National Renewable Energy Laboratory
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Office of Energy Efficiency & Renewable Energy
Innovation for Our Energy Future

Clone Bacterial Thermal Stable Enzymes in T. Reesei

Cooperative Research and Development Final Report

CRADA Number: CRD-01-00105

NREL Technical Contact: Michael E. Himmel

CRADA Report
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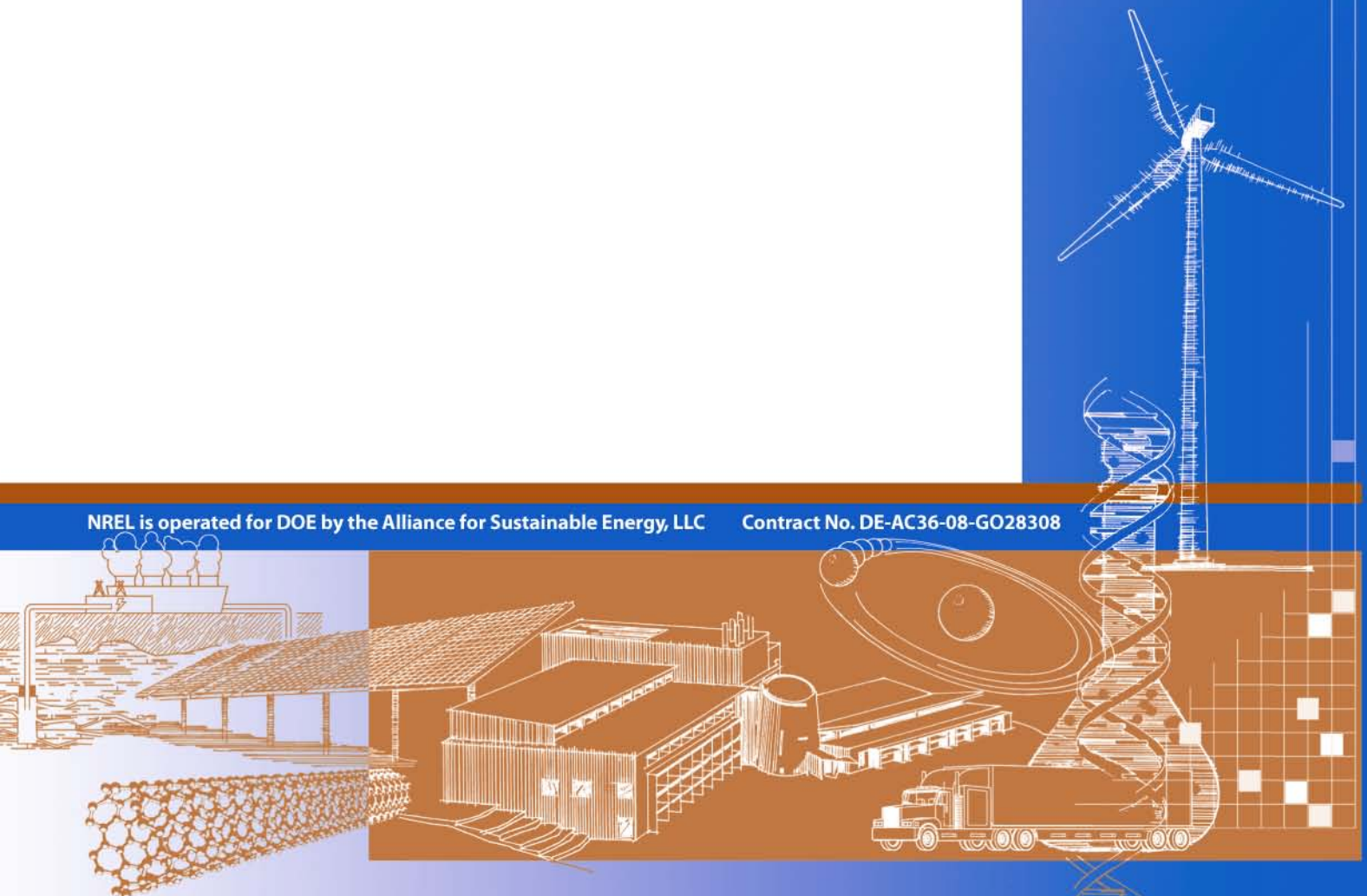

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Cooperative Research and Development Final Report

In accordance with Requirements set forth in Article XI.A(3) of the CRADA document, this document is the final CRADA report, including a list of Subject Inventions, to be forwarded to the Office of Science and Technical Information as part of the commitment to the public to demonstrate results of federally funded research.

CRADA number: CRD-01-00105

CRADA Title: Clone Bacterial Thermal Stable Enzymes in T. Reesei

Parties to the Agreement: Genencor Division, Danisco US, Inc.

