Journal of Industrial Microbiology, 1 (1987) 371–375
Elsevier

SIM 00050

Solubilization of coal by an extracellular product from *Streptomyces setonii* 75Vi2

G.W. Strandberg and S.N. Lewis

Chemical Technology Division, Oak Ridge National Laboratory, Oak Ridge, TN, U.S.A.*

Received 15 September 1986
Revised 12 December 1986
Accepted 12 December 1986

Key words: Coal; Coal solubilization; *Streptomyces*; Bacteria

SUMMARY

Several low-ranked coals were solubilized when placed on the surface of agar cultures of *Streptomyces viridosporous* T7A and *S. setonii* 75Vi2. When grown in submerged culture *S. setonii* 75Vi2 produced an extracellular component that was capable of solubilizing coals. The extracellular coal solubilizing component had a molecular weight of < 10 000 and was heat stable since, after 1 h at 121°C, only 30–40% of the activity was lost. Treatment with any of three proteases also appeared to be ineffective in decreasing activity. These results suggest that coal solubilization by *S. setonii* 75Vi2 is nonenzymatic.

INTRODUCTION

The ability of microorganisms to degrade coal and utilize coal constituents has been of interest for many years [3,5,6]. Recently, however, there has been a renewed interest in the possibilities for microbial processes to convert coal to other fuels and chemicals.

Cohen and Gabrielle [1] described lignite solubilization by two fungi, *Trametes versicolor* ATCC 12679 and *Poria monticola* ATCC 11538. Ward [9] and Scott et al. [7] found several other fungi capable of solubilizing coal. These organisms were found growing on lignites or in the vicinity of lignite deposits. The coal solubilization product is a highly polar, water-soluble material of moderate to high molecular weight and with a high degree of aromaticity [7]. Although it was suspected that coal solubilization was due to an extracellular enzyme(s), possibly ligninase, there has been no conclusive demonstration of extracellular coal-solubilizing activity in culture broths or cell-free extracts of these fungi. Coal solubilization by these fungi appears to be limited to conditions in which the coal must be in contact with aerial mycelia (i.e., fungal mats grown on the surface of static liquid or agar media).

* Research supported by the Fossil Energy Advanced Research and Technology Program, managed by the Pittsburg Energy Technology Center, U.S. Department of Energy, under Contract No. DE-AC05-840R21400 with Martin Marietta Energy Systems, Inc.

Correspondence: Dr. G.W. Strandberg, Chemical Technology Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, U.S.A.

Fakoussa and Trüper [2] found spectroscopic evidence for the solubilization of coal by a strain of *Pseudomonas* isolated from a mixed enrichment culture growing in the presence of finely ground bituminous coal. They suggested that both an enzyme and a surface-active agent produced by the organism were involved in attacking coal.

A primary interest of our laboratory has been the potential for developing a bioreactor system for coal solubilization. The advantages of a submerged culture process and the limitations imposed by the surface culture requirements for fungal activity led us to consider the use of bacteria. Furthermore, the high lignin content of low-ranked coals (up to 80%) and the general assumption that ligninases could be responsible for coal solubilization (several of the fungi are reportedly capable of lignin degradation) suggested lignin degradation as a likely criterion in searching for other organisms capable of coal solubilization. For this reason it was decided to examine known lignin-degrading bacteria. As a consequence of this work we can report the ability of two lignin-degrading strains, *Streptomyces viridosporous* T7A and *S. setonii* 75Vi2 to solubilize low-ranked coals when cultured on agar media and the production of an extracellular component with coal-solubilizing activity by *S. setonii* 75Vi2 when grown in submerged culture in the absence of coal.

MATERIALS AND METHODS

Streptomyces viridosporous T7A and *S. setonii* 75Vi2 (ATCC 39116) were kindly provided by D.L. Crawford, University of Idaho, Moscow, ID. These organisms were maintained by routine subculture on Sabouraud Maltose agar (Difco, Detroit, MI) slants at 30°C.

Three methods were used to demonstrate the solubilization of low-ranked coals:

(1) A known weight (0.3–0.5 g dry weight) of coal particles (1–2 mm in size, see below) was dispersed over the surface of 7–10-day agar cultures. Initial growth and subsequent coal solubilization were conducted at 30°C and at >80% relative humidity [7,8]. The extent of coal solubilization was determined by washing residual, nonsolubilized coal from the surface of a culture with water. The solids were recovered by centrifugation ($\approx$2000 × *g*, 10 min), washed several times with deionized, distilled water, and then dried for 16–18 h at 95–100°C. The amount of solids (cellular material) washed from the surface of control cultures without coal was negligible.

(2) The solubilization of coal in suspension cultures was accomplished by adding coal particles directly to 7-day cultures grown in shake flasks (100-ml medium in 250-ml Erlenmeyer flasks, 30°C, 100 rpm, 2-in stroke) of Sabouraud Maltose broth. Shaking was continued for 2 days after the addition of coal. The residual, nonsolubilized coal was separated from the cells by gentle agitation and decantation of the lighter, suspended cells using several volumes of water. The dry weight of the residue was determined as above.

