

STRATEGY FOR LARGE SCALE SOLUBILIZATION OF COAL - CHARACTERIZATION OF *NEUROSPORA* PROTEIN AND GENE

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INTRODUCTION

Coal represents an important source of energy (1,2). Its utilization for generating energy has been offset by environmental problem that it creates by the release of SO_x and NO_x, which are major causes of acid rain and deforestation. Some of these problems can be tackled by the use of industrial scrubbers. However, a biotechnological approach to these problems may prove more efficient and environment friendly. We have employed certain genetically characterized fungi for the biosolubilization of coal in order to yield chemicals that can be converted into utilizable energy and can be rendered free of SO_x and NO_x at the source. Here we describe the purification of a protein which is responsible for the biodegradation of low rank coal both *in vivo* and *in vitro*. We also report the characterization of the biochemical nature of the coal derived products obtained after the biosolubilization of coal by this protein *in vivo* and *in vitro*. Identification and characterization of this fungal protein is expected to help the cloning of the gene encoding this protein which is needed to construct a super strain of *Neurospora* capable of large scale solubilization of coal.

MATERIALS AND METHODS

Neurospora crassa is a very well characterized fungus (3). The wildtype strains of *Neurospora crassa* were used for the *in vivo* solubilization of coal and for the extraction and purification of proteins capable of coal solubilization *in vitro*. The low rank coal used was the North Dakota lignite generously supplied by Dr. Charles Scott of the Oak Ridge National Laboratory. The coal was ground to fine mesh 1-3 mm in size and autoclaved before use. The coal granules were not subjected to any other treatment. The *in vivo* solubilization of coal was determined by the liquefaction of coal granule sprinkled over the fungal mat on a Petri dish obtained after a 5-day growth of *Neurospora crassa*. The *in vitro* solubilization of coal was determined by changes in optical density of coal derived products as measured spectroscopically at 254 nm of UV light in a quartz cuvette after incubation of coal granules with *Neurospora* protein preparation in a microfuge tube containing Bis Tris buffer pH 6.5-7.0.

Protein Purification: *Neurospora* protein capable of coal solubilization was purified by ammonium sulfate precipitation, ion exchange chromatography, and HPLC. The amount of protein was determined by methods of Lowry or Bradford, or spectroscopically via absorption at 280 and 260 nm of light.

Determination of the biochemical nature of the coal derived products: Coal derived products obtained after *in vivo* or *in vitro* solubilization were separated by HPLC or by electrophoresis and then examined by mass spectroscopy to reveal their chemical nature. A large volume of liquid culture was grown with continuous aeration. After five days, the culture was treated with tritone X-100 (conc 0.1-0.5% of total volume). After allowing the reaction to run overnight, the mycelial mass was separated using Whatman 3MM paper. The filtrate (crude extract) was centrifuged to remove any debris; 500 ml of the crude extract was loaded directly on a DE-52 (DEAE cellulose) 3.8x11cm (Whatman Labsales, Hillsboro, OR) column which was pre-equilibrated with 0.1M phosphate buffer (pH 7.0). Proteins were eluted with a linear gradient of NaCl from 0.0 to 1M in 600 phosphate buffer. The protein profiles obtained from the ion exchange column showed several peaks. The enzyme peak eluted at 150 ml was equivalent to approximately 0.25M NaCl. The activity of two fractions was twice that obtained with the crude extract. The enzyme peak was pooled and concentrated by ultrafiltration. This solution was applied to a Bio Rad A 0.5M (BioRad, Richmond, VA) 1.8x68cm column which had been pre-equilibrated with phosphate buffer. Proteins were eluted using the same buffer. The enzymes were analyzed by SDS polyacrylamide gel electrophoresis.

RESULTS

It was found that the coal was biosolubilized both *in vivo* and *in vitro*. The possibility of the solubilization by the ingredients of the media was eliminated by performing the same treatment of coal with the ingredients used to make the media.

In vivo Solubilization: Vogel's and WM media were used to grow *N. crassa* on the plates. After five days of growth, coal particles were scattered on the fungal mat. The coal granules appeared as liquid droplets after three to five days (Fig. 1). Two wildtype strains of *N. crassa* viz. Yeehaw Junction and Everglades were used. It was observed that maximum growth was obtained with Yeehaw Junction and in Vogel's media.

