Development of novel microalgal and bacterial based production platforms

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A thesis submitted for the degree of Doctor of Philosophy in Biochemistry

University of Kent
Department of Natural Sciences
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Declaration

The work presented in this thesis is original, and was conducted myself (unless stated otherwise) under the supervision of Professor Colin Robinson. All sources of information have been acknowledged by means of references. None of this work has been used in any previous application for a degree.

I would like to thank the following people for their help in carrying out experiments and providing constructs used in this study:

Dr. Patai Charoonnart for undertaking and guiding me with the dsRNA shrimp challenge trials which were carried out at CENTEX Shrimp Centre, Mahidol University, within Dr Vanvimon Saksmerprome's lab, Bangkok, Thailand.

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Covid-19 Statement

This project was started on 1st March 2020 and finished 28th April 2023. After three weeks of starting the project, the Covid-19 lockdown started and the university was under full closure from: 24th March-1st July 2020. Upon reopening, part time work due to laboratory capacity, was allowed (3 days on 3 days off), this took place between 1st July to the beginning of October. There was also a second full closure in January 2021. This totalled to around 4-5 months off fully then around 4 months of working part time. This had an impact on what work could be carried out as well as causing disruptions to international collaborations. As both of the key collaborators on this project were based in SE Asia, there were numerous disruptions to the work and thus smaller projects were conducted alongside the main project.

Acknowledgements

Firstly, I would like to thank my supervisor, Colin Robinson for welcoming me into your lab as your final PhD student. Thank you for being a great supervisor to me throughout my PhD, providing guidance, support and fuelling coffee time with Colin the Caterpillar cakes. Despite only having three weeks in the lab before the start of lockdown and carrying out my PhD through the COVID-19 pandemic, you still pushed me and managed to get me out to Thailand for two great research trips in Bangkok.

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Abbreviations

°C	Degrees Celcius
Δ	Gene deletion
µl	Microlitre
µM	Micromolar
APS	Ammonium persulphate
DNA	Deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
DTT	1,4-Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
HSM	High salt medium
IMAC	Immobilized metal affinity chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kb	Kilo base
kDa	Kilo Dalton
LB	Luria broth
MQ	Milli-Q
OD_{600}/OD_{750}	Optical density at either 600 nm or 750 nm
PAGE	Polyacrylamide gel electrophoresis
PBST	Phosphate-buffered saline, 0.1% tween 20
PCR	Polymerase chain reaction
PGLB	Protein gel loading buffer
qPCR	Quantitative polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
dsRNA	Double stranded ribonucleic acid
SDS	Sodium dodecyl sulphate
SM6	Synthetic medium 6
TAP	Tris-acetate-phosphate
TB	Terrific broth
TBST	Tris-buffered saline, 0.1% tween 20
TEMED	Tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
Tween20	Polyoxyethylenesorbitan monolaurate
v/v	Volume per volume
w/w	Weight per weight
WT	Wild type

Abstract

The use of expression platforms is a critical aspect of biotechnology, enabling the production of proteins, enzymes and other molecules by introducing a gene of interest into a chosen expression host. Commonly used expression systems include bacterial, yeast, mammalian, plant and algal. Here we focus on the use of bacterial and microalgal expression systems specifically for the production of both proteins and double-stranded RNA (dsRNA).

The initial focus of this research was to establish a novel, microalgal-based, cost effective approach for shrimp disease control in South East Asia. This project utilised the microalgae *C. reinhardtii*, engineered to express two dsRNAs targeting the shrimp pathogen White Spot Syndrome Virus (WSSV), which was then incorporated into the shrimp feed. This feed was tested for its effectiveness in WSSV challenge trials. The results from these trials were inconclusive regarding its effectiveness as they showed no clear benefit of feeding WSSV specific dsRNA over the control dsRNA. However, the utilisation of this technology presents promising avenues for further exploration and development as it does not require downstream processing and purification prior to feeding. The second chapter aimed to express antibodies in *C. reinhardtii* to be used as an effective management tool for *Vibrio* control in shrimp. Expression and purification were achieved, with further testing required to measure its effectiveness in shrimp as a whole-cell feed. The third chapter aimed to express a fragment fusion protein in *E. coli* for use as a subunit vaccine in grouper fish for *V. parahaemolyticus* control. This fusion protein was used to vaccinate grouper fish and results highlighted it as a promising candidate for future experiments. Finally, the expression of the plastic degrading enzyme; PETase was successfully achieved in *E. coli* at both shake flask and batch fed fermentation scales. The protein was also successful purified and activity was confirmed through PET breakdown analysis using HPLC, demonstrating its efficacy in degrading polyethylene terephthalate (PET).

1 Introduction

1.1 Introduction to aquaculture

Aquaculture is the cultivation of fish and crustaceans from marine and inland waters under controlled or semi-natural conditions (Subasinghe, Soto and Jia 2009). Fish is a nutrient dense food, high in long chain omega-3 fatty acids and high in protein, therefore a key candidate for a healthy balanced diet across the world. The presence of long chain omega-3 fatty acids within the human diet is effective at preventing cardiovascular problems (Delgado-Lista *et al.* 2012). Other benefits of fish for human health include anti-oxidation, anti-inflammation, wound healing, and neuro protection (Chen *et al.* 2022). Fish proteins including immunoglobins act as defence against bacterial and viral infections. The aquaculture industry plays a key role in feeding the growing population and thus has led to a sharp increase in aquaculture production, as seen in figure 1, taken from the Food and Agriculture Organization (FAO 2020), which shows the dramatic increase of production between 1991 and 2020. It is predicted around 60% of people from developing countries depend on fish for over 30% of their animal protein (Sujatha, Anitha Joice and Senthilkumaar 2013). The aquaculture industry not only provides food security for a growing population but also provides jobs worldwide, consequently enhancing the economy particularly in developing countries.

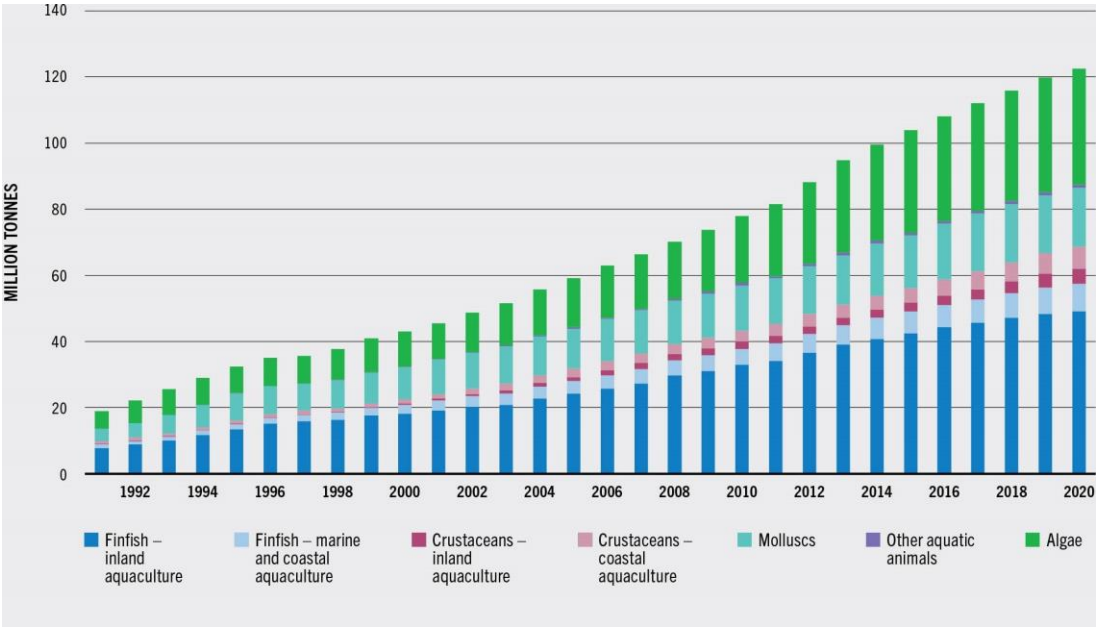

Figure 1. Aquaculture production between 1991 and 2020 (FAO 2020)
The millions of tons of aquaculture products produced per year between 1992 up to 2020.

Figure 1 highlights the sharp increase in aquaculture production over the past 30 years, as it is the fastest growing sector of the food industry (Fiorella *et al.* 2021), however with a growing industry there are increased areas for problems such as disease and economic loss.

1.1.1 Problems facing the aquaculture industry and current disease prevention methods

Aquaculture is currently the fastest growing food production sector and this provides many benefits such as economic development with particular impact on developing countries where traditional farming practices cannot sustain the increased population demand. Other benefits include a reduction in damage and pressure to wild fisheries which are often the cause for habitat destruction and overfishing, as well as minimising environmental impacts in comparison to traditional fishing methods.

The increased demand for food production has led to intensified practices within farming such as intensive aquaculture; the cultivation of aquatic species outside of their natural

ecosystem for example within a land based fishery. Intensive aquaculture farming means an increased risk of diseases due to the increased number of aquatic organisms within a smaller environment in comparison to wild fishing. The crowded conditions of the farm environment facilitates the spread of aquatic pathogens and disease which often has significant impacts on food production as it is suggested in farmed environments fish are farmed in densities over 1000 times higher in comparison to their natural environment (Pulkkinen *et al.* 2010).

1.1.2 Major shrimp diseases affecting the aquaculture industry; White Spot Disease and Acute Hepatopancreatic Necrosis Disease

This study focuses on collaboration with countries around the world with high production numbers within aquaculture with particular focus on shrimp farming in South East Asia. Asia is the largest producing region within aquaculture between 1990-2020 (FAO 2020). Figure 2 shows the world's major producers of crustaceans, one of the groups we are most focused on here.

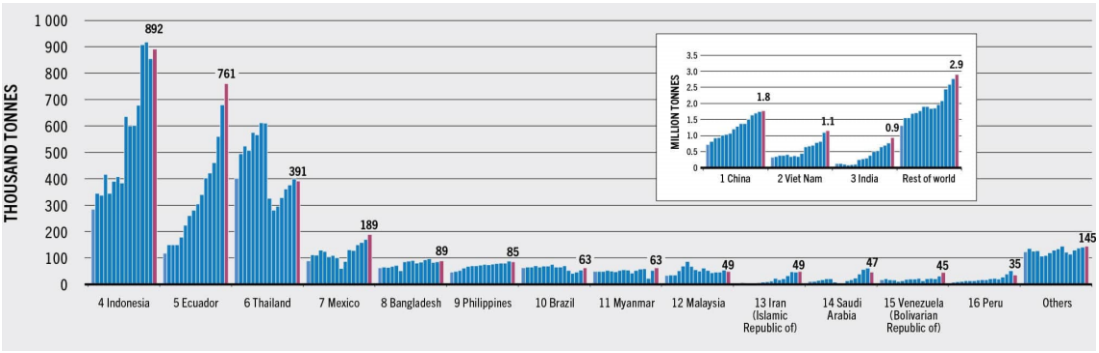

Figure 2. Worldwide marine and coastal aquaculture production of crustaceans by major producers (Food and Agriculture Organization (FAO), 2020).
Total amounts of marine and coastal aquaculture production of crustaceans over the world based on country.

Shrimp is the most traded aquaculture product in the world (WWF 2023). High demand around the world has resulted in high levels of intense farming of shrimp however disease poses a significant risk to farmers. Unlike vertebrates, shrimp rely heavily on their innate immune systems in defence against pathogens (Roy *et al.* 2020). Other mechanisms of

defence are restricted to physical defences such as the exoskeleton. The innate immune system relies on pattern recognition receptors (PRR) found on the haemocyte membrane which then activate the haemocytes to destroy the pathogen (Liu *et al.* 2006). Research into these defence mechanisms is key to understanding the response and using this information to develop effective disease management within the aquaculture industry (Aguirre-Guzman *et al.* 2009).

White Spot Disease (WSD) is a lethal viral infection caused by the White Spot Syndrome Virus (WSSV), affecting shrimp and other crustaceans. The disease is characterised by the white spots on the carapace and appendages of the body of the shrimp (Chou *et al.* 1995), as seen on the white leg shrimp *Litopenaeus vannamei (L. vannamei)* (figure 3).

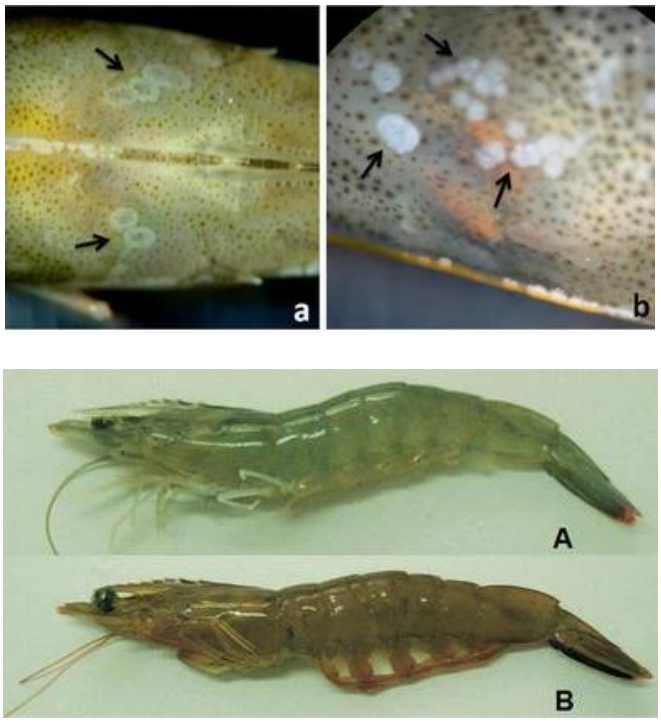

Figure 3. *L. vannamei* juveniles showing white spots
The upper images a and b show the white spots on the cephalothorax. The lower images A and B show the comparison of the WSSV-free shrimp (A) to the reddish body colouration of the WSSV infected shrimp. Image taken and modified from (Ramos-Carreño *et al.* 2014).

Other symptoms of WSSV in shrimp include lethargy, reduction in food consumption and a reddish-brown discolouration on the body (Sánchez-Paz 2010). The virus was first detected in 1992 in Asian shrimp farms and has since been reported worldwide, however there is currently no practical treatment of this disease (Feng *et al.* 2017). Routes of transmission include both horizontal transmission through ingestion of infected shrimp and through exposure of water-borne WSSV, as well as vertical transmission from the mother to offspring (Desrina *et al.* 2022). The WSSV is an enveloped virus with a 305 kbp double stranded circular DNA genome (Liu *et al.* 2006) and a member of the viral family *Nimaviridae*. Global shrimp production for 2023 was expected to be around 5.6 million metric tons, which is predicted to increase by 4.8% in 2024 (FAO 2023). However, diseases such as WSSV present a huge economic loss for fish and aquaculture farmers all over the world due to its high mortality rate of up to 100 % within ten days of infection in cultured shrimp (Liu *et al.* 2006). As of 2010, over 93 species of arthropods were reported as either hosts or carriers of WSSV (Sánchez-Paz 2010). The spread of this virus highlights the need for an economical treatment or prevention of viral spread.

Another disease of importance for this study is Acute Hepatopancreatic Necrosis Disease (AHPND), commonly referred to as Early Mortality Syndrome (EMS). This is a highly contagious bacterial disease which causes significant damage to shrimp farming businesses. The pathogen targets two main species of shrimp; the Pacific white shrimp (*L. vannamei*) and the black tiger shrimp (*Penaeus monodon*). The causative agent of AHPND is *Vibrio parahaemolyticus (V. parahaemolyticus),* a Gram-negative rod-shaped bacterium. This disease has cause serious global economic losses since its first outbreak in China in 2009. Figure 4 shows the global impact of AHPND. Since the initial outbreak the economic losses from AHPND have amounted to over 7 billion USD annually (FAO 2020).

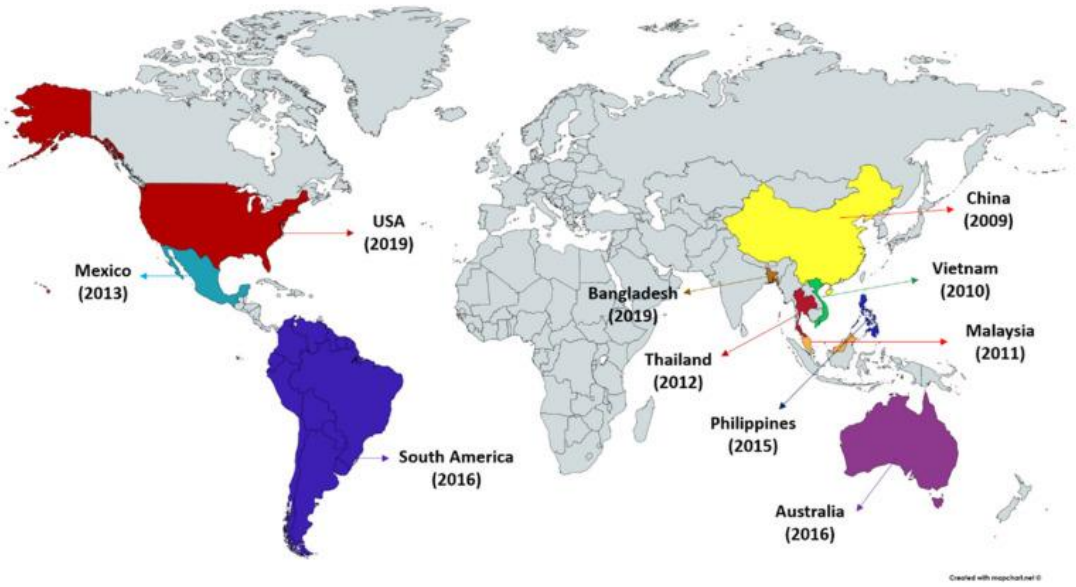

Figure 4. Occurrence of acuate hepatopancreatic necrosis disease (AHPND) in shrimp
Image taken from (Kumar *et al.* 2021) showing total coverage of countries where AHPND has been identified and dates it was first identified.

The bacterium contains a 70-kbp plasmid, named pAV1, which encodes toxins essential for disease pathogenesis (Lee *et al.* 2015). It has also been reported that other strains of Vibrio are also causative agents of AHPND in countries such as China and Northern Vietnam (Xiao *et al.* 2017), therefore highlighting a need for research into the diversity of the toxins genes of Vibriosis. The presence of these toxins encoded by the 70-kbp plasmid have been identified in all AHPND causing Vibrio (Han *et al.* 2015). AHPND is characterised by acute necrosis, cell death of the hepatopancreas which is a vital digestive organ within shrimp. The primary diagnostic method for AHPND is the identification of clinical signs which include a pale and shrunken hepatopancreas (HP) and gut emptying as seen in figure 5. Other symptoms of AHPND include lethargy and reduced weight and size, a large concern for the farming industry.

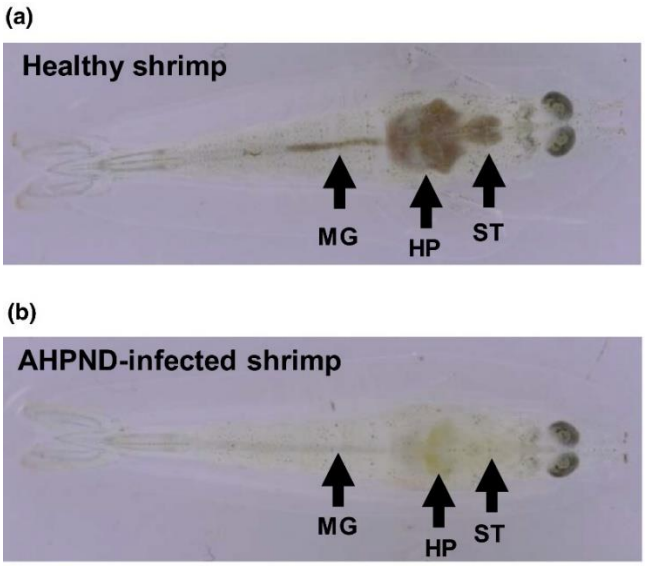

Figure 5. Gross clinical signs of acute hepatopancreatic necrosis disease (AHPND) infection in *L. vannamei*
The normally brown midgut (MG), hepatopancreas (HP) and stomach (ST) that as seen in (a) healthy shrimp all turn pale in (b) AHPND-infected shrimp. Image taken from (Kumar, Ng and Wang 2020)

The disease is often referred to as Early Mortality Syndrome (EMS) as the mortality rate for AHPND is up to 100% within 30-35 days of stocking (de la Peña *et al.* 2015). Due to the lack of an adaptive immune system, shrimp cannot combat diseases such as AHPND (Santos *et al.* 2020), and current prevention and control methods include the quarantine of shrimp prior to stocking and control of water conditions. The toxins of the pAV1 plasmid are of particular interest for current research to discover possible means of prevention and management of the disease.

1.1.3 Introduction to the fish disease Vibriosis

Other marine fish of economic importance are studied here such as grouper fish. One of the key bacterial pathogens posing significant risk to the fish farming industry is another *Vibrio* bacterium which causes fatal vibriosis; *Vibrio harveyi* (*V. harveyi*). This bacterium is a common cause of disease outbreaks in various marine and fresh water fish species, it is a Gram negative rod shaped bacterium which can thrive in a range of environments.

The current disease management strategies for controlling Vibrosis include the use of antibiotics, water quality control and increased biosecurity. *V. harveyi* has an extensive list of hosts in both aquatic vertebrates and invertebrates, from shrimp to grouper fish, posing it as a significant threat to the aquaculture industry. Fish possess an immune system that is distinct from that of shrimp. It is comprised of both the innate and adaptive components. The innate system consists of physical barriers such as the skin, gills and mucus layer in addition to cellular responses such as phagocytosis. In comparison the adaptive immune system is characterised by its specificity and can provide long term immunity. The presence of the adaptive immune system provides an avenue for disease control through the use of vaccines and thus avoiding strategies such as antibiotics. Upon vaccination, the immune response is initiated and memory B cells and T cells are generated, providing long term immunity.

1.1.4 Strategies for disease prevention

Prevention and control methods have relied heavily on the use of antibiotics and chemical drugs (Du *et al.* 2022). Although these methods may appear useful, such practices increase the risk of bacterial resistance and possible risk to the surrounding environment. Within the intensive farming setting, the enhanced transmission of pathogens is thought to promote pathogen evolution (Sundberg *et al.* 2016), creating a greater risk for the survival of the farm stock as without the correct treatment there can be major losses. The use of antibiotics by farmers helps to manage and control the spread of pathogens within fisheries however in recent years the overuse of antibiotics has led to the development of resistant pathogens. Areas of marine farmland have been found to contain an increase of resistance of bacterial pathogens where large amounts of antimicrobials have been used (Buschmann *et al.* 2012). The rise of resistant pathogens poses a risk to human health through the method of horizontal gene transfer from aquaculture pathogens to human pathogens rendering them

resistant to antimicrobials as many of the antimicrobials used in aquaculture are used for human health (Heuer *et al.* 2009).

To address the issue of antimicrobial resistance within the aquaculture farming industry other strategies of disease control must be implemented with the aim of reducing antimicrobial drug use. This has led to an emphasis on moving towards a greener method of disease control via the use of methods such as vaccines and immunostimulants. Within the industry there are vaccination programmes for economically important fish such Atlantic salmon, Rainbow trout and Nile tilapia, with a total of over 50 commercially available aquaculture vaccines for both bacterial and viral diseases (Du *et al.* 2022). Due to the vast spread of diseases within the farming industry, the aquaculture vaccines market surpassed 214.6 million USD in 2022 and is forecast to increase by 7.5% by 2032 (Faizullabhoy 2023), thus showing a positive increase and area for development in the industry.

Vaccines come in a variety of different forms such as the earlier used systems such as; live-attenuated vaccines, inactivated vaccines as well as genetically engineered vaccines which include subunit vaccines and DNA and RNA based vaccines. Inactivated vaccines use a form of the pathogen that has been inactivated to reduce virulence, this was an early form of aquaculture vaccine used by Duff in 1942 against *Aeromonas salmonicida* in trout (Du *et al.* 2022). Live attenuated vaccines contain a form of the living virus which has been weakened to avoid serious disease. This form of vaccine provides a good level of protection as they can induce cell mediated and humoral antibodies providing adaptive immunity for the fish (Mohd-Aris *et al.* 2019). Benefits of this method include low manufacturing costs, and rapid delivery however there are risks associated such as spread of the pathogenic strain to surrounding environment and possible reverse mutation (Munang'andu, Mutoloki and Evensen 2015), thus leading to an increase of genetically engineered vaccines in recent years.

The development of new biotechnological approaches includes the development of recombinant vaccines and DNA vaccines. Due to the engineering process they are more stable and well defined, thereby providing a safer option. Subunit vaccines mitigate this risk of reverse mutation as they are often developed to contain only the immunogen components therefore removing the pathogenic components which would in the natural environment cause disease. An example of a commercially available subunit vaccine is that used against Infectious pancreatic necrosis in salmonids, sea bass, sea bream, turbot and pacific cod. Other vaccines for Infectious pancreatic necrosis are available in the form of inactivated vaccines and recombinant vaccines, both of which improved survival rates of rainbow trout (Tamer *et al.* 2021). Recombinant vaccines are often used in aquaculture as the gene of interest can be expressed using a using a suitable host system either eukaryotic or prokaryotic, and purified so it can then be injected. This method provides successful vaccination in large fish such as grouper, against the viral disease nervous necrosis virus (Chen, Peng and Chiou 2015). Other genetically engineered vaccines include nucleic acid vaccines which require the introduction of a plasmid modified to encode an antigen under the expression of the host, providing enhanced immune protection. This approach has demonstrated successful application for species such as Salmonids against infectious hematopoietic necrosis virus (IHNV), as there is now a commercially available DNA plasmid vaccine (Ma *et al.* 2019).

The nature of aquatic farming means there are different methods of aquatic vaccination delivery such as injection; the most widely used method industry wide (Radhakrishnan *et al.* 2023), immersion immunisation and oral immunisation methods. One of the benefits of injection vaccination is that it is a targeted method, which does not have an impact on the surrounding environment such as pathogenic resistance caused by overuse of antibiotics. Fish are often vaccinated through injection into either the abdominal cavity or the

intramuscular tissue therefore, one of the drawbacks of this method is that it is not a viable method for vaccination of juvenile fish and shrimp due to their size. This impacts the vaccination process as aquatic organisms are often vaccinated at the juvenile stage of life to avoid spread of disease as they grow, as they are most susceptible to disease at the juvenile stage. Other issues with injection arise due to the intense handling and stress for the fish during the vaccination process (Radhakrishnan *et al.* 2023). Due to these issues, other vaccination methods are often used. Immersion immunisation requires the animal to be submerged in a solution containing the vaccine at a certain concentration. This method is often a cheap and easy in practise however there are issues regarding dosage of vaccine using this method and it often only produces short term protection.

The second option for vaccine delivery is oral immunisation; this is the process of delivering the vaccine through the digestive system. The process of oral vaccination has less impacts on fish stress and handling in comparison to injection. Benefits of this method include the use of formulating the vaccine into the animal feed, therefore it can be a cheaper option for the farmer. However, a higher level of processing is required as the vaccine antigens must be protected from the digestive system to make sure they have the desired effect. This could include different methods of encapsulation to avoid degradation within the gastrointestinal environment (Radhakrishnan *et al.* 2023).

Physical methods of disease control are also used throughout the aquaculture industry such as the use of nets and screens. One of the key entry routes for pathogens into fisheries is from other animals or predators entering the farm. The use of a net above the water is a physical barrier to stop other animals or predators from entering the water thereby controlling any contamination from above. Physical barriers can also be used below the water surface, for example being used to control areas of different aquatic animals therefore

stopping the physical contact between farmed groups, and thus reducing disease spread. These are often cheaper alternatives of disease control for farmers in developing countries however they do not contain or remove any disease once it is present in the farm therefore, they are not suitable as a single method of control.

1.1.5 Introduction to dsRNA and RNAi

The use of RNA interference (RNAi) technology provides a possible solution to targeting viral diseases. RNAi is the process of post transcriptional gene silencing (PTGS) through the use of double stranded RNA (dsRNA). This process occurs naturally in organisms including plants, animals and fungi as it is vital for processes such as defences against pathogens. The process of RNAi is outlined in figure 6. RNAi is initiated through the presence of dsRNA which is processed by an enzyme called dicer which cleaves the RNA molecules into smaller RNA fragments known as small interfering RNAs (siRNAs). The siRNA is then recruited by the protein complex known as the RNA-induced silencing complex (RISC) where it then binds to the complementary sequence of the mRNA. This binding causes the degradation and blockage of the mRNA molecule, further preventing protein translation.

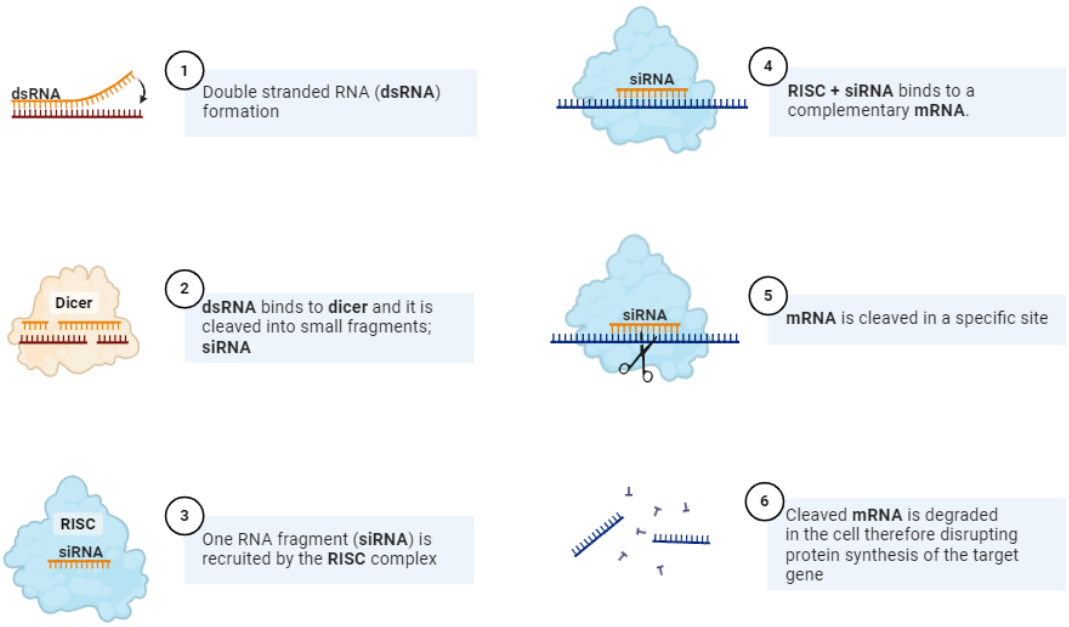

Figure 6. Outline of RNAi. Modified using BioRender
The first step in the RNAi process is the formation of dsRNA, followed by the binding of the dicer protein which cleaves the dsRNA into small interfering RNAs. The siRNA is then recruited by the RISC complex. The RISC and siRNA complex follows complementary binding to the mRNA which is then cleaved at a specific site. This cleaved mRNA is then degraded in the cell which disrupts protein synthesis of the target gene (Modified using BioRender).

This process can be used as a molecular tool as synthesised RNA has the potential to prevent translation and thus has a 'knockdown' effect on gene expression. Previous studies have proved RNAi to be a useful strategy against aquatic diseases, first applied in 2005 (Robalino *et al.* 2005) as the dsRNA can be introduced into the host organism and therefore either host or viral genes can be controlled (Itsathitphaisarn *et al.* 2017). This approach has also been successfully applied to combat Micropterus salmoides rhabdovirus (MSRV) the primary cause of mortality in the largemouth bass larvae. The use of RNAi was used to target one of the key virulence factors of the virus; the envelope glycoprotein. The success of this method demonstrates its potential to mitigate the impact of the virus by inhibiting viral replication (Yuan *et al.* 2023).

1.2 Introduction to microalgae as an expression host

Microalgae are typically unicellular, eukaryotic organisms that offer a promising technology to control diseases widespread in aquaculture. One frequently studied model organisms in this field is *Chlamydomonas reinhardtii* (*C. reinhardtii*), a unicellular green alga. *C. reinhardtii* is a motile alga with two flagella, other structures include a large chloroplast, large pyrenoid, and eyespot. *C. reinhardtii* is often used as a model organism due to its ease to cultivate and the ease of genetic manipulation (Mussgnug 2015). Growth can either be photoautotrophic using light as a sole energy source by using a simple medium containing inorganic salts, or they can be grown heterotrophically, using an acetate containing medium as an alternative carbon source. Under optimal conditions *C. reinhardtii* has a doubling time of 8 hours (Harris 2001). This alongside the ease to use either autotrophic or heterotrophic growth shows *C. reinhardtii* to be a suitable model organism within the laboratory. The manipulation of the *C. reinhardtii* genome has been well documented due to all three genomes; the nuclear, chloroplast and mitochondrial, being previously sequenced. Other advantages of microalgae as an expression system include their ability to perform post-translational modifications and their potential for large scale cultivation in photobioreactors. The use of microalgae in large bioreactors offers numerous potential applications not only across the aquaculture industry, but also in biofuel generation and pharmaceuticals.

There are two primary transformation routes for *C. reinhardtii;* chloroplast and nuclear transformation. Various methods are available for transformation; categorised as either biolistic or non-biolistic, each with its own advantages. The biolistic method describes the use of particle bombardment. This technique is versatile as it can be used for both chloroplast and nuclear transformation. In this method, the DNA is coated onto metallic particles which are then propelled to penetrate the target cells. In comparison, the non-biolistic approach encompasses techniques such as electroporation, *Agrobacterium tumefaciens*-mediated

transformation, and glass bead transformation, the latter being the method employed in this study. The glass bead transformation uses cell wall-deficient strains which are agitated in the presence of glass beads along with recombinant DNA to facilitate the transfer of genetic material.

After the transformation of *C. reinhardtii* cells, the identification of transgenic cells relies on the use of selection markers. Common selection methods include the use of antibiotics, selective media, and photosynthetic restoration techniques. As Chlamydomonas is capable of growing either photo-autotrophically or heterotrophically, the manipulation of the photosynthesis mechanism can serve as an effective selection marker. An example is the disruption and restoration of the gene, *psbH* (Cutolo *et al.* 2022), a gene involved in photosystem II (PSII) functioning. Therefore, transformed microalgae can be cultivated heterotrophically in media supplemented with acetate as a carbon source, allowing for the growth and identification of transgenic cells under non-photosynthetic conditions.

1.2.1 The use of microalgae as oral vaccines in aquaculture

Algae have gained significant attention in various fields due to their variety of beneficial components such as proteins, carbohydrates, vitamins, antioxidants, and lipids (Yaakob *et al.* 2014). This study specifically delves into harnessing the potential of microalgae for developing oral vaccines for use in aquaculture with particular focus on shrimp farming.

Shrimp lack an adaptive immune response, relying instead on innate immunity, thus posing a challenge for shrimp disease management. Both the cellular and humoral responses are vital to combat disease. The cellular response includes processes such as phagocytosis, encapsulation, and apoptosis. The humoral response involves the production of immune proteins such as cytokine-like factors, antimicrobial peptides (AMPs) and proteinase

inhibitors (Tassanakajon *et al.* 2013). This highlights the importance of developing novel techniques to enhance shrimp immunity and disease resistance.

The use of microalgae for oral vaccination is a beneficial area of biotechnology as many microalgae such as *C. reinhardtii* and *Chlorella vulgaris (C. vulgaris),* are generally recognised and safe (GRAS) (Torres-Tiji, Fields and Mayfield 2020), therefore they do not require purification and can be fed as a whole extract. Here the microalgae, *C. vulgaris*, has been used as an oral feed for WSSV prevention via recombinant protein expression. *C. vulgaris* was used to express WSSV protein VP28 (Kim *et al.* 2023). The expression of recombinant VP28 successfully reduced the mortality rate of shrimp suggesting its potential use within the aquaculture industry.

1.3 Introduction to solving the plastic pollution problem

1.3.1 The plastic pollution problem

Polyethylene terephthalate (PET) is a common type of plastic used in many single use items such as drinks bottles, plastic bags as well as items of clothing. PET is formed from the polymerisation of terephthalic acid and ethylene glycol. PETs low cost and properties, such as its strength, lightness and rigidity make it a good choice for many applications. However, the increase in demand is a risk for environmental health due to its non-degradability, resulting in a build-up in the environment. Plastic pollution arises from various sources, including incorrect disposal of plastic products, waste from plastic manufacturing and inadequate waste management. In 2021 the UK had a recycling rate of 44.6% which was only a 2.2% increase from the year prior (UK GOV 2023). This highlights the amount of waste that is not being recycled each year and is thus adding to the plastic pollution problem.

The use of PET plastic has caused a major concern over its ability to be broken down into smaller plastic particles referred to as microplastics. Microplastics are small plastic particles less than 5 mm in size, often generated from the fragmentation of larger plastic debris or the intentional manufacturing for products such as textiles. The risk to human health, occurs due to bioaccumulation within the environment. Recent studies using microplastics to evaluate the risk to gastrointestinal health has found that the presence of microplastics in the gut alter colonic microbiota which could promote biofilm formation and therefore impacts digestion health (Tamargo *et al.* 2022).

Due to the many uses of PET plastic, halting the production and use of PET products is not a viable option, however the increase of environmental contamination proposes a threat to our current ecosystems. One of the alternatives to this, is the use of a biological method of plastic reduction such as microorganisms. Biodegradation is the process of organic

substances being broken down into smaller ones by microorganisms. This is a technique which has been previously utilised and manipulated to our advantage for example the use of bacteria, fungi and other microbial strains to degrade pesticides. High levels of pesticides have been used throughout farming practices and the potential build-up of these in the environment poses a threat to the ecosystem, therefore producing a platform to degrade these pesticides using an ecological method was needed. The early methods of degradation were chemical degradation, physical degradation, and a mixture of the two which produced secondary pollution (Huang *et al.* 2018). The process of microbial degradation relies of the microorganism producing enzymes to degrade the pesticide into smaller non or less toxic molecules. This method has been exploited for other pollutants such as dyes, another compound mass produced for industries such as food, textiles and cosmetics due to their high solubility in water, dyes are hard to remove from the environment, therefore traditional methods are not successful at preventing pollution into the ecosystem. An example of this is the use of a Pseudomonas sp. SUKI which was shown to decolorised textile dyes to nontoxic products (Kalyani *et al.* 2009). The increase of plastic build up in the environment shows a significant cause for concern and the plastic pollution problem must be addressed.

1.3.2 Introduction to the plastic degrading enzyme; PETase

Biological control solutions have emerged as a promising for pollution removal, offering an environmentally friendly alternative to traditional methods. In particular, the use of microorganisms, including bacteria and enzymes, holds great potential reducing plastic wate accumulation. Microorganisms such as bacteria can be optimised to produce recombinant proteins with specific properties to target plastic degradation. Although the current methods of biodegradation of PET plastics are not yet at optimized for widespread use, there have been recent advancements. One notable discovery is the enzyme produced by *Ideonella sakaiensis* 201-F6 (*I. sakaiensis*), a bacterium isolated in 2016. This strain of Ideonellla has

demonstrated the ability to utilise PET as its sole carbon source from PET waste, making it a promising candidate for plastic degradation. The enzyme responsible for the breakdown of PET from *I. sakaiensis,* is known as PETase, which catalyses the initial step in PET degradation. *I. sakaiensis* also produces a second enzyme which together enable the bacteria to use PET as their sole carbon source (Maity *et al.* 2021). The enzyme PETase first catalyses the breakdown of PET to mostly mono(2-hydroxyethyl) terephthalic acid (MHET) with traces of terephthalic acid (TPA) and bis(2-hydroxyethyl)-TPA, see figure 7. The second enzyme produced by *I. sakaiensis*, known as MHETase, then converts MHET into the two monomers TPA and ethylene glycol (EG), (Austin *et al.* 2018).

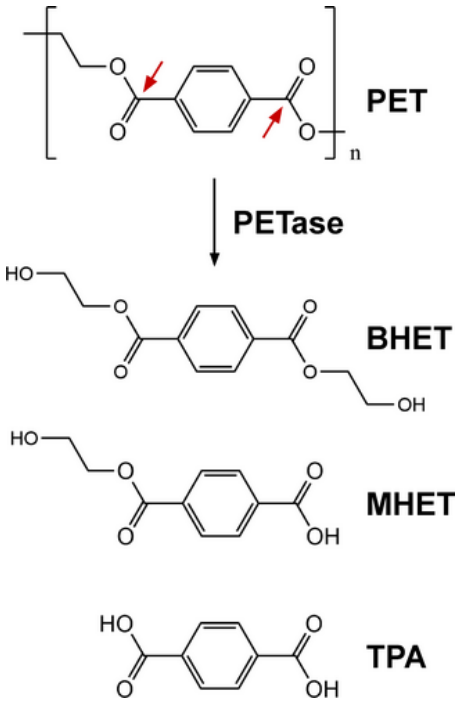

Figure 7. PET Degradation using the plastic degrading enzyme PETase.
The breakdown of PET plastic using PETase into the three breakdown products BHET, MHET and TPA. The potential PET cleavage sites are indicated by red arrows. Image from (Chen *et al.* 2018)

The discovery of this enzymatic processes shows a promising development in combating plastic pollution. The focus here is to use microorganisms as an innovative solution for bioremediation.

1.3.3 Bacterial expression systems in biotechnology

The use of bacterial systems to produce recombinant proteins is a well-established and highly researched area in biotechnology. Often chosen as the microorganism of choice is *Escherichia coli (E. coli)* due to its rapid growth rate, ease of genetic manipulation and low cost (Waegeman and Soetaert 2011). One of the key features of bacterial expression systems which make them an ideal candidate for protein production is the ease of upscale production.

The design of a bacterial plasmid for recombinant protein production is key for high levels of expression. They must contain as a minimum; an origin of replication, a selection marker then a promoter, gene of interest and terminator. The expression plasmid is then used in conjunction with secretion systems, based on the use of a signal peptide, to achieve high levels of protein expression.

There are many options for using signal peptides to guide the export of the PETase protein across the cytoplasmic membrane in bacteria. The choice of signal peptide determines the route of export, in bacteria there are two major pathways for export of proteins; the secretory pathway (Sec) which translocate proteins in their unfolded form so they can then be folded at the trans-side of the membrane, or the Twin-Arginine Translocase (Tat) pathway which translocate folded proteins across the membrane (Natale, Brüser and Driessen 2008). Signal peptides for both Tat and Sec transport, typically consist of three domains; the positively charge N-terminal region, the hydrophobic domain also known as the h-region and

the final region, C-terminal domain, known as the c-region. Together the three regions coordinate the recognition, targeting and translocation of proteins across the cellular membrane. The modification of signal peptides and help optimise protein secretion and expression levels.

1.3.4 Current export and purification of PETase

PETase was discovered as a promising candidate to combat the plastic pollution problem, however challenges have arisen when try to achieve high levels of expression. To address this issue, different signal peptides have been used in an attempt to enhance its efficiency in the organism *Bacillus subtilis* (*B. subtilis*). Researchers have tested various signal peptides; three Sec signal peptides, and two Tat signal peptides as well as the native signal peptide SP_{PETase}, in *B. subtilis* (Huang *et al.* 2018); results showed that the native SP_{PETase} gave the highest level of secretion. *I. sakaiensis* and therefore its native signal peptide, naturally secrete the enzymes into the extracellular space (Seo *et al.* 2019). Further research into the secretion pathways found that it was secreted via the Tat-independent pathway (Huang *et al.* 2018). The use of the SP_{amy} signal peptide in *B. subtilis* produced an increase in secretion of PETase in comparison to the native signal peptide SP_{PETase} when expressed alongside the P43 promotor (Wang *et al.* 2020).

The first report of active PETase exported in *E. coli* used Sec dependent signal peptides; SP_{MalE} and SP_{LamB}, in *E. coli* to achieve production of active extracellular PETase (Seo *et al.* 2019). Various different signal peptides were tested in *E. coli* and it was found that Sec specific signal peptides gave the best results and still produced a functioning enzyme after export.

The current expression systems for exporting PETase are focused on using bacterial systems, however extracellular expression of PETase has recently been reported using the microalgae

C. reinhardtii, (Kim *et al.* 2020). After three weeks of incubation of cell lysates of *C. reinhardtii* expressing PETase with PET powder at 30^{o}C, only traces of BHET were identified using a HPLC, however after four weeks, TPA was observed in the analysis indicating the full breakdown of PET. Marine microalgae has also been used to express PETase, *Phaeodactylum tricornutum* was used to produce and secrete PETase into the surrounding medium (Moog *et al.* 2019). The use of a marine microalga provides an alternative solution to clear PET polluted seawater.

