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Synthetic Remediation Biology: Asgard Detoxification of Synthetic Firefighting Foam for Environmental Sustainability

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14. ABSTRACT
Synthetic firefighting foam, especially aqueous film forming foams (AFFF), contains the toxic compounds perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS). Chronic exposure to these perfluoroalkyl toxins can lead to adverse health effects such as endocrine disruption, infertility and carcinogenesis. This project aims to use synthetic biology and synthetic enzymology means to address the growing problem of polyfluoalkyl substances (PFASs) contamination in the environment. It has been reported that PFASs can be degraded by enzyme catalyzed humification reactions (ECOHRs), but the limited degradation or defluorination capacity of wild-type microbes and enzymes necessitates engineering strategies to improve their potential for bioremediation. In this study, we endeavor to engineer novel enzymes, in particular laccases, with defluorinating activity towards PFOA and PFOS and deploy direct evolution strategies to enhance their biocatalytic performance. To facilitate downstream on-site bioremediation strategies, we also engineered recombinant laccases into the freshwater organism chassis, Chlamydomonas reinhardtii, employing several molecular designs to enhance transgene expression and extracellular protein production. The expressed laccases were shown to have functional catalytic activities and work is ongoing to examine their PFOA/PFOS-degradation capabilities. Subsequent whole-ecosystem approaches can be envisioned with the co-deployment of engineered AFFF-detoxifying enzymes (ADE)-expressing freshwater organisms and choice biosorption flora at sites of concern. Through this proposal, we seek to maintain planetary health by offering a bio-based approach to mitigation of anthropogenic activities in the aerospace industry.

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Final Report

SECTION I: DETAILS OF PROJECT

a) Award No.	FA2386-20-1-4040
b) Project Title	Synthetic Remediation Biology: Asgard Detoxification of Synthetic Firefighting Foam for Environmental Sustainability
c) Name of PI	Wen Shan YEW
d) Host Institution	National University of Singapore
e) Department	Biochemistry, and NUS Synthetic Biology for Clinical and Technological Innovation (SynCTI)
f) Approved Budget	USD$100,000.00
g) Duration of Project	1 year 9 months
h) Project Start Date	22 Jun 2020
i) Project End Date	21 Mar 2022

Section 2: Technical Report PDF Upload

Abstract

Synthetic firefighting foam, especially aqueous film forming foams (AFFF), contains the toxic compounds perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS). Chronic exposure to these perfluoroalkyl toxins can lead to adverse health effects such as endocrine disruption, infertility and carcinogenesis. This project aims to use synthetic biology and synthetic enzymology means to address the growing problem of polyfluoalkyl substances (PFASs) contamination in the environment. It has been reported that PFASs can be degraded by enzyme catalyzed humification reactions (ECOHRs), but the limited degradation or defluorination capacity of wild-type microbes and enzymes necessitates engineering strategies to improve their potential for bioremediation. In this study, we endeavor to engineer novel enzymes, in particular laccases, with defluorinating activity towards PFOA and PFOS and deploy direct evolution strategies to enhance their biocatalytic performance. To facilitate downstream on-site bioremediation strategies, we also engineered recombinant laccases into the freshwater organism chassis, *Chlamydomonas reinhardtii*, employing several molecular designs to enhance transgene expression and extracellular protein production. The expressed laccases were shown to have functional catalytic activities and work is ongoing to examine their PFOA/PFOS-degradation capabilities. Subsequent whole-ecosystem approaches can be envisioned with the co-deployment of engineered AFFF-detoxifying enzymes (ADE)-expressing freshwater organisms and choice biosorption flora at sites of concern. Through this proposal, we seek to maintain planetary health by offering a bio-based approach to mitigation of anthropogenic activities in the aerospace industry.

Research Objectives

Please list the main research objectives of this project.
List the major goals of the project including the scientific or technological objectives of this effort. Describe the proposed technical approach to obtain those goals. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Perfluorooctanoic acid (PFOA) and Perfluorooctanesulfonic acid (PFOS) are main chemical components of the aqueous film forming foam (AFFF) in synthetic firefighting suppressants. PFOA and PFOS are persistent pollutants due to the carbon-fluorine (C-F) bond, one of the strongest covalent bonds in organic chemistry. PFOA and PFOS threaten natural environments and human health because of their recalcitrance to breakdown. If accumulated at high levels, these perfluoroalkyl toxins can lead to severe toxicological effects, resulting in metabolic diseases, cancer and other human morbidities. Microorganisms prospected from polluted sites have been reported to show PFOA and PFOS degradation abilities through the action of enzymes such as peroxidases and laccases. It has been elucidated that these polyfluoalkyl substances (PFAS) are degraded by enzyme catalyzed humification reactions (ECOHRs) via free radical chain reaction processes. Successful biodegradation of PFASs, however, remains

limited, impelling the need to adopt engineering strategies to improve the degradation or defluorination capacity of wild-type microbes and enzymes for remediation purposes.

This project aims to develop recombinantly-expressed enzymes, particularly laccases, with defluorinating activity towards PFOA and PFOS (Figure 1) in a bid to break down these fluorinated polymers and reduce their toxicity. In comparison to peroxidases, laccases are more suitable candidates for potential in situ remediation application as they use oxygen instead of hydrogen peroxide as an electron acceptor and their activity is stable over a longer period of time. The novel enzymes will be engineered in *Pichia pastoris*, a widely used heterologous protein production workhorse, as well as in a freshwater chassis that can be deployed in areas of AFFF contamination. As part of a whole-ecosystem approach, we would like to demonstrate through proof-of-concept the co-deployment of biosorption flora to decrease environmental bioavailability of these toxic PFAS.

