Pacific Northwest
NATIONAL LABORATORY

PNNL-39101

Engineering Anti-Microbial Peptide Production in Fungi and Bioprocess Development (CRADA 502)

January 2026

1. Ziyu Dai
2. Yuqian Gao
3. Beth Hofstad
4. Kyle Pomraning

U.S. DEPARTMENT OF ENERGY

Prepared for the U.S. Department of Energy
under Contract DE-AC05-76RL01830

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Pacific Northwest National Laboratory
Richland, Washington 99354

Cooperative Research and Development Agreement (CRADA) Final Report

Report Date:

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, to be provided to PNNL Information Release who will forward to the DOE Office of Scientific and Technical Information as part of the commitment to the public to demonstrate results of federally funded research. **PNNL acknowledges that the CRADA parties have been involved in the preparation of the report or reviewed the report.**

Parties to the Agreement:

Battelle Memorial Institute, Management and Operator Contractor for the Pacific Northwest National Laboratory under its U.S. Department of Energy Contract No. DE-AC05-76RLO1830 and Alliance for Sustainable Energy, LLC Management and Operator Contractor for the National Renewable Energy Laboratory under its U.S. Department of Energy Contract No. DE-AC36-08GO28308 and Invaio Biosciences, Inc.

CRADA number: 502

CRADA Title: Engineering Anti-Microbial Peptide Production in Fungi and Bioprocess Development

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Name and Email Address of POC at Partner Company(ies): Davinia Salvachua davinia.salvachua@nrel.gov and Bhanua Harrison bharrison@invaio.com

Sponsoring DOE Program Office(s):

Joint Work Statement Funding Table showing DOE funding commitment:

Estimated Costs	DOE Funding	In-Kind
NREL	**$30,000	
Invaio		$102,500
PNNL	$410,000	
Total	$410,000	$102,500

** Initially the partnership was set up with PNNL budget at $380K, NREL at $30K and Invaio being In-Kind. At the end of the project, PNNL, NREL and Invaio discussed the benefit of transferring the remaining $30K from NREL to wrap up work at PNNL. This funding would allow PNNL to finish the last laboratory experiment of AMP purification/enterokinase digestion to complete the data enabling what is needed for a high-quality journal publication while addressing any potential IP and to complete the final report. BETO and funding transfer was made from NREL to PNNL

Provide a list of publications, conference papers, or other public releases of results, developed under this CRADA:
Publication in preparation by Ziyu Dai.
Presentations:
Peer Review presentation March 2023
Semiannual Review presentation March 2024

Provide a detailed list of all subject inventions, to include patent applications, copyrights, and trademarks:

Detailed list of all subject inventions to include patent applications, copyrights, and trademarks. Patents and patent applications to include the title and inventor names. When copyright is asserted, the Government license should be included on the cover page of the Final Report.

None: no subject inventions were generated under this CRADA.

Executive Summary of CRADA Work

Crop Protection, specifically controlling agricultural diseases and pests, is essential for crop production at every agricultural scale. Biologics hold enormous potential in this equation to decrease use of more toxic pesticides. Currently, the widespread use of biologics in crop protection is severely hampered by the lack of available tools for their mass production. To ensure biologics are regulated under EPA Biopesticide regulations, the following must be true: The biopesticide is a naturally occurring substance or structurally similar and functionally identical to a naturally occurring substance with a history of exposure to humans and the environment demonstrating minimal toxicity. Since this pertains to the marketed product it is also essential that fermentation hosts have a history of exposure and safe use by humans

This research focuses on developing a novel platform for the production and delivery of the cghSAMP peptide using the filamentous fungus *Aspergillus niger*. *A. niger* is a well-established industrial microorganism, widely recognized for its strong protein secretion capabilities, high-yield production of enzymes, and classification as a generally recognized as safe (GRAS) organism. By genetically modifying *A. niger* to express the cghSAMP peptide, we aim to leverage the fungus's robust fermentation system to produce a high volume of the antimicrobial agent. This genetically engineered system represents a potential breakthrough in sustainable disease control, offering an environmentally friendly method for localized application or large-scale production of a biopesticide designed to mitigate the effects of citrus greening disease (huanglongbing, HLB) on citrus production.

