct distillation bitumen 80/100 with a low content of saturated hydrocarbons and a high content of aromatic hydrocarbons and resins was more resistant to biodegradation.
Key words. Biodegradation; bitumen; radioactive waste.

Introduction

Bitumen is a residue of successive crude oil treatments. It is a mixture of high molecular weight hydrocarbons with such a complex chemical composition that it is still undefined. Because of their theoretical long term stability, bitumen materials are used in many countries[1–3] to immobilize low and intermediate level wastes. The bitumenized wastes are then disposed of in deep geological repositories.

Although the composition of bitumen is relatively complex and undefined, its organic content makes it susceptible to biodegradation. It is known that many microorganisms have the ability to degrade petroleum hydrocarbons[2, 7, 11, 16] although few studies have included the microbial degradation of bitumen, even when it has been shown that bituminous materials are subject to microbial attack and deterioration[3–5, 9, 10]. The presence in a repository of a large microflora which can undertake such geochemical processes[8] could affect the long-term stability of bitumen and the integrity of bitumenized wastes.

Rates of bitumen biodegradation depend on the chemical composition of the bitumen substrate, the metabolic specificity of the microorganisms involved and a large number of biological and physico-chemical parameters liable to come into play in a disposal site. Among the methods used to assess biodegradation rates, the measurement of CO_2 production is one of the most commonly used[3, 13].

Since the rates of oxidative degradation processes of hydrocarbons are greater than the ones of anaerobic decomposition[2], our experiments were therefore carried out under aerobic conditions.

In order to evaluate the microbial degradation of bitumen, investigations were made a) to quantify the rate of biodegradation from the CO_2 production, b) to show the possible alterations of bituminous material through chemical transformations such as the oxidation processes, c) to compare the activities of microorganisms from the point of view of metabolic specificity, and d) to determine whether the chemical nature of bitumen can affect

the degree of microbial utilization by comparison of two types of bitumen: blown Mexphalte R 90/40 and direct distillation Mexphalte 80/100.

Materials and methods

Substrate

Two types of bitumen were used, a blown Mexphalte R 90/40 and a direct distilled Mexphalte 80/100, kindly provided by Shell (Grand Couronne, France).

Organisms

Degradation was tested with various pure strains: *Pseudomonas aeruginosa, Pseudomonas putida, Arthrobacter* sp., *Saccharomycopsis lipolytica* (DSM Nos 1253, 50202, 312 and 1345, respectively); *Torulopsis holmii* was isolated from a fuel tank and a natural mixed culture enriched from a bituminous site which included a strain of *Pseudomonas aeruginosa.*

Culture conditions

The degradation of the bitumen was carried out in 500-ml Erlenmeyer flasks containing 200 ml of culture medium and about 100 mg bitumen. The medium composition is shown below and has been optimized for hydrocarbon degradation:

1.36 g · l^{-1} KH_2PO_4, 0.6 g · l^{-1} Na_2HPO_4, 70 mg · l^{-1} $MgSO_4$, $7H_2O$, 1 g · l^{-1} NH_4NO_3, 0.1 g · l^{-1} $CaCl_2$, 2 mg · l^{-1} $FeSO_4$, $7H_2O$, 1 mg · l^{-1} of yeast extract and 0.1 ml of trace elements solution (50 mg · l^{-1} $CuSO_4$, $5H_2O$, 100 mg · l^{-1} H_3BO_3, 100 mg · l^{-1} $MnSO_4$, H_2O, 100 mg · l^{-1} $ZnSO_4$ $7H_2O$, 1 g · l^{-1} $(NH_4)_6$ $Mo_7O_{24} \cdot 4H_2O$, 100 mg · l^{-1} Ca $(NO_3)_2$, $6H_2O$).

The enrichment precultures were obtained with 100 ml basic medium containing 1 ml of Arabian light petroleum. After an 8-day incubation period, the precultures were centrifuged and washed twice. The pellets were used to inoculate each culture. Cultures were incubated at 30 °C in a rotary shaker (at 120 rpm). Control flasks contained the inoculated basic medium without bitumen.