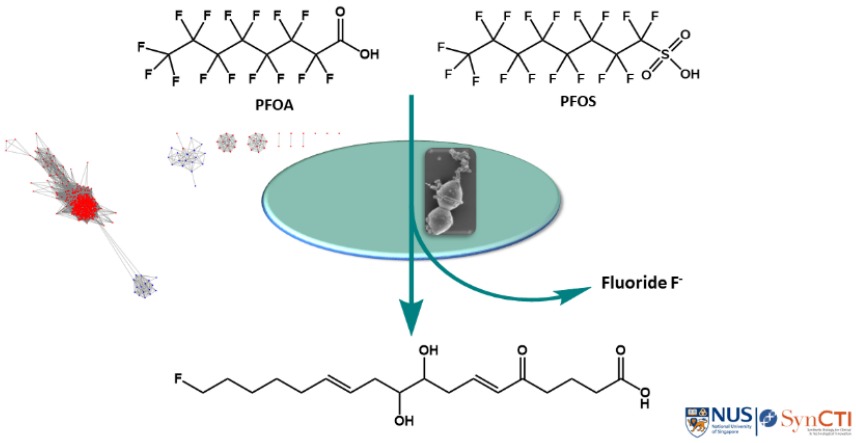

Figure 1. Engineering enzymes for biodegradation of recalcitrant PFAS.

Technical approaches

Enzymes such as laccases have been reported to catalyze the defluorination and partial degradation of PFOA and PFOS. Building on this information, we employed the Enzyme Function Initiative – Enzyme Similarity Tool (EFI-EST) web tool to generate protein sequence similarity networks (SSNs) that allows for visualization of relationships among protein sequences. SSNs reveal the closest neighbors of a target protein and directs exploration of the sequence-function space, presenting a highly useful tool in identification of potential candidates for enzyme engineering. Candidate enzymes are expressed, and their activity analyzed using an ion-selective electrode (ISE)-based enzymatic assay and ion-chromatography mass spectrometry. Recombinant enzymes that show good defluorinating activity will be used as precursors for synthetic enzyme evolution. This can be achieved using two approaches; the first deploys rational mutagenesis while the second involves mutant library screening. Mutagenesis by rational design is possible with known protein structures. Using

molecular visualization and modeling web tools, molecular docking scenarios can be performed to identify amino acids that likely interact with the ligand or mediator of interest. These sites become candidate targets for mutagenesis to improve enzyme function or specificity. The alternative approach uses Agilent QuikChange HT Protein Engineering System to target all non-conserved residues and creates a library of custom mutagenic oligonucleotides and primers to produce an engineered mutant plasmid library. These mutants are then screened for enhanced activity and/or stability using the protocols and workflows developed within the NUS BioFoundry.

With consideration of the practical deployment of AFFF-detoxifying enzymes (ADE) in mind, we also explored freshwater microorganisms as chassis for heterologous production of recombinant enzymes, which can be used at sites of contamination. Tapping on available genetic toolkits, we engineered the freshwater host organism, *Chlamydomonas reinhardtii*, to express recombinant laccases. A summary of the technical approaches is shown in Table 1.

If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Table 1. Task list and timeline.

	Project Year			
Research Activities/Milestones	Q1	Q2	Q3	Q4
Expression, purification and characterization of candidate enzymes using ion-selective electrode (ISE)-based enzymatic assay and ion-chromatography mass spectrometry				
Directed evolution of enzymes for enhanced activity				
Engineering of enzymes and accompanying pathways into the freshwater chassis *Chlamydomonas reinhardtii*				
Identification of compatible biosorption flora for possible co-deployment with engineered enzymes and freshwater chassis				

Key Outcomes

Please provide details of accomplishments during this reporting period.
1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); 4) other achievements. Include a discussion of stated goals not met.

This study harnesses the potential of laccases as green biocatalysts for the defluorination and/or degradation of PFOA and PFOS. Of particular interest are fungal laccases that are responsible for lignin decay in nature. They exhibit high redox potential (HRP) which broadens the range

of possible substrates for oxidation, making them ideal candidates for investigating AFFF-detoxifying capabilities. Laccases can be broadly categorized into classes 1, 2 and 3 in order of increasing redox potential based on three known possible amino acid residues (Met, Leu, Phe) at a critical position in the type 1 copper center.

I. Directed evolution of enzymes for enhanced activities

Sequence Similarity Network for laccase selection

A Sequence Similarity Network (SSN) was generated from the *Pleurotus ostreatus* laccase sequence (Figure 2). A total of 24,732 sequences similar to the *P. ostreatus* laccase sequence (e-value $\geq 10^{-5}$) and/or under the protein family PF00394 were identified and 4,407 were found to code for a laccase or laccase-like enzyme.

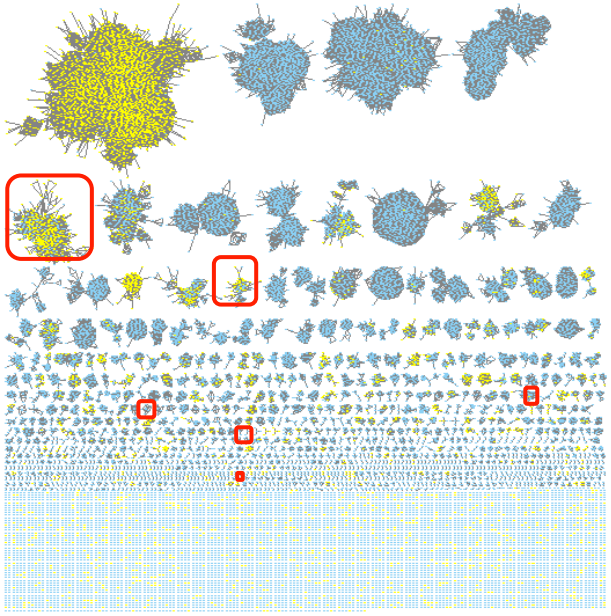

Figure 2. Sequence Similarity Network (SSN) of laccases and laccase-like enzymes. The SSN was generated from BLAST using *Pleurotus ostreatus* laccase sequence ((e-value $\geq 10^{-5}$, alignment score ≥ 200). Sequences from the protein family PF00394 were also included in the network. Nodes representing laccases or laccase-like enzymes identified by the SSN are indicated in yellow. Clusters from which laccases were chosen for further screening are indicated by the red boxes.

