Virginia Wine Board Grant
Final Report

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Title: Green Designer Enzymes to Eliminate Fungal Pathogens
Proposal Number: 20-29

Project Type: ☒ Research ☐ Education ☐ Marketing

Is this a multi-year grant? ☐ Yes ☒ No

Original Funding Amount: $50,000
Remaining Balance: $0

Objectives and Corresponding Achievements:

The aims of this proposal focused on further validating product performance under actual application conditions while ensuring other criteria for commercial feasibility were met, including scalability, cost-effectiveness for farmers, and compatibility with existing pesticide application practices. These objectives were divided into 3 major tasks in which the following progress has been made:

1) Optimizing parameters for maximal enzyme biomanufacturing yield.

At the start of 2019, we pursued production using an *E. coli* based expression system. Following receipt of support from the VWB grant and other private funding, we were able to further explore the viability of this expression system in terms of scalability, cost-effectiveness, and compatibility for production of an ag-food product. By onboarding experienced EPA consultant, Mark Wozniak, and discussing with potential industry partners, we learned that *E. coli* was not the ideal host organism to meet these aims, due to its production of endotoxins that would compromise the safety of a product applied to an agricultural crop. Otherwise, extensive downstream purification methods, which were being employed in our lab for current production, would lead to significant product costs.

To address this, we pivoted to the commonly used industrial host for food-grade enzyme production, *Pichia pastoris*, which qualifies as FDA GRAS (Generally Regarded as Safe). Thus, we designed and implemented an expression and purification protocol involving minimal downstream processing to reduce costs (Figure 1a), whereas with the previous, *E. coli* based-system, extensive downstream purification including lysis, centrifugation, chromatography, and filtration were required, accounting for over 70% of production costs. The new system developed with *Pichia* generates a secreted product, therefore ideally only requiring centrifugation and filtration steps to yield a finalized product concentrate.

Following strain development in *Pichia* and verification of expression, we pursued further optimization efforts in-house to develop a unique strain with high productivity. After modeling our manufacturing process at scale with input from industry partners, we determined a target expression of 5 g of enzyme per L of fermentation to achieve a material cost of under $200/kg (Figure 1b). This material cost, when combined with our projected packaging and distribution costs would yield a cost of goods sold (COGS) allowing a sale price competitive with existing biologics on the market, including Fracture and Cease.