1.4 Research aims

The overarching aim of this research was to develop novel microalgal based production platforms through the use of Chlamydomonas as well as the bacterial production host; *E. coli.* The first three areas of research covered in this thesis focus on the development of both microalgae and *E. coli* for use in aquaculture in the format of oral feeds and vaccines to generate a solution to the rising spread of diseases within the aquaculture industry:

1. Generate a transgenic strain of microalgae via chloroplast transformation, expressing two dsRNA cassettes to target gene silencing of the WSSV in shrimp. Once generated the microalgae expressing sequence specific dsRNA will then be tested in shrimp challenge trials carried out at CENTEX, Thailand, to test the effectiveness of feeding microalgae expressing dsRNA specific to WSSV when challenged with the virus.
2. Generate a transgenic strain of Chlamydomonas to express antibodies in the chloroplast, which target the two toxins produced by the pVA1 plasmid, the causative agent of AHPND. In collaboration with Professor Tae Sung Jung at the College of Veterinary Medicine, Gyeongsang National University in South Korea, the transgenic microalgae can then be used as an oral vaccine for shrimp.
3. Utilise *E. coli* as a host to express the fusion protein GAPDHf-OmpKf, in both small and large scale expressions. Once generated this protein can then be used as a vaccine for grouper fish against *Vibrio sp,* in collaboration with Dr Cahyo Budiman and Professor Zarina Amin at University Malaysia Sabah.

The final section of this thesis focuses on the development of a bacterial based production platform to express the plastic degrading enzyme, PETase to help combat the plastic pollution crisis. The main focus here was to improve the current export of PETase from *E. coli*

as the host organism due to the speed of protein production and purification as well as lower cost of production for future application.

4. Optimise PETase expression in *E. coli* through the use of small scale PETase expression using different cell lines and culture conditions to improve levels of PETase export, as well as larger scale expression in fed batch fermentation. Activity of the enzyme will be tested using HPLC to confirm plastic degradation.

2 Methods

2.1 Suppliers of chemicals, reagents and equipment used

All reagents, chemicals and equipment used throughout this study were supplied from the companies listed below unless stated otherwise. Centrifuge equipment was obtained from Beckman Coulter Inc (USA), Eppendorf and Thermo Fischer Scientific Inc (USA). All consumable plastic ware was obtained from Eppendorf, Thermo Fischer Scientific Inc (USA), Grenier, Starstedt, Starlabs and WVR. Oligonucleotides were obtained from IDT DNA. Gene strings were primarily obtained from GeneArt Thermo Fischer Inc (USA). DNA polymerase, restriction enzymes and competent cells were all obtained from New England Biolabs (NEB). Chemical reagents were obtained from Fischer Scientific Inc (UK), Sigma (Sigma-Aldrich, USA) and Melford Laboratories Ltd (UK). Sequencing was performed by either Genewiz or Eurofins Genomics.

2.2 DNA techniques

2.2.1 Preparation of plasmid DNA

QIAprep Spin Miniprep Kit (QIAGEN) was used to purify plasmid DNA. A 5 ml LB culture containing appropriate antibiotic was grown from either an isolated bacterial colony, or glycerol stock and incubated overnight at 37^{o}C, 220 rpm. The following morning the cells were pelleted at 4000 rpm for 5 minutes. Following centrifugation, the QIAprep Spin Miniprep Kit protocol was followed as per the manufacturer's instructions. DNA concentrations were determined using a NanoDrop 2000 (Thermo Fischer Scientific Inc).

2.2.2 DNA amplification by Polymerase Chain Reaction (PCR)

DNA fragments were amplified with a BioRad T100^{TM} Thermocycler, using the following reaction in figure 8.

Component	Volume
5 X Phusion Buffer	10 µl
10 mM dNTPs	1 µl
10 µM Forward primer	2.5 µl
10 µM Reverse primer	2.5 µl
Phusion Polymerase	0.5 µl
Template DNA (50-100 ng)	x µl
dH_2O	to 50 µl
Total volume	**50 µl**

Figure 8. Reagents for DNA amplification by PCR using Phusion

The protocol for PCR is shown in figure 9.

	30 Seconds	98°C	Initial denaturation
X 30 Cycles	10 Seconds	98°C	Denaturation
	30 Seconds	°C *	Annealing
	30 Seconds/kbp	72°C	Elongation
	5 Minutes	72°C	Final elongation
	∝	10°C	Pause

Figure 9. Thermocycler steps for DNA amplification by PCR *Temperature dependent, used lowest primer Tm°C for each cycle.

2.2.3 PCR Purification

Following PCR, if required, PCR products were purified using the Monarch PCR and DNA Clean-up Kit (NEB) according to the manufacturer's instructions.

2.2.4 Phosphorylation of primers

For amplifications of primers for overlap, to ensure direct ligation of the amplified DNA target, primer were 5' amplified prior to PCR amplification using T4 polynucleotide kinase. See below in table 1 for the mixture.

Table 1. Phosphorylation of primers mixture

Reagent	Volume
100 µM primer stock	1 µl
10X T4 DNA ligase buffer	1 µl
T4 Polynucleotide Kinase	0.5 µl
Sterile MQ water	7.5 µl

The reaction was incubation at 37^{o}C for 1 hour then heated to 75^{o}C for 10 minutes.

2.2.5 DNA Ligation

Ligations of DNA fragments were carried out using T4 DNA ligase with the reaction mixture below in table 2.

Table 2. Ligation reaction mixture
Components for a ligation reaction mixture/ * DNA volumes were calculated to produce a 3X molar excess of insert to vector.

Component	Amount
10X T4 DNA ligase buffer	2 µl
Vector DNA	*
Insert DNA	*
T4 DNA ligase	1 µl
Sterile MQ water	Up to 20 µl

2.2.6 Generation of plasmids by Gibson assembly

Gibson assembly was used in this study to generate plasmids. To amplify the chosen DNA, primers were designed with a 18-56 base pair annealing region with a 5' 25 base pair overhang complementary to the vector insert location. The primers designed for the vector amplification, only required the annealing portion. Once the chosen DNA had been amplified using the protocol mentioned earlier, the PCR reactions were treated with 0.5 µl DpnI and incubated overnight at 37°C. Following incubation at 37°C, the samples were heat inactivated at 80°C. PCR reactions were then purified using the Monarch PCR & DNA Clean-up Kit (NEB) following the manufacturer's instructions. Once purified, 5 ng of vector DNA alongside a 3X molar excess of insert DNA were both added to a 5 µl Gibson master mix (seen below in table 3). Once mixed, the reaction was incubated at 50°C for 1 hour, then 3 µl of the reaction was used to transform into NEB Turbo competent cells.

Table 3. Gibson Assembly master mix
Gibson Assembly mix on the left with the 5 x ISO buffer components on the right

Component	Amount
5 x ISO master mixture*	320 µl
10 U/µl T5 exonuclease	0.64 µl
2 U/µl Phusion polymerase	20 µl
40 U/µl Taq ligase	160 µl
Sterile MQ water	1.2 ml

*5 x ISO buffer component	Amount
1 M Tris-HCl pH 7.5	3 ml
2 M MgCl2	150 µl
100 mM dGTP	60 µl
100 mM dATP	60 µl
100 mM dTTP	60 µl
100 mM dCTP	60 µl
1 M DTT	300 µl
PEG-8000	1.5 g
100 mM NAD	300 µl
Sterile MQ water	6 ml

2.2.7 Generation of plasmids by Golden Gate

Plasmids were generated for microalgal transformation, using Golden Gate cloning, the reaction to generate a plasmid, can be seen in table 4 below.

Table 4. Reagents for Golden Gate assembly

Component	**Volume**
CutSmart Buffer 10x	2 μl
Vector	1 nM
Insert(s)	2 nM
10 mM ATPs	2 μl
T4 Ligase	1 μl
Enzyme	1 μl
dH_2O	to 200 μl
Total volume	**200 μl**

Golden gate cloning was set up using the reagents in table 4 and carried out in a BioRad T100™ Thermal Cycler, the cycle can be seen in figure 10.

	Initial digest	15 Minutes	37°C
X20-30 Cycles	Ligation	2 Minutes	16°C
	Digestion	2 Minutes	37°C
	Final digestion	15 Minutes	37°C
	Heat inactivation	20 Minutes	65°C

Figure 10. Golden gate protocol used for thermocycler

Three different enzymes were used for Golden Gate cloning, dependent on the level of plasmid being generated, seen in figure 11.

Enzyme name	Sequence
BsaI	GGTCTC
SapI	GCTCTTC
Esp3I	CGTCTC

Figure 11. Enzymes using in Golden Gate protocol

Following the reaction, the DNA was transformed and plated on selected agar, negative colonies appear as either red, purple, or blue due to the presence of selection markers.

2.2.8 Agarose gel electrophoresis

DNA was analysed using horizontal gel electrophoresis. 1-1.5 % (w/v) agarose gels were prepared using molecular biology grade agarose (Melford) gel in 1X TAE buffer. Samples were prepared using 6X Purple Loading Dye (NEB) and 1X SybrGreen (Invitrogen, Thermo Fisher Scientific Inc, USA), then run in either a Bio-Rad Mini-Sub DNA electrophoresis chamber or a Scie-Plas Midi-Tank (Scie-Plas, UK) containing TAE buffer for 45 minutes at 120 V. DNA was visualised using a Bio-Rad Gel doc.

2.2.9 Purification of DNA from agarose gels

DNA bands were visualised under UV lights using a Bio-Rad Gel doc, once selected, the required band was excised as an agarose gel slice. The DNA was then purified using the Monarch DNA Gel Extraction Kit (NEB, UK), according to the manufacturer's instructions.

2.2.10 Sequencing of plasmid DNA

After processing plasmid DNA, all cloning was confirmed by GENEWIZ using 5 μl sequencing plasmid and 5 μl plasmid DNA at a concentration between 80-100 ng/μl.

2.2.11 Construct and primer list

The list of all constructs (table 5) and primers (table 6) used in this study.

Table 5. Constructs used in this study
List of all constructs used in this study, name, vector type and source are all detailed.

Name	Vector type	Details	Source
pLvl0	Golden Gate Level 0	Level 0 acceptor vector for golden gate	Saul Purton UCL
pLvl1AZ	Golden Gate Level 1	Level 0 AZ acceptor vector for golden gate	Saul Purton UCL
pLvl0rbcl	Golden Gate Level 0	Level 1 with rbcl terminator vector for golden gate	Saul Purton UCL
pLvl0 16s/psaA	Golden Gate Level 0	Level 1 with 16s/psaA promotor for golden gate	Saul Purton UCL
P2xtrbl	Golden Gate Level 1	Double 16s vector for dsRNA production	Saul Purton UCL
pLvl2	Golden Gate Level 2	Level 2 with psbH flanks + mRFP high copy	Saul Purton UCL
pP03	*C. reinhardtii* expression vector	*ptxD* integration cloning plasmid for transformation (pP03)	Saul Purton UCL
pHS5	Golden Gate Level 2	ptxD-VP9 in p2xtrbl in Level 2 expression vector	This study
pHS6	Golden Gate Level 2 (modified from pP03)	ptxD-ORF366 in p2xtrbl in Level 2 expression vector	This study
pHS12	Golden Gate Level 2	B7C12 in Level 2 expression vector	This study
pHS13	Golden Gate Level 2	B9G10 in Level 2 expression vector	This study
pHS14	Golden Gate Level 2	B7C12 and B9G10 in Level 2 expression vector	This study
pHS1	pET30a	T7-OmpKf-His6	This study
pHS2	pET30a	T7-GAPDHf-His6	This study
pHS3	pET30a	T7- OmpKf-linker-GAPDHf-His6	This study
pHS4	pET30a	T7- GAPDHf-linker-OmpKf-His6	This study
pHS10	pET23	pET23-GAPDHf-linker-OmpKf	This study
pHS11	pET23	pET23-OmpKf-linker-GAPDHf	This study
pSB42	pEXTII	pEXTII-SP_{PhoDn}-SP_{PETase}-PETase-His6	Sarah Bischoff
pSB43	pEXTII	pEXTII-SP_{PhoD}-SP_{PETase}-PETase-CyDisCo-His6	Sarah Bischoff
pHS6	pEXTII	pEXTII-SP_{PhoD}-PETase-His6	This study
pHS7	pEXTII	pEXTII-SP_{PhoD}-PETase-CyDisCo-His6	This study
pHS8	pEXTII	pEXTII-PETase-CyDisCo-His6	This study
pHS9	pEXTII	pEXTII-PETase-CyDisCo-His6	This study

Table 6. Primers used in this study
List of all primers used in this study, primer name, sequence and the use it detailed.

Primer name	Sequence	Used for
Flank_1_F	GTCATTGCGAAAATACTGGTGC	PCR & sequencing
Seq_mCRH1	CTCTCGTCAGGCAATTTGCTTACACC	PCR & sequencing
RSeq_mCRH1	GGCAACAGGAACTTCTAAAGC	PCR & sequencing
HA_R	TTAAGCGTAATCTGGTACGTCG	PCR & sequencing
ORF366_F	AGGAAAATGACCTCTATGAAG	Cloning
ORF366_RNA_R	AGAAAGCGCGTGCTTTAGC	Cloning, PCR, RT-PCR and qPCR
ORF466_RNA_F	AGGAAAATGACCTCTATGAAGAAGA	PCR, RT-PCR and qPCR
VP9_F	TTAATGGCCACCTTCCAGAC	Cloning
VP9_R	ATGTTATTCTGTTGTTGGCAC	Cloning
VP9_RNA_F	TTAATGGCCACCTTCCAGA	PCR, RT-PCR and qPCR
VP9_RNA_R	ATGTTATTCTGTTGTTGGCAC	PCR, RT-PCR and qPCR
ptxD_F	ATGTTACCAAAATTAGTAATTACTCACCGT	Cloning
ptxD_R	TACATCCGCTTTAGTATGTTACTATTTCTT	Cloning
ptxD_VP9/ORF366_Seq_F	TAGCTCCACACTGTGAATTAATGAC	PCR & sequencing
ptxD_VP9/ORF366_Seq_R	TGACACTCCTTAAGCCATTGGC	PCR & sequencing
F3	TGGTCGTGGTTACTGGCAAGAA	PCR & sequencing
R4	CCCAACCTTGTAAACGGTCAGC	PCR & sequencing
R5	CGTCTACCATTCCGCCATATC	PCR & sequencing
GAP-Omp-pYU49 Gibson Overlap F	TGTCTAGACACAGAGGATCATATGAATGGCAG GTCAGGATATTGTTAG	Gibson cloning
Omp-GAP-pYU49 Gibson overlap F	GTCTAGACACAGAGGATCATATGAATGATCTT CGATCTGTATGGTTATG	Gibson cloning
pYU49 Gibson R	TCATATGATCCTCTGTGTCTAGAC	Gibson cloning
pYU49 Gibson F	CATCATCACCATCATCATTAAACTA	Gibson cloning
GAP-Omp-pYU49 Overlap R	TAGTTTAATGATGATGGTGATGATGAAACCAC GGAACCATAACAT	Gibson cloning

GAP-Omp-pYU49 Overlap F	TGTCTAGACACAGAGGATCATATGAGCAGGTCAGGATATTGTTAG	Gibson cloning
Omp-GAP-pYU49 overlap R	TAGTTTAATGATGATGGTGATGATGGGTCGGAACACAAATGCCA	Gibson cloning
Omp-GAP-pYU49 overlap F	GTCTAGACACAGAGGATCATATGAATCTTCGATCTGTATGGTTATG	Gibson cloning
PETase Seq F	AATGCAGCACCGAACTTTCAT	
PhoDn Petase Gibson F	TGTTTAACTTTAAGAAGGAGATATAATGGCCTATGATAGCCGCTT	Gibson cloning
PETase Gibson F	TGTTTAACTTTAAGAAGGAGATATAATGAACTTCCCCCGTGCCTC	Gibson cloning
PETase Gibson R	GCTCACTAGTGAATTCGGATCCTTATTAATGGTGATGGTGATGGTGG	Gibson cloning
pMJS162 Gibson F	TAAGGATCCGAATTCACTAG	Gibson cloning
pMJS162 Gibson R	TATATCTCCTTCTTAAAGTTAAACA	Gibson cloning
Remove PhoD F	ATGAACTTCCCCCGTGCCTC	Cloning
Remove PhoD R	GGATCCTCCTCTGTGGAATTCT	Cloning

2.3 Microalgae techniques

2.3.1 Media for Chlamydomonas maintenance

See table 7 below which details the media used to maintain *C. reinhardtii* cultures.

Table 7. List of media used for culturing and storing Chlamydomonas

Media	Components
TAP	2.42 g/L Tris, 25 ml Beijerinck salts, 0.62 mM/L K_2HPO_4, 0.4 mM/L KH_2PO_3, 1 ml of each trace element* (7 total), ~1 ml acetic acid to pH 7.0, MQ H_20 up to 1 L For solid agar: 20 g/L
TA	2.42 g/L Tris, 25 ml Beijerinck salts, 1 ml of each trace element* (7 total), ~1 ml acetic acid to pH 7.0, MQ H_20 up to 1 L
TAPhi	2.42 g/L Tris, 25 ml Beijerinck salts, 1 ml of each trace element* (7 total), ~1 ml acetic acid to pH 7.0, MQ H_20 up to 1 L After autoclaving 1 ml/L of 1 M $Na_2HPO_3 \bullet 5H_2O$ and 1 ml/L of 1 M KCL was added For solid agar: 20 g/L Bacto-Agar For soft agar: 5 g/L Bacto-Agar
HSM	25 ml Beijerinck salts, 4 mM/L K_2HPO_4, 2.6 mM/L KH_2PO_3, 1 ml of each trace element* (7 total), MQ H_20 up to 1 L For solid agar: 20 g/L Bacto-Agar For soft agar: 5 g/L Bacto-Agar

* Trace elements: Stock concentrations, 25 mM EDTA (Na salt), 28 µM $(NH_4)_6Mo_7O_{24}$, 0.1 mM Na_2SeO_3, Zn-EDTA (2.5 mM, 2.75 mM), Mn-EDTA (6 mM, 6 mM), Fe-EDTA (20 mM, 22 mM and 22 mM Na_2CO_3), Cu-EDTA (2 mM, 2 mM)

2.3.2 Storage of microalgae

C. reinhardtii cultures were maintained on solid TAP agar plates, at 25°C under continuous light at 15-20 µmol photons m^{-2} s^{-1}. Cultures were streaked every 4-6 weeks onto fresh TAP to maintain healthy stocks.

2.3.3 *C. reinhardtii* strains used in this study

The strains of *C. reinhardtii* used in this study can be seen below in table 8.

Table 8. *C. reinhardtii* strains used in this study

Name	Details	Source
TN72	CC-5168 cw15 Δ*psbH*, *psbH*::*aadA*, mCell wall deficient strain for chloroplast transformation using glass bead method. Contains *aadA* (SpcR) cassette, resistant to spectinomycin.	Saul Purton UCL
CCK10	TN72 background strain with *psbH* restored and *aadA* cassette removed, transformed with empty pASapI vectors to be used as photosynthetic control	(Zedler *et al.* 2015)
TN72-dsORF366	Background strain of TN72, expressing dsRNA targeting ORF366 in WSSV. *psbH* restored and *aadA* cassette removed.	Conner Webb
TN72-dsVP9	Background strain of TN72, expressing dsRNA targeting VP9 in WSSV. *psbH* restored and *aadA* cassette removed.	Conner Webb
TN72-dsVP9-ptxd-dsORF366	Background strain of TN72 expressing dsRNA targeting VP9 in WSSV as well as dsRNA targeting ORF36 with ptxD for phosphite selection. *psbH* restored and *aadA* cassette removed.	This study
TN72-B9G10	TN72 transformed with B9G10, *psbH* restored and *aadA* cassette removed.	This study

2.3.4 Chloroplast transformation

A 20 ml TAP pre culture was grown to late log phase and used to inoculate a 400 ml TAP transformation culture. The culture was left to grow to early/mid log phase (~1.5 x 10^6 cells/ml). For photosynthesis restoration transformations, the culture was harvested at this point. Transformations using *ptxD* selection marker, were pelleted at 4000 rm, 20^{o}C for 5 mins and resuspended in TA media to a cell density of ~1.5 x 10^6 cells/ml and put back in the

incubator for 24 hrs for a phosphate starvation step prior to transformation. Once ready to harvest, the 400 ml culture was centrifuged at 4000 rpm, 20°C for 5 mins. The pellets were resuspended to 2 x 10^8 cells/ml using either TAP or TAPhi, dependent on selection method. 300 μl of cells was then added to a 5 ml Eppendorf tube containing 300 mg, 425-600 μm acid washed glass beads. One of the tubes was left negative and the remaining tubes had 5- 10 μg plasmid DNA added to each. The mixture was then vortexed for 15 seconds at max speed and mixed with 3.5 ml of soft HSM or soft TAPhi agar, warmed to ~45°C, the agar and cell mixture was then poured onto HSM or TAPhi agar plates and left to dry for approx. 20 minutes. Once dry the plates were left overnight at 25°C in 2 μE of light then moved to moderate light, 50 μE the following day. Colonies from photosynthetic restoration transformations were picked after around 4-6 weeks incubation and streaked onto fresh HSM agar plates. Colonies from ptxD selection transformations were picked ~2 weeks after incubation and were streaked onto fresh TAPhi media. Once enough biomass had grown, a DNA extraction was performed and was screened for the presence of integrated DNA using PCR.

2.3.5 Freeze drying of microalgae

When required for shrimp challenge trials, *C. reinhardtii* cultures were centrifuged at 4,000 rpm for ten minutes to collect cells and the pellets were lyophilised overnight in an Edwards Freeze Dryer Modulyo. Samples were then stored at -20°C prior to the challenge trial.

2.3.6 Total DNA extraction

To screen *C. reinhardtii* transformants a total DNA extraction was performed. A single colony of algae was resuspended in 20 μl sterile MQ and 20 μl of 100% ethanol was added. The mixture was incubated at room temperature for one minute, then 200 μl of 5% Chelex resin was added. The samples were vortexed then boiled at 95°C for five minutes. Finally, samples

were cooled on ice and centrifuged for 2 minutes at 14,000 rpm at 4°C. The supernatant was transferred to a new tube for storage at -20°C.

2.3.7 dsRNA extraction of *C. reinhardtii*

All RNA work was carried out at CENTEX Shrimp Centre, within Mahidol University, Bangkok, Thailand. A 100 ml TAP culture was inoculated from a plate and grown at 25°C, 190 rpm, for 3-4 days, under continuous light at 15-20 µmol photons m^{-2} s^{-1}. 10 ml of this pre culture was then used to inoculate a 1 L flask containing 400 ml TAP media and growth was continued for 5 days. After growth the culture was centrifuged at 4,000 rpm, for 5 minutes at 4°C. The pellet was then resuspended in 2 ml of Trizol per 100 ml of cell pellet. The resuspended cells were then transferred to a 2 ml Eppendorf tube and followed by a short vortex. The samples were then left at room temperature for 15 minutes. 200 µl of chloroform was added and the sample was vortexed. After another 15 minute incubation at room temperature, the sample was centrifuged at 12,000 rpm for 15 minutes at 4°C. The aqueous phase (upper layer) was then collected and added to an Eppendorf tube containing 800 µl ethanol, 200 µl DEP-C treated 3 M NaOAc and was left to precipitate overnight at -20°C. The following morning, the samples were centrifuged at 12,000 rpm for 15 minutes at 4°C. The samples were washed with 1 ml 75% DEP-C treated ethanol and centrifuged at 12,000 rpm, 4°C, 10 minutes. The supernatant was discarded and the pellet was left to air dry at room temperature. The dry pellet was next resuspended in 200 µl DEP-C treated H_20. The DNA and RNA concentrations were then determined using a NanoDrop™ One/One^C Microvolume UV-Vis Spectrophotometer (Thermo Scientific™). The samples were treated with DNase to removed remaining DNA but adding 30 µl DNase buffer, 6 µl DNase I, 30 µl RNase A, in the presence of >300 mM NaCl, to ensure ssRNA degradation, and left to incubate for 6 hours at 37°C. After incubation, 600 µl of phenol:chloroform was added and the samples were vortexed until milky, followed by centrifugation at 12,000 rpm for 10 minutes at 4°C. Again the upper

layer was added to 800 µl ethanol and 200 µl DEP-C treated 3 M NaOAc and was left to precipitate overnight at -20°C. The following day the sample was centrifuged at 12,000 rpm for 10 minutes at 4°C. The pellet was washed with 1 ml 75% DEP-C treated ethanol and centrifuged at 12,000 rpm, 4°C, 10 minutes. The pellet was left to airdry and finally resuspended in 50 µl DEP-C treated H_2O. Samples were then checked for the presence of RNA by RT-PCR.

2.3.8 dsRNA Extraction of HT115 (DE3) *E. coli*

dsRNA was extracted from *E. coli* cells HT115 for use as a control. A single colony from a fresh transformation plate was used to inoculate 5 ml LB media, containing the appropriate antibiotic and grown over night at 37°C, 220rpm. The following morning 500 µl of the overnight culture was used to inoculate 50 ml fresh LB in a 250 ml flask, containing the appropriate antibiotic and was left to grow for 8 hours at 37°C, 220rpm. After incubation the cells were collect by centrifugation at 4,00 rpm, 4°C for 5 minutes. The pellet was then resuspended in 700 µl DEP-C treated H_2O. 700 µl of phenol:chloroform was then added and the samples were vortexed until milky, then heated at 65°C for 10 minutes. The samples were then centrifuged at 12,000 rpm for 10 minutes at 4°C. The upper layer was transferred to a tube containing 700 µl ethanol, 140 µl DEP-C treated 3 M NaOAc and was left to precipitate overnight at -20°C. After precipitating, the samples were centrifuged at 12,000 rpm for 10 minutes at 4°C. The pellet was next washed with 1 ml 75% DEP-C treated ethanol and centrifuged at 12,000 rpm, 4°C, 10 minutes. The supernatant was discarded and the pellet was left to airdry at room temperature. Once dry the pellet was resuspended in 200 µl DEP-C treated H_2O. The sample was then treated with DNAse to remove residual DNA by adding 30 µl DNase buffer, 6 µl DNase I, 30 µl RNase A in the presence of >300 mM NaCl, and left to incubate for 6 hours at 37°C. After incubation, 300 µl of phenol:chloroform was added and the samples were vortexed until milky, followed by centrifugation at 12,000 rpm

for 10 minutes at 4°C. The upper layer was transferred to a tube containing 700 µl ethanol, 140 µl DEP0C treated 3 M NaOAc and mixed by tapping. The sample was left to precipitate over night at 20°C and centrifuged the following morning at 12,000 rpm for 10 minutes at 4°C. The pellet was washed with 1 ml 75% DEP-C treated ethanol and centrifuged for a final time at 12,000 rpm for 10 minutes at 4°C. The supernatant was removed and the pellet left to air dry at room temperature, and finally resuspended in 100 µl DEP-C treated H_2O. The dsRNA samples was then run on an agarose gel stained by submersion in ethidium bromide and visualised under UV light. The dsRNA band was excised and then purified using a Qiagen extraction kit.

2.3.9 Detection of RNA by RT-PCR

RT-PCR was used to detect RNA in the sample extracted using the methods above. The RNA sample was first heated at 95°C for 3 minutes and then cooled on ice prior to amplification by RT-PCR using the reagents shown in table 9.

Table 9. Reagents used for RT-PCR amplification

Component	**Volume**
2X Master Mix	10 µl
10 mM dNTPs	0.4 µl
F primer	0.4 µl
R primer	0.4 µl
50 X RT Mix (PCR Bio)	1 µl
RNA Template (500 ng)	1 µl
dH_2O	to 20 µl
Total volume	**20 µl**

The samples were then run using a thermocycler using the following protocol in figure 12.

	30 minutes	45°C	Reverse Transcription
	2 minutes	98°C	Initial denaturation
X 30-35 Cycles	10 Seconds	98°C	Denaturation
	30 Seconds	°C *	Annealing
	30 Seconds/kbp	72°C	Elongation
	5 Minutes	72°C	Final elongation
	∝	10°C	Pause

Figure 12. General RT-PCR protocol

*Annealing temperature dependent on primer Tm.

2.3.10 RT-qPCR for dsRNA quantification

The amount of dsRNA was quantified using RT-qPCR using the following reagents, in table 10.

Table 10. Reagents used for RT-qPCR for dsRNA quantification

Component	**Volume**
2X iTaq universal SYBR Green reaction mix (Bio-Rad)	10 µl
10 µM forward primer	0.6 µl
10 µM reverse primer	0.6 µl
iScript reverse transcriptase	0.25 µl
RNA Template (500 ng)	1 µl
RNase free dH_2O	to 20 µl
Total volume	**20 µl**

The general protocol was used as seen in figure 13.

	10 minutes	50°C	Reverse transcription
	1 minute	95°C	Initial denaturation
X40 cycles	10 seconds	95°C	Denaturation
	30 seconds	55°C	Annealing/extension
	5 seconds (hold)	65-95°C	Pause

Figure 13. Protocol for RT-qPCR
General protocol used for RT-qPCR.

2.3.11 Shrimp challenge trial against WSSV

Shrimp trials were carried out at CENTEX Shrimp Centre, within Mahidol University, Bangkok, Thailand. Post larvae white shrimp *Litopenaeus vannamei* (*L. vannamei*) were provided by CPF (Thailand). For the initial set up of challenge trial experiment, the shrimp were cultured in large tanks of artificial seawater with a salinity of 5 parts per thousand (ppt) with aeration and a temperature of 28 ± °C. The shrimp were left for three days to acclimatise prior to the experiment. The water in the tanks was replaced every 3-4 days to ensure clean conditions throughout the experiment. To prepare a WSSV inoculum, juvenile shrimp were fed tissue infected with WSSV at 10% of shrimp body weight. The shrimp were then collected, homogenised, and analysed for WSSV in tissues and quantified using qPCR for copies. Following quantification, the infected shrimp meat was kept at -80°C.

After three days of acclimatisation, the post-larvae shrimp were divided into 5 groups, seen in table 11.

Table 11. List of groups used in the shrimp WSSV challenge trial
Outline of all groups used in the shrimp WSSV challenge trial, negative group, positive group, control dsRNA group, dsVP9, dsORF366 and dsVP9-ptxD-dsORF366

Group name	Shrimp feed	WSSV infection	Algal feed
Negative	Yes	No	No
Positive	Yes	Yes	No
Control dsRNA	Yes	Yes	Photosynthetically restored background strain; TN72-dsRFP
dsVP9	Yes	Yes	TN72-dsVP9
dsORF366	Yes	Yes	TN72-dsORF366
dsVP9-ptxD-dsORF366	Yes	Yes	TN72-dsVP9-ptxD-dsORF366

Each group contained 4 replicates of 25 shrimp each in the first experiment, totalling 100 shrimp per group. Once aliquoted out into the groups, the shrimps were fed respective commercial shrimp feed or microalgae-commercial shrimp feed blend as stated in table 11, for three days prior to challenging with WSSV. All groups were fed twice daily. Two challenge trials were carried out as part of this project, the first used a liquid algae feed and the second used freeze-dried algae blended with commercial shrimp feed to mimic a natural feed. To prepare the liquid feed, the selected 200 ml TAP cultures were grown until an OD_{750} of ~2.0 was reached, and pelleted at 4000 rpm for ten minutes. The cells were resuspended to achieve 1 X 10^9 cells/ tank per feed, consequently, the shrimp were fed with 250 µl algal resuspension per meal. The feed was kept at 4^{o}C until ready for use.

To prepare the freeze-dried algae shrimp feed mix, a 1:1 ratio of freeze-dried microalgae and finely blended commercial shrimp feed was prepared using 2 g of each and mixed in a pestle and mortar. Once mixed 2 ml of squid oil was used as an additional flavouring for the shrimp to encourage feeding. The mixture was left to dry ON and mixed again the following morning to form a dry fine powder mix. The feed was then left at room temperature until ready for use.

Shrimp in all groups apart from the negative group were challenged with the WSSV as an oral challenge at 50 % of the shrimp body weight on day 4 of the experiment. Shrimp numbers were counted each day from the challenge day and the trial was stopped once the positive group were recorded as 100 % mortality. The results were reported in percentage of shrimp cumulative mortality, which was calculated from remaining percentage of cumulative shrimp survival after oral challenge with WSSV infected tissue.

2.3.12 Protein sample preparation from *C. reinhardtii*

Unless stated otherwise, all cultures were grown at 25°C, 120 rpm, illuminated at 15-20 µmol photons m^{-2} s^{-1}, for the desired time until ready for sampling. Pre-culture samples were grown by inoculating a 20 ml TAP culture using microalgae from a TAP plate, into a 50 ml shake flask and left to grow for 3-4 days. Following incubation, 10 ml of the pre-culture was transferred to 100 ml TAP medium in a 250 ml flask and left to grow for a further 5 days. Samples equivalent to 10 OD units were taken and centrifuged at 4,000 rpm and left ON at -20°C to lyse the cells. The following morning the pellets were resuspended in 1 ml lysis buffer (50 mM Tris-HCl pH 8, 10 mM $MgSO_4$) and lysed by sonication at amplitude 8.0 amplitude for 20 seconds on 10 seconds off for a total of three cycles. After sonication a sample was taken and mixed with 5X PGLB and heated at 95°C for 5 minutes. All samples were stored at -20°C until required.

2.3.13 Purification of HA-tagged proteins from *C. reinhardtii*

HA-tagged proteins were expressed in *C. reinhardtii* and purified using the Pierce™ HA-Tag IP/Co-IP Kit (Thermo Scientific) following the manufactures instructions. 20 µl of anti-HA slurry was incubated with 500 µl of protein lysate in a spin column and left overnight at 4°C with end-over-end mixing. After incubation, the unbound protein was eluted by centrifugation for 30 seconds at 13,000 rpm, a sample of the flow through was collected and

prepared for SDS-PAGE. The column was then washed using 500 µl of TBS plus 0.05 % Tween-20, three times. All three wash samples were collected and SDS-PAGE samples were prepared. HA-tagged protein was eluted by adding 20 µl of the manufacturers Elution Buffer and centrifuged at 13,000 rpm for 30 seconds. 1 µl of 1M Tris, pH 9.5 was added to the elution to neutralize the eluent. The elution sample was then prepared for SDS-PAGE.

2.4 Maintenance of *E. coli* cultures

2.4.1 Media and supplements

See table 12 below for a list of media types used in the *E. coli* culturing section of this work, followed by table 13 showing all *E. coli* strains used.

Table 12. List of media used throughout this study for *E. coli* work

Media	**Components**
LB media	10 g/L sodium chloride, 10 g/L tryptone, 5 g/L yeast extract Bacto agar supplemented at 10 g/L, if required
TB media	24 g/L yeast extract, 12 g/L tryptone, 4 ml/L glycerol Potassium phosphate buffer (added after autoclaving separately): 125.4 g/L K_2HPO_4, 23.2 g/L KH_2PO_4
SM6 media	10 mm 10X SM6 trace elements, 95 g/L glycerol, 5.2 g/L (NH_4) SO_4, 3.83 g/L NaH_2PO_4, 4.16 g/L citric acid, 4.03 g/L KCL, 1.04 g/L $MgSO_4.7H_2O$, 0.25 g/L $CaCl_2.H_2O$
10X SM6 trace elements	104 g/L citric acid, 10.06 g/L $FeCl_3.6H_2O$, 5.22 g/L $CaCl_2.H_2O$, 2.72 g/L $MnSO_4.4H_2O$, 2.06 g/L $ZnSO_4.7H_2O$, 0.81 g/L $CuSO_4.5H_2O$, 0.42 g/L $CoSO_4.7H_2O$, 0.03 g/L H_3BO_3, 0.02 g/L $Na_2MoO_4.2H_2O$
Auto induction media	55.85 g/L auto induction media (FOREMEDIUM), 0.8% glycerol

2.4.2 *E. coli* strains

Table 13. *E. coli* strains used in this study

Name	Genotype	Source
NEB Turbo	*E. coli* K-12 strain. F' proA B lacIq ΔlacZM15 / /fhuA2 Δ(lac-proAB) glnV galK16 galE15 R(zgb-210::Tn10)TetS endA1 thi-1 Δ(hsdS-mcrB)5	NEB
BL21	*E. coli* B strain. F– ompT gal dcm lon hsdSB(rB–mB–) [malB+]K-12(λS)	ATCC
BL21 (DE3)	*E. coli* B strain. *F– ompT gal dcm lon hsdSB(rB–mB–) λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) [malB+]K-12(λS) pLysS[T7p20 orip15A](CmR)*	NEB
W3110	*E. coli* K-12 strain. F- λ- rph-1 INV(rrnD, rrnE)	ATCC
DH5α	*E. coli* K-12 strain. fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17	
HT115 (DE3)	*E. coli* K-12 strain. [F-, mcrA, mcrB, IN(rrnD-rrnE)1, rnc14::Tn10(DE3 lysogen: lacUV5 promoter -T7 polymerase]	Caenorhabditis genetics center (CGC)
MC4100	*Ara^R, F2 arD139 DlacU169 rspL150 relA1 flB5301 deoC1 ptsF25 rbs^R*	
MC4110 Δ*tatABCDE*	MC4110 with Δ*tatABCDEI* deletion	Tracey Palmer-Newcastle University

2.4.3 Bacterial transformation

Following cloning, DNA was first transformed into NEB Turbo cells for isolation and sequencing, once isolated from NEB Turbo cells, the DNA was then transformed into the selected cell line. For expression in cells lines MC4110, MC4100 *Δtat* and W3110, plasmid DNA was first passaged thought DH5α *E. coli* cells. To achieve transformation, 100 ng of isolated plasmid DNA was added to a 50 µl aliquot of competent *E. coli* cells and incubated on ice for 20 minutes. Following incubation, the cells were heat shocked at 42°C for 45 seconds and immediately placed on ice for 10 minutes. The cells were recovered in 100 µl of LB media and incubated at 37°C, 220 rpm, for 1 hour, before being plated on LBA containing

the appropriate antibiotics. Plates were incubated overnight at 37°C or left at room temperature for 2 days.

2.4.4 Dual transformation

For expression using a dual plasmid system, a dual transformation was used. DNA was isolated following section 2.2.1. Two plasmids were used; the first plasmid is a plasmid containing the protein of interest, the second plasmid contains the components for CyDisCo. 1 μl of each isolated plasmid was added to 50 μl of *E. coli* W3110 competent cells and kept on ice for 20 minutes, followed by a heat shock at 42°C for 45 seconds. After heat shocking the cell mixture was cooled on ice for ten minutes. Cells were then recovered in 100 μl of LB media and incubated at 37°C, 200 rpm for 60 minutes. After incubation, the whole cell mixture, was spread onto LB agar plate containing both selection antibiotics; chloramphenicol (35 μg/ml) and either ampicillin (100 μg/ml) or kanamycin (25 μg/ml) for plasmid containing protein of interest. Once dry, plates were incubated at 37°C ON.

2.4.5 Competent cells

One colony was collected from a fresh streak of the desired strain on LB media, placed into 5 mL LB media and grown overnight at 30°C, 220 rpm. After incubation, 10 ml fresh LB media was inoculated from the pre-culture and allowed to grow at 37°C, 220 rpm, until OD~0.4 was reached. At this point the cells were left on ice for 20 mins, after cooling, the cells were centrifuged at 3000 rpm for 10 minutes at 4°C. The supernatant was discarded, and the pellet was gently resuspended in 10 ml of ice cold 0.1 M $CaCl_2$. Following a second incubation on ice for 30 minutes, the cell suspension was re-pelleted at 3000 rpm for 10 mins at 4°C. The remaining pellet was resuspended in 1 ml ice cold 0.1 M $CaCl_2$ containing 15% glycerol. The prepared competent cells were then stored at -80°C, ready for use.

2.4.6 Storage of *E. coli* cells

One colony was selected from a transformation plate and used to inoculate 5 ml LB media containing the appropriate antibiotic, and grown overnight at 37°C, 220 rpm. Following incubation 800 µl of the culture was mixed with 400 µl 50 % (v/v) glycerol in a cryovial, mixed and transferred to -80°C for long term storage.

2.5 *E. coli* protein expression and cell fractionation

2.5.1 Cell culture and plasmid induction

For all cell growth assays a 5 ml LB pre culture containing appropriate antibiotic, was set up using a single colony from a freshly streaked LBA plate and incubated ON at 220 rpm 37°C. The following morning cultures were diluted to OD_{600} 0.05 to a volume of 50 ml in a 250 ml flask using fresh medium containing appropriate antibiotics. For large scale shake flask growth, cultures were grown in 400 ml media in a 2 L flask. Dependent on strain, cultures were either incubated at 30°C or 37°C at 220 rpm until the OD_{600} reached mid log phase at around 0.4-0.6. Gene expression was induced using 100 µM IPTG, the cultures were then allowed to continue growth for a further 3 hrs.

2.5.2 Dual plasmid expression

One to three colonies were selected from the dual transformation plate and used to inoculate 5 ml LB containing 2 g/L glycerol in a 50 ml tube. Cultures were left to grow for five hrs at 30°C at 250 rpm. After 5 hrs, 100 µl of the pre culture was used to inoculate 10 ml autoinduction media containing 0.8% glycerol, in a 100 ml flask. The culture was incubated for 24 hrs at 30°C, 250 rpm. Following incubation, the cultures were ready to harvest following the fractionation method (section 2.5.7).

The dual plasmid system was also used for other media types including TB and LB. One to three colonies were selected from the dual transformation plate and used to inoculate at 5 ml LB culture containing the antibiotics of both strains and left to grow at 37°C ON. The following morning, the pre culture was used to inoculate 50 ml of either TB or LB media containing required antibiotics, in a 250 ml flask to an OD_{600} of 0.05. Cultures were left to grow at 37°C, 220 rpm until OD_{600} of ~0.4 was reached. Samples were then induced with 100 µM IPTG and left to grow at 30°C, 220 rpm for 3 hrs. Cells were harvested after three hours using the fractionation method (2.5.7).

2.5.3 *E. coli* isolation of soluble and insoluble proteins from small scale shake flask cultures

Throughout expression, samples were taken at specified time points, to the equivalent of 10 OD_{600} units. The culture was centrifuged at 3,000 rpm, 4°C for 10 minutes, and the supernatant was discarded. From this point all samples were kept on ice. Following centrifugation, pellets were resuspended in 1 ml resuspension buffer (50 mM Tris-acetate, 2.5 mM EDTA pH 7.0). Samples were then lysed using sonication on ice at amplitude 8.0 for 6 repetitions of 10 seconds sonicating, 10 seconds resting. The lysed suspensions were then centrifuged at 14,000 rpm for 15 minutes at 4°C. The supernatant was collected as the soluble fraction and the remaining pellet was resuspended in a further 1 ml resuspension buffer, sonicated on ice at amplitude 8.0 for 10 seconds, then collected as the insoluble fraction. All samples were then ready to prepare for SDS-PAGE gels by mixing 80 µl of sample with 20 µL 5XPGLB + β-mercaptoethanol and boiled for 5 minutes at 95°C. All samples were stored at -20°C until required.

2.5.4 *E. coli* isolation of soluble protein from large scale shake flask cultures

Following incubation, the 400 ml of culture was centrifuged at 10,000 rpm for 30 minutes at 4°C. The pellets were then resuspended in 5-10 ml binding buffer (20 mM sodium phosphate 0.5 M NaCl), at approximately 2 ml per g of wet cell weight. Following resuspension, the cells were sonicated at amplitude 8, for 3 seconds on, 7 seconds off for 5 minutes, followed by 2.5 minutes on ice, followed with a further 3 seconds on, 7 seconds off for 5 minutes. After sonication, the samples were centrifuged at 15,000 rpm for 30 minutes at 4°C. The supernatant was then collected as the soluble fraction and filter sterilised using a 0.45 µL filter.