Fifteen wild-type laccases were subsequently selected for further expression and screening, of which 14 were chosen from the SSN. These laccases were shortlisted as they contain a phenylalanine (F) amino acid residue in the fungal laccase copper-binding domain consensus sequence 'HCHNNNHNNNN[FLM]NN'. Laccases with 'F' in this amino acid position belong to the Class 3 category consisting of high-redox-potential laccases (HRPLs). Laccases with leucine (L) in this amino acid position fall under Class 2 that generally have lower redox potentials. Also included in the study are evolved variants that have been lab-engineered to increase their redox potentials and catalytic ability

towards various laccase-specific substrates [1–4]. An increase in the redox potentials of laccases has been reported to result in a concomitant increase in the redox activity of laccase-mediator systems [5]. HRPLs could therefore potentially be more effective in overcoming the energy barrier of the C-C or C-F bonds of PFOA and PFOS and confer enhanced capability for biodegradation. In total, 20 wild-type or evolved laccases were selected for further expression and catalytic activity screening in *P. pastoris* (Table 2). All selected laccases originate from fungi and are secreted endogenously.

Table 2. List of laccases from SSN selected for heterologous expression and screening of PFOA and PFOS degradation capability.

Name/ UniProt ID	Organism/ Origin	Wild-type	Evolved	Class
O59896	*Pycnoporus cinnabarinus*	✓		3
3PO-PCL	Evolved from O59896		✓	3
Q99044	*Trametes villosa*	✓		3
Q99046	*Trametes villosa*	✓		3
Q99055	*Trametes villosa*	✓		3
Q99056	*Trametes villosa*	✓		3
Q12717	*Trametes versicolor*	✓		3
Q12718	*Trametes versicolor*	✓		3
Q12719	*Trametes versicolor*	✓		3
D0VWU3	*Trametes maxima*	✓		3
Q02497	*Trametes hirsuta*	✓		3
O60199	*Pleurotus ostreatus*	✓		3
1H6C	Evolved from O60199		✓	3
Q12571	*Basidiomycete* PM1	✓		3
6H5Y	Evolved from Q12571		✓	3
OB1-PM1L	Evolved from Q12571		✓	3
DOOKU	Evolved from OB1-PM1L		✓	3
H8ZRU2	*Botrytis aclada*	✓		2
P56193	*Rhizoctonia solani*	✓		3
Q02081	*Rhizoctonia solani*	✓		3

Structure and molecular docking analysis of laccases

Laccases oxidise mediators at the Type 1 (T1) copper site and these mediators form free radicals upon oxidation which, in turn, can potentially cleave the C-C or C-F bonds in PFOA and PFOS. Through molecular docking analysis with the laccase 1H6C, amino acids that

interact with the mediator ligand were identified (Figure 3). Amino acids C452, H395 and H457 were disclosed to be in proximity to the T1 copper but were conserved across all screened laccases. Of particular interest were amino acids near the T1 copper which are not heavily conserved and could be suitable candidates for subsequent mutagenesis to evolve laccases with higher redox potentials. Currently, additional molecular docking analysis are being conducted with a range of synthetic mediators as the target ligands of interest.

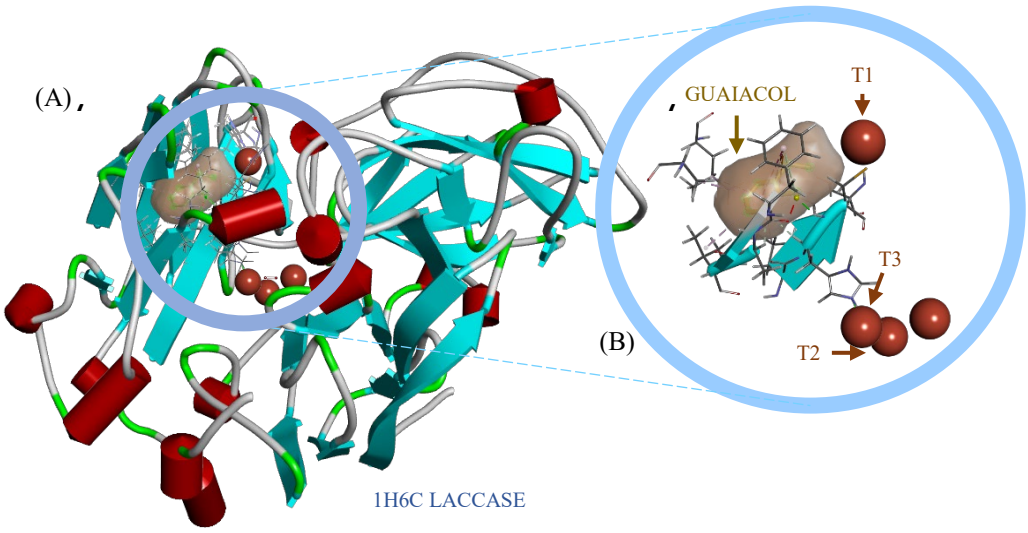

Figure 3. Molecular docking analysis of laccases. (A) Predicted structure of laccase 1H6C generated via SWISS-MODEL with four copper centers at the active site. (B) Guaiacol docked unto 1H6C using Autodock 4.2.6 showcasing the amino acids interaction with the ligand.

Heterologous expression of laccases in *Pichia pastoris*

The yeast *Pichia pastoris* was chosen as the cellular host for the expression of recombinant laccase as it is a known workhorse for high heterologous protein production. Importantly, *P. pastoris* is a eukaryote and is capable of post-translational modifications required for the functionality of the selected fungal laccases. The genes of interest were cloned using Golden Gate Assembly into a pFAi2 vector that facilitates transgene integration into the host genome at the *his4* locus (Figure 4). Gene expression was driven by *P. pastoris* constitutive glyceraldehyde 3-phosphate dehydrogenase (GAP) promoter and targeted for extracellular secretion using the α-Mating Factor (α-MF) secretion signal from *Saccharomyces cerevisiae*.