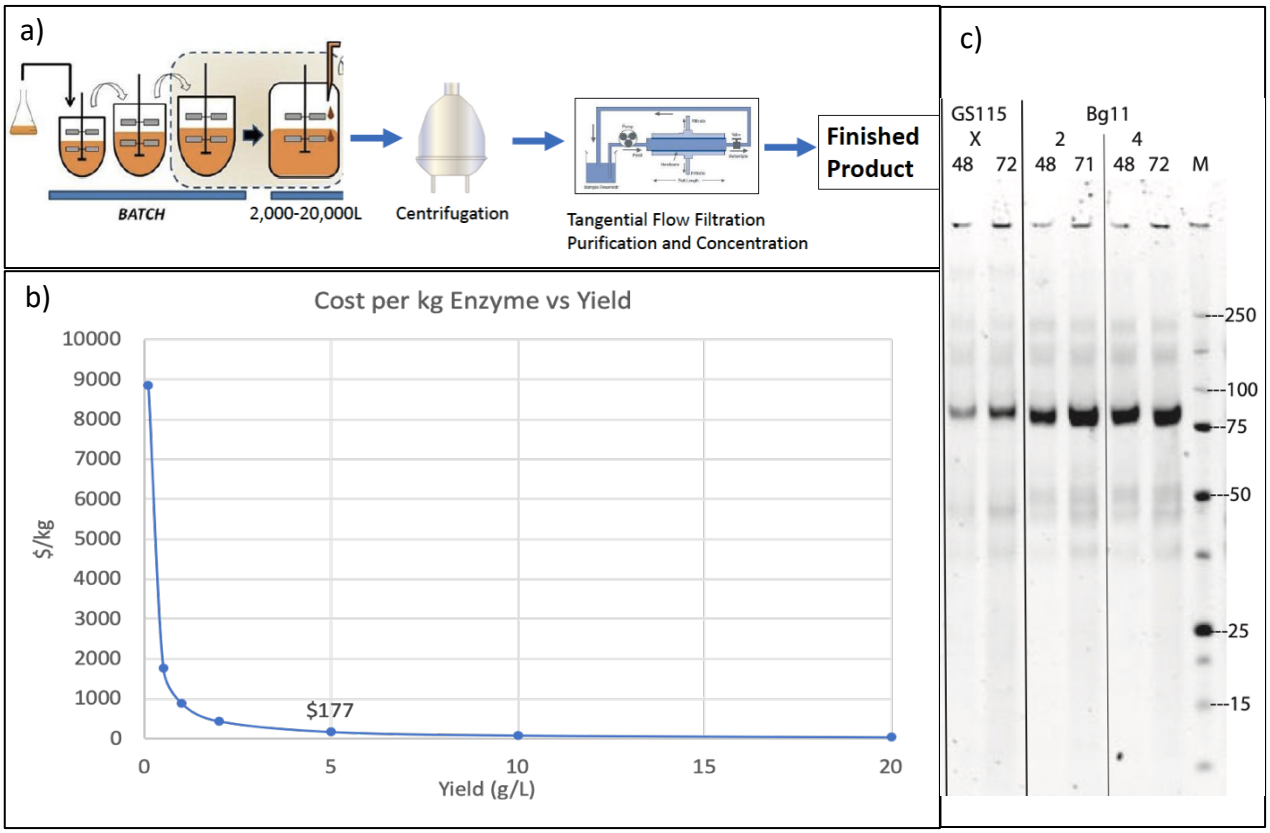

Figure 1: Manufacturing Process Design (a) Developed minimal manufacturing process with *P. pastoris* and Tangential Flow Filtration to yield a finished product, (b) Modelled enzyme cost using the designed process relative to yield in fermentation indicates a 5 g/L yield target to achieve an economically viable product, (c) Enzyme expression levels in shake flask after 48 and 72 hrs in a *Pichia*, methanol inducible system. Strain produced in collaboration with BioGrammatics (Bg11) demonstrates yield improvements over Lytos in-house strain (GS115)

Initial in-house shake-flask experiments yielded up to 0.1 g/L titers, and no significant improvement was achieved upon scale-up to the bioreactor. Considering that an optimized fermentation process is expected to yield 10X yields over shake-flask expression due to improved cell density, we sought external partners to further optimize our strain and fermentation process.

To achieve or surpass our target fermentation productivity, we are currently working through two external contracts for scale-up optimization and strain optimization in parallel. University of Georgia's Bioexpression facility is performing fermentation optimization of our *Pichia* strain in 4L batches. The optimized process will then be transferred to 100L pilot scale, a critical milestone to demonstrate equivalent manufacturing yields per liter at eventual commercial scale. Project completion was anticipated in April of 2020, but has been delayed until Fall 2020 due to COVID-19.

Further strain optimization to maximize productivity per cell, is being performed by BioGrammatics, a leader in *Pichia* optimization with over 20 years of experience who has successfully developed high productivity *Pichia* strains for other successful companies. Initial strain optimization efforts show significant improvement over our methanol-inducible strain in shake flasks (FIgure 1); we have now established a work plan for further strain optimization and fermentation efforts to achieve our target 5 g/L yield.

2) Determine Optimal Fungicide Formulations

An enzyme formulation compatible with our manufacturing process was determined to maximize product activity and stability. By benchmarking against existing products and extrapolating application rates from prior field trials, we determined that a liquid concentrate containing 50 g/L enzyme in a 20 mM phosphate buffer, pH= 8.25 and 100 mM NaCl was ideal and could be directly generated through our minimal purification process to yield a packaged product. Optimal formulation components, including salt and pH, was determined via Schales' Assay, where a greater change in A_{420} indicates greater activity. By referencing common pesticide application practices by growers, we determined that addition of surfactants directly to our product was not necessary, as growers typically add Cohere or other products to their tank prior to application.

Still, product compatibility was tested in the presence of other agents that would commonly be added together in a tank sprayer. In terms of sticker-spreaders, our product remained active in the presence of 0.1% Cohere, 0.1% Tween, 0.1% Therm-X 70 surfactants as determined by the TBA activity assay, in which measurement of A_{540} corresponds to enzyme activity (Figure 2). The Therm-X 70 compound would be recommended to growers as compatible sticker-spreaders for their organic certification. In field trials, Cohere was added to all products prior to application.