2.5.5 *E. coli* fed-batch fermentation

Typically, a 5 ml TB culture was used as a pre-inoculant using a single colony from a fresh transformation containing the desired gene for protein expression. The 5 ml culture was grown for 6 hrs at 37°C, 250 rpm. Following incubation, 1 ml of the pre-inoculant was transferred to 200 ml SM6Gc media, containing the appropriate antibiotic, in a 1 L baffled flask and incubated ON at 30°C, 250 rpm. The fermenters used for this this study were 1.5 L Minifors 2 by Infors, with a total volume of 500 ml. The fermenter parameters were set to pH 7.0, regulated by automatic addition of either 25% sulphuric acid or 25% ammonia, 40% pO_2, total flow 1.5-2.0 L/min and stirrer speed was 900-1600 rpm, both regulated by pO_2.

The following morning, the 500 ml SM6Gc media in the fermenter was inoculated using the ON culture, to a volume of 300 OD_{600}/L. The fermenter was inoculated through the sample port and the appropriate antibiotic was added through the septum, to ensure sterile conditions were maintained. The cultures were grown at 30°C until both the stirrer and airflow were maxed, then the temperature was lowered to 25°C. Supplements were added once certain OD_{600} were reached; at OD_{600} 38-42, an 8 ml shot of 1 M $MgSO_4 \cdot 7H_20$ was added,

at OD_{600} 54-58, a 5 ml/L shot of 1.687 M NaH_2PO_4 was added and once reached OD_{600} 66-70 a final 5 ml of 1.687 M NaH_2PO_4 was added. At this point in the run, the glycerol feed for a carbon source, was set at a rate of 0.7 % pump capacity of 80% w/w glycerol. Once the cultures reached a desired OD_{600} usually around 75+, protein expression was induced using 9 ml/L 0.0181 M IPTG. Following induction, the samples were left to grow and were harvested dependent on protein expression monitored though regular sampling. Throughout the fermentation run, samples were taken through the sample port. A pre-induction sample was taken, then following induction, samples were taken at regular time intervals. Antibiotics were added daily throughout the run and antifoam was added if required.

2.5.6 *E. coli* fermentation using Ambr250

In preparation for fermentation a 5 ml LB pre culture containing appropriate antibiotic was set up using one colony from freshly transformed cells and incubated at 37°C, ON at 250 rpm. The following morning, a 50 ml TB day culture was inoculated using the pre-culture to an OD_{600} of 0.1 in a 250 ml flask. The culture was incubated for 6 hrs at 37°C, 250 rpm. After 6 hrs the culture was used to inoculate a fresh 400 ml SM6Gc media, in a baffled flask, to an OD_{600} of 0.1 in a 2.5 L baffled flask and left to grow ON at 30°C, 250 rpm.

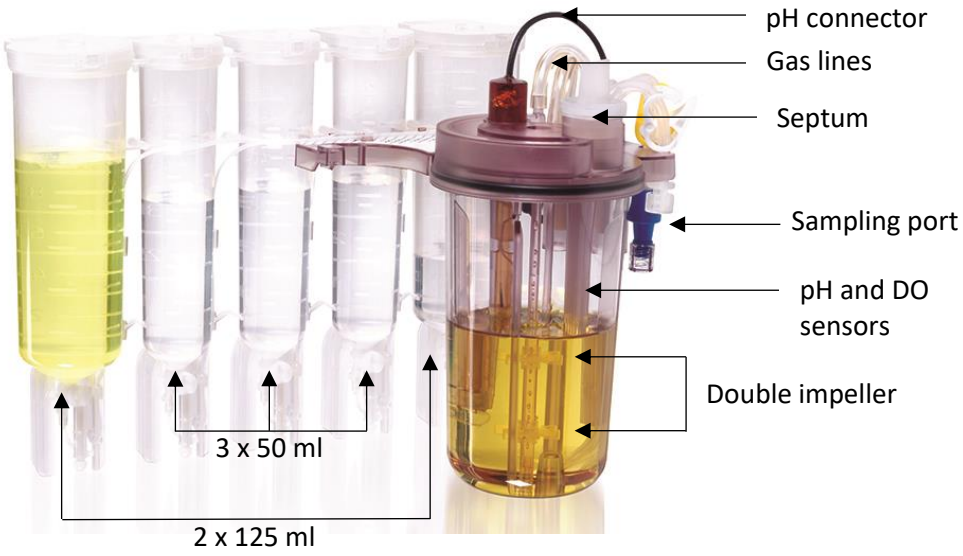

Figure 14. Ambr250 bioreactor vessel set up (Sartorius 2024)**.**
Ambr250 bioreactor with labels to show two 125 ml reservoirs, and three 50 ml reservoirs, the culture compartment with a double impeller, pH and DO sensors, as well as a sampling port and septum and gas lines.

On the day of fermenter inoculation, the overnight culture is used inoculate the Ambr250 vessel which contained 100 ml sterile media to an OD_{600} of 0.3. Parameters were set according to the expression test and grown for the desired amount of time. Samples equivalent to 10 OD units were taken and processed for SDS-PAGE.

2.5.7 *E. coli* fractionation from shake flask cultures

Following plasmid induction for 3hrs (section 2.5.1), 10 OD units were harvested ($10/OD_{600}$) and centrifuged at 3,000 rpm at 4°C. From this point onwards all samples were kept on ice. Pellets were resuspended in 500 µl ice cold Buffer 1 (100 mM Tris-acetate pH 8.2, 500 mM sucrose, 5 mM EDTA pH 8.0). 500 µl of ice cold mqH20 and 40 µl lysozyme (1 mg/mL stock) were added. After mixing by inverting the sample was left to incubate on ice Following incubation, to stabilise the inner membrane. After 5 mins, 20 µl $MgSO_4$ (1 M stock) was added, and the sample was inverted. The sample was then centrifuged at 14,000 rpm, 4°C for 2 mins. The periplasmic sample was taken from the supernatant and prepared ready for

SDS-PAGE analysis, and the rest discarded. Pellets were gently resuspended in 1 ml ice cold buffer 2 (50 mM Tris-acetate pH 8.2, 250 mM sucrose, 10 mM $MgSO_4$). Followed by another centrifugation at 14,000 rpm, 4°C for 5 mins, the pellets were resuspended in 1 ml ice cold buffer 3 (50 mM Tris-acetate pH 8.2, 2.5 mM EDTA pH 8.0) and sonicated on ice for 6 repetitions of 10 seconds on 10 seconds off at amplitude 8.0. Samples were next separated into cytoplasmic fraction and insoluble fraction, using ultracentrifugation. 500 µl of the sample was spun at 70,000 rm for 30 mins at 4°C. The cytoplasmic fraction was taken from the supernatant and the rest discarded. The final pellet was resuspended in buffer 3 (50 mM Tris-acetate pH 8.2, 2.5 mM EDTA pH 8.0) and the insoluble fraction was taken from this suspension. All samples were prepared for SDS-PAGE gels at the point of preparation and then the resultant sample was stored at -20°C.

2.5.8 *E. coli* fractionation from fed batch fermentation cultures

For cell fractionations from fermentation cultures, a larger scale protocol was used due to the size of the bioreactors. A total of 500 ml culture was harvest from each bioreactor was centrifuged at 4,000 rpm, 3°C, for 20 mins using a Beckman Avanti JA.10 rotor. From this point onwards all samples were kept on ice. Pellets were resuspended in 15 ml ice cold buffer 1 (100 mM Tris-acetate pH 8.2, 500 mM sucrose) without EDTA to avoid interference with down-stream purification. Following a 5-minute incubation on ice, 15 ml of sterile dH_20 was added, then left on ice for a further 10 minutes. 1.4 ml MgS04 from a 1 M stock was added and inverted. The sample was then centrifuged at 14,000 rpm for 20 mins at 4°C in a JA25.5 rotor. The periplasmic sample was taken from the supernatant and filter sterilised using a 0.45 µM filter, prior to being stored at -80°C, 1 cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail tablet was added.

2.5.9 *E. coli* fractionation from Ambr250 cultures

The 100 ml culture from the Ambr250 was harvested by centrifugation at 8,000 rpm for 20 minutes at 4°C. The pellets were then resuspended in 50 ml Buffer 1 (-EDTA) and left on ice for ten minutes. After incubation, 50 ml dH_20 and 1 ml of 2 mg/ml lysozyme was added. Following a second 10-minute incubation on ice 2 ml 1 M $MgSO_4$ was added, and the samples were centrifuged at 14,000 rpm for 20 mins at 4°C. The supernatant was collected and 1 cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail tablet was added before storing at -80°C.

2.6 Protein purification and quantification

2.6.1 Immobilised Metal Affinity Chromatography (IMAC) protein purification using AKTA Pure Systems

Nickel Immobilised Metal Affinity Chromatography (IMAC) purification was caried out for 6x Histidine-tagged proteins. Following an *E. coli* fractionation, the periplasmic fraction was collected in binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4).

For purification of PETase proteins from a large scale expression, cultures were centrifuged at 4000 rpm for 20 mins at 4°C using a Beckman Avanti JA.10 rotor the pellets were then resuspended in 15 ml of EDTA free buffer (100 mM Tris-acetate pH 8.2, 500 mM sucrose). After a 5 minute ice incubation, 15 ml dH_20 was added and incubated for a further 15 minutes. To stabilise membranes 1.4 ml $MgSO_4$ was added prior to centrifugation at 14,000 rpm for 20 minutes using a Beckman Avanti JA25.5 rotor. The supernatant was then collected and 1 crushed Complete EDTA-free protease inhibitor tablet was added. Prior to loading on the column, the periplasmic fraction was filter sterilised using a 0.45 µL filter.

The programme UNICORN 7.3 was used on the AKTA system with a 5 ml HisTrap HP ™ column (Cytiva). The column is first equilibrated with 5 column volumes of binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4), the sample is then loaded on the column and then washed again using binding buffer. A total of three wash samples were collected from the column; W1, W2 and W3. For protein elution, a gradient was chosen to allow for the protein to be eluted at an increasing imidazole concentration in different fractions. A total of 14x 1.8 ml fractions were collected from the purification from 5 column volumes. After collection, a small volume of the samples were diluted with 5X PGLB and all stored at -20C.

2.7 Protein electrophoresis

2.7.1 SDA poly-acrylamide gel electrophoresis (SDS-PAGE)

This study used the Bio-Rad Mini-PROTEAN Tetra System, SDS-PAGE gels were either prepared in lab or purchased as pre-cast SDS-PAGE gels. The lab prepared gels were made according to the manufacturer's instructions, consisting of 15 % acrylamide, 375 mM Tris-HCL pH 8.85, 0.1 % SDA, 0.1 % APS and 0.06 % TEMED, which was then layered using a stacking gel composed of 5 % acrylamide, 125 mM Tris-HCL pH 6.8, 0.001 % SDS, 0.6 % APS and 0.06 % TEMED. Once the protein samples were prepared following expression, they were mixed with 5 % β-mercaptoethanol and heated for 10 minutes at 90 °C before loading. Gels were typically run at 60 mA for 40 minutes in protein gel running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS, pH 8.3).

2.7.2 Coomassie Brilliant Blue staining

Following the running of the gel, proteins were visualised using Coomassie Brilliant Blue staining. The SDS-PAGE gels were submerged in 50 ml Coomassie Brilliant Blue Stain (1 g/L Coomassie Brilliant Blue, 10 % acetic acid, 40 % ethanol) for 1 hour at room temperature on

an orbital rocker. Once stained, the excess stain was removed using de-staining solution (5 % ethanol, 7.5 % acetic acid) and left on the orbital rocker for a minimum of 2 hours.

2.8 Protein detection

2.8.1 Western blotting

Proteins were transferred onto PVDF membrane using the Invitrogen Power Blotter Semi-Dry Transfer System. The PVDF was first activated in 100 % methanol, and 2 sheets of Whatman paper per membrane and SDS-PAGE gel were submerged in 1x Power Blotter 1-Step™ Transfer buffer, for 10 minutes. The gel sandwich was assembled in the Power Blotter cassette with Whatman paper, PVDF membrane, SDS-PAGE gel on top, followed by second sheet of Whatman paper. The conditions of the transfer were selected according to protein size on the Power Blotter. Once transferred the membrane was blocked for 1 hr at room temperature or ON at 4°C in a 2.5 % skimmed milk solution in 1X PBS-tween (1X PBS, 0.1 % tween20) on an orbital rocker. The membrane was then washed for 15 minutes followed by 3 X 5-minute washes in fresh 1X PBST before incubation with primary antibody for 1 hr, see table 14 for list of antibodies used. The membrane was then washed again for 15 minutes in fresh PBST followed by 3 X 5-minute washes. The appropriate secondary antibody was then applied for a further hour. After antibody incubation, the membrane was washed 8 times for 5 minutes each before imaging using the ECL™ kit. Immunoreactive bands were visualised using Bio-Rad Gel-doc XR+ chemiluminescence imager.

2.8.2 Antibodies used in this study

The table below, lists all the antibodies used in this study including their working concentrations.

Table 14. Antibodies used in this study

Antibody Name	Working concentration of antibody to PBS-tween	Source
Anti-His (C-terminal), mouse monoclonal	1:5,700	Invitrogen
Anti-Mouse HRP conjugate, goat polyclonal	1:5,700	Promega
6X His Tag Monoclonal Antibody. (C & N terminal mouse monoclonal) (4E3D10H2/E3)	1:2,000	Invitrogen
Anti-HA, rabbit polyclonal	1:909	Sigma
Anti-rabbit HRP conjugate, goat polyclonal	1:5,700	Promega

2.9 PETase activity analysis

2.9.1 HPLC analysis

PETase activity was analysed and quantified by HPLC by collaborators at the University of Portsmouth.

PETase with first grown by fed-bath fermentation following the protocol above in section 2.5.4 and fractionated following the protocol in 5.5.7. The next step was to purify the periplasmic fraction containing the protein as detailed in 2.6.1. A total of 4 x 2 ml elution fractions were sent to Portsmouth for analysis. The 2 ml samples were dialyzed using a PD 10 column equilibrated with 50 mM sodium phosphate pH 8, to remove imidazole which was present in the purification buffer. Following this the samples were concentrated to 500 µl. The reaction was then set up as follows: 250 µl of protein sample, 200 µl of buffer (50 mM sodium phosphate, pH8), 50 µl DMSO with a 13 mm x 3 mm; 10.5 mg piece of PET plastic. The reaction was incubated at 30^{o}C for 120 hours, then analysed by HPLC according to methodology from collaborators (Avilan et al., 2023).

2.10 Fragment vaccine expression and vaccination methods from collaborator

The following methods from section 2.10.1 to 2.10.12, were used by collaborators at the Faculty of Science and Natural Resources, University Malaysia Sabah, to express the fusion protein designed here in this study, and for grouper fish vaccination trials. Methodology taken directly from work by Iffah Hayani Binti Md Daud.

2.10.1 Growth Media and Bacterial Culture

Terrific Broth (TB) (Miller, Merck KGaA, 64271 Danstadt Germany) was used as culture broth for expression and overexpression of *E. coli* Rosetta cells. *V. parahaemolyticus* were cultivated on Nutrient Agar (NA) (Oxoid LTD., Basingstoke, Hampshire, England) with 2% (w/v) NaCl overnight at 28°C. Thiosulfate citrate bile salts sucrose agar (TCBS) agar (BD™ Difco™, 38800 Le Pont de Claix, France) was employed to confirm the presence of *V. parahaemolyticus.*

2.10.2 Oligonucleotides used in this study

Table 15. The oligonucleotide of primers and plasmids used in this study

Target primer/plasmid name	**Primer sequence (5' – 3')**	**Purpose (reference)**
Interleukin-2 (IL-2) forward	GCCGACCTGGTTGTAATCCTCA	qPCR (He *et al.* 2021)
IL-2, reverse	ATCTCAAAGCCTGTCTCATTGGT	qPCR (He *et al.* 2021)
Interleukin-6 (IL-6), forward	AGGAAGGTCTGGCTGTCAGGA	qPCR (He *et al.* 2021)
IL-6, reverse	GCCCTGAGGCCTTCAAGATT	qPCR (He *et al.* 2021)
Interferon-gamma (IFN-ϒ), forward	CCACCAACATGGAGGCTAAC	qPCR (He *et al.* 2021)
IFN-ϒ, reverse	CTGCCACCTCACCATTGCT	qPCR (He *et al.* 2021)
B-actin, forward	GCGACCTCACAGACTACCT	Reference gene for qPCR (Yang *et al.* 2022)
B-actin, forward	CTGGGCAACGGAACCT	Reference gene for qPCR (Yang *et al.* 2022)
Plasmid	pET30a-OmpK/GADPH	Fusion fragment protein over expression (Budiman *et al.* 2022)

2.10.3 Overexpression of fusion fragment protein

Fusion fragment protein vaccine candidate expression and overexpression were conducted following the protocol established by Zhang et al., (2007). Briefly, 3 ml of pre-cultured *E. coli* Rosetta containing GAPDH-OmpK (GAPDH-OmpK) the fusion fragment protein was inoculated into 100 ml Terrific Broth (TB) at a ratio of 1:100 (v/v) Terrific broth (TB) with 100 µL of kanamycin antibiotic. The culture flask containing TB inoculated with *E. coli* Rosetta containing fusion fragment protein was vigorously shaken and incubated at 37^{o}C until an optical density (OD_{600}) of 0.6 was reached. The culture containing TB inoculated with *E. coli* Rosetta containing fusion fragment protein then underwent induction with 100 µL of IPTG. Upon IPTG induction, the culture containing TB inoculated with *E. coli* Rosetta containing fusion fragment protein was left to incubate overnight (12 – 16 hours) at 18^{o}C.

For identification of fusion fragment protein expression by SDS-PAGE, 1 ml of 100 ml cultured TB inoculated with *E. coli* Rosetta containing fusion fragment protein pre and post IPTG induction was transferred into a sterile 1 ml microcentrifuge tube and stored on ice prior to SDS-PAGE.

2.10.4 Harvesting of fusion fragment protein

To harvest the fusion fragment protein, approximately 98 ml of cultured TB inoculated with *E. coli* Rosetta containing fusion fragment protein underwent centrifugation method for 10 min at 8000 rpm and 4^{o}C [Beckman Coulter Avanti J-E centrifuge (J-10 rotor) (United States)]. The resulting supernatant was discarded while the resulting pellets of the fusion fragment protein were resuspended in 100 ml MiliQ water. Subsequently, cell disruption was carried out on the resuspended pellet of fusion fragment protein, with the addition of approximately 100 ml of 10 mM Tris–HCl (pH 7.6) containing 1 mM EDTA using an Ultrasonic processor

(Sonic, Danbury, CT, USA) for 6 min under the following conditions: 30% amplitude, 10 s sonication and a 30 s pause each time with cooling on ice.

The disrupted resuspended pellet of fusion fragment protein was then subjected to ultracentrifugation (Beckman Coulter Optima™ L-100K Ultracentrifuge, United States) at 35, 000 rpm at 4^{o}C for 30 min as outlined by Ningqiu et al., (2008). This process aimed to isolate the fusion fragment protein contained in the supernatant. Both supernatant and pellet obtained from this ultracentrifugation procedure, respectively. Approximately 50 ml of supernatant containing fusion fragment protein obtained from this method was then further used in purification method. The Beckman Coulter Optima™ L-100K Ultracentrifuge (United States) used in this procedure is as shown in the Figure 3.3 below. To identify protein solubility using SDS-PAGE, 1 ml of the 50 ml supernatant and pellet obtained from ultracentrifugation procedure was removed. The pellet obtained from ultracentrifugation undergoes sample preparation procedure first before can be used for SDS-PAGE analysis. Briefly, the pellet was resuspended with 50 µL MiliQ water as a sample preparation for fusion fragment protein solubility identification.

Approximately, 1 ml of 50 µL of prepared resuspended pellet was isolated for solubility identification. Both isolated samples; 1 ml of supernatant and 1 ml of prepared resuspend pellets were then subjected to SDS-PAGE analysis.

2.10.5 Purification of fragment vaccine

The harvested 50 ml of supernatant contain fragment vaccine from method 2.10.4 then underwent purification of fragment vaccine using the Cytivia ÄKTA pure™ chromatograph, following the method outlined by (Ningqiu *et al.* 2008) with minor adaptation. In this

method, affinity chromatography (AC) was employed with a series of system washes, sample application, column washes, elution and collection of samples.

Briefly, the procedure involved the use of the distinct buffers: Buffer A, designated as the binding or washing buffer and Buffer B, recognized as the elution buffer, Buffer A maintained at pH 7.5, consisted of 50 mM Tris HCL, 100 mM NaCl, 50 mM Imidazole and 1 mM DTT. Meanwhile, Buffer B (pH 7.5), comprised 50 mM Tris HCL, 100 mM NaCl, 500 mM Imidazole, and 1 mM DTT. Upon completion of purification, 1 ml of 15 ml purified fusion fragment protein was removed for analysis of protein purification using SDS-PAGE.

2.10.6 Vaccination of Hybrid TGGG Grouper *(Epinephelus fuscoguttatus x E. lanceolatus)*

Animal experimentation of hybrid TGGG grouper was approved by the University of Malysia Sabah Animal Ethics Committee reference number AEC 0018/2023. Groups of 5-inch length hybrid TGGG grouper weighing 40 ± 6 g were received and acclimatized for 1 week at the UMS Fish Hatchery in a controlled environment of 28 ppt salinity, 6.0 mg/L dissolved oxygen, 30oC and pH of 7.9, residing in 100 L 45 fiberglass-reinforced plastic (FRP) tanks filled with aerated recirculating saltwater. Daily diet of TGGG fish comprised commercial dry pellets containing crude protein (50%), crude fat (>8.4%), crude fibre (<2.9%), ash content (<16.0%), and moisture (<10%), administered twice daily.

Daily assessments of hybrid TGGG grouper health and regular monitoring of water quality in each tank were conducted. Parameters such as water salinity, dissolved oxygen, pH water and water temperature were monitored using the ProDSS Multiparameter Digital Water Quality Meter.

2.10.7 TGGG Vaccination

2 groups of 5-inch hybrid TGGG grouper (n=42), were selected for the vaccination procedure. The first group (n=21) was immunized with pure fragment vaccine GADPH-OmpK at a dosage of 50 µL/100 g while another group (n=21) served as a control with 50 mM Tris HCl at pH 7.5 at a dosage of 50µL/100g by using intraperitoneal injection (IP) using 23G needle. This procedure was performed at the UMS Fish Hatchery as guided by the trained (Institut Penyelidikan Marin Borneo) IPMB research assistant. For day 7 and 14 post vaccination sampling the hybrid TGGG grouper was anesthetized to euthanized using 250 mg/L l−1 of MS-222 (Tricaine®- S, Western Chemical).

2.10.8 Vaccinated TGGG Challenge with *Vibrio parahaemolyticus;* Bacterial Culture and Challenge

For bacteria challenge, the method outlined by Huang *et al*. (2019) was followed with slight modifications. Briefly, *V. parahaemolyticus* were first cultivated on NA agar with 2% NaCl overnight at 28°C. Upon incubation, single colonies were selected and cultured for 18 hours at 25°C in 5 ml of TB Broth containing 2% NaCl.

Approximately 1 ml of TB Broth containing 1X108 CFU/mL of the *V. parahaemolyticus* culture was isolated and harvested by centrifugation at 5000 rpm for 10 min. The bacterial pellet was then added with 1 ml of normal saline (0.8% NaCl). The challenge test employed unvaccinated and vaccinated groups of hybrids TGGG, following previously defined protocols (Chin *et al.* 2020) (Zhang, Yu and Qian 2007), with slight modifications. Briefly, TGGG were firstly anaesthetized with 50 mg/L l−1 of MS-222 (Tricaine®-S, Western Chemical). The skin of fish was then subjected to injury by grazing using a 23G needle, before being immersed into the tank containing bacterial culture. TGGG was then left in the tank for 12 hours before exchange with fresh clean water. On day 7 post challenge, the procedure was repeated for a second bacterial exposure of TGGG. For control purposes, 1 ml of water from both

unvaccinated and vaccinated tanks containing *V. parahaemolyticus* were grown on thiosulfate citrate bile salts sucrose agar (TCBS) agar (BD™ Difco™, 38800 Le Pont de Claix, France). For the culture, approximately, 50 µL of the tank water sample were spread on the TCBS agar and incubated at 28^{o}C for overnight (12-16 hours) according to research done by (Kim *et al.* 2012).

2.10.9 Fish tissue harvest for immune gene expression analysis

The tissue harvest method followed the protocol by Zhang et al., (2007) with slight modifications. Briefly, one fish was lethally anesthetized to euthanasia with 250 mg/L l−1 of MS-222 (Tricaine®-S, Western Chemical) for 20 minutes from each tank. Using a scalpel, fish spleen was excised under RNase-free conditions and on ice. The sample was then homogenized using mortar and pestle on ice. Approximately 3 volumes of Invitrogen by Thermo fisher scientific RNAlater ™ solution was subsequently added to 1 volume of the homogenized sample for storage at -80^{o}C. This method was repeated for day 0, 7 and 14 post vaccination and day 7 and 14 post challenge test.

2.10.10 RNA extraction for immune gene expression analysis

The RNA extraction method followed the RNeasy® Plus Mini Kit protocols with some modifications. Approximately 30 mg of the extracted sample was transferred to a new tube and centrifuged for 3 mins at maximum speed to separate the sample from the previously used RNA later. After removing the RNA later, 200 µL of Trizol reagent was added to the tube containing the sample, followed by disruption and homogenization of the lysate. An additional 300 µL of Trizol and 100 µL of chloroform were introduced into the tube, and the lysate was mixed by inverting the tube a few times. To facilitate the reaction, the sample was allowed to stand for 1 minute, after which the lysate was centrifuged for 5 mins at 10, 000 rpm, leading to the separation of aqueous, interphase, and organic phases. The RNA, located

in the aqueous phase (supernatant), was carefully transferred to a new tube. Following the acquisition of the aqueous phase, approximately 350 µL of 70% ethanol was added to the tube containing the transferred supernatant and mixed thoroughly by pipetting. Subsequently, the sample was transferred to an RNeasy spin column placed in a 2 mL collection tube and centrifuged for 15 seconds at 13, 000 rpm. The discarded flow-through was then replaced with 700 µL of Buffer RW1, followed by another 15-second centrifugation at 13, 000 rpm, with the flow-through being discarded. This step was repeated once. Next, 500 µL of Buffer RPE was added to the RNeasy spin column, and centrifugation for 2 minutes at 13, 000rpm ensued, with the subsequent discarding of the obtained flow-through. The RNeasy spin column was then placed in a new 2 ml collection tube, and 20 µL of RNase free water was directly added to the spin column membrane, allowing it to stand for 1 minute. The RNeasy column was centrifuged for 1 minute at 13, 000 rpm to elute the RNA, and this step was repeated once. The resulting RNA, collected in the flow-through, was stored on ice and preserved at -80°C until further use.

2.10.11 Quantitative Real – Time Polymerase Chain Reaction (qRT-PCR) for immune gene expression analysis

Real time – polymerase chain reaction (qRT-PCR) procedure was performed using the ViPRimePLUS One Step AtTaq RT-qPCR Green Master Mix I (SYBR® Green Dye) kit. A standard 20 µL reaction mix was prepared for all sample (10 µL AtTaq one Step RT-qPCR Green Master Mix I, 1 µL forward primer, 1 µL reverse primer followed by 5 µL of RNA sample and 3 µL of distilled water. 50 Amplification was performed using the C100™ Thermal Cycler (Bio-Rad) with the following program conditions: reverse transcription cycle at 42^{o}C for 10 minutes followed by 95^{o}C for 8 minutes (enzyme activation), 95^{o}C for 10 seconds (denaturation) and 60^{o}C for 60 seconds (data collection). The C100™ Thermal Cycler (Bio-Rad) used in this study.

3 Results

3.1 The use of RNAi in microalgae to combat WSSV in shrimp

This study focuses on creating an oral feed using microalgae to be used as a preventative method for WSSV control in shrimp, in collaboration with Dr Vanvimon Saksmerprome's lab at CENTEX Shrimp Centre, Mahidol University, Bangkok, Thailand. WSSV is highly virulent in commercially important species of penaeid shrimp (Pradeep *et al.* 2012). The current control methods for WSSV, rely on the use of prevention, to avoid initial introduction of the virus, through the use of nets over outdoor farms and maintenance of biosecurity through clean water practices. To address this issue, this study seeks to develop a prevention method which is cost effective and specific to the virus to prevent initial infection and combat the spread if it was to enter the farm.

The method of using dsRNA as a therapeutic has previously been used when it was expressed in bacteria as a method of producing the dsRNA prior to extraction and used as a treatment for WSSV. One of the earlier targets used for RNAi against WSSV was the major envelope protein VP28 of WSSV (Sarathi *et al.* 2008), which had been previously identified as being involved in the systemic infection in shrimp (van Hulten *et al.* 2001). The method of administering this dsRNA was via intramuscular injection which proved to be successful, and no physical signs of WSSV were visible. 100 % survival was reported in both groups injected with VP28dsRNA and the point of WSSV infection was not a factor as one group was injected with the WSSV and VP28dsRNA simultaneously whereas the other group was injected with VP28dsRNA 24 hours prior to infection. The use of bacterially expressed dsRNA targeting other structural genes of WSSV has also been successful. dsRNA corresponding to structural genes of WSSV; VP26, VP24, VP19 and VP15, was successful in reducing the mortality rate of shrimp (Sarathi *et al.* 2010).

The drawback of this form of dsRNA production is the host organism, as it was expressed in the RNase III-deficient strain of *E. coli*, therefore it must be isolated and purified prior to use as a therapeutic. Therefore, this shows the gap in the market for dsRNA to be expressed in an organism which is 'generally recognised as safe' (GRAS) and could therefore be used as a commercial feed.

Previous work by collaborators showed an effective tool of using dsRNA initially produced by *E. coli* to protect white leg shrimp, against Yellow Head Virus (YHV) another lethal shrimp disease (Saksmerprome *et al.* 2009). This tool was then developed further by using microalgae as an expression system so the microalgae can be used as a whole feed. This development improved survival rate of shrimp as the mortality observed in the control groups was 84.1 % in comparison to the groups fed with dsRNA targeting key YHV genes had a 50 % survival rate (Charoonnart *et al.* 2019). This approach was then used to target WSSV using another key viral gene found in the WSSV, a major envelope protein; VP28 (Zhang *et al.* 2002), previously reported successful in improving survival rates in shrimp when expressed in *E. coli* (Thammasorn *et al.* 2015), however this is not commercially applicable due to the unsuitability of the host organism. dsRNA targeting VP28 was expressed in *C. reinhardii,* and it was shown that shrimp fed with feed supplemented with dsVP28, had a 95.23±0.32 % survival rate in comparison to the positive control group which received a WSSV challenge and no dsRNA feed - this group had a survival rate of only 50 %. Surprisingly, the other group fed with dsRNA not targeting WSSV, also showed a high survival rate, possibly indicating the benefits of feeding microalgae (Charoonnart *et al.* 2023), or possibly the impacts of dsRNA on upregulation of immunity. Using these tools developed by our collaborator this work expands on the use of *C. reinhardtii* expressing dsRNA to combat WSSV control in shrimp.

3.1.1 Aim: Generating a transgenic microalgae expressing two dsRNA specific to WSSV

This project aimed to generate a transgenic strain of microalgae via chloroplast transformation, expressing two dsRNA cassettes, harnessing the mechanism of RNAi, to target gene silencing of the WSSV in shrimp alongside a selection marker which reduces the need for antibiotics when culturing *C. reinhardtii*. Once generated the microalgae expressing sequence specific dsRNA will then be fed to shrimp as an oral feed in challenge trials carried out at CENTEX, Thailand. The results from this can then be used to determine the effectiveness of feeding microalgae expressing dsRNA specific to WSSV when challenged with the virus.

3.1.2 WSSV dsRNA targets used in this study

The two dsRNA cassettes used here were named ORF366 and VP9, both of which were used to target genes of the WSSV using RNAi. WSSV vaccines have already been and are still being researched; however, there has been no complete successes, therefore here we harness the mechanisms of RNAi and express two dsRNA virus specific cassettes in *C. reinhardtii,* a GRAS organism, to target the WSSV and therefore silence the viral genes, thus halting infection. The use of a GRAS organism means the dsRNA expressed by *C. reinhardtii* does not need to be isolated and purified prior to feeding, it can instead be used as a whole organism oral feed. The transformations of *C. reinhardtii,* were targeted to the chloroplast genome, as it lacks the RNAi machinery (Maul *et al.* 2002), therefore if generated in the nucleus, disruption of dsRNA production would be expected. This is a method used previously which showed high levels of dsRNA production (Charoonnart *et al.* 2019). The use of specific dsRNA allows for direct targeting of the WSSV and therefore reduces undesirable toxicity to the host (Sarathi *et al.* 2008). VP9 is a non-structural WSSV protein, predicted to be required for replication of the viral genome, production of viral particles and inhibition of host cell functions (Alenton *et al.* 2016). Targeting a protein required for viral replication is key to

stopping the virus taking hold within the organism. To further enhance viral prevention a second dsRNA was used, in this case; ORF366. ORF366 is open reading frame 366 in the WSSV genome, known to encode a fragment of the WSSV capsid protein (Utari *et al.* 2017). This combination of dsRNA was designed to prevent viral infection of the host when used as an oral vaccine.

In standard techniques, selection markers are used when transforming microalgae, often in the form of antibiotics, auxotrophic selection or fluorescence; however, there are concerns surrounding these methods. Concerns include the possibility of horizontal gene transfer to other organisms in the environment, although the risk is thought to be very low (Miki and McHugh 2004). To avoid these issues, recent advances have used ecologically safe selection markers such a purple chromoprotein, which offers section based on phenotypic coloration (Shih *et al.* 2015).

To avoid the use of antimicrobial selection markers, this study utilises a media selection marker taken from *Pseudomonas stutzeri* WM88; *ptxD*, a bacterial gene which encodes phosphite oxidoreductase which uses NAD+ as a cofactor to oxidise phosphite (Phi) to phosphate (Pi) (Costas, White and Metcalf 2001). In standard practice, phosphate is used when preparing TAP media to culture *C. reinhardtii,* which is the sole source of phosphorus used by the microalgae. Microalgae can actively import phosphite from media however they are unable to use it as a source of phosphorus, therefore microalgae which have been transformed with this selection marker acquire the ability to metabolise Phi (López-Arredondo and Herrera-Estrella 2012). The use of this selection marker also reduces the amount of false positives and enhances the culture sterility as many microorganisms are unable to utilise Phi. This sterile environment would be maintained as it has been shown that it provides a metabolic advantage to the organism and therefore outcompetes

contaminating organisms (González-Morales *et al.* 2020). Previous expression of the *ptxD* gene in *C. reinhardtii* has shown that microalgae are able to use Phi as their sole source of phosphorus and it was later used as a successful selection marker for chloroplast transformations (Sandoval-Vargas *et al.* 2019). This selection marker was chosen due to the possibility of using this media without the need for sterilisation, therefore offering a cheaper alternative which would be an advantage for its use in countries throughout SE Asia.

3.1.3 Plasmid construction to produce dsRNA in microalgae

The initial stage of the project aimed to produce two strains of microalgae, the first is referred to as TN72-ORF366, which then underwent a second chloroplast transformation to express a second cassette of dsRNA in the form of VP9 with the selection marker *ptxD*. The second is the reverse; a background strain of TN72 already expressing dsRNA specific to *VP9*; TN72-dsVP9 which was subject to chloroplast transformation to introduce the second dsRNA cassette of dsORF366 alongside the *ptxD* selection marker. Both strains were generated to establish whether there was a difference between expression. The background strains used here (TN72-dsVP9 and TN72-dsORF366) were generated by a previous laboratory member; Conner Webb, using strain CC-5168 (*cw15,* Δ*psbH*), referred to here as TN72.

To generate dsRNA, a method using p2XTRBL was used, this is a system of inverted promotor and terminator elements to produce dsRNA from a linear sequence, provided by Prof Saul Purton at UCL. The construct consists of mRFP; monomeric Red Fluorescent Protein, under the control of two inverted 16S promoters and terminators to generate dsRNA. This vector was used previously to generate the TN72-dsVP9 and TN72-dsORF366. However, for this study, this vector was modified further, the 2XTRBL elements were amplified and assembled into a vector containing *ptxD* as well as an ampicillin resistance marker, provided by UCL (plasmid pP03). This was used as the backbone strain and amplified using Phusion PCR,

primers used were ptxD_vector_F & ptxD_vector_R. The next step was to insert the 2XTRBL elements which were also amplified from a storage vector using primers; 2XTRBL_overlap_F & 2XTRBL_overlap_R. All primers were designed using an overhang technique so the assembly can be generated using Gibson Assembly. As this plasmid was to be used for insertion into the chloroplast, the usual method of chloroplast transformation in the lab used the insertion site of *aadA* coupled with *psbH* to restore photosynthesis, however the background strains used here; TN72-dsVP9 and TN72-dsORF366 had already been photosynthetically restored therefore another insertion site was needed. The plasmid generated (figure 15) contained the *ptxD* gene, flanked either side by the homology arms *psaA-3* and *trnL ccsA*, and therefore inserted the construct containing *ptxD* and 2XTRBL into a neutral location on the *C. reinhardtii* plastome between *psaA-3* and *trnL.*

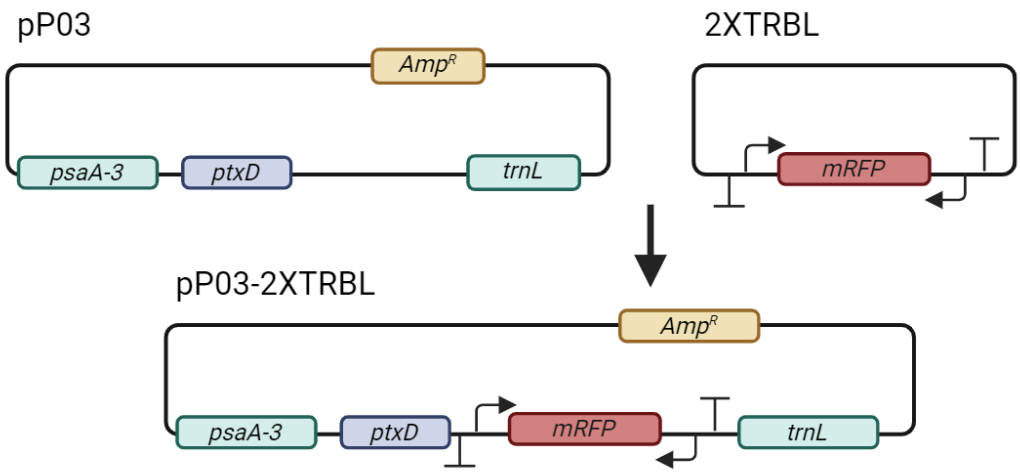

Figure 15. Generation of ptxD-2XTRBL plasmid for dsRNA expression.
The pP03-2XTRBL plasmid constructed using backbone pP03 with mRFP under the control of inverted 16s promotor and terminator regions used to generate dsRNA. The final construct consists of the selection marker ptxD and mRFP which can be modified using Golden Gate due to restriction sites between the promotor and gene. (Image designed in BioRender).

The gene under the control of the 2XTRBL elements was mRFP, see figure 15, which generates a pink pigment in *E. coli*, hence pink colonies would be expected. The next step in this cloning process was to replace the mRFP by the gene of interest in this case either VP9

or ORF366. The design of this vector for use by Golden Gate cloning allows for rapid generation of different dsRNAs in place of mRFP. Both fragments of DNA were amplified using VP9_F & VP9_R and ORF366_F & ORF366_R respectively. Once amplified the VP9 fragment was introduced into the ptxD-2XTRBL plasmid in place of the mRFP using a one pot Golden Gate reaction with the type IIS restriction enzyme Esp31. The mRFP works here as a selection marker as if the gene of interest; VP9, has been successfully integrated into the cut site the colonies would be white as the mRFP would have been removed and replaced by VP9, whereas negative colonies would be pink, therefore producing a colorimetric method of selection (Figure 16).

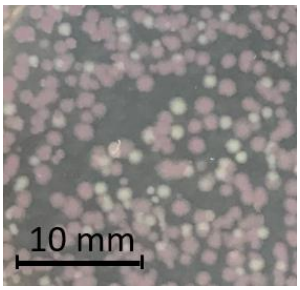

Figure 16. Pink white selection on LB agar from Golden Gate reaction
LB agar plate showing pink white selection from a Golden Gate reaction. Red colonies indicate unsuccessful cloning and white colonies indicate positive cloning as the RFP has been disrupted and replaced.

The outline of the constructs made can be seen in figure 17, which shows the convergent 16s promoters which produces dsRNA.

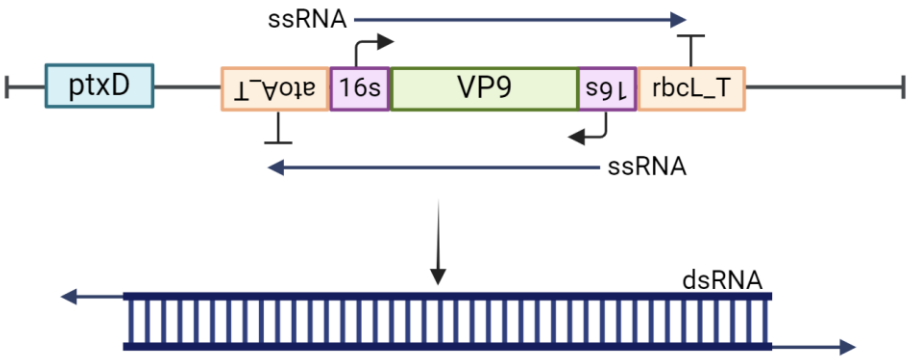

Figure 17. Outline of dsRNA construct used in this study
Schematic of the dsRNA construct used in this study to express dsRNA, *ptxD* selection marker alongside *VP9* under the control of two convergent 16s promotors which produces two strands of complementary ssRNA. (Image designed in BioRender).

Prior to *C. reinhardtii* chloroplast transformation the plasmids had to be generated, one using Golden Gate and the other using Gibson Assembly. Following construction, the plasmids were sequenced prior to use in chloroplast transformations.

3.1.4 *C. reinhardtii* chloroplast transformation and screening

In order to produce a strain of *C. reinhardtii* expressing two different dsRNAs to target WSSV, a background strain was used first, in this case TN72-dsVP9. As discussed this strain was generated using photosynthetic restoration based on the *psbH* knockout. This strain was then used for the next transformation performed for this study. The plasmid used was pHS6; ptxD-ORF366 in pPO3, which was transformed into TN72 dsVP9 using the glass bead method. The transgenic microalgae were selected due to the *ptxD* insertion based on their ability to grow in the presence of phosphite. Due to the nature of the selection marker, it was important to ensure all traces of phosphate were removed from the media. A 20 ml pre culture was grown in TAP media, and this was used to inoculate a 400 ml TAP culture. Once the cell density had reached around 1.5-2 X 10^6 cells/ml, the culture was centrifuged to remove TAP media which was replaced with TA media, lacking phosphate. The culture was then incubated for a further 24 hrs, for a phosphate starvation period, and was then ready

for transformation. After unsuccessful transformations without the phosphate starvation step, this 24 hr period proved crucial for achieving successful gene integration. Multiple rounds of transformation were performed, and colonies were selected for based on *ptxD* selection. Many colonies produced false positives, and grew on TAPhi media but were negative when screened by PCR. See figure 18 for transformation screening.

