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Project Title: Large Scale Solubilization of Coal and Bioconversion to Utilizable Energy

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Introduction

In order to develop a system for a large scale coal solubilization and its bioconversion to utilizable fuel, we plan to clone the genes encoding *Neurospora* protein that facilitate depolymerization of coal. We also plan to use desulfurizing bacteria to remove the sulfur *in situ* and use other microorganisms to convert biosolubilized coal into utilizable energy following an approach utilizing several microorganisms (Faison, 1991). In addition the product of coal solubilized by fungus will be characterized to determine their chemical nature and the mechanism of reaction catalyzed by fungal product during *in vivo* and *in vitro* solubilization by the fungus or purified fungal protein.

Main Objectives

1. Cloning of *Neurospora* gene for coal depolymerization protein controlling solubilization in different host cells, utilizing *Neurospora* plasmid and other vector(s).
2. a. Development of a large scale electrophoretic separation of coal drived products obtained after microbial solubilization.
   b. Identification of the coal derived products obtained after biosolubilization by *Neurospora* cultures or obtained after *Neurospora* enzyme catalyzed reaction in *in vitro* by the wildtype and mutant enzymes.
3. Bioconversion of coal drived products into utilizable fuel.
4. Characterization of *Neurospora* wildtype and mutant CSA protein(s) involved in solubilization of coal in order to assess the nature of the mechanism of solubilization and the role of *Neurospora* proteins in this process.

Methods:

Only experimental approaches for objective #1 are presented here since experiments were performed during this period in this area.

Objective #1 Cloning of gene for *Neurospora* CSA-protein

The following methods will be used to clone the gene for *Neurospora* protein with coal solubilization activity (CSA).

A. Identification of *Neurospora* CSA protein gene in a DNA library:

In case the above approach to clone *Neurospora* gene in yeast or *Neurospora* cells is not successful, alternative methods will be used. The wildtype CSA protein has been purified in my laboratory. The purified wild type CSA protein obtained from the SDS PAGE (Laemmli, 1970) will be electroblotted onto Immobilon P, polyvinylidene diflouride (PVDF) membrane (Millipore Corporation, Bedford, MA) using the method of Matsudaira (1987). The membrane will then be given to Dr. Ishikawa of the Protein Microanalyses Facility of the Carolina Institute for Biological Research and Technology (IBRT), University of South Carolina, Columbia campus for microsequencing. Based on about the first nine amino acid sequences at the N-terminus (Matsudaira 1987) or internal amino acid sequence (Huang, 1983), an oligonucleotide probe will be synthesized at the oligonucleotide synthesis facility of the USC IBRT facility located in our Department. This oligonucleotide probe will then be used to screen a cDNA library (as well as a genomic library of *Neurospora*) to identify the clone carrying *Neurospora* CSA protein gene.

Alternatively, immunoblotting will be used to identify clone with *Neurospora* CSA gene. We will prepare polyclonal antibody against the *Neurospora* CSA protein in rabbit. The antibody so prepared will be used to screen a *Neurospora* cDNA library to

identify a clone carrying the gene for the *Neurospora* CSA protein. This method should work since the genes in the cDNA library are known to be expressed. Once the clone containing the gene for the *Neurospora* CSA protein is identified, the gene will be transferred to suitable vector such as pYELeu-10 or to *Neurospora* pstp 2.2, a mt DNA plasmid to which benomyl resistance (Ben^R) gene has been added as a selectable marker (our unpublished results).

These chimeric plasmid containing the *Neurospora* CSA protein gene will be used to transform yeast cells or *Neurospora* (sol) mutant cells (see Figure 2), which are devoid of ability to biosolubilize coal *in vivo*. The transformants will be identified by their ability to solubilize coal *in vivo* when assayed in Petri plates. The yeast or *Neurospora* transformants will be further examined for multiple copy of the CSA protein gene and for their possible autonomous existence by the method of Southern hybridization or by amount of the CSA protein produced.

