Lab Report

Laccase enzyme as a potential solution to the negative effects of glycosylation reaction when oxybenzone is metabolized by coral*

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Reviewed on 4 May 2024; Accepted on 10 June 2024; Published on 26 October 2024

Addressing issues such as coral bleaching and subsequent coral death through synthetic biology broadens and improves methods to mitigate anthropogenic consequences on marine wildlife. This project focuses on using synthetic biology to combat the negative effects of the common sunscreen ingredient, oxybenzone, on corals without harming other organisms found in reef environments. Previous studies have used the oxidase enzyme, laccase, to prevent the metabolic reaction that turns oxybenzone phototoxic and therefore harmful to corals. We propose using an alginate matrix with immobilized laccase from the fungi species Trametes versicolor to release into environments affected by oxybenzone. The laccase enzyme will be produced by inserting the Trametes versicolor strain of DNA into a T7 plasmid vector, which will produce the enzyme when expressed. Once isolated, the enzyme will be immobilized and ready for use.

Keywords: Oxybenzone, laccase, glycolysis, coral, alginate

Our research addresses the health of coral in reef environments and the impact of sunscreen on coral. Oxybenzone is a common sunscreen ingredient that undergoes a glycosylation reaction when metabolized by coral, resulting in phototoxicity (Vuckovic et al., 2022). This can lead to damage to coral in the form of growth deformities, DNA mutations, and bleaching (Lenzie, 2022). We plan to combat the effects of this glycosylation reaction using the oxidative enzyme laccase, which can react with the oxybenzone before it undergoes this reaction (Luisa, n.d.). We inserted a gene for laccase expression into a T7-P14 plasmid vector and fused it into a chimeric protein with a green fluorescent protein expressor. We ran a cell-free gene expression experiment comparing the fluorescence levels of cells containing our engineered DNA with and without a DNA inducer, with a control cell of just TxTl Mastermix. Our findings suggest that there may be expression of laccase in the cell-free environment, but further testing is needed to confirm this.

Materials and methods

When oxybenzone is metabolized by coral, it undergoes a glycosylation reaction in which its hydroxyl group is removed and replaced with a glucose, turning the oxybenzone into a

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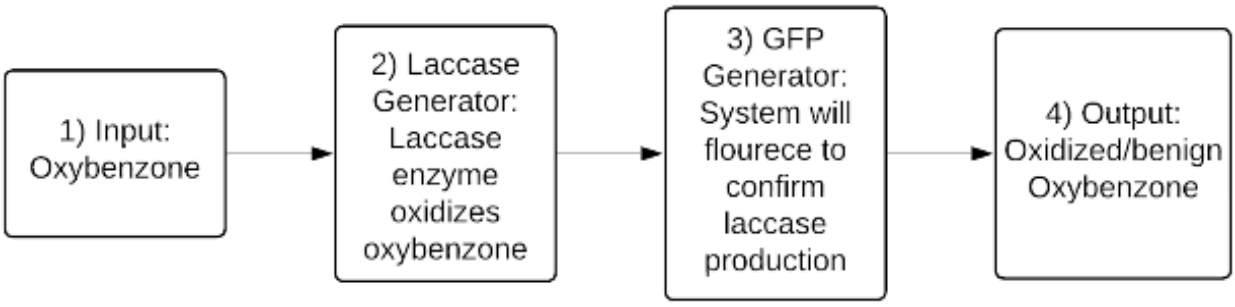

Figure 1. Proposed pathway system to produce laccase and oxidation of oxybenzone.

glucoside conjugate with phototoxic properties. To inhibit this chemical reaction, the enzyme laccase derived from the fungi species *Trametes versicolor* can be used to oxidize oxybenzone, thus preventing coral metabolization and mitigating its negative effect on coral (Figure 1; Garcia et al., 2010). To produce laccase, we modified a T7-P14 GFP plasmid vector obtained from Arbor Scientific. After enzyme production, we plan to develop a delivery system using alginate beads, which would deliver laccase and a mediator in an environmentally friendly manner (Zhang et al., 2018). The use of a mediator expands the oxidative range of laccase. The beads would be prepared through ionotropic gelation, dripping a solution of sodium alginate, laccase, and acetosyringone into a calcium chloride solution, causing the drops to form a porous hydrogel shell. After production, the beads would be lyophilized to improve stability, transportation, and delivery of the system to the surface of the corals. After release into the coral reefs, the beads will rehydrate and diffuse laccase and acetosyringone into the environment, catalyzing the oxidation of oxybenzone.