(3) Cell-free activity was demonstrated in filter-sterilized (0.22- or 0.45-μm pore size, Millipore Corp., Bedford, MA) culture broths. The sterilized broth with added coal was shaken at 30°C (100 rpm, 2-in stroke). The nonsolubilized coal was recovered by centrifugation (2000 × *g*, 10 min) or on tared filters and washed several times with deionized, distilled water. The dry weight of the oven-dried material was determined as before.

Several coals were tested, including North Dakota, Mississippi, and Texas lignites and a Wyoming-Dakota (Wyodak) subbituminous coal. The origins and properties of these coals have been described previously [7,8]. Also, some of these coals were pretreated with 8 M HNO_3 to enhance their susceptibility to solubilization [8]. The coals were sterilized by autoclaving (121°C, 45 min) prior to contact with the organisms or cell-free broth.

The active fraction in cell-free broths was tested for its susceptibility to proteases. Filter-sterilized culture broths were adjusted with HCl to pH 4.0 prior to the addition of 0.5 mg/ml of *Aspergillus* acid proteinase (EC 3.4.23.6, Type XIII, 0.32 units/mg) or to pH 7.5 prior to the addition of 0.5 mg/ml of either trypsin (porcine pancreatic, Type IX, EC 3.4.21.4, 16950 BAEE units/mg) or crude

pancreatic protease (Type 1, 8 units/mg). The enzymes, obtained from Sigma Chemical Co., St. Louis, MO, were filter-sterilized before being added to the culture broths. The samples were then shaken for 24 h at 30°C. Prior to the addition of coal, the pH was readjusted with NaOH to the original pH of the broth (8.1–8.8). The coal-solubilizing activity in these broths was determined as above.

RESULTS AND DISCUSSION

Streptomyces viridosporous T7A and *S. setonii* 75Vi2 both solubilized North Dakota, and nitric-acid-treated Texas and Mississippi lignites and Wyodak subbituminous coals when they were added to the surface of agar cultures. Coal solubilization was evidenced, in a manner similar to that observed with fungi, by the appearance of a black liquid surrounding the pieces of coal, within 24–48 h after addition to 7-day cultures. The initial rate of solubilization was more rapid than with fungi [7] but the degree of solubilization was not as extensive. For example, the solubilization of North Dakota lignite by *Candida* sp. ML13 progressed slowly over a period of 7–10 days ($\approx$12% solubilization), eventually reaching 80–90% solubilization after 14 days [7]. In contrast, from 25–55% of the coal was solubilized within 3–5 days by the two streptomycetes, but further solubilization was not evident beyond this period. The optimization of conditions using the surface culture procedure could lead to more extensive solubilization.

When coal was added to a 7-day shake flask culture of *S. setonii* 75Vi2, solubilization was soon apparent, as indicated by a blackening of the culture broth within 3–4 h. However, no solubilization occurred under similar conditions with *S. viridosporous* T7A. The extent of solubilization of North Dakota lignite and nitric-acid-treated Wyodak subbituminous coal after 2 days exposure to a culture of *S. setonii* 75Vi2 is shown in Table 1.

The rapid (a few hours) onset of solubilization and the apparent absence of a requirement for a direct association of the cells with the coal particles (i.e., there was no attachment of the cells to the coal) indicated that the coal-solubilizing activity was already present in the culture broth. This conjecture was readily demonstrated by effecting coal solubilization with filter-sterilized (0.22-μm pore size filter) broths from 7-day cultures of *S. setonii* 75Vi2 grown in the absence of coal. Within 10–15 min after addition to the broths, the coal was seen to be dissolving. Typical results showing the decrease in dry weight of two coals after 2 days contact with different volumes of cell-free culture broths of this organism are presented in Table 2. The consistency of the data, although obtained from several experiments, indicates that the extent of solubilization was due to inherent properties of the coals rather than a limiting factor in the cell-free broths. No cell-free activity could be demonstrated in culture broths from *S. viridosporous* T7A.

As coal solubilization proceeded, there was a substantial loss in the integrity of the coal particles (originally 1–2 mm in size). Most of the residual, nonsolubilized coal appeared as fine particulates (less than 0.1 mm in size). However, filtered culture

Table 1

The solubilization of coal by a submerged culture of *Streptomyces setonii* 75Vi2

Conditions	Coal[a]	Dry weight of coal (mg)		Weight change (%)
		initial	final[b]	
Sterile medium	NDV	542	517	− 4.6
	Wyodak-PT	509	518	+ 2.0
Submerged cultures[c]	NDV	508	289	−43.0
		511	371	−27.5
	Wyodak-PT	513	98	−81.0

[a] NDV is a North Dakota lignite obtained from American Colloid Co., Skokie, IL. Wyodak-PT is a subbituminous coal obtained from Amax Coal Co., Indianapolis, IN. It was pretreated by soaking for 48 h in 8 M HNO_3 (see Ref. 8).

[b] Final dry weights of residual coal after 48 h incubation with cultures.