Bitumen separation

Asphaltenes, the heptane-insoluble material, were retained by filtration through a Büchner funnel and weighed after drying. The heptane-soluble compounds, the maltenes, were fractionated into saturated and aromatic compounds, and in resins by liquid chromatography on an activated aluminium column (neutral aluminum oxide 90 Merck, 17 × 2.0 cm) by successive elutions with 300 ml of heptane, 300 ml of toluene and 200 ml of toluene-methanol. The solvents were then evaporated and the weight of each fraction was determined.

Table 1. Elementary composition of the two types of bitumen used

Bitumen	Elementary composition weight %				
	C	H	O	N	S
Mexphalte R 90/40	84	10.5	1.5	0.5	3.7
Mexphalte 80/100	83.6	10.3	1.1	0.4	Nd

Analysis

Consumption of oxygen and generation of CO_2 were determined with a VARIAN GC 3400 gas chromatograph. The flasks were hermetically sealed and aerobic conditions were maintained by a sequential renewal of air. Gas was withdrawn and injected into the gas chromatograph with a Hamilton Syringe. Krypton was used as internal standard allowing the determination of the CO_2 released. To determine the total amount of CO_2 generated, the values were corrected with a factor B (dependent on the pH medium of each culture)[17]

$$B = \left[1 + \frac{RT}{H} \cdot \frac{V_L}{V_G}(1 + 10^{(pH - pK)})\right]$$

V_L: liquid volume; V_G: gas volume

where the pK for pKH_2CO_3/HCO_3^- is 6.46.

Gas liquid chromatographic analysis was performed with a GIRDEL (series 30) chromatograph with a flame-ionization detector (FID). The column used was a 50 × 0.3 mm ID glass capillary coated with OV1 (methylated silicon). Column conditions were: carrier gas, He 0.9 bar; initial temperature, 110 °C; programmed temperature, 3 °C min^{-1}; final temperature, 290 °C.

IR spectra were recorded in carbon-tetrachloride with IR NICOLET SDX. Protein was determined with the Pierce Micro BCA protein assay reagent.

Results

a) Mexphalte R 90/40 biodegradation under air renewal

Several microorganisms were tested for their bitumen biodegradation capabilities. The cumulative carbon dioxide released and oxygen consumed were measured for *Pseudomonas aeruginosa, Pseudomonas putida, Arthrobacter* sp., *Saccharomycopsis lipolytica, Torulopsis holmii,* a natural mixed culture (mixture I) and two combinations of pure strains (mixture II: *P. putida, S. lipolytica, T. holmii*; mixture III: P. *aeruginosa, Arthrobacter* sp., *T. holmii*). Figure 1 shows that oxygen consumption is related to CO_2 production. The rates of biodegradation are very low and the activities of different pure strains are all very similar.

Consumption of oxygen, although constantly supplied, ceased after some time with all the microbial strains indicating that biological activity was very small. In batch cultures oxygen is neither the main nor the only limiting factor. A nutrient limitation or accumulation of metabolic by-products might also inhibit the biodegradation potential of the microflora[2,12].

The total amount of CO_2 released at the end of growth after approximately 2400 h is used to determine the percentage of carbon released as CO_2 from the bitumen,

