Development of new vaccines to combat aquaculture pathogens in South East Asian countries

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Declaration

The work presented in this thesis is original, and was conducted by myself (unless started 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.

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Abbreviations

°C	Degrees Celsius
µL	Microliter
µM	Micromolar
A2M	Alpha-2-macroglobulin
APS	Ammonium persulphate
BSA	Bovine serum albumin
C. reinhardtii	*Chlamydomonas reinhardtii*
CIEX	Cation exchange chromatography
CV	Column volume
DNA	Deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
DTT	1,4-Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
GapA	Glyceraldehyde-3-phosphate dehydrogenase
HSM	High salt medium
IMAC	Immobilized metal affinity chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Lysogeny broth
MQ	Milli-Q
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBST	Phosphate-buffered saline, 0.1% tween20
PCR	Polymerase chain reaction
PGLB	Protein gel loading buffer
PPG	Polypropylene glycol
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SEA	South East Asia
SDS	Sodium dodecyl sulphate
SM6	Synthetic medium 6
SPF	Specific pathogen free
TAP	Tris-acetate-phosphate
TB	Terrific broth
TEMED	Tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
Tween20	Polyoxyethylenesorbitan monolaurate
WSSV	White spot syndrome virus
WT	Wild type
YHV	Yellow head virus

Abstract

Aquaculture is a rapidly growing sector, with large economic value and potential. However, aquaculture is severely hampered by disease outbreaks, which cause large losses in production. Antibiotics are often used in South East Asian countries to control disease outbreaks, due to their low cost and ease of use, nevertheless they are ineffective against viral infections and give rise to antibiotic resistant strains. This research sought to develop more convenient and low-cost alternatives, using bacteria and microalgae to express proteins and double stranded RNA (dsRNA) which could then be administered orally. Soluble and insoluble expression of Glyceraldehyde-3-phosphate dehydrogenase (GapA) from *Streptococcus agalactiae*, a bacterial pathogen which causes high mortality in fish, was achieved in *Escherichia coli* in shake flask and fed-batch fermentation. Purified soluble and insoluble protein were administered orally to red hybrid tilapia fish and challenged with *S. agalactiae* resulting in a 51.1% and 75.6% survival respectively, compared to 13.3% in the control group. GapA was expressed in the chloroplast of the microalgae *Chlamydomonas reinhardtii* and later optimised with yields of up to 18 mg/L being achieved. Freeze dried transgenic *C. reinhardtii* showed that protein could be detected up to 54 days when stored at room temperature, showing large potential for microalgae for the production and oral application of vaccines. In addition, two dsRNA expressing *C. reinhardtii* strains were generated targeting key viral genes of the white spot syndrome virus, which causes large losses in shrimp aquaculture. Part of the VP9, thought to be involved in viral replication, and ORF366, part of the nucleocapsid, genes were expressed in the chloroplast as dsRNA reaching yields of 3.86±1.07 µg/L (VP9) and 11.63±5.84 µg/L (ORF366). However, challenge trial data appeared to show no real benefit to survival rates to shrimp over the control (dsRFP expressing) strain. Finally, an alternative strategy to help identify biomarkers for disease resistance was also developed using an antibody generated to target Alpha-2-macroglobulin (A2M) in Brown-marbled grouper. Blood serum samples from fish more resistant

to infections of *Vibrio parahaemolyticus* showed higher levels of A2M, indicating A2M as a potential disease resistance biomarker.

Chapter 1: Introduction

1.1 Background

1.1.1 General background to aquaculture

Aquaculture is the practice of farming aquatic animals and plants in freshwater, brackish and marine environments. The aquaculture sector is one of the fastest growing food producing sectors with an average annual percentage growth rate (APR) of 5.3% between 2001 to 2018 (Ahmad *et al.*, 2021). In 2020 aquaculture production had an estimated value of US$265 billion, of this farmed finfish made up a total of US$146.1 billion with US$109.8 billion being from inland aquaculture, crustaceans also made up a significant amount of the value at US$81.5 billion (FAO, 2022). Due to the significant increase of growth in the sector over the last 50 years it has become a major contributor to many countries' economies, especially in South-East Asia (SEA). The industry contributes to the livelihoods of a large number of individuals, particularly in rural communities, not only does it contribute to food security but also generation of income through employment and services in these areas. Technological advancements alongside an increased demand for fish as a source of protein are likely reasons for the industry's growth. Indeed, the health benefits of aquatic food products have been long known, they contain low-fat high quality protein, are a rich source of omega-3 polyunsaturated fatty acids alongside a wide range of vitamins and minerals (Lund, 2013). Omega-3 fatty acids are often taken as a supplement in the form of fish oils or directly through the consumption of fish products, there is significant evidence that the consumption of omega-3 fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), have been linked to a reduction in cardiovascular problems (Lee *et al.*, 2008). Given the trend of economic growth and taking into consideration the growth of the global population it appears as though the sector will continue expanding and demand for aquatic products will continue to rise which the capture fisheries will be unable to supply. However, the industry faces substantial challenges, such as climate change, limited water sources and disease. The industry is under constant threat from

infectious diseases which can cause huge mortalities in animals which has a significant impact on businesses, with there being an estimated loss of several billion US$ globally per year (Bank, 2014). Pathogenic bacteria are more adept at growing in aquatic environments independent of the host and can therefore reach high densities around the animals which can then ingest them while feeding or drinking. Bacterial infections most notably affect fish, with infections from species of *Streptococcus*, *Pseudomonas*, *Vibrio* and *Aeromonas*. In addition to bacterial infections, there have also been a number of problematic viral diseases in fish which have an impact on farms, for example nervous necrosis virus (NNV), infectious spleen and kidney necrosis virus (ISKNV), and red sea bream iridovirus (RSIV). Shrimp aquaculture is more concerned with viral diseases, infections from White Spot Syndrome Virus (WSSV) and Yellow Head Virus (YHV) being able to wipe out an entire farm's stock within several days of an outbreak (Chantanachookin *et al.*, 1993; Chou Hsin-Yiu *et al.*, 1995). The impact of disease in shrimp aquaculture in Thailand can clearly be seen in Fig. 1, where production was severely impeded by outbreaks of several diseases including, YHV and WSSV between 1994-2000, Monodon slow growth syndrome (MSGS) between 2000-2002 (Lightner *et al.*, 2012) and lastly a clear drop in 2013 attributed to Early Mortality Syndrome (EMS) (Songsangjinda and Polchana, 2016).

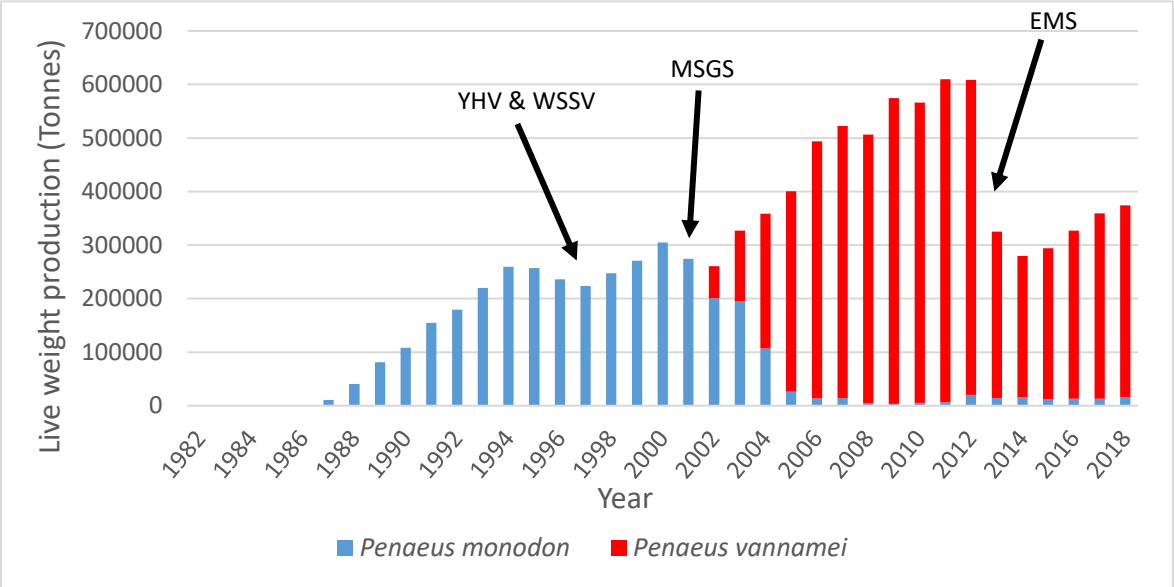


Figure 1. Thailand shrimp aquaculture production for *Penaeus monodon* and *Penaeus vannamei* from 1981 to 2018 Production is given in live weight (tonnes). Drops in production are indicated with the given disease attributed to the reduction (Source: FAO Global Aquaculture Production Statistics from FishstatJ Software for Fishery and Aquaculture Statistical Time Series).

The most sustainable solution for disease control is vaccination of the animal. Vaccination has already been demonstrated to be an effective method of protecting animals from infection by viral and bacterial pathogens. Although effective, vaccination does come with technical, regulatory, and cost associated challenges. For instance, the development and production of vaccines can be expensive, and this is only further exacerbated by the requirement of 'cold chain' storage and transport of the product. Furthermore, vaccinating large numbers of fish by injection is time consuming and requires skilled labour to carry out, also the vaccination process can also be stressful for the animal even when carried out correctly (Sharpe, 2007). As such, there is a real need for novel methods for production and administration of the vaccine, which can both prevent loss and be carried out at a reduced cost.

1.1.2 Major pathogens in aquaculture

The intensive farming practices in the aquaculture sector creates an ideal environment for the proliferation of viral and bacterial infections. Coupled with the fact the animal's immune system can become compromised as a result of the stress experienced. A range of factors contribute to

outbreaks such as fluctuations in temperature, hypoxia and high population densities (Shimon-Hophy and Avtalion, 2021). A large range of bacterial and viral pathogens have been identified, the prevalence and impact of these diseases vary widely depending on region, species being cultivated, farming practices and biosecurity protocols being implemented. Bacterial infections from a range of species have been reported in a number of farmed aquatic organisms. Notably, only a few pathogens have been identified to be responsible for a large portion of the global economic loss in production, infections from *Streptococcus* spp., *Aeromonas* spp., *Edwardsiella* spp., and *Vibrio* spp. have all been shown to cause major issues in aquaculture (Irshath *et al.*, 2023). A common pathogen to appear in freshwater, tropical aquaculture is from species of *Aeromonas*. Animals which are infected with species of *Aeromonas* can present symptoms such as tail and skin rot, internal and external haemorrhaging, skin ulcers and ultimately can lead to high mortality rates (Janda and Abbott, 2010). *Aeromonas* spp. are widely distributed in aquatic environments making it difficult to control infections. Although good practices and high quality water management can keep numbers down, producers prefer to use antibiotics to control infections but these can be ineffective and cause more issues, natural microbial flora can be reduced allowing for the pathogen to take over and increases the risk of antibacterial resistance (Pauzi *et al.*, 2020). *Aeromonas salmonicida* affects salmonids and is one of the oldest identified fish pathogens, being reported to cause disease in brown trout in 1894 (Bartkova *et al.*, 2017). *Aeromonas hydrophila* causes disease outbreaks in farmed freshwater fish including carps, catfish and tilapia (Nhinh *et al.*, 2021) and has also been identified as an issue in shrimp aquaculture (Zhou *et al.*, 2019). *Edwardsiella tarda* is a Gram-negative bacterium which has been found in a wide range of cultured fish species including, bass, catfish, tilapia, sea bream and koi (Park, Aoki and Jung, 2012). Symptoms can present differently depending on the host, however common symptoms include haemorrhaging on the fin and skin, swollen organs, swelling of the abdomen and pigment loss (Pandey *et al.*, 2021). *Vibrio* species are a Gram-negative bacterium found in marine environments, which are known to cause vibriosis in fish, shellfish and shrimp (Liu *et al.*, 2016). The bacteria can survive independent of the host in the correct environmental conditions, with high salinity and high temperatures allowing for a higher

bacterial load, as such infections are known to occur in the summer and in more tropical climates (Di *et al.*, 2017). A large range of species of *Vibrio* are able to cause disease in aquatic systems (Sanches-Fernandes, Sá-Correia and Costa, 2022), *V. harveyi* has been identified as one of the largest causes of vibriosis and is able to be found to affect both fish and penaeid shrimp (Pavlinec *et al.*, 2022). In fish, the bacteria can cause gastroenteritis, skin ulcers, tail rot, ulcers and without treatment fish will become blind (Zhang, He and Austin, 2020). Fish have also been observed to become lethargic, disoriented with reduced feeding (Hashem and El-Barbary, 2013). The bacteria are a large problem for shrimp cultures, where up to 100% mortality can occur (Jiravanichpaisal, Miyazaki and Limsuwan, 1994). The disease can be characterised by lethargy, tissue necrosis, reduced growth and bioluminescence of the animal (Manefield *et al.*, 2000). The Gram-positive *Streptococcus* spp. commonly affect fish species, the two main species of the bacteria causing disease are *S. iniae* and *S. agalactiae*. Infections from both species are characterised by spinning on the water due to loss of equilibrium, ulcers and skin haemorrhages, eye protrusions and opacity in the cornea (Legario *et al.*, 2020). Reduction of feeding and mortality associated with infection reduce total produce of farmed fish. Streptococcosis is prevalent in SE Asia and is often found in tilapia, an important cultured species in the region. *S. agalactiae* has been isolated in a range of SE Asian countries including Thailand (Kannika *et al.*, 2017), Vietnam (Kayansamruaj *et al.*, 2019), Malaysia (Syuhada *et al.*, 2020) and Indonesia (Anshary *et al.*, 2014).

Viral diseases are of more concern in shrimp aquaculture, it's estimated that around 60% of disease loss is a result of viral infections compared to only 20% being from bacteria (Flegel *et al.*, 2008). This could be due to the difficulty in vaccinating shrimp against viral diseases due to their high numbers, size, the cost-benefit ratio, and the fact invertebrates lack a traditional adaptive immunity. Several viral diseases are of high concern in shrimp aquaculture, especially in the SE Asia who are the largest producers of shrimp (Chen, 1996). The World Animal Health Organisation (OIE), lists several viral diseases which are of large concern to the industry, including YHV, WSSV, Taura Syndrome virus (TSV) and infectious myonecrosis virus (IMNV) which have caused major losses in

Asia. The total estimated losses due to these four viruses in the region are US$8-8.5 billion between 1981 to 2006 (Lightner *et al.*, 2012). Outbreaks of viral diseases are incredibly difficult to control with no commercial therapeutics currently available. Viruses spread quickly in ponds and can often be transmitted by shrimp cannibalising infected shrimp tissue. YHV is a ssRNA virus first reported in shrimp in Thailand in 1990. The virus causes a light yellow colouring in the cephalothorax and gills, causes shrimps to swim erratically at the water's surface and gather at the ponds edges and can ultimately lead to 100% mortality in affected populations within 3-5 days (Boonyaratpalin *et al.*, 1993). WSSV is a large DNA virus which has become the biggest contributor to the economic impact on farmed shrimp, causing an estimated global loss of US$8 billion since first being reported in 1992 (Lightner *et al.*, 2012). Although shrimp can develop white spots on their bodies, they are not always present in infected shrimp and white spots are also known to develop in other bacterial infections, meaning that it can be an unreliable indicator of infection. Symptoms include, shrimp becoming lethargic with a reduced feeding rate, gathering at the ponds edges and also exhibit a red discolouration with cuticles becoming loose (Sánchez-Paz, 2010). Mortality rates are also incredibly high with infections resulting in 100% mortality within 2-7 days from the onset of infection (Chou Hsin-Yiu *et al.*, 1995). TSV is an ssRNA virus which often occurs in juvenile shrimp, but is also known to affect adult and post larvae shrimp (Lightner *et al.*, 1995). There are three distinct stages of the infection, acute, transition and chronic. In the acute stage, shrimp show a red colour which is more prominent in the tail fin and pleopods, the animal's shells become soft and can die during moulting with mortalities being able to reach up to 95% (Erickson, Zarain-Herzberg and Lightner, 2002). Shrimps surviving the acute phase begin recovering in a transition phase, the behaviour of the shrimp is normal however, they do present dark lesions on the tail, appendages, and cephalothorax. After this, shrimp then move into the chronic phase of the disease where shrimp display no clear clinical signs but are still carriers of the virus for 8-12 months or even the life-span of the shrimp (Hasson *et al.*, 1999), providing potential pools of virus for subsequent outbreaks. IMNV is a small dsRNA virus and is a more recently emerging virus, being first reported in Brazil in 2004 and was later detected in shrimp in Indonesia in 2006, likely as a result of

inadequate biosecurity protocols while transporting live animals (Senapin *et al.*, 2007). Currently the disease is less widespread and mortality rates are lower than other viral infections of shrimp, being around 40-60% (Tang *et al.*, 2005). The virus causes lesion on the skeletal muscle with a white opaque appearance, and shrimp can become lethargic, with muscles and appendages showing a reddish colour making the animal appear "cooked" (Prasad *et al.*, 2017). This overview shows that currently there are a whole host of diseases and emergence of new pathogens in aquaculture which highlight a real need for the ongoing development of both diagnostic tools and therapeutics to detect and manage outbreaks.

1.2 Strategies for disease prevention

Certain management processes can be taken on farms to mitigate the risk of infections such as increasing water quality through filtration, the use of nets to prevent contamination from infected vectors and specific diets to increase overall health of the stock. Some farmers have also started to use specific pathogen free (SPF) animals to stock their ponds, this is most common in shrimp farms and helps to alleviate loss from disease, by ensuring that certain pathogens aren't introduced to the ponds from new shrimp. The SPF shrimp come from a facility which is able to physically exclude the pathogens and reliably diagnose for certain pathogens which are of most concern. For SPF populations of shrimp these include WSSV, YHV, Taura syndrome virus (TSV), infectious hypodermal and hematopoietic virus (IHHNV) and infectious myonecrosis virus (IMNV) (Lightner *et al.*, 2009). Currently there are only a handful of SPF shrimp populations commercially available, but this will likely expand when new diagnostic tools become available. Despite these measures being in place disease outbreaks do still occur.

Currently infections are often treated with the use of antibiotics or other chemotherapeutics which target bacterial pathogens or eukaryotic parasites. Antibiotics offer a convenient method of treating the animals and are readily available at an affordable price, indeed, a large proportion of farmers will use antibiotics prophylactically to alleviate risk of outbreaks (Holmström *et al.*, 2003). Although, antibiotics are able to accumulate in the water systems of aquaculture facilities; this

could be likely due to the fact fish are unable to effectively metabolize antibiotics (Sun *et al.*, 2020), increasing the risk of environmental and health problems. The frequent and widespread use of these drugs can also result in the development of resistant strains of the pathogen for example several tetracycline-resistant *Aeromonas sp*. strains have being found in catfish (Nawaz *et al.*, 2006), resulting in the use of antibiotics becoming less effective. The rise of antibiotic resistant bacteria can be seen globally with some of the worst affected areas being within Asia (Reverter *et al.*, 2020). Countries that are the major producers of aquaculture products also appear to have little regulation around the use of antibiotics (Howgate, 2003) which further exacerbates the issue, along with a lack of viable alternatives, the problem will continue to grow.

1.2.1 Vaccination in general

Another issue with antibiotics is they offer no protection against viral diseases; therefore, vaccination could offer a better solution to control infections. Vaccination is a safe and effective method for preventing disease and can offer long term protection from both viral and bacterial infections. Vaccination harnesses the animal's natural immune system to help fight infections through recognition of certain immunogenic proteins present in the bacteria or virus particles. There is no risk of drug resistance with vaccination and herd-immunity offers protection to unvaccinated animals. Vaccination has long been investigated in aquaculture with vaccination against *Aeromonas salmonicida* in Cutthroat trout being carried out in 1942 (Duff, 1942). Vaccination has shown success in Norway where vaccines have successfully helped reduce the amounts of antibiotics administered in Norwegian salmon farming while still controlling infections (Midtlyng, Grave and Horsberg, 2011). The use of vaccines in aquaculture presents a number of different challenges when compared to terrestrial animals. Concerns around the practical methods of administration, the cost to benefit ratio and the lack of a traditional adaptive immunity in invertebrates such as shrimp all need to be considered.

1.2.2 Types of vaccines

Traditionally vaccines are made up of killed/inactivated whole organisms or attenuated varieties of the pathogen that would be administered to elicit an immune response. Live vaccines are forms of weakened pathogens that are able to replicate within the host and provoke an immune response with a lowered risk of causing disease or adverse reactions seen in the disease it is intended to protect from. Some attenuated vaccines have been reported to cause symptoms especially in hosts which are immunocompromised (Ljungman, 2012). As a result of the risk of causing symptoms it is important to consider the dosing of the vaccine striking the correct balance of raising a strong immune response while avoiding harmful symptoms, as such many attenuated vaccines are given at lower doses which require repeat boosters to gain long-term immunity. Killed vaccines consist of pathogens which are inactivated which are then administered as an antigen to elicit an immune response without risk of replication. Organisms are often inactivated through the use of chemicals such as formaldehyde or even physically denatured using heat, pH, UV light or even gamma radiation (Nunnally, Turula and Sitrin, 2015). Killed vaccines are easy to design, cheaper and safer than their attenuated counterparts due to their lack of ability to replicate and gain function. These vaccines also cause a comprehensive immune response due to the whole pathogen being presented to the immune system. Killed vaccines are also stable, making them easier and cheaper to store and transport. The large drawback of these vaccines is that they often don't produce an immune response to intracellular pathogens, meaning there are limitations to which diseases are able to be targeted using this platform (Munang'andu, 2018).

Most commercial vaccines presently used in aquaculture consist of killed vaccines (see Table 1). Advancements in vaccine technology allows the targeting of specific components of the pathogen through the use of modern approaches such as subunit, recombinant vector, genetically modified, synthetic peptide, RNA and DNA vaccines. These new developments in vaccine research are promising however application in aquaculture is limited due to the aqueous environment and practical challenges of mass administration required in commercial fish farms. Nevertheless,

research continues to be carried out into these new approaches with some commercial vaccines already being established using these technologies (see Table 1). The subunit vaccine approach relies on the use of specific antigenic proteins being produced, and then administered to elicit an immune response to those proteins. Through manufacturing a vaccine with specific proteins undesirable symptoms can be avoided while targeting a specific immune response, the product can also remain consistent allowing for fine tuning of dosing. The subunit proteins can be produced recombinantly using established bacterial, yeast or mammalian systems allowing for a relatively cheap manufacture cost. Due to the limited number of compounds able to stimulate a response in this system, it is sometimes necessary to use less immunogenic antigens in their formulation. To help increase the effectiveness of the vaccine highly antigenic proteins are identified and used in their construction, they are also often formulated with adjuvants to stimulate the immune system and multiple boosters are administered.

DNA vaccines produce antigen-specific proteins through use of plasmids containing optimised gene sequence specific to the antigen. After the plasmid has been delivered, the hosts own cellular machinery can be utilised to produce foreign peptides which will then be recognised by the immune system generating an immune response. DNA vaccines are cheap to produce with the plasmids being purified from bacterial expression systems. The platform can be quickly adapted for new or emerging diseases by rapidly cloning in new targets. There is no requirement for temperature controlled storage or transport due to the stability of DNA. Finally, multivalent vaccines can easily be produced by cloning plasmids containing multiple genes of interest or by mixing plasmids together. Research into DNA vaccines for protection of salmon against Infectious hematopoietic necrosis virus (IHNV) has been carried out. In fact, the plasmid based vaccine APEX-IHN® has been approved for use in Canada to protect Atlantic salmon against IHNV. In one study the APEX-IHN® was shown to be highly effective, vaccinated fish challenged with the IHNV showed a survival of >97% compared to unvaccinated fish being around 3% (Long *et al.*, 2017). Some more examples of licensed vaccines available for use and the different technologies used can be seen in Table 1.

Table 1. Examples of currently licensed vaccines in aquaculture. IP: Intraperitoneal injection IM: Intramuscular injection, IMM: immersion. Table adapted from (Ma *et al.*, 2019)

Vaccine name	Disease	Pathogen	Major Fish Host	Vaccine Type	Delivery Method
Viral Diseases					
APEX-IHN	Infectious hematopoietic necrosis	IHNV *Rhabdovirus*	Salmonids	DNA	IM
ALPHA JECT IPNV-Flavo 0,025	Infectious pancreatic necrosis	IPNV *Birnavirus*	Salmonids, sea bass, sea bream, turbot, Pacific cod	Inactivated	IP
AquaVac IPN Oral				Subunit	Oral
NorvaxMinova -6				Subunit	IP
ALPHA JECT micro 7 ISA	Infectious salmon anaemia	ISAV	Atlantic salmon	Inactivated	IP
ALPHA JECTS 1000	Pancreatic disease virus	SAV *alphavirus es*	Salmonids	Inactivated	IP
-	Spring viremia of carp virus	SVCV *Rhabdovirus*	Carp	Subunit	IP
-				Inactivated	IP
KV-3	Koi herpesvirus disease	KHV *Herpesvirus*	Carp	Attenuated	IMM or IP
AQUAVAC IridoV	Infectious spleen and kidney necrosis	ISKNV *Iridovirus*	Asian seabass, grouper, Japanese yellowtail	Inactivated	IP
Bacterial diseases					
AquaVac ERM	Enteric redmouth disease (ERM)	*Yersinia ruckeri*	Rainbow trout	Inactivated	IMM
Norvax Minova 6	Vibriosis	*Vibrio anguillarum; Vibrio ordalii; Vibrio salmonicida*	Salmonids, ayu, grouper, sea bass, sea bream, yellowtail, cod, halibut	Inactivated	IP
Norvax Minova 6	Furunculosis	*Aeromonas salmonicida* subsp.*salmonic ida*	Salmonids	Inactivated	IP
Renogen	Bacterial kidney disease (BKD)	*Renibacterium salmoninarum*	Salmonids	Avirulent live culture	IP
ALPHA JECT Panga 2	Enteric septicaemia of catfish (ESC)	*Edwarsiella ictaluri*	Catfish	Inactivated	IP
AQUAVAC-COL	Columnaris disease	*Flavobacterium columnaris*	All freshwater finfish species, bream, bass, turbot, salmon	Attenuated	IMM
ALPHA JECT 2000	Pasteurellosis	*Pasteurela piscicida*	Sea bass, sea bream, sole	Inactivated	IMM

ICTHIOVAC LG	Lactococciosis	*Lactococcus garviae*	Rainbow trout, amberjack, yellowtail	Inactivated	IP
AQUAVAC Strep Sa	Streptococcus infections	*Streptococcus* spp.	Tilapia, yellow tail, rainbow trout, sea bass, sea bream	Inactivated	IP
AQUAVAC Strep Sa1					IP
AQUAVAC Strep Si					IP or IMM
ALPHA JECT micro 3	Salmonid rickettsial septicemia	*Piscirickettsia salmonis*	Salmonids	Inactivated	IP
ALPHA JECT Panga 2	Motile *Aeromonas* septicemia (MAS)	*Aeromonas* spp.	Striped catfish	Inactivated	IP
ALPHA JECT micro 6	Wound Disease	*Moritella viscosa*	Salmonids	Inactivated	IP
ICTHIOVAC TM	Tenacibaculosis	*Tenacibaculum maritimum*	Turbot	Inactivated	IP

1.2.3 GapA as a target antigen

As previously mentioned, a subunit vaccine requires an immunogenic protein to be used which can create an immune response within the host. Identifying such proteins can be difficult to carry out due to lack of conservation of in protective antigens amongst pathogens (Wang *et al.*, 2017) and so preliminary research steps are required for identification of good vaccine candidates. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH/GapA) is a highly conserved ubiquitous protein (Malhotra *et al.*, 2017), which may well be a vaccine candidate that could help to reduce research in identification of vaccine candidates.