The main objectives of this project were:

1. Developing a high-yield and cost-efficient bioprocess to produce a cghSAMP in a current ABF host or readily onboarded hosts.

2. Scale up the process in stirred tank reactors and produce at least 100 grams of cghSAMP at 1- 5g/L.

Benefit to PNNL/DOE Through this project, the capabilities to produce an innovative biologically active AMP at a scale that has not previously been achieved, will be realized. These capabilities will add to the core capabilities of the PNNL/DOE and should be extendable to other peptides, proteins, and potentially metabolites. The challenges facing US agriculture are national issues. This project can help build a genetic toolbox with the potential to impact food security in the US and globally.

Benefits to CRADA Participant Peptides are historically challenging to produce and are often made through chemical synthesis. This is very expensive and given the price restrictions that agricultural products impose on input costs, peptides have had very little market application in agriculture to date. We believe in partnership with the PNNL/DOE, the development of a set of production strains and fermentation scale up capabilities that do not exist at Invaio, can be used in the development of safer products for crop protection and could prove revolutionary for agriculture. As a small company, our ability to partner with the PNNL and together to create novel technological solutions, which could have benefits beyond those realized by Invaio, is an opportunity that would not be possible within the private sector, or through individual sponsored research programs with academic collaborators.

Use Body Text for paragraphs in this section. PNNL reports use http://www.chicagomanualofstyle.org/home.html for document style. Right-click and choose open hyperlink to view the style guide.

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 five (5) years from the date it was produced except as expressly provided for in the CRADA."

The CRADA with Invaio was fully executed and therefore began on 4/28/2021 with a period of performance of 24 months. A No Cost Extension (NCE) was approved during project for an end date of April 27, 2024.

Project kickoff with NREL, PNNL and Invaio was May 5, 2021. We received the antimicrobial peptide on 5/15/2021. Two project goals existed: Goal 1: Develop a high-yield and cost-efficient bioprocess to produce cghSAMP in a current ABF host. Goal 2: Scale up the process in stirred tank reactors and produce at least 100 grams of AMP at 1-5g/L. The must haves were:

- GRAS (Generally Recognized as Safe) host or a host widely used in the production of food products
- The cghSAMP fermentation product is identical to what is found in nature
- Bioprocess can be scaled up for eventual commercial production

Listed below are the four PNNL tasks and outcomes.

Task 1: Host sensitivity to AMP (PNNL): 5/2021 completed 6/2021.

Higher levels of CghSAMPa protein were found to interfere with fungal spore germination. Further testing with pre-germinated spores, more reflective of peptide production conditions, concluded that the growth of both *A. niger* and *Aspergillus oryzae* was good at low levels of CghSAMPa, but were partially inhibited at higher CghSAMPa levels. Actual production of the CghSAMPa *in situ* is expected to have a different and lesser effect vs. the growth inhibition testing done by addition of exogenous CghSAMPa at the outset. PNNL's research experience in *A. niger* for small molecules, chemicals, and heterologous protein production has established excellent molecular tools, so we determined this strain as the first choice. **PNNL will proceed in building transgene expression constructs into *A. niger* for Task 2, reserving *A. oryzae* as a backup system.**

- All strains showed some extracellular sensitivity at low levels of cghSAMP but were inhibited at the higher levels
- Actual production of the cghSAMP *in situ* is expected to have a different and lesser effect
- **Outcome: *Aspergillus niger* was the best choice.**

Task 2: Design and Build of Multiple AMP expression strains (PNNL): 5/2021 completed 11/2021.

The backbone transgene expression vectors were constructed by Gibson assembly with the DNA fragments of 1 kb 5'-*gla1*, *gpdA* or *gla1* promoter, the first 22 amino acids of Gla1 signal peptide, HpaI restriction enzyme site, *gpdA* transcriptional terminator, *hph* selection marker, and 1 kb 3'-*gla1* fragments. Then the fragment containing 6x-HN tag, enterokinase protease site, and the cghSAMP coding sequence with codon-optimized for *A. niger* was prepared by de novo synthesis. The fragment was inserted into the above backbone expression vectors. The DNA

sequences of those transgene expression constructs were confirmed by DNA sequencing prior to be used for the transformation. The linearized plasmid DNA was transferred into *A. niger* wild type ATCC11414 via protoplast transformation. Two additional transgene expression vectors are being prepared for fusion of CghSAMP into the gla1 coding region at the position of 170 amino acids or 510 amino acids. The backbone constructs were prepared.