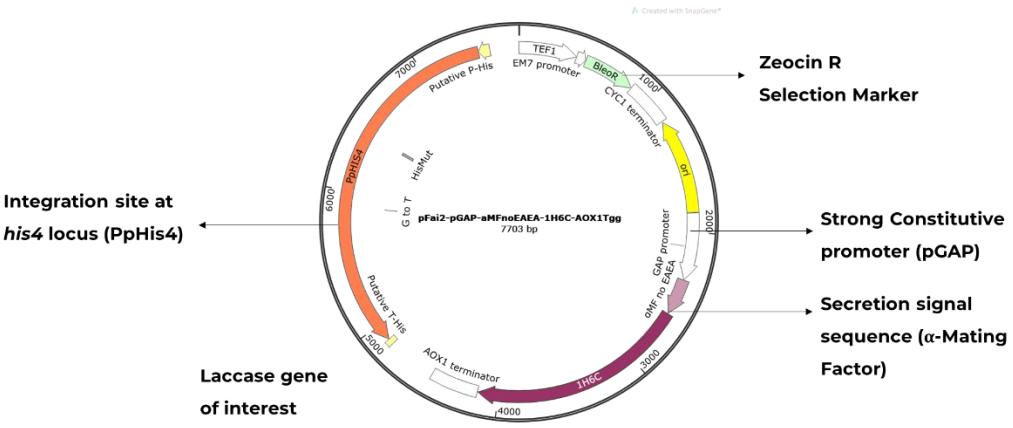

Figure 4. Plasmid map of vector construct containing pGAP constitutive promoter, α-Mating Factor, and laccase gene of interest. The resultant vector construct was integrated into the *P. pastoris* genome at the *his4* locus.

For quick screening of functional enzyme activity, positive transformants were screened on culture plates supplemented with 0.02% guaiacol and 0.2 mM $CuSO_4$ and observed for development of a reddish-brown colour zone indicating oxidation of guaiacol. Of the 20 selected laccases for screening, seven laccase variants showed oxidative activity towards guaiacol, with POXa1b, 1H6C and DOOKU displaying strong oxidative potentials as indicated by the intense browning compared to the others (Figure 5). Interestingly, the laccase from *Botrytis aclada* displayed stronger oxidative potential compared to the other class 3 laccases even though it falls under class 2. The better performing recombinant laccases were further examined for their catalytic activity.

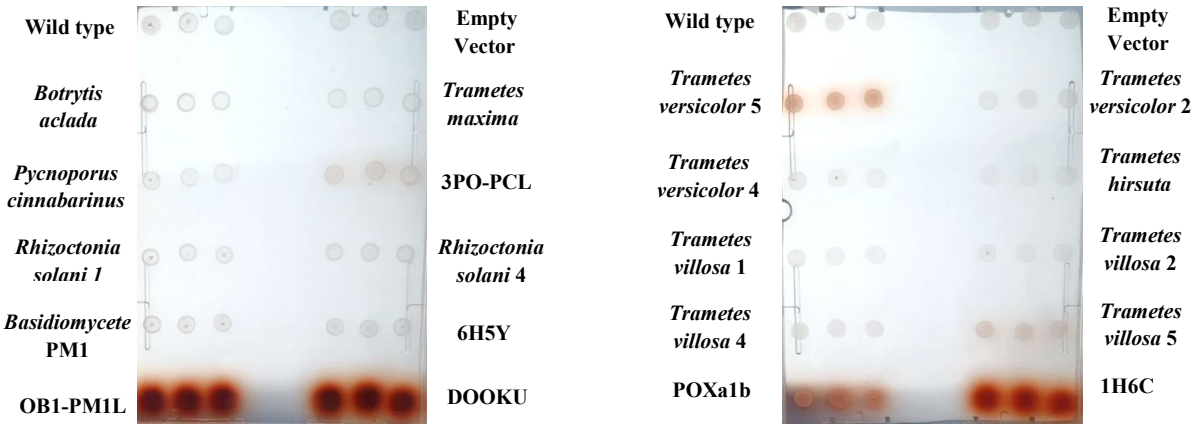

Figure 5. Enzyme activity plate assay of *P. pastoris* expressing selected laccases. *P. pastoris* over-expressing selected laccase genes, along with empty vector pFAi2 and GS115 strain, grown on Buffered Minimal Media (BMM) agar plates. Oxidation of guaiacol by laccases, as indicated via browning on the agar plates, is shown. The assay was performed in technical triplicates.

Laccase activity and free radical generation upon laccase-catalyzed oxidation of mediators

Supernatant fractions of *P. pastoris* transformant cultures was incubated with three laccase-specific substrates, namely, guaiacol, 2,6-Dimethoxyphenol (2,6-DMP or Syringol) or 2,2'-azino-bis(3-

ethylbenzothiazoline-6-sulfonic acid) (ABTS) and analyzed spectrophotometrically. Enzyme activities and specific enzyme activities were calculated for each laccase towards the substrates using the following equation:

$$\mathrm{U\,L^{-1}} = \frac{(\Delta A)(V_t)(D_f)(10^6)}{(t)(\varepsilon)(d)(V_s)}$$

where U L^{-1} = Enzyme activity (μmol min^{-1} L^{-1}), ΔA = Change in absorbance, V_t = Total reaction volume (mL), D_f = Dilution factor, 10^6 = Correction factor (μmol mol^{-1}), t = Reaction time (min), ε = Molar extinction coefficient (M^{-1} cm^{-1}), d = Optical path length (1 cm) and V_s = Sample volume (mL).

Against the three laccase-specific substrates, POXa1b and 1H6C showed the highest enzyme and specific enzyme activities (Figure 6). Overall, the results mostly coincided with that from the plate assay although some laccases, such as *Trametes villosa* laccase-1 and 3PO-PCL, showed relatively high enzyme and specific enzyme activities towards guaiacol (Figure 6c, 6d) despite no observable results on the guaiacol plate assay. The laccases with high oxidative potential showed high enzyme and specific enzyme activities across all three substrates. Contrastingly, the negative controls and low performing laccases showed almost negligible activity towards all three substrates compared to control.