GapA is a major housekeeping enzyme involved in the energy generation through the conversion of glyceraldehyde-3-phosphate to 1,3-biphosphoglycerate in the presence of NAD+ and inorganic phosphate. However, the protein is also multifaceted and has been implicated in a range of different functions including DNA repair, control of gene expression and regulation of apoptosis (Nicholls, Li and Liu, 2012).

Furthermore, although GapA is a cytosolic protein, it has been shown to be present on the surface of many bacteria and is thought to be involved in the virulence of several pathogens, by not only

facilitate binding to the hosts extracellular matrix but also through avoidance of phagocytosis (Kopeckova, Pavkova and Stulik, 2020).

Given that GapA is a ubiquitous protein, present on the cell surface and is involved in the virulence of some bacteria it is a strong candidate for vaccine development. Indeed, several vaccines having been tested using GapA as an antigen and shown success at generating an immune response (Tsai *et al.*, 2013; Cao *et al.*, 2015; Salcines-Cuevas *et al.*, 2021). One such study where GapA was trialled against infections of *S. agalactiae* in Tilapia fish. The protein combined with common adjuvants and administered via intraperitoneal injection, showed a relative survival percentage of up to 63%, 14 days post-challenge compared to fish treated with PBS alone (Zhang *et al.*, 2017).

1.2.4 Methods of administering vaccine

One of the major challenges regarding vaccination of fish is the method of administration. For vaccination to be successful the correct dose of the vaccine must be delivered appropriately along with any subsequent booster doses which would be required to offer complete protection. Vaccines are most commonly delivered by injection which generally offers the best protection, with injection being able to deliver specific doses of the vaccine to be delivered and allows for the antigen to be mixed with adjuvants which can enhance the immune response. Since injection is so effective it also means that minimal doses of vaccine can be administered meaning overall less vaccine is required to be produced. However, vaccination by injection does come with drawbacks, the process is labour intensive and requires a skilled workforce to be carried out, injection can be difficult for smaller fish particularly for shrimp, removal of the animals to carry out injections can also be stressful and damaging to the animals with abdominal lesions and vertebral deformities being present after injection (Grini *et al.*, 2011). Immersion vaccination addresses some of the problems associated with injection. Immersion vaccination is the process of spraying or dipping the fish in a concentrated vaccine solution for a short period of time so that antigen can be taken up via the gills and skin (Kiryu *et al.*, 2000). This allows for a more convenient method of vaccinating smaller animals using a less skilled method of administration and causes less stress than injection.

However, it is difficult to ensure the correct dosage is given, the method is still labour intensive and can be expensive with extra equipment being required and large amounts of vaccine solutions being required.

Oral vaccination is the most sought-after method for immunisation in aquaculture. Oral vaccination works by the animal ingesting the antigen which is then taken up by epithelial cells of the hindgut to gut associated lymphoid tissue, where an immune response can be generated (Rombout and van den Berg, 1989). The vaccine can be combined with the regular feed during production which makes administration more convenient, less labour intensive, requires no technical skill, avoids stress in animal and repeat dosing can be easily carried out. Even with these advantages there are few oral vaccines commercially available, attributed to their poor performance when compared to other available vaccines (Mutoloki, Munang'andu and Evensen, 2015). This poor performance is likely as a result of degradation of the antigen, which must pass through the hostile stomach environment, before reaching the hindgut where uptake can take place. Processes for encapsulation of the antigen to help reduce degradation are being investigated. Liposomes coated in chitosan showed a reduction in premature drug release when exposed to conditions similar to those found in the stomach and small intestine, but effective in conditions resembling the large intestine (Barea *et al.*, 2012). Chitosan-alginate microcapsules have also been shown to have a similar pH dependent release of proteins *in vitro* (Polk *et al.*, 1994). This technique has shown to be effective in largemouth bass, when testing the encapsulated MSRV glycoprotein from the *Micropterus salmoides* rhabdovirus compared to the antigen alone an increased immune response was observed along with an improved survival rate increasing from 25.8% to 54.8% post challenge (Xu *et al.*, 2022)**.** An alternative method of encapsulation could be through employing the use of microalgae. Microalgae would be able to produce the antigen within the cells which would then be harvested and dried effectively encapsulating the antigen within the cells, allowing for the antigen to be protected from degradation. Some research has been carried out in the use of microalgae for oral vaccination already. For instance, the VP28 protein from WSSV has been successfully expressed

in *C. reinhardtii* and allowed for a survival of 70% compared to 0% of the untreated control when challenged (Lanh *et al.*, 2021). Beyond degradation further challenges should be considered for the development of oral vaccines. Dosage is an important function of vaccination, and this is difficult to control for individuals undergoing oral vaccination, currently there are no solutions to this problem. Immunity from oral vaccination is often short-lived and would require subsequent boosters, this is relatively simple with oral vaccination however there is a risk of oral tolerance occurring. Oral tolerance is when the immune system becomes unresponsive to ingested antigens, due to repeat exposure, this is a mechanism in which the immune system does not generate an adverse immune response to food and the commensal bacteria found in the digestive system (Tordesillas and Berin, 2018). Many factors can influence oral tolerance including dosage, time between doses, the antigen used and even genetic factors (Joosten *et al.*, 1997), which should be taken into account when designing and testing oral vaccines. In addition to this the use of oral adjuvants may help to both increase the immune response and resist oral tolerance (Van der Weken, Cox and Devriendt, 2021).

1.2.5 An overview of the immune system of fish and shrimp

The immune systems of fish and higher vertebrates are similar although there are some notable differences. Just as in mammals there are two major components of the immune system within fish, the innate and adaptive immune system. The innate immune systems role is to prevent pathogen entry into the host through physical barriers and to prevent the growth and proliferation of a pathogen within the host. When a pathogen infects a host the innate immune response elicits a nonspecific response, which is activated by recognition of non-self-expressing elements known as pathogen associated molecular patterns (PAMPs) by the innate immune cells pathogen recognition receptors (PRRs). Upon recognition of a pathogen the innate immune system begins expression of a host of different components including cytokines, chemokines, cell adhesion molecules and immunoreceptors. Expression of these immune molecules begin the initial immune

responses which includes inflammation and production of antimicrobial compounds and also plays a crucial role in linking to the adaptive immune response (Mokhtar *et al.*, 2023).

The second major component of the immune system is the adaptive immune response which acts in a more specific manner than the innate immune response. The adaptive immune response is mainly conducted by T and B-lymphocytes through the production of immunoglobulins and cytotoxic T-cells**.** The primary focus for vaccine development, is the adaptive immune response, where memory to antigens present in a vaccine can be generated. This process is largely carried out in the same way as in mammals. In brief, antigen presenting cells (APCs) take up antigens and present them to T-cells and immature B-cells. T-cells differentiate into helper T-cells which associate with the antigen bound immature B-cells. T-cells then release cytokines which initiates proliferation and differentiation of immature B-cells into plasma cells and memory B-cells. B-cells then produce soluble antibodies specific to the antigen. Memory B-cells persist in the host and produce low levels of antibodies, recognition of the antigen again, from a pathogen, will result in a quicker and more comprehensive secondary immune response (Muñoz-Atienza, 2021).

Although the immune system of fish and mammals are broadly similar the immune system varies widely amongst fish with different nuances existing (Mokhtar *et al.*, 2023). With that being said, major differences in the immune systems found in fish compared to mammals is firstly the absence of certain immune organs such as lymphatic nodules and bone marrow, instead the head kidney and thymus takes the role of lymphocytes and erythrocyte production (Salinas, Zhang and Sunyer, 2011). Fish skin is not keratinised like mammalian skin, and as a result fish secrete mucus on the skin which regularly sloughs off and contains a host of different antimicrobial compounds such as lysozyme, proteases, antimicrobial peptides, immunoglobulins, and lectins (Nigam *et al.*, 2012). Although there are large similarities in the complement system of fish and mammals, fish have a well-developed complement system, with higher diversity and many isotypes of complement proteins which increases complement activity (Holland and Lambris, 2002).

The adaptive immune response in contrast is less complex. Fish produce immunoglobins such as IgM, IgD, IgZ and IgT, whereas mammals have a higher range of sophisticated immunoglobins including IgM, IgG, IgA, IgE, and IgD (Mashoof and Criscitiello, 2016). Fortunately, despite a slightly less developed adaptive immune system found in fish, vaccines are still effective with several commercial vaccines being available (see Table 1).

Conversely, shrimp and other crustaceans are invertebrates with no traditional adaptive immune system and therefore rely heavily on their innate immune system, which can be very effective at defending against pathogens. As such the crustaceans employ many of the same mechanisms as vertebrates in their innate immune system along. The immune system conducts processes such as encapsulation, nodule formation, phagocytosis, and release of AMPs to fight infections, much of which is controlled by the hosts haemocytes (Kulkarni *et al.*, 2021).

One unique feature of the immune system in crustaceans is the prophenoloxidase (proPO) system which is not present in vertebrates and is well characterised in shrimp (Sritunyalucksana and Soderhall, 2000). The proPO system results in melanisation, encapsulation, phagocytosis and cytotoxic reactions. Therefore, the system helps to entrap parasites, kill microbes and is involved in the healing of wounds (Amparyup, Charoensapsri and Tassanakajon, 2013). ProPO is synthesised in haemocytes and released during degranulation. The active phenoloxidase (PO) is then produced when proteolytic cleavage of proPO is achieved by a cascade of serin proteinases upon recognition of microbial cell wall components, including lipopolysaccharides (LPS), β-1,3-glucans or peptidoglycans by PRPs. Once active PO has been created, melanin can be produced at the site of infection. During the proPO system intermediates including quinone-like substances, reactive oxygen or nitrogen compounds are generated which are toxic to invading pathogens (Amparyup, Charoensapsri and Tassanakajon, 2013).

Given that there is no traditional adaptive immune system in shrimp and other crustaceans' vaccination, which relies on harnessing the adaptive immune response, is likely to be ineffective.

Therefore, different approaches should be considered when designing treatments for infections which affect shrimp.

1.3 RNA interference

Vaccination of shrimp is not routinely carried out, as previously mentioned, they lack the adaptive immunity seen in vertebrates and as such vaccination is likely to be ineffective. RNA interference (RNAi) has the potential to play a major role in treating viral infections in shrimp populations in the future. RNAi is a mechanism of post-transcriptional gene silencing, double-stranded RNA (dsRNA) specific to a gene is recognised within the cells and initiates degradation of equivalent mRNA. The mechanism for degradation of mRNA by the RNAi mechanism can be seen in figure 2. Double stranded RNA is processed by Dicer, an RNase III like enzyme which processes the dsRNA into small interfering RNA (siRNA). The siRNA then binds to a series of proteins known as the RNA-induced silencing complex (RISC), which is then able to unwind the siRNA creating single stranded molecules. After being loaded into RISC the sequence from the siRNA's targets mRNA through complimentary base pairing, the mRNA is then degraded.

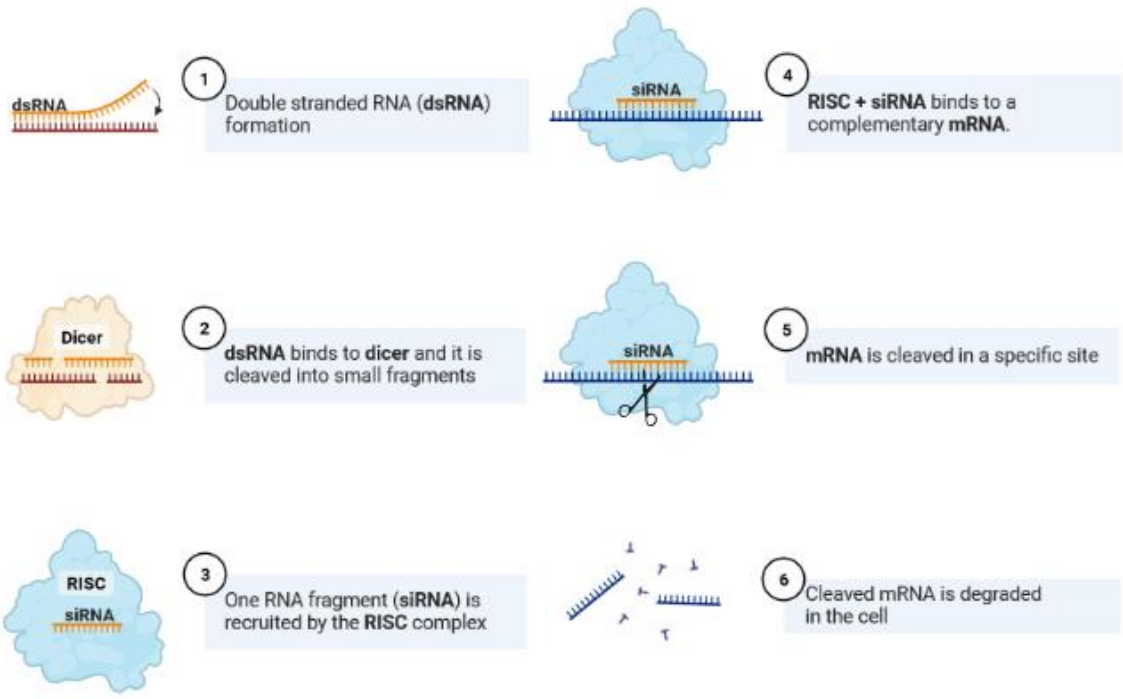


Figure 2. Overview of RNAi mechanism. Created with BioRender.com

RNAi can be used to target key viral genes to knockdown expression and inhibit the virus's ability to replicate (Stram and Kuzntzova, 2006). Several studies have shown that introduction of dsRNA specific to key viral genes have been able to inhibit replication of viral particles within shrimp. This inhibition can offer significant protection when targeting certain genes, RNAi has already been demonstrated to be effective against YHV (Yodmuang *et al.*, 2006), WSSV (Mejía-Ruíz *et al.*, 2011) and Laem-Singh Virus (LSNV) (Saksmerprome *et al.*, 2013). Production of dsRNA for therapeutic use can be carried out *in vitro* through the use of a reverse transcriptase or *in vivo* using a plant or modified bacterial system. Commercially available kits allow for rapid production of high purity dsRNA by *in vitro* methods; however, they are costly often making them less practical for generation of large amounts of dsRNA required for application in aquaculture. A more practical method is to use a modified strain of *E. coli*, HT115 (DE3), which lacks the dsRNA specific RNase III and has the T7 RNA polymerase integrated into the genome. Through the introduction of a plasmid with T7 promoters into HT115 (DE3) cells, dsRNA can be produced and accumulate within the cells which can later be purified for experimental use. The purification and verification of dsRNA produced in bacterial cells is an important consideration for this method, it is important to remove any bacterial toxins, non-specific RNAs and other nucleic acids which could be harmful to the target organism or reduce the effectiveness of the dsRNA. This also brings into question the scalability of this technology, there are significant technical challenges to the largescale production and purification of dsRNA to gain the volumes needed for use in aquaculture.

Delivery is the major drawback of this method of treating infections, experiments using dsRNA use injection to achieve introduction of dsRNA to the animal. Injection can be highly effective, reducing exposure of the RNA, which is susceptible to degradation, although injection is not seen as practical for large scale use in farms which contain thousands of these animals. Injection is also difficult to carry out on shrimp due to their size, it is stressful to the animal and the cost to benefit ratio means it is not a feasible strategy. Oral delivery of dsRNA would be a far more attractive manner of delivering dsRNA due to the low skill and reduced labour requirements. Delivery by the oral route

can be achieved either by feed coated with purified dsRNA or using inactivated bacteria used in the production of dsRNA. Feed coated with purified dsRNA exposes the RNA to potential degradation in the environment and gut of the animal whereas inactivated bacteria may protect the RNA from degradation. For instance, In one study inactivated bacteria producing dsRNA targeting the VP28 gene of WSSV were more effective at protecting shrimp from the virus than dsRNA encapsulated in chitosan nanoparticles when challenged, with survival rates of 68% and 37% respectively (Sarathi *et al.*, 2008). Although the use inactivated bacteria seems to give promising results the prolonged use of *E. coli* in shrimp hasn't been investigated and could cause issues as a result of endotoxins associated with the bacteria (Erridge, Bennett-Guerrero and Poxton, 2002). A safer solution could be engineering probiotics for the production and delivery of dsRNA. The probiotic bacteria *Lactobacillus plantarum* has shown to successfully produce dsRNA targeting the YHV, however the quantity produced was far lower than that of dsRNA produced in HT115 (DE3) cells (Thammasorn *et al.*, 2017). The yield of dsRNA could be improved by disruption of the RNase machinery within these cells in the same manner as the HT115 (DE3) strain. However, the plasmid used by Thammasorn *et al* still contained an antibiotic resistance cassette which are undesirable due to the risk of horizontal gene transfer. A more desirable and sophisticated solution would be to produce dsRNA in microalgae.

The microalga *C. reinhardtii* lacks RNAi machinery within the chloroplast (Maul *et al.*, 2002), and new strains can be selected for by restoration of photosynthesis (Economou *et al.*, 2014), in addition to this *C. reinhardtii* has a Generally Regarded as Safe (GRAS) status granted by the US FDA (Triton Algae Innovations, 2018). These benefits have already been recognised with the generation of a marker less strain of *C. reinhardtii* producing dsRNA targeting the RNA dependent RNA polymerase (RdRp) of the YHV, whereby restoration of photosynthesis in a photosynthetic deficient strain of *C. reinhardtii* was used to generate clones in place of selection by antibiotic resistance. The dsRNA targeting the RNA dependent RNA polymerase (RdRp) of the YHV, was able to knockdown expression of RdRp preventing viral replication and therefore helping to protect shrimp

from the infection (Charoonnart *et al.*, 2019). The power of RNAi as a tool to prevent disease in shrimp has been demonstrated but further research into delivery strategies and range of targets is still required for it to be widely adopted within the industry.

1.4 Microalgae cell factories

Recombinant protein production for use in vaccines and other therapeutics is predominantly carried out using microbial cells such as yeast, bacteria, insect and mammalian cells (de Pinho Favaro *et al.*, 2022). These platforms are well understood with a whole host of techniques available making them highly accessible and easy to utilise. Recently microalgae have been gaining traction as a host for production of therapeutic proteins and present advantages for use in aquaculture over more traditional cell factories. Microalgae consist of a diverse group of unicellular photosynthetic microorganisms, which includes both eukaryotic and prokaryotic organisms. They are able grow rapidly and in a range of conditions including terrestrial, freshwater, and marine environments. Microalgae already play an important role in aquaculture as a natural food source and are often produced for feed due to their nutritional benefits. Species such as *Spirulina* sp., *Dunaliella* sp., *Isochrysis* sp., *Pavlova* sp. are routinely use in hatcheries for feeding fish in the larvae stage (Ahmad, W. Hassan and Banat, 2022). Research is also being conducted in the use of other species for alternatives to standard fish meals. For example, feeding Nile Tilapia with a mixture of *Nannochloropsis oculata*, and *Schizochytrium* sp., was shown to improve the growth of the animal as well as enhance the nutrient profile (Sarker *et al.*, 2020). Microalgae also contain useful pigments and micronutrients valuable to the industry for instance, *Haematococcus pluvialis* is a green microalga rich in astaxanthin, a carotenoid which give fish the characteristic pink-red colour (Borowitzka, Huisman and Osborn, 1991). As a result of being utilised for feed, the methods for culturing and large-scale growth of cells are already well known with them being able to be grown using cheap and widely available nutrients in either large open systems such as raceway ponds or in closed photobioreactors (PBRs). Downstream processing of microalgae is also already well characterised and easy to achieve, several drying processes are available and since many

microalgae are GRAS and could be used without any further purification this could greatly reduce cost. Figure 3 shows an outline of the process of using microalgae to produce therapeutic proteins might look like.

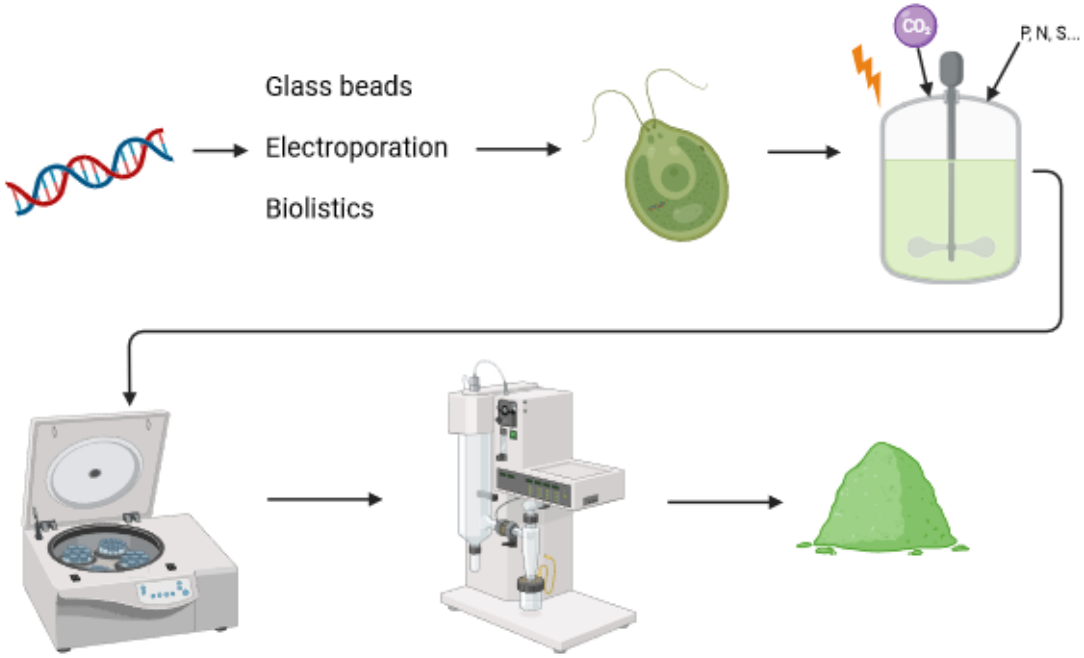


Figure 3. A proposed process outline for production of microalgae. Foreign DNA can be introduced to the cells through glass beads, electroporation or biolistics (gene gun). Once clones are confirmed the cells can be grown in a large bioreactor requiring only light, CO_2 and simple nutrients to grow. After large scale growth cultures can be centrifuged and dried. Dried biomass can then be taken directly or formulated into feeds. Created with BioRender.com

The potential of microalgae in biotechnology has already been identified because of their ability to be a "green" alternative to other production platforms. Many green microalgae have a generally regarded as safe status (GRAS) which make them ideal candidates for oral applications. So far over 40 species of microalgae have been transformed, with the freshwater algae *Chlamydomonas reinhardtii* being the best studied and having the largest molecular toolkit available (Gangl *et al.*, 2015). Microalgae can be transformed using biolistics, electroporation, *Agrobacterium* or even agitation using glass beads or silicon carbide whiskers (Ortiz-Matamoros, Villanueva and Islas-Flores, 2018). Exogenous genes can also be inserted into the nuclear, mitochondrial and chloroplast genome. Although nuclear transformation allows for glycosylation of proteins, insertion of DNA is

essentially random and can result in gene silencing (Cerutti *et al.*, 1997). The chloroplast is the preferred target, allowing for precise integration of DNA, high accumulation of recombinant proteins and the use of photosynthetic restoration as a marker. The chloroplast of *C. reinhardtii* resembles it's bacterial ancestry and is much like a prokaryotic cell containing a eubacterial-like RNA polymerase and 70S ribosome (Manuell *et al.*, 2005). Because of its popularity, molecular tools for chloroplast transformation of *C. reinhardtii* are more widely available and are still being developed. Several mutants with deletions in photosynthetic genes have been investigated including *psbA, atpB* and *psbH* (Boynton *et al.*, 1988; Economou *et al.*, 2014; Bertalan *et al.*, 2015). As a result of *C. reinhardtii*'s ability to grow heterotrophically, photosynthetic deficient strains can be generated allowing for integration and selection of new mutants by photosynthetic restoration. Through this selection method antibiotic resistance cassettes can be avoided removing the risk of horizontal gene transfer in the event biomass is used without further purification, reducing costs associated with purification.