Joint Work Statement Funding Table showing DOE commitment:

Estimated Costs	**NREL** Shared Resources
Task 1	
Loaded Labor	$ 10,000.00
Other Costs	$ 1,000.00
Task 2	
Loaded Labor	$ 170,000.00
Other Costs	$ 19,000.00
Task 3	
Loaded Labor	$ 00.00
Other Costs	$ 00.00
Task 4	
Loaded Labor	$ 90,000.00
Other Costs	$ 10,000.00
TOTALS	$ 300,000.00

Abstract of CRADA work:

Cellulose, an unbranched β-1,4 linked homopolymer of glucose, is the most abundant renewable fuel resource on Earth, accounting for about half of the organic material in the biosphere, and is the major polysaccharide found in plant biomass. The hydrolysis of cellulose, aided by endocellulase, exocellulase, and β-glucosidase catalysis, produces glucose, an easily fermentable monosaccharide. Intense research is aimed at the conversion of cellulose to ethanol because, as a source of renewable fuel, the process has great economic potential and is environmentally friendly. Cellulose is insoluble and crystalline, hence it is largely resistant to microbial attack. In many biomass utilization schemes the raw material is first treated with dilute acid at moderate temperatures to remove lignin and to speed up cellulose hydrolysis. The pretreated biomass can then be subjected to carefully chosen mixtures of endo- and exoglucanases for maximum cost effectiveness.

Highly thermostable cellulase enzymes are known to be secreted by the cellulolytic thermophile *Acidothermus cellulolyticus gen. nov.*, *sp. nov.*, a bacterium originally isolated from decaying wood in an acidic, thermal pool at Yellowstone National Park (Mohagheghi et al. 1986. Int. J. System. Bacteriol. 36:435-443). The cellulase complex produced by this organism is known to contain several different cellulase enzymes with maximal activities at temperatures of 75^{o}C to 83^{o}C. In addition to demonstrating the useful property of cellobiose (end product) inhibition resistance, the cellulases from *A. cellulolyticus* are active over a broad pH range centered about pH 5, the pH at which yeasts are capable of fermenting cellobiose and glucose to ethanol. A high molecular weight cellulase isolated from growth broths of *A. cellulolyticus* was found by SDS-PAGE of approximately 156,600 to 203,400 daltons was described by U.S. patent #5,110,735.

A novel cellulase enzyme, known as the E1 endoglucanase and also secreted by *A. cellulolyticus* into the growth medium, is described in detail in the U.S. Patent #5,275,944. The *A. cellulolyticus* E1 endoglucanase is one of the most active cellulases known. In combination with the exocellulase CBH I from *Trichoderma reesei,* E1 gives the highest saccharification and degree of synergism of all cellulases tested at NREL. This useful endoglucanase demonstrates a temperature optimum of 83^{o}C and a specific activity of 40 μmole glucose release from carboxymethylcellulose/min/mg protein. E1 endoglucanase was further identified as having an isoelectric pH of 6.7 and a molecular weight of 81,000 daltons by SDS polyacrylamide gel electrophoresis. The gene coding EI catalytic and cellulose binding domains and linker peptide were protected by US Patent # 5,536,655.

The NREL has achieved expression of active E1 catalytic domain in *E. coli* using a variety of expression vectors and this host is currently used for routine protein engineering work. Full length E1 is best expressed from *Streptomyces lividans* TK24, presumably because fewer serine proteases are produced by this most. NREL has tested a strain of *Pichia pastoris* designed for expression and secretion of the *A. cellulolyticus* E1 endoglucanase. In this construction, the mature E1 coding sequence was joined in the same translational reading frame to the yeast alpha factor signal sequence present in pPIC9. Transformants have not yet been analyzed for gene copy number, but unoptimized fermentations have already yielded 1.5 g/L of E1. This is by far the highest expression level for any strain yet developed at NREL for any gene. E1 has been expressed in an active and thermal stable enzyme from a variety of plants, including *Arabidopsis*, tobacco, cotton, barley, and duckweed.

NREL has recently identified and characterized a series of related and important glycosyl hydrolases produced by *A. cellulolyticus*. We expect that these high GC coding sequences will be similarly well expressed in a wide range of commercial hosts, but this is yet unproven. We also expect that all enzymes secreted from *A. cellulolyticus* will be thermal tolerant and synergistic with E1 and each other, as well as the known *T. reesei* cellulases.

Summary of Research Results:

The focus of this CRADA was on improving the efficacy of GCI's primary host for commercial cellulase production, *Trichoderma reesei*. We employed a diversity of technologies in modern protein biotechnology to clone and express selected genes initially from the thermophilic bacterium, *A. cellulolyticus*, in experimental and commercial strains of *T. reesei*. As the work progressed, either partner identified other promising genes from the same or other bacterial sources that warranted expression research and these were considereded for inclusion in the project.

Subject Inventions listing: None.

Report Date: 4/30/10 Responsible Technical Contact at Alliance/NREL: Himmel, Michael E.

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