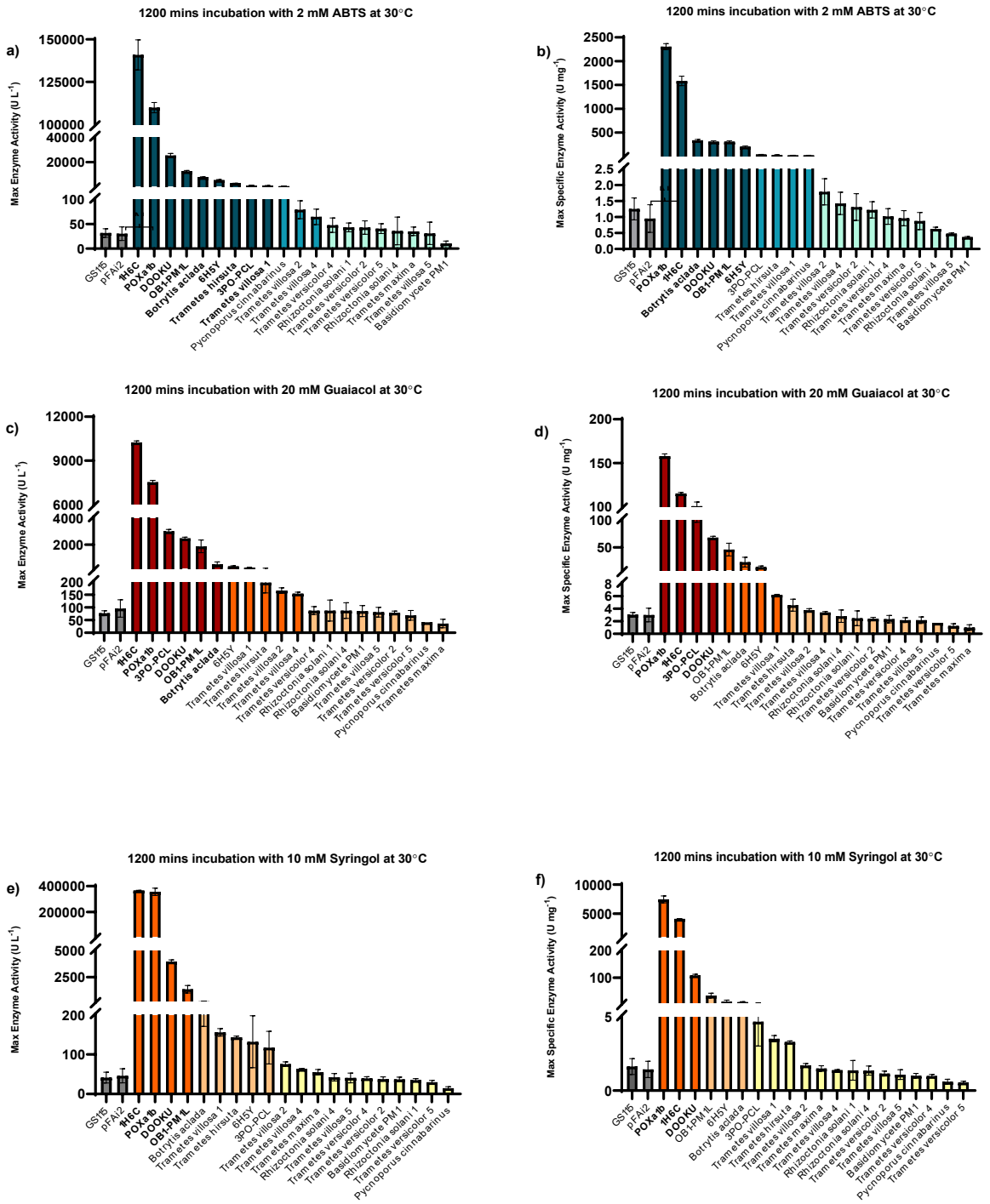

Figure 6. Enzyme activity in culture supernatants of *P. pastoris* transformants expressing selected laccases. Enzyme activities (U L^{-1}) and specific enzyme activities (U mg^{-1}) of selected laccases were tested against (a, b) 2 mM ABTS, (c, d) 20 mM Guaiacol and (e, f) 10 mM Syringol over 1200 minutes, respectively. *P. pastoris* strain GS115 and transformant containing the empty vector pFAi2 were used as negative controls. Specific Enzyme activities (U mg^{-1}) were calculated by dividing enzyme activities with concentration of total secreted proteins determined by Bradford assay. The assay was performed with technical triplicates.

As part of the effort to characterize the recombinant laccases, their relative redox potentials were determined using redox titration with potassium hexacyanoferrate. The absorbance readings were fitted to a Nernst curve (Figure 7) and the midpoint potential calculated using the following equation:

$$Y = \frac{A}{1 + e^{\frac{nF}{RT}(Em-E)}} + B$$

with n = 1, F =96486 C mol^{-1}, R = 8.314 J mol^{-1} K^{-1}, T =293K and E_m =Midpoint potential.

A + 18.3 mV difference in relative redox potentials was shown between POXa1b and *Trametes versicolor* laccase (Table 3). Work is in progress to measure the relative midpoint potentials of the other laccases with varying enzyme activities. To verify the rigor of the current relative redox potential measurement method, we are also exploring alternative assays, such as the cyclic voltametric method, to obtain absolute, rather than relative, redox potentials.

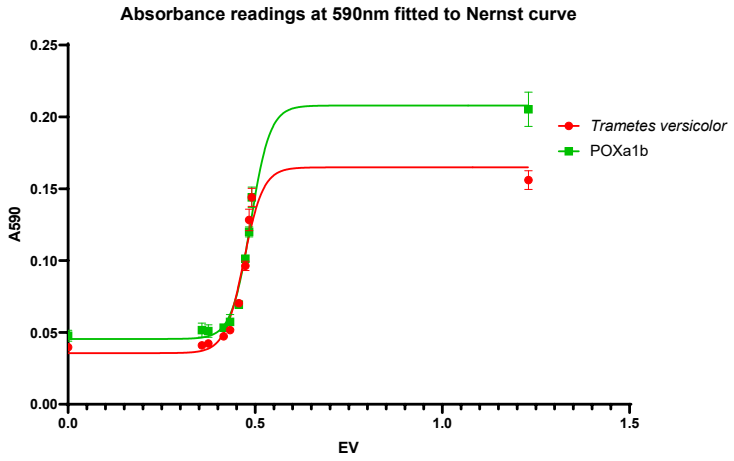

Figure 7. Relative redox potential measurement of laccases through absorbance change fitted to the Nernst equation. Absorbance changes were measured at 590 nm corresponding to the reduction of the T1 copper of laccases. The relative redox potential of recombinant POXa1b expressed from *P. pastoris* was compared against commercial *Trametes versicolor* laccase.