A novel biocontainment tool has also been demonstrated in *C. reinhardtii*, the cold-inducible translational read-through in the chloroplast (CITRIC). The CITRIC system utilises an unused stop codon (TGA) in the chloroplast genome to help contain and prevent translation of exogenous DNA which could escape to the environment. Introducing an altered tryptophan tRNA which is able to recognise the UGA sequence. The system is also inducible as the engineered tRNA is able to fold at 22°C but not 37°C, resulting in protein production when cultures are grown at 22°C (Young and Purton, 2018). This codon re-assignment tool has also permitted further progress to be made in making microalgae more practical to grow at larger scales, where contamination can cause issues. Introduction of a phosphite oxidoreductase gene (ptxD), under the control of the tRNA codon assignment system, into the chloroplast of *C. reinhardtii* permitted growth of cultures using phosphite in place of phosphate. By replacing phosphate with phosphite in cultures, contamination in cultures was found to be far lower than that of cultures grown in normal media (Changko *et al.*, 2020). Utilising this strategy would allow for growth of strains to be achieved under non-sterile

conditions which often lead to increased costs or could even be used as another antibiotic free selection marker, making microalgae an even more attractive host for recombinant protein production. The previous section mentioned that dsRNA has already been produced in the chloroplast of microalgae and conferred protection against YHV in shrimp. With reference to protein production, the p57 protein from *Renibacterium salmoninarum* was expressed in *C. reinhardtii* and was able to induce antibody production in iris fish larvae after supplementation of feed (León R., 2007). In addition to this two genes from *Aeromonas salmonicida*, *acrV* and *vapA* were successfully expressed in the chloroplast of *C. reinhardtii* (Michelet *et al.*, 2011). The use of microalgae in aquaculture looks promising but more research should be carried out to progress the area further.

1.5 Aims

The aims of this project were to investigate a low-cost production and delivery system for oral vaccine or dsRNA treatment for the South East Asian aquaculture industry, which the industry desperately needs to help continue support it's increasing growth. Low-cost solutions which are practical to carry out are especially valuable to South East Asian countries where not only infections are widespread but also, the expense and availability of more traditional vaccines and administration methods are too high to be considered sustainable.

The GapA protein was previously shown to be effective when administered intraperitoneally. The aim of chapter 3 was to demonstrate expression and purification of GapA in *E. coli* which as a low-cost expression system and to upscale this to small scale fermentation as a proof of concept for any subsequent upscale processes. In addition to this oral delivery of proteins would be preferred to reduce cost and labour in administering vaccines. The objective with the GapA protein was to evaluate if it could be used as an oral subunit vaccine as a soluble protein and if the effectiveness of the vaccine could be increased by producing and feeding the protein to the animals as inclusion bodies.

Chapter 4 aimed to build further on the work in the previous chapter by using microalgae as a host for expression of GapA which would allow encapsulation and oral delivery of the protein. The use microalgae as a combination of both host and delivery vector would decrease downstream processing costs and simplify production of vaccines. Testing of the dried biomass allows for gaining an idea of the "shelf life" and storage conditions of the product. While the cells encapsulate the protein could not just protect the protein from degradation while in the gut of the animal but may also serve to prolong the shelf life of the protein at ambient temperatures further decreasing costs by negating the need for cold-chain transport and storage.

Chapter 5 sought to improve on previous studies that had utilised a gene knockdown approach to control viral infections in shrimp by utilising dsRNA synthesis in the *C. reinhardtii* chloroplast. A new purpose-built dsRNA vector for the generation of dsRNA targets could speed up the reactions to new emerging threats. The aim was to use this new construct to generate dsRNA producing *C. reinhardtii strains* which could increase total dsRNA yields, compared to the previous strain, while also being effective at treating WSSV infections. This would allow for quicker generation of strains to be assessed for their effectiveness against viral infections in shrimp, bringing a much-needed treatment option to shrimp aquaculture.

Finally, the project aimed to investigate alternative to treatments by studying a biomarker for disease resistance in Brown-marbled grouper in chapter 6. Using biomarkers to identify fish which are naturally resistant to infections can help to produce more robust stocks which can then be farmed. Using biotechnology to build tools such as this makes breeding programs more efficient, increasing the chances of generating disease resistant stock while also reducing the time invested into breeding programs.

Chapter 2: Materials and Methods

2.1 Suppliers of chemicals, reagents and equipment used

All reagents, chemicals and equipment were supplied from the companies listed below unless otherwise stated. Centrifuge equipment was obtained from Beckman Coulter Inc (USA) and Thermo Fisher Scientific Inc (USA). QPCR equipment was obtained from Thermo Fisher Scientific Inc (USA). PCR, electrophoresis, and imaging machines were obtained from Bio-Rad (USA). Oligonucleotides (primers) were sourced primarily from IDT DNA. DNA polymerase, restriction enzymes and competent cells were obtained from New England Biolabs (NEB; UK). QPCR reagents were obtained from Bio-Rad (USA). RT-PCR reagents were obtained from PCR Biosystems Ltd (UK). Chemicals and consumable plastic-ware were obtained from Fisher Scientific Inc (UK), Sigma (Sigma-Aldrich, USA), Melford Laboratories Ltd (UK) and Formedium (UK). Sequencing was performed by Eurofins Genomics or Genewiz.

2.2 DNA techniques

2.2.1 Preparation of Plasmid DNA

Purification of plasmids were carried out using a QIAprep Spin Miniprep Kit (QIAGEN) as per the manufacturer's instructions. Plasmids were eluted in 50 µL elution buffer (10 mM Tris-Cl pH 8.5) and DNA concentrations determined using a NanoDrop 2000 (Thermo Fisher Scientific Inc).

2.2.2 Amplification of DNA by Polymerase Chain Reaction (PCR)

DNA fragments were amplified using a BioRad T100^{TM} Thermal Cycler machine. Reactions mixtures were made up as per Table 2. Plasmid DNA concentrations ranged from 50-100 ng. For screening microalgae transformations ¼ of the recipe shown in Table 2 was used (final volume of 12.5 µL) and 2 µL of DNA extraction from *C. reinhardtii*. For checking DNA contamination in RNA extracts 500 ng of RNA template was mixed with a 2x Phusion High-Fidelity PCR Master Mix (NEB, UK), 0.5 µM of forward and reverse primers then made up to a final volume of 12.5 µL.

Table 2. Reagents for DNA amplification by PCR.

Reagent	Volume
5X Phusion HF buffer	10 µL
10 mM dNTPs	1 µL
10 µM forward primer	2.5 µL
10 µM reverse primer	2.5 µL
Vector DNA (50-100 ng)	X µL
Phusion polymerase	0.5 µL
MQ H_2O	to 50 µL

A general PCR protocol can be seen below. Annealing temperature was determined using primer pair Tm.

Step	Temperature	Time	
Initial denaturation	98˚C	2 minutes	
Denaturation	98˚C	10 seconds	X 30-35 cycles
Annealing	X˚C	30 seconds	
Elongation	72˚C	30 seconds/kbp	
Final elongation	72˚C	5:00 minutes	
Hold	4˚C	∞	

2.2.3 Agarose gel electrophoresis

Agarose gels were prepared by adding 1-1.5% (w/v) agarose to TAE buffer and microwaved until fully dissolved. Samples were prepared by addition of 6X purple loading dye (NEB, UK) and 20x SYBR Green I (Invitrogen, Thermo Fisher Scientific Inc, USA). Electrophoresis was carried out in either Bio-Rad Mini-Sub® gel tank or Scie-Plas Midi-Tank (Scie-Plas, UK) at 120 V for 45 minutes. DNA was visualised using a Bio-Rad Gel doc.

2.2.4 Purification of DNA from agarose gels

DNA was visualised under UV light and the required band excised as a gel slice. DNA was purified from the gel slice using Monarch® DNA Gel Extraction Kit (NEB, UK) according to the manufacturer's instructions.

2.2.5 Generation of plasmids by golden gate

To generate a plasmid via golden gate the recipe in Table 3 was used. Enzymes used in the reaction can be seen in Table 4.

Table 3. Reagents for golden gate protocol.

Component	**Amount**
Vector	1 nM
Insert/inserts	2 nM
Cutsmart Buffer 10x	2 µL
10mM ATPs	2 µL
T4 Ligase	1 µL
Enzyme	1 µL
Sterile MQ H_2O	Up to 20 µL

Table 4. Enzymes used golden gate protocol.

Enzyme name	**Sequence 5' -> 3'**
BsaI	GGTCTC (N1)/(N5)
SapI	GCTCTTC (N1)/(N4)
Esp3I	CGTCTC (N1)/(N5)

A general golden gate protocol can be seen below and was carried out in a BioRad T100™ Thermal Cycler machine. Following transformation of DNA from golden gate and plating on selective agar negative colonies will either present as red, purple, or blue dependent on their selection marker.

Stage	Temperature	Time	
Initial digest	37°C	15 minutes	
Ligation	16°C	2 minutes	X 20-30 cycles
Digestion	37°C	2 minutes	
Final digestion	37°C	15 minutes	
Heat inactivation	65°C	20 minutes	

2.2.6 Generation of plasmids by Gibson assembly

To generate a plasmid by Gibson assembly vector and insert DNA was first amplified by PCR. Inserts were amplified using a 5' 25 base pair overhang complimentary to vector insert location. Following amplification, PCR reactions were treated with 0.5 µL DpnI overnight at 37°C, then heat inactivated at 80°C for 20 minutes. Vectors and inserts were purified using Monarch® PCR & DNA Cleanup Kit (NEB) according to the manufacturer's instructions. To 5 µL of Gibson master mix (see Table 5) 50 ng of vector DNA was added along with a 3x molar excess of insert. The Gibson reaction was then left at 50°C for 1 hour then 3 µL transformed into NEB Turbo competent cells.

Table 5. Reagents for Gibson assembly mix.

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 H_2O	1.2 mL
5 x ISO buffer	
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 H_2O	6 mL

2.2.7 Transformation of competent *E. coli* cloning cell lines

Following cloning DNA was transformed into *E. coli* NEB Turbo cloning cell line by mixing 5-10 µL DNA with 50 µL cells, incubating on ice for 30 minutes followed by a 45 second heat shock at 42°C in a heat block (AccuBlock Digital Dry Bath, Labnet International, USA), incubation on ice for a further 20 minutes before adding 100 µL LB medium and incubating for 1 hour at 37°C, 200 rpm in a shaking incubator (Multitron Pro shaking incubator, Infors, Switzerland). Cell suspensions were spread onto LB agar medium containing selection antibiotic. In the case of golden gate protocols that used lacZ as a negative marker a selection antibiotic and 1 µL of X-Gal Solution (20 mg/mL) 1 µL of 100 mM IPTG were added to 20 mL of agar. Plates were incubated overnight at 37°C in a static incubator (MIR-162, Panasonic, Japan).

2.2.8 Chloroplast transformation

400 mL of an early log-phase TN72 culture (1-2 x 10^6 cells/mL) was centrifuged at 4000 rpm (Megafuge 16R Thermofisher, USA), 20°C for 5 minutes to concentrate the cells. Cells were resuspended in 4 mL TAP culture and 300 µL added to 5 mL eppendorfs with 300 mg, 425 - 600 µm acid washed glass beads. A total of 5 – 10 µg plasmid DNA was added to each tube except for one tube left as a negative control. The mix of culture, plasmid and glass beads was vortexed at max speed for 15 seconds. 3.5 mL of HSM soft agar at ~45°C was added per tube and inverted to mix. Tubes mixed with HSM, and cell suspensions were poured onto HSM agar plates and left for 20 minutes until dry. Plates were then left overnight at 25°C in 2 µE of light. The following day plates were moved to moderate light at 50 µE. After 4-6 weeks' colonies were picked and re-streaked onto fresh HSM. Once colonies have grown a DNA was extracted and subjected to PCR for screening.

2.2.9 Total DNA extraction of *Chlamydomonas reinhardtii*

Colonies picked from HSM plates were resuspended in 20 µL sterile MQ H_2O, 20 µL ethanol added and incubated at room temperature for 1 minutes. 200 µL 5% Chelex-100 (w/v) was added to the tube and vortexed at high speed for 10 seconds. Samples were boiled on a heat block at 95°C for 5 minutes, then cooled on ice for 5 minutes. Finally, samples were centrifuged at 14,000 rpm (Eppendorf centrifuge 5417R, Hamberg), 4°C, 2 minutes and supernatant transferred to a new tube for storage at -20°C. PCRs were carried out using specific primers to check for integration of the gene of interest.

2.2.10 Sequencing of plasmid DNA and gel extracts

Single colonies from transformations were used to inoculate into 5 mL LB medium containing antibiotic then incubated overnight at 37°C, 200 rpm in a shaking incubator. The following day, the plasmid DNA was isolated using a QIAprep Spin Miniprep Kit (QIAGEN) and the concentration of the DNA determined using a Nanodrop 2000. In the case of sequencing of microalgal constructs, PCRs were carried out using primers specific for the insertion site. PCRs were run on an agarose gel and

DNA was purified from the gel slice using Monarch® DNA Gel Extraction Kit (NEB, UK) according to the manufacturer's instructions. Sequencing was carried out by either Eurofins Genomics (Germany) or GeneWiz (UK). Sequencing mixes were prepared according to manufacturer's guidance.

2.2.11 Constructs used in this study

Table 6. Constructs used in this study.

Name	Vector type	Details	Source
pLvl0	Golden gate level 0	Level 0 entry level plasmid for golden gate	Saul Purton UCL
pLvl1AZ	Golden gate level 1	Level 0 AZ for golden gate cloning	Saul Purton UCL
pLvl0rbcL	Golden gate level 0	Level 1 with rbcL terminator	Saul Purton UCL
pLvl0 16S/psaA	Golden gate level 0	Level1 with 16S/psaA promoter	Saul Purton UCL
pLvl2	Golden gate level 2	Level 2 with psbH flanks + mRFP high copy	Saul Purton UCL
pCLW1	pET23/ptac	pET23/ptac GapA-His6	This study
pCLW2	pET23/ptac	pET23/ptac A2M-His6	This study
p2xTRBL	Golden gate level 1	Double 16s vector for dsRNA production	Saul Purton UCL
pCLW10	Golden gate level 1	YHV-RdRp in p2xTRBL Level 1 plasmid	This study
pCLW11	Golden gate level 1	VP9 in p2xTRBL Level 1 plasmid	This study
pCLW12	Golden gate level 2	YHV-RdRp in p2xTRBL in Level 2 expression vector	This study
pCLW13	Golden gate level 2	VP9 in p2xTRBL in Level 2 expression vector	This study
pCLW14	Golden gate level 1	ORF366 in p2xTRBL Level 1 plasmid	This study
pCLW15	Golden gate level 2	ORF366 in p2xTRBL in Level 2 expression vector	This study
pCLW17	pEXT22	pEXT22 A2M-His6	This study
pCLW18	pET26b	GapA in pET26b - His6	This study

2.2.12 Primers used in this study

Table 7. Primers used in this study.

Primer name	Sequence 5' - 3'	Purpose
A2MGibF	GTCTAGACACAGAGGATCATATGAATGAAAAACCTGGACGGC	Gibson cloning
A2MGibR	GTTAGCAGCCGGATCACTAGTTTAATGATGATGGTGATGATGACCTTC	Gibson cloning
GapAGibF	GTCTAGACACAGAGGATCATATGAATGGTGGTGAAAGTGGGCATTA	Gibson cloning
GapAGibR	GTTAGCAGCCGGATCACTAGTTTAATGATGATGGTGATGATGTTTGGC	Gibson cloning
A2M_PEXTII_GibF	GAATTCCACAGAGGAGGATCCAAGAATGAAAAACCTGGACGGC	Gibson cloning
A2M_PEXTII_GibR	CTTATTAATGGTGATGGTGATGGTGACCTTCAACACACGGATAGG	Gibson cloning
PEXTIIGIB1	GGATCCTCCTCTGTGGAATT	Gibson cloning
PEXTIIGIB2	TCTTGGATCCTCCTCTGTGGA	Gibson cloning
PYU49Gib1	TCATATGATCCTCTGTGTCTAGAC	Gibson cloning
PYU49Gib2	ACTAGTGATCCGGCTGCTAAC	Gibson cloning
T7F	GCTAGTTATTGCTCAGCGG	Sequencing
T7R	TAATACGACTCACTATAGGG	Sequencing
pET26b_BsaI_F	TGAGTGGGTCTCCAAAGATCCGGCTGCTAACAA	Golden gate cloning
pET26b_BsaI_R	TGAGTGGGTCTCCTATGTATATCTCCTTCTTAAAGTTAAACAA	Golden gate cloning
GapA_Ggate_F	TGAGTGGGTCTCCCATATGGTGGTGAAAGTGGGC	Golden gate cloning
GapA_Ggate_R	TGAGTGGGTCTCCCTTTAATGATGATGGTGATGATGTT	Golden gate cloning
YHVF	GATCATCGTCTCCCGTCCGCATGTC	Golden gate cloning
YHVR	GATCATCGTCTCGCAAGGGGTGAAT	Golden gate cloning
VP9F	GATCATCGTCTCCCGTCCTTAATGG	Golden gate cloning
VP9R	GATCATCGTCTCGCAAGGATGTTAT	Golden gate cloning

Flank1	GTCATTGCGAAAATACTGGTGC	PCR, sequencing
Rseq_mCRH1	GGCAACAGGAACTTCTAAAGC	PCR, sequencing
Seq_mCRH1	CTCTCGTCAGGCAATTTGCTTACACC	PCR, sequencing
Seq_p2xTRBL	GGTGAGAATGGTCTCAGGAGCAGTA	Sequencing
Seq_Lvl1	GGATTTGTTCAGAACGCTCGGTTGC	Sequencing
HA_R	TTAAGCGTAATCTGGTACGTCG	Screening PCR
JX01_R	CCGGGTCTCCTGTGGCTCTTCGTTA	Golden gate cloning
JX01_F	CCGGGTCTCCTGTGGCTCTTCCATG	Golden gate cloning
GapA_F	CCATGGTTGTAAAAGTAGGT	Screening PCR
YHV_RNA_F	GCATGTCCTGTTCTCTCACTGAATT	PCR, RT-PCR, qPCR
YHV_RNA_R	GGTGAATTCTAGCCATGCGGTGTTG	PCR, RT-PCR, qPCR
ORF366_RNA_F	AGGAAAATGACCTCTATGAAGAAGA	PCR, RT-PCR, qPCR
ORF366_RNA_R	AGAAAGCGCGTGCTTTAGC	PCR, RT-PCR, qPCR
VP9_RNA_F	TTAATGGCCACCTTCCAGA	PCR, RT-PCR, qPCR
VP9_RNA_R	ATGTTATTCTGTTGTTGGCAC	PCR, RT-PCR, qPCR

2.3 Maintenance of *E. coli* cultures

Table 8. *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)

2.3.1 Media and supplements

Cultures were grown in the medium specified. For colony growth, LB agar was used with the LB recipe outlined in Table 9 along with the addition of 10 g/L bacto-agar. When growing cultures containing a plasmid, cultures were supplemented with an appropriate antibiotic: Ampicillin (100 µg/mL), kanamycin (50 µg/mL), Chloramphenicol (35 µg/mL) or Tetracycline (10 µg/mL).

Table 9. Media and supplements for bacterial cells.

Media	Components
Luria Broth (LB) medium	10 g/L sodium chloride, 10 g/L tryptone, 5 g/L yeast extract
Terrific Broth (TB) medium	24 g/L yeast extract, 12 g/L tryptone, 4 mL/L glycerol Potassium phosphate buffer: 125.4 g/L K_2HPO_4, 23.2 g/L KH_2PO_4
SM6Gc medium	10 mL 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 $Na_2MoO_4.2H_2O$

2.3.2 Preparation of competent cells

A single colony of the desired *E. coli* strain was inoculated into 5 mL LB media with appropriate antibiotic and grown overnight at 37°C, 200 rpm in a shaking incubator. The following day 400 mL of LB with appropriate antibiotic was inoculated using the overnight culture (1:100). Cells were grown until OD_{600} = 0.3-0.4. Cells were left to incubate on ice for 20 minutes and then pelleted by centrifugation at 4000 rpm, 4°C for 5 minutes. Supernatant was discarded and the cells resuspended gently in 30 mL of ice cold 0.1 M $CaCl_2$ and incubated on ice for 30 minutes. Cells were then centrifuged at 4000 rpm, 4°C for 5 minutes. Supernatant was discarded and the cells resuspended gently in 20 mL of ice cold 0.1 M $CaCl_2$ containing 15% glycerol. Cells were aliquoted into Eppendorf tubes and used immediately or stored at -80°C until required.

2.3.3 Transformation of competent *E. coli* expression cell lines

Plasmid DNA shown to be positive from sequencing was purified using a QIAprep Spin Miniprep Kit (QIAGEN). The DNA was then transformed into either competent *E. coli* cells. For transformation of W3110 *E. coli* cells, plasmid DNA was purified from DH5α *E. coli* cells. Plasmid DNA was transformed into *E. coli* cells by mixing 2 µL DNA with 50 µL cells, incubating on ice for 30 minutes followed by a 45 second heat shock at 42°C in a heat block, incubation on ice for a further 20 minutes before adding 100 µL LB medium and incubating for 1 hour at 37°C, 200 rpm in a shaking incubator. Cell suspensions were spread onto LB agar medium containing selection antibiotic. Plates were incubated overnight at 37°C in a static incubator.

2.3.4 Storage of *E. coli* cells

Plasmids were stored in NEB Turbo at -80°C. A single colony of transformed *E. coli* was inoculated into 5 mL LB media with appropriate antibiotic and grown overnight at 37°C, 220 rpm in a shaking incubator. The following morning, 500 µL of cell culture was combined with 500 µL of 50% (v/v) glycerol in a 2 mL cryovial, vortexed, then transferred to -80°C.

2.4 Maintenance of algal cultures

Table 10. *C. reinhardtii* strains used in this study.

Name	Details	Source
TN72	CC-5168 cw15 ΔpsbH. Cell wall deficient strain for chloroplast transformation using glass bead method. Contains aadA (SpcR) cassette, resistant to spectinomycin.	Saul Purton UCL
dsORF366	Strain expressing double stranded RNA targeting ORF366 in WSSV. TN72 background strain with psbH restored and aadA cassette removed.	This study
dsVP9	Strain expressing double stranded RNA targeting VP9 in WSSV. TN72 background strain with psbH restored and aadA cassette removed.	This study
dsRdRp	Strain expressing double stranded RNA targeting RNA dependant RNA polymerase (RdRp) in YHV. TN72 background strain with psbH restored and aadA cassette removed.	This study
PYP	Strain expressing double stranded RNA targeting RNA dependant RNA polymerase (RdRp) in YHV as a hairpin RNA. TN72 background strain with psbH restored and aadA cassette removed.	(Charoonnart *et al.*, 2019)
GapA	Strain expressing GapA from *Streptococcus agalactiae*. 16S/psaA fusion promotor and C-terminal HA tag. TN72 background strain with psbH restored and aadA cassette removed.	This study
CCK10	Empty pASapI vector transformed strain for use as a photosynthetic control. TN72 background strain with psbH restored and aadA cassette removed.	(Zedler *et al.*, 2015)

2.4.1 Media and supplements

Table 11. Media and supplements for microalgae.

Component	TAP medium (1 L)	HSM medium (1 L)
Tris	2.42 g	-
Beijerinck salts	25 mL	25 mL
Phosphates	0.62 mM K_2HPO_4, 0.4 mM KH_2PO_3	4 mM K_2HPO_4, 2.6 mM KH_2PO_3
[a]Trace Elements	1 mL each	1 mL each
Glacial acetic acid	~ 1 mL to pH 7.0	-
For solid medium	20 g Bacto-Agar	20 g Baco-Agar
For Soft-Agar	-	5 g Bacto-Agar
MQ H_2O	Up to 1 L	Up to 1 L

[a]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)

2.4.2 Storage of *C. reinhardtii* cells

Microalgae cultures were maintained on solid TAP agar plates and streaked every 4-6 weeks to maintain a healthy viable stock. Cultures were incubated at 25°C under continuous light at 15-20 µmol photons m^{-2} s^{-1}.

2.5 *E. coli* protein production and cell fractionation

2.5.1 Cell culture and induction of plasmids

Protein expression in shake flask consisted of using a single colony from a fresh transformation plate into 5 mL LB with antibiotic and left overnight to grow at 37°C, 220 rpm. The following morning 50 mL LB or TB, with antibiotic, in a 250 mL Erlenmeyer flask was inoculated with the overnight culture to an OD_{600} of 0.05. Cultures were grown at the stated temperature, 220 rpm. Once the culture reached a density of OD_{600} 0.5, gene expression was induced by adding 100 µM IPTG to the culture and continued to grow. Samples were harvested samples 3 hours post induction of after being expressed overnight.

2.5.2 *E. coli* isolation of soluble and insoluble proteins from shake flask cultures

Samples taken at specified time points were harvested to the equivalent of 10 OD_{600} units. Samples were centrifuged at 4000 rpm, 5 minutes, 4°C. Cell pellets were resuspended in 1 mL resuspension buffer (50 mM Tris-acetate, 2.5 mM EDTA pH 7.0) and lysed by sonication (Soniprep 150 plus, Sanyo Gallenkamp, Loughborough, UK) 4-6 times at amplitude 8.0 for 10 seconds with 10 seconds resting between rounds, samples were kept on ice at all times. After sonication lysed cells were centrifuged at 14,000 rpm, 15 minutes, 4°C yielding the soluble fraction as the supernatant. The cell pellet was then resuspended in 1 mL resuspension buffer resulting in the insoluble cell fraction.