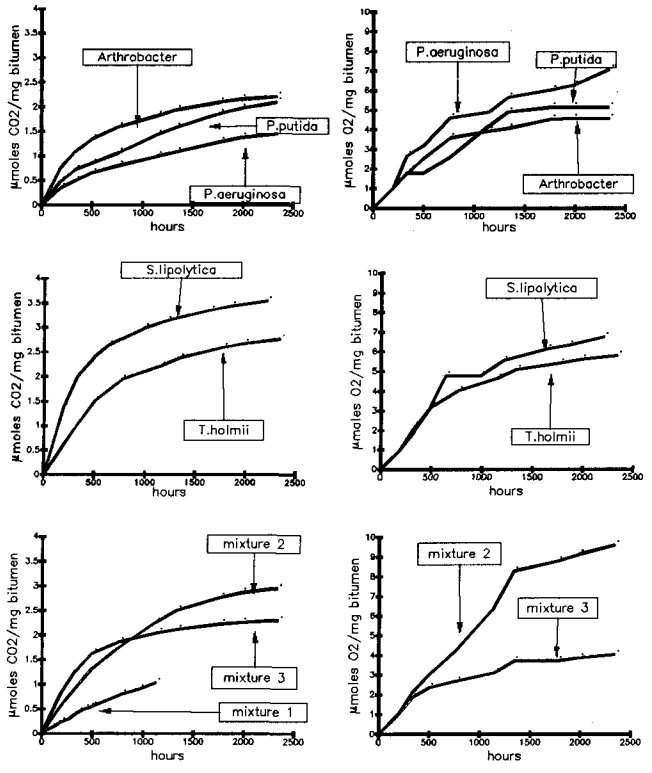

Figure 1. CO_2 production and O_2 consumption from Mexphalte R 90/40 by different microbial cultures (mixture 1: natural mixed culture; mixture 2: *P. putida, S. lipolytica, T. holmii*; mixture 3: *P. aeruginosa, Arthrobacter* sp., *T. holmii*).

and the total percentage of degraded bitumen, assuming that 60% of carbon is converted to CO_2 during biodegradation, and that bitumen R 90/40 carbon content is 84%. The results are summarized in table 2.

Degradation was observed in all the microbial cultures; about 10% of Mexphalte R 90/40 was degraded after 2200 h and about 100 ml of CO_2 per g of bitumen were generated.

The natural mixed population consisting of *P. aeruginosa*, isolated from a bituminous site and the mixture of pure strains had no increased activity when compared to single pure strains. We calculated therefore that biodegradation activity is similar for both bitumens and independent of the microbial culture used. To evaluate the long term stability of bitumen in a deep storage, a theoretical extrapolation of data has been made. Such an extrapolation depends heavily on the conditions of the experiment. The calculations are presented from data with *S. lipolytica*. Assuming that the end of gas production is real and ignoring the environmental parameters of a repository, the cumulative data of CO_2 production of *S. lipolytica* were analysed by non-linear regression of a kinetic model derived from the Monod equation.

The gas production can be written as follows:

$$Q_i = Q_m - A^{(-kti)} \quad (1)$$

where Q is the CO_2 amount at t, A a corrective factor associated with the integration constant, and k the first order kinetic constant.

Parameter values were calculated as $k = 2.47 \cdot 10^{-3}\ h^{-1}$; $A = 3.72$; $Q_m = 3.78 \cdot 10^{-6}$ moles mg^{-1}. The maximal percentage of degraded bitumen by *S. lipolytica* was deduced as 9.0%.

Maximal biodegradation by *S. lipolytica* was calculated to be about $3.2 \cdot 10^{-3}$ g/cm^2 or $3.1 \cdot 10^{-3}$ cm. The limiting conditions in batch cultures do not permit the expression of a biodegradation rate per year.

Bitumen is a solid material and its biodegradation involves a microbial colonization of the bitumen surface. Biofilm formation has been observed by scanning electron microscopy and epifluorescence techniques[17,18] and is even observed visually (fig. 2).

Protein has been determined from the liquid culture medium and from the biofilm after its removal from the solid support by washing during growth of *S. lipolytica* on Mexphalte R 90/40. Figure 3a shows that biofilm formation is closely related to the CO_2 production. The biomass in suspension increased parallel to the CO_2 released at the beginning, then decreased, probably due to adsorption of the cells on the bitumen surface (fig. 3b). The biofilm is characterized by $1 \cdot 10^5$ CFU/cm^2 equal to 9.9 mg cells/cm^2.