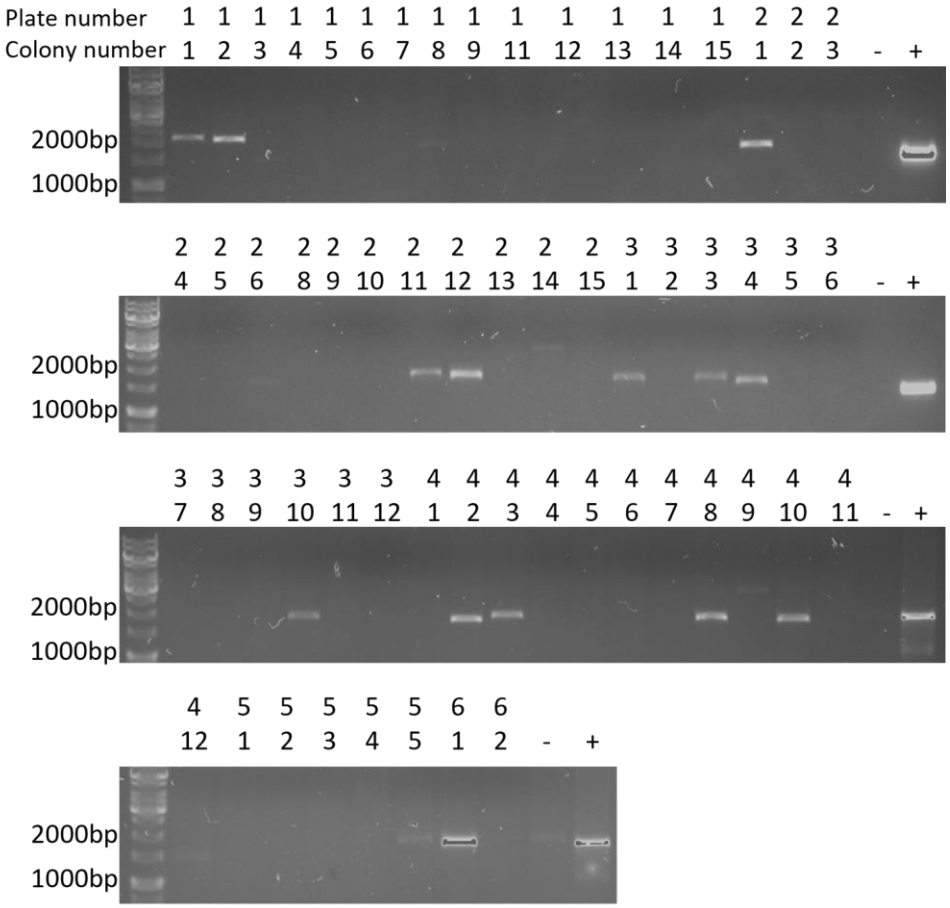

Figure 18. PCR amplification of ptxD from suspected transformants
PCR amplification of suspected transformed *C. reinhardtii* with ptxD-dsVP9 using primers to amplify *ptxD* region. Expected product size of 1466bp. The top number corresponds to the plate number the colony was taken from and the bottom number is the colony number. Negative sample: TN72-dsORF366.

Figure 18 highlights the varying number of false positives taken from transformation selection plates. Prior to DNA extraction, single colonies were streaked out onto a fresh selection plate to increase biomass ready for DNA extractions. All of the extractions and PCRs

were performed on colonies which had enough biomass for DNA extraction on the second selection plate and therefore, should contain the *ptxD* gene; however the results from the PCR do not support this. There are however, some possible transformants as seen from the PCR. The positive sample used here is the plasmid used to transform the microalgae, as it amplifies a fragment of 1466 bp. This band is seen in multiple samples; plate 1 colony 1 (1/1), plate 2 colony 2 (2/2), 1/1, 1/2, 2/1, 2/11, 2/12 etc.

The colonies which amplified a fragment using the ptxD primers, were re-streaked onto fresh selective media and following a week's incubation a second DNA extraction was performed. A repeat PCR was then performed, using the primers used previously ptxD_F & ptxD_R as well as ptxD_F & VP9 R, which amplifies both the start of ptxD through to the end of VP9. The results from this PCR can be seen in the figure 19.

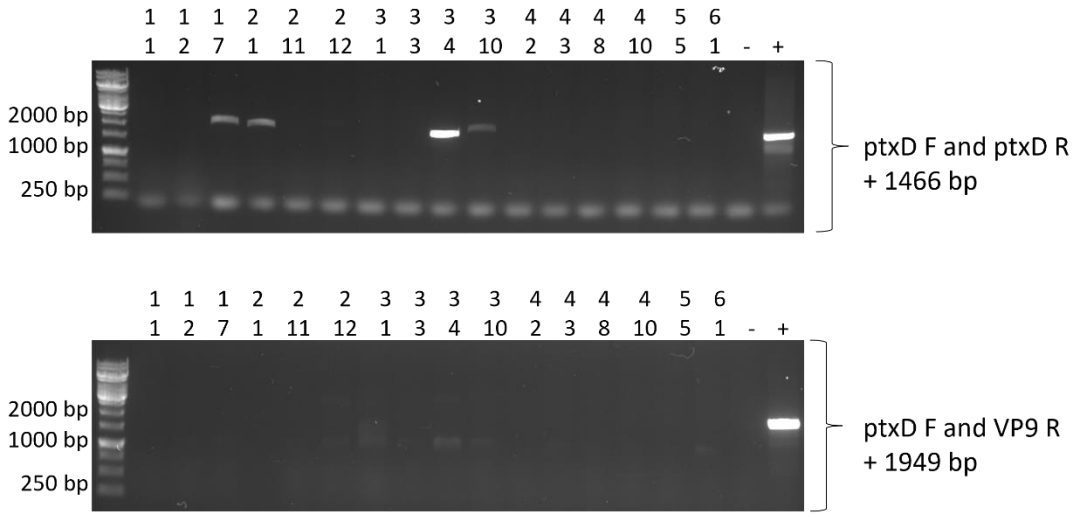

Figure 19. PCR amplification of TN72-dsRNA transformed with ptxD-dsVP9
PCR amplification of suspected transformed *C. reinhardtii* with ptxD-dsVP9 using two primer sets. The negative (-) sample was a total DNA extraction from the background strain of TN72 dsORF366, and the positive (+) sample was the construct used for chloroplast transformation.

As seen in figure 19, many of the colonies which previously amplified using the *ptxD* specific primers in the figure 18, did not amplify using ptxD_F & VP9R, possibly indicating a loss of

the transgene after re-streaking onto fresh selection media as many of them were also negative for the repeated ptxD_F & R.

The reverse transformation was also performed; TN72-dsVP9 with ptxD-dsORF366. Following transformation, the colonies were transferred to fresh selection media to build biomass. A total DNA extraction was performed and a PCR conducted using primer set ptxD_F and ORF366_R which amplifies a fragment of 1946 bp, another primer set was also used ptxD_VP9/Orf366_Seq_F & R which amplifies the region starting in *ptxD* and ends after either the *VP9* or *ORF366* insert, which is 2122 bp for ORF366 insertion, see figure 20.

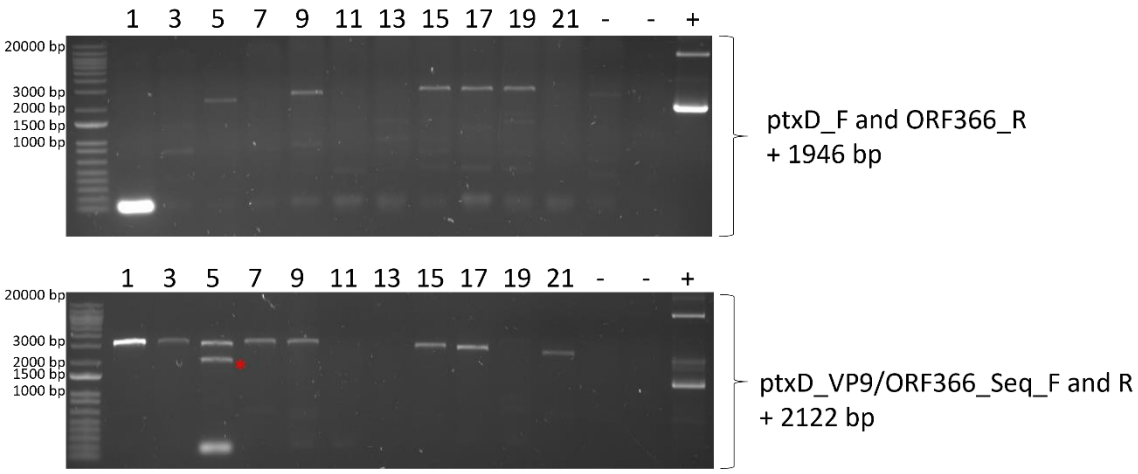

Figure 20. PCR amplification of TN72dsVP9 + ptxd-dsORF366
PCR amplification of suspected transformed *C. reinhardtii* TN72-dsVP9 with ptxD-dsOFR366 using two primer sets. The negative (-) sample used here was from the background strain of TN72 dsVP9, and the positive (+) sample was the construct used for chloroplast transformation. *possible band of interest for ptxD_VP9/OFR366_Seq_F & R amplification.

Following multiple transformations, the use of a phosphate starvation period proved essential to generating a positive transformant. The results from a PCR to screen for the presence of dsORF366 in a suspected TN72-dsVP9-ptxD-dsORF366 are presented in figure 21.

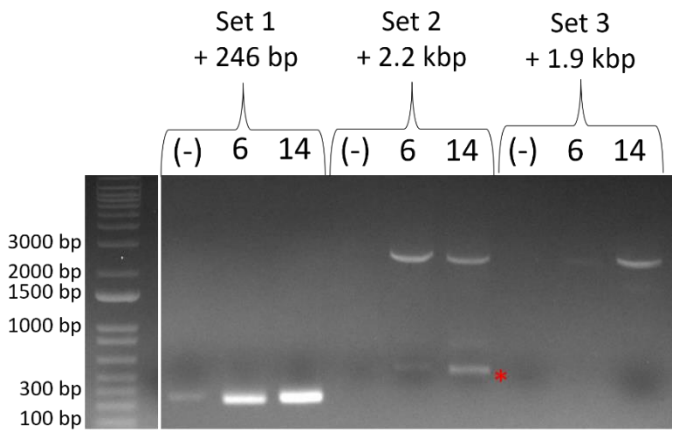

Figure 21. PCR to screen for insertion of ptxD-dsORF366 in TN72-dsVP9
PCR amplification of suspected transformed *C. reinhardtii* TN72-dsVP9 with ptxD-dsOFR366 using three primer sets; set 1) ORF366_F & OFR366_RNA_R, set 2) ptxD_VP9/OFR366_Seq_F & ORF366_RNA_ R and set 3) ptxD_F & ORF366_RNA_R. The negative (-) sample used here was the background strain of TN72 dsVP9. *possible non-specific binding.

Three primer sets were used to screen the two suspected transformation set 1 using primers ORF366_F & OFR366_RNA_R which gives a positive amplification of 246 bp, set 2) ptxD_VP9/OFR366_Seq_F & ORF366_RNA_ R which amplifies a fragment of 2122 bp, set 3) ptxD_F & ORF366_RNA_R which amplifies a fragment of 1946bp. All three primer sets are specific to the ORF366 fragment and thus no amplification was expected in the negative control which was the background strain used for this transformation; TN72-dsVP9. There is however a faint band in the negative control of the first primer set. There also appears to be non-specific amplification in both samples 6 and 14 when amplified using the second primer set, as highlighted by the asterisk. After PCR the DNA extractions were amplified and sent for sequencing which confirmed insertion of *ptxD* and ORF366.

3.1.5 Double stranded RNA detection and quantification from transgenic *C. reinhardtii*

Once the construct was confirmed as a stable transformant in *C. reinhardtii*, the next step in the analysis was to confirm expression of dsRNA from both the dsVP9 and dsORF366. A total RNA extraction was performed using the method detailed in section 2.3.7. Following total

RNA extraction, the samples were treated with DNase I and RNase A to remove both DNA and ssRNA. DNase I treatment was often performed twice to confirm total removal of DNA. A PCR was then completed to confirm that all DNA had been removed from the sample, as seen in figure 22.

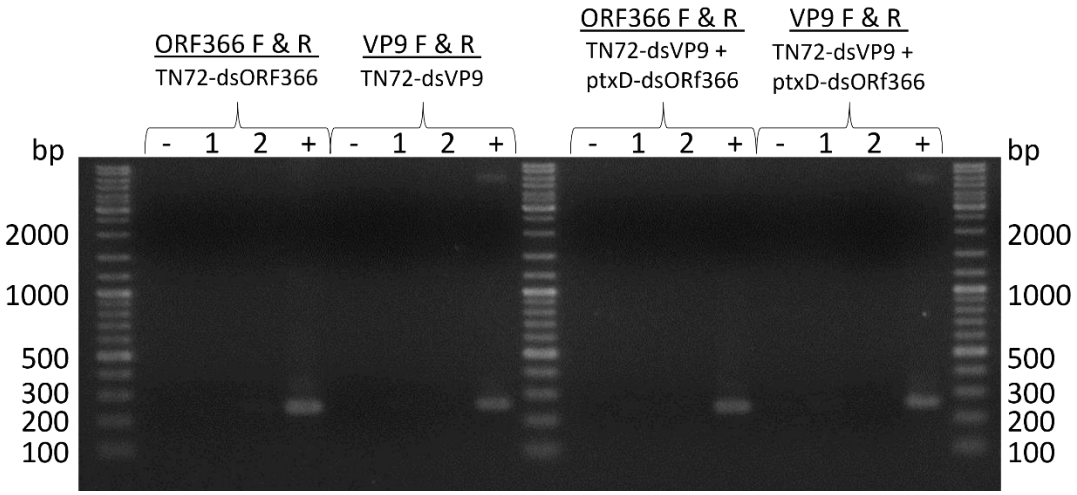

Figure 22. PCR to confirm absence of DNA in RNA extractions from *C. reinhardtii* expressing dsRNA
PCR to confirm removal of DNA following DNAse I treatment of total RNA extraction from both the background strains; TN72-dsORF366 and TN72-dsVP9 as well as TN72-dsVP9 + ptxD-dsORF366. Negative sample is TN72 total RNA extraction and positive sample is the DNA used for chloroplast transformation. ORF366 F and R amplifies a fragment of 246 bp and VP9 F and R amplifies a fragment of 255 bp.

As seen above, the PCR to confirm there was no DNA remaining in the samples following total RNA extraction, showed a clear result following a repeated DNAse I digest. Presence of RNA was then confirmed by RT-PCR (figure 23). RNase III digests were also performed to confirm the presence of dsRNA as RNase III degrades dsRNA.

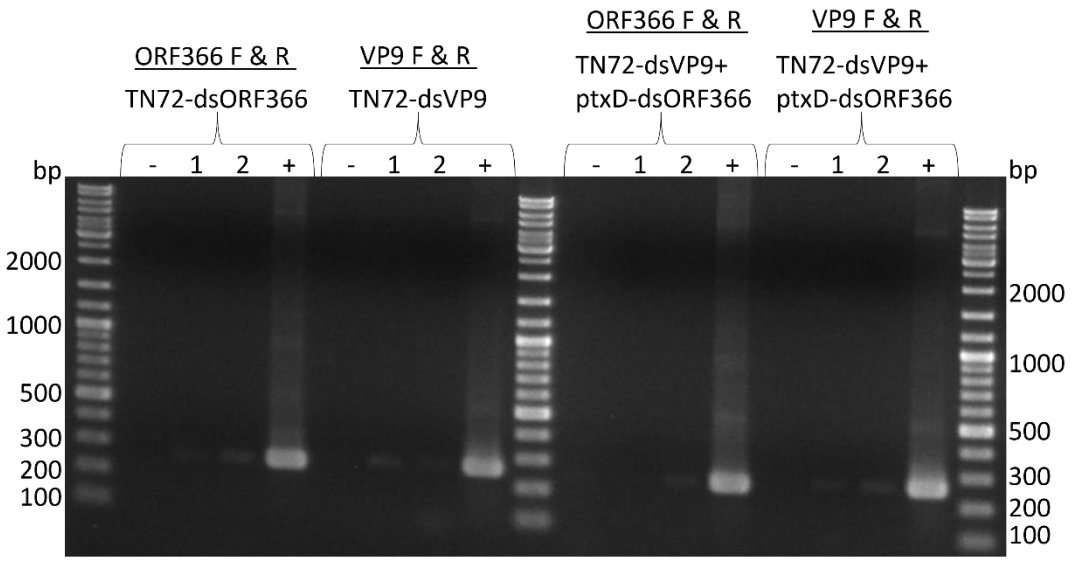

Figure 23. RT-PCR to confirm presence of dsRNA in *C. reinhardtii*
RT-PCR to confirm the presence of dsVP9 and dsORF366 in both the parental strains TN72-dsORF366, TN72-dsVP9 as well as the dual dsRNA strain; TN72-dsVP9 + ptxD-dsORF366. Negative sample used here was the TN72 strain to show no presence of ORF366 and VP9 specific dsRNA, positive controls were plasmids used for transformation.

RT-PCR was used to confirm the presence of dsRNA of both VP9 and ORF366 in both the background strains; TN72-dsORF366 and TN72-dsVP9 as well as the dual transformant; TN72-dsVP9 + ptxD-dsORF366 which was screened by RT-PCR using both primer sets. The negative sample used here is TN72 which does not have any dsRNA cassettes, as well as the positive sample used here as DNA. Both TN72-dsORF366 and TN72-dsVP9 show amplification in both replicates 1 and 2, as expected as this was the background strain used for the second transformation. The presence of dsRNA specific to both the VP9 and ORF366 in the dual transformation was also shown in figure 23.

Following confirmation of dsRNA from both VP9 and ORF366, this strain of microalgae was used in RT-qPCR to quantify the amount of dsRNA produced by the microalgae. The first step in RT-qPCR was to generate standards from *E. coli,* to produce a standard curve which can then be used to calculate the amount of dsRNA produced in the microalgae. The 2XTRBL

constructs; one containing dsVP9 and one containing dsORF366, were transformed into the mutant *E. coli* strain HT115 (DE3) which is defective in RNAse III which targets and degrades dsRNA, therefore the deficient strain allows for the production of dsRNA. RNA extractions were performed from these transformed *E. coli* to produce dsRNA to be used for standard curve generation for RT-qPCR seen later in the results. The same process as above was carried out, first step was isolating the RNA, running an RT-PCR, then RT-qPCR.

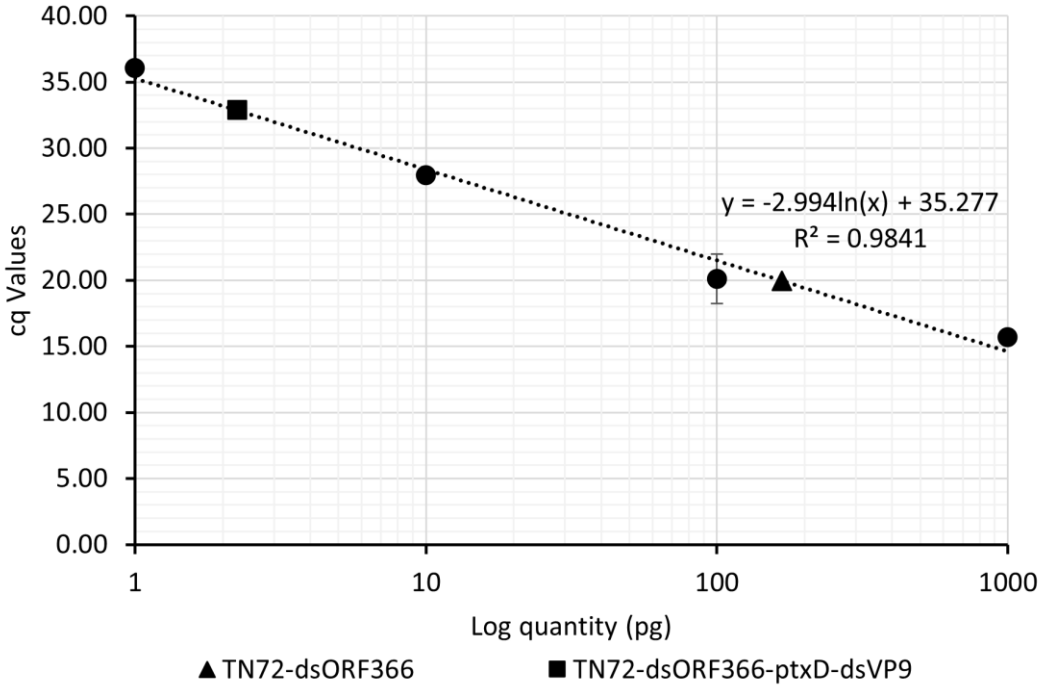

Figure 24. Standard curve from RT-qPCR for dsORF366
Standard curved of dsORF366 produced using dsRNA expressed in the RNase III deficient HT115 *E. coli* strain. Logarithmic scale of quantity from 1 pg to 1 ng. R^2 value of the curve is shown at 0.9841. Both samples of microalgae expressing dsRNA ORF366 are plotted here.

The dsRNA produced in the RNase III deficient strain HT115 were used to generate a standard curved of dsRNA ranging from 1 pg to 1 ng, by RT-qPCR. This was then used to plot the dsRNA extracted from both TN72-dsORF366 and TN72-dsORF366-ptxD-dsVP9. Following this, the amount of dsRNA was calculated using the volume of culture used to express the dsRNA. The TN72-dsORF366 strain was used here as a comparison of a 'single' dsRNA transformant to the dual transformant; TN72-dsORF366-ptxD-dsVP9. TN72-dsORF366 produces 483.16±0.64

ng/L of dsORF366 and the dual transformant produces less; 6.5±0.32 ng/L. This can be seen in figure 26.

The standard curve process was also carried out for dsVP9, and plotted below in figure 25.

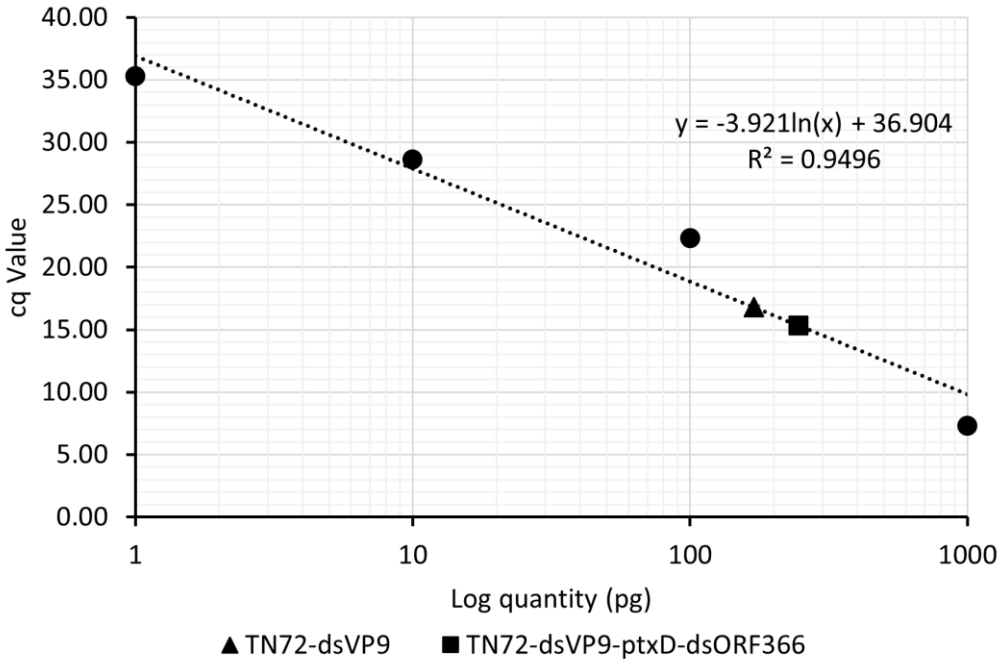

Figure 25. Standard curve from RT-qPCR from dsVP9
Standard curved of dsVP9 produced using dsRNA expressed in the RNase III deficient HT115 *E. coli* strain. Logarithmic scale of quantity from 1 pg to 1 ng. R^2 value of the curve is shown at 0.9496. Both samples of microalgae expressing dsRNA VP9 are plotted here.

E. coli HT115 was used to generate a standard curved of dsRNA VP9, ranging from 1 pg to 1 ng, by RT-qPCR. This was then used to plot the dsRNA extracted from both TN72-dsVP9 and TN72-dsORF366-ptxD-dsVP9. From this experiment the yields were calculated which indicated a much higher yield of dsVP9 than dsORF366. Per 1 L of culture of TN72-dsVP9, 491.18±0.17 ng of dsRNA were calculate and from the TN72-dsORF366-ptxD-dsVP9 strain it was calculated as 713.31±0.02 ng/L, figure 26.

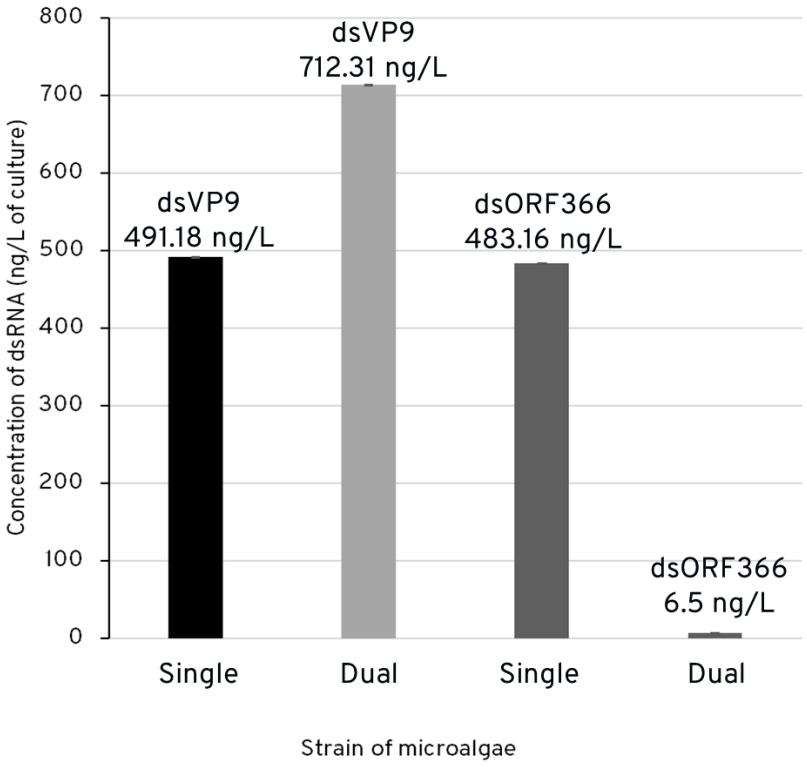

Figure 26. Comparison of dsRNA yields from TN72-dsVP9, TN72-dsORF366 to the dual expressing microalgae strain TN72-dsORF366-ptxD-dsVP9.
Concentration of dsRNA per L of microalgae culture, calculated from RT-qPCR of both the single expressing strains TN72-dsVP9 and TN72-dsORF366 compared to the dual expressing strain TN72-dsORF366-ptxD-dsVP9.

Figure 26 displays the total level of dsRNA calculated per L of microalgae, comparing the single dsRNA expressing strain (either dsVP9 or dsORF366) in comparison to the yields of the respective dsRNA from the dual strain; dsVP9-ptxD-dsORF366. A two sample t-test was conducted to compare the difference between using the single and the dual dsRNA expressing strain. The mean performance from the single dsVP9 group (M= 491.18, SD= 0.170) was significantly lower that the dual dsVP9 strain (M= 712.31, SD= 0.020), t (1.03) = -1830.76, p= 0.00028, therefore confirming the dual expressing strain generates more dsVP9 than the single dsVP9. This test was also carried out for the single dsORF366 (M=483.16, SD=0.636) was significantly higher than the dual dsORF366 group (M=6.5, SD=0.204), t(1.20)=1009.82, p=0.00016. Here we can conclude that there is a significant difference in the dsRNA produced from the single dsRNA group to the dual expressing dsRNA group.

3.1.6 WSSV challenge trials

Two challenge trials were carried out during this work, both conducted at CENTEX, Thailand. Both of these challenge trials were carried out under the supervision of Dr Patai Charoonart to test the effectiveness of dsRNA expressing microalgae strains produced at the University of Kent. The challenge trials were conducted to determine if feeding shrimp microalgae expressing dsRNA to target WSSV can be used as a viral control strategy, utilising the RNAi mechanism.

3.1.6.1 WSSV challenge trial using liquid feed containing dsRNA

This challenge trial was the first conducted for this project, consisted of challenging shrimp which have been fed a 'liquid' microalgae feed which contained microalgae cells suspended in media, expressing dsRNA specific to WSSV.

The selected microalgae biomass from a selection plate was used to inoculate TAP media which was then grown until ready for preparation prior to feeding. The cultures were pelleted once OD_{750} 2.0 was achieved, and resuspended to achieve a cell density of 4 X 10^9 cells/ml. The liquid feed was maintained at 4^{o}C throughout the trial to ensure the stability of the dsRNA. Six groups were used in this study; positive (no algal treatment), negative (no algal treatment), control dsRNA (fed photosynthetically restored microalgae, dsRFP) ORF366 (fed algae expressing dsORF366), VP9 (fed algae expressing dsVP9), VP9-ORF366 (fed algae expressing both dsVP9 and dsORF366). Shrimp were fed twice daily with commercial shrimp feed and the treated shrimp were given an additional feed of algae expressing the stated dsRNA. On day 0 all groups other than the negative control were challenged with WSSV. WSSV inoculum was prepared by feeding juvenile shrimp with WSSV infected tissue at 10% of shrimp body weight. The shrimp were then collected, homogenised and stored at -80^{o}C. This infected tissue was used to infect shrimps at 50% body weight of the shrimp. Following

viral challenge, shrimp numbers were observed daily for mortality and signs of infection. It was concluded the shrimp should be challenged again with WSSV on day 4 due to a low initial viral inoculum. The results from this shrimp challenge trial were plotted in figure 27 using an average shrimp survival rate in percentage recorded on the day of challenge with WSSV and monitored for ten days.

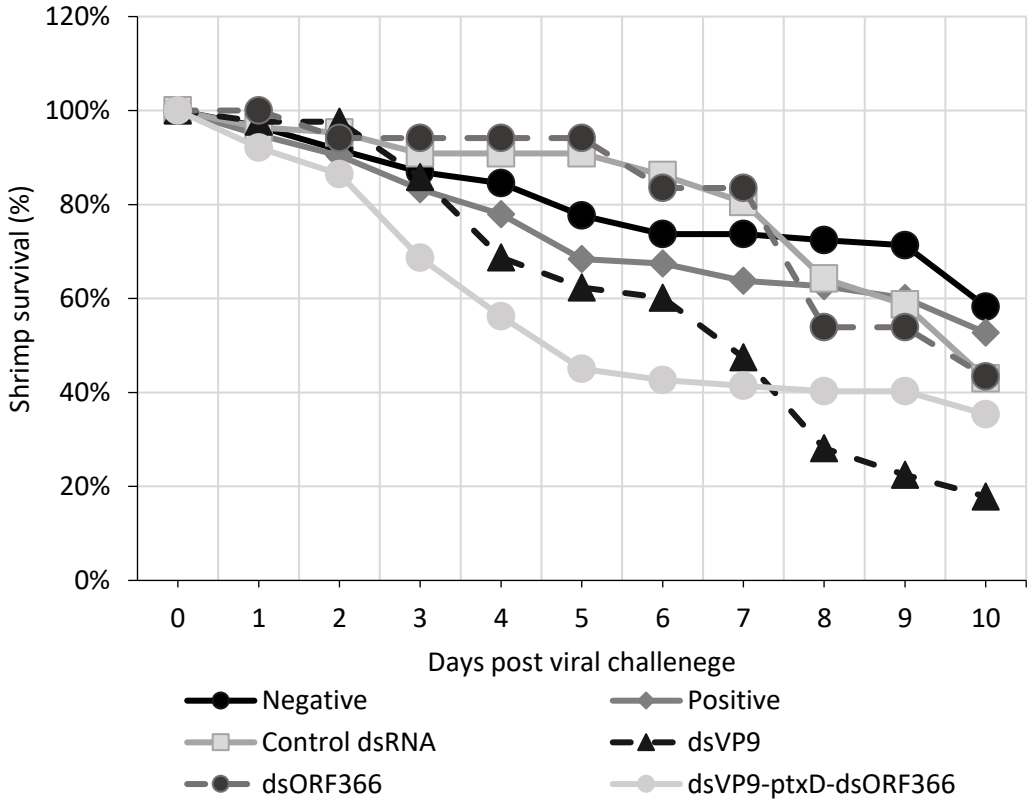

Figure 27. Trial 1- Shrimp survival rate post infection with WSSV
Average shrimp survival rate from 4 replicates per group over the ten days post challenge with WSSV. In addition to commercial feed, groups control dsRNA, dsVP9, dsORF366 and dsVP9-ptxD-dsORF366 were fed with ~1 x 10^9 cells/tank of the stated microalgae feed. Shrimp mortality was observed daily following WSSV challenge on day 4.

The results above show the average percentage survival rate from all four replicates of each group. As seen above, the group which had the lowest survival rate was the group fed with dsRNA VP9. As anticipated the group which had the highest survival rate was the negative group, however they did experience a drop to 60 % survival rate which is lower than

expected, therefore indicating the health of the shrimps prior to challenging was not at an optimum, as the negative group were not challenged with the WSSV therefore would be expected to have a higher survival rate.

All groups followed the general trend of a drop of survival rate especially after day three, however the group fed with the control microalgae and the negative group, which received no algal feed, experienced this drop later at around day 7. This could be due to the re-challenge of the virus at day 4, which was carried out due to a lower viral rate on the first challenge at day 0. Although there was expected to be a large difference in survival rate between the negative and positive group, it can be observed that there was a very similar trend of survival rate between them. The negative group had not been challenged with the virus therefore, it can be assumed the low survival rate was due to another factor impacting the shrimp's health. These factors could include water quality, shrimp health prior to the trial, however both of which were mitigated against as the water was changed every 3-4 days to ensure a clean environment and the shrimp were also acclimatised in quarantine tanks prior to the experiment to ensure they were healthy. The results recorded on day 10 were reported in a box and whisker plot, to see the distribution of the data, as seen in the figure 28.

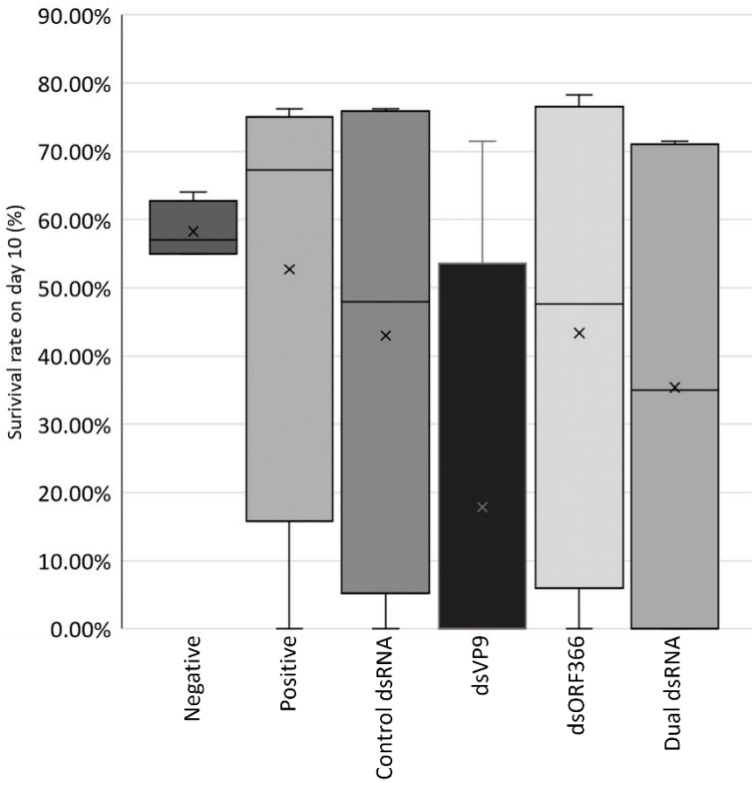

Figure 28. Trial 1- Box and whisker plot of shrimp survival rate from WSSV challenge trial on day ten

Shrimp survival rate recorded on day 10 day post viral challenge with WSSV. Six groups for the trial; negative group which received commercial shrimp feed and no viral challenge, positive group which were fed commercial shrimp feed and WSSV viral challenge, then four dsRNA groups which received WSSV challenge; control dsRNA group which were fed microalgae without dsRNA, dsORF366, dsVP9 and the dual dsRNA of dsVP9-ptxD-dsORF366.

The results from the box and whisker plot show that all the groups from this experiment do not show a normal distribution of the data. Figure 28 highlights the distribution of the data, of all the groups but the negative, which suggests that there is great variability within each group.

This experiment was carried out using four replicates of each condition to help obtain an average between the separate tanks, however there was a wide range within the results, therefore, the raw data showing the individual tank survival number has been recorded here in table 16.

Table 16. Raw data showing shrimp survival number post challenge with WSSV.

Days post infection (dpi)	N 1	N 2	N 3	N 4	P 1	P 2	P 3	P 4	C 1	C 2	C 3	C 4	V 1	V 2	V 3	V 4	O 1	O 2	O 3	O 4	VO 1	VO 2	VO 3	VO 4
0	20	20	25	22	21	21	25	19	21	24	20	21	24	21	21	24	21	21	21	23	24	21	21	20
1	20	20	25	19	21	20	21	19	20	24	19	20	24	19	21	24	21	21	21	23	21	19	19	20
2	19	17	25	19	19	20	19	19	20	23	19	20	24	19	21	24	20	21	19	22	19	16	19	20
3	18	16	24	18	19	19	13	19	19	21	19	19	16	19	19	23	20	20	19	22	10	11	19	18
4	18	15	24	17	19	19	9	18	19	21	19	19	3	17	18	23	20	20	19	22	5	7	18	17
5	15	15	22	16	18	19	2	17	19	21	19	19	0	16	18	21	20	20	19	22	0	3	18	16
6	15	15	18	16	18	19	1	17	16	20	19	19	0	15	18	20	17	19	19	22	0	1	18	16
7	15	15	18	16	18	19	0	15	15	18	18	18	0	7	18	17	15	17	19	21	0	0	18	16
8	15	14	18	16	17	19	0	15	5	15	17	18	0	3	18	3	3	12	18	20	0	0	17	16
9	15	14	18	15	17	18	0	14	3	12	17	18	0	0	18	1	1	9	17	20	0	0	17	16
10	11	11	16	13	15	16	0	12	0	5	15	16	0	0	15	0	0	5	15	18	0	0	15	14

In the table above, the negative group are labelled as N1- 4, the positive group are labelled P1-4, the control dsRNA group are labelled as C1-4, the group fed algae expressing VP9 dsRNA are labelled as V1-9, as with ORF366 labelled as O1-4. Followed by the double dsRNA group which were fed with algae expressing both sets of dsRNA ORF366 and VP9 was labelled as VO1-4.

This table was included to be able to analyse each group within the experiment separately due to the wide variance in results. At the start of the experiment, 25 shrimp of similar size were put into each 2 L tank, a total of four replicates per group. The shrimp were then fed commercial shrimp feed for four days prior to challenging with WSSV to ensure they acclimatise to the tanks. The shrimp number is first recorded on the day of challenging at the start number, however as seen in the raw data above most of the groups had lost shrimp over these four days, some groups lost up to 5 shrimp such as N1 and N2. As both groups are from the negative group, which received no viral challenge, they were expected to have a high survival rate after the ten days of feeding with commercial shrimp feed, however three of the tanks lost nearly 50% of their total population over this period. Therefore, supporting the suggestion that the health of the shrimp may not have been at an optimum prior to infection with WSSV. When comparing the raw data from the negative to the positive group, three of the groups share a similar trend, as three of the positive tanks number of surviving shrimp only dropped to 12, 15 and 16, which was unexpected for the positive group. The LD 50 is used when preparing inoculum, it is the amount of viral feed required to reduce the shrimp survival rate to below 50%. The positive group is the one we would expect to see a clear drop to less than 50% survival rate in all four groups, however this was only seen in group P3 which experienced the LD50 on day 4.

The control dsRNA group was used to show the effect of feeding non-specific dsRNA whole algal to shrimp as previous research has shown some benefits of feeding algae as a feed (Charoonnart *et al.* 2023). As seen from this group, there is also a large range between the results on day 10. C1 experiences a dramatic drop between day 7-8 and group C2 also showed this drop from 12-5 on days 9-10. In comparison to the other two groups, C3 and C4, both of these remained at a steady rate throughout the experiment and finished with a loss of only 5 shrimp from each group.

The remaining three groups are those fed with *C. reinhardtii* expressing dsRNA, either dsVP9, dsORF366, or the combination of microalgae expressing both VP9 and ORF366 dsRNA. The dsVP9 group ended with the lowest survival rate from the whole experiment, with only one out of the four tanks having any surviving shrimp after the ten-day trial. V1 had a significant drop in survival over days 3-4, V2 had this drop over days 6-7 and V4 on days 7-8. V3 had large amount of shrimp surviving at the end of the trial, losing only 6 shrimp, therefore ending with a survival rate of 71.43 %. This is a very large difference when compared to V1, V2, and V4 which was 0 % survival. Both dsORF366 group and dsVP9-dsORF366 group had a better overall survival rate of both 43.37 % and 35.36 % respectively. Group O1 had an overall survival of 0 %, after a steep drop from 15-3 over days 7-8, similar to O2 which dropped to just 5 shrimp remaining after 10 days. O3 and O4 had a much higher survival rate of 71.43 % and 78.26 % respectively, possibly showing a positive impact after being fed with ORF366 dsRNA. A similar survival rate is also shown in the VP9-ORF366 groups. The first two tanks, VO1 and VO2, dropped to 0 % survival on day 5 for group 1 and day 7 for group 2. However, the second two tanks only lost 6 shrimps each over the ten days, and achieved a survival rate of 71.43 % and 70 % respectively, therefore indicating the feed containing dsRNA could provide a benefit to the shrimp to help tackle the WSSV infection.

The results from all groups fed with *C. reinhardtii* expressing dsRNA, could indicate that the inoculum of viral shrimp meat could have been too high however when comparing the results to the positive group which achieved a relatively high survival rate, with only one of the groups achieved LD50, therefore the strength of the inoculum can be ruled out as a factor contributing to the low survival rate.

The overall results from this challenge trial, showed no clear benefit of feeding microalgae expressing dsRNA VP9 however, the other microalgae groups had a higher survival rate with on average, two out of the four tanks having a relatively high survival rate. However, the positive group also maintained a relatively high survival rate, therefore questioning the efficiency of the dsRNA feed. In summary it is evident that there are issues with this experiment and the cause of variation between the results remains unclear and would thus require repeated challenge trials.

3.1.6.2 WSSV challenge trial using dried feed containing dsRNA

The second challenge trial used dried algae mixed with commercial shrimp feed to form a ground feed in comparison to the liquid feed as used previously. The commercial shrimp feed was ground and mixed with the freeze-dried algae; shrimp oil was also added for additional flavour to encourage the shrimp to eat the feed. Images of the shrimp feed can be seen in figure 29.

Figure 29. Shrimp feed for WSSV challenge trial
Freeze-dried algae were blended in a pestle and mortar with commercial shrimp feed using a 1:1 ratio, following this, squid oil was added to improve attractiveness of the feed.

The results showing the overall trend of survival rate from day 0 to 6 post challenge with WSSV can be seen in figure 30.

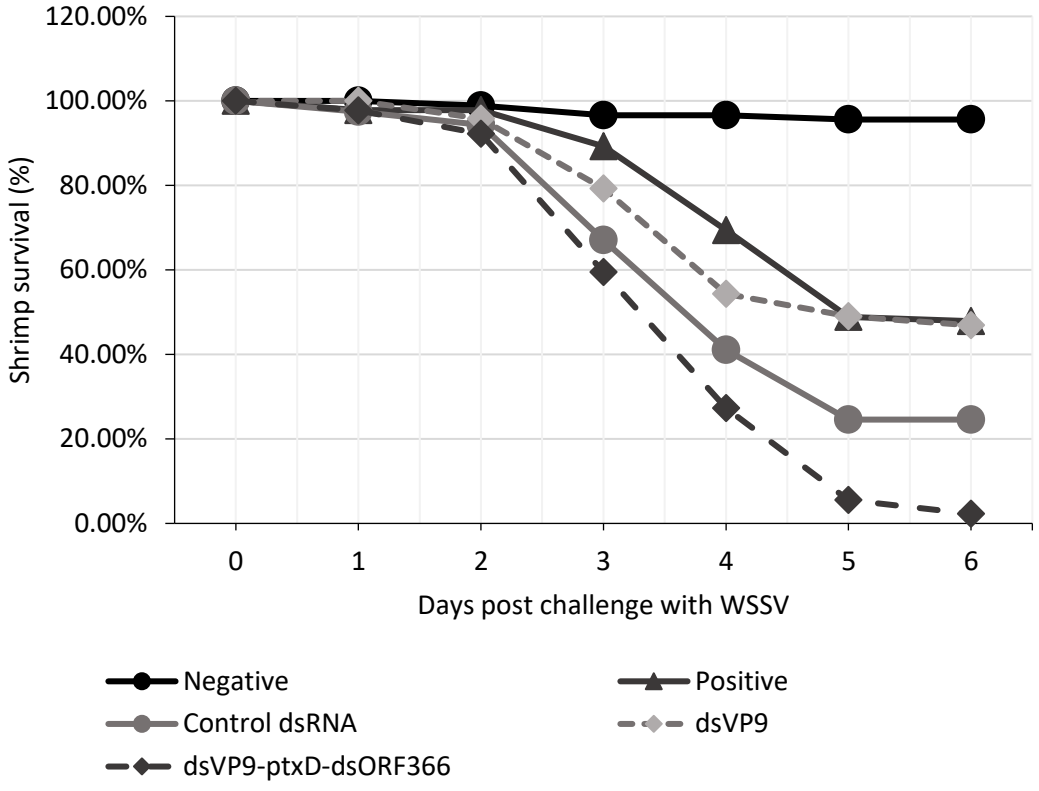

Figure 30. Trial 2- Shrimp survival rate post infection with WSSV
Average shrimp survival rate from 4 replicates per group over the 6 days post challenge with WSSV. In addition to commercial feed, groups dsVP9, and dsVP9-ptxD-dsORF366 were fed with a commercial feed/dried microalgae feed.