2.5.3 *E. coli* fed-batch fermentation

Pre-cultures for the fermenters were inoculated by adding a single colony from a freshly transformed *E. coli* LB agar plate into 5 mL TB with antibiotic and grown over the day, for 6-8 hours at 37°C, 220 rpm. Following this, 2 mL of the over-day culture was transferred into 200 mL SM6Gc media with antibiotic in a 1 L baffled flask and left to grow overnight at 30°C, 250 rpm. The following day 300 OD_{600}/L of the overnight culture were used to inoculate 500 mL SM6Gc with antibiotic into the Minifors 2 1.5 L fermenter (Infors AG, Switzerland) prior to inoculation the equivalent volume of media to inoculum was disposed of to keep the volume consistent at 500 mL. PPG 2000 was added as an antifoam at 1 mL/L and the parameters set to keep pH 7.0, 40% pO_2 controlled via a cascade with stirrer speed at 800-16000 rpm and then total flow 1.5-2.0 L/min. Cultures were grown at 30°C until stirrer speed and total flow had consistently reached maximum capacity, temperature was then reduced to 25°C. Fermenters were supplemented at specific OD_{600} measurements to aid in growth: At OD_{600} 38-42, an 8 mL/L shot of 1 M $MgSO_4 \cdot 7H_2O$ was injected. At OD_{600} 54-58, a 5 mL/L shot of 1.687 M NaH_2PO_4 was added, followed by a further shot of 7 mL/L of 1.687 M NaH_2PO_4 at OD_{600} 66-70 and a glycerol feed (80 % w/v glycerol) was set at a rate of 0.7 % pump capacity (equates to 0.245 mL/min). When the culture had reached OD_{600} 75, protein expression was induced by addition of 9 mL/L 0.0181 M IPTG and grown for the remainder of the experiment. During the

course of the experiments OD_{600} was monitored for growth and cells harvested to monitor protein expression. Fermenter runs were terminated once cultures reached their max OD_{600}.

2.5.4 *E. coli* isolation of soluble and insoluble proteins from fed-batch fermentation cultures

Cultures were harvested at the end of the fermentation run, 500 mL of culture was collected in 500 mL Nalgene centrifuge bottles and centrifuged at 10,000 rpm (Beckman Avanti J-25 JA10 rotor), 30 minutes at 4 °C. The pellet was stored at -80 °C for later processed or protein extracted the same day. Protein was extracted by resuspending pellets in a resuspension buffer (50 mM Tris-acetate pH 7.0) at 2 mL/g wet pellet weight. Cells were lysed by sonication at amplitude 8 for 3 seconds with 7 seconds resting for a total of 2.5 minutes. The sample was then rested for 2.5 minutes before repeating the sonication step, samples were kept on ice at all times. The samples were then centrifuged at 15,000 rpm (Beckman Avanti J-25 JA25.5 rotor), 4°C, 30 minutes, supernatant was kept as the soluble fraction and the pellets resuspended given the insoluble fraction. Samples were then purified by IMAC or visualised by SDS PAGE.

2.5.5 Preparation and denaturation of inclusion body proteins small scale for A2M

A single colony of freshly transformed *E. coli* was used to inoculate 5 mL LB and antibiotic and left overnight to grow at 37°C, 220 rpm. The following morning a 1 L shake flask with 200 mL TB and antibiotic was inoculated with the overnight culture and left at 30°C, 220 rpm. The culture was induced with 100 µM IPTG when OD_{600} was between 0.4-0.6. 16 samples standardised to 10 OD_{600} units each were harvested 3 hours post induction and processed into the insoluble fraction as described in *E. coli* isolation of soluble and insoluble proteins from shake flask cultures. The samples were washed in resuspension buffer (50 mM Tris-acetate, 2.5 mM EDTA pH 7.0) by resuspending the insoluble fraction and immediately centrifuging at 14,000 rpm, 15 minutes, 4°C with the supernatant discarded. The samples were then resuspended in 1 mL of a range of buffers containing 2-8 M urea, 1 M NaCl, 5 mM DTT, at pH 6-12 (buffering solution changed depending on pH required). Details of the buffers used can be seen in Table 12. Once resuspended samples were left in beakers

overnight stirring. The following morning samples were centrifuged at 14,000 rpm, 15 minutes, 4°C. The pellets were resuspended in the same buffer used to solubilise the inclusion bodies and both were prepared for SDS PAGE by adding 5 x PGLB and boiling on a heat block at 95°C for 10 minutes.

Table 12. Buffer compositions for resuspending inclusion bodies.

Buffer Name	**NaCl (5 M)**	**DTT (1 M)**	**Urea**	**Buffering solution (100 mM)**	**pH**
1	5 mL	0.25 mL	2 M	Na_2HPO_4 (13.69 mM), $NaH_2PO_4H_2O$ (86.31 mM)	6
2	5 mL	0.25 mL	2 M	Tris	8
3	5 mL	0.25 mL	2 M	$NaHCO_3$	10
4	5 mL	0.25 mL	2 M	$NaH_2PO_4H_2O$	12
5	5 mL	0.25 mL	4 M	Na_2HPO_4 (13.69 mM), $NaH_2PO_4H_2O$ (86.31 mM)	6
6	5 mL	0.25 mL	4 M	Tris	8
7	5 mL	0.25 mL	4 M	$NaHCO_3$	10
8	5 mL	0.25 mL	4 M	$NaH_2PO_4H_2O$	12
9	5 mL	0.25 mL	6 M	Na_2HPO_4 (13.69 mM), $NaH_2PO_4H_2O$ (86.31 mM)	6
10	5 mL	0.25 mL	6 M	Tris	8
11	5 mL	0.25 mL	6 M	$NaHCO_3$	10
12	5 mL	0.25 mL	6 M	$NaH_2PO_4H_2O$	12
13	5 mL	0.25 mL	8 M	Na_2HPO_4 (13.69 mM), $NaH_2PO_4H_2O$ (86.31 mM)	6
14	5 mL	0.25 mL	8 M	Tris	8
15	5 mL	0.25 mL	8 M	$NaHCO_3$	10
16	5 mL	0.25 mL	8 M	$NaH_2PO_4H_2O$	12

2.5.6 Larger scale shake flask preparation and denaturation of inclusion body proteins for rabbit immunisation

A single colony of freshly transformed *E. coli* was used to inoculate 5 mL LB and antibiotic and left overnight to grow at 37°C, 220 rpm. The following morning a 2 L shake flask with 400 mL TB and antibiotic was inoculated with the overnight culture and left at 37°C, 220 rpm. The culture was induced with 100 µM IPTG when OD_{600} was between 0.4-0.6. Cells were harvested 3 hours post induction by centrifuging at 10,000 rpm, 4°C, 30 minutes. Cells were then resuspended in ~50 mL resuspension buffer (50 mM Tris-acetate pH 7.0) and lysed by sonication at amplitude 8.0 for 3 seconds with 7 seconds resting for a total of 2.5 minutes. The sample was then rested for 2.5 minutes before repeating the sonication step, samples were kept on ice at all times. The sample was then centrifuged at 15,000 rpm, 4°C, 30 minutes, with the supernatant was discarded and the pellet resuspended in the resuspension buffer (50 mM Tris-acetate pH 7.0), and centrifuged again at 15,000 rpm, 4°C, 30 minutes. The supernatant was again discarded, and the pellet resuspended in binding buffer (4 M urea, 100 mM $NaH_2PO_4H_2O$, 0.5 M NaCl, 20 mM imidazole, pH 12) and left stirring overnight at 4°C. The following morning the sample was purified by IMAC.

2.5.7 Overexpression of protein at large scale shake flask to produce inclusion bodies

A single colony of freshly transformed *E. coli* was used to inoculate 5 mL LB and antibiotic and left overnight to grow at 37°C, 220 rpm. The following morning a 2.5 L baffled shake flask with 500 mL TB and antibiotic was inoculated with the overnight culture and left at 37°C, 220 rpm. The culture was induced with 100 µM IPTG when OD_{600} was between 0.4-0.6 and left overnight to express the protein. Cells were harvested by centrifuging at 10,000 rpm, 4°C, 30 minutes. Cells were resuspended in ~50 mL resuspension buffer (50 mM Tris-acetate, 2.5 mM EDTA pH 7.0) and lysed by sonication at amplitude 8.0 for 3 seconds with 7 seconds resting for a total of 2.5 minutes. The sample was then rested for 2.5 minutes before repeating the sonication step, samples were kept on ice at all times. The sample was washed by being centrifuged at 15,000 rpm, 4°C, 30 minutes, the supernatant was discarded, and the pellet resuspended in the wash buffer (50 mM Tris-acetate,

2.5 mM EDTA, 0.5% triton X-100 pH 7.0). The wash step was repeated an addition 2 times with the triton x-100 increasing to 1% then 2% respectively, an additional 2 wash steps were carried out in the resuspension buffer with no triton x-100. Finally, pellets were lyophilised overnight in an Edwards Freeze Dryer Modulyo.

2.6 Protein expression in *C. reinhardtii*

2.6.1 Expression and isolation of soluble and insoluble proteins from shake flask cultures

All strains were grown at 25°C, 120 rpm illuminated at 15-20 µmol photons m^{-2} s^{-1}, unless stated otherwise. Pre-cultures of strains were grown by inoculating 20 mL of TAP with from an agar plate in a 50 mL Erlenmeyer flask and left 3-4 days to grow. Larger strains were inoculated with 10 mL of pre-culture in 100 mL TAP medium in a 250 mL Erlenmeyer flask and left 5 days. Cultures were pelleted by centrifugation at 4,000 rpm, 4°C for 5 minutes. Cell pellets were resuspended in a sonication buffer (50 mM Tris-HCl pH 8, 5 mM $MgCl_2$) and lysed by sonication 2 times at amplitude 8.0 for 20 seconds with 30 seconds resting between rounds, samples were kept on ice at all times. After sonication lysed cells were centrifuged at 14,000 rpm, 15 minutes, 4°C yielding the soluble fraction as the supernatant. The cell pellet was then resuspended in 1 mL resuspension buffer resulting in the insoluble cell fraction.

2.6.2 Growth and protein expression of *C. reinhardtii* in 15 L hanging bags

Hanging bags were made from food grade plastic tubing sealed at both ends using a heat sealer. A small cut was made in the top of the bags to allow for sampling, a tube connected to a sterile pipette tip was used to pierce the bottom of the bag allowing bubbling of the culture using compressed air with an inline filter. Hanging bags were hung from a railing and filled with 15 L of sterile TAP medium. The bags were inoculated to OD_{750} 0.03 from a 400 mL preculture and left at 25°C, with constant aeration and illuminated at 60-80 µmol photons m^{-2} s^{-1}. Samples were taken by removing culture using a serological pipette through the sampling hole, OD_{750}, pH, Quantum yield and dry weight were measured daily.

2.7 Protein purification

2.7.1 Nickel affinity purification

Purification was carried out on an ÄKTA Pure refrigerated system using a GE LifeSciences 5 mL HisTrap HP pre-packed column. Proteins were expressed and extracted as described in chapter 2.5, the resuspension buffer for purification was 20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4. Prior to loading on the column, the protein suspension was filter sterilised through a 0.45 µM filter. The column was first equilibrated with 5 column volumes (CVs) of the resuspension buffer, after which the protein suspension was loaded onto the column and the flow through collected. The column was washed with 10-15 CVs of wash buffer (20 mM sodium phosphate, 500 mM NaCl, 50-100 mM imidazole, pH 7.4) and the elutions from the wash step collected. Finally, the protein was eluted from the column using 1-5 CVs of elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4) separated into 2 mL fractions. All steps were carried out at a 5 mL/min flow rate. For insoluble proteins, the same protocol was used with the resuspension buffer being changed to 4 M urea, 100 mM $NaH_2PO_4H_2O$, 0.5 M NaCl, 20 mM imidazole, pH 12, the same buffer with 100 mM imidazole used for washing the column and 500 mM imidazole used for eluting proteins. The fractions were then visualised by SDS PAGE and staining with Coomassie blue to determine their purity.

2.7.2 Gel-filtration chromatography

Purification was carried out on an ÄKTA Pure refrigerated system using a GE LifeSciences Superdex 200 Increase 10/300 column. The column was first washed with 1.5 CV of filtered and degassed H_2O and then equilibrated with the resuspension buffer (4 M urea, 100 mM $NaH_2PO_4H_2O$, 0.5 M NaCl, 20 mM imidazole, pH 12). A 500 µL sample of protein first purified by IMAC was then loaded onto the column through a 1 mL capillary loop. After the protein was applied 1.5 CV of resuspension buffer was applied to the column at a flow rate of 0.8 mL/minute and elution samples collected in

1 mL fractions until no further peaks were visible on the UV trace. The elution samples were visualised by SDS PAGE and staining with Coomassie blue to determine purity.

2.7.3 Purification of HA-tagged proteins

HA-tagged proteins expressed in *C. reinhardtii* were purified using Pierce™ HA-Tag IP/Co-IP Kit (Thermo Scientific) following the manufacturer's instructions. In a spin column, 500 µL of soluble protein lysate was left to incubate in 20 µL of anti-HA agarose slurry at 4°C overnight. The following unbound protein was eluted by centrifugation at 13,500 rpm, 1 minute. The spin column was then washed 3 times with 500 µL TBS-Tween (0.05%). Bound HA-tagged protein was finally eluted with 20 µL of the provided elution buffer and was repeated a further 2 times. Each elution had 1 µL 1 M Tris pH 9.5 added.

2.8 Protein quantification

2.8.1 Protein quantification by Bradford Assay

Purified protein was quantified using Thermo Scientific™ Pierce™ Coomassie (Bradford) Protein Assay Kit, following the manufacturer's instructions. Samples were prepared by mixing 1 mL of reagent with 20 µL of the purified protein sample and left to incubate for 10 minutes before reading on the spectrophotometer at OD_{595}. The samples were then compared to a standard curve made up with dilution of BSA standards provided with the assay kit.

2.8.2 Protein quantification using densitometry

Protein samples and BSA standards produced to a known concentration were run on a SDS gel stained with Coomassie blue and imaged. For densitometry by western blot, a known concentration of a protein expressing the same affinity tag and the unknown sample were run on an SDS gel, a western blot carried out and imaged for analysis. Densitometry was carried out using image lab software (Bio-Rad), and a standard curve was generated when using dilutions of known concentrations or a relative quantity estimated by comparison to a band of a known concentration.

2.8.3 Protein quantification using Nanodrop

For quantification of purified protein samples with small volumes the quantification setting on the NanoDrop 2000 (Thermo Fisher Scientific Inc) were used following the manufacturer's instructions. The instrument was first blanked with 1 µL of buffer used to elute the protein sample followed by a measurement using 1 µL of purified protein sample. Extinction coefficients of the protein were estimated using ProtParam (ExPASy), and input into the software to increase accuracy.

2.9 Protein electrophoresis

2.9.1 SDS poly-acrylamide gel electrophoresis (SDS PAGE)

SDS poly-acrylamide gels were prepared and run using the Bio-Rad Mini-PROTEAN Tetra system. Gels were made at a thickness of 0.75 mm with 10 or 15 wells. SDS gels composed of a resolving gel (15 % acrylamide, 0.3 % bis-acrylamide (37:5:1), 375 mM Tris-HCl pH 8.85, 0.1 % SDS, 0.1 % APS and 0.0 % TEMED) and with a layer stacking gel (5 % acrylamide, 0.0375 % bisacrylamide, 125 mM Tris-HCl pH 6.8, 0.001 % SDS, 0.6 % APS and 0.06 % TEMED) on top. Samples were mixed with a 5X PGLB (125 mM Tris-HCl pH 6.8, 20 % glycerol, 4 % SDS, 0.02 % bromophenol blue, 5 % β-mercaptoethanol) and boiled at 95°C for 10 minutes before loading. Electrophoresis was typically performed at 40 mA (for 1 gel) or 60 mA (for 2 gels) for 50 minutes in protein gel running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS, pH 8.3).

2.9.2 Coomassie Brilliant Blue staining

Proteins were visualised on SDS PAGE gels by incubating with 50 mL Coomassie Brilliant Blue stain (10 % acetic acid, 40 % methanol, 1 g/L Coomassie Brilliant Blue) for at least 1 hour at room temperature on an orbital rocker. Following this, gels were destained using a destaining solution (5 % ethanol, 7.5 % acetic acid) and incubating for at least 2 hours at room temperature on an orbital rocker.

2.10 Protein detection

2.10.1 Western-blotting

Following SDS PAGE, proteins were transferred onto a PVDF membrane by Western blot. The membrane was first activated by soaking it in 100 % methanol for 1 minute. When using the Bio-Rad Mini Trans-Blot® Cell the gel and activate membrane were sandwiched between two sheets of Whatman paper and two sponges, pre-soaked in Western blot transfer buffer (192 mM glycine, 25 mM Tris and 10 % ethanol). The sandwich was placed in a cassette between electrodes in the gel tank and submerged with Western blot transfer buffer, along with an ice pack to keep the sandwich from overheating. The blot was then run at 40 V (for 1 blot) or 80 V (for 2 blots) for 1 hour. Alternatively, a semi-dry blot was carried out using the Invitrogen Power blotter. For the semi-dry blot, the PVDF membrane was activated as previously described and was soaked in Power Blotter 1-Step™ Transfer Buffer (5X) along 2 sheets of Whatman paper for 5 minutes. The blot was then run for 8.5 minutes with the amperage adjusted accordingly for the number of blots following the manufacturer's instructions. Once transfer was complete, the PVDF membrane was blocked for 1 hour in a solution containing 5% skimmed milk powder in 1X PBS-tween (1X PBS, 0.1 % tween 20) on an orbital shaker, the membrane was then washed 3x for 5 minutes each in PBS-tween before the incubation of primary antibody for 1 hour. The membrane was washed again 3x for 5 minutes each in PBS-tween before incubation with the appropriate secondary antibody for 1 hour. Finally, the membrane was washed 8x for 5 minutes each before imaging. Immunoreactive bands were detected using the ECLTM kit according to the manufactures instructions and visualised using Bio-Rad Gel-doc XR+ chemiluminescence imager with associated software.

2.10.2 Antibodies used in this study

Table 13. Antibodies used in this study. Usage concentration is a ratio of antibody to PBS-tween.

Antibody	Usage concentration	Source
Anti-His (C-terminal), mouse monoclonal	1:5,700	Invitrogen
Anti-Mouse HRP conjugate, goat polyclonal	1:5,700	Promega
Anti-HA, rabbit polyclonal	1:909	Sigma
Anti-Rabbit HRP conjugate, goat polyclonal	1:5,700	Promega
Anti-A2M, rabbit polyclonal	1:5700	This study

2.11 dsRNA extraction, detection, and quantification

2.11.1 dsRNA extraction of *C. reinhardtii*

A 100 mL TAP pre-culture 250 mL Erlenmeyer flask was inoculated from a plate and left for 4-5 days. A 1 L Erlenmeyer flask containing 400 mL of TAP was inoculated using 10 mL of pre-culture and were incubated at 25°C under continuous light at 15-20 µmol photons m^{-2} s^{-1}. After 5 days the cells were centrifuged at 4,000 rpm, 4°C for 5 minutes. The supernatant was discarded, and cell pellets were resuspended in Trizol, with a total of 1 mL Trizol used per 100 mL cell pellet. 1 mL of resuspended cells were transferred into a 2 mL Eppendorf tube and vortexed at full speed and incubated at room temperature for 15 minutes. After incubation 200 µL of chloroform was added to each sample, vortexed at full speed and incubated at room temperature for a further 15 minutes. Samples were centrifuged at 14,000 rpm, 4°C, 10 minutes. The upper layer was then mixed, by inverting, in a 2 mL centrifuge with 800 µL absolute ethanol and 200 µL Dep-C treated 3 M NaOAc and left at -20°C overnight to precipitate. The following day samples were centrifuged at 14,000 rpm, 4°C, 10 minutes. The supernatant was discarded, and 1 mL 75% DEP-C treated ethanol added. The samples were centrifuged at 14,000 rpm, 4°C, 10 minutes, the supernatant discarded, and the

pellet left to air dry for 30 minutes. Pellets were resuspended in 400 µL RNase free H_2O and the DNA and RNA concentrations determined using a NanoDrop 2000 (Thermo Fisher Scientific Inc). Samples were cleared of DNA by treating with 30 µL DNase I, 60 µL of 10x DNase buffer and left to incubate for 5 hours at 37°C. After 5 hours ssRNA was cleared by a further addition of 20 µL RNase A although with 60 µL of RNase free 3 M NaCl and left to incubate at 37°C for a further hour. After incubation, 600 µL of phenol/chloroform was added and samples vortexed at full speed. Samples were centrifuged at 14,000 rpm, 4°C, 10 minutes. The upper layer was then mixed, by inverting, in a 2 mL centrifuge with 800 µL absolute ethanol and 200 µL Dep-C treated 3 M NaOAc and left at -20°C overnight to precipitate. Pellets were resuspended in 400 µL RNase free H_2O. DNase I treatment was repeated a further 2 times to remove any remaining DNA. DNA contamination was checked by PCR by using 500 ng of dsRNA sample described in chapter 2.2.2. Samples were then checked for presence of RNA by RT-PCR.

2.11.2 dsRNA extraction of HT115 (DE3) *E. coli*

Expression of dsRNA in *E. coli* consisted of using a single colony from a fresh transformation plate into 5 mL LB with antibiotic and left overnight to grow at 37°C, 220 rpm. The following morning 50 mL LB, with antibiotic, in a 250 mL Erlenmeyer flask was inoculated with the 500 µL of overnight culture. Cultures were grown at 37°C, 220 rpm. After 8 hours' cultures were harvested by centrifuging at 4000 rpm, 4°C, 5 minutes. The supernatant was discarded, and the cell pellet resuspended in 700 µL RNase free H_2O. The samples were transferred into a 2 mL Eppendorf tube and 700 µL of phenol/chloroform added. Samples were mixed by vortex at full speed at heated on a heat block set to 65°C for 10 minutes. Samples were then centrifuged at 14,000 rpm, 4°C, 10 minutes. The upper layer was then mixed, by inverting, in a 2 mL centrifuge with 700 µL absolute ethanol and 140 µL Dep-C treated 3 M NaOAc and left at -20°C overnight to precipitate. The following day samples were centrifuged at 14,000 rpm, 4°C, 10 minutes. The supernatant was discarded, and 1 mL 75% DEP-C treated ethanol added. The samples were centrifuged at 14,000 rpm, 4°C, 10 minutes, the supernatant discarded, and the pellet left to air dry for 30 minutes. Pellets

were resuspended in 200 µL RNase free H_2O. Samples were cleared of DNA by treating with 20 µL DNase I, 30 µL of 10x DNase buffer and left to incubate for 5 hours at 37°C. After 5 hours ssRNA was cleared by a further addition of 10 µL RNase A although with 30 µL of RNase free 3 M NaCl and left to incubate at 37°C for a further hour. After incubation, 300 µL of phenol/chloroform was added and samples vortexed at full speed. Samples were centrifuged at 14,000 rpm, 4°C, 10 minutes. The upper layer was then mixed, by inverting, in a 2 mL centrifuge with 700 µL absolute ethanol and 140 µL Dep-C treated 3 M NaOAc and left at -20°C overnight to precipitate. Pellets were resuspended in 100 µL RNase free H_2O. The following day samples were centrifuged at 14,000 rpm, 4°C, 10 minutes. The supernatant was discarded, and 1 mL 75% DEP-C treated ethanol added. The samples were centrifuged at 14,000 rpm, 4°C, 10 minutes, the supernatant discarded, and the pellet left to air dry for 30 minutes. Pellets were resuspended in 100 µL RNase free H_2O. The resulting dsRNA was then run on an agarose gel which was stained with SYBR™ Gold nucleic acid gel stain (Invitrogen™) visualised under UV light and the required band excised as a gel slice. dsRNA was purified from the gel slice using Monarch® DNA Gel Extraction Kit (NEB, UK) according to the manufacturer's instructions.

2.11.3 RT-PCR for detection of RNA

RNA fragments were amplified using a BioRad T100™ Thermal Cycler machine. Reactions mixtures were made up as per Table 14. Prior to RT-PCR RNA was heated at 95°C for 3 minutes then cooled on ice for 2-3 minutes.

Table 14. Reagents for RNA amplification by RT-PCR.

Reagent	Volume
2x High-Fidelity PCR Master Mix	6.25 µL
10 µM forward primer	0.625 µL
10 µM reverse primer	0.625 µL
RNA template (500 ng)	1 µL
RTase Go reverse transcriptase (PCR Biosystems)	0.625 µL
RNase free H_2O	to 12.5 µL

A general RT-PCR protocol can be seen below. Annealing temperature was determined using primer pair Tm.

Step	Temperature	Time	
Reverse Transcription	45°C	30 minutes	
Initial denaturation	98˚C	2 minutes	
Denaturation	98˚C	10 seconds	X 30-35 cycles
Annealing	X˚C	30 seconds	
Elongation	72˚C	30 seconds/kbp	
Final elongation	72˚C	5:00 minutes	
Hold	4˚C	∞	

2.11.4 RNase III treatment of dsRNA

To confirm dsRNA in the sample samples were treated with RNase III. To 30 µL RNA sample 10 µL of ShortCut RNase III (NEB), 10 µL of 10x reaction buffer, 10 µL of 10x $MnCl_2$ and 40 µL RNase free H_2O and left to incubate at 37°C for 2 hours. A control sample with 10 µL of RNase free H_2O was used in place of the RNase III was also set up. After incubation samples had 200 µL RNase free H_2O added. The samples were then mixed with 300 µL of phenol/chloroform by vortex at max speed. Samples were centrifuged at 14,000 rpm, 4°C, 10 minutes. The upper layer was then mixed, by inverting, in a 2 mL centrifuge with 700 µL absolute ethanol and 140 µL Dep-C treated 3 M NaOAc

and left at -20°C overnight to precipitate. The following day samples were centrifuged at 14,000 rpm, 4°C, 10 minutes. The supernatant was discarded, and the pellet left to air dry for 30 minutes. Pellets were resuspended in 20 µL RNase free H_2O and the DNA and RNA concentrations determined using a NanoDrop 2000 (Thermo Fisher Scientific Inc). Samples were then checked for presence of RNA by RT-PCR.