We modified a T7-P14 GFP plasmid vector to produce the enzyme laccase. We were granted permission by Arbor Scientific to use the plasmid vector. When expressed in its original form, the T7-P14 plasmid produced green fluorescent protein, which serves as a useful indicator of gene expression. Using the original T7-P14 GFP plasmid as a template for our new design, we used restriction enzyme cuts to insert the sequence for laccase. When expressed in a cell-free system, the recombinant plasmid is intended to produce both green fluorescent protein and laccase through the expression of one chimeric protein.

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In order to make the engineered plasmid compatible with the Arbor Scientific cell-free expression kit, we used a T-7 promoter and terminator. The expression genes for laccase and green fluorescent protein have been combined into one chimeric protein expression gene (Figure 2).

In order to be an effective combatant to oxybenzone, the plasmid needs to be immobilized in an alginate matrix. The laccase must be encapsulated properly due to its high oxidative potential. Because of this quality of the enzyme, other biological mechanisms are at risk of being disturbed by the reaction with laccase. In order to ensure this does not happen, we must test the ability of the alginate to encapsulate the laccase fully without lowered reactivity once delivered to oxybenzone. Testing might take the form of releasing the alginate matrix into a control environment with chemical indicators of any reactions the laccase might undergo aside from oxybenzone. To ensure safety while testing, experimenters will wear proper

Figure 2. Flow chart representing gene expression of Laccase GFP Fusion plasmid.

protective clothing. Another important safety concern is the impacts the device has on users and their communities. This further provides an incentive for effective encapsulation; it is necessary to ensure that exposure to the loose laccase enzyme does not induce negative health effects in the communities in which it is utilized.

Results

Data analysis

Using ImageJ, we compared cell fluorescence across tubes in greyscale images of our assays taken in dark settings (Figure 3). Fluorescence was measured in Mean Gray Value, which is the sum of Gray Value pixels divided by the number of pixels in a selected area (Table 1). Larger values indicate greater fluorescence. Since fluorescence is correlated with GFP expression, and the laccase and GFP genes were fused in our plasmid, positive results would look like significant fluorescence in the Induced-laccase tube (ILT).

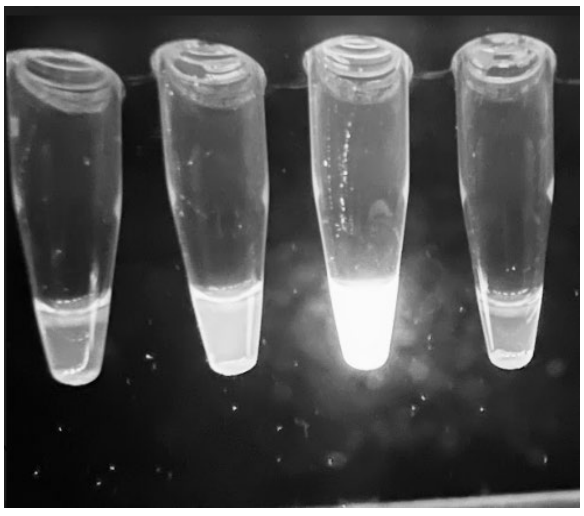

Figure 3. Photo of results after T = 48 Hours (Left to right: Complete Control, PCT, PCT, ILT)

Discussions

We found that the mean Gray Value for the complete control tube (only TxTl) was 142.346 in an area of 609. This number is significantly less than the Gray Value for the ILT was 176.854 in an area of 609. This denotes some level of expression of the GFP.Because the GFP and Laccase expression genes were fused, this would also indicate the expression of laccase. However, the mean Gray Value of the ILT was less than the Positive Control Tube (PCT) , which had a mean of 250.921 in an area of 609. The PCT had TxTl, GFP DNA, and inducer DNA. This could mean that the expression of chimeric Laccase/GFP protein is less strong, or the expression of laccase is somehow lessening the expression of GFP. Further tests using chemical indicators or electrophoresis could help to determine the presence of laccase, as well as how effectively it has been expressed in the cell-free kit.

