Cooperative Research and Development Agreement (CRADA) Final Report

Report Date: November 6, 2024

In accordance with Requirements set forth in the terms of the CRADA, this document is the CRADA Final Report, including a list of Subject Inventions. It is to be forwarded to the DOE Office of Scientific and Technical Information upon completion or termination of the CRADA, as part of the commitment to the public to demonstrate results of federally funded research.

Parties to the Agreement: Lawrence Berkeley National Laboratory and Protein Evolution, Inc.

CRADA number: FP00016789

CRADA Title: Development of Enzyme Production Host

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Sponsoring DOE Program Office(s): DOE-BER

LBNL Report Number: LBNL-2001619

OSTI Number: *[SPO to complete]*

Joint Work Statement Funding Table showing DOE funding commitment:

DOE Funding to LBNL	
Participant Funding to LBNL	$255,207
Participant In-Kind Contribution Value	$2,602,000.00
Total of all Contributions	$2,857,207.00

Provide a list of publications, conference papers, or other public releases of results, developed under this CRADA:
(Publications must include journal name, volume, issue, Digital Object Identifier)

No publications to date

Provide a detailed list of all subject inventions, to include patent applications, copyrights, and trademarks:
(Patents and patent applications are to include the title and inventor(s) names. When copyright is asserted, the Government license should be included on the cover page of the Final Report

No subject inventions to report.

Executive Summary of CRADA Work:

This CRADA aims to assess the effectiveness of various enzyme production hosts in efficiently expressing and producing proprietary enzymes for plastic recycling applications. The research includes screening different microbial hosts—notably Bacillus subtilis, Pichia pastoris, and E. coli—to determine their suitability for high-yield enzyme production. Additionally, the project focuses on optimizing scalable fermentation processes tailored to these hosts, with the goal of enabling efficient enzyme production at commercially viable scales.

Summary of Research Results:

Host Comparison and Selection:

- *Bacillus subtilis* and *Pichia pastoris* were both effective at secreting target enzymes into the supernatant, which facilitated easier purification.
- *E. coli*, while capable of limited secretion with specific secretion tags, primarily produced enzymes either as insoluble aggregates or as soluble, intracellular products. This made purification more challenging compared to the other hosts.
- Although *Pichia pastoris* produced the cleanest supernatant, it yielded lower titers than *Bacillus subtilis* and required longer production times.
- **Decision**: Based on secretion efficiency, titer potential, and production time, *Bacillus subtilis* was selected as the preferred production host for further development toward a scalable fermentation and downstream process.

Fermentation Process and Strain Development

In the development of a scalable fermentation process for *Bacillus subtilis*, we identified aeration and nitrogen source selection as crucial parameters for optimizing growth and enzyme production.

1. **Aeration**:
    - Proper oxygenation proved essential for supporting robust cell growth and maximizing enzyme expression. By implementing controlled bioreactor conditions with continuous pH and oxygen monitoring, we achieved significantly improved production compared to shake flask cultures.
2. **Nitrogen Source Selection**:
    - We evaluated various complex nitrogen sources, including tryptone, casamino acids, and yeast extracts from multiple vendors, to determine the most effective supplement for enzyme production.
    - *Lallemand Bio-Ingredients FNI 100 yeast extract* was identified as the most effective nitrogen source, increasing titers by approximately 2x over the next best candidate, tryptone. Consequently, this specific yeast extract has become a key component in the *Bacillus subtilis* fermentation process.
3. **Secretion Tags**:
    - In collaboration with an external CDMO (Aciies Bio), we tested several secretion tags to enhance enzyme yield and solubility. The optimal secretion tag, proprietary to the CDMO, significantly improved enzyme secretion into the

supernatant, thereby simplifying downstream processing and reducing purification challenges.

Fermentation Process Results

Initial fermentation trials demonstrated promising results for enzyme production in *Bacillus subtilis* compared to *E. coli*, with specific insights into the effects of pH, aeration, and feeding profiles.

1. **Bacillus subtilis Fermentation**:
    - In initial bioreactor runs, we achieved optical densities (OD) of approximately 60, which significantly outperformed shake flask cultures. This improvement was attributed to the enhanced pH and oxygen control in bioreactor settings.
    - Production levels reached ~1.8 g/L, demonstrating the potential of *Bacillus subtilis* as an effective host for enzyme production.
    - *Parameters Tested*: We primarily tested batch fermentation conditions. Results showed that higher dissolved oxygen (dO) levels (maintained at 50%) produced better outcomes than 30% dO. Additionally, maintaining pH control around 7 yielded higher production rates than uncontrolled pH conditions.
    - **Note**: Further experiments are planned to refine production conditions in *Bacillus subtilis*, which should provide additional data on optimizing this host.
2. **E. coli Fermentation**:
    - Through optimization of pH, aeration, and feeding profiles, we achieved production levels of approximately 400 mg/L of secreted protein in *E. coli*.
    - Despite this optimization, *E. coli* produced significantly lower titers compared to *Bacillus subtilis*, with production primarily as intracellular or insoluble forms, which increased purification complexity.

These findings underscore *Bacillus subtilis* as the more suitable production host, with the potential for higher yields and more straightforward downstream processing. Further optimization of *Bacillus subtilis* fermentation conditions will continue as we gather additional data.

APPENDIX A (Reference Only)

*This appendix has been developed by DOE to assist DOE Labs in drafting the **Executive Summary** and **Summary of Research Results** sections of the CRADA Final Report.*

Executive Summary of CRADA Work:

Include a discussion of 1) how the research adds to the understanding of the area investigated; 2) the technical -effectiveness of the materials, methods or techniques investigated or demonstrated,

and their economic feasibility, if known; and 3) how the project is otherwise of benefit to the public. The discussion should be a minimum of one paragraph and written in terms understandable by an educated layman.

Summary of Research Results:

- *INCLUDE, IF APPLICABLE: "This product contains Protected CRADA Information, which was produced on [DATE] under CRADA No. [##-####] and is not to be further disclosed for a period of [up to and not to exceed] five (5) years from the date it was produced except as expressly provided for in the CRADA."*
- *Summarize project activities for the entire period of performance, including original hypotheses, approaches used, problems encountered, any departure from planned methodology, and an assessment of their impact on the project results. Incorporate technical data, e.g. facts, figures, analyses, and assumptions used during the life of the project to support the technical conclusions of the work. It is acceptable to incorporate the technical data by reference to other publicly available sources, such as a publications or other reports, but not websites. Provide a comparison of the actual accomplishments with the goals and objectives of the project. Where possible, the summary should cover each task listed in the Statement of Work (SOW) and should note any deviations from the project plan, or lack of technical data.*
- *Identify products, potential applications, and technology transfer activities developed under the CRADA, including those completed and anticipated at the time of the report. These include, but are not limited to: 1) networks or collaborations fostered; 2) technologies/techniques/methodologies; 3) other products that reflect the results of the project, such as commercial products, internet sites, data or databases, physical collections, audio or video, software, models, educational aid or curricula, and instruments or equipment.*