2.11.5 RT-qPCR for quantification of dsRNA

RNA was quantified by using the QuantStudio™ 3 Real-Time PCR system (Applied Biosystems™) and associated software. Reactions were made up as per Table 15. Prior to running RNA was heated at 95°C for 3 minutes then cooled on ice for 2-3 minutes. A standard curve from dsRNA extracted and purified was first created in duplicate and the samples were also run in duplicate. Data collected by real time PCR was then analysed by the software provided with the QuantStudio system to generate a melt curve and quantify the dsRNA in relation to the standard curve.

Table 15. Reagents for dsRNA quantification by RT-qPCR.

Reagent	**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
RNA template (250-500 ng)	1 µL
iScript reverse transcriptase	0.25 µL
RNase free H_2O	to 20 µL

A general RT-qPCR protocol can be seen below.

Step	**Temperature**	**Time**	
Reverse Transcription	50°C	20 minutes	
Initial denaturation	95˚C	1 minute	
Denaturation	95˚C	15 seconds	X 40 cycles
Annealing / Extension	55˚C	30 seconds	
Step and hold melt curve (0.5 °C/second)	65-95 °C	5 seconds (hold)	

2.12 Fish challenge trials

This study was carried out at the Universiti Putra Malaysia (UPM) and approved by UPM Institutional Animal Care and Use Committee (UPM/IACUC/AUP-R054/2022). Juvenile red hybrid tilapia (*Oreochromis spp.*) fish 80-90g (acquired from a fish hatchery at Taman Pertanian Universiti) 80-90g were acclimatised in 2000 L tanks for two weeks with constant feeding while observed for abnormalities in behaviour and health. The fish were fed a basal diet of 2% of their body weight twice daily in the morning and afternoon. The basal diet consisted of 40% fish meal, 21% soybean meal, 25% corn starch, 2.5% mineral premix, 1.0% multivitamin, 10% fish oil and 0.5% fibre. The water quality parameters were maintained at 20−22°C, pH 6.9–7.4, 6.5–7.8 mg L^{-1} dissolved oxygen and 0.25−0.3 parts per trillion (salinity). After becoming acclimatised fish were randomly separated into three groups in 100 L tanks. Each group was made up 15 fish within a tank. The immunisation routine was the same as used by Tu *et al* (Tu *et al.*, 2010). Two vaccinated groups were fed basal diets coated with purified soluble GapA or GapA inclusion bodies for five consecutive days on week 0 and 2. The quantity of the feed was 1% body weight, with the vaccine dosage of 20 µg per g of tilapia. The third experimental group was run as a control being fed the basal diet without any vaccine. All groups were challenged with *Streptococcus agalactiae* on week 4 of the experiment. Bacterial cultures were inoculated in tryptic soy broth and incubated at 28°C. The cultures were then centrifuged at 800 *g* for 15 minutes. Cell pellets were washed in PBS and the required dose then resuspended in PBS. Fish were immersed in 1 x 10^8 cfu/mL of bacteria for 3 hours. After challenging the fish were transferred to a clean tank and monitored for 7 days for signs of infection and mortalities.

2.13 Shrimp challenge trials

This study was carried out at CENTEX Shrimp Centre, Mahidol University, Bangkok, Thailand. Post larvae (0.03 g) and juvenile (20 g) specific pathogen free shrimp (*P. vannamei)* were acquired from CPF (Thailand) for use in challenge trials and preparation of WSSV inoculum. Shrimp were acclimatized for three days prior to experiments. Throughout the experiment shrimp were kept in

artificial seawater at 15 parts per thousand salinity with aeration at 28±0.5°C. WSSV inoculum was prepared by feeding juvenile shrimp WSSV infected tissue at 10% body weight. Moribund shrimps were collected and sacrificed for muscle collection. DNA was extracted from the shrimp and WSSV detected using IQ2000™ kit (Farming IntelliGene Tech. Corp). Infected shrimp muscles were homogenized and quantified by qPCR and stored at -80°C. Post larvae shrimp were divided into five groups each consisting of three replicates of 20-25 shrimp. The groups consisted of negative control (no algae, no challenge), positive control (no algae, WSSV challenge), wild type (algae with dsRFP cassette, WSSV challenge), VP9 (algae with dsVP9 cassette, WSSV challenge) and ORF366 (algae with dsORF366 cassette, WSSV challenge). All groups were fed twice daily with commercial shrimp feed at 10% body weight, treated groups were fed an extra feed consisting of 1×10^9 of the appropriate algal culture. To prepare the algal feed cultures were grown in 200 mL of TAP media under constant light for 5 days. Cells were centrifuged at 4000 rpm for ten minutes and resuspended to achieve 4×10^9 cells/mL. Microalgae suspensions were stored at 4°C until being used. Shrimp were challenged on day 4 of the experiment by feeding WSSV infected tissue at 50% body weight of the shrimp. Shrimp were observed daily for mortality and signs of infection and data recorded.

Chapter 3: Expression of GapA in *E. coli*

3.1 Introduction

Disease outbreaks in aquaculture pose a severe problem for future growth of the sector (Bostock *et al.*, 2010). Pathogenic bacteria are an issue due to the aquatic environment being supportive of their growth, allowing for the pathogens to grow to high densities which are then ingested by the animals. Antibiotics are currently used to control bacterial infections in aquaculture however their widespread use can give rise to antibiotic resistant strains, because of this vaccination against bacterial pathogens has become more favourable, with vaccines being available for up to 17 species of fish combatting over 22 different bacterial diseases (Brudeseth *et al.*, 2013). Previously fish vaccines have been made up of formalin-killed microorganisms and some live attenuated vaccines. Subunit vaccines pose a cheaper, more scalable and a safer alternative, as there is no risk of infecting the host organism, integrating with host DNA and a reduced risk of non-specific effects.

Streptococcus agalactiae is a bacterial pathogen which infects a wide variety of fish species including Nile tilapia (*Oreochromis niloticus*). Infections are of concern due to the high mortality rates as a result of septicaemia and meningoencephalitis (Bowater *et al.*, 2012). There is a real need for alternative treatments and vaccines to the widespread use of antibiotics used for controlling *S. agalactiae* infections. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH/GapA), a cell surface protein, has been demonstrated as a good vaccine candidate against *S. agalactiae* infections in Tilapia, when combined with adjuvant and administered as an intraperitoneal injection with a relative survival percentage of up to 63% 14 days post-challenge, while those injected only with PBS had a 0% survival amongst challenged animals (Zhang *et al.*, 2017). While injection does give the best immunity, it is impractical and costly requiring a large skilled workforce to administer. In addition to this capture and removal of stock for injection causes stress on the animal (Evensen, 2009), which present issues specifically with the use of subunit vaccines as they often require

multiple injections to achieve desired long term protection. Oral vaccinations have been long sought after as an ideal low-tech solution to the drawbacks of traditional vaccination, given their ease of use which allows for repeated treatments and is best for the animal's wellbeing due to the reduced handling.

The repeat treatments needed from subunit vaccines to provide effective long-term protection along with the requirement of higher doses needed for oral vaccination means that low-cost methods for production of large amounts of antigen are key to making oral vaccines feasible for use in the industry. Recombinant protein production in *E. coli* is a widely used and already well-established platform, expression in *E. coli* is cheap and easy with the ability to produce large amounts of protein. In industry, proteins are most commonly produced in a bioreactor in a process known as fermentation. Fermentation allows for high cell densities to be achieved in favourable conditions which help to achieve an overall higher yield of product.

Once fermentation processes are optimised for high protein concentrations it is necessary to extract and purify the protein of interest using downstream processes like column purification. A commonly used technique is immobilized metal ion chromatography (IMAC), which takes advantage of the engineered poly histidine tags (His6/His tag) often added to proteins allowing for visualisation that will also strongly bind to metallic cations such as Ni^{2+}, allowing for purification. IMAC purification can achieve purities of up to 95% and their resins are re-usable (Burden and Whitney, 1995).

Despite the clear advantages and demand for oral vaccinations in the industry there are currently only a limited number available on the market, due to their poor performance (Mutoloki, Munang'andu and Evensen, 2015). The reduced efficacy of oral vaccines could be due to the degradation of the product as it passes through the harsh conditions of the stomach before reaching the intestine where absorption takes place. Ongoing research looks at encapsulation techniques which involves encapsulating the antigen in a carrier to protect it from the hostile environment of the stomach (Romalde *et al.*, 2004). Inclusion bodies may offer a solution which

protect from degradation and also avoid the extra processing costs of encapsulation. Inclusion bodies are protein aggregates which are made up of misfolded or partially misfolded proteins (Carrió, Cubarsi and Villaverde, 2000). A number of different factors can influence the formation of inclusion bodies such as, high protein expression temperatures, overexpression of proteins and misfolding of proteins due to the environmental conditions in the cytosol or lack of eukaryotic chaperone proteins (Singh *et al.*, 2015). Nevertheless, inclusion bodies are more stable and have an increased resistance to proteases than their soluble counterpart (van Beek et al., 2021) so may be more resistant to degradation in the stomach. Using inclusion bodies over whole cell lysate increases the concentration of target antigen while reducing the immune response/reactions from irrelevant proteins within the vaccine (Kęsik et al., 2004). Although vaccines comprising of soluble protein can have a high level of target antigen with low or no contaminating proteins, use of the insoluble extract directly would also negate the need for any column purification and reduce the cost of production. This is primarily due to the fact that inclusion bodies can easily be concentrated by centrifugation removing the majority of the soluble native proteins and further centrifugation and wash steps can be added to lower the amount of some of the undesirable insoluble native proteins.

The approach taken here was to attempt to form GapA as inclusion bodies by overexpression of the protein using a high copy plasmid with a strong T7 promoter. Plasmids with a T7 promoter take advantage of the T7 RNA polymerase which is under the control of the IPTG inducible lacUV5 promoter, once IPTG has bound the T7 RNA polymerase is transcribed providing a high level of expression, with up to an 8-fold increase of transcription when compared to the native *E. coli* RNA polymerase (Sun *et al.*, 2021).

This chapter aims to outline a process to produce GapA in both a soluble and insoluble form which can easily be translated to an industrial process which would be appropriate for large scale production of the protein. The chapter will also compare and outline the effectiveness of the

protein when administered orally through the use of challenge trials in red hybrid tilapia fish (*Oreochromis spp.*).

3.2 Results

3.2.1 Expression of GapA protein in shake flask cultures

The GapA gene from *Streptococcus agalactiae* was first codon optimised for expression in *E. coli*, was cloned into a pET23/ptac vector using Gibson assembly generating pCLW1 with a 6xHis tag at the C-terminus for detection and purification. To assess expression of the protein the vector pCLW1 was expressed in both W3110 and BL21 cells at 37°C and 30°C. W3110 (a K12-strain derivative) and BL21 (a B-strain derivative) were used due to the fact they are both industrially used strains of *E. coli*. Both strains are able to produce large amounts of protein but sometimes differ in protein expression levels, amount of soluble protein and total protein yields (Kang, Kim and Cha, 2002; Arauzo-Aguilera *et al.*, 2023). Cells were grown to mid log phase and induced with 10 µM IPTG and were harvested 3 hours post induction and after expression overnight. The cells were then processed into their soluble and insoluble fractions and visualise by SDS PAGE and immunoblotting.

The GapA protein was successfully expressed in both W3110 and BL21 cells at the different temperatures with a band appearing in the immunoblots (Fig. 4A and 4C) at around 36 kDa which is the predicted size of the GapA protein. The majority of the protein expressed seems to be soluble protein with small amounts of insoluble protein being detected in all conditions. The results appear to show that optimal condition for soluble protein to be expression is at 37°C with cells harvested after being expressed overnight. This is further supported when comparing the coomassie stained gels which also show a large band in the soluble fraction at the size corresponding to the GapA protein (Fig. 4B and 4D).

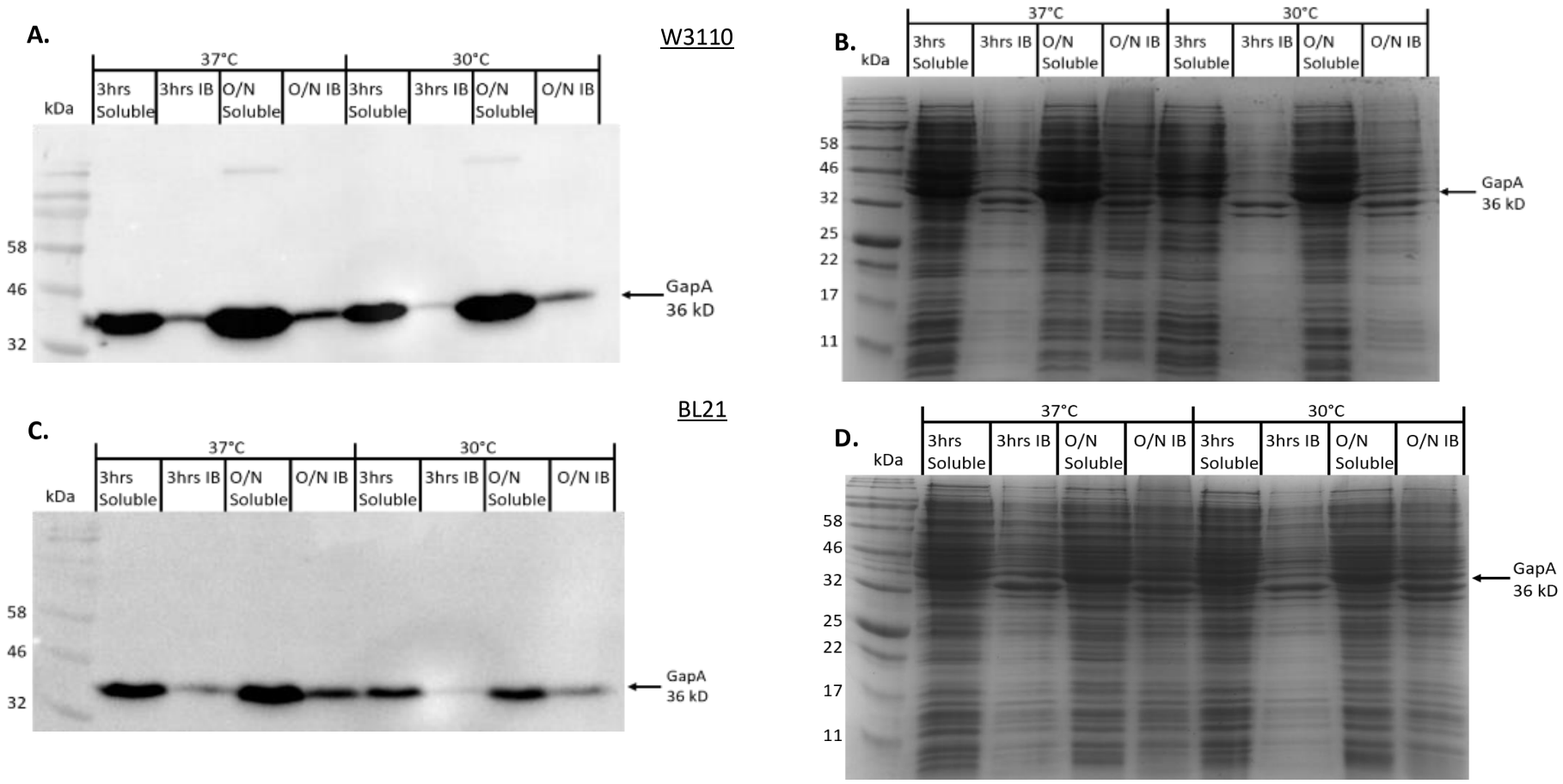


Figure 4. Shake flask culture of W3110 and BL21 cells expressing GapA constructs at different temperatures. Cultures were grown and expressed at both 37°C and 30°C in LB medium. Cells were harvested 3 hours post induction or after overnight expression then processed into both insoluble and soluble fractions. Following extraction proteins were separated by SDS PAGE and visualise by **A.** Anti-HIS western blot of W3110 cells expressing GapA **B.** Coomassie stained gel of W3110 cells expressing GapA **C.** Anti-HIS western blot of BL21 cells expressing GapA **D.** Coomassie stained gel of BL21 cell extracts expressing GapA. Samples were standardised to 10 OD_{600} units of cells and equal volumes (15 µL) of samples loaded in each well.

3.2.2 Fed-batch fermentation and purification of GapA

Following successful expression at shake flask scale the next step was to test expression by fed-batch fermentation. W3110 cells were chosen to express the protein due to the seemingly larger amounts of protein expressed in the shake flask experiments. Large scale fermentation is the chosen method of protein production when producing commercial proteins, successful expression at fed-batch fermentation would make GapA a more viable vaccine candidate. A 1.5 L Minifors bioreactor with 500 mL SM6 defined media was inoculated with the same pCLW1 vector expressing GapA transformed into W3110 cells. The immunoblot (Fig. 5A) shows successful expression of GapA during fed-batch fermentation. The majority of the protein produced was soluble, with a noticeable increase in protein production during the fermentation run. The growth curve (Fig. 5B) shows that high cell densities can be achieved with a maximum OD_{600} of 134 being reached when harvesting, higher cell densities may be possible with more optimisation. By looking at band intensity of the immunoblot (Fig. 5A), the best time point for harvesting was determined to be 72.5 hours after induction, this sample has some of the higher levels of protein when compared to earlier timepoints while also being when the culture was at its highest density, allowing for the higher total yield of protein to be achieved.

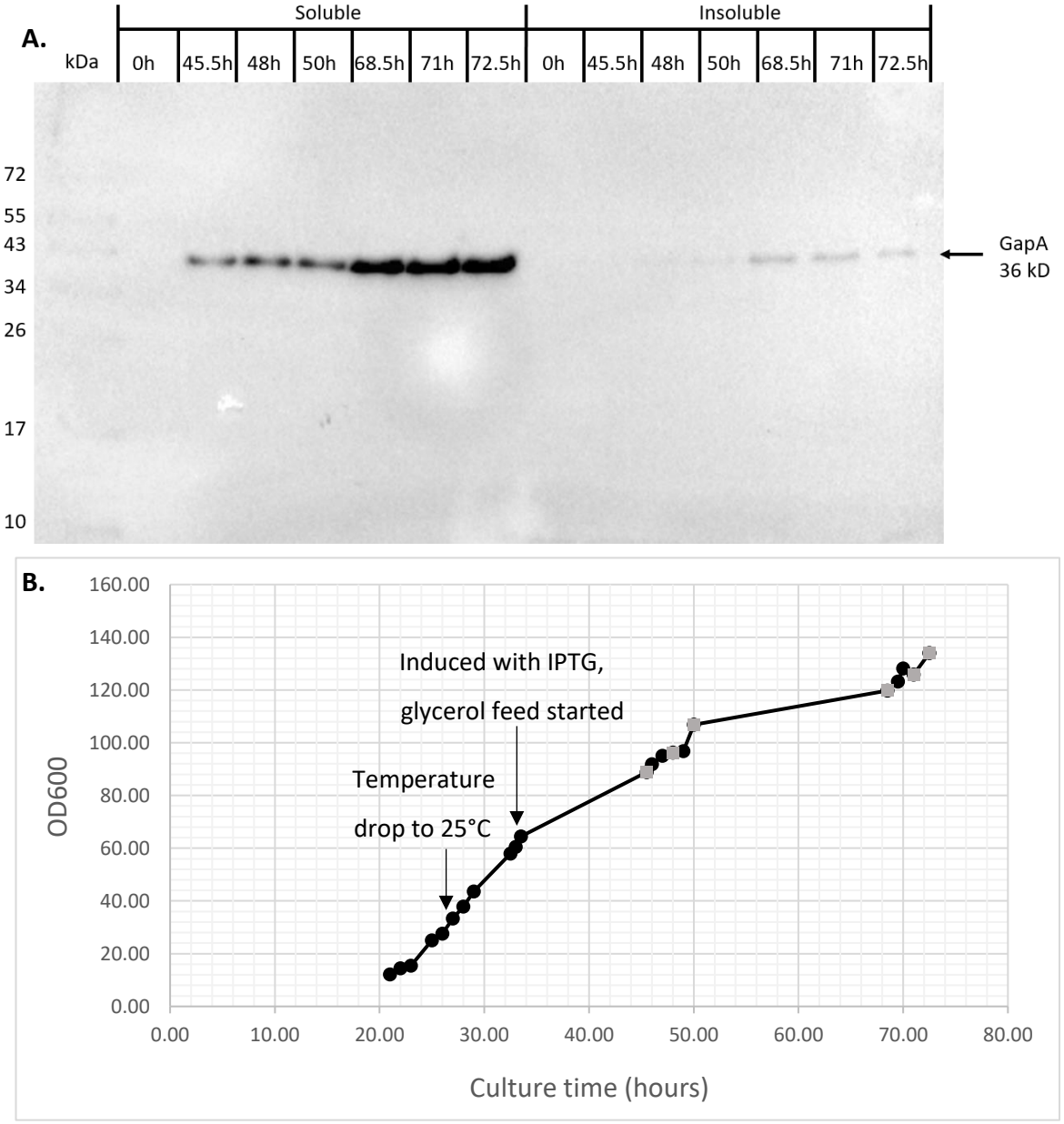


Figure 5. Fed-batch fermentation of W3110 *E. coli* cell expressing pCLW1 in SM6. Following inoculation cells were growth at 30°C. Temperature was dropped when stir speed and airflow reached max capacity. Shortly after the culture was induced and the glycerol (80% w/w) feed started at 0.245 mL/min. **A.** Anti-HIS western blot for samples taken during the run with samples processed into soluble and insoluble protein standardised to 10 units at OD_{600} and 15 µL of sample loaded, sample times are times post inoculation. **B.** Growth curve of W3110 *E. coli* cells expressing GapA during fed-bath fermentation. Grey squares indicate where samples were taken for Western Blot imagining.

The next stage was to purify enough protein that could be later used for challenge trials which would determine how effective GapA at protecting Tilapia against infection. IMAC was chosen as the purification method due to its affinity for the C-terminal His6 tag and high levels of purity.

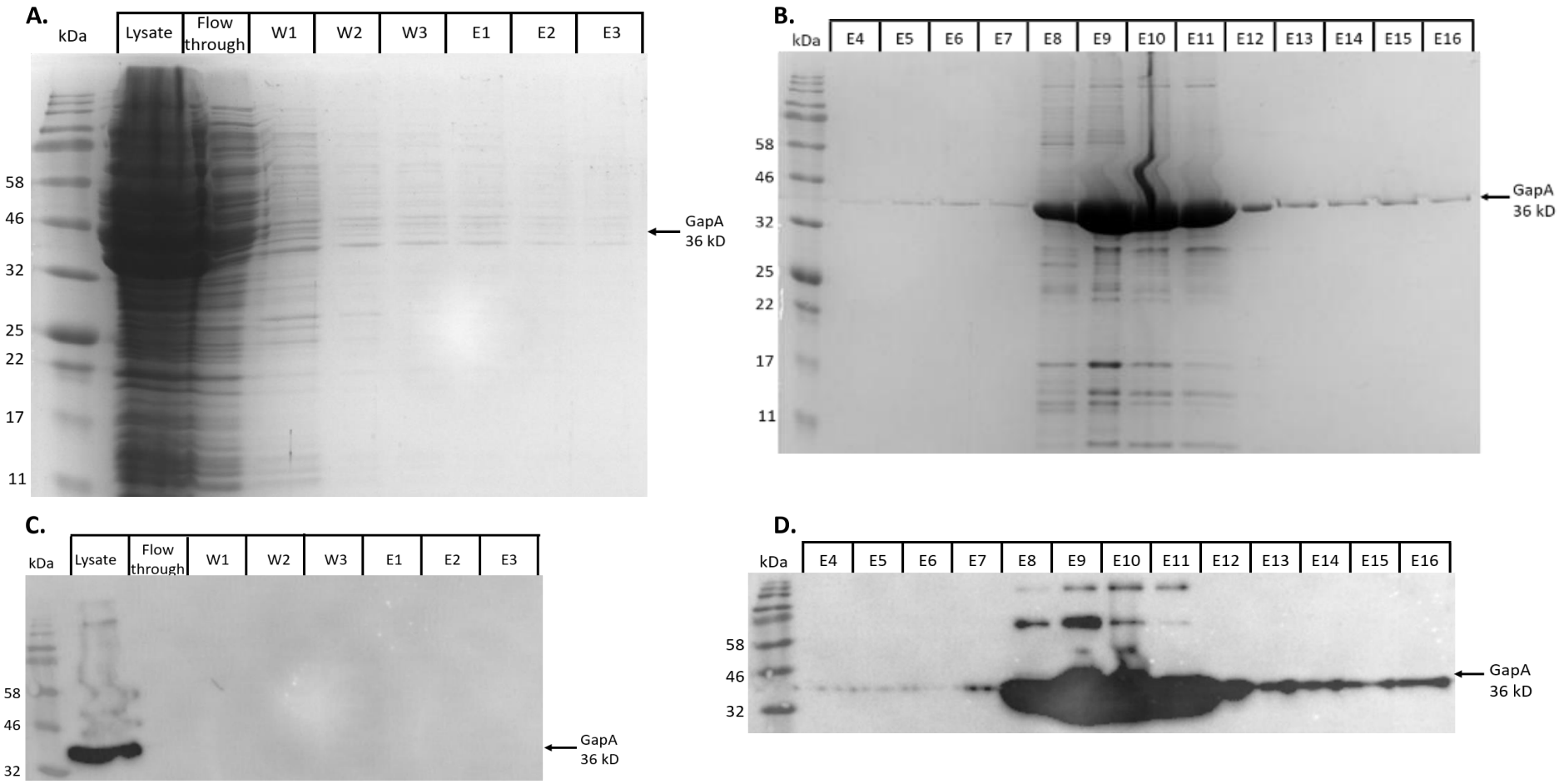


Figure 6. Purification of cell lysates from fed- batch fermentation of W3110 *E. coli* cell expressing GapA. Protein was extracted from a 10 g (wet weight) sample of the pellet from fed-batch fermentation and purified by IMAC using an AKTA automated purification system fitted with a GE LifeSciences 5 mL HisTrap HP pre-packed column. The lysate was run through the column and the flow through collected proceeded by wash steps carried out using 40 mM imidazole. Protein eluted using a gradient elution from 0-500 mM imidazole over 16 elution steps. Following purification proteins were separated by SDS PAGE and visualised by **A.** & **B.** Coomassie staining and **C** & **D.** Anti-HIS western blot.