In vitro Solubilization: Liquid cultures were grown with Vogel's minimal media with constant aeration. After five days, the mycelium was separated using Whatman 3MM paper. The filtrate was used for further experiments. 30mg of coal powder was weighed in an Eppendroff tube and 1ml of filtrate was added to it. Absorbance increased with time with maximum increase during the first 4-6 hours. The filtrate was kept in cold room and ammonium sulfate was added slowly to it to a concentration of 90%. Centrifuged at 6000 rpm for 20 minutes. Pellet obtained was collected by mixing with 50mM Bis Tris (pH = 7.0). Supernatant left after centrifugation did not show any activity after being treated with coal. Treatment of coal with the sample (pellet + Bis Tris) showed considerable activity. The sample was passed through Sephadex G-25 and dialyzed against 50mM Bis Tris (pH = 7.0) overnight to remove any salts or low molecular weight substrates, if present. By running a pH gradient, it was observed that low and high pH aggravates the biosolubilization reaction and that pH has minimum effect on the reaction between the range of 6.5-7.0. Elevated temperatures also have similar effects on the solubilization and hence the experiments were carried at room temperature.

Protein Purification: The protein was purified using column-chromatography (as described) and at each stage of purification the samples were subjected to solubilization assay, thus discarding the ones without any activity. Finally, the protein was concentrated by collection in a dialysis bag and then covering it with PEG (4000-8000) from all sides. The concentrated protein was dialyzed overnight against the buffer to remove impurities. Bradford test and the Lowry test performed with this sample gave the concentration to be around 1 μ g/ μ l. The protein was analyzed for temperature stability and it was found that it was stable even after treatment to higher temperatures like 80°C for half an hour or more. The amino acid composition analysis shows that it is an unusual protein in which tyrosine was absent but a sugar moiety was present. The SDS gel containing the protein could not be stained with Coomassie blue. But, it was stained with the silver stain.

Product of Coal Solubilization: Nature of the products obtained from biosolubilization of coal was established using HPLC and mass spectroscopy. The products so obtained are described in Table 2. The products obtained by biosolubilization were extracted by Pentane. In the future, extraction will also be performed with acetone, etc., in order to establish the identity of the productions. The products were compared with ones in NBS library for establishment of chemical structures. The products of coal solubilization are mostly hydrocarbons of low molecular weights (see Table 2).

DISCUSSION

A number of fungi have been described to biosolubilize coal (3-7), however, we are the first one to demonstrate the role of a genetically characterized fungus such as *Neurospora* in this process. Here we describe a method for the large scale purification of a protein from a genetically characterized fungus capable of solubilization of coal. The availability of purified proteins in abundant amount will help in determining the N-terminus sequence of the protein (8) required for the generation of the oligonucleotides for the cloning of the gene encoding this protein capable of coal solubilization. Alternatively the large amount of purified proteins will be used to develop antibody against this protein which will be useful for the screening of the *Neurospora* genomic library harboring a clone containing the gene for this protein. Also, here we describe the identification of the coal solubilization product for the first time. Such information will provide an insight into the mechanism of the action of *Neurospora* protein during coal solubilization. These data can be used to develop a bioreactor capable of conversion of coal product into utilizable chemicals (9).

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Table 1. *In vivo* biosolubilization

STRAIN	CULTURE MEDIUM	NO. OF COAL GRANULES		% SOLUBILIZATION
		TOTAL	SOLUBILIZED	
Yeehaw	Vogel's Medium	50	47	94
	WM	50	05	10
Everglades	Vogel's	50	13	26
	WM	50	01	02

Table 2. Characteristics of coal derived products

MOLECULAR WT.	POSSIBLE FORMULAS	INFERENCE
84	C_6H_{12}	may be a contaminant sugar molecules present in growth medium
82	C_6H_{10}	hydrocarbons
326	$C_{23}H_{26}$ or $C_{24}H_{38}$ or $C_{26}H_{34}$	hydrocarbons
278	$C_{16}H_{22}O_4$	probably a contaminant from the plastic tube used during the handling of samples

Figure 1. Coal solubilization by *Neurospora crassa*