As seen in the results above, the group which finished the trial, 6 days post challenge, with the highest survival rate was the negative group which was the only group which was not exposed to WSSV. This result was as expected, suggesting the other conditions of the experiment were at an almost optimum condition, such as water quality, shrimp health prior to challenge and food consumption of commercial shrimp feed. The positive group, which would be expected to have the lowest survival rate had the second highest after the negative group at 47.87 %. This was similar to the VP9 group which finished the trial with a survival rate of 46.88 %. The final two groups had much lower survival rates of 24.57 % for the group fed photosynthetically restored microalgae (control dsRNA) and 2.27 % for the group fed dsRNA VP9-ORF366. Although the dsVP9 group had a relatively high survival rate compared to the other groups being fed dsRNA, the result is very similar to the positive group which were only fed commercial shrimp feed therefore, the results indicate no clear improvement in survival when fed with microalgae expressing dsRNA specific to WSSV.

Similar to the visual representation of the data from the first challenge trial, the data from the last day of the challenge trial was plotted using a box and whisker plot (figure 31).

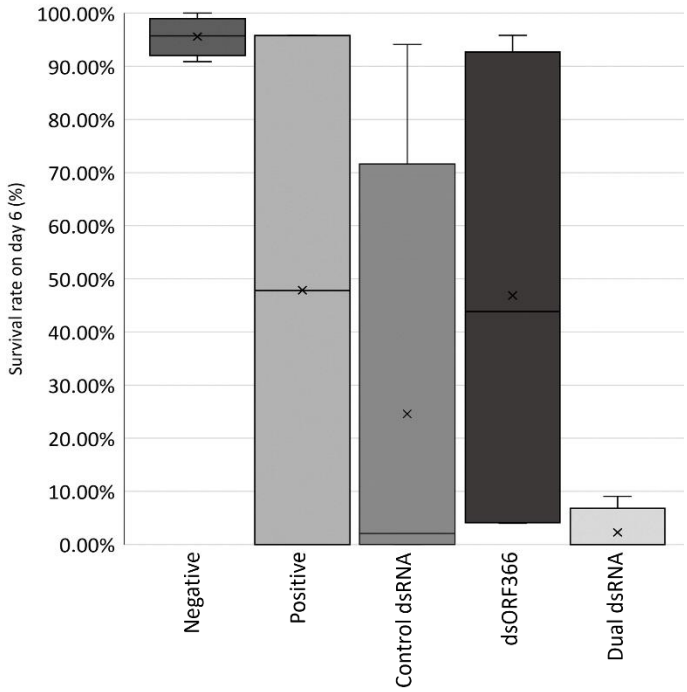

Figure 31. Trial 2- Box and whisker plot of shrimp survival rate on day 6 post infection with WSSV

Shrimp survival rate recorded on day 6 post viral challenge with WSSV. Five groups for the trial; negative group which received commercial shrimp feed and no viral challenge, positive group which were fed commercial shrimp feed and WSSV viral challenge, then three dsRNA groups which received WSSV challenge; control dsRNA group which were fed microalgae with non-specific dsRNA, dsVP9 and the dual dsRNA of dsVP9-ptxD-dsORF366.

The results from the box and whisker plot highlight the normal distribution of the negative control group, which had a high median survival rate as expected. This can be used to indicate good conditions of the experiment such as water quality and feeding rate. The positive, control dsRNA and the dsORF366 group all show a skew in the data. The results for the dual expressing dsRNA group shows less variation in the group.

Table 17. Viable shrimp count raw data over 6 day WSSV challenge trial

Days post infection (dpi)	N 1	N 2	N 3	N 4	P 1	P 2	P 3	P 4	C 1	C 2	C 3	C 4	V 1	V 2	V 3	V 4	V O 1	V O 2	V O 3	V O 4
0	22	22	22	25	23	26	24	23	24	23	25	17	25	24	23	24	22	23	22	25
1	22	22	22	25	22	26	24	22	24	22	25	16	25	24	23	24	22	23	20	25
2	22	22	21	25	22	26	24	22	24	21	23	16	24	23	21	24	21	22	18	24
3	22	21	20	25	15	25	24	22	14	11	17	16	15	21	16	24	12	15	11	17
4	22	21	20	25	3	19	23	22	4	4	9	16	2	20	6	24	8	12	1	4
5	22	21	20	24	0	1	23	22	1	0	0	16	1	20	2	24	2	3	0	0
6	22	21	20	24	0	0	23	22	1	0	0	16	1	20	1	23	2	0	0	0

Table 17, shows the raw data obtained from this WSSV challenge trial. Similar to the previous table the negative group are labelled as N1- 4, the positive group; P1-4, control dsRNA group; C1-4, the group fed algae expressing VP9 dsRNA; V1-9, as with the double dsRNA group which were fed with algae expressing both sets of dsRNA ORF366 and VP9 were labelled as VO1-4. From the raw data, the spread between each replicate in the groups can be seen. The negative group showed similar results and achieved an average percentage survival of 95.59 %. When analysing the survival rate of the positive group; 47.87 %, this result seems to be

quite high, due to the difference between the replicates, groups 1 and 2 dropped to 0 viable shrimp by day 6 of the trial, whereas group 3 and 4 only dropped by 1 shrimp each and finished with 23 and 22 viable shrimp respectively. The results from P1 and P2 are what we would expect from a positive group as this group were challenged with WSSV and were only fed commercial shrimp feed however P3 and P4 simulate results expected from the negative group. The other group which achieved similar results was the shrimp fed with algae containing VP9 dsRNA, this condition finished with an average survival rate of 46.88 % however, over the 6 day trial, group V2 only dropped by 4 shrimp and V4 only dropped by 1 shrimp, when in comparison, V1 started with 25 shrimp and ended with 1 and V3 started with 23 shrimp and also ended the trial with 1 shrimp. Due to no clear improvement in survival rate between the positive group and the VP9 group, it was expected that the group fed with VP9-ORF366 dsRNA would experience a similar loss in shrimp number, which they did, as this group finished with an overall survival rate of 2.27 %, with only group VO1 having any viable shrimp left on day 6. The control dsRNA group also had a wide variance in the results with two groups C2 and C3 dropping to 0 shrimp on day 5, and C1 finishing with just 1 shrimp. However, C4 which was within the same condition as the others finished, with 16 viable shrimp.

In summary the results from this WSSV challenge trial cannot be used to draw and firm conclusions regarding the use of WSSV specific dsRNA to shrimp to help control disease due to the variations of the control groups therefore further trials would be required.

3.1.7 Expression of dsRNA in microalgae for WSSV control in shrimp discussion

This chapter aimed to develop a tool for controlling WSSV in shrimp through the use of feeding microalgae expressing two dsRNAs specific to WSSV to utilise the RNAi mechanism in shrimp as a form of disease control.

The initial cloning needed for this project consisted of generating a vector which contained WSSV targeting dsRNA under the control of convergent promotors as well as *ptxD* which could be used as an alternative to antibiotic selection. This cloning was a new technique in the laboratory as the 'background' strain used for the chloroplast transformation had already undergone the standard transformation process and thus had restored photosynthetic capability. Therefore, *psbH* restoration could not be used for this project, and hence another chloroplast genome insertion site had to be selected. The plasmid used for transformation; pPO3, was provided by Prof Saul Purton at UCL. This plasmid contained the *ptxD* gene, flanked either side by the homology arms *psaA-3* and *trnL ccsA*, and would therefore insert *ptxD* into a neutral location on the *C. reinhardtii* plastome between *psaA-3* and *trnL.* The *ptxD* gene found on this plasmid, is considered a bio-containment system as it contains codon optimised ptxD which has two internal TGA stop codons as well as a chloroplast specific tRNA gene which had been modified to recognise the stop codon as a tryptophan codon. The use of this strategy ensures that any escape of the *ptxD* to other microorganisms, is very unlikely (Changko *et al.* 2020). This was the plasmid used to introduce either the dsVP9 unit or dsORF366, both were completed and sequenced. Once generated these plasmids were used for chloroplast transformation of TN72-dsVP9 or TN72-dsORF366, with the complementary *ptxD* dsRNA plasmid, to generate TN72-dsVP9-ptxD-dsORF366 and TN72-dsORF366-ptxD-dsVP9. This chloroplast transformation process was one which was conducted multiple times in an attempt to optimise the process. The first rounds of transformation were conducted and plated on TAPhi media and thus it was expected only transgenic microalgae which are expressing *ptxD* would be able to utilise the phosphite and convert it to phosphate. However, many colonies grew post transformation, which once a PCR was conducted, did not show signs of successful gene insertion. It was also recorded that many colonies once re-streaked onto fresh media, appeared to have 'lost their phenotype' and were unable to grow on TAPhi media. Many different methods were optimised such as

cell density and DNA quantity prior to glass bead agitation. Following unsuccessful rounds of screening, the method of phosphate starvation was adopted. This required the pre transformation culture to be grow to the correct density using TAP media, then once at the correct OD_{750}, cells were pelleted via centrifugation and respected in an equal volume of TA media, the culture was then placed back into the incubator to undergo a phosphate starvation period. This incubation period, ensured all traces of phosphate are removed and used during this process and thus should remove the possibility of false positives post transformation. This step was optimised further with different incubation times. It was concluded that 24 hrs in TA media was sufficient as any further reduced cell density. Once optimised this protocol was used as routine for transformations containing *ptxD*. This method proved successful to generate the strain used in this study TN72-dsVP9-ptxD-dsORF366. This strain was confirmed as transgenic by both PCR and sequencing.

Once generated the strain was cultured prior to the first challenge trial carried out in CENTEX, and the dsRNA was quantified from the 'liquid feed'. Total dsRNA of dsORF366 and dsVP9 was quantified from both the single expressing strains and was compared to the dual expressing strain to determine if there was an improved or reduced expression level between them. The results from this was only conducted once while visiting CENTEX in Bangkok and therefore further quantification and analysis would be required to confirm whether these results are significant. Due to the loss of the transgenic strains phenotype, it was not possible to quantify dsRNA after returning from Thailand, from the dried feed used in the second trial however the cultures were grown to the same cell density in comparison to the 'liquid feed' trial and therefore cell biomass was consistent. Although both challenge trials were conducted using microalgae shown to be expressing the dsRNA, quantification would improve this analysis.

The next step in this project was to test the efficacy of using WSSV dsRNA as an oral feed for shrimp against WSSV. Two challenge trials were conducted during two visits to CENTEX at Mahidol University, Bangkok, one using the microalgae as a liquid feed where the culture was cultured whilst visiting CENTEX and one using a dried microalgae feed, which was cultured and freeze dried at the University of Kent. As seen from the results of this trial, further testing would be required to determine if feeding WSSV specific dsRNA to shrimp provides a form of protection against the virus. The presence of the negative group was used in this trial to confirm the health of the shrimp, thus to be used as a negative control, and as seen from the trials, the negative group which had a predicted survival rate of around 90-100% ended with a much lower rate in both experimental trials. Another issue with the trial was the positive group, used here to show the lethality of the viral dose given during the trial, however in both trials the positive groups ended the trial with a survival rate of around 50% therefore indicating issues with the trial set up. Due to the unreliable results from both trials, further experiments would have to be conducted to draw any conclusions.

3.2 Expressing antibodies in *C. reinhardtii* for disease control in shrimp against AHPND

Shrimp farming and production mostly involves three species; *Penaeus monodonm*, *Macrobrachium rosenbergii* and *L. vannamei*, the white leg shrimp also known as pacific white shrimp, one of most important for this study. Expansion of aquatic farming has caused significant environmental and sociological disturbances (Walker and Mohan, 2009). These environmental impacts include the emergence of aquatic diseases, often responsible for mass livestock destruction, one of which is Acute Hepatopancreatic Necrosis Disease (AHPND), also known as early mortality syndrome (EMS). This disease is caused by the bacterium *V. parahaemolyticus,* a gram negative bacteria, found in warm marine environments (Lazarte *et al.* 2021). *Vibrio* bacteria are considered opportunistic for shrimp, which typically target the hepatopancreas which is a vital organ for digestion.

The *Vibrio* bacterium encodes a 63-70 kDa plasmid that encodes binary toxins $PirA^{vP}$ and $PirB^{vP}$, according to previous research (Lee *et al.* 2015) these toxins are suspected to be the primary virulence factors for causing AHPND, therefore are suitable targets for a vaccine.

The utilisation of microalgae as a platform for drug/protein delivery system has gained significant interest in recent years. One of the most widely used hosts for recombinant protein production is *C. reinhardtii*, due to its rapid growth rate, the use of both photo-autotrophic and heterotrophic growth, and amenability to genetic engineering. The use of proteins expressed by microalgae has spread to pharmaceuticals and the biotechnology industry, here we focus on the use of microalgae expressing compounds of interest to be used as a whole cell feed in aquaculture.

3.2.1 Aim: Generate a strain of microalgae to be used as a vaccine against AHPND in shrimp

The aim of this project was to generate a transgenic strain of *C. reinhardtii*, which expressed antibodies which targets both the toxins which are produced by the pVA1 plasmid, the causative agent of AHPND. This work was carried out in collaboration with Professor Tae Sung Jung at the College of Veterinary Medicine, Gyeongsang National University in South Korea. The project aimed to generate the strain of microalgae at the University of Kent and then tested for its efficacy as an oral vaccine in shrimp by Prof Tae Sung's group in South Korea.

3.2.2 Generating *C. reinhardtii* expressing antibodies against AHPND

This work was initiated through communication with Professor Tae Sung Jung regarding *Vibrio* shrimp disease control. An ELISA assay was carried out by Prof Tae Sung Jung's group at the College of Veterinary Medicine, Gyeongsang National University, South Korea, which identified two antibodies which displayed high binding capacity, labelled; Anti-PirA^{vP} (7C12) and Anti-PirB^{vP} (9G10) (Lazarte *et al.* 2021). Following on from this, it was proposed by collaborators that engineering the construct to contain internalin B at the N terminal of the sequence, increased its stability, therefore from here on out the constructs will be referred to as B7C12 and B9G10. Internalin B are surface proteins, variable lymphocyte receptors found on the surface of *Listeria monocytogenes*. Work by collaborators utilised the host *E. coli* BL21 to generate antibodies, however this is not an optimum delivery system as it requires purification. The aim of this project was to produce a strain of *C. reinhardtii* which expressed antibodies against AHPND which could then be used as a commercial feed for shrimp and thus a vaccine. This method of disease control is a lower cost option as the immunisation is carried out through the feeding process.

The work process for this work was as follows; codon optimisation of the antibodies for expression in the chloroplast of *C. reinhardtii*, amplification of the genes using PCR, Golden Gate cloning to produce vectors containing promotor sequence, gene and terminator sequences which were then cloned into the expression vector which contains *psbH* for photosynthetic restoration, for use in *C. reinhardtii*. Non-transformed microalgae, cannot grow on media without acetate, once transformed, microalgae are plated onto HSM media and thus the transgenic microalgae are selected due to their ability to photosynthesise due to the integration of *psbH* (Figure 32). Once the constructs were produced, the glass bead method was used to introduce antibodies B7C12 and B9G10 into separate TN72 recipient strains.

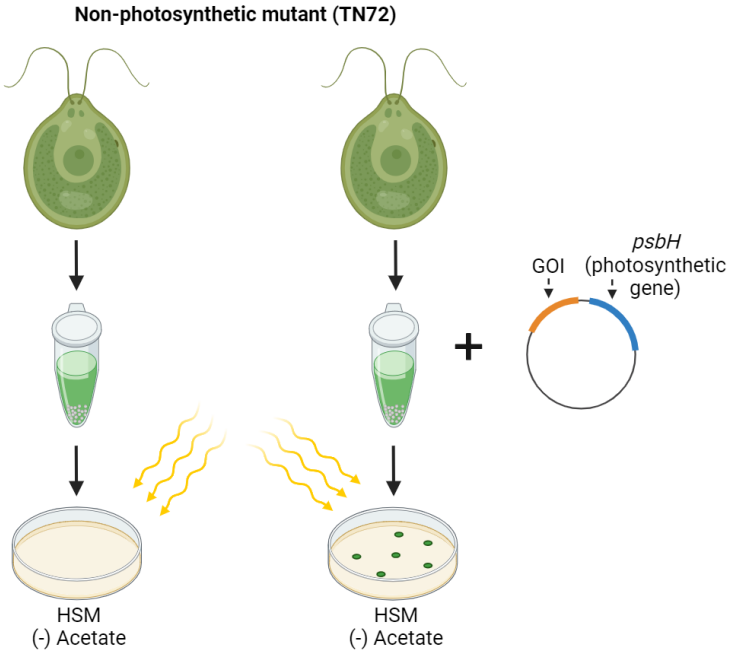

Figure 32. Outline of chloroplast transformation using photosynthetic restoration
Outline of chloroplast transformation selection via photosynthetic restoration. Transgenic microalgae are selected based on *psbH,* and thus able to grow photosynthetically. (Image designed in BioRender)

Following transformation, the transformation culture was spread onto HSM plates and incubated overnight at 25°C in dim light (2 µmol photons $^{m^{-2}\,s^{-1}}$). The next day, plates were

moved to moderate light; 50 µmol photons$^{m-2\ s\ -1.}$ Following incubation, colonies appear around 4 weeks post transformation. Once colonies appeared they were selected onto fresh HSM media to build up biomass. This was repeated at least once more before the colonies were screened by PCR. A total DNA extract was taken from each suspected transgenic colony and a PCR was performed using primers specific to the gene of interest. The results from a screening PCR can be seen in figure 33.

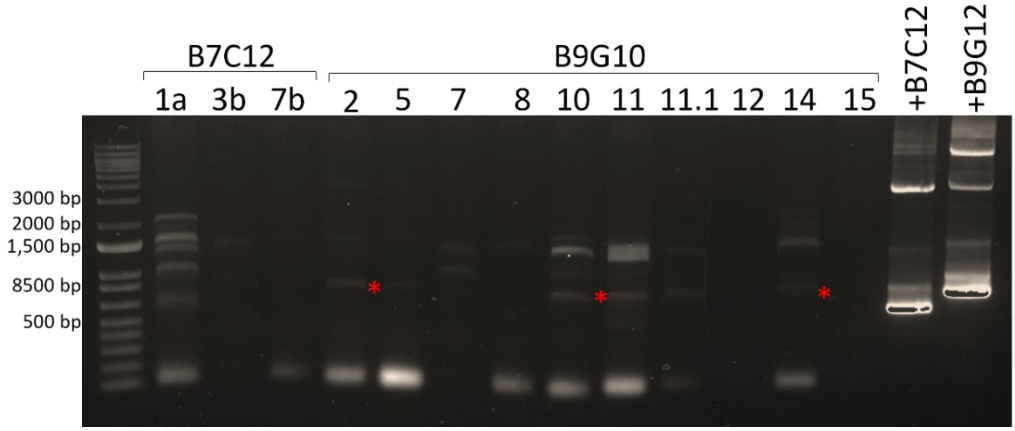

Figure 33. Screening PCR using Internalin B & R primers specific to B7C12 and B9G10.
PCR amplification of suspected transformants using Internalin B F & R. Positive controls using plasmid DNA +B7C12 is 633bp and +B9G10 is 795bp. Samples split into either B7C12 or B9G10 as indicated above.

This PCR was conducted to target the specific sequence of either B7C12 or B9G10 using primers which annealed at the beginning of the internalin B sequence just after the promotor sequence and at the end of the B7C12/B9G10 sequence. As seen above, there appears to be multiple bands in the samples such as 1a, 10, 11, etc. There are however, a few samples such as 2, 10, 11, 14 which show a band at the correct size, as highlighted by an asterisk in the image. These samples were then used to build biomass and streaked onto fresh selection media. Further colonies were then screened for presence of gene insertion in the chloroplast genome, in figure 34.

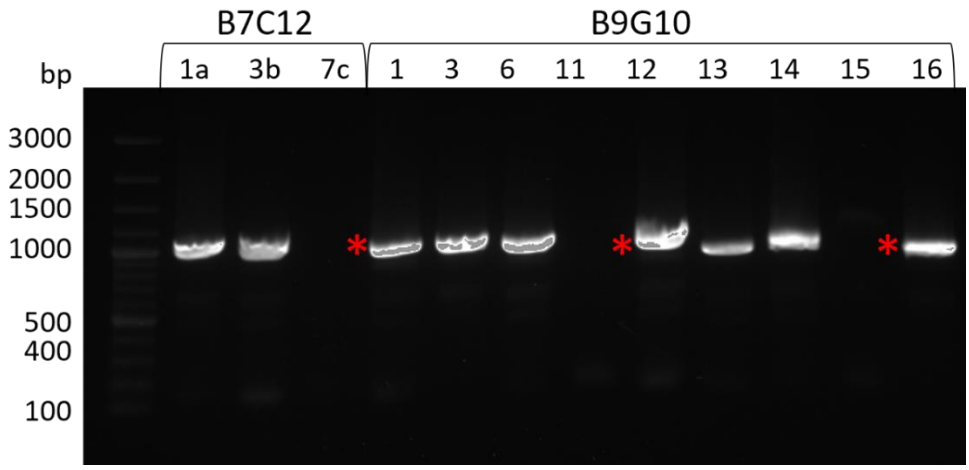

Figure 34. Screening PCR to detect insertion of B7C12 and B9G10 into the chloroplast of *C. reinhardtii*
PCR using Flank 1 F and RbcL.F which produces a 850 bp product of strains which still contain the *aada* gene. Samples without a product are suspected of being positive transformants.

The primer set used here was Flank 1 F and RbcL.F, which amplifies upstream of the gene of interest insertion site and to the end of the *aadA* cassette. The *aadA* gene is the gene in place of the *psbH* region in the background strain TN72, therefore presence of a band shows presence of *aadA* and thus a unsuccessful transformation or non homoplasmic strain as this method of transformation inserts the gene in the place of *aadA* and restores *psbH*. Complete removal of *aadA* suggests the strain is homoplasmic for the gene of interest. The results above show a lack of a band in samples 7c, 11 and 15 as seen by the asterisk, indicating the strain is homoplasmic. Following this, a PCR was run to screen for the gene of interest, figure 35.

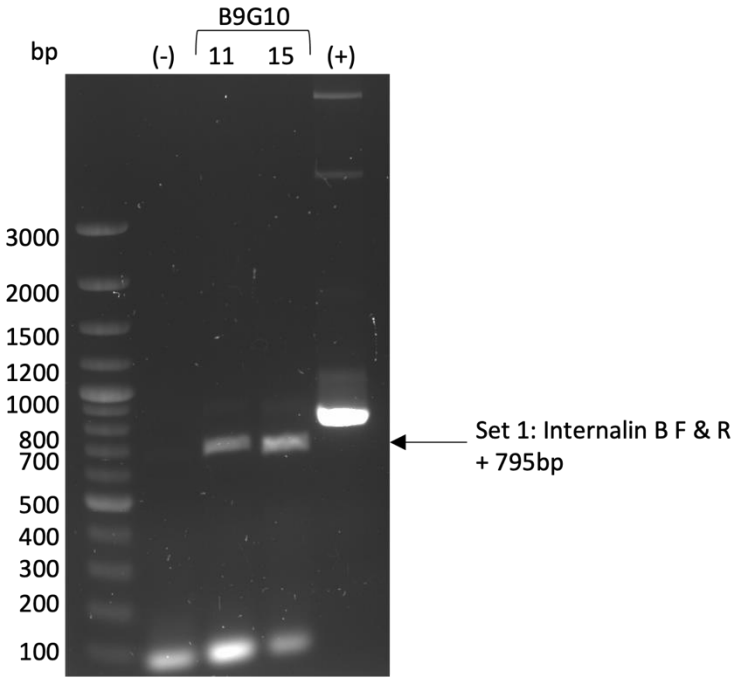

Figure 35. PCR to show gene amplification of B9G10 from *C. reinhardtii* transformants. PCR to check for gene insertion of B9G10, using primers Internalin B F and Internalin B R. A positive control was performed using the plasmid used for transformation. The expected gene size is 795 bp.

After a positive result from this PCR (figure 35), the cells were streaked onto fresh media to reach homoplasmy. A second total DNA extraction was taken and PCR was performed. This is a necessary step when screening microalgae as *C. reinhardtii* is polyploidy, and has around ~ 80 copies of its chloroplast genome, however growth of transformant lines under selective conditions, should result in homoplasmic cells (Purton 2007).

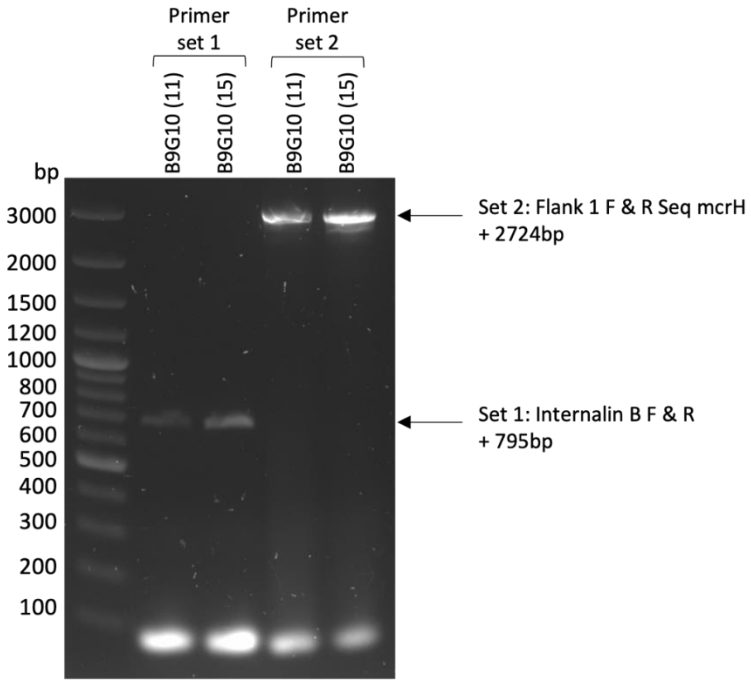

Figure 36. PCR amplification of B9G10 from TN72 transformant samples 11 and 15.
PCR to check for gene insertion of B9G10, using two primer sets. Set 1) Primers Internalin B F and Internalin B R were used to amplify the gene B9G10. Set 2) Primers Flank 1 F and R Seq mcrH were used to amplify B9G10 from a total DNA extract.

The primers used in set 1 are specific to the B9G10 gene and produce a band of 795 bp. As seen in figure 36, both samples; 11 and 15 show the presence of this band. The second primer set is non-specific to the GOI however amplified either side of the insertion site, and amplifies a positive band of 2724 bp, as seen in both samples. After multiple round of selection screening using HSM medium, a positive transformant for B7C12 was not maintained. Therefore, from here on out this work focuses on the TN72-B9G10 transformants.

After confirmation by PCR to confirm the B9G10 gene in the *C. reinhardtii* chloroplast genome, the antibody was expressed in liquid culture and processed for SDS-PAGE (figure 37) to screen for protein expression via its HA tag. A total of 5 suspected positive transformants were grown as well as TN72 to serve as a negative control.

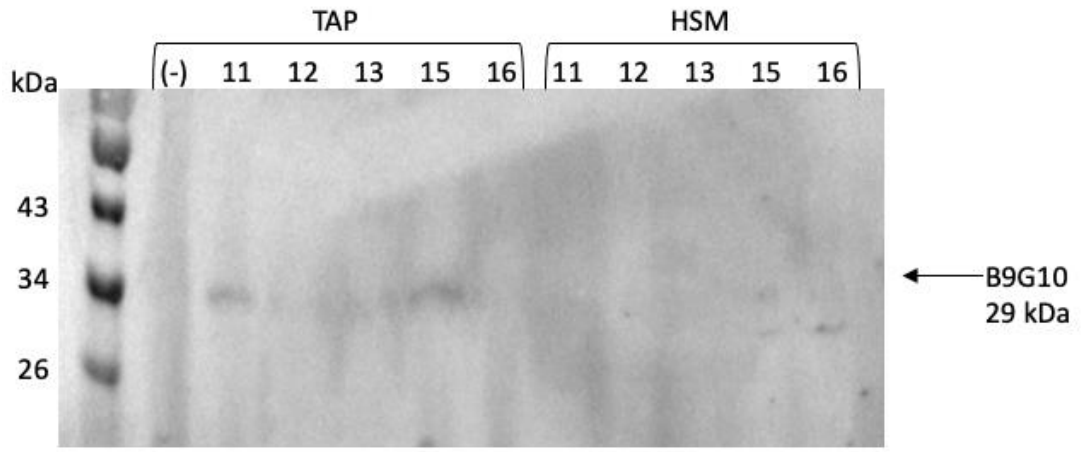

Figure 37. Screening expression of transformants of *C. reinhardtii* expressing antibody B9G10 in TAP and HSM
Anti-HA western blot of potential transformants expressing antibody B9G10, grown in either 2 ml TAP or HSM media using a 6 well plate 25°C for 7 days. Negative control is TN72 an untransformed strain of *C. reinhardtii.*

Once transformed cell lines expressing the B9G10 antibody were identified, the two which expressed the best; 11 and 15 from the blot above (figure 37), the biomass was built up through a process of streaking regularly on fresh HSM media until homoplasmic and then plated on TAP media which contains acetate for a carbon source. Once enough biomass was achieved a larger culture of 100 ml TAP was grown using a 20 ml pre culture. The 100 ml TAP was inoculated to OD_{750} 0.04 and sampled each day to record a growth curve seen in figure 38.

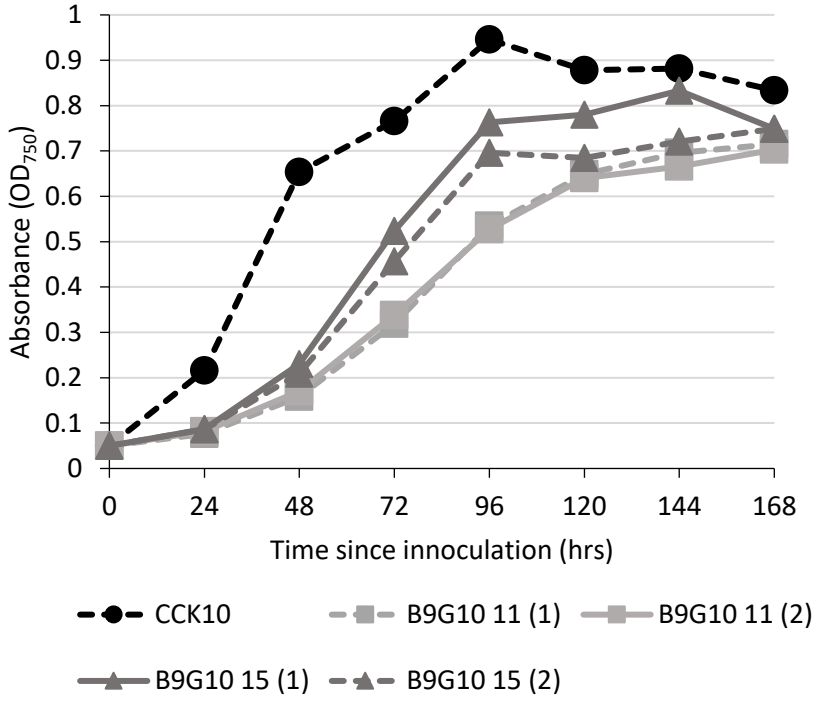

Figure 38. Growth curve of *C. reinhardtii* expressing antibody B9G10
Growth curve results from three strains of *C. reinhardtii*, the control strain; CCK10 is a photosynthetically restored strain, the following two B9G10 11 and B9G10 15, two positive transformants expressing the antibody B9G10. The cultures were grown at 25°C for 7 days, growth was monitored in duplicates, represented after the sample name.

Three strains were used for this experiment: CCK10, a photosynthetically restored strain and two transformants of TN72-B9G10 (11) and TN72-B9G10 (15), both cultured in duplicates. The strain CCK10 was used here as a negative control over TN72 because it has undergone transformation to restore its photosynthetic capability and can therefore be grown in the same conditions as the transformed B9G10 strain. The cultures were grown for 7 days post inoculation and a protein sample was taken equivalent to 10 OD units on days 5 and 7. All strains grew in a similar patter however, as seen above, the photosynthetically restored strain, which was grown as a control comparison, overall achieved a higher OD750. Both replicates of CCK10 had a much higher increase in OD over the first 48 hrs compared to both of the B9G10 strains.

Samples were taken from the cultures equivalent to 10 OD units, following section 2.3.12. All samples were separated by SDS-PAGE and analysed via an anti HA western blot as seen in figure 39.

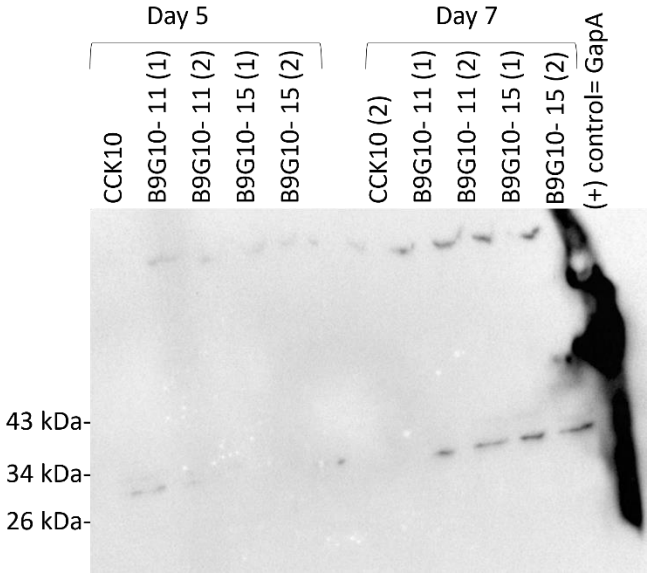

Figure 39. Expression of two transformants of *C. reinhardtii* expressing antibody B9G10
Anti-HA western blot of suspected transformants expressing antibody B9G10 using duplicates, samples 11 and 15 in duplicate, alongside a negative control strain CCK10.

As seen above there is an increased level of protein expression in the day 7 samples compared to day 5, with B9G10 expected to be ~ 29 kDa, although the day 7 samples appear higher due to the running conditions.

After expression of HA-tagged B9G10 in *C. reinhardtii*, the protein was purified using a Pierce™ GA-Tag IP/Co-IP kit (Thermo Scientific), samples were prepared for SDS-PAGE and were immunoblotted, see figure 40.

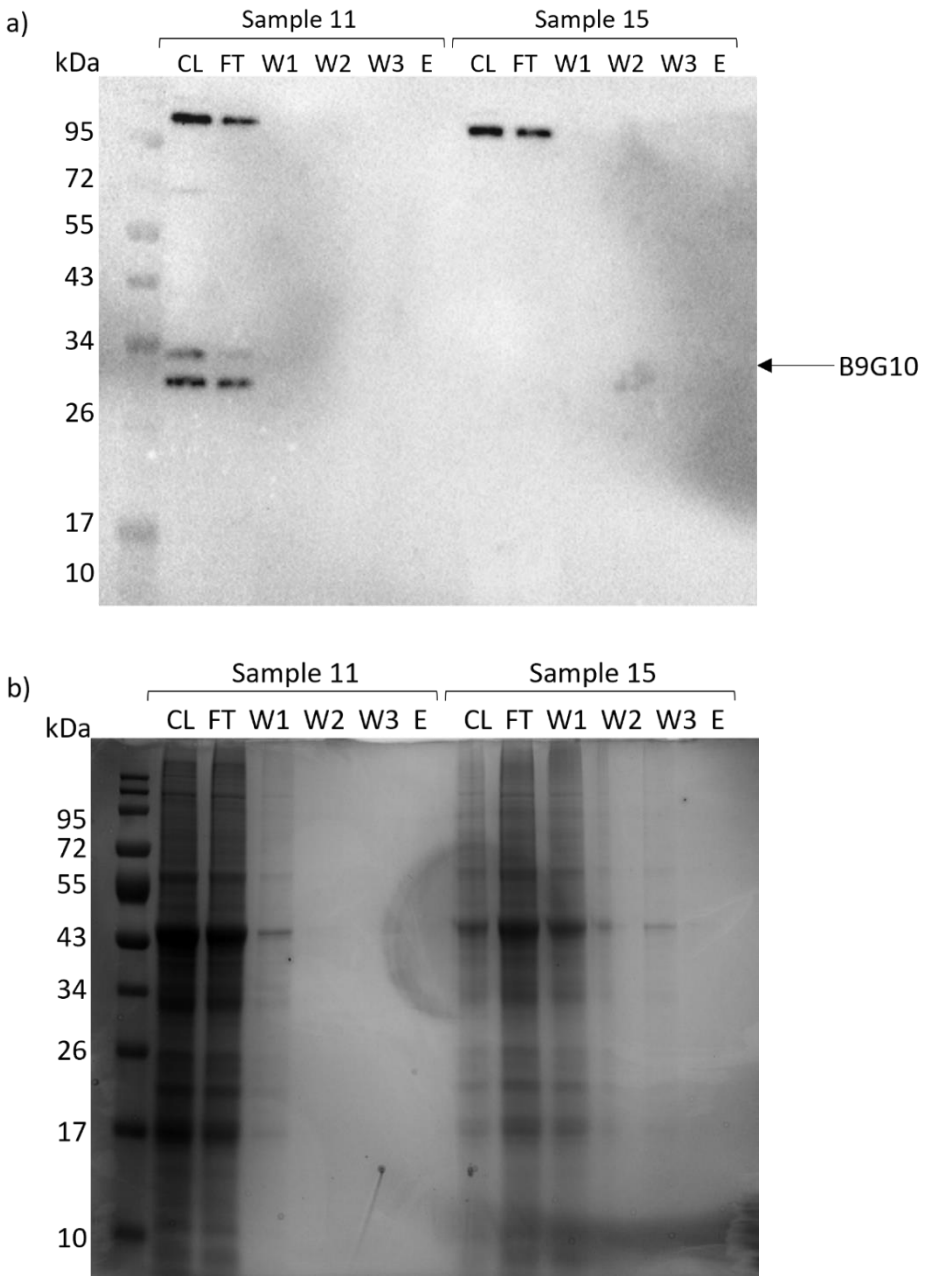

Figure 40. Purification of HA tagged B9G10 from *C. reinhardtii*
Proteins were purified using the Pierce™ GA-Tag IP/Co-IP kit (Thermo Scientific) and a) separated by SDS-PAGE and stained with Coomassie Brilliant Blue for visualisation. (b) The corresponding gel was used for immunoblotting and proteins were visualised using anti-HA antibody.

As seen in figure 40, it appears that the B9G10 protein comes off the column in the flow through seen in (a). The protein is 29 kDa, which corresponds to the lower band on the anti-HA western blot. Other bands are present in the western blot, at the high molecular weight at the top of the membrane showing possible protein aggregation at around >95 kDa. There

is also a doublet band above the target band. The reason for these non-specific bands remains unknown and would require further purification experiments.

3.2.3 Protein expression in microalgae for shrimp disease control discussion

This project aimed to express the antibodies B7C12 and B9G10 in microalgae which reduces the need for antibody purification. The sequences were first codon optimised for the chloroplast genome and used for transformation in *C. reinhardtii.* The presence of the transgene was confirmed by PCR. Due to the polyploidy nature, possible transformants required many rounds of streaking onto the minimal media HSM, to ensure autotrophic growth. After multiple rounds of selection results showed insertion of B9G10, however no positive transformant for B7C12, suggesting issues with gene insertion or culturing conditions. An avenue to explore would be transformation method as electroporation has been shown to provide higher transformation efficiency rates in comparison to the glass bead method (Shimogawara *et al.* 1998). Another area to explore would be the strain used for expression, as previous studies have found a variation in protein expression between *C. reinhardtii* strains (Braun-Galleani, Baganz and Purton 2015).

Growth assays were performed using *C. reinhardtii* expressing HA tagged B9G10, and samples were separated by SDS-PAGE and immunoblotted using a HA tag western blot. Here the B9G10 antibody was successfully expressed in *C. reinhardtii*, although levels of protein expression were low. This is a possible constraint of using microalgae to express recombinant proteins, however there still remain areas to be optimised for example, the use of a bioreactor could greatly increase cell biomass and protein yield. This is a method which has been optimised for use in *C. reinhardtii* which has been able to achieve recombinant protein yields of as high as 50 mg/L of the recombinant human protein ICAM-1 (Torres-Tiji *et al.*

2022). Further optimisation such as changing light and growth conditions could also be adopted to improve protein yield. The purification method of HA tagged proteins showed non-specific bands, therefore, further optimisation on purification would be beneficial to assess protein yields, although purification was not necessary for use in shrimp trials as this project aimed to produce a whole cell oral feed, which reduces downstream costs, thus producing a more industry relevant disease control method.

This project had aimed to generate two strains of microalgae, each expressing one of the antibodies, however as both B7C12 and B9G10 appeared to be good vaccine candidates, cloning in *E. coli* was also performed to create a construct containing both B7C12 and B9G10 ready for transformation via *psbH* restoration in *C. reinhardtii*. Although the transformation construct was generated, it was not used for chloroplast transformation. Due to time constrains of the project, the single expression constructs and TN72-B9G10 microalgae, was sent to our Prof Saul Purton's lab at UCL for continued expressions and animal trials with Prof Tae Sung Jung's group at Gyeongsang National University, South Korea.

3.3 Generating a subunit vaccine in *E. coli* for disease control in grouper fish

Previous areas of research in this work have shown the effective use of *C. reinhardtii* for both dsRNA and recombinant protein production for the purpose of using the microalgae as a whole feed. However, this next project utilises *E. coli* to produce immunogenic proteins which can then be purified to be used as a vaccine in the form of injection. *E. coli* was chosen as a host due to its possibility of high levels of protein expression.

The use of recombinant proteins for aquaculture is a widely researched area. This project aimed to produce a linker protein for expression in *E. coli* which can be used as an aquaculture vaccine for grouper fish. Grouper are a type of marine fish, which belong to the family Serranidae, found in both tropical and subtropical environments. Many species of grouper fish such as *Epinephelus coioides* (Orange-Spotted grouper), *Epinephelus fuscoguttatus* (Tiger grouper), and *Epinephelus lanceolatus* (Giant grouper) are farmed for consumption due to their large size and good growth rate (Dennis *et al.* 2020). One of the key challenges for grouper farming is the presence of *Vibrio* sp. infections. The information regarding *Vibrio* infections in grouper remains limited, however prevalence studies have shown the high risk of *Vibrio* infections amongst grouper. Of 270 grouper samples taken from 9 different farms across Malaysia, 72% indicated presence of *Vibrio* spp. 25% of which were *Vibrio parahaemolyticus* (*V. parahaemolyticus)* (Amalina *et al.* 2019). *V. parahaemolyticus* is an aquatic zoonotic pathogen which causes vibriosis in multiple species of marine fish as well as gastroenteritis, wound infection and sepsis in humans, through the consumption of seafood (Zhang *et al.* 2016). The presence of a zoonotic bacteria found within aquaculture farms is of great concern not just for farmers but also human health. One control method for *Vibrio* is the use of antibiotics, however there are concerns around their use regarding antibiotic resistance as bacteria have been found to show high resistance to antibiotics such as colistin and ampicillin (Mok *et al.* 2021). The development of non-antibiotic control

methods is required to combat this bacterial infection within aquaculture farming as *Vibrio* spread not only infects grouper fish but many other aquatic animals (Austin 2010), highlighting the ease of spread once in a farm. The immune system of fish, different to that of shrimp, protects them against pathogens, it consists of both an adaptive and innate system. The innate immune system is comprised of physical barriers such as the skin, gills and mucus layer as well as cellular responses such as phagocytosis. The adaptive immune system is highly specific and can provide long lasting immunity, it is comprised of immunoglobulins, T cell receptors and major histocompatibility complex (Smith, Rise and Christian 2019). Methods such as vaccination provide an alternative prevention method, as the introduction of antibodies initiates an immune response and thus provides protection against the pathogen. Outer membrane proteins (OMPs) which are unique to Gram negative bacteria, have been discovered as good candidates for vaccine development as they are highly immunogenic (Lin, Huang and Zhang 2002). Outer membrane protein (OMP) immunisation has been shown to be effective against *V. parahaemolyticus* in other species of fish such as zebrafish, where 17 OMPs were identified as immunogens, four of which were effective at protecting against *V. parahaemolyticus.*

3.3.1 Aim: Express a fusion protein in *E. coli* for use in aquaculture disease control

This study aimed to use this method of antigen immunisation to induce an immune response and thus with further validation, protection against *V. parahaemolyticus* in grouper fish. This project was in collaboration with Dr Cahyo Budiman and Professor Zarina Amin at University Malaysia Sabah. The initial aim of this project was to design and generate the fusion construct for expression in *E. coli* at the University of Kent, which could then be sent to researchers in Malaysia. The next aim was to develop a successful expression system in *E. coli* in parallel to researchers in Malaysia. The downstream steps of this project aimed to confirm its suitability as a vaccine through the injection of the fusion protein to detect if an

immune response is detected in grouper fish challenged with Vibrio, carried out by collaborators at University Malaysia Sabah.