Table 2. CO_2 production and % of degraded Mexphalte R 90/40 by biodegradation with different microbial cultures after about 2400 h when no further growth was observed

Microbial cultures	µmoles · mg^{-1}	Production** ml g^{-1}	Percentage of carbon released as CO_2 % w/w	Percentage of degraded bitumen % w/w
P. aeruginosa	1.45	64.8	4.2	7.2
P. putida	2.09	93.6	6.0	10.2
Arthrobacter sp.	2.22	95.2	6.4	10.8
S. lipolytica	3.54	88.1	5.7	9.5
T. holmii	2.76	68.0	3.9	7.3
Mixed natural* culture (mixture I)	0.90	40.4	3.4	4.4
Mixture II	2.30	69.5	5.7	9.5
Mixture III	2.95	89.2	4.5	7.6

*1100 h of growth; **values including the corrective factor: $\left[1 + RT \cdot \frac{V_L}{V_G}(1 + 10^{(pH - pK)})\right]$.

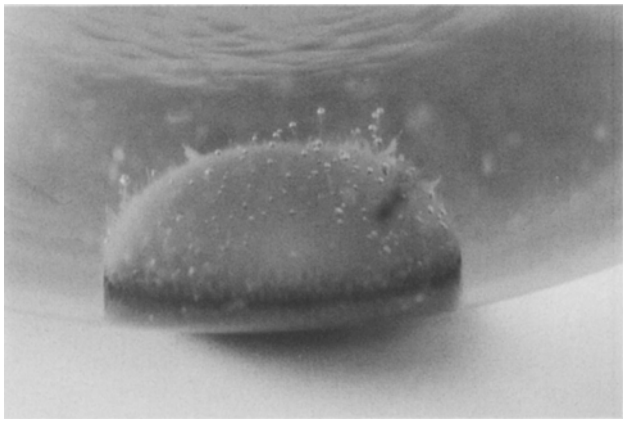

Figure 2. Visual observation of *S. lipolytica* biofilm on Mexphalte R 90/40.

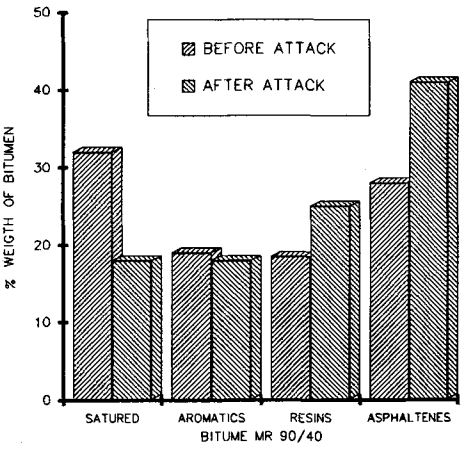

Figure 4. Chemical composition of Mexphalte R 90/40 before and after *S. lipolytica* attack.

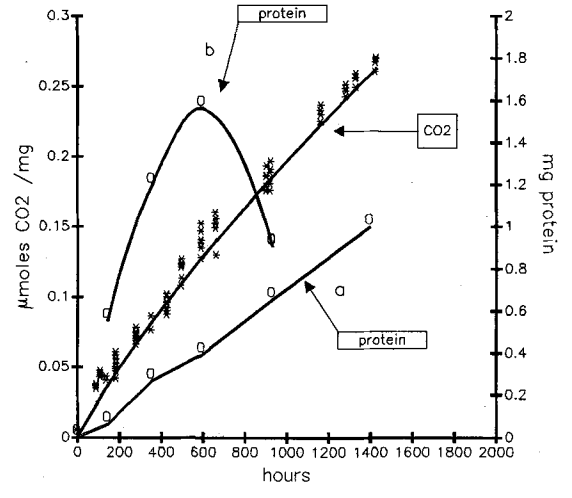

Figure 3. Relationship between CO_2 production from Mexphalte R 90/40 and amount of *S. lipolytica* adsorbed (a) and in supernatent (b).

b) Chemical changes of Mexphalte R 90/40 as a result of the action of S. lipolytica

Bitumen can be separated by column chromatography into different fractions: 1) saturated or paraffinic hydrocarbons; 2) aromatics, containing from one to six benzenoid rings; 3) resins or polar compounds, and 4) asphaltenes insoluble in n-heptane or n-hexane.