Table 3. Relative midpoint potential of laccases after Nernst curve fit.

Laccase	E_m (V)
Commercial *Trametes versicolor* laccase	0.4699
Recombinant POXalb	0.4882

II. Expression, purification and characterization of candidate enzymes using ion-selective electrode (ISE)-based enzymatic assay and ion-chromatography mass spectrometry

A trial study was conducted to investigate the biocatalytic activity of the recombinant laccases against PFOA. POXalb-expressing *P. pastoris* were incubated with 1 µM PFOA with synthetic mediators, guaiacol, ABTS, syringol and 1-HBT, added to the culture weekly. At the end of one month, the supernatant was retrieved and analyzed for PFOA using liquid chromatography-mass spectrometry (LC-MS). Among the mediators, 1-HBT-laccase catalysed oxidation showed potential PFOA degradation after 1-mth incubation when compared against the no-cell control (Figure 8). However, reduction in PFOA levels was also observed in the samples without mediator, albeit with high variability across replicates. Further optimization of the LC-MS method for PFOA and PFOS is in progress to improve analytical reproducibility and the study is repeated for other recombinant laccases over a longer period of incubation.

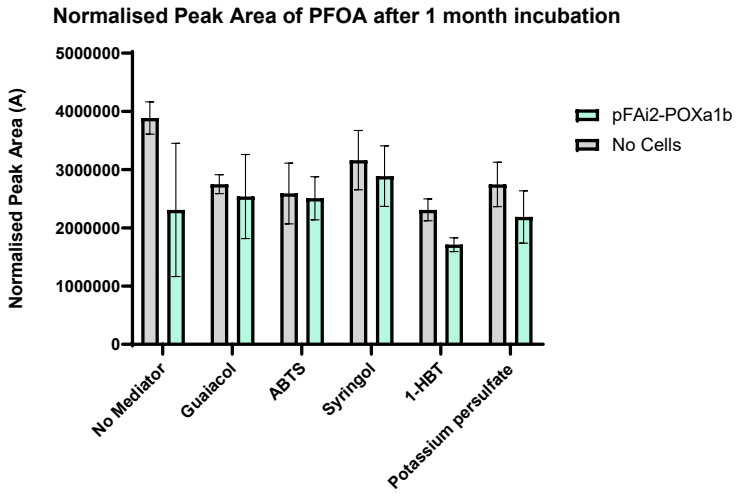

Figure 8. Analysis of PFOA degradation after 1-month incubation with POXa1b expressing *P. pastoris.* Peak areas of PFOA were determined by Liquid Chromatography-Mass Spectrometry (LC-MS) and normalized against culture volume.

How were the results disseminated to communities of interest? If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest for this reporting period. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Results from this research was shared with the scientific community at the Singapore Catalysis Forum 2022 held in Singapore on May 26. The poster presentation—Heterologous production of fungal laccases for radical generation—generated questions on the potential use of enzymes for environmental remediation and added to the growing conversations on emerging pollutants in our environment, particularly in the waters.

III. Engineering enzymes and accompanying pathways into the freshwater chassis *Chlamydomonas reinhardtii*

To engineer the freshwater, unicellular algae *Chlamydomonas reinhardtii* for green biotechnological application, we used a Modular Cloning (MoClo) toolkit composed of more than 60 genetic elements (eg. promoters, UTRs, targeting peptides, purification tags and reporter genes) codon-optimised for *C. reinhardtii*. Based on the Golden Gate approach, the toolkit offers high modularity and allows permutations of multiple genetic parts for iterative design and testing [6]. The deployment of microalgal platform for environmental bioremediation necessitates that the AFFF-detoxifying enzymes (ADE) are targeted for extracellular secretion. For this reason, heterologous expression of the genes of interest were directed to the nuclear genome, instead of the chloroplast genome, even though transgene expression is known to be higher in the latter.

In a bid to enhance nuclear transgenes expression and secretion of recombinant enzymes in microalgae, several genetic strategies were deployed in the expression vector design. Firstly, all heterologous genes of interest had to be codon-optimized for *C. reinhardtii* as the nuclear genome of *C. reinhardtii* has an elevated overall GC content of nearly 65%, as well as narrow codon bias. As codon usage bias of genes is known to be positively correlated with gene expression levels, the selection for a preferred set of codons can enhance translational efficiency in recombinant genes. The second strategy involves using transcriptionally fused transgene design to circumvent gene silencing. The Ble resistance protein (BleR) confers resistance to DNA double-strand breaking by the bleomycin antibiotic through binding and sequestration such that only cells expressing relatively high levels of Ble protein survive zeocin selection. As the recombinant gene-of-interest is transcriptionally fused to ble in the same open reading frame, selection of high ble expression effects a corresponding high transgene expression. The resulting heterologous protein is then cleaved from the fusion protein via the foot-and-mouth-disease-virus (FMDV) 2A self-cleavage peptide (Figure 9). To design for practical deployment of this microalgal platform for environmental bioremediation, the AFFF-detoxifying enzymes must be targeted for extracellular secretion. Here, an endogenous secretion signal sequence from *C. reinhardtii* gametolysin was utilized to drive extracellular secretion.

Relative to other unicellular eukaryotes and land plants, the nuclear genome of *C. reinhardtii* has a high percentage (88%) of endogenous introns. To facilitate innate splicing efficiency, endogenous introns of *C. reinhardtii* Rubisco small subunit 2 (rbcS2-i1, i2, i3) was systematically inserted at optimal insertion sites (…NG/GN…) throughout the codon-optimized coding sequences at exon lengths of ~500 bp as a strategy to enhance transgene expression. For a comparative study on expression efficiency, we varied the number of endogenous intronic sequences integrated (Figure 9).