It can be seen from the results that the majority of the GapA protein binds the column with the flow through fraction and wash step fractions (W1-W3) having no protein being detected in the immunoblot (Fig. 6C). In both Figure 6B and 6D it can be seen that large amounts of GapA are eluted between fraction's 8 and 11 (around 250-345 mM imidazole) with later fraction's still showing some protein being eluted. The coomassie strained gel show that later fractions (Elution 12-16) (Fig. 6B) have a much higher purity of the GapA protein but also seem to have a lower total yield. Although fractions 8-11 don't seem to be completely free of contaminants the vast majority of the protein present is the desired GapA protein and should be acceptable for use as an oral vaccine. After purification, the purified soluble protein was lyophilized ready for use in challenge trials.

Overall, this data proves that GapA can be produced on a larger scale using fed-batch fermentation and subsequently purified to yield a large concentration of relatively pure protein. Showing that GapA, if effective, is easily scalable for use at a commercial level, with further optimisation a higher yield and purity of the protein could be easily achieved, through addition of further purification steps.

3.2.3 Quantification of soluble GapA from fed-batch fermentation

To help gain an understanding of the feasibility of using GapA as a vaccine on a commercial scale it was necessary to estimate the concentration of GapA produced at fed-batch fermentation. A further 10 g (wet cell weight) of pellet was purified by IMAC and visualised by a coomassie staining and immunoblot, bands for the correct size of GapA can be seen and is confirmed by looking at the immunoblot (Fig. 7).

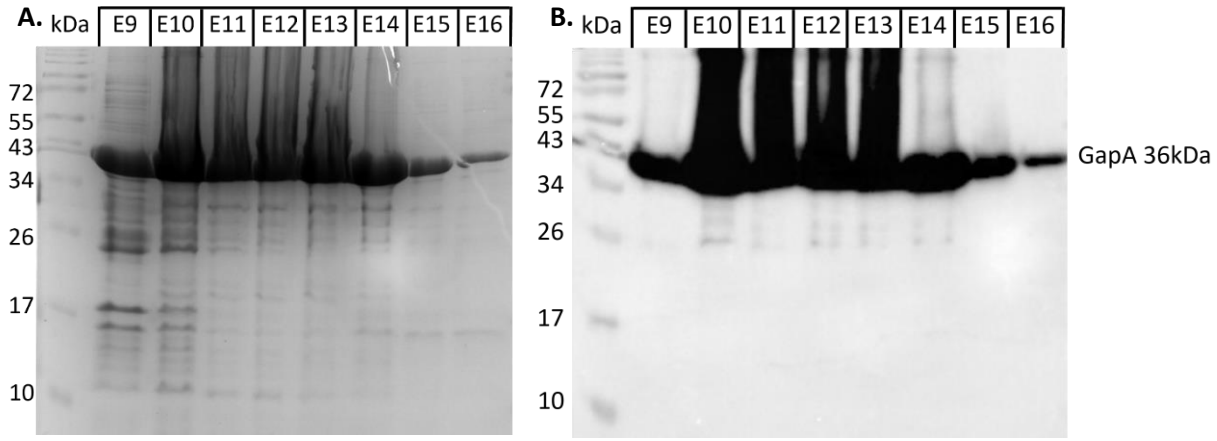


Figure 7. Purification of cell lysates from fed- batch fermentation of W3110 *E. coli* cell expressing GapA. Protein was extracted from a 10 g (wet weight) sample of the pellet from fed-batch fermentation and purified by IMAC using an AKTA automated purification system fitted with a GE LifeSciences 5 mL HisTrap HP pre-packed column. The lysate was run through the column and the flow through collected proceeded by wash steps carried out using 40 mM imidazole. Protein eluted using a gradient elution from 0-500 mM imidazole over 16, 1.8 mL elution steps. Following purification proteins were separated by SDS PAGE with 10 µL of sample loaded to each well and visualised by **A.** Coomassie staining and **B.** Anti-HIS western blot. E: elution. Size of GapA: 36 kDa.

Quantification was carried out using a combination of two methods, Bradford assay for elution 11-15 and then later densitometry software was used to estimate elution 9 and 10 relative to the known concentration of the other bands.

A standard curve was first generated for the Bradford assay using known concentrations of BSA (Fig. 8). The standard curve was then used to determine the concentration of elution 11-15, samples which were higher than the range of the standard curve were diluted 1:10 in elution buffer and later adjusted to determine the concentration. Later, densitometry software was used to analyse elution 9 and 10 and estimate their concentration. From this data, it was estimated that GapA had a concentration of 2.347 mg/g of wet cell weight (see table S1). Since 1 OD_{600} unit of *E. coli* cells typically produces 1.7 g of wet cell weight (Glazyrina *et al.*, 2010) and the fed-batch fermentation

in section 3.2.2 reached an OD_{600} of 134, an approximal concentration of 0.53 g/L of GapA was calculated.

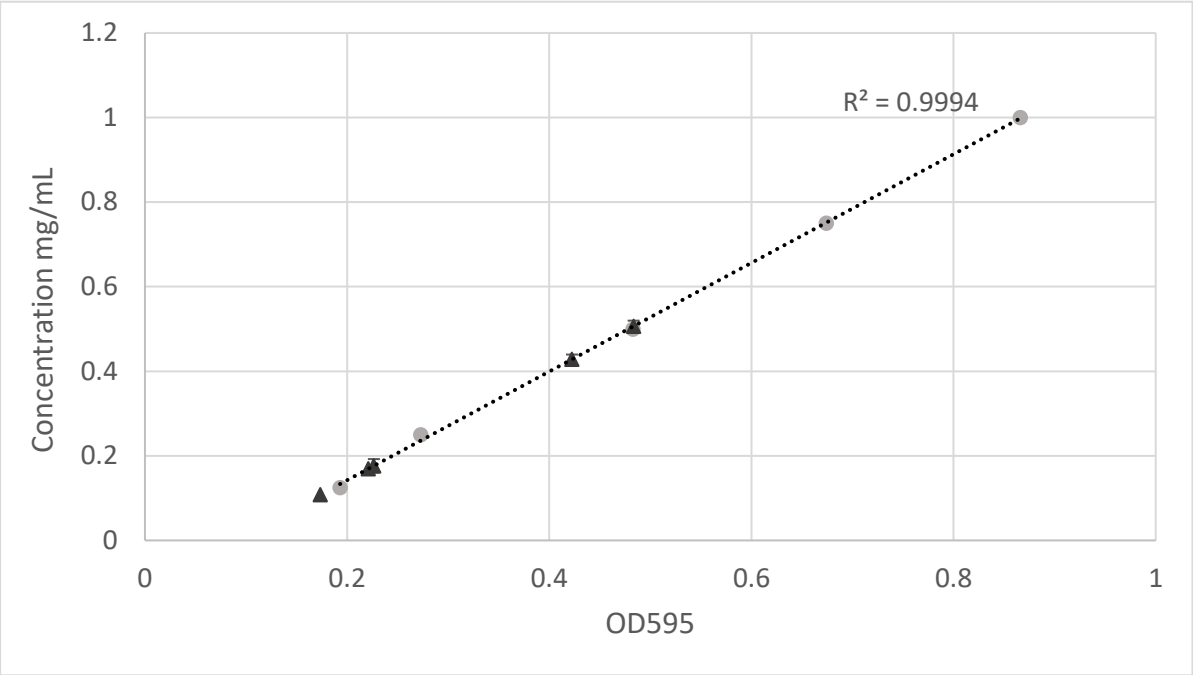


Figure 8. Standard curve of Bradfords assay. BSA standards were mixed with Bradford reagent and measured at OD_{595} to generate a standard curve (grey circles). Purified GapA was mixed with Bradford reagent and OD_{595} recorded. A 1:10 dilution was conducted on samples which with readings above the standard curves range. Concentration was worked out and the GapA samples plotted (black triangles). All samples were carried out in duplicate. The R^2 of the curve is displayed at 0.9994.

3.2.4 Expressing GapA as inclusion bodies

As previously discussed, inclusion bodies could offer advantages over soluble protein when used for oral vaccination, the work here will look to compare soluble protein and inclusion bodies to see if one offers more protection than the other when used as an oral vaccine. The next stage of work therefore looked to express the GapA protein as inclusion bodies so they could be later used in challenge trials. Inclusion bodies can be formed in many different ways; one such way is often due to the overexpression of the protein (Palmer and Wingfield, 2012). The method used here was to achieve overexpression of GapA by cloning into a high expression pET26b vector with a T7 promoter. Whereby, the increased strength of the T7 promoter compared to the tac promoter coupled with the increased plasmid copy number would allow for higher expression of the protein.

This was first trialled at a small scale by transforming the resulting vector pET26b_GapA vector (pCLW18) into BL21 (DE3) cells and growing the cells in a rich media (TB medium) for high cell densities, the experiment was carried out in both baffled and standard Erlenmeyer flasks.

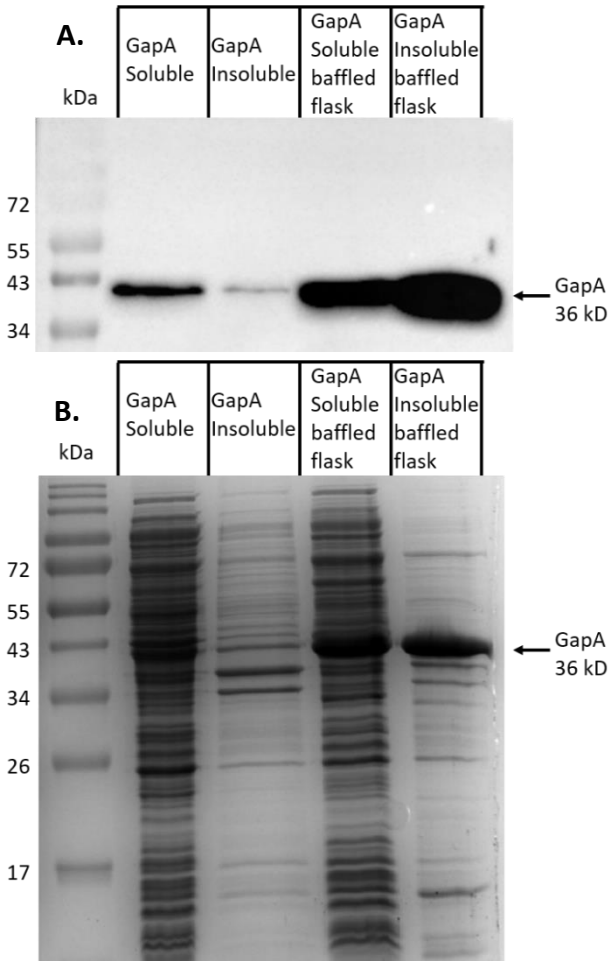


Figure 9. Small scale expression of GapA inclusion bodies in W3110 *E. coli* cells Cultures were grown in TB medium at 37°C in baffled and standard Erlenmeyer flasks and harvested 3 hours post induction. Protein was extracted and processed into soluble and insoluble protein. Following extraction proteins were separated by SDS PAGE and visualise by **A.** Anti-HIS western blot **B.** Coomassie staining.

Figure 9 shows the successful expression of GapA as inclusion bodies. Cultures in baffled flasks show a much higher level of protein than in the standard flask, this is expected as baffled flasks have increased levels of aeration resulting in higher protein production. The use of baffled flasks also has the added benefit of a higher cell density, being over twice as high as that of the standard flasks with an OD_{600} of 7.32 and 2.98 when harvested, respectively. Although the amount of GapA in the insoluble protein fraction (Fig. 9B) accounts for >50% the total protein in the sample (estimated using densitometry), the amount of contaminating insoluble proteins could be reduced by adding wash steps using buffer containing Triton X-100. Figure 10A shows that the addition of wash steps cleans up the final insoluble fraction, with the first wash (W1) removing the majority of contaminating bands, in addition to this none of the protein of interest appears to be lost with the added wash steps (Fig. 10B). A densitometry analysis estimates that GapA accounts for >60% of the total protein in the final inclusion body fraction after washing, this improvement on the previous experiment can be seen when comparing the two gels (Fig. 9B and Fig. 10A).

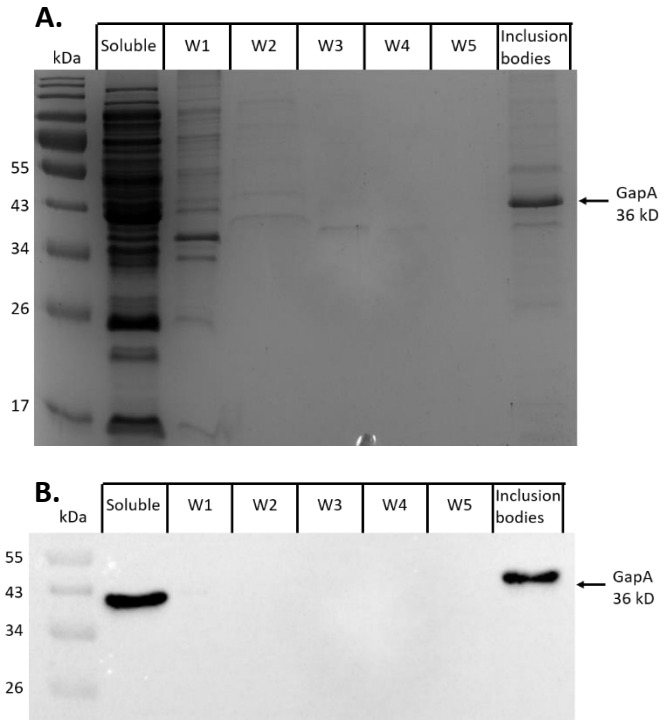


Figure 10. Small scale washes for GapA inclusion bodies Cultures were grown in TB medium at 37°C in baffled Erlenmeyer flasks and harvested 3 hours post induction. Wash steps 1-3 were performed using a wash buffer (50 mM Tris-acetate, 2.5 mM EDTA, 2% Triton X-100) pH 7.0, wash steps 4 & 5 used the wash buffer without Triton X-100. Supernatant from the washes were loaded onto a SDS gel along with the soluble and insoluble protein and separated by SDS PAGE and visualise by **A.** Coomassie staining. **B.** Anti-HIS western blot.

3.2.5 Large scale growth and processing of GapA Inclusion bodies

Following on from small scale expression of inclusion bodies it was necessary to generate enough protein at large scale so that it could be used for challenge trials. Large scale production of GapA inclusion bodies was carried out in 2.5 L shake flasks. Wash fractions on a larger scale were shown to contain more contaminating bands than at a smaller scale (Fig. 10A and Fig. 11). This is likely to be as a result of the higher concentration of proteins present which is clear when looking at figure 11 W1-W3 which are overloaded with protein. The larger scale washes (W1-W5 Fig. 11) show the importance of using the wash procedures to eliminate these contaminating bands. The inclusion bodies fraction in figure 11 show a band at the correct size for GapA which appears to be the most prevalent protein in this fraction.

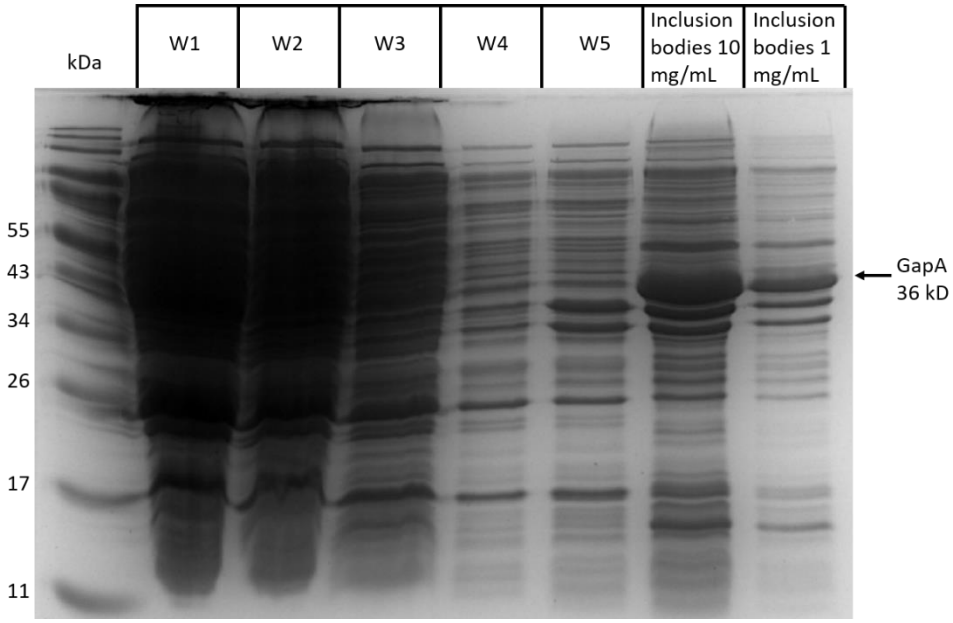


Figure 11. Large scale washes for GapA inclusion bodies Cultures were grown in 500 mL TB medium at 37°C in 2.5 L baffled Erlenmeyer flasks and harvested after overnight expression. Wash steps were carried out using the same buffer as the small-scale washes. Total insoluble protein was estimated by Bradfords assay prior to loading. Supernatant from the washes were loaded onto a SDS gel along with insoluble protein and separated by SDS PAGE and visualised by Coomassie staining.

The band at 36 kDa was previously determined to be GapA by immunoblotting with a His specific antibody. To further confirm that this band is GapA an empty pET26b vector was also expressed at large scale. Absence of a band in the empty pET26b vector lane (Fig. 12) suggests the protein band at 36 kDa is the GapA protein.

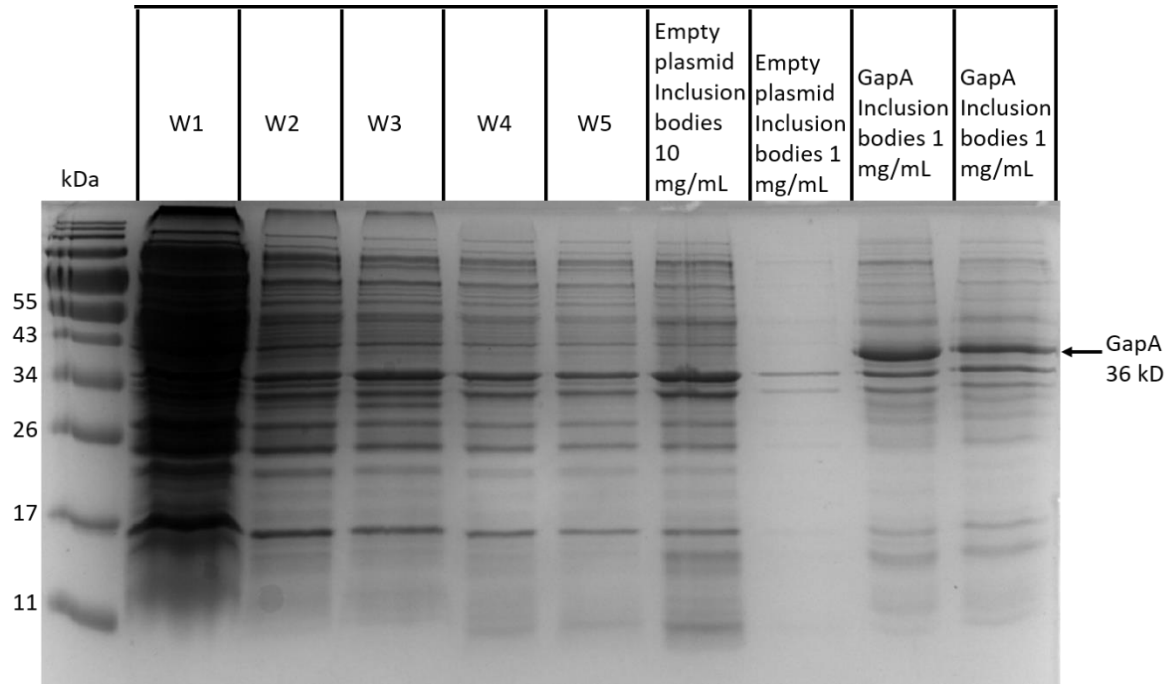


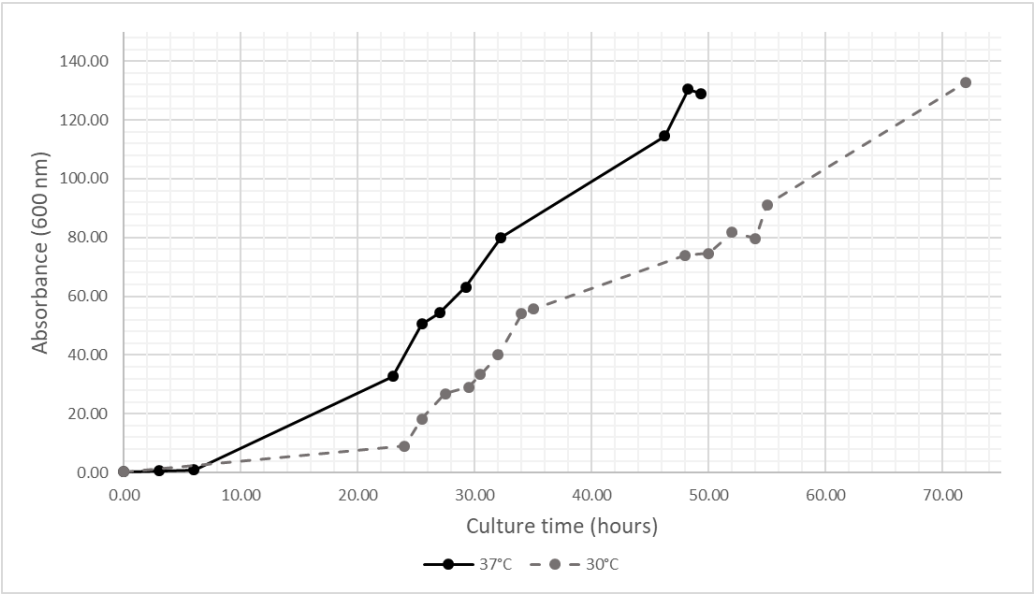
Figure 12. Expression of GapA inclusion bodies and empty plasmid used as a control. An empty pET26b vector was grown, expressed, and extracted using the same protocol to produce GapA inclusion bodies at large scale. The washes and final insoluble protein were loaded onto a SDS gel and separated by SDS PAGE and visualised by Coomassie staining.

3.2.6 Fed-batch fermentation of pET26b-GapA in BL21 (DE3)

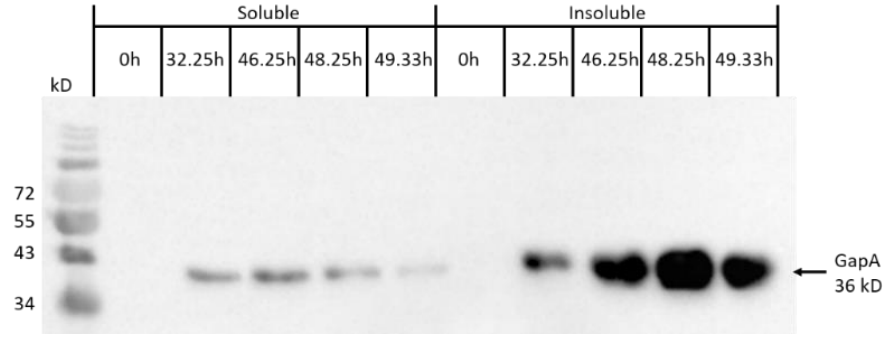
Large scale production of proteins in shake flask is often impractical, with lower cell densities and lower protein yield, and because of this it was decided to investigate the production of the protein using fermentation. Attempts had been previously made to grow the cells containing pET26b-GapA, however this resulted in soluble protein (Fig. 13C). The new construct was therefore run in an Ambr250 system to optimise conditions for protein production. The Ambr250 is a modular small scale fermentation system which allows for the optimisation of fermentation conditions which can then be replicated on a larger scale. To achieve expression of inclusion bodies the only change required was an increase in temperature to 37°C for both growth and protein expression compared to culturing at 30°C and expression at 25°C. Figure 13 shows a comparison between the previous attempt at fed-batch fermentation and the optimised conditions used in the Ambr250 system. Increasing the temperature led to a greater formation of inclusion bodies, with little soluble protein in culture grown at higher temperatures (Fig. 13B and 13C). Fig 13A shows a comparison of the growth of the cells at different temperatures, the previous fermentation run at 30°C showed a

similar trend to the optimised run at 37°C although the culture at 37°C unsurprisingly generated higher cell densities faster than at the lower temperature. The largest accumulation of inclusion bodies in the Ambr250 system was around 48.25 hours post inoculation (Fig. 13B), around 19 hours post induction.