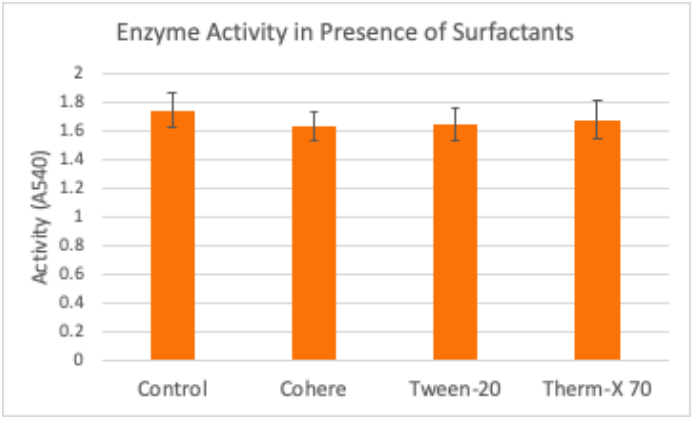

Figure 2: Enzyme activity in the presence of surfactants. Standard error reported with samples performed in triplicate

3) Comprehensive field trials at Afton Mountain Vineyards, AHS Agricultural Research and Extension Center

Scaled field trials to further validate initial results from the 2018 growing season were pursued in parallel at Afton Mountain Vineyards and Virginia Tech's Agricultural Research and Extension Center in partnership with Damien Blanchon and Dr. Tony Wolf respectively. These trials aimed to 1) scale results to larger segments of crop, 2) more directly reflect actual application techniques, and 3) compare product performance to existing organic and chemical fungicides while exploring different application timings.

The Afton Mountain trial contained three half-acre plots where product was applied using a traditional tank sprayer. One half acre was treated with Lytos Biofungicide, one half acre was treated with Afton's normal fungicide rotation including products like Elevate and organic teas, and one half acre was left untreated. Three product applications for *Botrytis* were performed from May through July and incidence and severity of fungal proliferation were monitored.

In contrast to 2018 field trials where significant rainfall led to nearly all observed bunches displaying infection by *Botrytis,* summer of 2019 yielded exceptionally dry conditions. As a result, little or no manifestation of *Botrytis* was observed prior to harvest and significant results on incidence or severity could not be determined. However, qualitative observations by Damien Blanchon on mildew affecting the leaf surface indicated significantly reduced proliferation and sporulation of mildew in the Lytos Biofungicide treated plot.

Further exploration on the effects of enzyme on powdery mildew are being explored in spring of 2020 through greenhouse studies in partnership with Dr. Tony Wolf to verify and quantify product activity on Mildews.

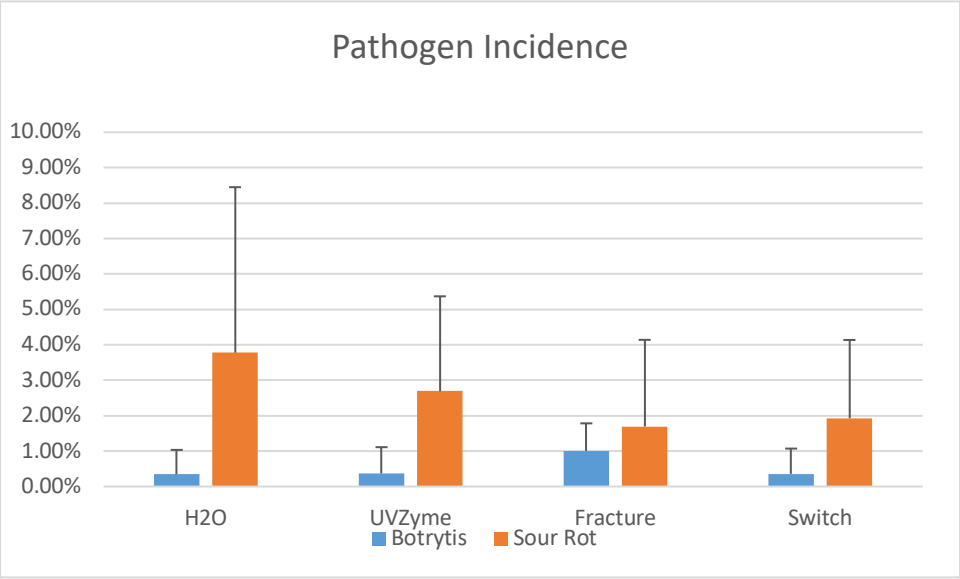

Figure 3: Incidence of Botrytis cineria and Sour Rot in treatment groups at harvest

The Virginia Tech trial was conducted in collaboration with Dr. Tony Wolf who aided in statistically significant trial design, product applications, and data collection. 16 plots throughout the vineyard were designated for treatment with random assortment of vine treatment within each of those plots. In each plot, the enzyme treatment was compared to a water treatment as a negative control, and Fracture and Switch treatments as positive organic and chemical controls respectively. Cohere was added to all products, which were applied as a fruit-directed spray using a backpack sprayer. Product was applied on two to four occasions on each panel, as different application timings were also studied including combinations of application at bloom, cluster closure, veraison, and preharvest.