3.3.2 Fusion protein design for bacterial expression

The fragments used here for this study were glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the outer membrane protein K (OmpK) of *Vibrio* spp. Outer membrane proteins can be used as vaccine candidates due to their immunogenic properties such as their exposed epitopes on the bacterial cell. They also carry pathogen associated molecular patterns (PAMPs) which are recognised by the pathogen recognition receptors on host cells, thus allowing antigen processing and presentation for the adaptive immune response (Maiti *et al.* 2020). The fragment of GAPDH chosen here is from *V. parahaemolyticus,* however the fragment used shares 100 % sequence identity to other strains of *Vibrio* such as *V. harveyi* and *V. campbelli,* suggesting possible use against other strains. The second fragment used here is from OmpK. This is another protein, shared amongst the *Vibrio* species, which has previously been identified as possible vaccine candidate (Li *et al.* 2010). This protein was used as a recombinant vaccine in orange spotted groupers and provided elevated levels of protection against *Vibrio* strains (Li *et al.* 2010). Previous research had shown that using the fusion of OmpK-GAPDH offered increased protection of the large yellow croaker to *V. harveyi* (Zhang, Yu and Qian 2007) and thus the fragments were designed for use as a fusion fragment sequence. Fragments of each of these proteins were selected and used to generate a fusion protein containing a flexible linker between the two fragments (figure 41).

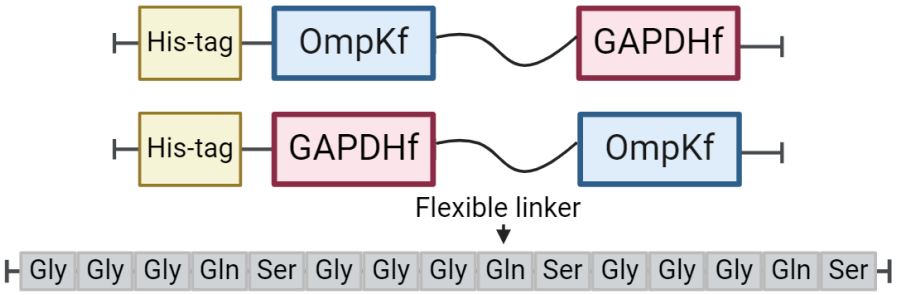

Figure 41. Design of fragment sequences for vaccine production in *E. coli*
Schematic of the constructs designed for expression in *E. coli* to produce OmpKf and GAPDHf. Both constructs were made with a N terminal His tag, then followed by either OmpKf with a flexible linker then GAPDHf, or GAPDHf with a flexible linker to OmpKf. (Image designed in BioRender)

The constructs, as seen in figure 41 were designed with either OmpKf with a flexible linker to GAPDHf or the reverse; GAPDHf connected with a flexible linker to OmpKf, both of which were designed for the pET30 vector which contains an N terminal His-tag. The linker as proposed by our collaborators contained a repeating unit of glycine, glutamine and serine, as seen in figure 41. The design of this experiment involved codon optimisation and ordering of gene strings from GeneArt™, then cloning into expression vectors, in this case pET30 vector which contains the N terminal his tag. The next step was optimising expression of the fusion proteins, followed by purification. Once expressed these fragments were used as a vaccine in both purified and non-purified forms, for grouper fish against *Vibrio sp,* in collaboration with Dr Cahyo Budiman and Professor Zarina Amin at University Malaysia Sabah. The rationale behind testing both purified and non-purified protein was if the non-purified protein gave a successful response in fish testing, it would remove the need for purification when upscaling, and thus reduce the cost, a critical part when developing a sustainable tool for aquaculture disease in developing countries.

3.3.3 Expression optimisation of GAPDHf, OmpKf and GAPDHf-OmpKf and OmpKf-GAPDHf fusion proteins

The first step in this project was to express the single genes in *E. coli* as seen in figure 42. The constructs used were generated in a pET30 vector which contains a T7 promotor, therefore a compatible cell line was crucial for expression. In this case *E. coli* BL21 DE3 cells were chosen.

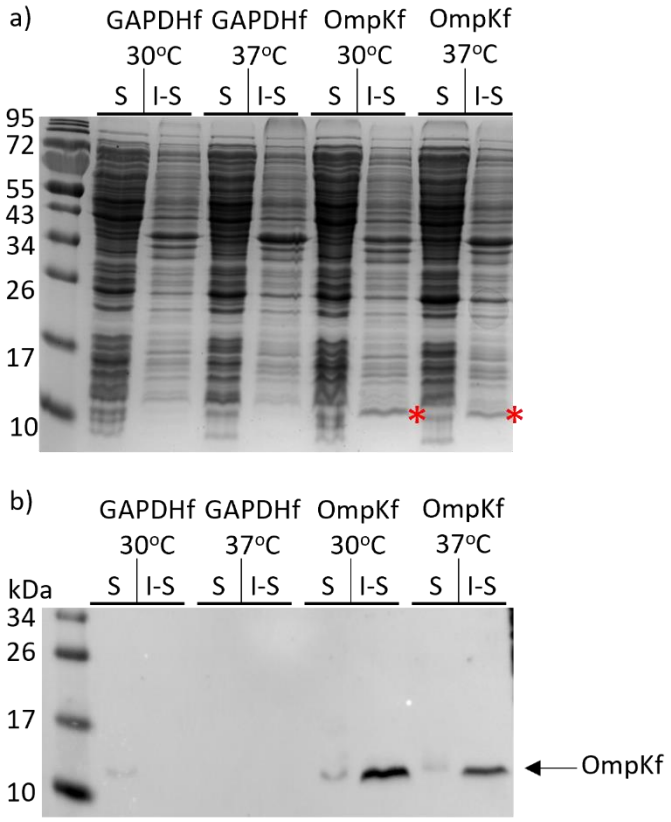

Figure 42. Expression of GAPDHf and OmpKf in BL21 DE3 cells
Expression of both single fragments GAPDHf and OmpKf in BL21 DE3 cells, grown at either 30°C or 37°C post induction. Following the expression protocol in section 2.5.1. Samples were processed into soluble (S) and in-soluble (I-S) fractions using methods in 2.5.3. Samples were separated by SDS-PAGE and visualised by a) Coomassie staining and b) immunoblotted using an Anti-his N and C term antibody (1:2000).

The results above show clear expression of the single OmpKf fragment in *E. coli* BL21 DE3, the blot indicates relatively high levels, the same band which can also be seen in the SDS-PAGE gel as highlighted with an asterisk. The results also show a much higher level of insoluble protein compared to soluble.

After testing multiple expression conditions for GAPDHf, the was no successful expression, only for that of OmpKf. Following expression at shake flask level using the single fragments of OmpKf, the next step was to express the fusion proteins of both GAPDHf-OmpKf and OmpKf-GAPDHf as these were the chosen targets for a vaccine candidate.

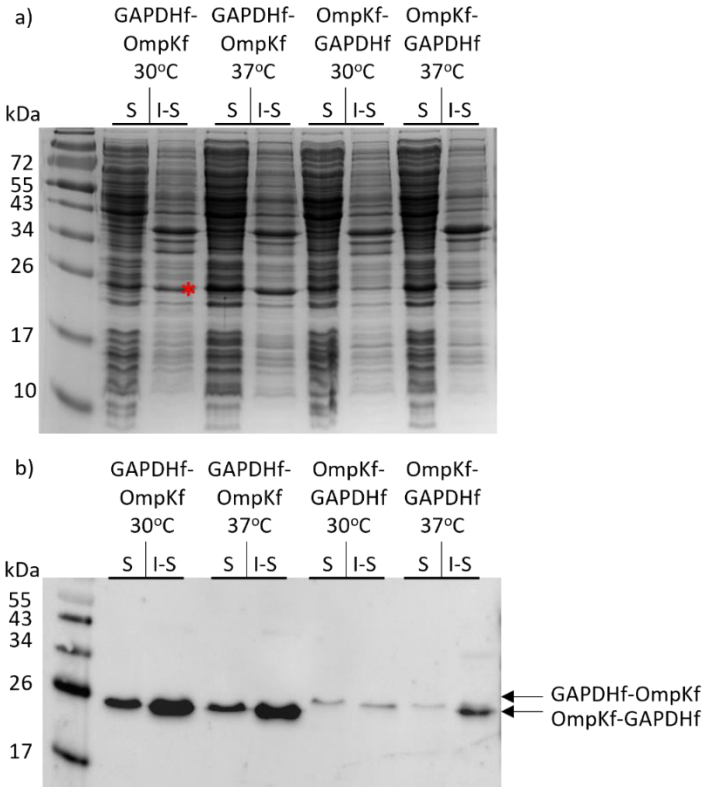

Figure 43. Expression of fusion linker proteins GAPDHf-OmpKf and OmpKf-GAPDHf using BL21 DE3 cells in TB media

Expression of both fusion protein fragments GAPDHf-OmpKf and OmpKf-GAPDHf in BL21 DE3 cells. Cells were grown in a 50 ml TB shake flask at 30°C and 37°C following the expression protocol in section 2.5.1. Samples were processed into soluble (S) and in-soluble (I-S) fractions using methods in 2.5.3. Samples were separated by SDS-PAGE and visualised by a) Coomassie staining and b) immunoblotted using an Anti-his N and C term antibody (1:2000).

In figure 43, it is evident that there is a higher level of expression of GAPDHf-OmpKf compared to OmpKf-GAPDHf. The size of the fusion protein is around 23 kDa. The GAPDHf-OmpKf fragment appears to express the best in this experiment, in the insoluble fraction, this level of protein is also seen in the SDS-PAGE gel marked by an asterisk. Although a higher

amount of insoluble protein, there is also an increase in the amount of soluble protein for this experiment.

After obtaining both soluble and insoluble fractions of GAPDHf-OmpKf and OmpKf-GAPDHf in small scale experiments, large scale expression was carried out for downstream purification by IMAC, figure 44. Here the GAPDHf-OmpKf (23 kDa) was expressed in 400 ml TB media following section 2.5.1 at 37°C. Following growth, the cells were harvested and processed according to section 2.5.4.

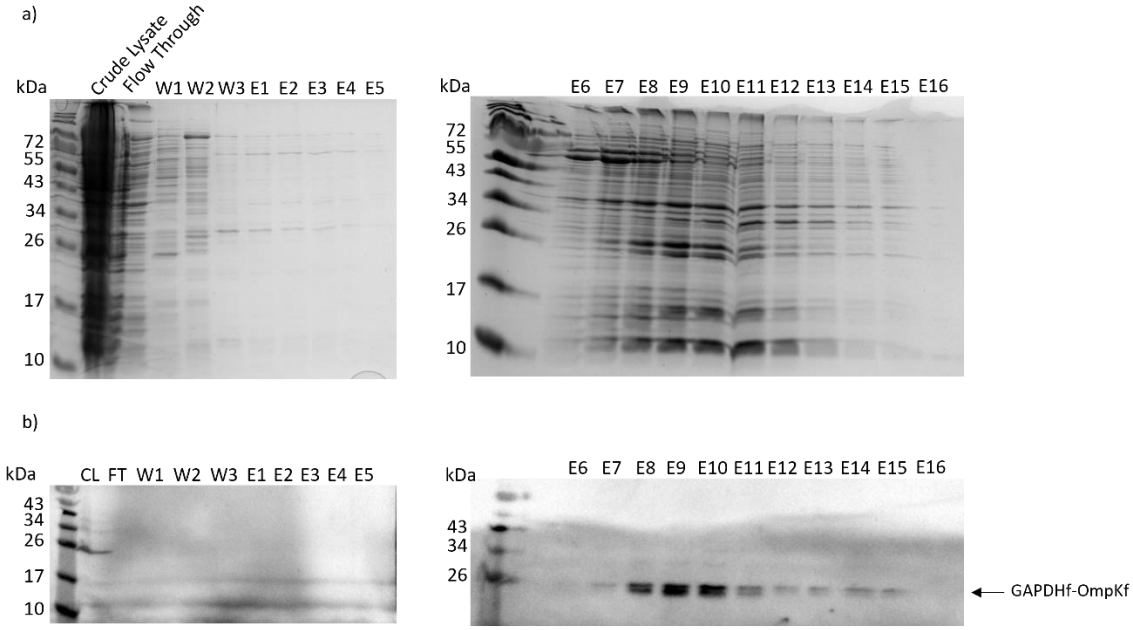

Figure 44. Purification of GAPDHf-OmpKf from large scale expression in BL21 DE3
The soluble protein fraction was applied to a 5 ml HisTrap HP ™ column and the flow through was collected (FT). A pre purification crude lysate (CL) sample was also collected as well as three wash fractions (W1-3). The bound proteins were then eluted using a gradient elution of 0-500 mM imidazole, into 2 ml fractions (E1-16). a) Fractions were separated by SDS-PAGE and stained using Coomassie Brilliant Blue. b) The corresponding gels were immunoblotted following SDS-PAGE, using an Anti-his N and C term antibody (1:2000).

As previously mentioned, this project required both purified and non-purified material to test effectiveness of both. The large culture was processed and purified using a HisTrap HP™ column using an AKTA. The size of the fusion protein; GAPDHf-OmpKf is 23 kDa, so expected to be just below the 26 kDa marker. The SDS-PAGE gel in a) shows a messy purification,

however there is a band in both the flow through and the first wash, indicating that some of the protein could have been lost from the column, this is also reflected in the western blot. The western blot shows a clearer image of the elution steps, it appears the protein is eluted between E7-16, this band is reflected in the SDS-PAGE in a) however, the Coomassie shows multiple bands in the elution, showing a possible issue with the protein binding to the column. As there was a poor purification from GAPDHf-OmpKf the reverse fusion protein was also expressed in BL21 DE3 at large scale for purification as seen in the figure 45. OmpKf-GAPDHf (23 kDa) was expressed in 400 ml TB media following section 2.5.1 at 37°C. Following growth, the cells were harvested and processed according to section 2.5.4.

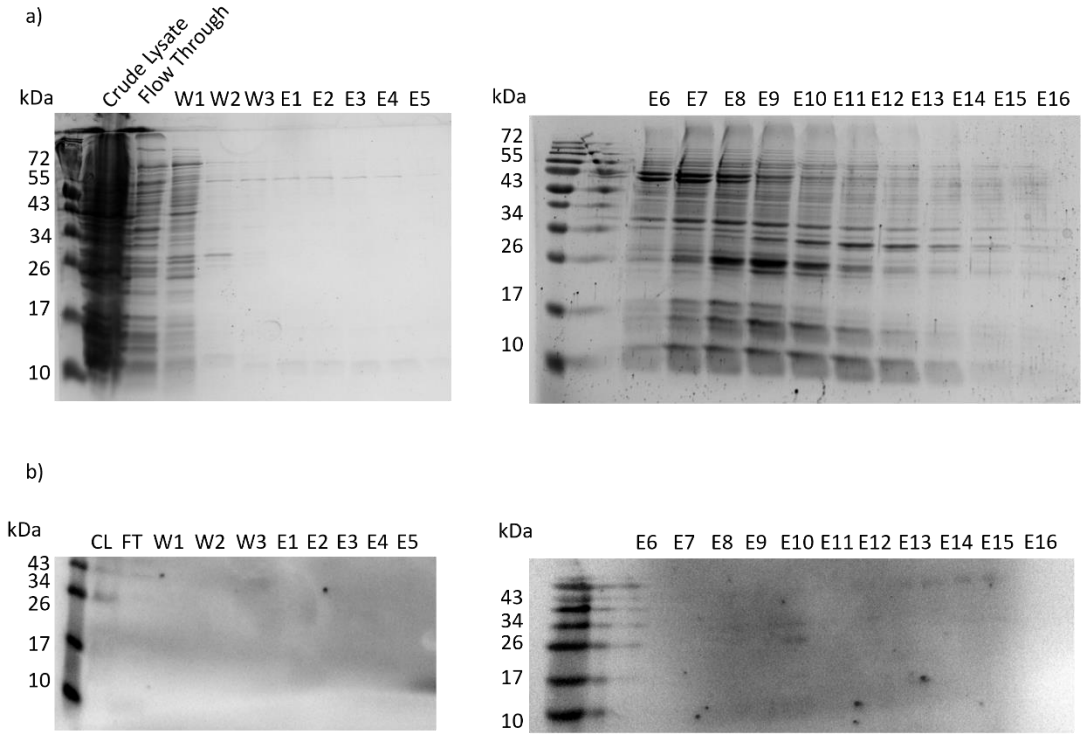

Figure 45. Purification of OmpKf-GAPDHf from large scale expression in BL21 DE3
The soluble protein fraction was applied to a 5 ml HisTrap HP ™ column and the flow through was collected (FT). A pre purification crude lysate (CL) sample was also collected. The column was washed with buffer containing 40 mM imidazole to remove impurities (W1-3). The bound proteins were then eluted using a gradient elution of 0-500 mM imidazole into 2 ml fractions (E1-16). a) Fractions were separated by SDS-PAGE and stained using Coomassie Brilliant Blue. b) The corresponding gels were immunoblotted following SDS-PAGE, using an Anti-his N and C term antibody (1:2000).

Similar to the purification of GAPDHf-OmpKf, it appears the sample does not bind well to the column as there is a band in the flow through however this time the western blot did not detect any protein in the elution's.

In order to improve soluble protein levels, the incubation temperature post induction was altered. Here in figure 46, samples were harvested 3 hrs post induction at 18°C and harvested after overnight incubation at 18°C, as well as after 3 hrs post induction at 25°C.

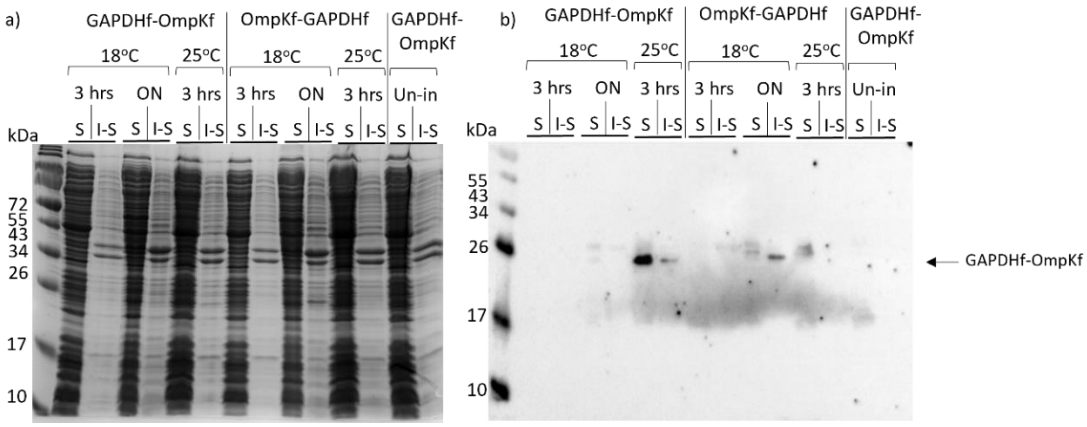

Figure 46. GAPDHf-OmpKf and OmpKf-GAPDHf expression in BL21 DE3 at lower temperatures
Both fusion proteins were expressed at small scale (section 2.5.1) in TB media at 37°C. Cells were grown at either 18°C or 25°C post induction and samples were either collected 3 hours post induction or the following morning. (section 2.5.3). Uninduced control also included. Samples were then separated into soluble (S) and insoluble fractions (I-S). Proteins were separated by SDS-PAGE (a) and immunoblotted (b) using an Anti-his C and N term antibody (1:2000).

As incubation at 25°C after induction gave a higher level of soluble protein compared to insoluble protein, a large scale culture was grown under these conditions and then purified, results seen in figure 47. GAPDHf-OmpKf (23 kDa) was expressed at large scale in 400 TB media following section 2.5.1. Following growth, the cells were harvested and processed according to section 2.5.4.

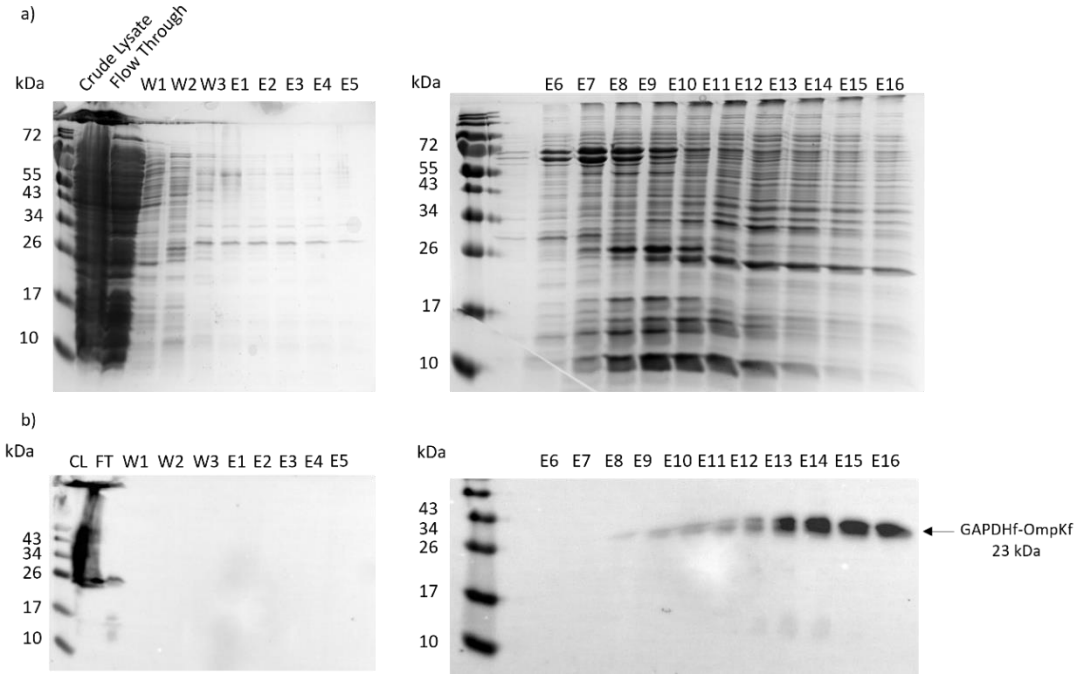

Figure 47. GAPDHf-OmpKf purified from 400 ml TB at 25°C
The soluble protein fraction was applied to a 5 ml HisTrap HP ™ column and the flow through was collected (FT). Samples include a pre purification crude lysate (CL), three washes (W1-3) and elutions. The bound proteins were then eluted using a gradient elution of 0-500 mM imidazole into 2 ml fractions (E1-16). a) Fractions were separated by SDS-PAGE and stained using Coomassie Brilliant Blue. b) The corresponding gels were immunoblotted following SDS-PAGE, using an Anti-his N and C term antibody (1:2000).

The results from this purification were an improvement from figure 45, as there is protein detected in the western blot between elutions 8 to 16 this provides evidence of clear binding of the protein to the column. The SDS-PAGE results show multiple bands for the elutions; an unexpected result from a purification. It remains unclear why there are contaminating bands in this purification.

Results from multiple expressions showed clear production of the fusion protein GAPDHf-OmpKf, therefore the next step for this experiment was to test its use as an aquaculture vaccine in grouper fish. The constructs were generated at the University of Kent for this study and were sent to collaborators in Malaysia for grouper fish studies.

3.3.4 Grouper fish vaccine trial using purified GAPDHf-OmpKf

This vaccine candidate GAPDHf-OmpKf showed clear signs of expression when produced using *E. coli* strain BL21 DE3. The possibility of using this fusion protein as a vaccine candidate requires testing in grouper fish. Using the fusion proteins produced for this study, our collaborators conducted grouper fish experiments to analyse immune gene expression following injection and bacterial challenge. The results presented here were performed by collaborators at the Faculty of Science and Natural Resources, University of Malaysia Sabah and taken directly from work by Iffah Hayani Binti Md Daud.

The fish used in this study were Hybrid TGGG Grouper fish (*Epinephelus fuscoguttatus* x *Epinephelus lanceolatus*). For the experiment, the fusion protein was overexpressed, harvested and purified. Vaccination and bacterial challenge was then conducted. Immune gene expression was finally analysed by qPCR. For this study conducted in Malaysia, GAPDHf-OmpKf was expressed in *E. coli* Rosetta cells, the outline of the expression and purification methodology can be seen in the methods section.

Prior to the experiment, hybrid TGGG grouper fish, 5 inches in length, weighing 40 ± 6 g were acclimatised for 1 week in a controlled environment of 28 pp salinity, 6 mg/L dissolved oxygen, 30^{o}C and pH 7.9, in tanks. The fish were fed with a daily diet of commercial fish feed. The fish were separated into five groups (n=21). The first group was immunised with purified GAPDHf-OmpKf at a dosage of 50 µl/100 g, the second group was a control group which received 50 mM Tris HCL, pH 7.5 at a dosage of 50 µl/100 g. Immunisation was caried out by intraperitoneal injection (IP) using a 23G needle. The grouper were then subjected to bacterial challenge, the fish were firstly anaesthetised, then the skin was subjected to grazing using a needle. The fish were then challenged by via submersion in a tank containing *V.*

parahaemolyticus, and left for 12 hours. The fish were subjected to a second bacterial challenge on day 7.

Immune gene expression was analysed through tissue harvest followed by RT-qPCR. On days 0, 7 and 14 post vaccination, 1 fish per group was euthanised and the spleen was excised, and an RNA extraction was performed. The results presented here are taken from work by Iffah Hayani Binti Md Daud. The quantity and purity of RNA of the unvaccinated, vaccinated, unvaccinated and challenged and vaccinated and challenge samples can be seen in the appendix.

Three immunity genes; Interleukin 2 (IL-2), Interleukin 6 (IL-6) and Interferon-gamma (IFN-γ), were analysed for expression in both the unvaccinated sample and vaccinated samples at both day 7 and 14, as seen in figure 48. β-actin was used as a reference gene to normalise data. IL-2 is a cytokine involved in the differentiation of T-helper cells and activation of effector lymphocytes (Wang *et al.* 2018). IL-6 is a multifunctional cytokine, found to be an essential part of the innate immune response in orange spotted grouper (Lin *et al.* 2022). IFN-γ is another cytokine involved in the activation and differentiation of cells such as T cells, B cells and macrophages (Hu *et al.* 2021).

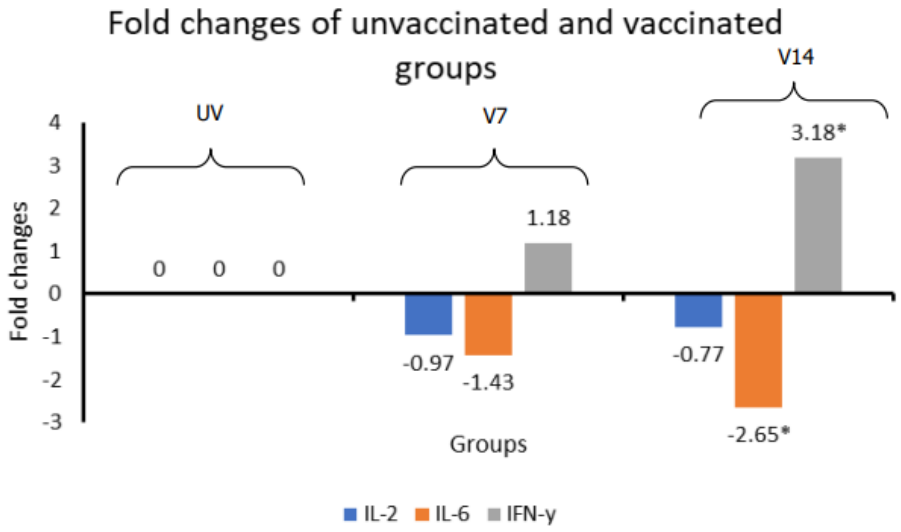

Figure 48. Fold changes of gene expression for IL-2, IL-6 and IFN-Ɣ for unvaccinated and vaccinated spleen tissue from TGGG
UV represent unvaccinated group while V7 and V14 represent vaccinated day 7 group and vaccinated day 14 group, respectively. Asterisk (*) Represent a significance expression as value greater than 2 considered as significantly differences. Results provided by collaborator and presented as given to me.

The unvaccinated group is seen here as the baseline as it is evident there is no change in gene expression of either IL-2, IL-6 or IFN-Y. The Livak method was used to quantify (Livak and Schmittgen 2001), and a fold change of >2 is accepted as significant expression. Gene IL-2 is shown in blue, which shows a fold change of 0 in the unvaccinated group compared to -0.97 in the group vaccinated on day 7 and -0.77 on the group vaccinated on day 14. There is also a fold change in IL-6 expression, -1.43 in the sample vaccinated on day 7 and -2.65 in the sample vaccinated on day 14. Another fold change is also seen in the IFN-Y gene, a 1.18 fold change and 3.18 fold change for the groups vaccinated on day 7 and 14 respectively, showing a significant upregulation of gene expression in the day 14 sample. Another significant result taken here was the downregulation of IL-6 in the group vaccinated on day 14.

The following results measured the fold changes for the groups challenged with *V. parahaemolyticus*. UVC is unvaccinated and challenged day 7, VC7 is the group vaccinated and challenged on day 7. The next two groups are UVC14; unvaccinated, and challenge on

day 14 and VC14; vaccinated and challenged on day 14. The same three genes were measured for expression.

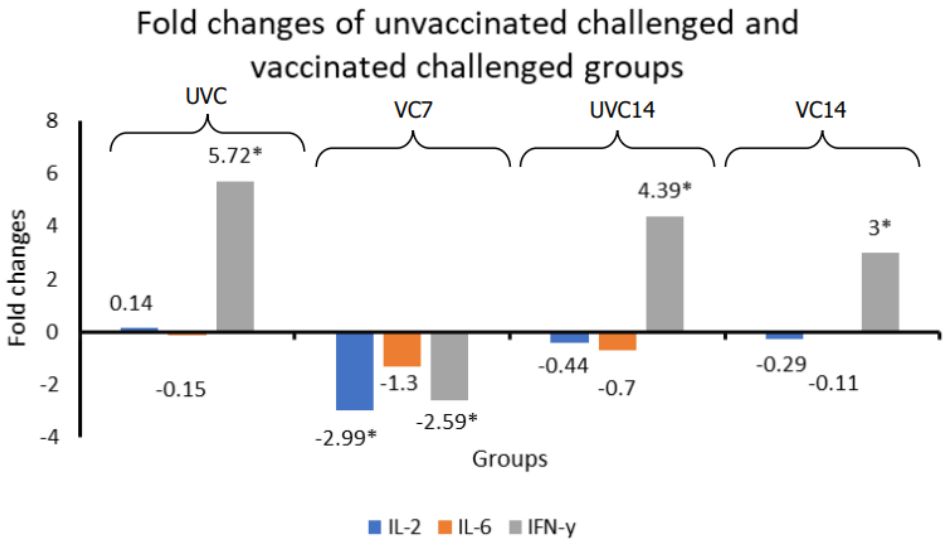

Figure 49. Fold changes of gene expression for IL-2, IL-6 and IFN-Ɣ for unvaccinated challenged and vaccinated challenged spleen tissue from TGGG.
UVC7 represent unvaccinated challenged day 7, VC7 represent vaccinated challenged day, UVC14 represent unvaccinated challenged day 14 while VC14 represent vaccinated challenged day 14. Asterisk (*) represent significance difference as value greater than 2 considered as significantly differences. Results provided by collaborator and presented as given to me.

The results from this gene expression show more varied results compared to the unchallenged samples. There are multiple significant upregulations; IFN-Y showed significant upregulation in all groups other than VC7, in this sample IFN-Y showed significant downregulation. Another significant fold change in expression here was in IL-2 showing significant downregulation in VC7, with a fold change of -2.99 compared to the UVC.

To conclude the results from gene expression analysis, it can be summarised that the fusion fragment protein elicited an immune response in TGGG grouper fish. Results comparison between the vaccinated and challenged day 14 and unvaccinated challenged day 14, shows

a difference in upregulation in IFN- Y, and possible reduced immune response as there is a lower IFN-Y expression in the vaccinated group compared to the unvaccinated group, which had a much higher fold change in upregulation, showing immune response modulation.

3.3.5 Summary of generating a subunit vaccine in *E. coli* for use in disease control in grouper fish

This project aimed to express fusion proteins in *E. coli* to be used as a subunit vaccine for TGGG grouper fish to prevent *V. parahaemolyticus* infection. This fusion protein was composed of fragments of GADPH and OmpK, from the pathogen *V. harveyi*. Cloning was performed to generate this fusion protein which consisted of the use of a pET30 vector which contained an N terminal his tag. The first construct was OmpKf-GAPDHf, where OmpKf was at the N terminus and fused to GAPDHf at the C terminus, through a flexible linker, the reverse was also generated; GAPDHf-OmpKf, whereby GAPDHf was at the N terminus, fused to OmpKf at the C terminus through a flexible linker.

Following cloning of the fusion protein, it was successfully expressed in *E. coli* in its soluble form, following optimisation using the cell line BL21 DE3. The cell line BL21 is a strain used for routine protein expression. The BL21 DE3 is a cell line which contains a chromosomal copy of the T7 RNA polymerase, for efficient expression under a T7 promotor (Joseph, Pichaimuthu and Srimeenakshi 2015). Early testing showed that there was a higher level of insoluble protein compared to soluble, therefore the expression process was optimised further to improve soluble protein yields, which was achieved by lowering incubation temperature post induction. Purification results showed a high presence of contaminating bands in the elution samples however, this project had set out to use both the purified and non-purified crude extract from *E. coli*.

Following expression trials at the University of Kent, the constructs were sent to collaborators; Professor Zarina Amin and Dr Cahyo Budiman at the Faculty of Science and Natural Resources, University of Malaysia Sabah. The aim of this project was to generate these fusion proteins in *E .coli* which could then be purified and administered to grouper fish in Malaysia. All work carried out during this later stage of the project was conducted by Iffah Hayani Binti Md Daud. As previously mentioned this project was carried out in parallel to researchers at University Malaysia Sabah, who had achieved optimised purification methods using *E. coli* Rosetta cells. Following protein purification, grouper fish were vaccinated via intramuscular injection prior to bacterial challenge of *V. parahaemolyticus.* This challenge trial used RNA screening from body tissue to screen for gene upregulation or down regulation rather than survival rate as this was used to screen for the immune response caused by injection of this fusion protein and to see what impact it has on the fish, both in the presence of the pathogen and without. This subunit vaccine was designed using specified fragments of the pathogen with the aim of eliciting an immune response. The three immune genes analysed by RT-qPCR were IL-2, IL-6 and IFN-γ. Expression was analysed and compared to the control which was the unvaccinated which showed fold changed of 0 for all three genes. There was a significant upregulation of the IFN- γ gene in challenged groups; with the most significant fold change in the unvaccinated and challenged day 7 group, in comparison to the vaccinated and challenged day 7 group. The upregulation of IFN-Y, was not surprising however, there was no significant upregulation of both IL-2 and IL-6 in the unvaccinated challenged day 7 group, this could be an indication that the challenging method was not sufficient as it can be expected that the presence of the pathogen would cause initiation of the immune response. The method used for bacterial challenging with the abrasion and submersion method, this is the most comparative to methods of infection in farms (Decostere, Lammens and Haesebrouck, 2000), however cannot be quantified when in a controlled study. Therefore, a method such as injection of the pathogen may prove suitable

to monitor the gene expression change in the unvaccinated group post bacterial challenge. There was significant down regulation in the vaccinated group, showing possible immune response, however this was not seen in the day 14 groups as both these showed significant up regulation between the unvaccinated and vaccinated, showing possible modulation of the immune system. There was also clear down regulation of both the IL-2 and IL-6 genes in the unvaccinated groups compared to the vaccinated groups, suggesting activation of the immune response, a possible downregulation effect to reduce extreme inflammation.

In summary the results from this study can be used to develop this vaccine further as there is evidence of a varied immune response seen in the grouper fish both the vaccinated and challenged compared to the unvaccinated and challenged, therefore further testing regarding higher levels of bacterial challenging and further immune response analysis would expand this project further.

3.4 PETase expression in *E. coli* to combat plastic pollution

There are several different organisms currently utilised for recombinant protein production, amongst them are bacteria, yeast, and algae. Algae had been previously used in other projects within this work and was selected due to its potential as an oral feed for both shrimp and fish. PETase has previously been successfully exported using a range of different organisms such as *C. reinhardtii* (Kim *et al.* 2020)*, B. subtilis* and *E. coli* (Maity *et al.* 2021). Previous studies have demonstrated that *C. reinhardtii* engineered to produce PETase, obtained the enzyme in its active form. After incubating with microplastics after 2-4 weeks, HPLC revealed evidence of PET degradation (Kim *et al.* 2020). The breakdown products screened for were TPA, for 30 mg of PET plastic, 9.12 mg of TPA was generated (Kim *et al.* 2020). Other areas of research have used yeast as an expression system; here the use of *Pichia pastoris* as a whole cell biocatalyst by expressing PETase on the surface of the cell (Chen *et al.* 2020). This method discovered that expressing the protein on the surface of the cell, improved pH and thermal stability of the enzyme, as well as increasing enzyme activity 36 fold- compared to their previously purified PETase (Chen *et al.* 2020). Microalgae could be considered a 'green process' due to its environmental benefits however the yields of recombinant protein production in microalgae are often lower in comparison to bacterial expression systems.

Bacterial expression systems offer high protein yields, low cost production whilst still being able to achieve high cell density cultures as well as ease of relatively simple genetic manipulation. This project aimed at improving current yields of PETase protein expression, therefore choosing an organism which could achieve high expression levels was crucial, subsequently *E. coli* was the expression system used here. Not only can *E. coli* can be cultured using standard laboratory techniques such as Erlenmeyer shake flask expression, it can also be used in large scale cultivation using bioreactors to achieve a higher cell density and

protein production; a major draw for this study. Other benefits of using *E. coli* as a host organism include cheaper price of upstream and downstream processing proving the expression to be more cost effective.

3.4.1 Aim: Express PETase using a bacterial expression host

The aim of this research was to express PETase using an economical bacterial system due to its properties such as rapid growth rates and scalability. The aim of this project was to develop an optimised expression system using *E. coli* to express the plastic degrading enzyme PETase. Upon optimisation at small scale, the next aim was the upscale growth and purification of the protein, followed by activity analysis using HPLC.

3.4.2 PETase expression project outline

A challenge with protein production is the possibility of the host organism to fully fold the exported proteins, this was a parameter that had to be accounted for when producing PETase using a host organism as PETase has two disulfide bonds as seen in figure 50.

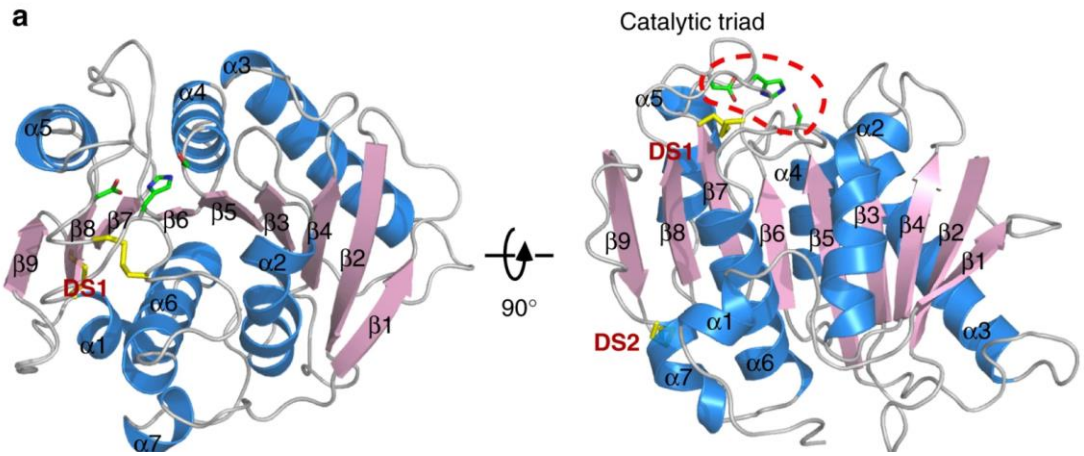

Figure 50. Overall structure of PETase showing the catalytic triad and disulphide bridges
Structure of PETase showing the catalytic triad in the red circle and the disulphide bridges, highlighted in red. Image taken from (Han *et al.* 2017).

Export was targeted to the periplasmic space due to the presence of the two disulfide bridges in PETase. It is well documented that the oxidative environment of the periplasm helps the formation of disulfide bonds (Seo *et al.* 2019), therefore targeting PETase to export to the periplasm could aid with the proper folding of the protein and therefore full functionality.

This project was undertaken alongside microalgal work with the aim of improving expression levels of PETase to the periplasm. Work conducted by previous laboratory members had not shown any export of the protein therefore, the first plan was to optimise expression using different cell lines then modify the plasmid. Several different constructs are used throughout this chapter, seen in table 18. Another benefit of using *E. coli* as an expression system is the use of different signal peptides to direct export via either the Sec or Tat transport pathway. This would not have been an option available if using a microalgal based expression system. Once folded PETase contains 2 disulfide bonds, one within its active site; vital for the hydrolase activity of this enzyme (Fecker *et al.* 2018). To support the formation of these disulfide bonds in the cytoplasmic environment, both protein disulfide-isomerase (PDI) and sulfhydryl oxidase (ErV1p) were expressed on the same plasmid as PETase. This system is named CyDisCo; 'cytoplasmic disulfide bond formation in *E. coli'*. This is a system which has proved effective at exporting antibody fragments to the periplasm via the tat pathway in *E. coli* (Alanen *et al.* 2015) .

One of the key considerations for this project was export of the protein, therefore it was vital to explore different signal peptides to ensure export to the periplasm. The first construct used in this work was a plasmid containing PETase with its native signal peptide in place as well as SP_{PhoDn}. PhoD is a Tat specific signal peptide, as it contains to twin arginine motif, originally found from *B. subtilis*, however the signal peptide used here contains a mutation within the h region of the peptide, referred to as PhoDn. The mutation here is of the alanine

in position 51 to asparagine. The native signal peptide from *I. sakiensis* is present in this construct, which has been shown previously to produce the highest levels of protein secretion in *B. subtilis* (Huang *et al.* 2018). A range of different constructs were used in this study containing a mixture of the native signal peptide (SP_{PETase}) and PhoDn signal peptide (SP_{PhoDn}), as seen in table 18.

Table 18. List of signal peptides used in this study for PETase export

Construct name	Signal peptide (s) present	Details
SP_{PhoDn}-SP_{PETase}-PETase	SP_{PhoDn} + SP_{PETase}	Construct containing gene for PETase and both PhoDn and the native signal peptide
SP_{PhoDn}-SP_{PETase}-PETase-CyDisCo	SP_{PhoDn} + SP_{PETase}	Construct containing gene for PETase and both PhoDn and the native signal peptide as well as CyDisCo
SP_{PhoDn}-PETase	SP_{PhoDn}	Construct containing gene for PETase and PhoDn signal peptide
SP_{PhoDn}-PETase-CyDisCo	SP_{PhoDn}	Construct containing gene for PETase and PhoDn signal peptide as well as CyDisCo
SP_{PETase}-PETase	SP_{PETase}	Construct containing gene for PETase and the native signal peptide for PETase
SP_{PETase}-PETase-CyDisCo	SP_{PETase}	Construct containing gene for PETase and the native signal peptide for PETase as well as CyDisCo

Different cell lines of *E. coli* were used throughout this study to explore the best one for high protein yields. These include *E. coli* W3110, a K-12 derivative, BL21 DE3, a derivative of BL21, MC4100, another K-12 strain and MC4100 Δ*tat*, which has tat machinery deleted. All of which are used for protein expression.