The composition of Mexphalte R 90/40 before and after 41 days of growth of *Saccharomycopsis lipolytica* is given in table 3 and figure 4, and includes the elementary analysis of the different fractions. A clear decrease in the saturated and aromatic hydrocarbons compared with the more polar and oxidized fractions such as resins and asphaltenes is seen, confirming that microbial attack starts with hydrocarbon oxidation as demonstrated by the significant increase of oxygen in the asphaltenic and resins fractions.

The dominant metabolism of the n-saturated fraction in Mexphalte R 90/40 is shown in the profiles of gas-liquid chromatography (fig. 5).

IR analyses on the bitumen before and after *S. lipolytica* growth were performed on samples from the surface and from the middle of the particles. The spectra of the initial (fig. 6a) and of the degraded bitumen (fig. 6b, c) provide evidence of significant oxidation of the surface of the substrate (fig. 6b). New bands appear or increase, especially O–H (3640–3100 cm^{-1}) and C = O stretching absorption (1720–1690 cm^{-1}). Some changes are observed in the aromatic C–H absorption (900–750 cm^{-1})

Table 3. Distribution of the different bitumen MR 90/40 constituents and their elemental composition before and after degradation by *Saccharomycopsis lypolytica* (41 days)

Constituent fractions		% Weight of bitumen	% Weight of fraction C	H	O	N	S
Before growth	Asphaltenes	25.3	82.4	7.5	2.5	1.1	4.0
	Saturated oils	35.5	87.3	12.5	0.4	nd	nd
	Aromatic oils	19.6	85.5	9.8	1.0	0.2	3.3
	Resins	17.6	81.1	7.5	43	0.7	3.4
	Residues	2.3	–	–	–	–	–
After growth	Asphaltenes	44.3	79.5	7.3	6.1	< 0.7	6.2
	Saturated oils	20.5	87.1	13.1	< 0.1	< 0.7	nd
	Aromatic oils	13.2	83.6	10.5	0.15	nd	5.7
	Resins	19.1	73.4	8.9	9.5	1.5	6.7
	Residues	2.8	–	–	–	–	–

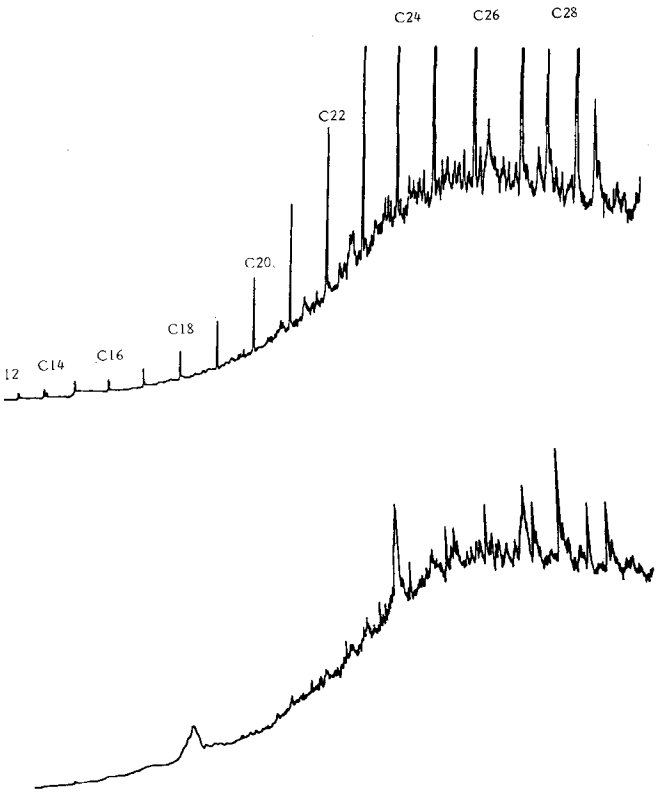

Figure 5. GLC profile of the n-saturate fraction of Mexphalte R 90/40 before and after growth of *S. lipolytica*.