The plasmids pYEleu-2 and pstp 2.2 and the genomic and cDNA libraries of *Neurospora* and PCR machine are available in my laboratory. All methods of molecular cloning, transformation, and identification and charcterization of transformants will be as practiced in my laboratory (Schablik et al., 1982; Almasan and Mishra, 1988; 1990; 1991) or as described previously (Hinnen et al., 1978; Maniatis et al., 1989; Yadav and Mishra, 1994; Feher and Mishra, 1994).

B. Cloning of *Neurospora* gene in yeast

As yeast cannot solubilize coal this provides the easiest way to clone *Neurospora* gene by shotgun experiment in which *Neurospora* DNA segment (obtained after restriction enzyme digestion) will be ligated to pYEleu-2 plasmid and then

introduced into $CaCl_2$ treated competent pYEleu-2$^-$ yeast cells (Hinnen et al., 1978). The Leu$^+$ yeast transformants will be examined for their acquisition of ability to solubilize coal in plate assays. The transformed yeast colonies containing the chimeric plasmid carrying *Neurospora* DNA segment encoding CSA-protein will be thus identified and further characterized. The success of this shotgun-transformation experiment using yeast recipient cell will depend on the expression of the *Neurospora* gene encoding CSA protein in yeast cells. Since a number of heterologous genes have been expressed in yeast, it is therefore expected that *Neurospora* CSA protein gene could be expressed in yeast (Mishra, 1985; 1991).

The wildtype *Neurospora* gene for CSA will also be directly cloned in *Neurospora* mutant cells deficient for CSA activity. In this approach wildtype *Neurospora* DNA will be shotgunned into a *Neurospora* plasmid pst 2.2 Ben^R (Ben^R confers resistance to antibiotic benomyl); the chimeric plasmid will be used to transform *Neurospora* mutant lacking CSA. First the Ben^R transformants will be picked up by their ability to grow on plates containing benomyl and then these will be examined for coal solubilization activity (CSA).

Results:

Following experiments have been performed to achieve the proposed goals:

A. **Major break through in purification of *Neurospora* CSA protein and determination of its enzyme activity.** A purification of the *Neurospora* protein with coal solubilization activity (CSA) was undertaken in order to determine its N-terminal or internal amino acid sequence or to prepare the antibody against this protein in rabbit. The amino acid sequence will be used to prepare the oligonucleotides to identify the clone carrying *Neurospora* CSA gene among cDNA organomic libraries. Alternatively, the antibody will be used to identify the clone carrying *Neurospora* CSA gene via immunoblotting. Even though this protein was purified in microscale in my laboratory by a previous graduate student (Brian Odom) who worked on this aspect, he has left, after receiving his Ph.D. to join as Assistant Professor at Georgia Southern University, and this project is taken over by another graduate student, Ashish Patel. Ashish Patel has made a major breakthrough by purifying the protein to homogeneity and identifying this *Neurospora* protein with coal solubilization activity to be tyrosinase; a phenol oxidase.

We have finally devised a methodology to purify the *Neurospora* CSA protein on a large scale. The purification protocol includes fractionation of a large volume (3-10 litre) of culture filtrate in which *Neurospora* has been grown by DEAE cellulose chromatography, Biogel chromatography, and phenyl Sepharose chromatography. At each step of chromatography, the peak fractions with high coal solubilization activity (CSA) was pooled and then utilized

for subsequent chromatographic procecdures. The coal solubilization is monitored via an increase in absorption at 254 nm due to release of UV absorbing material from coal added to the reaction mixture containing *Neurospora* protein. We are prompted to try this new approach to purify CSA protein because of our recent success in purifying another difficult protein (Feher and Mishra, 1994). We have recently made some other modification in our purification procedure and use this new protocol for protein purification two homogenous peak of protein with CSA property has been obtained (Figure 1). The first *Neurospora* protein peak preparation was found to possess more than one subunit of protein when analyzed on SDS polyacrylamide gel, whereas the other protein peak possessed only one subunit (42 Kdal) (Figure 2). We have confirmed the solubilization process of the coal by *Neurospora crassa* and have developed a protocol for the purification of the enzyme which shows intense solubilization of coal by both protein purification. We have now obtained a large amount of *Neurospora* CSA protein (i.e., in mg amount) which is being used for the preparation of antibody in the rabbit. We have also designed the oligonucleotides for the primer to be used in the experiments for the cloning of the *Neurospora* gene for protein with coal solubilization/phenol oxidase activities. These oligonucleotide sequences (P1 and P2) have been synthesized in our departmental facility. The structure of these primers are:

P1 - 5'TTTTCCAATGGGCACCGACATCAAATTT3'

P2 - 5'TTTTCTGGAGAGTTGGCGCGCAGATGCGGGA3'

Methods of preparing mRNA are now developed in order to prepare cDNA. These primers are used in the cDNA cloning (via RT-PCR) of the *Neurospora*

gene encoding the protein with CSA and phenol oxidase activity. The scheme for cloning is presented in Figure 2.

B. **Determination of the N Terminus Sequence of the *Neurospora* CSA protein.** The purified protein (see Figure 3) has been analyzed by microsequencing at the University of Georgia, Athens. However, such sequencing of the N-terminus amino acid was not successful leading to the conclusion that the protein is heavily glycosylated and the N-terminus is blocked. We are now trying to sequence the internal amino acids after digestion of the protein with endopeptidase.

The fact that the *Neurospora* CSA protein is glycosylated suggests that it is different than tyrosinase or common phenol oxidase even though these proteins resemble in enzymatic activity and molecular weight. We have also tried to remove glucose moiety from the purified protein using deglycosylation kit (Oxford, Glycosylation, Rosedale, NY). This is a chemical method for glycosylation and does not interfere with the protein structure and would be useful for determination of N-terminus of the protein.

C. **Nature of the enzymatic activity of the *Neurospora* CSA protein.** We have shown that the purified *Neurospora* CSA protein can catalyze the solubilization of coal and produce substances which differ by one carbon atom. This finding, as well as the results of other mass spectrophotometric analysis clearly

establish the enzymatic role of the enzyme and rules out the possibility that *Neurospora* protein may solubilize the coal by acting as a metal chelator.

D. **Analysis of the product of coal solubilization.** Coal samples were solubilized biologically by *Neurospora crassa* mycilium and enzymatically by *Neurospora* protein or by commercial tyrosinase. The products after solutilization were separated by gel electrophoresis and then extracted with pentene and then analyzed by mass spectroscopy. The product of coal solubilization has been identified by mass spectroscopy and demonstrated to be low molecular substance such as hexane, alcohol, and esters. Thus the fact that the coal products obtained after biosolubilization are small molecular weight substances clearly provides evidence for the enzymatic activity of the *Neurospora* protein. We have further demonstrated that coal solubilized by commercial tyrosinase also yield products similar to that produced by the *Neurospora* CSA protein.

E. **Desulfurization of coal.** We have also shown by X-ray analysis that the coal treated by *Neurospora* protein is significantly reduced in its sulfur content. We have shown a 4-6 fold reduction in sulfur content of the coal.

F. **Personnel.** The work is carried by Mr. A. Patel and Dr. Mishra (PI). Dr. Y.P. Chen has also joined the project as a collaborator. The data reported here was presented at the annual meetings of the DOE contractors in Nashville and at the meetings in Chicago this summer of the Chemical Society of America for which the PI was invited. During this period we were also joined by Ms. Jennifer Jones, a summer intern selected and supported by the Department of Energy

(ORISE). She was trained in protein chemistry and enzymology during this period.

G. This work was presented on invitation at the Annual UCR Contractors Meeting in Pittsburgh. I am in the process of seeking patent rights to this *Neurospora* protein with CSA activity.