- Multiple copies of AMP transgene expression cassettes should integrate into chromosomes randomly and 6xHN tag-purification was utilized to determine CghSAMP production in transgenic *A. niger* strains.
- **Outcome: 8 different transgenic strains for *A. niger* and 1 for *Trichoderma reesei* were built for CghSAMP Expression.**

Task 3: Test strains for CghSAMP Production and Quantitate (PNNL):11/2021 completed 6/2023.

- Many strains were tested for CghSAMP Production. Two *A. niger* transgenic strains expressed CghSAMP peptide, which was detected, confirmed, and quantified. The fusion protein production in the culture supernatants was confirmed by liquid-chromatography tandem mass spectrometry-based global and targeted proteomics analyses.
- **Outcome: About 0.33 mg/L *gla1*-AMP was detected in the culture supernatant, therefore, further production improvement is essential for field application.**

Task 4: Culture Optimization and Scale-Up (PNNL):11/2022-end of project 4/2024

- Buffering, nitrogen source and other media component optimization; scale-up to bench bioreactors. Maintaining neutral pH and adding soy protein improved CghSAMP titer to 100 mg/L gla1-AMP at 2L scale
- **Outcome: About 100 mg/L AMP peptides were detected after the optimization of culture conditions.** The protein blotting analysis confirmed that the AMP peptides can be effectively cleaved and released from the gla1-AMP fusion protein. **Further optimization of the gla1-AMP protein production and methods for effective purification of AMP peptide need to realize the potential commercial application.**

Citrus greening disease (huanglongbing, HLB) poses a significant threat to global citrus production, which there is no cure for this disease currently, stimulating the demand for sustainable and efficient biopesticides. This research aimed to partner the industrial development of a novel platform for producing and delivering the cghSAMP antimicrobial peptide (AMP) produced by an organism recognized for its industrial utility due to its robust protein secretion capabilities and status as a Generally Recognized as Safe (GRAS) organism. By genetically engineering *A. niger* to produce the cghSAMP, we sought to utilize its fermentation efficiency for scalable cghSAMP production.

The project was conducted in collaboration with NREL, PNNL, and Invaio under the guidelines of a Cooperative Research and Development Agreement (CRADA) for 24 months, initiating in May 2021, with a subsequent no cost extension (NCE) until April 2024. Two primary objectives guided the research: (1) development of a high-yield and cost-effective bioprocess for AMP production and (2) scaling the process to stirred tank reactors for large-scale synthesis, targeting quantities exceeding 100 grams at a concentration of 1–5 g/L. Progress included four critical tasks: identifying a host organism suitable for cghSAMP production, strain engineering

and transgene integration, examining the cghSAMP production, and optimizing culture conditions for scale-up.

Key findings revealed *A. niger* as the optimal host, resulting in successful integration of cghSAMP expression cassettes into eight transgenic *A. niger* strains. Initial culture supernatants produced detectable cghSAMP concentrations (0.33 mg/L), later optimized to 100 mg/L at the 2-liter scale through medium formulation adjustments, including pH stabilization and soy protein supplementation. Protein analysis confirmed effective cleavage and release of AMP from fusion proteins; however, further advancements in production efficiency and purification techniques are required to meet commercial application standards.

However, planned scale-up targets of 100 grams of AMP were not achieved due production efficiency, and abrupt direction changes in the project's scope by Invaio management team, leading to premature project termination. To facilitate completion of key tasks and ensure actionable outcomes, remaining funds of $30,000 from NREL were transferred to PNNL with BETO approval in December 2023. These funds were allocated for final experiments focusing on AMP purification and enterokinase digestion, critical for generating high-quality data for a journal publication and addressing potential intellectual property.

This study marks a critical step toward leveraging *A. niger* for sustainable, large-scale cghSAMP production as a biopesticide for citrus greening disease (HLB) mitigation. The outcomes demonstrate the viability of fungal bio-factories while emphasizing the necessity for continued improvement in bioprocess scalability and cost efficiency, offering significant implications for environmentally friendly disease management and agricultural innovation.

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