3.4.3 Tat pathway vs Sec pathway

To first investigate the levels of export of PETase to the periplasm, PETase with its native signal peptide still present (SP_{PETase}), was expressed using a pEXTII plasmid also containing

the PhoDn signal peptide (SP_{PhoDn}), with and without CyDisCo. PhoDn was assumed to be a Tat specific signal peptide, therefore, the first experiment was to achieve export which could then be optimised. The cell line used for this experiment was a K-12 strain of *E. coli;* W3110, under two conditions, one grown in LB media and the other grown in TB media. Cultures were incubated at 30°C until ready for induction with IPTG, which was followed by a temperature reduction to 30°C for protein expression. The cultures were processed following the fractionation procedure (2.5.7) and shown in figure 51.

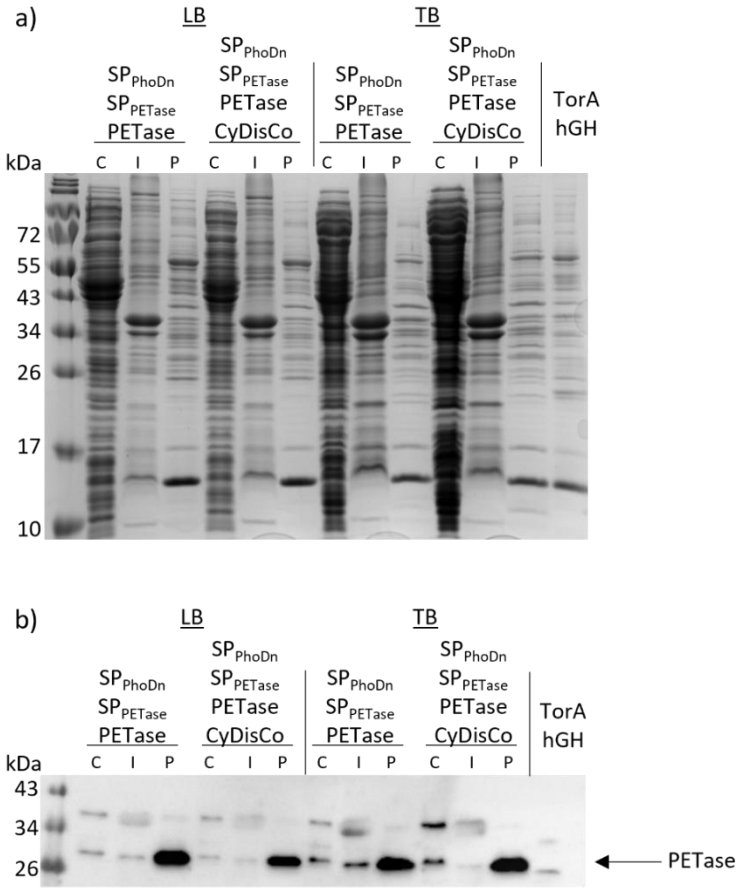

Figure 51. Fractionation of SP_{PhoDn}-SP_{PETase}-PETase with and without CyDisCo in W3110 from shake flask expression

Two constructs; SP_{PhoDn}-SP_{PETase}-PETase and SP_{PhoDn}-SP_{PETase}-PETase-CyDisCo were expressed in W3110 at shake flask in both LB and TB media at 37°C, according to section 2.5.1. Cells were grown at 30°C post induction and harvested following method reported in 2.5.7. (a) The cytoplasmic (C), insoluble (I) and periplasmic (P) fractions were separated by SDS-PAGE and stained with Coomassie Brilliant Blue for visualisation. (b) The corresponding gel was used for immunoblotting and proteins were visualised using antibodies to the C-terminal His tag.

The first six lanes are expression results from W3110 expressing SP_{PhoDn}-SP_{PETase}-PETase and SP_{PhoDn}-SP_{PETase}-PETase-CyDisCo respectively, in LB media. These constructs were also expressed in TB media, seen in the next 6 lanes. Figure 51 highlights that co-expression of CyDisCo does not have an impact on the export of the PETase protein in both media types. This was an unexpected results due to PETase containing two disulfide bonds, therefore it was predicted that the CyDisCo strain would improve export. The co-expression system of CyDisCo has been used to effectively express many disulfide bonded proteins of intertest (Yu *et al.* 2022), and therefore was expected to improve expression of the PETase protein which contains two disulfide bonds. There is a clear presence of PETase protein in the periplasmic fraction of all conditions however, there is no clear improvement with the addition of CyDisCo. Both media types showed clear export of PETase to the periplasm however there was variation between the presence of a higher band between 34 and 43 kDa in the cytoplasmic and insoluble fraction. When grown in TB media there appears to be an increase in the amount of PETase in both the cytoplasmic fraction and insoluble fraction when compared to the LB cultures, therefore it was concluded the best condition for successful export of SP_{PhoDn}-SP_{PETase}-PETase to the periplasm is LB media without CyDisCo, as the total protein was mostly found in the periplasmic fraction. Consequently, SP_{PhoDn}-SP_{PETase}-PETase expressed in W3110 was upscaled to a 400 ml culture using a baffled flask to see if there would be an improvement of expression once purified.

3.4.4 SP_{PhoDn}- SP_{PETase}-PETase large scale growth

SP_{PhoDn}- SP_{PETase}-PETase was grown at larger scale to improve protein export (figure 52). A 400 ml TB culture was grown following the cell culture and plasmid induction protocol detailed earlier (section 2.5.1). The culture was harvested, and the periplasmic fraction was purified

using a 5 ml HisTrap HP ™ column on the AKTA system. The results from this purification can be seen in figure 52.

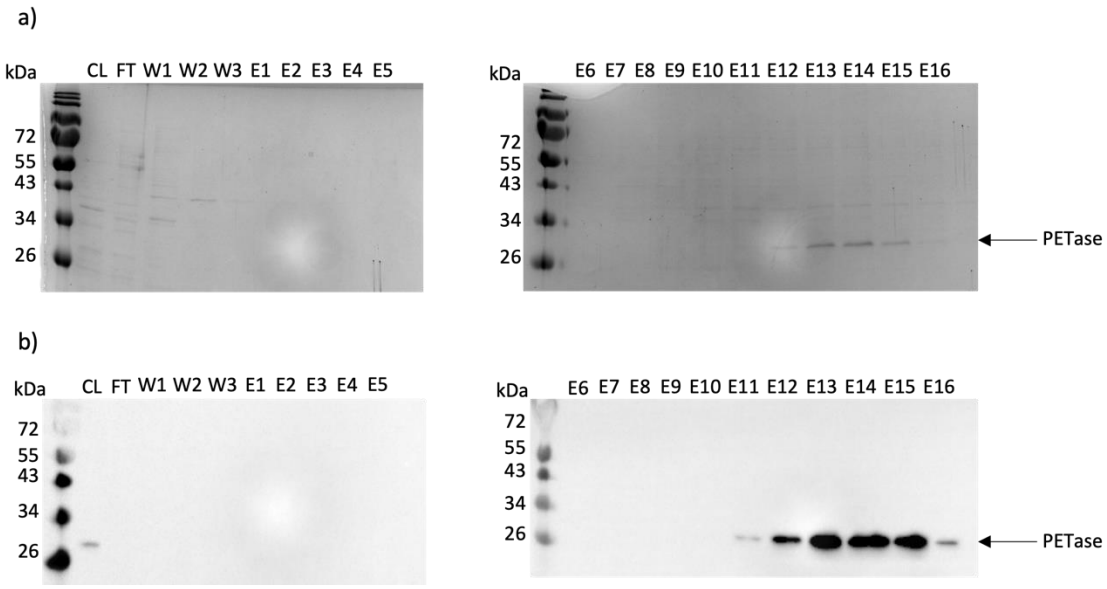

Figure 52. Purification of SP_{PhoDn}-SP_{PETase}-PETase from a periplasmic fraction from large scale shake flask expression
SP_{PhoDn}-SP_{PETase}-PETase was expressed in W3110 at large scale in TB media following section 2.5.1. The periplasmic fraction was collected and applied to a 5 ml HisTrap HP™ column. Samples collected include crude lysate (CL), flow through (FT), three washes (W1-3) and 16 protein elutions (E1-16). a) Fractions were separated by SDS-PAGE and stained using Coomassie Brilliant Blue. b) The corresponding gels were immunoblotted following SDS-PAGE, using antibodies to the C terminal His tag on PETase.

The samples from purification were separated by SDS-PAGE and western blotted. The presence of PETase protein was detected in the Anti-his western blot, seen in figure 52 b. The protein was eluted between elution fractions 12 (E12) and elution 16 (E16). Although the western blot has a clear signal at the expected size of PETase, the Coomassie stained gel shows a much lower amount, however it does still confirm the presence of protein, and a clear purification with limited number of non-specific bands. The crude lysate (CL) sample shows a small level of protein prior to purification and no protein in either the flow through (FT) or wash fractions (W1-3).

To further investigate whether PETase was being exported via Sec or Tat, an expression and fractionation was performed at small scale to compare what impact the native signal peptide

has, as it is predicted to be Tat specific (Huang *et al.* 2018). This fractionation can be seen in figure 53.

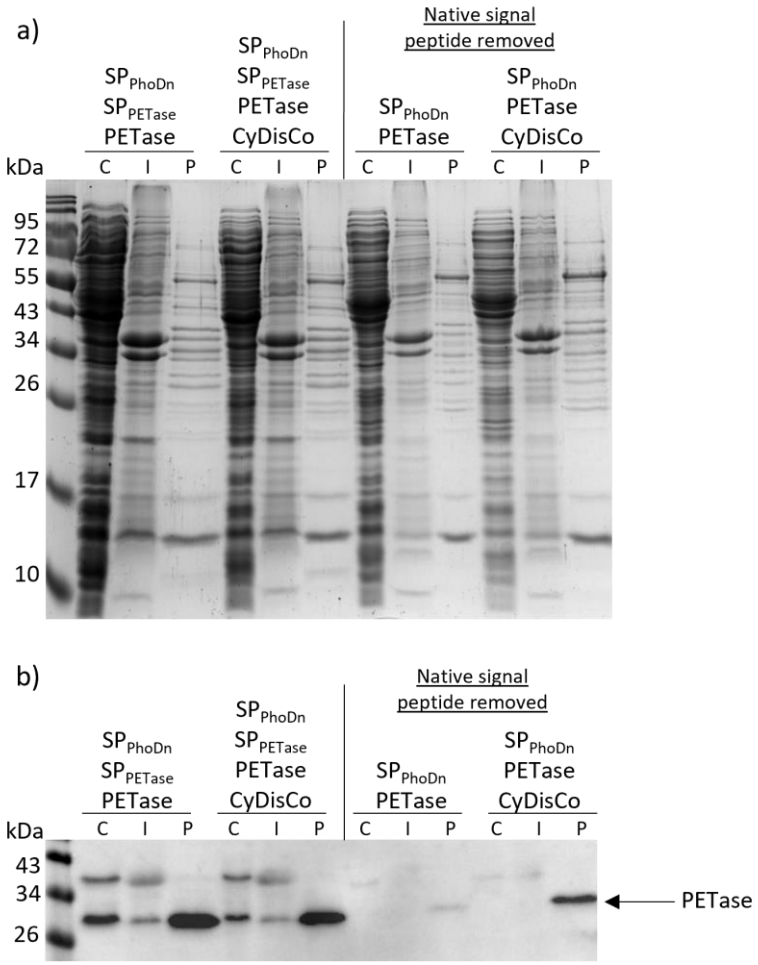

Figure 53. Expression and fractionation for comparison of SP_{PhoDn}-SP_{PETase}-PETase and SP_{PhoDn}-PETase, both with and without CyDisCo at shake flask in W3110
All four constructs were expressed in W3110 in LB at 37°C (section 2.5.1). Post induction cells were grown at 30°C before fractionating into cytoplasmic (C), insoluble (I) and periplasmic (P) fractions (section 2.5.7). a) Fractions were separated by SDS-PAGE and stained with Coomassie Brilliant Blue for visualisation. (b) The corresponding gel was used for immunoblotting and proteins were visualised using antibodies to the C-terminal His tag.

The first two fractionations (first six lanes) in the blot and Coomassie stained gel show the constructs with the SP_{PETase} still in place after the SP_{PhoDn}. The following six lanes show the constructs with SP_{PETase} removed, and thus just the PhoDn signal peptide. The overall result from this fractionation shows a clear reduction in the export when the PETase native signal peptide has been removed, leaving just SP_{PhoDn} in place. Although the construct with the native signal peptide shows a higher level of export, the results from the constructs without

SP_{PETase} show a the first improvement in periplasmic export of PETase when CyDisCo is present. This improvement is not visible in the fractionation with the native signal peptide present, indicating that the native signal peptide is more important in the efficient export of PETase in comparison to the PhoDn signal peptide. This suggests there is more reliance on CyDisCo for disulfide bond formation when the native signal peptide is not present.

Following the improvement of CyDisCo seen above when the native signal peptide was absent, this theory was applied to the construct containing the native signal peptide to see if this yield could be increased through the use of a dual plasmid system containing CyDisCo. The dual plasmid system consisted of one plasmid which contains the CyDisCo components in addition to a second plasmid which contains the protein of interest, in this case SP_{PETase}-PETase. Figure 54 shows results from an expression and fractionation of PETase using the dual plasmid system in cell line W3110, comparing two media types: LB and TB with both standard shake flasks and baffled.

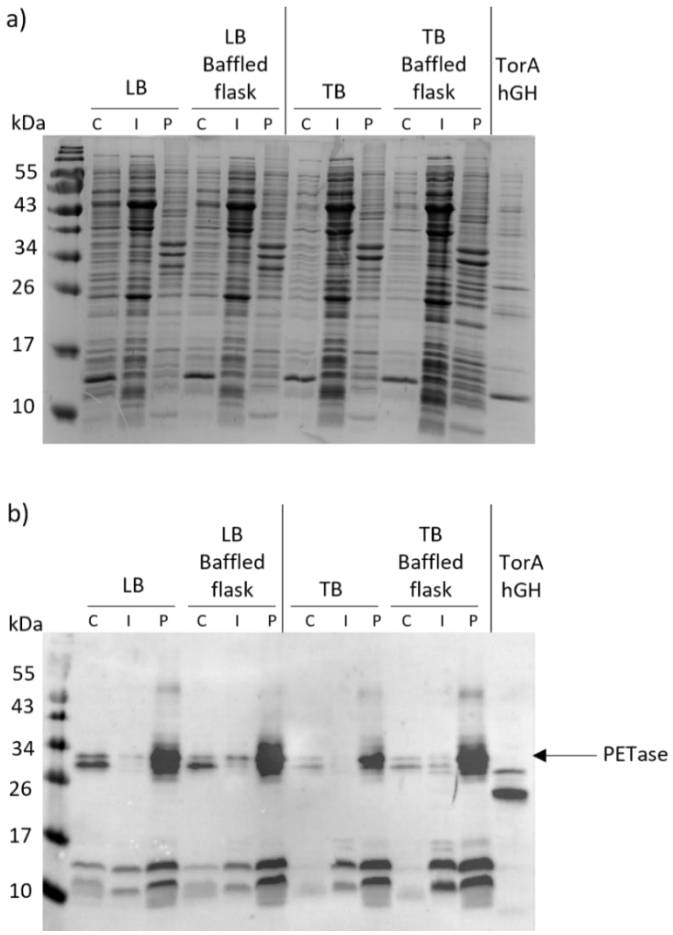

Figure 54. Expression of fractionation of SP_{PETase}-PETase using a dual plasmid system containing CyDisCo in W3110

Expression and fractionation of SP_{PETase}-PETase in a dual plasmid system with CyDisCo in W3110 (section 2.5.2) at 37°C, 250 rpm. Following induction cells were grown at 30°C, before fractionating into cytoplasmic (C), insoluble (I) and periplasmic (P) fractions (section 2.5.7). a) Fractions were separated by SDS-PAGE and stained with Coomassie Brilliant Blue for visualisation. (b) The corresponding gel was used for immunoblotting and proteins were visualised using antibodies to the C-terminal His tag.

The results from this small-scale expression show very similar levels of export of PETase in all conditions, both media and flask type. To maximise cell density, an increase in oxygen availability is key, therefore flask type was altered for this expression as the presence of baffled edges in a flask, increases aeration (Rosano and Ceccarelli 2014). As seen above there is an increase of cytoplasmic protein compared to periplasmic protein that has been seen in previous experiments. It can also be noted that there is some protein detected on the western blot between 10 and 17 kDa, which could be an indication of protein degradation

and cleavage. There is no clear definition as to whether LB or TB was the preferred media for increased levels of PETase export in this study, therefore further evidence was needed.

As seen previously, there appeared to be high levels of PETase exported to the periplasm, the next step was to determine which pathway was being used in order to optimise the use of the pathway. Therefore, expressions and fractionations were performed using MC4100 delta *tatABCDE* (Δ *tatABCDE*) cell line, this cell line has all Tat components removed to ensure that protein export can take place by the Sec pathway alone. There are three key membrane proteins involved in the Tat system; TatA, TatB and TatC, all encoded by the *tatABCD* operon. This operon also encodes TatD, which is not used in the Tat pathway as it codes for a DNase. The results from this expression can be seen in figure 55.

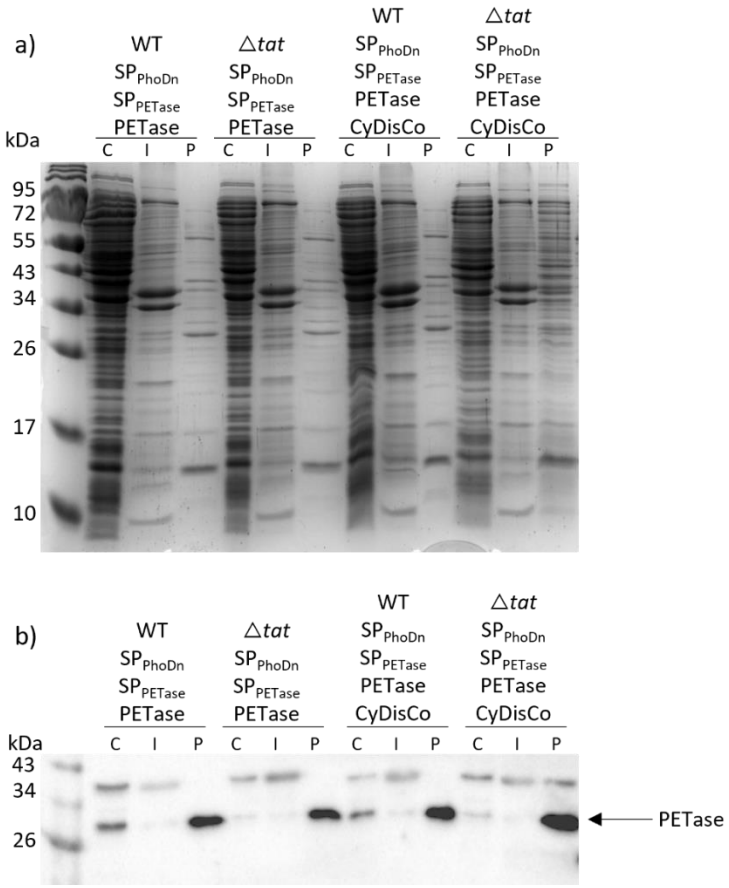

Figure 55. Expression and fractionation of SP$_{PhoDn}$-SP$_{PETase}$-PETase and SP$_{PhoDn}$-SP$_{PETase}$-PETase-CyDisCo from MC4100 WT and MC4110 △*tat* cells
Expression and fractionation of SP$_{PhoDn}$-SP$_{PETase}$-PETase and SP$_{PhoDn}$-SP$_{PETase}$-PETase-CyDisCo in MC4100 WT and MC4100 △*tat* cells in TB media at 37°C (section 2.5.1). Post induction, cultures were grown at 30°C before fractionation into cytoplasmic (C), insoluble (I) and periplasmic (P) fractions. a) Fractions were separated by SDS-PAGE and stained with Coomassie Brilliant Blue for visualisation. (b) The corresponding gel was used for immunoblotting and proteins were visualised using antibodies to the C-terminal His tag.

The results of the MC4100 △*tat* strain above indicate there is no real impact when the Tat machinery is not present in the host cell as we are still seeing export to the periplasm, in both MC4100 WT and MC4100 △*tat*, suggesting that translocation is occurring via the Sec pathway. However, we cannot confirm which pathway is being used in the wild type (WT) cell line. The constructs used here contain the SP$_{PhoDn}$ and well as SP$_{PETase}$. Due to their being no clear difference in export of PETase between the four fractionations we can conclude that the export of PETase in cell line MC4100 is not reliant on the sole use of the tat machinery.

The same experiment was also conducted using PETase with the native signal peptide removed to test just the PhoDn signal peptide (SP_{PhoDn}-PETase) and determine the export in Δ*tat* cells. As seen previously when using the PETase construct with SP_{PETase} still present, there is no clear difference in the export of PETase to the periplasm between wild type cells and Δ*tat* cells, indicating export of the PETase protein was via the Sec pathway when the SP_{PETase} was present. The results from the experiment without SP_{PETase} can be seen in figure 56.

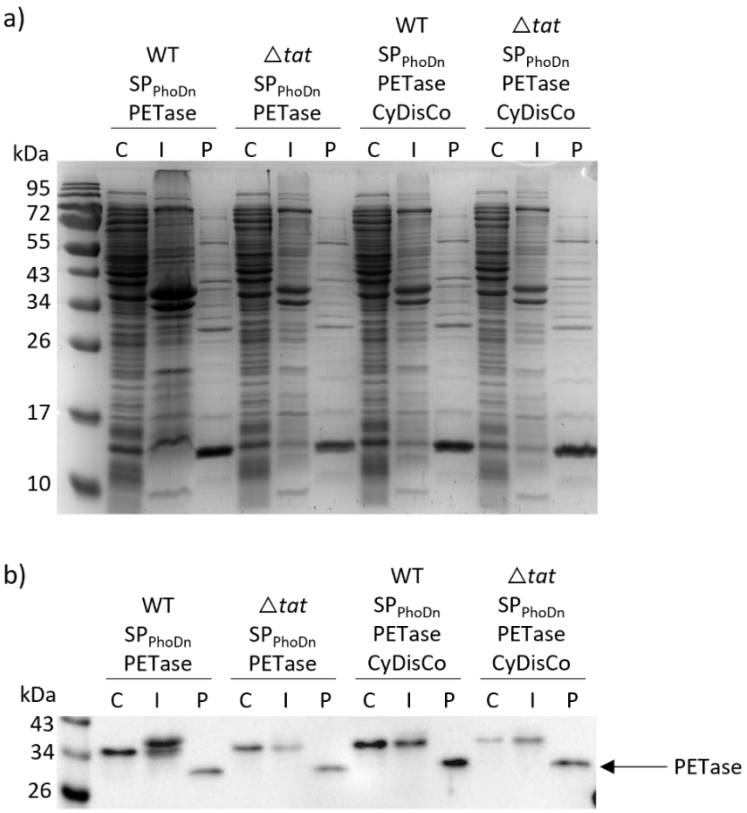

Figure 56. Expression and fractionation of SP_{PhoDn}-PETase and SP_{PhoDn}-PETase-CyDisCo from MC4100 WT and MC4110 Δ*tat* cells

Expression of SP_{PhoDn}-PETase and SP_{PhoDn}-PETase-CyDisCo in MC4100 WT and MC4100 Δ*tat* cells in TB media (section 2.5.1). Post induction cultures were grown at 30°C before fractionation into cytoplasmic (C), insoluble (I) and periplasmic (P) fractions, (section 2.5.7). a) Fractions were separated by SDS-PAGE and stained with Coomassie Brilliant Blue for visualisation. (b) The corresponding gel was used for immunoblotting and proteins were visualised using antibodies to the C-terminal His tag.

As previously found, the level of export of the PETase protein was not altered between the WT and Δ*tat* cell lines (figure 55). This is a similar finding to the results from this assay (figure

56), however, it is noted that there appears to be a higher level of PETase protein in the cytoplasmic and insoluble protein fraction compared to the periplasmic fraction. This is a finding that has not been seen in the earlier fractionations, suggesting some difference in export to the periplasm when SP_{PETase} is not present.

It can be concluded that PETase expressed in a Δ*tat* cell line with either SP_{PhoDn}-SP_{PETase}-PETase or SP_{PhoDn}-PETase, can be exported using the Sec system. However, when expressed in the WT cell line it is still unknown whether the protein is being exported by the Sec pathway alone or the protein can utilise both pathways.

3.4.5 Optimising cell lines

The following work continued with the PETase plasmid containing only SP_{PETase}, without SP_{PhoDn}, as this achieved the greatest export, an expression and fractionation were carried out using different cell lines; BL21 DE3 and W3110, see figure 57, to determine if expression levels could be optimised before expressing the protein using fed-batch fermentation. *E. coli* BL21 DE3 is a B strain which is often the strain of choice for recombinant protein production whereas W3110 is a K-12 strain.

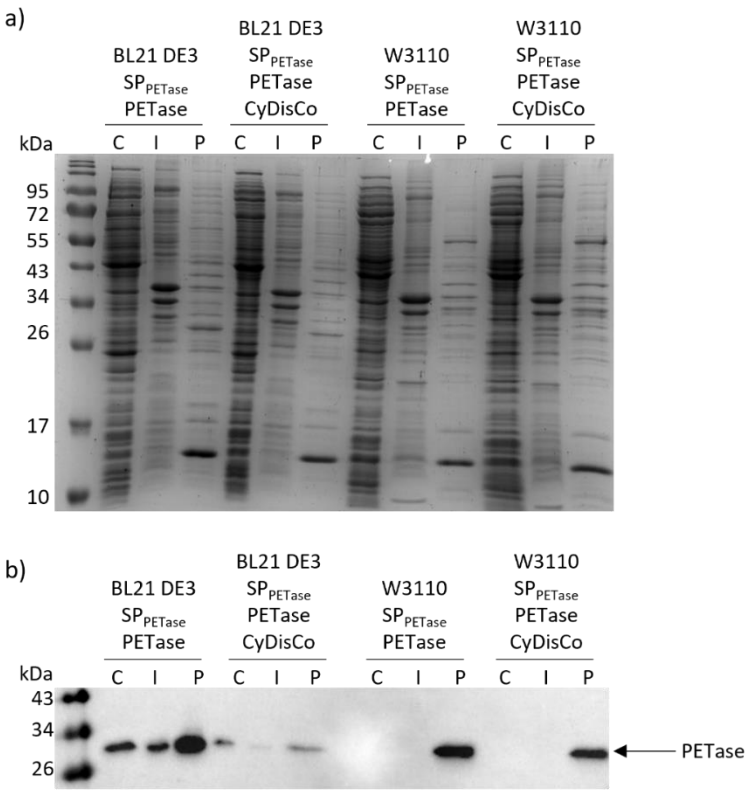

Figure 57. Expression and fractionation of SP$_{PETase}$-PETase and SP$_{PETase}$-PETase-CyDisCo in BL21 DE3 and W3110

Expression and fractionation of SP$_{PETase}$-PETase and SP$_{PETase}$-PETase-CyDisCo from cell lines BL21 DE3 and W3110 in TB media (section 2.5.1). Post induction, cultures were grown at 30^{o}C before fractionation into cytoplasmic (C), insoluble (I) and periplasmic (P) fractions (section 2.5.7). a) Fractions were separated by SDS-PAGE and stained with Coomassie Brilliant Blue for visualisation. (b) The corresponding gel was used for immunoblotting and proteins were visualised using antibodies to the C-terminal His tag.

The results in figure 57 show highest levels of PETase export when expressed in W3110 cell line, BL21 DE3 showed a similar level of expression however, there is protein present in both the cytoplasmic and insoluble fractions of the BL21 DE3 cell line in comparison to W3110. Focusing on the BL21 DE3 expression, there is a much higher level of PETase expression without the CyDisCo machinery. This difference is not seen in W3110 as there appears to be no difference between periplasmic PETase expression between the constructs containing the CyDisCo components. As we were focused on exporting PETase to the periplasm due to the presence of disulfide bonds required for accurate folding of the protein, the cell line used in further experiments including fermentation, was W3110.

3.4.6 Fed-batch fermentation of PETase

This project sought to express the PETase in *E. coli* which was enzymatically functional, at amounts high enough for purification to be used in plastic degradation, therefore once conditions to export PETase protein were optimised at shake flask level, the next step was to run fermentation experiments to increase protein yield. The fermentation vessels used here were 1.5 L Infors Minifors 2 bioreactors containing 500 ml SM6 media. The cell line used for expressing PETase with SP_{PETase}, was W3110, the results can be seen in figure 58. The experiment was run in duplicate using two vessels under the same conditions, labelled as vessels 1 and 2 in the growth curve below in figure 58. The fractionation was performed on samples taken from vessel 1, protein was separated by SDS-PAGE and Coomassie stained and immunoblotted, figure 58.

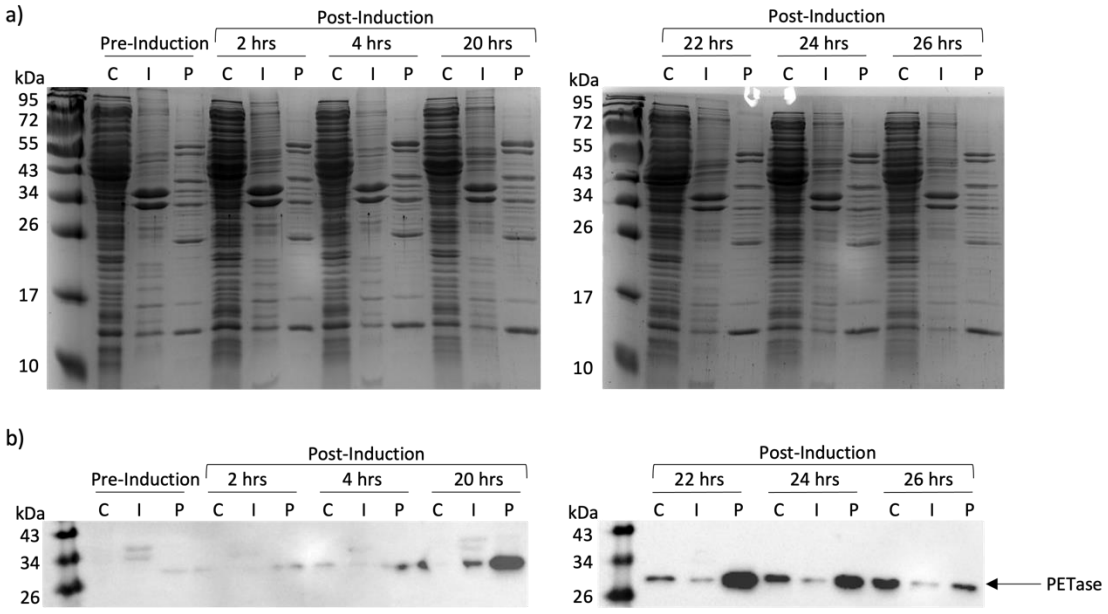

Figure 58. Fractionation of SP_{PETase}-PETase from fed batch-fermentation in W3110
SP_{PETase}-PETase was expressed according to section 2.5.4. in W3110. Expression was induced at 26 hours with IPTG and was then grown for a further 26 hours at 25°C. Samples were fractionated into cytoplasmic (C), insoluble (I) and periplasmic (P) fractions (section 2.5.7). a) Fractions were separated by SDS-PAGE and stained with Coomassie Brilliant Blue for visualisation. (b) The corresponding gel was used for immunoblotting and proteins were visualised using antibodies to the C-terminal His tag.

As seen in figure 58, samples were taken pre induction, 2 hrs, 4 hrs, 20 hrs, 22 hrs, 24 hrs and 26 hrs post induction. These samples were taken equivalent to 10 OD_{600} units and processed using the fractionation protocol. The OD_{600} of the cultures from both bioreactors was measured and plotted on the growth curve (figure 59). The western blot indicated that the highest protein levels were achieved at 22 hrs post induction (48.5 hrs post inoculation). This highest protein level corresponds to the highest OD_{600} recorded on the growth curve below. From this point onwards the OD_{600} of vessel 1 started to drop, the drop in OD_{600} was slightly less in vessel 2.

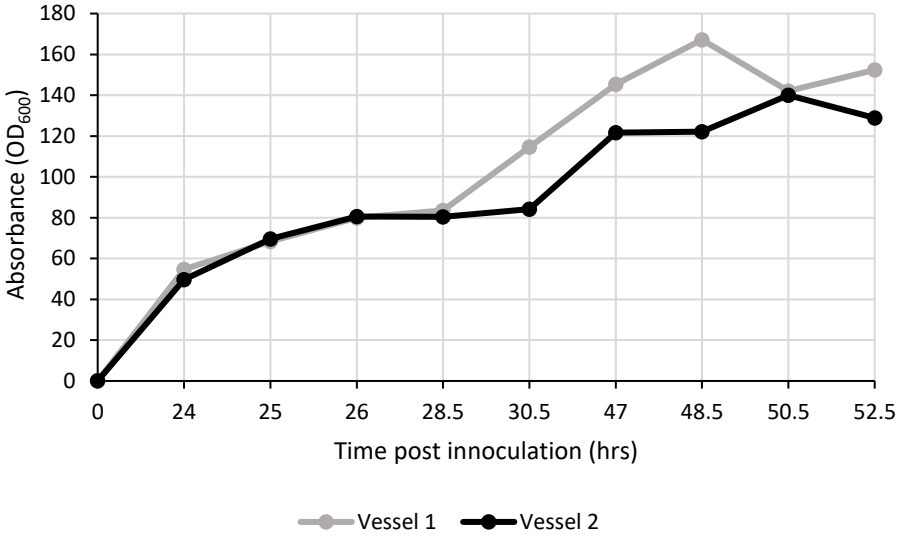

Figure 59. Growth curve of PETase from batch-fed fermentation.
Growth curve of SP_{PETase}-PETase expressed in W3110 from batch-fed fermentation using Minifors 2, the fermentation was run in duplicates indicated here by vessel 1 and vessel 2.

Following fermentation, the 500 ml 26-hr post induction culture was harvested and processed to collect the periplasmic fraction using the large scale periplasmic fractionation protocol, the sample was then purified using an IMAC 5 ml HisTrap HP ™ column, the results from this purification can be seen in figure 60.

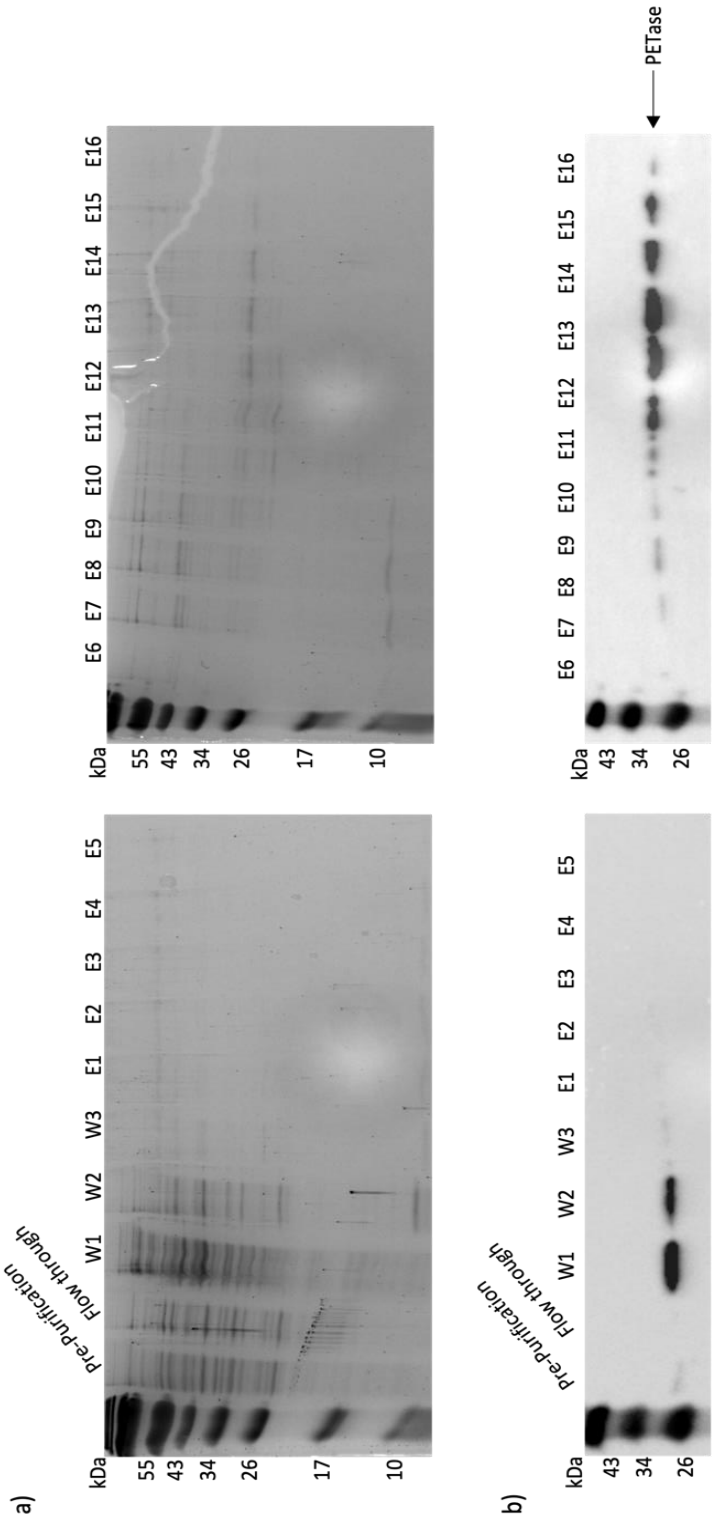

Figure 60. Purification of SP_{PETase}-PETase periplasmic fraction from batch fed fermentation. A 500 ml culture of SP_{PETase}-PETase expressed in W3110 from the previous batch fed fermentation was harvested after 26 hrs and fractionated (section 2.5.8). The 500 ml periplasmic fraction was collected and applied to a 5 ml HisTrap HP ™ column. Samples collected include crude lysate (CL), flow through (FT), three washes (W1-3) and 16 protein elutions (E1-16). a) Fractions were separated by SDS-PAGE and stained using Coomassie Brilliant Blue. b) The corresponding gels were immunoblotted following SDS-PAGE, using antibodies to the C-terminal His tag on PETase.

After achieving successful PETase export to the periplasm at fed batch fermentation, the construct containing just the native signal peptide SP_{PETase}, was used further in a repeat

fermentation using a different media, this time TB media, supplemented with glycerol as a carbon source. The results from this fermentation can be seen in figure 61.

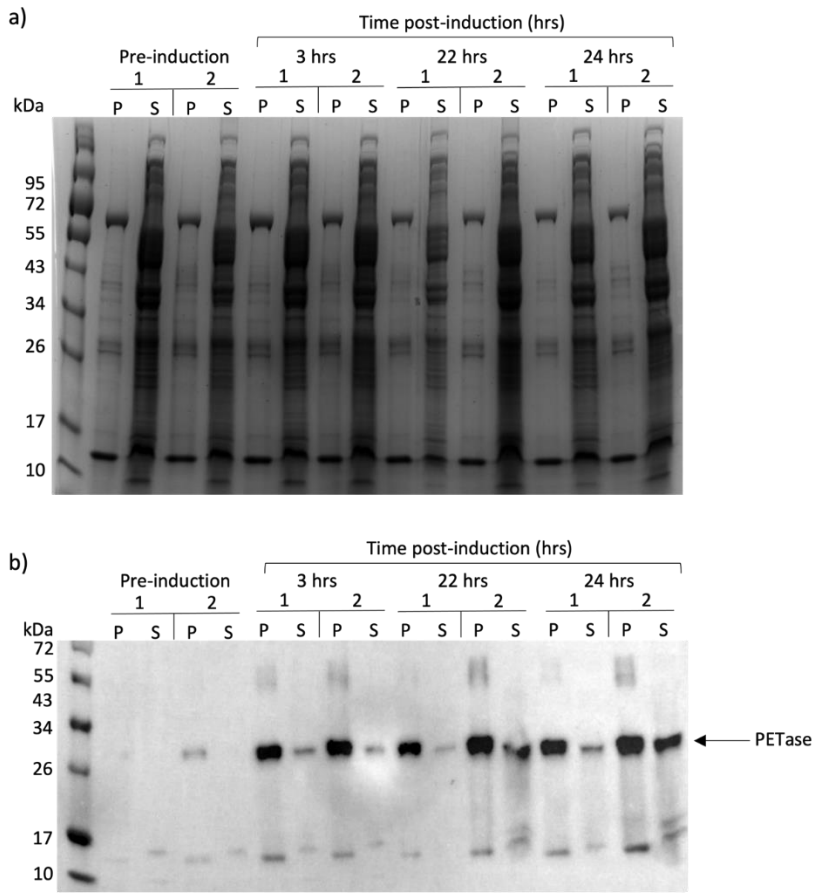

Figure 61. Fractionation into spheroplast and periplasmic fractions from batch fed fermentation of SP_{PETase} PETase using TB media.
Fractionation of SP_{PETase}-PETase from W3110 using samples equivalent to 10 OD units from two 500 ml TB fermentation vessels (labelled 1 & 2). Plasmids were induced after growing for 26 hours and was then grown for a further 24 hours at 25°C. Samples were fractionated into spheroplast (S) and periplasmic (P) fractions. a) Fractions were separated by SDS-PAGE and stained with Coomassie Brilliant Blue for visualisation. (b) The corresponding gel was used for immunoblotting and proteins were visualised using antibodies to the C-terminal His tag.

This experiment was used to evaluate the impact media choice had on protein expression at fed-batch fermentation as the standard media used is the defined media SM6. The samples from this fermentation were processed from 10 OD units similar to the previous fractionations however these samples were processed into periplasmic fractions and

spheroplasts as we were focused here on the periplasmic export. From the immunoblot it can be assumed there would be no impact between harvesting at 3 hrs post induction or 24 hrs post induction. This is a very different finding in comparison to the previous fed-batch run using SM6 media which had an increase in high cell density and protein expression.

Continuing the use of SP_{PETase}-PETase in W3110, a variety of conditions were used for fermentation using 100 ml Ambr250 bioreactors to optimised fed-batch conditions. A total of four different conditions were tested, all using the same construct and media. All bioreactors were incubated at 30°C prior to induction however post induction, two were dropped to 25°C. In each temperature condition, the stirrer speed was also dropped to alter the dissolved oxygen from the standard of 40 % to 30 %, detailed in table 19.

Table 19. Vessel conditions of the four bioreactors used in fed batch fermentation using the Ambr250

Vessel Name	Temperature	Dissolved oxygen (%)
Vessel 1	Post induction 30°C	DO 40
Vessel 2	Post induction 30°C	DO 30
Vessel 3	Post induction 25°C	DO 40
Vessel 4	Post induction 25°C	DO 30

These conditions were chosen as 30°C appeared to be the best temperature for PETase expression at small scale, therefore 25°C was also picked to ensure a range of temperatures was used. The dissolved oxygen was also modified with the aim to achieve a higher level of protein expression.

To monitor the growth of each culture, a growth curve was recorded using the absorbance measure OD_{600} was recorded post inoculation, as seen in figure 62.