9. Literature References

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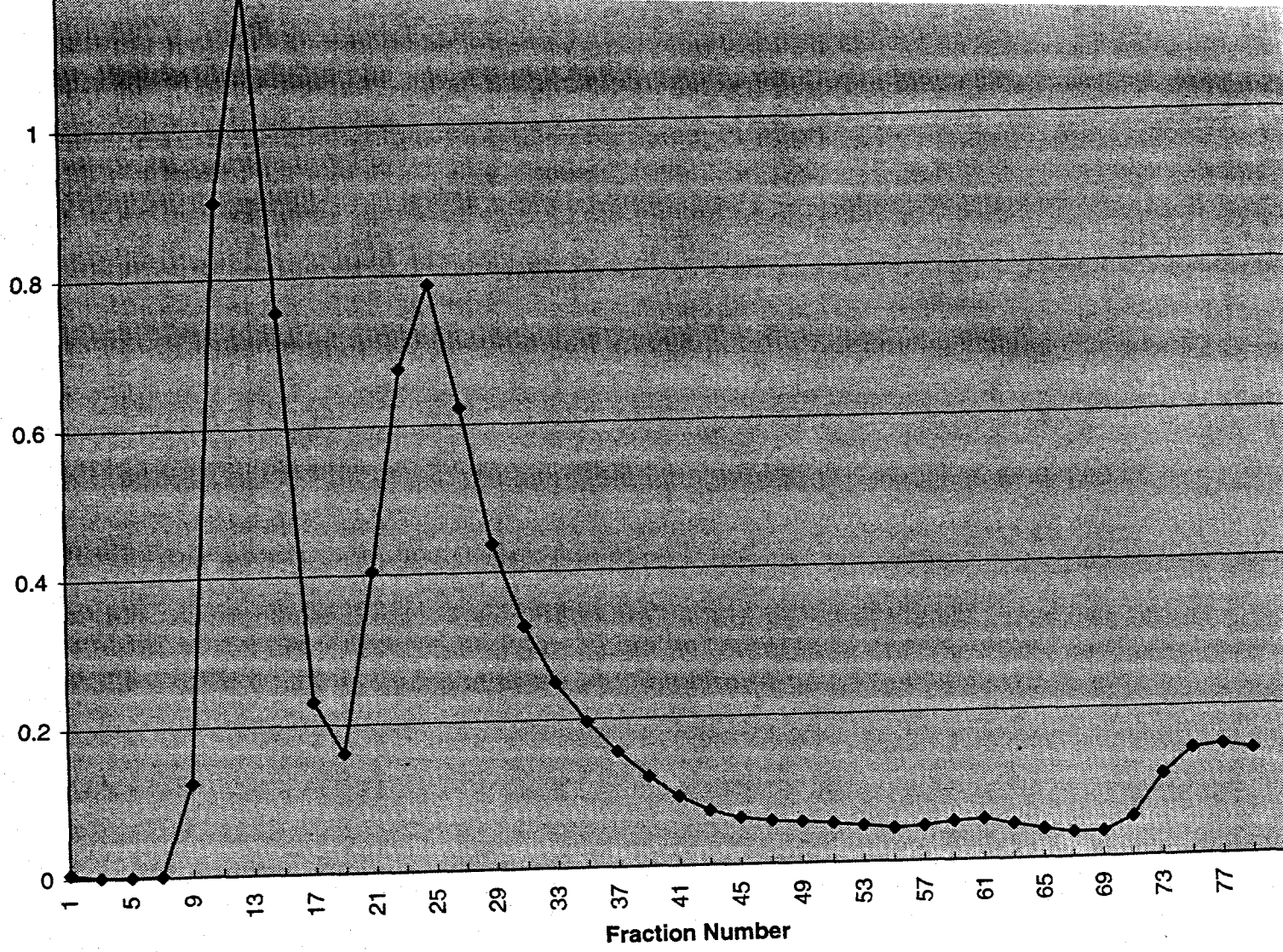

Fig. 1. Purification of *Neurospora* protein with coal solubilization activity.

Fig. 2. SDS gel electrophoresis of *Neurospora* protein with coal solubilization and tyrosinease active showing purification to homogeneity.