The tubes which we analyzed in ImageJ are shown in Figure 3. The first tube on the left is the Complete Control Tube, which contains no GFP DNA or Inducer DNA. Because of this, there was no visible fluorescence, and the Mean Gray Value

Table 1. Results of fluorescence intensity test. Area measured in pixels and Mean Gray Value.

Tube	Area (Pixels)	Mean Gray Value
1 (Complete Control Tube)	609	142.346
2 (Positive Control Tube)	609	220.332
3 (Positive Control Tube)	609	250.921
4 (Induced-Laccase Tube)	609	176.854
1 (Complete Control Tube)	609	142.346

calculated from the tube served as a baseline. The second and third tubes from the left are the Positive Controls. These tubes expressed only the GFP gene and had visible fluorescence. The fourth tube is the Induced-Laccase Tube, which contains TxTl Mastermix, Inducer DNA, and DNA for the expression of the Laccase-GFP fused protein.

Next steps

Using the results of our experiment, we analyzed our data to confirm whether laccase was successfully expressed in our cell-free system. We first used spectrophotometry to compare fluorescence across assays. To indicate that there may be expression of laccase, yet another method might be running electrophoresis or using a chemical indicator (e.g. acid-base indicators) to ensure laccase expression. Once the expression of laccase is confirmed, we will test its ability to react with Oxybenzone effectively.

Finally, We would like to design an alginate matrix with immobilized laccase. Specifically, the use of a Copper (II) Alginate matrix may be most effective. We may use the already-tested method of dissolving isolated laccase in a Cu^{2+} solution before gelation in a sodium alginate solution. The dissolution of Laccase in Cu^{2+} solution should increase its effectiveness (Zhang et al., 2018).

However, immobilization would most likely require a mediator to the laccase. This is a current limitation to our design, as we have not decided whether to use Synthetic Mediator 2,2'- Aceto-bis(3-ethylbenzthiazoline-6sulphonic acid) diammonium salt (ABTS) or natural mediator Acetosyringone (ACE). Both have previously been shown to be most efficient at the removal of oxybenzone (Garcia et al., 2010), however using synthetic mediators such as ABTS could pose bioethical concerns. Similarly, limiting ACE requires higher concentration to meet the same effectiveness as ABTS. The mediator used will most likely impact other components of the matrices, like the concentration of immobilized laccase and other factors relating to proper encapsulation.

After designing the matrix, we will need to test its effectiveness to ensure immobilization is the best method. We will also need to measure its ability to encapsulate laccase in such a way that reactivity with other marine organisms or mechanisms does not occur when delivered to the substrate.

Author contributions

M.C., S.E., M.H., W.K., L.N., S.P., E.S., A.S., and C.W. conducted preliminary research. M.C., M.H., W.K., L.N., S.P., E.S., and C.W. further researched the Laccase enzyme. M.C. lead research into alginate matrices, with help from S.P. and E.S. S.P. completed plasmid engineering in SnapGene. W.K., S.P., and E.S. carried out the experiment, and S.P. was responsible for initial photographs of the results. S.P. carried out image interpretation in ImageJ.

M.C, S.P., and E.S. took the lead in preparing for publication, communicating with *BioTreks*, and writing the manuscript. All authors played a substantial role putting work into the research and publication process.

Acknowledgements

We sincerely thank our mentor, Alberto Donayre Torres at UTECH, for his expert guidance; Arbor Scientific, for allowing us to modify their T7-P14 cloning vector and providing us with Cell-Free Gene Expression Kits; and K2 Scientific, for the generous donation of a -25° cold storage unit.This project was made possible through the BioBuilderClub. We are grateful to the BioBuilder Educational Foundation for providing us with the tools and opportunities to pursue our research in the field of synthetic biology. Lastly, we graciously thank our club sponsor Matthew Kirkpatrick for his endless support and for providing us the opportunity to be a part of an incredible organization.

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