and in the characteristic band of long linear paraffinic chains (720 cm^{-1}), confirming the biodegradation of saturated and some aromatic hydrocarbons. The IR analysis from the middle of the sample of bitumen (fig. 6c) is unchanged indicating that *S. lipolytica* acts only at the surface.

c) Influence of the chemical composition of bitumen

The different fractions of Mexphalte R 90/40, bitumen and each generic family of it were inoculated in batch experiments with *S. lipolytica* for 60 days and the percentage of degraded substrate calculated from the cumulative CO_2 production. The saturated fraction was preferentially attacked (10% w/w) while the aromatics were only slightly (1% w/w) and asphaltenes not degraded at all agreeing with the general observations [1, 2, 6, 15].

Asphaltenic and resin fractions had been considered before to be recalcitrant to biodegradation [2], but the asphaltenic fractions of crude oil have been shown to be partially degraded by a mixed marine bacterial population with saturated hydrocarbons as cosubstrate [14].

The mixed culture (mixture I) was inoculated on the resin and asphaltene fractions extracted from Mexphalte R 90/40 in the presence and absence of the saturated fraction as cosubstrate. The biodegradation rates of these mixtures were compared with the one obtained on the saturates alone. Figure 7 shows the biodegradability of

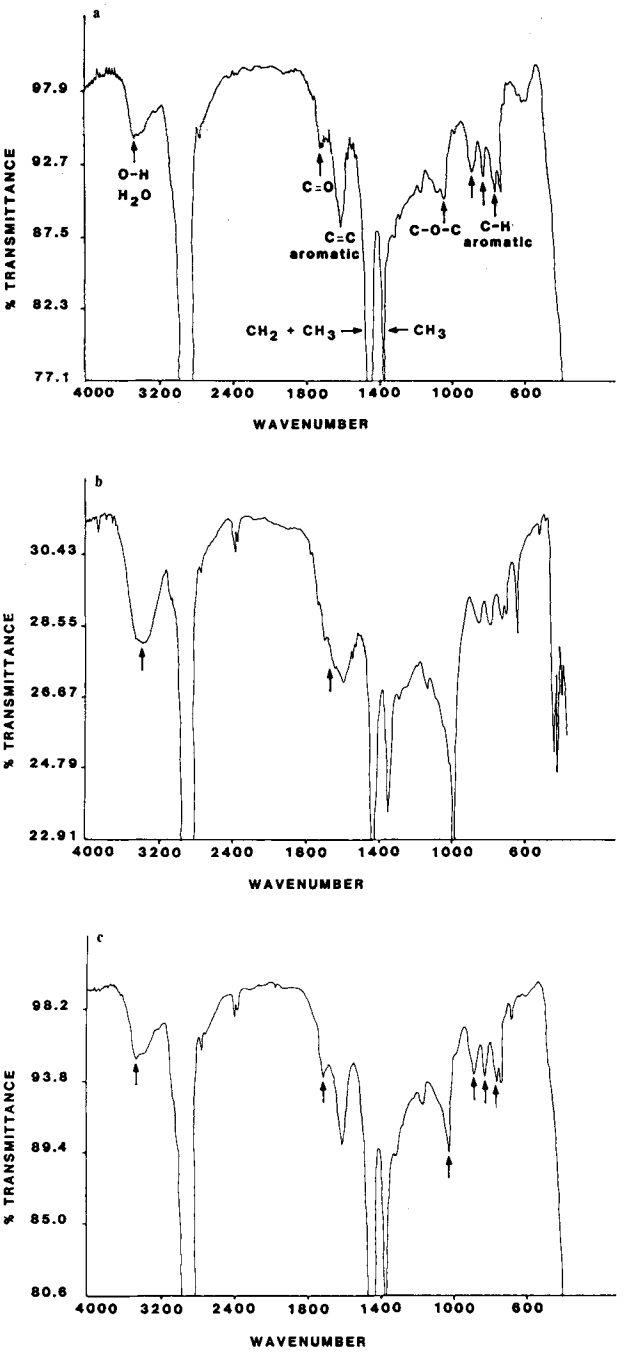

Figure 6. Spectrum of the initial Mexphalte R 90/40 (a); of the surface of bitumen sample (b) and the middle of bitumen sample (c) after *S. lipolytica* growth.