Figure 9. Schematic diagram of *Chlamydomonas* heterologous gene expression cassette (pCMH). (A) Transcriptional fusion of the recombinant gene-of-interest to bleomycin resistance gene. The resulting heterologous protein is cleaved from the fusion protein via the FMDV 2A self-cleavage peptide and targeted for extracellular secretion through the endogenous secretion signal. Inclusion of a histidine tag allows for protein purification. (B, C) Targeted insertion of the introns of *C. reinhardtii* Rubisco small subunit 2 (rbcS2-i1, i2, i3) throughout the transgene sequences. PPSAD+5'UTR; TPSAD – promoter+5'untranslated region and terminator of *C. reinhardtii* PsaD gene, GL – secretion signal sequence from *C. reinhardtii* gametolysin, His – octa-histidine purification tag.

Laccases can be broadly categorised into classes 1, 2 and 3 in order of increasing redox potential. As such, the microalgae *C. reinhardtii*, was engineered for the heterologous expression of five Class 3 HRP wild-type and evolved laccases from *Trametes versicolor*, *Pycnoporus cinnabarinus*, *Pleurotus ostreatus* and basidiomycetes PM1 (Table 4). Of these, four were variants modified through directed evolution to improve their catalytic efficiency and/or thermostability in previous studies [1–4]. To screen for stable transgene integration, positive transformants had to be passaged on selective media for several generations. To perform this at greater ease, we utilized the automated platforms available at SynCTI NUS BioFoundry (Figure 10). As transgene expression levels in *C. reinhardtii* are known to be variable across populations, hundreds of individual clones were maintained before they were screened for functional enzyme activity.

Table 4. Heterologous laccases expressed in *C. reinhardtii*.

Organism	**Wild-type gene**	**Evolved gene**	**Number of intronic sequences**
Trametes versicolor	lcc2	-	*i*0, *i*3
Pleurotus ostreatus	POXA1b	**1H6C**	*i*2
Pycnoporus cinnabarinus	PcL	**3PO**	*i*3
Basidiomycete PM1	PM1 lacc	**7D5**	*i*3
Basidiomycete PM1	PM1 lacc	**Dooku**	*i*2

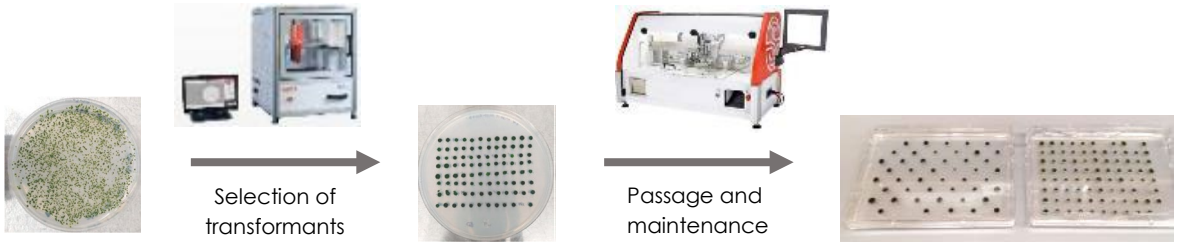

Figure 10. Automation-assisted screening of transformed *Chlamydomonas*. Positive transformants were selected using the Singer PIXL, which automates imaging, colony recognition, colony selection, and picking from culture plates. Transformants were continually passaged on culture media containing 10 mg/L zeocin using the Singer RoToR HDA pinning robot designed for rapid picking and re-plating of microorganism colonies. Photos: Singer Instruments.

Screening of transformants for laccase activity was performed using the ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) colorimetric assay, which is based on ABTS cation radical formation catalyzed by laccase. The ABTS cation radical exhibits a change of color from slightly yellow to an intense turquoise color, making the assay a convenient method for mass screening for laccase activity. Individual clones were inoculated in liquid culture, grown over two weeks and centrifuged to retrieve the supernatant. The extracellular culture fluid was then applied unto microtiter plates containing 0.5% agarose supplemented with 2 mM ABTS and incubated at 37 °C for 24–72 h until color development. Of all the positive transformants examined, none of those harbouring the heterologous lcc2, with (*i*3) or without (*i*0) intronic spacers, showed detectable laccase activity. This is likely due to the fact that lcc2 is the only wild-type enzyme, while the rest are evolved laccases with enhanced activity. The strongest laccase activity was found in 1H6C- and Dooku-expressing transformants, with moderate color development at just 3 h of incubation. Recombinant 3PO gave significantly fewer transformed colonies and exhibited the weakest activity even after 24 h incubation. Part of the results of the screening assay is shown in Figure 11.

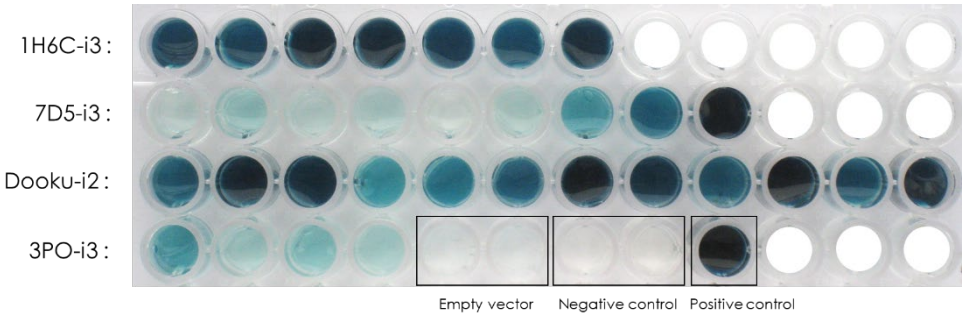

Figure 11. ABTS screening assay for laccase activity in *Chlamydomonas* transformants. Extracellular culture fluids of *C. reinhardtii* cells transformed with pCMH harbouring recombinant laccases, 1H6C-i3, 7D5-i3, Dooku-i2 and 3PO-i3, was applied unto 0.5% agarose containing 2 mM ABTS. Negative controls include culture media only and transformants carrying the empty vector pCMH. A commercial laccase from *Trametes versicolor* was used as positive control.

