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TECHNICAL PROGRESS REPORT

Kind of Report: Quarterly

Period: January-March, 1994

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

DE-FG22-93PC93224

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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. Cloning of *Neurospora* gene in yeast

As yeast cannot solubilize coal this provides the easiest way to clone *Neurospora* gene by shortgun 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 shortgun-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 (see Figure 2). 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).

b. <u>Identification of *Neurospora* CSA protein gene in a DNA library:</u>

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 (<u>Ben</u>^{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* (<u>sol</u>) 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).

Results:

Following experiments have been performed to achieve the proposed goals:

A. **Further Purification of *Neurospora* CSA protein.** We have also started purification of the 84 Kdal CSA protein from *Neurospora* 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 Mississippi Women's University). I have therefore attempted this purification of CSA protein on a large scale for determining the amino acid sequence of this protein for preparation of oligonucleotide probe and for raising antibody against this protein needed for use by future graduate students during the cloning of CSA gene. I am also trying to minimize the purification procedure by adopting ammonium sulfate precipitation steps. The ammonium sulfate precipitation may also help in separating the two CSA proteins, the 10 Kdal and 84 Kdal. At present I have found that at 80% Ammonium Sulfate concentration, all *Neurospora* protein with CSA activity is precipitated. The CSA activity was checked by examining the ability of (ammonium sulfate precipitated) *Neurospora* protein to solubilize coal *in vitro*. The coal solubilization was monitored by increase in absorption at 254 nm due to release of UV absorbing material from coal added to the reaction mixture containing *Neurospora* protein. I have undertaken this short cut in purification of CSA protein encouraged by our recent success in purification of another difficult protein (Feher and Mishra, 1994). I have used this methodology to purify the *Neurospora* cell free extract with coal solubilization activity and am awaiting the arrival of Mr. Ashish Patel to continue the project as part of his graduate training program. Also Mr. Brasher has found that certain microorganisms that he has obtained from nature on charcoal sediment were able to solubilize coal *in vivo*.

B. **Development of transformation system.** This work has been undertaken to make Sphaeroplasts of *Neurospora* mycelia so that it can be used as recipient cells during molecular cloning experiments. The sphaeroplasts were made using cell wall digesting enzyme (Novozyme) in isotonic buffer to avoid the lysis of the cells.

C. **Preparation of the vector DNA.** We have described a plasmid (pstpb 2.2) (to which a selectable marker gene for resistance to benomyl has been added). We have prepared this vector plasmid for the molecular cloning expeirments. We have developed methodology to prepare this plasmid in microgram amounts. Upon arrival of our new graduate student, we plan to shotgun *Neurospora* genomic DNA into this vector plasmid and then transfer the mutant strain fo *Neurospora* lacking CSA) into colonies capable of CSA (i.e., coal solubilization activity).

D. Mr. Ashish Patel has been offered a research assistantship and he has accepted the position and will join the project as a full time graduate student beginning the middle of April, 1994. As reported earlier, we have recruited two undergraduate students, Mr. Shawn Brasher and Ms. Tracy Scarborough (both biology majors), to work on the project under provision for undergraduate research experience credit at the University of South Carolina. They have joined my lab in January 1994 and are currently working on the project. Ms. Tracy Scarborough is supported by a scholarship from the Howard Hughes Foundation. Much of the effort was devoted toward the training of these students. Mr. Brasher is trying to learn and develop his skill for fungal transformation which will be used for gene cloning whereas Ms. Scarborough has learned to prepare the plasmid which will be used as vector in the future gene cloning experiments. Ms. Scarborough will work through this summer under our EPSCoR program and will be funded by EPSCoR and will be funded by EPSCoR. Furthermore, I plan to work 100% on this project during the summer of 1994.

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