saturates by this mixed culture and the recalcitrant character of asphaltenes and resins. The presence of saturates with the asphaltenic and resin fractions did not increase the biodegradation of the latter; on the contrary, the recalcitrant fractions seemed to slow down the rate of biodegradation of the saturates.

Bitumen materials vary greatly in their chemical composition resulting from their origin and the manufacturing, e.g. blowing or direct distillation. The effect of the nature

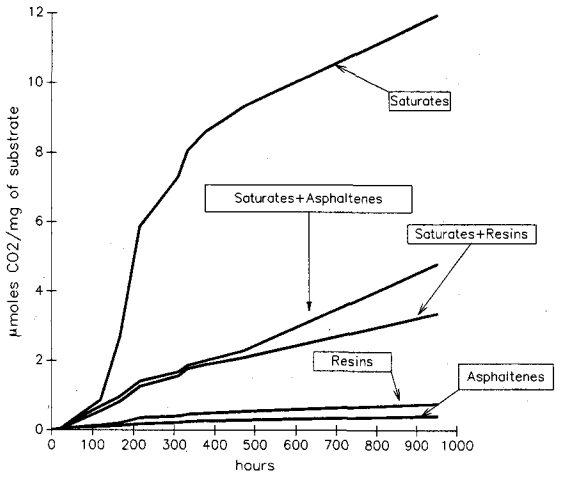

Figure 7. Biodegradation rates of saturates, asphaltenes and resins extracted from Mexphalte R 90/40 and asphaltenes and resins associated with saturates.

of bitumen material on the degradation rates was studied using blown Mexphalte R 90/40 and direct distilled Mexphalte 80/100. The cumulative CO_2 evolved (fig. 8) shows that *Saccharomycopsis lipolytica* attacks both Mexphalte R 90/40 and 80/100 with the same initial rate, but that biodegradation ceases after a short time on bitumen 80/100. The low amount of saturates (9.9% w/w) and the high content of recalcitrant compounds (aromatic oils 30.1% w/w; resins 46.9% w/w) explain the poor Mexphalte 80/100 biodegradability.

From CO_2 production rates, the maximal degradation of bitumen 80/100 by *Saccharomycopsis lipolytica* in batch culture was calculated from equation (1) to be about $1.7 \cdot 10^{-3}$ g cm^{-2} or $1.65 \cdot 10^{-3}$ cm.

Conclusions

The biodegradation potentials of four pure cultures, bacteria and yeasts, and three natural or reconstituted mixed cultures on a Mexphalte R 90/40 in aerobic batch conditions were found to be very similar. From CO_2 generation, maximal degradation of bitumen R 90/40 was estimated to be 9.0% w/w, $3.2 \cdot 10^{-3}$ g · cm^{-2} and $3.1 \cdot 10^{-3}$ cm for *Saccharomycopsis lipolytica*. It was assumed that the cessation of CO_2 production is real, that bitumen degradation occurs through homogeneous surface attack and that the surface area of the bitumen is homogeneous. Metabolic activity in batch culture is arrested probably due to inhibiting conditions present, e.g. accumulation of metabolic byproducts [10]. In a continuous culture using the same bitumen biodegradation is expected to be greater.

The Mexphalte R 90/40 degradation rate by *S. lipolytica* as seen by the CO_2 generation is closely linked with a biofilm formation at the surface of the bitumen sample. Of the various chemical fractions of Mexphalte R 90/40 the saturated and the aromatic fractions were lost with about a 40% and 25% reduction in these fractions respectively. A complete disappearance of n-paraffins, an increase of oxygen content in the resins and the asphaltenes characterize bitumen oxidation by *Saccharomycopsis lipolytica*.