A. Growth curve comparing growth of BL21 (DE3) expressing GapA at 37°C and 30°C



B. Expression of GapA at 37°C optimised fermentation experiment



C. Expression of GapA at 25°C, cells grown at 30°C prior to induction

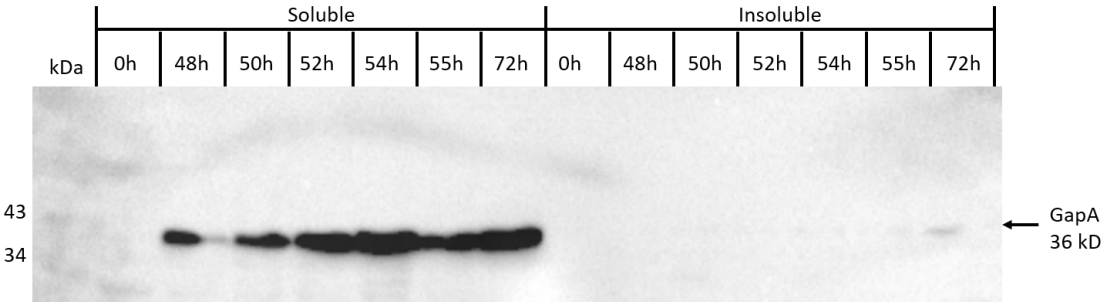


Figure 13. Comparative fed-batch fermentation of BL21 (DE3) *E. coli* cell expressing pCLW18 in SM6 at different temperatures. Cultures were grown under the same conditions as chapter 3.2.2, with the exception of temperature which started and remained at 37°C during the optimised experiment. Optimised fermentation experiment was induced 29.25 hours post inoculation, the The initial fermentation experiment at 30°C/25°C was induced 35 hours post inoculation. **A.** Growth curve of BL21 (DE3) *E. coli* cells expressing GapA during fed-bath fermentation at 30°C and 37°C **B.** Anti-HIS Western Blot for samples taken during optimised run at 37°C **C.** Anti-HIS Western Blot for samples taken during initial run with cells cultured at 30°C and expressed at 25°C. Samples processed into soluble and insoluble protein were standardised to 10 OD units at OD_{600} with 15 µL of sample loaded.

3.2.7 Challenge trials with GapA

To test if the proteins produced would protect from infections of *S. agalactiae* a challenge trial was conducted. The fish challenge trial was performed under the supervision of Dr Chong Chou Min at the Universiti Putra Malaysia, GapA protein previously expressed as both a soluble and insoluble protein was used in the challenge trials. Three groups were made up of 15 juvenile red hybrid tilapia fish (*Oreochromis spp.*) of 80-90g, a control group with no treatment, a group fed soluble GapA, and a group fed GapA inclusion bodies, each group was repeated in triplicate. Fish were first acclimatised for two weeks with constant feeding on a basal diet at 2% of their body weight to ensure stable behaviour and no signs of abnormality. The two vaccinated groups were then fed basal diets coated with purified GapA or GapA inclusion bodies for five consecutive days on week's 0 and 2. The quantity of the feed was 1% body weight, with the vaccine dosage of 20 µg/ g of fish weight. The control group was fed the basal diet without any vaccine. At week four fish were challenged with *Streptococcus agalactiae,* the fish were immersed in 1×10^8 cfu per mL of bacteria for 3 hours. After being challenged, fish were transferred to clean aquariums and monitored for 7 days. The cumulative mortalities and signs of the infection were observed daily.

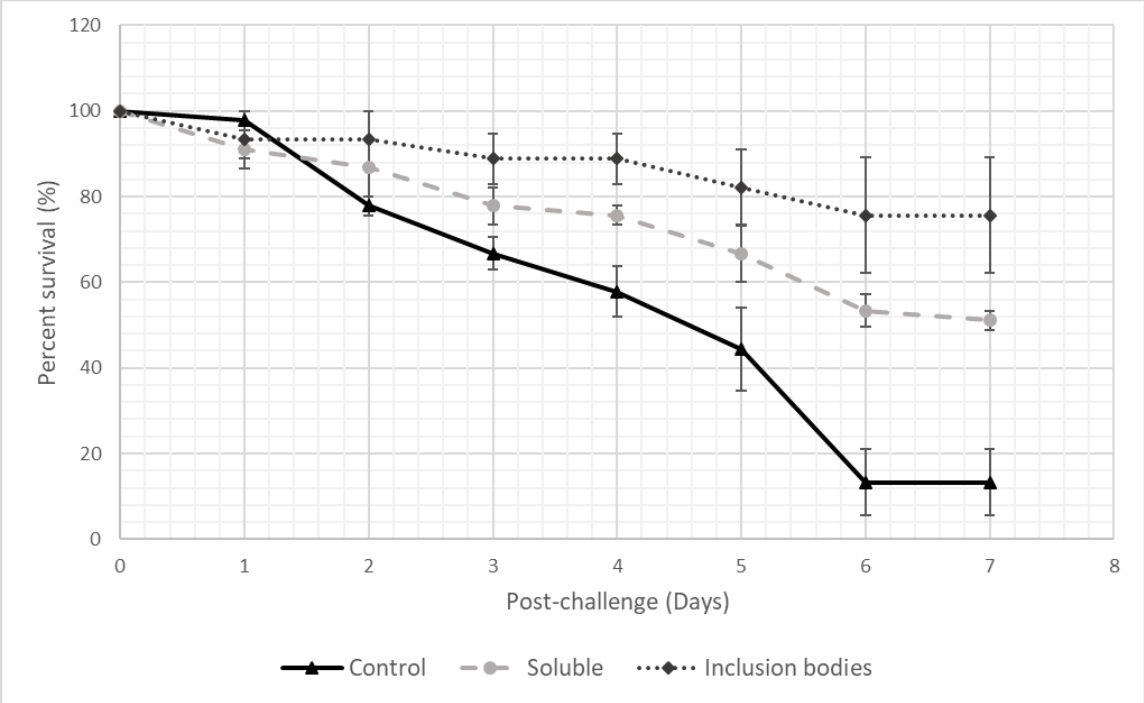


Figure 14. Percentage of survival of groups challenged with *Streptococcus agalactiae*. Each group was made up of 15 fish in triplicate. The control group had no treatment and were fed as normal. In addition to normal feed the soluble and inclusion body groups were fed for 5 days on week 0 and week 2 with 20 µg/g of fish weight added to feed for immunisation. The fish in all groups were challenged on week 4 with *S. agalactiae* by immersion in 1×10^8 cfu/mL of bacteria for 3 hours. Fish were transferred to clean aquariums and monitored for 7 days.

Figure 14 shows that both the soluble protein and the inclusion bodies, when fed orally, have a protective effect on Tilapia 7 days' post infection. Soluble protein conferred a 51.1% (± 2.2) survival amongst challenged fish, with the inclusion bodies outperforming this at 75.6% (± 13.5), finally as expected the control group had the worst outcome with only 13.3% (± 7.7) of fish surviving 7 days after being challenged with *S. agalactiae.*

3.3 Conclusions

This study set out to investigate and compare the effectiveness of soluble and insoluble GapA produced in *E. coli* when used as an oral vaccine.

Expression in shake flask showed that W3110 coupled with the pET23 vector when grown at 37°C resulted in a large accumulation of soluble protein, as a result there was no need to do any further optimisation of conditions for production of soluble GapA. The success of expression at shake flask

permitted fed-batch fermentation of the protein. The fermentation of GapA yielded high cell densities and a respectable amount of protein that could then be purified by IMAC to yield large concentrations of protein which could then be delivered for challenge trials. Soluble GapA was also quantified and the gave an approximate yield of 0.53 g/L, which given a vaccine dose of 20 µg/g of fish weight was used in challenge trials, would equate to 297-334 vaccine doses per litre of culture.

After successful production of the soluble protein the next step involved generation of insoluble GapA, this was first achieved by using a the T7 driven pET26b vector in baffled shake flasks. After washing the insoluble fractions with a wash buffer containing Triton X-100 to reduce contaminating bands moderate amounts of insoluble protein were able to be produced that could later be used in the challenge trials so that soluble protein could be compared alongside the insoluble GapA. It was later discovered that fed-batch fermentation could also yield insoluble GapA when adjusting the temperature settings for growth and expression from 30°C/25°C to 37°C using BL21 (DE3) cells transformed with the pET26b vector, making this process more practical for use at a large scale. The growth at 37°C also had the benefit of being able to accumulate a higher cell density more quickly than the culture grown at a lower temperature.

The challenge trial provided valuable insight into whether oral immunisation would be an effective way to increase fish survival rates against infection. The results from the challenge trial show that feeding the GapA antigen to red hybrid tilapia fish allows for a relative survival 7 days' post infection of ~51.1% when using the soluble antigen and ~75.6% when using inclusion bodies. As previously mentioned, inclusion bodies are more stable than soluble protein and less susceptible to degradation by proteases, this may explain their better performance when compared the soluble protein, as they may have been able to resist degradation in the harsh conditions of the gut. These results are very promising especially when looking at the insoluble protein which would require less processing and therefore create a low-cost convenient method of immunising fish against *S. agalactiae* infections. An extended challenge trial could be useful to determine if fish survival rates persist after 7 days and could further serve to optimise the amount of antigen required. An

extended challenge trial would also be useful to determine if the protection of the protein could be upheld and further improved by increased dosing.

It was demonstrated in this chapter that GapA could be purified as a soluble protein. Figures 6 and 7 showed contaminating bands in several of the purified elutions. Contaminating bands were also present in insoluble fractions in figures 9, 10, 11 and 12. Although here the aim was to provide a proof of concept, the study should also consider biosafety and regulations surrounding the potential commercialisation of this technology especially given the success of the vaccine in the challenge trial. Although veterinary vaccines tend to be less pure than vaccines produced for human use, it is still important to analyse vaccine products for any traces of compounds which could cause adverse effects or impact the safety of the product (Gifford *et al.*, 2011). Firstly, contaminating proteins in purified protein fractions would need to be evaluated, this could be determined using mass spectrometry to establish exactly what proteins are present and long-term effects of these on the host should also be investigated. This is especially important when considering the insoluble GapA where additional insoluble *E. coli* proteins were present, and it was theorised that these could be acting as potential adjuvants. For example, in the case of soluble protein extra chromatography steps could be carried out to further purify the protein fractions, eliminating contaminating products. The exact amount of the target protein (GapA) should also be measured, and further animal trials should be undertaken with this knowledge to help determine the optimal dosage.

Given that one of the primary purposes for using vaccines over antibiotics was to reduce the risk of antimicrobial resistant occurring the use of antibiotics as a selectable marker would need to be considered. Testing for residual DNA encoding for antimicrobial resistance could be carried out to verify if these cassettes still reside in the final product, alternatively genes could be stably integrated onto the genome, removing the need for the selectable marker (Seco and Fernández, 2022). Much of these experiments would need to be carried out prior to licensing the vaccine. However, there would also be a need for regular testing to be carried out on the product to ensure that the vaccine is consistent, with no batch-to-batch variation, and the is safe for use.

Overall, a commercially relevant process has been demonstrated for the production of GapA in both soluble and insoluble form, which could be easily taken on for large scale production of the protein, if found to be cost effective. Although protein yields demonstrated here are respectable, the process may benefit from further optimisation of conditions. The proteins have also been shown to be effective at increasing survival rates of fish when fed orally, allowing for a convenient method of administration. The methods used here could reduce the impact of infections by *S. agalactiae* with the benefit of keeping costs down for farmers and the need for antibiotics.

Chapter 4: Expression of GapA in *C. reinhardtii*

4.1 Introduction

The previous study gave encouraging insight into how effective GapA is at increasing the survival rates of Tilapia against *S. agalactiae* infections when used as an oral vaccine. Although the results from this study were positive there still remains alternative methods for the delivery of the protein to the animal. One such method would be to employ the use of microalgae to produce the protein which could then be dried and serve to encapsulate the protein for oral delivery.

Microalgae make up a highly diverse group of prokaryotic and eukaryotic organisms and offer several advantages over other platforms when looking at oral vaccination in aquaculture. For instance, microalgae naturally make up a large part of the diet of many small aquatic animals and several species are already used in the aquaculture industry as a feed additive as a result of the health benefits provided, providing the right balance of lipids, proteins and other micronutrients (Roy and Pal, 2015). They also contain various compounds which have been reported to function as immunostimulants, improving the animal's resistance to pathogens by supporting the innate immune system (Cerezuela *et al.*, 2012). Additionally, some species of microalgae have been shown to produce anti-microbial compounds that may work against particular bacterial pathogens within aquaculture (Austin, Baudet and Stobie, 1992).

The cultivation of microalgae is already well established and can be achieved at a relatively low cost only requiring simple nutrients, an abundant light source and CO_2. Several species are already classified as GRAS (Generally Recognised as Safe) for human consumption by the US FDA, meaning there is no detected endotoxins or infection agents, as such there is no requirement for purification (Triton Algae Innovations, 2018). The GRAS status of these species allows for the simplification of downstream processing only requiring centrifugation or flocculation of the product which can later

be dried for use in feed, which greatly reduces costs. Genetically modified stains can also be grown at a large scale under sterile conditions in closed photobioreactors which reduces the risk of release of transgenes and environmental contamination, although growing in these conditions increases production costs.

Despite the high prospect of microalgae's use as a protein production platform especially with relevance as a functionalised feed additive in the aquaculture sector there are only a handful of species that are routinely transformed (Gong *et al.*, 2011). The freshwater alga *Chlamydomonas reinhardtii* has all the aforementioned advantages and is the best studied chlorophyte being used as a model system for molecular studies and coming with perhaps the largest molecular toolkit available (Wannathong *et al.*, 2016). The chloroplast is the preferred site of protein expression, with exogenous DNA being precisely integrated into the chloroplast by homologous recombination. Protein production in the chloroplast is usually higher than that of the nuclear genome (Potvin and Zhang, 2010) and in addition to this, proteins expressed in the chloroplast are retained within the organelle (Tran *et al.*, 2013) allowing for compartmentalising and another added layer of membrane protection potentially protecting the products from being degraded by the harsh conditions of the gut. New strains can also be generated through selection of restoration of essential photosynthetic genes such as *psbA* or *psbH* (Bertalan *et al.*, 2015; Wannathong *et al.*, 2016), creating marker-less mutants which reduce the risk of horizontal gene transfer of antibiotic resistant genes. The strain used here is TN72/CC-5168, a cell wall deficient mutant that has a deletion in the middle of the *psbH* region and is replaced with the *aadA* gene which codes for spectinomycin resistance, insertion of the GOI and a functional *psbH* is carried out and selection carried out on minimal medium, without acetate, and removes the *aadA* gene when strains become homoplasmic (Economou *et al.*, 2014). Indeed, the potential of the chloroplast of *C. reinhardtii* as a host or production of subunit vaccines and other therapeutics has been investigated with one study expressing the E2 gene from classical swine fever virus and being able to produce antibodies in mice by injection but no immune response was detected when administered orally (He *et al.*, 2007). Another group was able to

successfully express endolysins targeting *S. pneumoniae* in the chloroplast of *C. reinhardtii* which showed activity against the bacteria when used as a crude lysate (Stoffels *et al.*, 2017).

This study outlines the generation of a homoplasmic GapA expressing strain of *C. reinhardtii*. The amount of protein produced was estimated, and the strain was taken through steps of optimisation for light intensities and harvesting time and applied to a larger scale production for the generation of dried biomass which could be used in further experiments such as challenge trials. Finally, the shelf life of the biomass was tested to determine if the protein was still detectable after an extended period of time at different storage temperatures.

4.2 Results

4.2.1 GapA transformation and expression in *C. reinhardtii*

The GapA gene was first codon optimised for expression in the *C. reinhardtii* chloroplast, after which the gene was then transformed into the chloroplast by the glass bead method and selected for by restoration of photosynthesis. Due to the fact *C. reinhardtii* is polyploidy with ~50-80 copies of its plastome it is necessary to check for homoplasmic insertion of the gene of interest (GOI) (Gallaher *et al.*, 2018). Positive transformants were screened by PCR using primers specific to the GOI. These strains were then tested for homoplasmy by PCR using primers targeting the gene being replaced, in this case the *aadA* cassette. Figure 15 shows an agarose gel of these PCRs and the successful integration of the GOI into the chloroplast is seen due to a band displayed at approximately 1040 bp. This strain was also confirmed to be homoplasmic due to the lack of band at 850 bp in the last lane which would correspond to the *aadA* cassette, this 850 bp band can be seen in the TN72 background strain. PCRs were also carried out with DNA from TN72 using the primers specific for the GOI to check for nonspecific amplification, no amplification can be seen in TN72 suggesting the band at 1040 bp is the GOI. The band at 1040 bp was also excised and sequenced to further confirm that it is the GapA sequence.

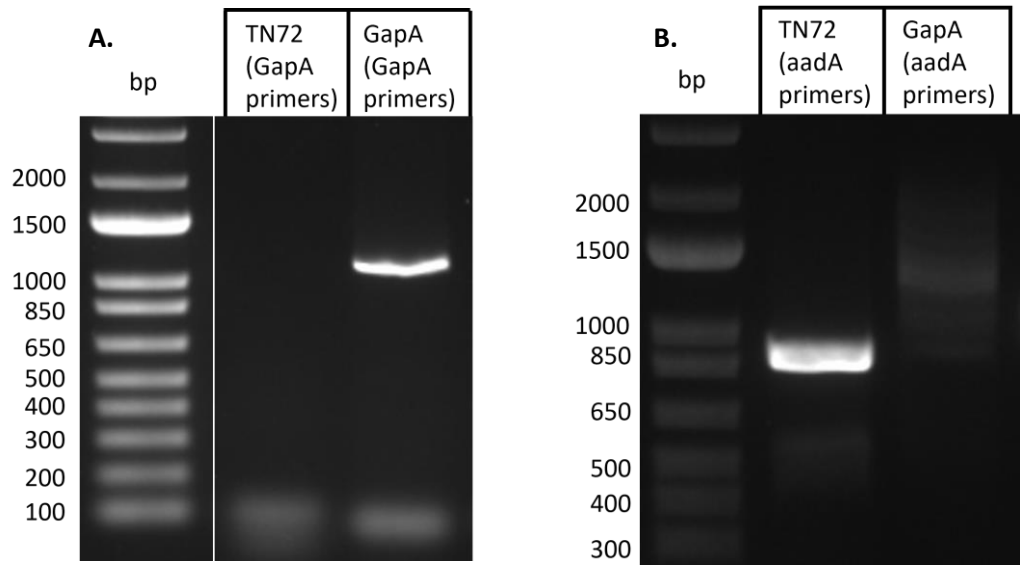


Figure 15. Agarose gels of PCRs confirming homoplasmic integration of the GapA gene into the chloroplast. Flank1 primer anneals downstream of the gene of interest insertion site when combined with the rbcL.F primer which anneals to the end of the *aadA* cassette it produces an 850 bp product in strains carrying the *aadA* cassette. GapA_F anneals to the start of the GapA gene and the HA_R primer anneals to the HA tag on the C –terminus of the gene, producing 1040 bp product in strains carrying the GapA gene. The agarose gel shows integration of the GapA gene in the new strain. A lack of band at 850 bp in the GapA strain when using the primers for the *aadA* cassette show the strain is homoplasmic. The parental strain TN72 was also run as a negative control. **A.** PCR using GapA primers on the GapA expressing clone and TN72 parental strain. **B.** PCR using aadA primers on the GapA expressing clone and TN72 parental strain.

After generation of a homoplasmic strain, the strain was then grown and the protein expressed in shake flask to check for expression of the protein. A 100 mL culture of the GapA strain, parental TN72 strain and a photosynthesis restored strain (CCK10) were set up by inoculating from plate. These strains were grown in mixotrophic conditions in continuous light at 20 µmol photons m^{-2} s^{-1}. The 16S-psaA fusion promoter used is a constitutive promoter and required no induction. After cultures reached late log phase 10 OD_{750} units were harvested from each culture and the cells fractionated into soluble and insoluble protein and separated and visualised by SDS PAGE and immunoblot. Figure 16 shows a band at approximately 36 kDa corresponding to the correct size of GapA. It can also be seen that the protein is both in a soluble and insoluble form, although given the results of the previous study where insoluble protein appeared to confer protection to infection, this does not seem to be an issue that needed addressing by further optimisation. It can

also be seen that no bands are present in the two control strains loaded, confirming that the band seen on the western blot is likely the GapA protein.

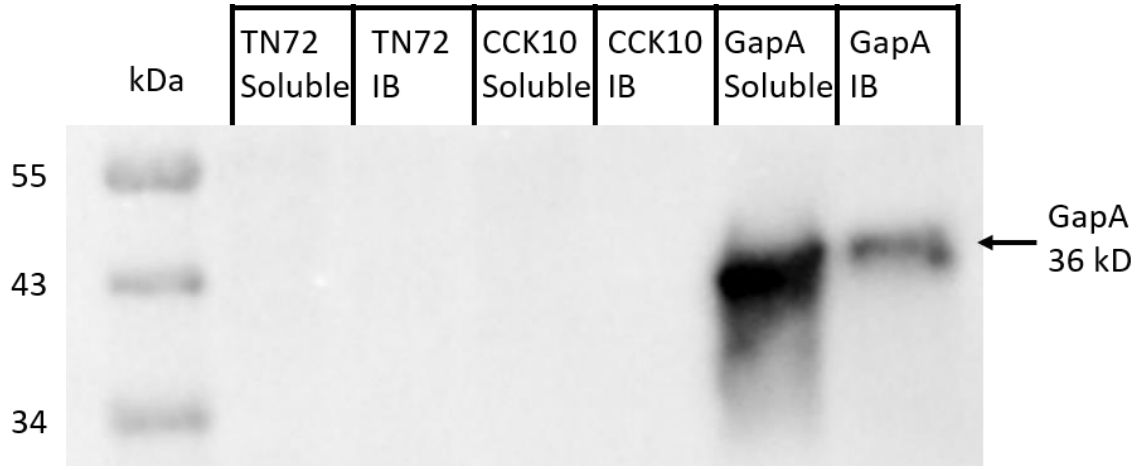


Figure 16. Shake flask expression of the GapA strain showing successful expression of the GapA protein. Cultures were grown at 25°C for 5 days, 10 OD_{750} cells were harvested by centrifugation, lysed, and processed into soluble and insoluble fractions. Protein was separated by SDS PAGE and immunoblotted using anti-HA antibody and visualised using the Bio-rad gel doc. The blot shows successful expression of the GapA protein at the expected size of 36 kD with the parental strain also loaded as a negative control. IB: Inclusion bodies. TN72: Parental control strain. CCK10: Photosynthetic restored control strain, with no GOI inserted.

4.2.2 Purification and quantification of GapA

The protein was purified to then gain an estimation of the amount of GapA produced. For any future challenge trials or use of the GapA producing microalgae as an oral vaccine it would be useful to know the amount of protein produced. Microalgae are known to produce low yields of protein when comparing to other protein production platforms (Sproles *et al.*, 2021). As a result estimating protein concentration would be crucial to knowing the amount of protein that could be delivered to the animals. Estimation of GapA concentration was carried out by purifying the protein using a HA purification kit and quantifying the amount of the target protein using the NanoDrop 2000 spectrophotometer. This protein could then be visualised by immunoblot and serve as a reference band that would allow for other bands loaded onto the blot to be quantified using densitometry software.

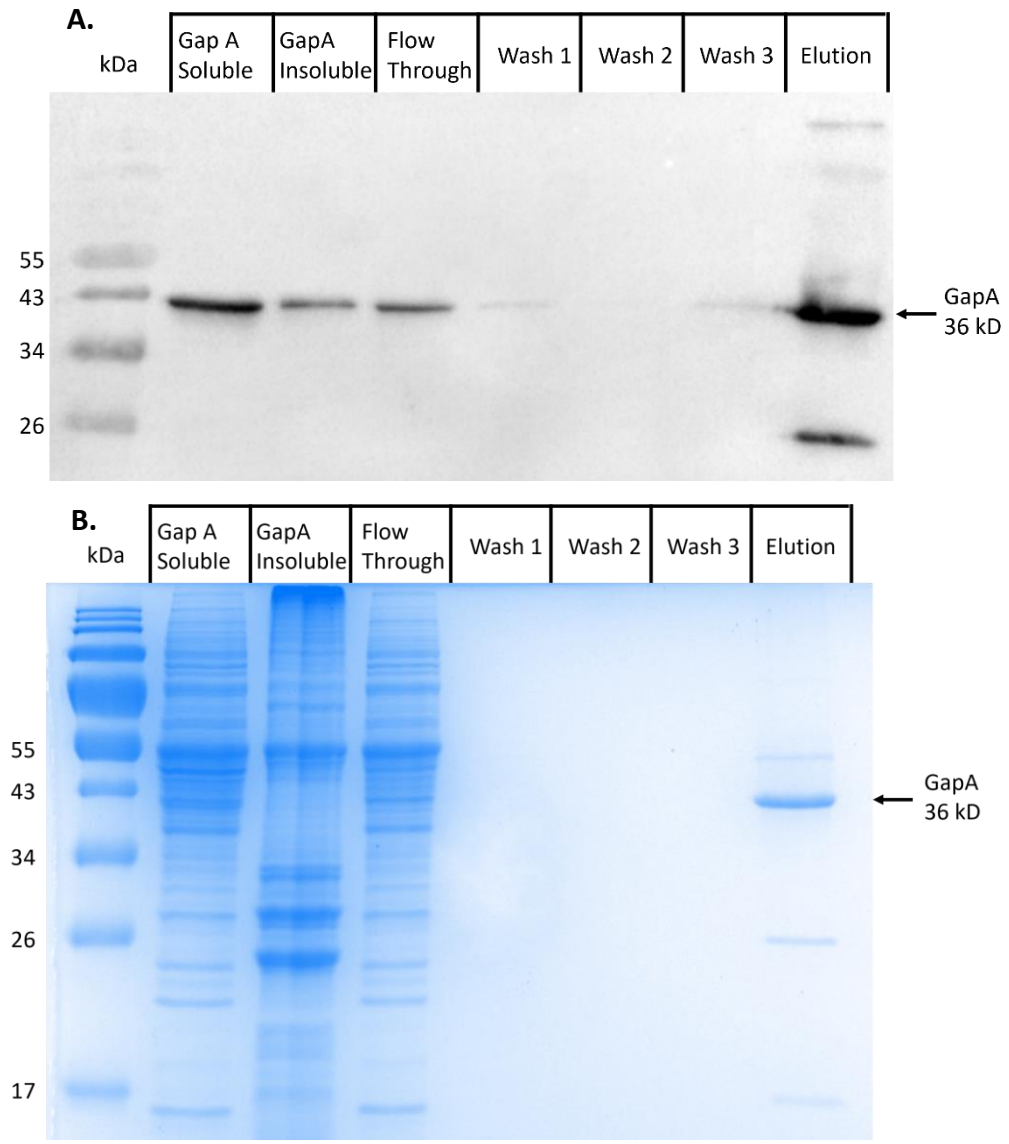


Figure 17. Purification and quanitfication of GapA from GapA microalgae strain. Soluble and insoluble protein extracts from the equivalent of 10 OD_{750} culture, along with purification samples from equivalent of 10 OD_{750} culture were loaded and visualised by **A.** Western blot using anti-HA antibody and **B.** Coomassie stain.