[c] Coal was added to 7-day shake cultures (100 ml medium in 250-ml Erlenmeyer flasks, Sabouraud Maltose medium, 30°C, 100 rpm, 2-in stroke).

Table 2

The solubilization of coal by cell-free broth from *Streptomyces setonii* 75Vi2

Conditions	Volume (ml)	Coal[a]	Dry weight of coal (mg)		Weight change (%)
			initial	final[b]	
Sterile medium	25	NDV	542	517	− 5
	50		536	495	− 8
	50		536	497	− 9
	50		535	504	− 6
	25	Wyodak-PT	509	518	+ 2
	25		522	494	− 5
	50		504	508	+ 1
	50		520	536	+ 3
Spent culture broth[c]	25	NDV	550	423	−23
	60		538	455	−15
	16		517	483	−26
	50		1036	806	−22
	25	Wyodak-PT	513	162	−69
	25		543	305	−44
	50		529	232	−56
	50		510	237	−54

[a] See Table 1, for description of coals, and Ref. 8.

[b] Weight of residual coal determined after 48 h incubation (30°C, 100 rpm, 2-in stroke).

[c] Broth from 7-day culture (see Materials and Methods) was filter sterilized using a 0.22-μm pore size Millipore filter.

broths retained the intense dark brown to black coloration and were free of particulates when examined microscopically (400 ×). Further characterization of the water-soluble products was not attempted.

While we have not identified the active coal-solubilizing component(s), results indicate that it is not an enzyme. The active component(s) in the culture broth showed extreme heat stability, since heating the culture broth in boiling water or even autoclaving (121°C) for 10 min did not measurably affect activity. Autoclaving for 1 h reduced activity by 30–40%, whereas no solubilization occurred with a broth heated for 3 h at 121°C.

The active component(s) does not appear to be a protein. No significant loss in activity was observed following treatment of the broth with either an acid protease (Type XIII) or two alkaline proteases (Type I and Type IX). Furthermore, the molecular weight was shown to be between 1000 and 10000 as determined by ultrafiltration (Diaflo PM10 and UM2 membranes, Amicon Corp., Lexington, MA).

The pH of the active culture broth was between 8 and 9, and coal-solubilizing activity was shown to be maximal between 8 and 10. We found that certain coals were susceptible to solubilization in mild alkaline buffers (e.g., tris(hydroxymethyl)aminomethane, glycylglycine, pH 8.5) (Strandberg and Lewis, manuscript in preparation). Furthermore, there were indications that coal-solubilizing activity was dispersed among several fractions obtained by gel permeation chromatography (Sephadex G25, Pharmacia, Inc., Piscataway, NJ) of the moderate

molecular weight portion of the culture broth. These results suggest that the active component(s) could be basic polypeptides or polyamines.

Pometto and Crawford [4] recently reported that the oxidative depolymerization and solubilization of lignin by *S. viridosporous* was maximal in the pH 8.4–8.8 range. No attempts at characterizing the nature of the active material were reported. One further note of interest is that the soluble product produced by the action of several fungi on coal was also alkaline (pH 7.8) [7]. Thus, there is the possibility that both lignin and coal solubilization occur by similar mechanisms in these bacteria and fungi.

REFERENCES

1 Cohen, M.S. and P.D. Gabrielle. 1982. Degradation of Coal by the Fungi *Polyporous versicolor* and *Poria monticola.* Appl. Environ. Microbiol. 44: 23–27.

2 Fakoussa, R.M. and H.G. Trüper. 1983. Köhle als Microbielles Substrat unter Aeroben Bedingungen. In: Kolloquium in der Bergbau-Forschung GmbH (Rehm, H.J., ed.), pp. 41–49, Steinkohlenbergbauverein, Essen.

3 Korburger, J.A. 1964. Microbiology of Coal: Growth of Bacteria in Plain and Oxidized Coal Slurries. Proc. W. Va. Acad. Sci. 36: 26–30.

4 Pometto, A.L., III and D.L. Crawford. 1986. Effects of pH on Lignin and Cellulose Degradation by *Streptomyces viridosporous.* Appl. Environ. Microbiol. 52: 246–250.

5 Rogoff, M.H. and I. Wender. 1957. The Microbiology of Coal in Bacterial Oxidation of Phenanthrene. J. Bacteriol. 73: 264–268.

6 Rogoff, M.H., I. Wender and R.B. Anderson. 1962. Microbiology of Coal. U.S. Bureau of Mines, Information Circular 8075.

7 Scott, C.D., G.W. Strandberg and S.N. Lewis. 1986. Microbial Solubilization of Coal. Biotechnol. Prog. 2: 131–139.

8 Strandberg, G.W. and S.N. Lewis. 1986. A Method to Enhance the Microbial Liquefaction of Lignite Coals. Biotechnol. Bioeng. Symp. Ser. 17: 153–158.

9 Ward, H.B. 1985. Lignite-degrading Fungi Isolated from a Weathered Outcrop System. Appl. Microbiol. 6: 236–238.