Work is in progress to test the best performing transformants for biocatalytic activity on PFOA and PFOS. The recombinant microalgae are incubated in media containing 1 uM PFOA or PFOS supplemented with a mediator, 1-HBT (1-hydroxybenzotriazole), required for free radical generation. Due to the expected chemical recalcitrance of PFAS, a very long period of incubation (i.e., weeks or months) would likely be required to facilitate biodegradation. Efficiency of PFOA/PFOS degradation by the recombinant laccases will be analyzed through changes in the PFOA-PFOS concentrations, as well as the by-product formation of partially fluorinated compounds and fluoride.

IV. Identification of compatible biosorption flora for possible co-deployment with engineered enzymes and freshwater chassis

Degradation of PFOA and PFOS through laccase-mediated ECOHRs results in the formation of partially fluorinated compounds and fluoride [7]. While complete decomposition of the toxic perfluorinated parent compound into its constituent, i.e., carbon dioxide, may not be attainable using a biological approach, the partially fluorinated products, as well as shorter chain-length compounds, are believed to be more environmentally benign, less bioaccumulative and may be more amenable to further degradation [8]. Although fluoride is naturally present in soil and water and is considered an essential microelement for human well-being, excessive levels as a result of geogenic and anthropogenic activities can lead to detrimental effects on ecological and human health. Hence it is relevant to explore possible approaches for cleanup of microbiologically-released fluoride to mitigate the risk of fluoride pollution at the site for in situ bioremediation of PFAAs.

Conventional processes for the removal of fluorides from water, such as chemical precipitation, ion exchange, membrane filtration and electrodialysis, often involve high operational and maintenance costs. Of these, sorption using activated carbon/alumina, synthetic materials and biomass-based adsorbents have been widely reported to be an effective and more economical defluorination technique [9]. The adsorption method, however, requires complex technicalities and generates sludge waste in the treatment process. Phytoremediation offers a promising alternative as a low-cost and eco-friendly strategy for decontaminating fluoride in aqueous environments through the use of green plants which have high tolerance, translocation efficiency, and bioconcentration factor towards fluoride ions. Plants that are tolerant and resistant to fluoride have been extensively reviewed and investigated for fluoride remediation from contaminated environment [10–12]. Although certain crops like rice and sugarcane, and vegetables such as cabbages and spinach, are reported to bioaccumulate fluoride, edible plants are ill-suited for remediative application as they pose a food risk. Instead, ornamental hydrophytes or aquatic plants that have been shown to be fluoride hyperaccumulators are most applicable for phytoremediation as they are often easy to cultivate and confer aesthetic pleasure [13–15] (Figure 12). Aquatic plants in particular have a high propensity for accumulating soluble fluorides and can be easily utilized to remove fluoride from contaminated water bodies.

Figure 12. Potential plants for use in bioremediation of fluoride. (A) Indian shot, *Canna indica*, (B) umbrella palm, *Cyperus alternifolius*, (C) nut sedge, *Cyperus rotundus*, (D) money plant, *Epipremnum aureum*, (E) water hyacinth, *Eichhornia crassipes*, (F) water lettuce, *Pistia stratiotes*, (G) hornwort, *Ceratophyllum demersum*, and

(G) water thyme, *Hydrilla verticillate*. The left panel comprises hydrophytes, or terrestrial plants adapted to living in aquatic environment, while the right panel features floating (E, F) and submerged (G, H) aquatic plants. Photos: Online sources

The potential use of flora for attenuation of fluoride contamination invites the application of constructed wetlands in the treatment design. Constructed wetlands or smaller-scale bioretention systems utilize the natural processes involving wetland vegetation, soils or substrates, and their associated microbial assemblages to assist in treating surface runoffs or wastewaters. These green infrastructures have a growing presence in urban landscapes as they serve the dual purpose of pollutant reduction and aesthetics. A pilot-scale hybrid system involving an absorption component and a vertical-flow constructed wetland have been reported to show promising results in the removal of fluoride and arsenic from wastewater [16].

Impact

Development of the principal discipline(s) of the project

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that a lay audience can understand (e.g., Scientific American style).

The discovery of microorganisms with the ability to defluorinate perfluorinated compounds ushered in the proposition of a biological approach to mitigating PFAS contamination. While it remains a nascent quest, a bio-based alternative to the breakdown of PFASs presents an attractive strategy for environmental remediation as conventional chemical treatment processes can be infrastructure-intensive and cost-prohibitive. Enzyme catalyzed oxidative humification reactions (ECOHRs) via a laccase-mediator mechanism have been demonstrated to induce PFOA-PFOS degradation in water and soil systems in previous studies, albeit at limited effectiveness and requiring extensive periods of incubation. Hence, in order to tap onto ECOHRs as a mechanism for remediation of PFAs in the environment, it is imperative that the enzyme catalysts and/or mediators have to be engineered to improve degradation capabilities. The study of recombinant laccases and their characterization allows for better knowledge on their catalytic activity towards PFASs and lead the way for the use of synthetic biology to engineer the enzymes for improved target specificity and/or degradation efficiency by rational design. In addition, the ability to express functionally active laccases in microalgae is a step towards practical deployment of engineered microorganisms for remediating high-risk or polluted sites.

Describe the impact in this reporting period on the development of human resources

For example, how has the project provided opportunities for research and teaching in the relevant fields; improved the performance, skills, or attitudes of members of underrepresented groups that will improve their access to or retention in research, teaching, or other related professions; provided scholarships; provided exposure to science and technology for practitioners, teachers, young people, or other members of the public?

The project has been undertaken by a postgraduate student as part of his research towards a doctoral degree. The research offers the student extensive learning opportunities in the areas of genetics, biochemistry and enzymology as well as to acquire skill sets in molecular, analytical and computer-aided techniques.

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Declaration by PI

I declare that all of the above information provided is true and complete to the best of my knowledge.

Name **Wen Shan YEW**

Signature 

Date **June 17 2022**