Growth on individual bitumen fractions confirms the preferential utilization of the n-saturates fractions. Resins and asphaltenes are resistent to *Saccharomycopsis lipolytica* attack and their presence inhibits the biodegradation of n-saturates. The chemical composition of a bitumen has a marked effect on its biodegradability. A direct distillation bitumen Mexphalte 80/100 retained for the coating of LLW wastes shows a greater resistance to degradation by *Saccharomycopsis lipolytica* than Mexphalte 90/40 probably due to its low content of saturates. The composition and content of other bitumen components may also influence the biodegradability [16]. It has been shown that bituminous materials are subject to microbial attack and deterioration in many of their technical applications [3, 5, 8, 9]. Therefore the use of bitumen materials for the coating of low and intermediate level wastes requires a deeper knowledge of the effects that the environmental conditions of a repository can have on its biodegradation. Also, how such biodegradation could affect the long-term stability and integrity of bitumized wastes requires further investigation.

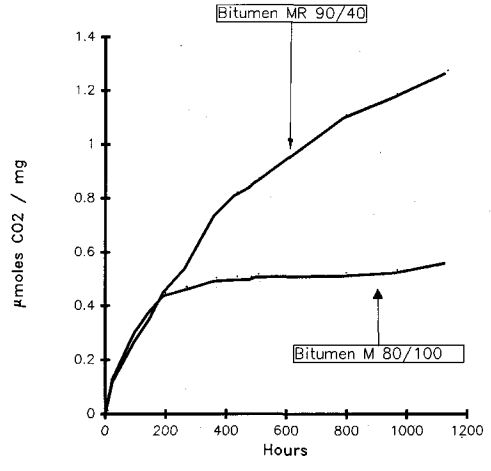

Figure 8. Cumulative CO_2 released by *S. lipolytica* from Mexphalte R 90/40 and Mexphalte 80/100.

Acknowledgments. We thank Mrs V. Celi for technical support.

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Biodegradation of bitumen used for nuclear waste disposal

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Summary. Studies have been carried out to test microbial degradation of bitumen used for encapsulating radioactive waste in Sweden. Microorganisms have been isolated that degrade bitumen. In long-term tests under conditions simulating those in the silo part of the final repository for low- and intermediate-level radioactive waste, both aerobic and anaerobic degradation of bitumen has been found, equivalent to 0.6–1.5 μmoles CO_2/month · mg bitumen and 1.1–1.5 μmoles CO_2/month · mg bitumen, respectively.
Key words. Microorganism; bacteria; bitumen; biodegradation; gas production; oil degradation; hydrocarbons; radioactive waste.

Introduction

In Sweden some of the low- and intermediate-level radioactive waste from nuclear power plants will be encapsulated in bitumen, put into steel drums and placed in mined rock caverns 50 m below the Baltic sea bed. After the repository is finally closed it will be filled with water. The question has been raised whether microorganisms can degrade bitumen under the prevailing conditions and contribute to increased gas production. Apart from this direct production due to microbial decomposition of bitumen, it is also assumed that the microbial activity may decrease the pH so that hydrogen-evolving corrosion will occur with large amounts of gas being produced. For safety reasons it is therefore important to estimate the microbial activity in the repository and the rate of microbial degradation of bitumen[1–9,15–17,20]. This paper summarizes experimental investigations carried out in Sweden on aerobic and anaerobic degradation of bitumen used for encapsulation of radioactive waste[11].

The literature available shows that the knowledge is very limited on anaerobic degradation of hydrocarbons including asphaltenes (bitumen). Biochemical pathways are in most cases unknown. Most studies are limited to single compounds and not concerned with complex mixtures like bitumen. It has been claimed that anaerobic degradation of asphalt occurs, e.g., anaerobic conversion of thiophene and other sulphur-bearing compounds of petroleum (asphaltenes, polysulphides, heavy residues) with production of H_2S has been demonstrated[6].

The growth of microorganisms on the surface of bitumen has been shown using electron microscopy[2,13,19]. A