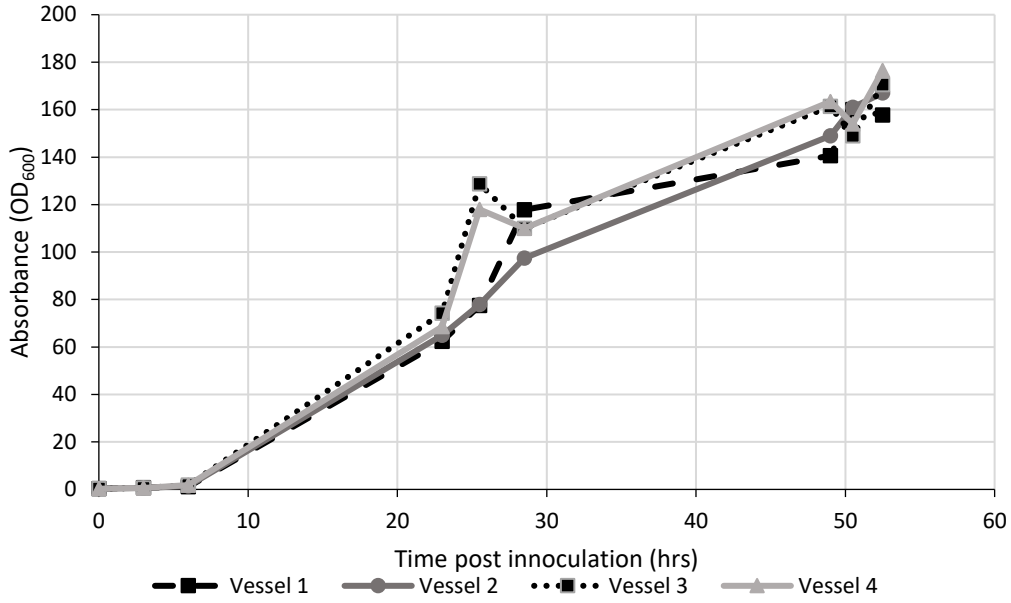

Figure 62. Growth curve of W3110 expressing PETase in Ambr250 fed batch fermentation. Absorbance was measured by OD_{600} from vessels 1-4, at various time points through the fermentation run to check for culture density and growth. Each vessel was inoculated to OD_{600} 0.3, and harvested at 52.5 hrs.

Samples were taken throughout the fermentation run and fractionated, then separated by

SDS-PAGE, as seen in figure 63.

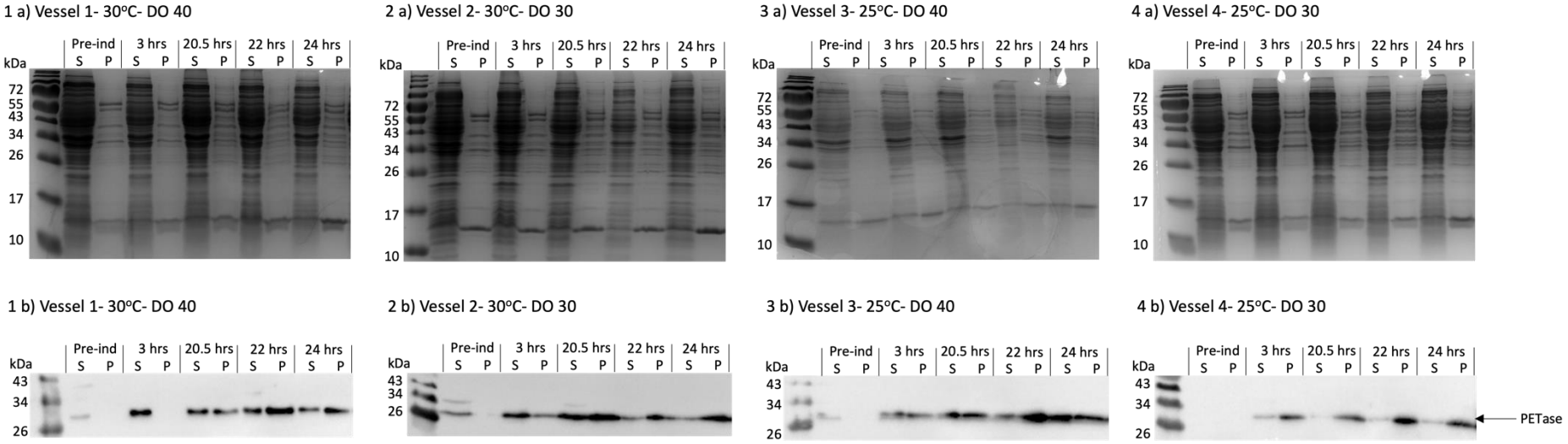

Figure 63. Expression and fractionation of SP_{PETase}-PETase in W3110 from fed batch fermentation using an Ambr250 set up.

SP_{PETase}-PETase was expressed in W3110 using an Ambr250 vessel for fed batch fermentation (section 2.5.6). 1) Culture grown at 30°C pre and post induction, dissolved oxygen content was set to 40 %. 2) Culture grown at 30°C 30°C pre and post induction, dissolved oxygen content was set to 30%. 3) Culture grown at 30°C pre and 25°C post induction, dissolved oxygen content was set to 40%. 3) Culture grown at 30°C pre and 25°C post induction, dissolved oxygen content was set to 30%. Pre induction sample was collected (pre-ind). Cultures were grown for 24 hours post induction and samples equivalent to 10 OD units were taken at time points; 3 hrs, 20.5 hrs, 22 hrs and 24 hrs post induction. Samples were fractionated into spheroplast (S) and periplasmic (P) fractions.

For this experiment samples were separated into spheroplast and periplasmic fractions, as the periplasmic fraction was the focus here for export analysis. Samples were taken pre-induction to check for any expression before inducing with IPTG, as well as regular time intervals post-induction. As seen in figure 63, the level of expression at the 3 hrs post-induction in vessel 1, only had protein present in the spheroplast, while in comparison to vessels 2, 3 and 4, there was a low level of expression in all conditions. The best time point post-induction appeared to be 22 hrs as this achieved the highest level of protein in all conditions. As a comparison could not be made between the four western blots, the samples were run on one membrane so conclusions could be made, see figure 64.

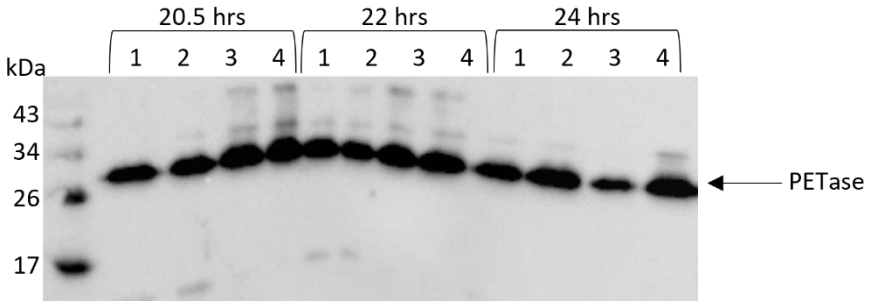

Figure 64. PETase fractionation of periplasmic fractions of all vessels from Ambr250 fermentation in W3110
Periplasmic fractions of SP_{PETase}-PETase in W3110 from the previous fed batch fermentation using the Ambr250 (figure 63), were separated using SDS-PAGE and the proteins were immunoblotted using antibodies to the C-terminal His tag. Samples include 20.5 hrs post induction, 22 hrs post induction, 24 hrs post induction and the large-scale processing of periplasmic fractions 24 hrs after induction.

Although the immunoblots from figure 63 showed possible differences in expression of PETase, once compared using one membrane, the results of periplasmic export from all four conditions look very similar for the three time points of 20.5 hrs, 22 hrs and 24 hrs.

3.4.7 Purification of PETase from Ambr250 fermentation

To determine the effect of protein export using the Ambr250 system, the 100 ml culture of SP_{PETase}-PETase expressed in W3110 from vessel 2 which appeared to have a sufficient level of protein present was fractionated (section 2.5.9) and purified using the AKTA system, the results can be seen in figure 65.

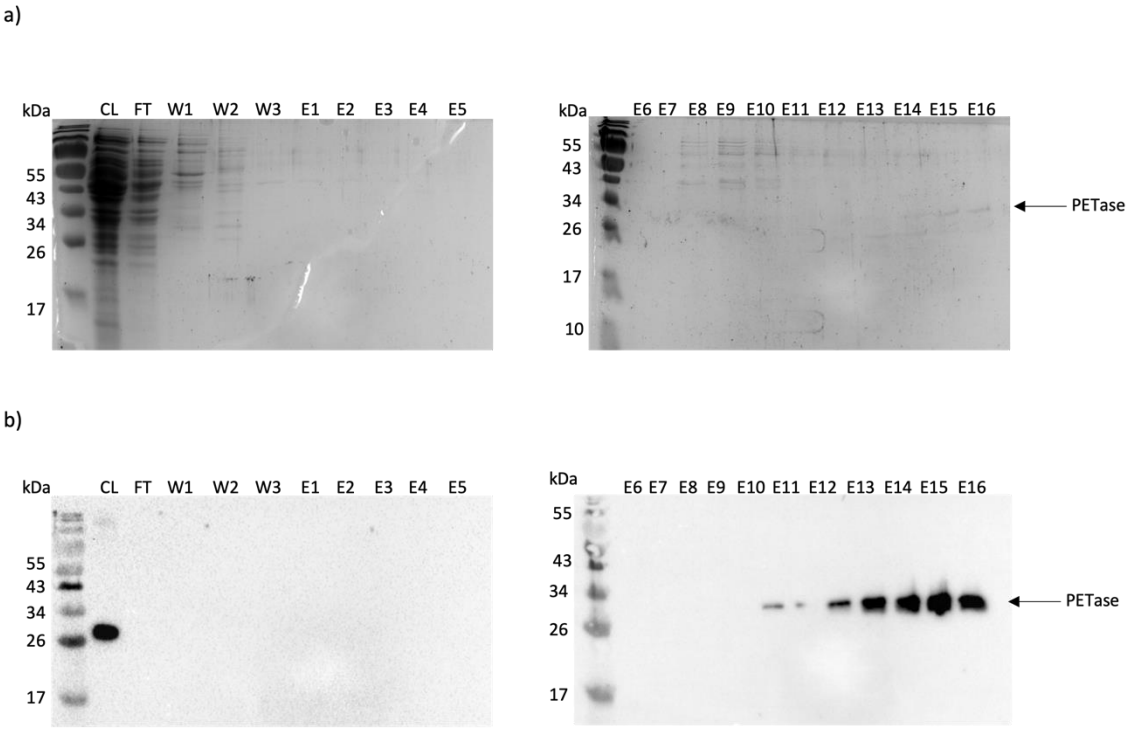

Figure 65. PETase periplasmic purification using culture from Ambr250 fermentation
The periplasmic fraction was collected and applied to a 5 ml HisTrap HP ™ column. Samples collected include crude lysate (CL), flow through (FT), three washes (W1-3) and 16 protein elutions (E1-16). a) Fractions were separated by SDS-PAGE and stained using Coomassie Brilliant Blue. b) The corresponding gels were immunoblotted following SDS-PAGE, using antibodies to the C terminal His tag on PETase.

The results in figure 65, show the SDS-PAGE and Anti-his blot from a purification of PETase from the periplasmic fraction from vessel 2 of the previous Ambr250 fed-batch fermentation (figure 63). The first column contains the crude lysate prior to purification which shows presence of the protein, there also appears to be no protein lost in the flow through or three wash steps. The protein is then detected in elutions 10 to 15, (E10 to E16). Although a good

signal is seen on the Anti-his blot, only a very faint band just above 26 kDa can be seen in the Coomassie stained SDS-PAGE gel.

3.4.8 Activity Analysis of PETase using HPLC

Following fermentation, periplasmic samples were purified and analysed via HPLC to detect activity of PETase. Prior to HPLC, the 2 ml samples were dialyzed using a PD 10 column equilibrated with 50 mM sodium phosphate pH 8, to remove imidazole which was present in the purification buffer. Samples were processed using HPLC by collaborators at University of Portsmouth see figure 66 for PET breakdown analysis.

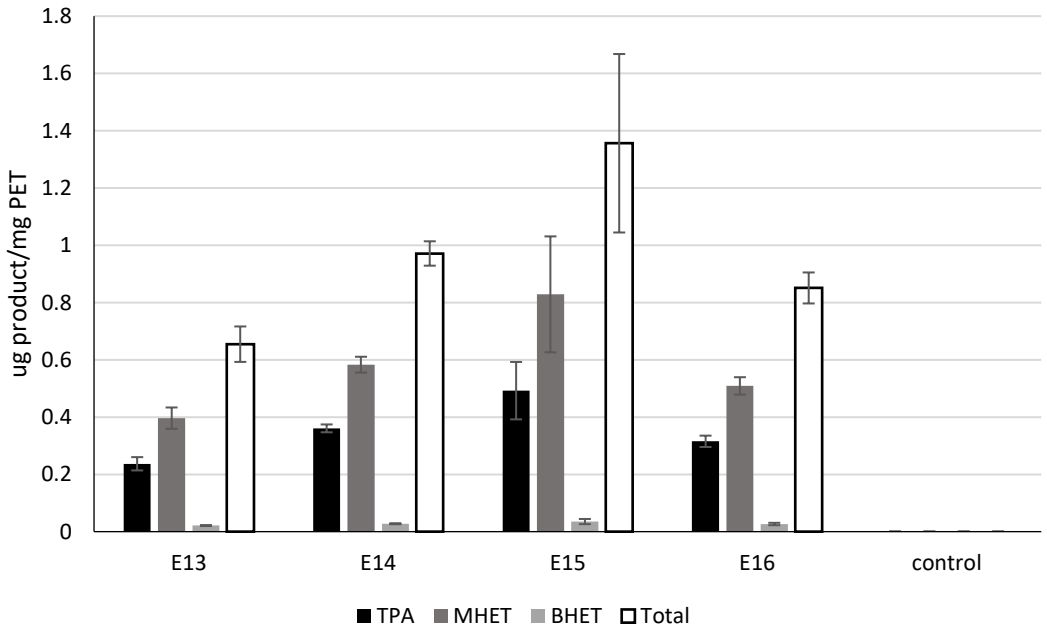

Figure 66. HPLC analysis of SP_{PETase}-PETase activity from fed-batch fermentation
HPLC analysis showing breakdown products detected following incubation of PET plastic with purified PETase from fed batch fermentation. Construct used here is SP_{PETase}-PETase Breakdown products recorded by µg product/mg PET.

During PETase activity, PET is broken down into three products, all of which were detected on the HPLC. Total amounts of each breakdown product TPA, MHET and BHET was calculated per mg of PET. The highest total breakdown recorded here was from sample elution 15 (E15)

of around 1.35 µg product per mg PET. The sample used here for breakdown analysis was elution 15, seen in figure 60.

The results from activity analysis using fermentation samples showed a much higher increase of enzyme activity when compared to protein purified from a 400 ml shake flask expression of SP_{PhoDn}-SP_{PETase}-PETase in figure 52. The results from HPLC analysis for shake flask can be seen in figure 67.

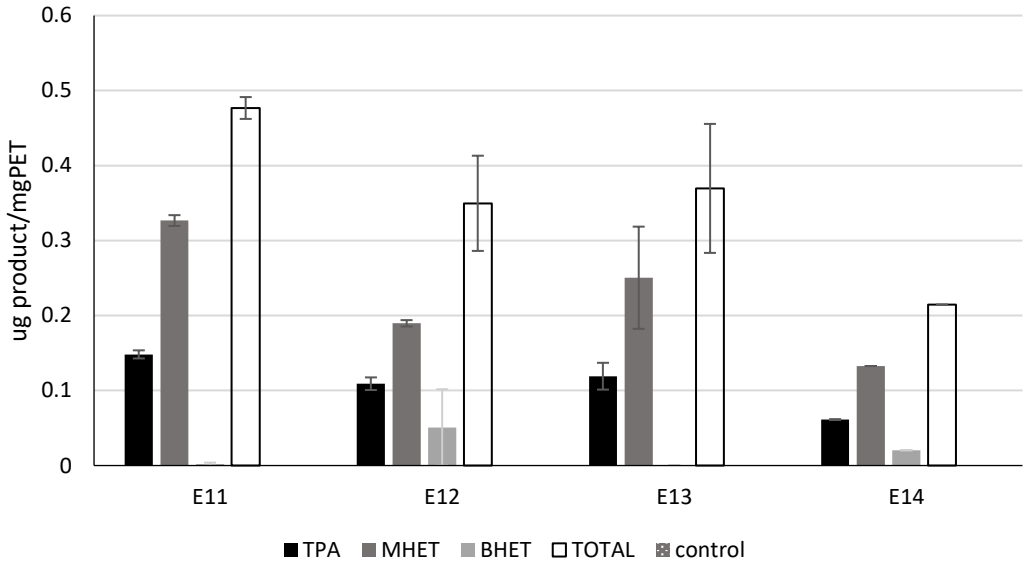

Figure 67. HPLC analysis of SP_{PhoDn}-SP_{PETase}-PETase from 400 ml shake flask expression
HPLC analysis showing breakdown products detected following incubation of PET plastic with purified PETase from 400 ml shake flask expression. Construct used here is SP_{PhoDn}-SP_{PETase}-PETase. Breakdown products recorded by µg product/mg PET.

Total amounts of each breakdown product TPA, MHET and BHET were calculated per mg of PET. The highest total breakdown recorded here was only ~0.45 µg product per mg PET, in sample E11, a much lower amount of total breakdown products compared to the fed batch fermentation showing a clear improvement in active protein production from fed batch fermentation.

3.4.9 Summary and discussion of PETase expression and optimisation in *E. coli* to combat plastic pollution

At the start of this project, the first step was to analyse current expression and optimise expressions in the lab with an overall aim of developing *E. coli* as a recombinant host for high yield PETase expression. The first construct tested here was SP_{PhoDn}-SP_{PETase}-PETase +/- CyDisCo in a pEXTII expression plasmid. Altering media for expression proved an option for improving expression of PETase. The aim of this project was to produce enough active protein in a commercial process which could be used for plastic degradation via optimising the expression process. PhoDn was predicted to be a tat dependent signal peptide, however when tested in Δ*tat* strains, it was evident that the protein could still be exported and thus via the sec pathway. The use of the SP_{PhoDn}, resulted in protein in both the cytoplasmic and insoluble fractions, however, as this protein had disulfide bonds, it need to be exported to the periplasm for correct folding thus, the signal peptides were altered to optimise periplasmic export. This was achieved by removing the SP_{PhoDn} and just using the native signal peptide; SP_{PETase}. Previous research in *E. coli* had shown that expressing PETase via Sec dependent signal peptides was successful (Seo *et al.* 2019). Optimisation was continued using different cell types; both BL21 DE3 and W3110, it was concluded that W3110 performed the best for exporting PETase to the periplasm and was thus used in fed batch fermentation. Previous research had achieved 6.2 mg of PETase per L of culture medium, using the signal peptide SP_{LamB} in *E. coli*, (Seo *et al.* 2019), therefore fermentation was used here to improve current yields. Two different fermenters were used in this study, both batch fed fermentation; a 500 ml Infors bioreactor and a 100 ml Ambr 250 bioreactor. The 500 ml bioreactor was used first using the conditions optimised at shake flask level, the periplasmic fraction was then obtained and purified. The increase in vessel size, meant a much higher biomass could be achieved as the cell density reached over OD_{600} 160. The results from this showed a clear improvement on the level of protein exported and isolated ready for activity

analysis. One of the key components of this study was to produce functionally active PETase which could then be used in industrial processes. To test activity, purified protein was incubated with PET plastic and analysed by HPLC for breakdown products of PET. Two runs were conducted on HPLC by Dr Luisanna Avilan at the University of Portsmouth. Both runs detected the PET breakdown products; TPA, MHET and BHET and activity was calculated in amount of breakdown product per mg of PET. The highest total breakdown recorded here was 1.35 µg product per mg PET. This outcome showed that expressing PETase in fed batch fermentation was an effective method to achieve high cell density and active PETase once purified. Further analysis for this project would use microscopy to visualise PET degradation after incubation with purified PETase which could then be used to quantify degradation further. However, HPLC is the most established method to detect PET degradation as it is accurate and sensitive to the different products released during plastic breakdown. Other possible methods could include the use of absorbance assays to which would be a quicker and easier method to show enzyme activity (Zhong-Johnson, Voigt and Sinskey 2021).

4 Discussion

This thesis has outlined the development and application of both microalgal and bacterial based production platforms to produce commercially relevant products either for use in disease control and prevention in aquaculture or addressing the issue of plastic pollution in the environment.

The first three results chapters of this thesis (chapters 3.1, 3.2 and 3.3) aimed to improve current disease control methods in shrimp, through the use of RNAi for WSSV control and the expression of recombinant proteins against AHPND. The surge in aquaculture, driven by the growing global population, has applied significant pressure to the farming industry which has in turn experienced development of diseases and control issues. Both of these projects used microalgae as an expression system. In contrast the third results chapter details the use of *E. coli* as an expression host for production of protein fragments to be used as an intramuscular vaccination against *Vibrio*.

The final results chapter in this thesis was conducted due to restrictions experienced with collaborators in SE Asia, due to the Covid pandemic. This project aimed to build upon current expression of the plastic degrading enzyme PETase using the host; *E. coli*.

4.1 Generation of *C. reinhardtii* expressing dsRNA for WSSV control in shrimp

This chapter worked to develop an RNAi based whole cell feed to be used in defence against WSSV in shrimp. This work built upon the initial work of another laboratory member, which was the generation of two strains of TN72 expressing either dsVP9 or dsORF366. Both of these dsRNAs have been previously discussed as a potential target in WSSV, therefore this

project aimed to generate a single strain of *C. reinhardtii* which expressed both dsRNAs, and would thus potentially provide better protection against the virus, and therefore reducing the need to cultivate multiple strains of microalgae. The co expression of genes is a technique which has previously been conducted in *C. reinhardtii* using a co expression vector (Jia *et al.* 2016).

This project started by using a strain of microalgae which was already genetically modified to express dsVP9 or dsORF366, a transformation which had been carried out using the photosynthetic restoration gene *psbH.* Due to this, the second gene insertion had to be inserted into a different site in the chloroplast, however this also required a second selection method. To avoid the use of antibiotics and thus the risks associated such as transfer of antibiotic resistance genes, this study used a media selection marker; *ptxD*. As mentioned, the use of this marker is considered to be a biocontainment tool as the gene is contains two internal stop codons alongside a chloroplast specific tRNA which has been previously modified to recognise the stop codon as a tryptophan, therefore the transfer of this gene to other organisms is unlikely. As this gene provides the microalgae with the ability to utilise phosphite and convert it to phosphate, the TAP media used to culture the microalgae had phosphite in place of the phosphate, however this was not used for the control strains used in this study, the dsVP9 and dsORF366 as well as the background strain TN72, which could have explained issues with culture growth.

Although results show clear successful gene insertion, the strain was particularly poor growing and required regular re-streaking onto fresh TAPhi media to maintain *ptxD*. This posed many issues when trying to achieve high biomass levels for example for use in challenge trials. Prior to the second WSSV challenge trial, TN72-dsVP9-ptxD-dsORF366 was grown at large scale with help from collaborators in Prof Saul Purton's lab at UCL. The aim of

this visit was to use hanging bags for large scale growth. See the picture in figure 68, which shows the hanging bag set up.

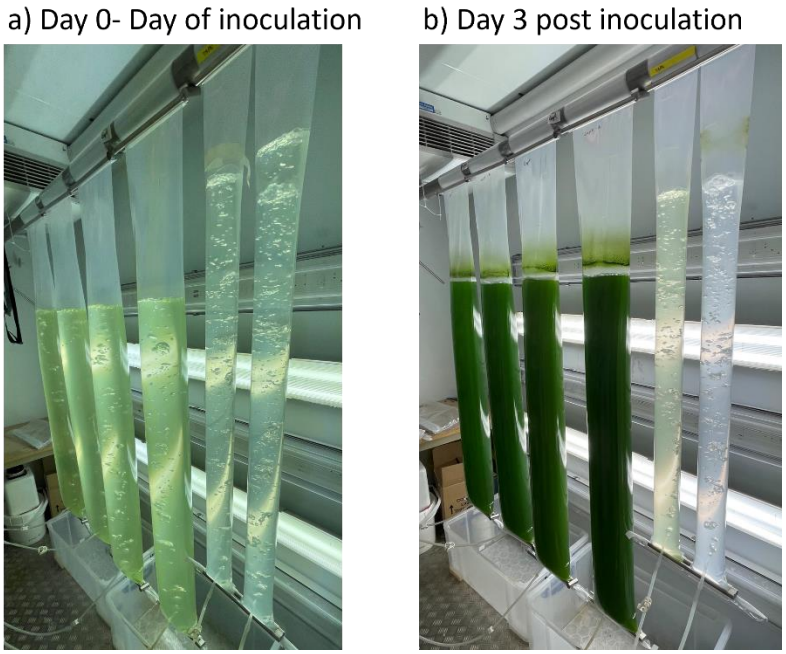

Figure 68. Hanging bag growth experiment set up
Hanging bag growth set up at UCL. From left to right; first 4x 15 L hanging bags are cultures grown by another laboratory member, shown here as a comparison of ideal growth. The last two are both 2x 3 L cultures of TN72-dsVP9-ptxD-dsORF366. a) Hanging bags on day of inoculation and b) day 3 post inoculation.

The figure above shows the hanging bag set up in Prof Saul Purton's lab at UCL. From left to right; the first four hanging bags contain 15 L TAP media, inoculation with a another students culture, shown here as a comparison. The last two hanging bags on the right contain 3 L TAPhi media, inoculated with TN72-dsORF366-ptxD-dsVP9 on the left and TN72-dsVP9-ptxD-dsORF366 on the right. This image is shown here to highlight the lack of cell density and culture growth of this strain of microalgae. The volume of the hanging bag had to be reduced to 3 L due to a lack of initial inoculum and were therefore only inoculated to an initial OD_{750} of 0.01, the optimum initial OD750 was 0.05, therefore this could have been the cause of reduced growth. The culture was monitored for the full 5 days however, by day 5 the culture had been contaminated and thus could not be used for further studies. It was concluded that

there was an issue with the original inoculum culture which then caused issues downstream such as lack of growth. The grow bag set up required a hole at the top of the bag for initial inoculum, this is suspected to be the source of contamination. There was a clear presence of contamination in this set up of the hanging bags however this would require repeating as this is a method which has been previously optimised as it utilises the industry applicable process of a large scale, contained growth unit, which is single use but low cost (Cui, Purton and Baganz 2022).

Following an unsuccessful large scale growth experiment, conditions for growth were optimised at the University of Kent at small scale, in preparation for the second challenge trial performed at Centex, Bangkok.

As seen in the first results chapter, the construct was generated and transformed into the *C. reinhardtii* chloroplast and integration was confirmed by PCR and sequencing. This strain was then taken forward for WSSV challenge trials conducted in Bangkok, under the supervision of Dr Patai Charoonart. Upon arrival, the first step prior to the shrimp challenge trial was to confirm the presence of dsRNA in the microalgae by RT-PCR and RT-qPCR. Although the levels of dsORF366 from the TN72-dsVP9-ptxD-dsORF366 appeared lower than the other samples such as TN72-dsORF366, the presence was still confirmed. The dsRNA was quantified to show that the TN72-dsVP9 strain generated 491.18 ng/L, and the TN72-dsORF366 strain generated 483.16 ng/L. The dual transformant was also screened for both dsVP9 and dsORF366 and was calculated as 713.31 ng/L and 6.5 ng/L, respectively. As seen here the yields from the single dsRNA expressing strains were much higher compared to the dual however, it can be noted that the yield of dsVP9 was much higher in TN72-dsVP9-ptxD-dsORF366 in comparison to TN72-dsVP9. This is a major limitation of this project as the yield of dsRNA produced by the microalgae, as the influence of the dsORF36 would presumably

not produce as greater effect as the dsVP9. The use of *C. reinhardtii* to generate dsRNA over the *E. coli* strain HT115 was used as it is generally considered a safe organism and does not bring the risk of toxin production like other host organisms such as bacteria (Riet *et al.* 2021). Possible optimisation of this dual dsRNA expressing *C. reinhardtii* strain could enhance its ability to express high levels of dsRNA and thus induce the RNAi machinery and reduce impact of WSSV. This is not a novel mechanism, the systemic induction of RNAi in shrimp, through the use of oral feed containing dsRNA has been shown previously (Attasart *et al.* 2013), it has also been shown effective in shrimp against WSSV (Riet *et al.* 2021). Optimisation could include optimising the dsRNA sequence for example the length of the dsRNA, screen for possible secondary structures of the ssRNA which could inhibit the formation of dsRNA.

A WSSV challenge trial was used to determine the effectiveness of feeding whole cell microalgal feed expressing dsRNA specific to the WSSV in both the single and dual transformants strain, in preventing or reducing WSSV in shrimp. A key step in the experiment to ensure the health of the shrimp was acclimatisation before the experiment. An initial number of 25 shrimp was used in each tank however this dropped to as low as 19, a reduction of 6 shrimp, possibly showing an unhealthy stock of shrimp, which could explain the reduced survival rates later on in the experiment. This challenge trial was conducted using a 'liquid feed'; microalgae cells expressing dsRNA, pelleted and resuspended to a high cell density which can then be injected into the shrimp tank water so the shrimp can feed on the microalgae present in the water, alongside their daily diet of commercial shrimp feed. This method of feeing is perhaps an inconsistent method as it cannot be quantified as to how much microalgae the shrimp are consuming and therefore the dsRNA cannot be quantified. Although this has not been quantified, microalgae has been used in shrimp feeds (Ahmad,

Hassan and Banat 2022) and has been shown to improve the fatty acid profile of shrimp and fish, a benefit for the aquaculture industry (Chen *et al.* 2019).

The group which had the highest survival rate at the end of the experiment was the group fed with control microalgae, this microalgae was the strain which had dsRFP and thus non-specific to the WSSV. This group was used here as a control, as they still received the viral challenge but were also being fed a non-specific dsRNA feed. It can be suggested from these results that feeding non-specific dsRNA has an upregulation of the immune system of the shrimp which has possibly helped protect against the WSSV. This conclusion however, does not cover all the results as the other groups fed with dsRNA; dsORF366, dsVP9 and dsVP9-ptxD-dsORF366, had the three lowest final survival rates, and therefore the same conclusion cannot be drawn. For example, if the introduction of dsRNA was enough to upregulate the shrimp immune system, we would expect all four groups fed with dsRNA to have higher survival rates. The group used here to show the lethality of WSSV on 'unprotected' shrimp was the positive group, however this group ended up being one of the healthiest groups, with the highest survival rate. The negative group used here; the group which received a daily diet of commercial shrimp feed and no WSSV challenge also had a drop in survival to 58% which is much lower than expected. The lack of consistency with the control groups in this experiment, highlights clear error with either the experimental design or other variables such as health of the shrimp prior to viral challenge. Overall, it can be concluded that there were clear experimental issues with this experiment, as the results could not be used to draw any conclusions regarding efficacy of feeding microalgae expressing dsRNA to target WSSV in shrimp.

Following unreliable results from the first challenge trial, another shrimp WSSV challenge trial was conducted. This trial aimed at producing a method which would be more applicable

to the shrimp farming industry as maintaining a microalgae culture at 4°C which is then fed in the form of liquid feed is not feasible for shrimp farms in the tropical climate. Therefore, the method of freeze drying algae was used here as this can be stored in the freezer prior to use and can be kept long term. The microalgae was grown in liquid culture first, freeze dried at the University of Kent, then sent to Bangkok for use in the challenge trial. Upon arrival the microalgae was mixed and ground in a pestle and mortar with commercial shrimp feed plus squid oil for improved taste, and stored at room temperature for the duration of the challenge trial. The addition of squid oil, was a suggestion from our collaborator following previous shrimp experiments. The use of a dried feed is a more practical option for shrimp farms across SE Asia. The same groups were used as per the last shrimp challenge trial minus the TN72-dsORF366 group.

The results of the negative group from this challenge trial showed must better overall conditions of the shrimp as the survival rate after 6 days had only dropped to 95.59%, a much better survival rate for the non- viral challenged group, compared to the previous trial. This indicates a better overall health of the shrimp. The other four groups survival rate, all of which received viral challenge, all dropped to below 47%. The group with the lowest survival rate was those fed with the dual targeting dsRNA; TN72-dsVP9-ptxD-dsORF366, therefore not supporting the hypothesis that the dual target of dsRNA to two essential genes of the WSSV would improve chance of shrimp survival against WSSV. The other two groups fed with dsRNA microalgae; TN72-dsVP9 and TN72-dsRFP (control dsRNA) group, had a survival respectively of equal to and lower than that of the positive group which was expected to have the lowest survival rate. This is a result which does not reflect previous research in the use of dsRNA as an oral feed for WSSV control (Charoonnart *et al.* 2023). Overall the results from this challenge trial cannot be used to evaluate the effectiveness of feeding microalgae expressing dsRNA as the control groups were unreliable. The presence of wide variation in

the results suggest improvements are required to achieve a higher level of accuracy. Further improvements would include feed analysis as the current methods of feeding the shrimp in both liquid and dried feed format, do not offer a standardised approach to the level of feed fed each day and therefore, it cannot be confirmed that all shrimp are consuming the microalgae and thus the dsRNA. Another important improvement required would be the stability of the dsRNA in both the liquid and dried feed format to ensure the quantity of dsRNA fed on day 1 of the trial was the same as day 7. Similar studies have been conducted using liquid microalgae expressing dsRNA in this method before with success (Charoonnart *et al.* 2023), however the dsRNA in the dried microalgae in this study was not tested for stability however collaborators have predicted its stability at room temperature (Charoonnart *et al.* 2023), however further testing would be required to validate this and allowing a full cost analysis to be developed for commercialisation. The freeze dried method of microalgae feeding proposes a more industry relevant technique as it is easier to store, used to feed and reduces risk of environmental contamination with GM species (Charoonnart *et al.* 2023).

This research was a challenging task to generate a dual dsRNA expressing *C. reinhardtii*, after rounds of failed transformation attempts, once generated the strain was very slow growing and unable to achieve high biomass levels. This strain also showed evidence of losing its phenotype when not regularly maintained on fresh media, posing possible constraints for industry application. This was the case during the final few months of this project. Upon returning from Thailand following the challenge trials, the strain had not been maintained on fresh media after the usual 14 day re-streaking method, and appeared to lose its phenotype after just 4 weeks. Following multiple re-streaks onto fresh media after returning to the University of Kent, the phenotype was lost and could not be revived. Due to time constraints this strain could not be reproduced via transformation and thus no further

analysis was carried out. The analysis planned upon return was to improve dsRNA yield by optimising growth conditions and quantify dsRNA at different stages of the growth to optimise harvest time.

To conclude, the outcome of both of the shrimp challenge trials proved unsuccessful in determining the efficiency of feeding dsRNA to target WSSV as a shrimp feed due to the unreliable results, thus further analysis and repeats of this experiment would be required to draw conclusions regarding its potential as a disease control strategy in shrimp as the method has the potential to be suitable for the aquaculture farming industry.

4.2 Expression of antibodies in *C. reinhardtii* for use in shrimp against *Vibrio*

This chapter aimed to explore the use of microalgae as a vector to deliver antibodies to shrimp via an oral feed to protect them against the AHPND causing pathogen *V. parahaemolyticus.* This was a project which developed from communication with Professor Tae Sung Jung at Gyeongsang National University, South Korea. Previous work by this group had developed antibodies specific to $PirA^{vp}$ and $PirB^{vp}$. The antibodies were expressed and purified from *E. coli* BL21 and used to passively immunize shrimp to neutralise the toxins $PirA^{vp}$ and $PirB^{vp}$ which are associated with AHPND (Lazarte *et al.* 2021). The antibodies of $PirA^{vp}$ and $PirB^{vp}$ were labelled as 7C12 and 9G10 respectively. The purified antibodies, were mixed with commercial shrimp feed and fed prior to infecting the shrimp with *V. parahaemolyticus,* those fed $PirB^{vp}$-9G10 had a significantly higher level of survival; 60% in comparison to the control group; 0%, however the group fed with $PirA^{vp}$-7C12 also had a low survival, similar to that of the control group; 3% (Lazarte *et al.* 2021). The use of bacteria to generate antibodies, poses issues such as purification requirements therefore, the use of a host organism to express these antibodies would provide an easier and cheaper alternative.

Here we aimed to express these antibodies in *C. reinhardtii,* both were codon optimised for expression in *C. reinhardtii,* in addition to a HA-tag for purification. The glass bead transformation method was used and after rounds of transformation using minimal media to ensure autotrophic growth, we were not able to confirm a successful B7C12 gene insertion, only B9G10. After confirming this strain the B9G0 expressing strain was used for growth assays and expression of the protein was analysed.

The B9G10 antibody was successfully expressed here in *C. reinhardtii,* however further optimisation would be required to improve the yields of the protein and develop the

purification process further. The HA-tag western blot showed low levels in the purification, however for the use of this study, purification was not required for the final product as the aim was to generate a whole feel in *C. reinhardtii* as this organism does not require purification prior to feeding. Other areas of optimisation of either the growth or the purification method from a larger culture density could help improve protein production. Additionally, the generation of the B7C12 expressing strain would have been an advantage to draw comparisons between expression.

A construct was generated which contained both the B7C12 and B9G10 ready for transformation into *C. reinhardtii,* this would be a more desirable strain to be taken forward for commercialisation however was not successfully inserted in *C. reinhardtii.*

Overall, this work aimed to produce a microalgae whole feed to be taken forward with shrimp challenge trials, however due to time constraints this was not possible. This work has great potential of development into an oral vaccine as the use of these antibodies in *E.c oli* proved promising for use as a vaccine candidate. Due to this, the work using *C. reinhardtii* is set to be continued by Prof Saul Purton's lab at UCL, in collaboration with Prof Tae Sung Jung.

4.3 Expression of subunit vaccines in *E. coli* for use in grouper fish

This project used the expression host *E. coli* for recombinant protein production for use in aquaculture. As the previous project showed, protein yield in *C. reinhardtii* is often lower than *E. coli*, as *E. coli* recombinant protein yields can achieve up to several grams per litre. There are also many options for optimisation in *E. coli* such as temperature, media, aeration, induction time and concentration of inducer, proving it to be a suitable option for high protein yields. Here we aimed to express a fusion protein which could be used as a subunit vaccine in grouper fish against *V. parahaemolyticus* infection. This is one of the many challenges which the aquaculture industry faces as this a zoonotic pathogen, therefore not only infecting aquatic species it causes gastroenteritis and septicaemia in humans from consuming seafood (Li *et al.* 2019).

These fusion protein constructs, selected due to their specific protein components of the pathogen, were generated and confirmed by sequencing prior to use in protein expression experiments. GAPDH was chosen due to its role in glycolysis and its role in modulation of host-immune responses (Perez-Casal and Potter 2016). The outer membrane protein, OmpK was chosen as a vaccine candidate as these proteins have been identified for their role in bacterium-host interactions (Lin, Huang and Zhang 2002).

The results from expression trials using cell line BL21 DE3, showed successful expression of the GAPDHf-OmpKf however there were very low levels of expression of OmpKf-GAPDHf, therefore the GAPDHf-OmpKf was taken forward for purification. The cell line used for expression was BL21 DE3, under the control of a T7 promotor, providing high selectivity and activity (Rosano, Morales and Ceccarelli 2019), which appeared to show good levels of protein expression. Large scale growth expressions were then carried out at shake flask prior to purification by IMAC. Here IMAC purification was used using a HisTrap™ column, although

this method of purification is used routinely in protein purification, non-specific binding can occur which results in contamination of the final product (Bornhorst and Falke 2000). Although his-tagged protein was confirmed by immunoblotting, the purification showed a high level of contaminating bands in the Coomassie stained SDS-PAGE. It was concluded that the binding of the protein to the column was not an issue as the protein was present in the later elutions which had a higher imidazole concentrations and thus was bound to the column. Therefore, further purification would be required to test the protein purified as well as the whole cell extract.

This project was carried out in collaboration with researchers in University Malaysia Sabah however, due to the pandemic international collaboration was restricted. Towards the end of the project collaborators had concluded that the Rosetta cell line was successful in expressing the fusion protein GAPDf-OmpKf in levels high enough to purify and were able to achieve a high purity sample which could then be used for challenge trials. The challenge trials used purified protein which was injected into the grouper fish via intraperitoneal injection. The fish were then challenged with *V. parahaemolyticus,* via a submersion technique which simulates the real world scenario in aquaculture farming. Following this, RNA screening was used to evaluate gene regulation rather than survival rate. The results from this study can be used a promising tool in a step towards controlling *V. parahaemolyticus* in aquaculture farms. Further testing in the use of these vaccine candidates could reveal the benefit of using a fusion protein vaccine. Although the use of *E. coli* as an expression system here has proved to be a good host, the use of an algal system would reduce the need for purification as well as reducing the need for vaccination. The vaccination process is one which requires trained workers to hand deliver the vaccine into the fish via injection without causing damage, and thus an increased stress response (Liu *et al.* 2019), therefore, production of a microalgae expressing this fusion protein provides the

possibility of using it as an oral feed. The use of a mucosal route vaccine is more practical and affordable and thus applicable to farms however, there remains a limited number of mucosal vaccines commercially available (Adams 2019). There remains debate about the stability of mucosal route vaccines due to possible digestion in the gut. The aim would be to deliver to the mid gut which has the ability to absorb antigens (Meng-Han 2024).

In summary the results from this study have shown a possible step towards controlling *V. parahaemolyticus* in aquaculture farms. With optimisation and expression in different organism such as *C. reinhardtii*, this project could develop a successful vaccine for use in grouper fish.

4.4 PETase expression developments for plastic degradation

The final chapter presented here was the development of PETase expression in the host organisms *E. coli*. This was a project undertaken as a 'side project' in the early stages of this PhD project, as international collaborations were unable to go ahead due to the Covid-19 pandemic. A pilot expression had been carried out by another laboratory member which showed no level of expression of SP_{TorA}-PETase in BL21 DE3 Tat Express strain, however expression was seen when using the HyaA signal peptide. This work aimed to build on this research and optimise PETase expression in *E. coli* for purification and activity analysis.

Overall, this project proved successful in expressing functionally active PETase in *E .coli* at a high enough level to show activity. Future prospects of this project would include the combination of the second enzyme produced by *I. sakaiensis*; MHETase. This is the second enzyme in the complete breakdown of PETase in the host organism *I. sakaiensis* (Maity *et al.* 2021). PETase breaks down PET into BHET, a small molecule and MHET. MHET can then be broken down further by MHETase; into TPA and ethylene glycol. Therefore engineering a strain which could express both of these enzymes; PETase and MHETase, and thus a complete breakdown of PET plastic would help tackle the plastic problem. Another area of future work would be the use of other host organisms to express this enzyme. As seen in other projects, microalgae with particular interest in *C. reinhardtii* is a suitable host for recombinant protein production. Expressing this protein in *C. reinhardtii* would be a suitable area as the ability to perform post-translational modifications in *E. coli* is much lower in comparison to eukaryotes, therefore in *E. coli,* inclusion bodies are often formed which can affect functionality. Therefore, the use of *C. reinhardtii* would be a suitable option and considered a ‘greener’ option as this microalgae is considered a safe organism, which is also able to perform post-translational modifications (Dehghani *et al.* 2022). However, this

project aimed to improve yields, and as seen above high yields of recombinant proteins in *C. reinhardtii* remains a limiting factor when using microalgae (Taunt, Stoffels and Purton 2018).

4.5 Final discussion and future perspectives

In summary the aquaculture industry is an ever growing one, required to feed the growing population, therefore cost effective disease control methods are essential to reduce mass stock loss in aquaculture farms. The use of microalgae and *E. coli* for dsRNA production and recombinant protein production provides an effective method to control disease. Here, we showed that the system of using microalgae to express dsRNA specific to WSSV provides a good insight although improvements regarding dsRNA yield and challenge trial optimisation would be required. The expression of antibodies in *C. reinhardtii* proved a promising area of disease control as the microalgae can be fed as a whole feed and thus a cheaper and easier alternative to antibiotics. *E. coli* also proved to be an effective host for generating vaccines for use in grouper fish. Further work using challenge trials and exploring different vaccination routes would develop this technique further. Finally, the expression of the plastic degrading enzyme; PETase proved successful in *E. coli* at high enough levels to measure PET breakdown by HPLC.

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Appendix

4.5.1 Results from collaborators at University of Malaysia, Sabah for Grouper fish challenge with *V. parahaemolyticus*

Table 1: The concentration and purity of the RNA of unvaccinated, vaccinated, unvaccinated and challenge and vaccinated and challenge sample

Sample	RNA Concentration (ng/mL)	RNA Purity ratio (A260/280)	RNA Purity ratio (A260/230)
Unvaccinated	451.9	2.11	1.86
Vaccinated day 7	571.0	2.09	2.06
Vaccinated day 14	15.3	1.867	0.20
Unvaccinated and challenge day 7	608.7	1.81	0.97
Vaccinated and challenge day 7	32.6	1.93	0.22
Unvaccinated and challenge day 14	705.2	2.04	1.42
Vaccinated and challenge day 14	186.5	2.04	1.40