Due to overall dry conditions, data was collected only at harvest after observing a lack of sporulating Botrytis in the crop. Incidence and severity ratings were collected for both *Botrytis* and Sour Rot on all vines. Incidence results as a percentage of all clusters are displayed in figure 3. As seen, no significant variation in incidence between treatment groups was observed. Variation in severity ratings were also insignificant, as severity on each infected bunch was typically below 5%. Quality control data, including fruit mass, pH, and brix was also collected at harvest by processing representative clusters. No significant variation of quality metrics in any treatment group was observed. However, significant fruit scarring or russeting was observed in the Switch group, whereas no such deformation was observed in the enzyme treated group.

Overall Benefit for Virginia Wine Industry:

With nearly 300 active wineries driving rapid growth in Virginia's wine industry, as well as a growing state-wide emphasis on biotechnology startup formation, our centrally-located company in Charlottesville provides an ideal environment to bring novel biofungicides to market. The economic impact of wineries and vineyards in the state is $1.37 B. In conversations with several local growers such as Afton Mountain Vineyards, we have found a strong emphasis from growers on continually seeking green, organic and sustainable approaches to crop management and a passion for ensuring the highest product quality. Unfortunately, the relatively humid and warm climate of Virginia provides an ideal environment for fungal pathogens such as Botrytis to thrive and severely reduce product yields, while existing organics lack efficacy.

Initial data from 2018 indicated that our product addresses this unmet need for growers, with demonstrated stability and robust activity in the presence of significant rainfall when applied directly in the field to vines. Furthermore, as a product which is OMRI- and USDA organic eligible, our product appeals directly to local growers for its safety to growers, consumers and the environment while having no effect on measured product quality or taste.

Through the Virginia Wine Board project, we have taken major steps towards the commercialization of our product to achieve the intended impact on Virginia's wine industry. In the past year we have made progress including (1) successfully completing development of our optimized antifungal enzyme and a formulation to file a PCT patent application, (2) completing experimental validation of core product safety claims, (3) designing a viable manufacturing process using an EPA-accepted host organism, (4) developing a minimum cost strategy through the EPA registration process, and (5) building out our core team and infrastructure. While there remain challenges in manufacturing, we have partnered with

leading organizations to maximize our chances of achieving our target process productivity and ensuring that we can bring an effective, safe, and affordable product to market.

Considering our initial focus on the Virginia wine industry in our go-to-market strategy, the local economy will be the first to benefit from a product that improves crop yields while allowing organic product certification that may justify an increased sale price or provide a competitive edge for Virginia based wine.

Publications and Activities Associated with Project:
Filed PCT patent: PCT/US2019/057797 titled "Antipathogenic Polypeptides"

Future Work:
In order to enter the EPA registration process in 2021, we are working to complete the following milestones:

- Developing an optimized strain of Pichia capable of achieving 5 g/L yields in fermentation in partnership with BioGrammatics
- Performing fermentation optimization and scaling the process to 100L in partnership with University of Georgia
- Conducting additional field and greenhouse trials to demonstrate product efficacy on Botrytis and mildew compared to existing organic and synthetic products
- Performing ongoing stability studies to demonstrate product stability for at least 1 year at room temperature
- Raising an additional $1.5 M to $2 M in order to begin EPA testing
- With additional strain optimization, extended EPA review time, and delays due to COVID-19, we are anticipating market entry in summer of 2023.

Final Budget and Justification:

Item Type	Original Awarded Amount	Final Amount Spent
Personnel	$25,000	$25,000
Fringe	$0	$0
Travel	$800	$800
Supplies & Materials	$19,500	$19,500
Contractual	$4,700	$4,700
Other	$0	$0
Total	$50,000	$50,000

Other Supporting Funds:

- Seed funding from angel investors: $405,000
- UVA Catalyst Accelerator Grant: $20,000
- Charlottesville City Match Grant: $25,000
- Virginia Catalyst Grant: $500,000