The purification of GapA gave a band at approximately the correct size of the protein (Fig. 17B), this is further confirmed by the HA immunoblot (Fig. 17A). The purification contains few contaminating bands with the 36 kDa band accounting for >80% of the protein present. There does seem to be a lower band on the Coomassie stained gel which may be as a result of the protein N-terminal degradation as the band is also present in the immunoblot (Fig 17A) it would be necessary to perform further analysis such as mass spectrometry to confirm this. However, this band has not been seen in other gels or blots. The purified elution fraction was subjected to quanitifcation using the Nanodrop 2000 giving an estimation of 263.61 µg/mL. The purified band was then compared to

the soluble and insoluble protein bands in the immunoblot (Fig. 17A) using densitometry. Once the protein concentrations of the soluble and insoluble bands were determined the volume of the culture loaded was used to work out the total protein concentration, which was estimated to be 13.84 mg/L of soluble protein and 4.33 mg/L of insoluble protein.

4.2.3 GapA light optimisation

Although the amount of protein had been determined it was decided to see if the yield of protein could be optimised prior to large scale growth of the culture. Cultures were grown in TAP medium with three different light intensities used, low at 20 µmol photons m^{-2} s^{-1}, moderate (mid) at 50 µmol photons m^{-2} s^{-1} and high light at 145 µmol photons m^{-2} s^{-1}. Although cultures tend to grow better and gain a higher biomass using high light levels, higher levels of expression may be achieved using lower light levels (Stoffels *et al.*, 2017). Figure 18 demonstrates how light intensity can influence both growth and protein production. The growth curve shows that that, unsurprisingly, higher light levels increase the total biomass of cultures (Fig. 18A). However, the lower light levels do appear to have a higher accumulation of the protein of interest (POI) (Fig. 18B). This difference is most clearly seen in the soluble protein on day 5, where the band intensity in the low light culture is far higher than that of the moderate and high light samples. It can also be seen that high light conditions seem to give a higher level of insoluble protein compared to soluble protein (Fig. 18B & Fig. 18C). The low light level conditions gave a higher amount of soluble protein when comparing

to the higher light conditions (Fig. 18B) and allowed for an earlier harvesting time at only 5 days' post inoculation.

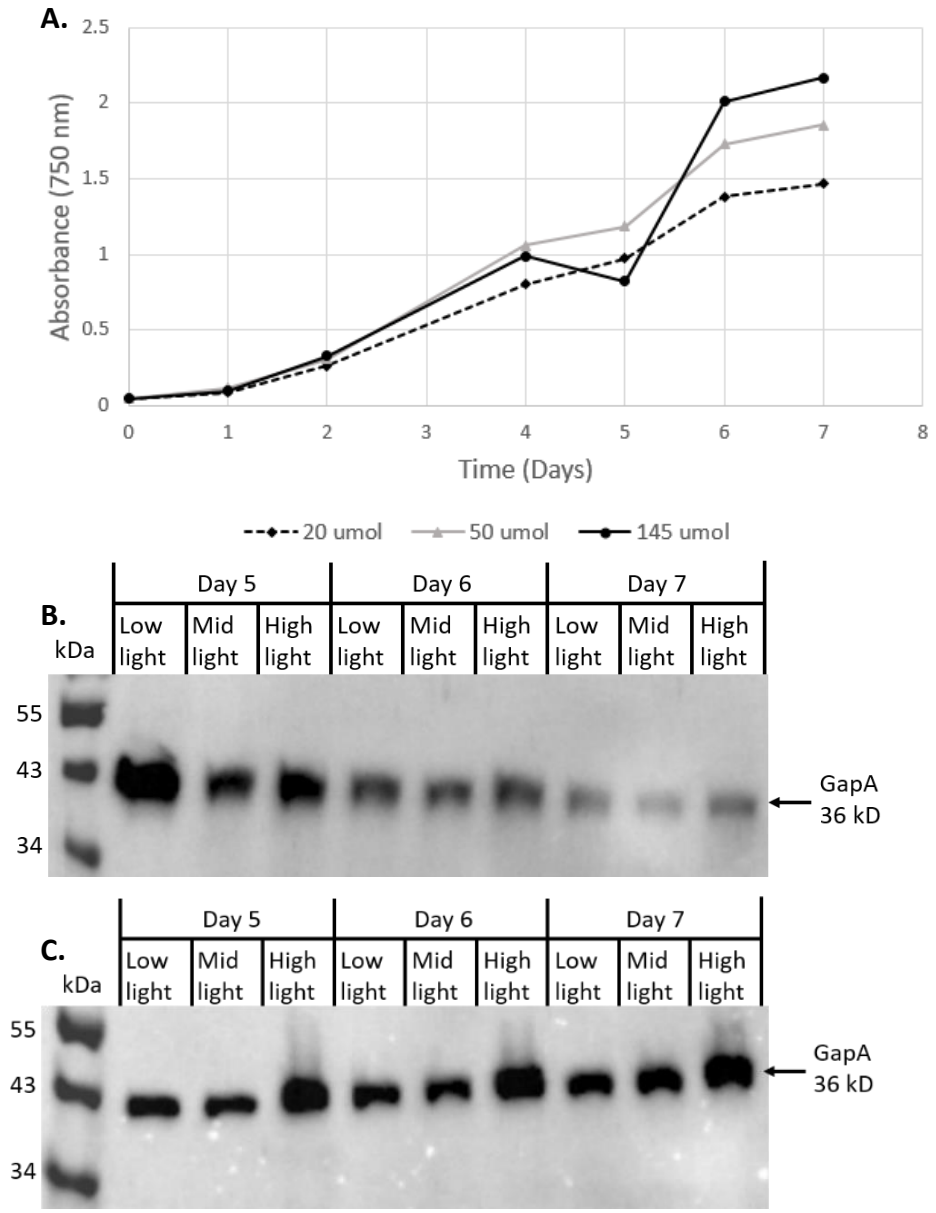


Figure 18. Optimisation of light and harvest time for GapA. Samples were harvested each day and protein samples extracted in soluble and insoluble fractions for separation by and visualisation by Western blot using anti-HA antibody. The equivalent of 10 OD_{750} culture were loaded. Growth of cells was also measured by optical density at OD_{750} **A.** Growth curve of cultures **B.** Soluble protein extracts from cells **C.** Insoluble extracts from cells.

4.2.4 Growth and expression of GapA in hanging bags

To gain enough biomass for any later studies and to determine the growth characteristics of the strain at a larger scale the next experiment was to culture the strain in 15L hanging bags. Hangings bags are a cheap effective way of growing microalgae when compared to expensive

photobioreactors (PBRs), they provide an easy modular system which can be quickly set-up with a small footprint. Hanging bags consist of sterile single use polythene tubing sealed at both ends, that are then filled with culture and mixed via bubbling of compressed air which can be supplemented with CO_2. The experiment compared the growth of the new strain to a photosynthesis restored strain (CCK10) and the expression of GapA was also checked via immunoblot. When looking at the growth curve (Fig. 19A) the growth of the new strain seems comparable to that of the *psbH* restored strain, this can be further seen when comparing the final biomass of the two cultures with the total biomass of the GapA strain being approximately 29.72 g and the CCK10 strain 32.58 g. After 4 days of growth the bags were harvested by flocculating with chitosan and centrifugation to concentrate the cells, the resulting pellets were then lyophilised before being stored. The protein from lyophilised samples was extracted and visualised by immunoblot (Fig. 19B) to confirm the expression of GapA at a larger scale, the immunoblot also shows a faint band in the CCK10 sample from the hanging bags at the same size of GapA. This band from the negative control was not seen previously (Fig. 16) and could be from some cross contamination of the cultures when being grown or processed, the band present in the sample from the GapA strain is also far more prevalent than that of the band in the CCK10 sample giving increased confidence that it is the protein of interest.

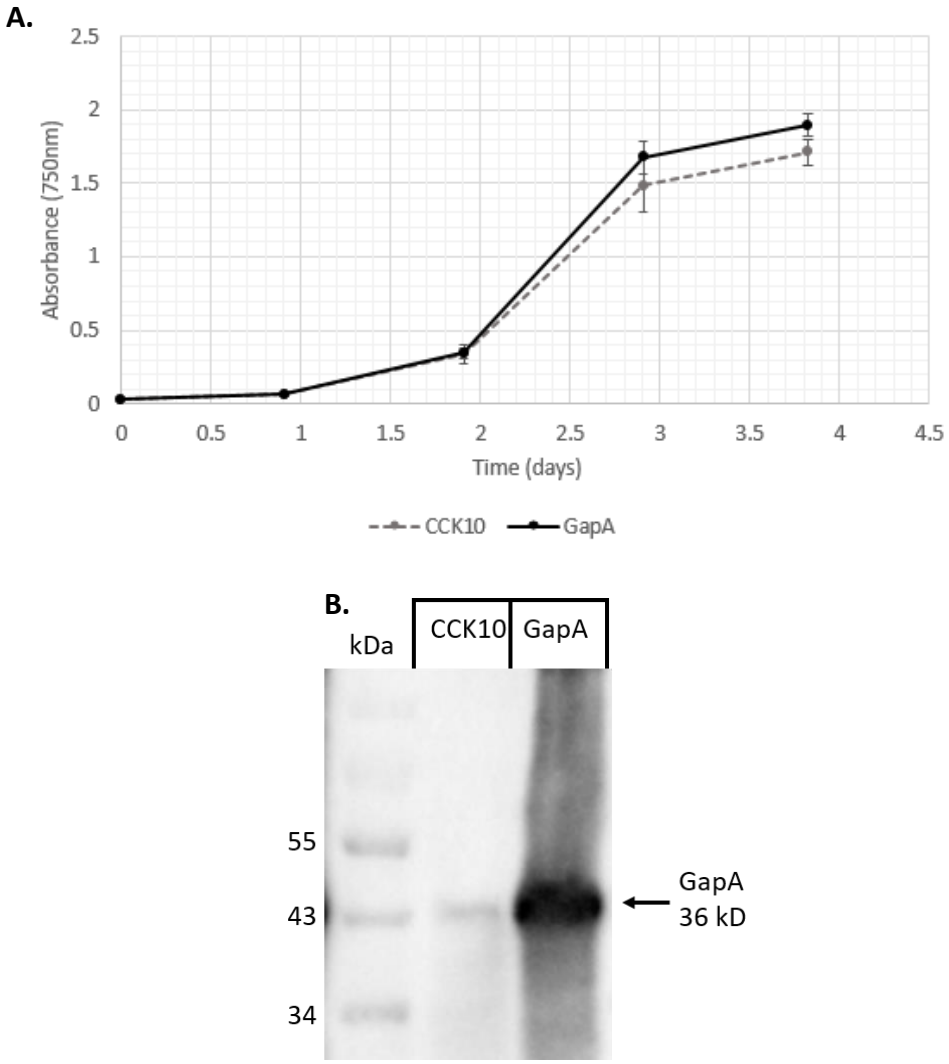


Figure 19. Growth and expression data for GapA strain and CCK10 in hanging bag system. 10 mg of both the freeze dried GapA and CCK10 strain were resuspended in 1 mL MQ H_2O, and the proteins extracted for visualisation by immunoblot using anti-HA antibody. Growth of cells was also measured by optical density at OD_{750} throughout the culture time **A.** Growth curve of cultures **B.** Western blot using anti-HA antibody of whole cell protein extracts.

4.2.5 GapA freeze dried stability trial

Cold chain transport and storage is a major contributor to the expense of conventional vaccines. The use of dried microalgae provides an attractive solution to the requirement of cold chain supply by preserving the antigen in the dried host cells preventing its degradation. One study has found that antigens can be stored at room temperature for up to 6 months (Specht and Mayfield, 2014). To determine the shelf life of the GapA protein produced here within the freeze-dried cells a stability experiment was carried out. Samples standardised to 10 OD_{750} units were harvested from the same culture and lyophilized and stored at either room temperature (RT), 4°C or -20°C. Samples

were then lysed and the proteins extracted and prepared on the stated days before being visualised by immunoblot later. The protein is still detectable in samples up to 54 days being stored at all temperatures (Fig. 20). Control samples were loaded, with a sample before being lyophilised (GapA pre-FD) and immediately after being lyophilised so that they could be compared to samples being extracted at later dates. Unsurprisingly samples extracted earlier on (day's 7 and 14) show a higher intensity than those samples left for longer (day's 28 and 54). The band intensity does also appear to show that the temperature the protein is stored at seems to have little effect. The samples do show some variability which could be caused by the difficulty in resuspending and extracting the dried biomass.

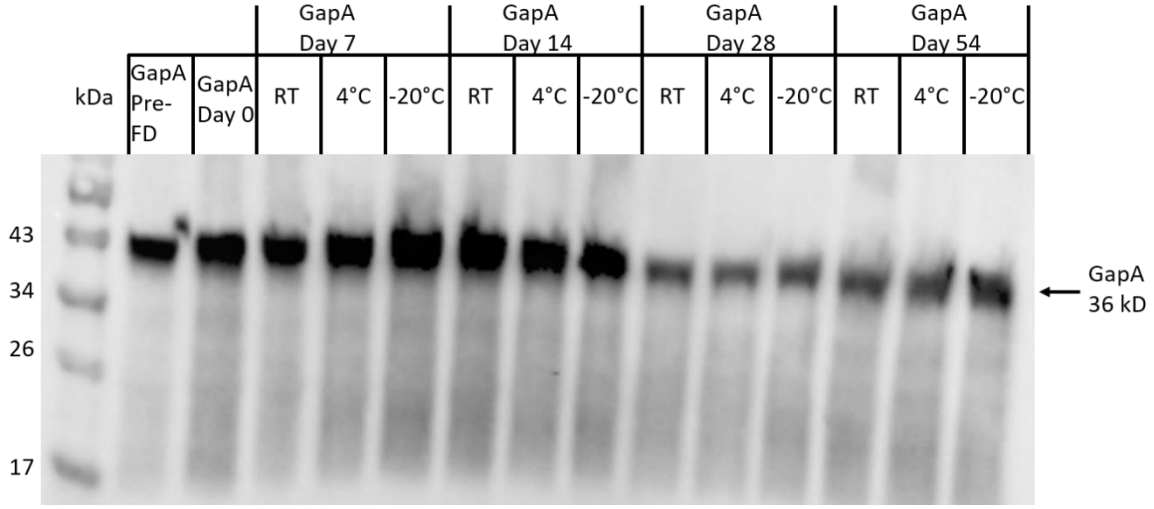


Figure 20. Stability of GapA protein in lyophilised *C. reinhardtii* cells in different storage conditions over time. Samples standardised to 10 OD_{750} unit of the culture were harvested from the same 400 mL culture after 4 days of growth. The samples were centrifuged and whole cells lyophilised and stored at either room temperature (RT), 4°C or -20°C. Samples were taken out at different time points, the cells were lysed, and the proteins extracted for separation and visualisation by Western blot using anti-HA antibody. A sample was taken and extracted prior to freeze drying (Pre-FD) and a sample was prepared at day 0 after the cells has been freeze dried.

4.3 Conclusions

This study has shown integration of the GapA gene into the chloroplast of *C. reinhardtii*, generating a marker-less strain, a strain in which no antibiotic resistance was used for selection of clones. The marker-less strain reduces the risk of horizontal transfer of undesirable antibiotic resistance genes often used as selection markers. The concentration of protein produced was estimated at around 18 mg/L which was a relatively high level of protein and high enough to be used for oral vaccination

of animals. For future use it would be more appropriate to determine the protein concentration as a measurement of dry weight given that the product is intended to be used as a dried product.

The strain was also demonstrated to be able to grow and express the protein at a larger scale with seemingly no impact on the growth of the strain. In addition to this the protein was still detectable in lyophilised biomass after being stored at room temperature for up to 54 days, although there does appear to be some loss of the band intensity of the protein after 14 days of storage. More experiments should be carried out to determine the exact concentration of protein remaining after being lyophilised and stored at room temperature.

The effectiveness of the dried microalgae containing the GapA protein at protecting fish from *S. agalactiae* would need to be investigated through the use of challenge trials in the same way in which was carried out in the previous chapter. Challenge trials that are to be carried out will also need to use the photosynthetic restored strain as a control as it has been reported that some microalgae already contain compounds that could protect against bacterial pathogens (Falaise *et al.*, 2016).

The study shown here presents an alternative method for the production of the GapA protein. The protein remains encapsulated in the cells and could be utilised as an oral feed with very minor downstream processing. In addition to having the protein of interest, which has already demonstrated a protective effect from *S. agalactiae* infections, microalgae provide a range of different valuable nutrients which could help support the animals overall health (Molino *et al.*, 2018).

Chapter 5: dsRNA expression in *C. reinhardtii*

5.1 Introduction

The last decades have seen a substantial growth in the shrimp farming industry with a nearly 10-fold growth of production from 0.673 million tons in 1990 to 6.004 million tons in 2019 (Lee *et al.*, 2022), however this growth has been plagued with several viral diseases which lead to large economic losses. There are currently seven viral pathogens which affect penaeid shrimp listed by the World Organisation for Animal Health, which include yellow head virus (YHV) and white spot syndrome virus (WSSV) (Walker and Mohan, 2009). YHV has been a major concern in Thailand since it caused devastation in 1990 in cultured *P. monodon* and has also been reported in other shrimp-farming countries in Asia, infections can result in 100% mortality within 3-5 days of the onset of disease (Chantanachookin *et al.*, 1993). WSSV has also been of increasing concern in the region contributing to the loss of production in Thailand and presents an almost equally rapid rate of mortality, causing 100% loss of product within 2-7 days of infection (Chou Hsin-Yiu *et al.*, 1995).

One of the major challenges when considering the prevention of viral diseases in shrimp is that they lack the traditional adaptive immunity seen in vertebrates, meaning that vaccination is likely to be an ineffective method of preventing disease. RNA interference (RNAi) using sequence specific double stranded RNA (dsRNA) may provide a novel and attractive solution to combating viral infections in these animals. The natural abilities of the animal can be harnessed by designing dsRNA targeting key viral genes which could then utilize RNAi to silence these genes, which may inhibit the pathogens ability to replicate and slow down or prevent infection. For instance, one study was able to inhibit YHV replication through injection of dsRNA targeting the viral protease gene (Yodmuang *et al.*, 2006). Another study was also able to protect shrimp from WSSV by injecting dsRNA specific

to the VP28 gene, with a survival of 97.5% in treated shrimp over 10 days compared to 100% mortality in those treated with either PBS alone or GFP dsRNA (Nilsen *et al.*, 2017).

Although RNAi technology has shown promise in survival of virally infected shrimp, administering dsRNA through injection is not practical in a commercial farm containing thousands of shrimps, which would likely need treatment in their post larvae stage where the size of the shrimp ranges between 2.2-11.0 mm. As such oral delivery of dsRNA has been recently investigated, for example, one study showed that ingestion of bacterial expressed dsRNA targeting Laem-Singh Virus (LSNV) reduced the amount of detectable virus by 20-60% (Saksmerprome *et al.*, 2013). Another study was able to reduce levels of *Penaeus monodon* densovirus (PmDNV) by 88% when used as a prophylactic and curing 64% of infected animals by mixing feed with inactivated bacteria containing dsRNA targeting PmDNV (Chimwai *et al.*, 2016). Still, the use of *E. coli* in shrimp feed additive has not yet been investigated for the long-term effect on the animal or its impact on the environment.

Production and oral delivery of dsRNA would be better served by microalgae. In addition to the benefits of using microalgae as a production platform and delivery mechanism mentioned in previous chapters, the chloroplast of *C. reinhardtii* also lacks RNAi machinery which could interfere with dsRNA produced there, allowing for large accumulation of dsRNA within the organelle (Maul *et al.*, 2002). This strategy has been applied previously, shrimps fed *C. reinhardtii* cells containing dsRNA targeting the RNA dependent RNA polymerase (RdRp) of the YHV had a 50% mortality rate 8 days' post infection compared to an 84.1% mortality rate in the control group (Charoonnart *et al.*, 2019). This study looks to improve the system used by Charoonnart *et al* and generate new strains targeting the WSSV. This will be done by using an improved purpose fit construct design that uses the 16S promotor previously demonstrated to be the most transcriptionally active promoter within the chloroplast of *C. reinhardtii* (Rasala *et al.*, 2011). The ability of this promoter has been previously demonstrated where a more than 5 fold increase in expression of Luciferase has been achieved when using the 16S promoter compared to the psaA promoter (Taunt *et al.*, 2023). In

addition to the improved promoter the construct now has the 5’ UTR removed and an addition of a terminator on the antisense strand which was not used in the previous design. Addition of the second terminator prevents generation of any long ssRNA tails which would be produced on the anti-sense ssRNA as a result of termination of transcription only occurring when a native terminator was reached after the DNA cassette was integrated into the chloroplast genome. The 5’ UTR’s function is to recruit ribosomes which drive translation of mRNA transcripts, since only ssRNA is required to be synthesised which will later form dsRNA, the 5’ UTR was removed. The new purpose fit dsRNA expressing cassette was termed p2xTRBL, the differences between the two designs are outlined in figure 21. The new system came with other benefits over the old expression system including integration into an easy cloning system which utilizes golden gate cloning allowing for rapid generation of new targets and the ability for the 16s promoter being able to be expressed in *E. coli* for the purpose of generating a positive control which could be employed in later quantification experiments without any further cloning being required. This chapter will quantify dsRNA produced from both systems to determine if there is an improvement in overall yield and generate two new strains which target WSSV. This virus still threatens the aquaculture industry and is generally regarded as one of the most damaging in the sector, not just in South East Asian but globally (Sánchez-Paz, 2010). There are no commercial treatments available for WSSV which can help control and delay infections spreading and due to the fact in can wipe out entire farms within days, leaving little opportunity to intervene before losing an entire stock. With that being said, if successful this technology could be applied to multiple infectious diseases which currently affect shrimp aquaculture.

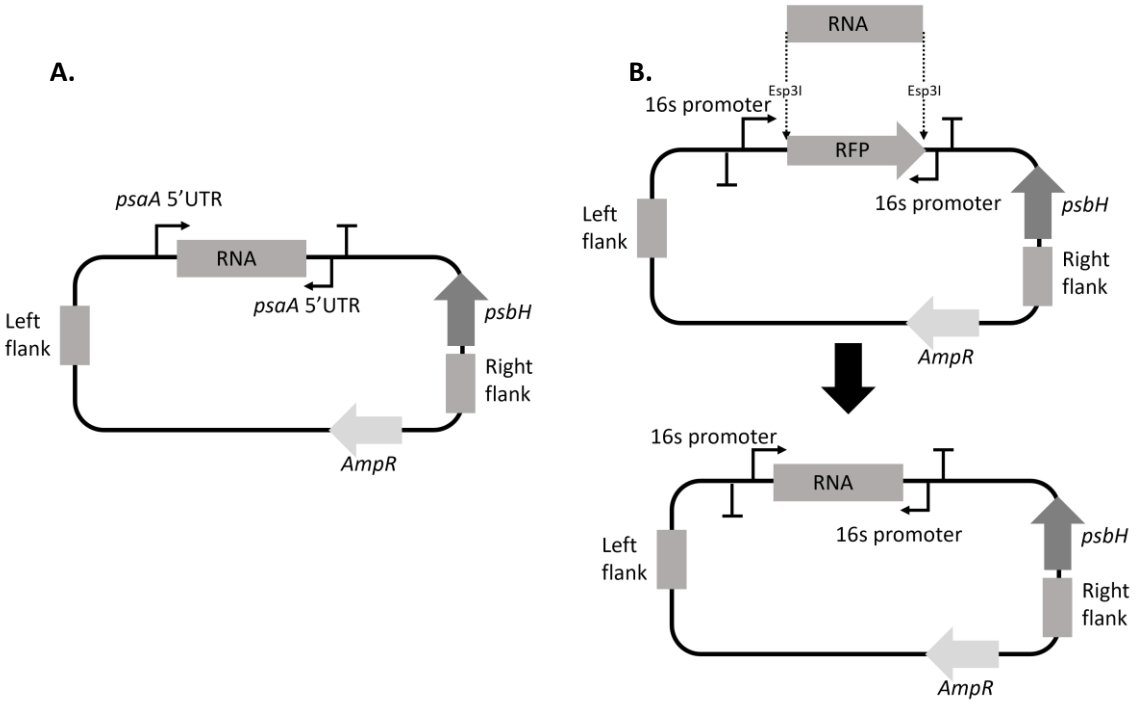


Figure 21. Comparison between new p2xTRBL design and previous dsRNA producing vectors. **A:** Previously used dsRNA vector. **B:** The new p2xTRBL cassette shows the introduction of the improved 16s promoter without the 5'UTR required for translation along with the addition of a terminator on the antisense 3' to 5' strand, along with Esp3I golden gate sites for easy insertion of new dsRNA sequences.

The chosen targets for use in RNAi were VP9 and ORF366 from WSSV. VP9 has shown to be involved in the early replication of the virus and has an essential role in WSSV replication (Alenton *et al.*, 2016a). One group has already shown that injection of dsRNA targeting VP9 elicits a survival of 80% in challenged shrimps compared to 0% survival in those injected with PBS alone (Alenton *et al.*, 2016a). A hypothesis set out by one study is that is that shrimp are able to integrate small sequences of viral DNA/RNA within their own genome to produce anti-sense RNA that can aid in fighting viral pathogens through the use of RNAi (Utari *et al.*, 2017). The study highlights ORF366, a part of the nucleocapsid, as a sequence thought to be important for infections from WSSV and as such ORF366 was selected as a potential target for RNAi.

5.2 Results

5.2.1 The p2xTRBL construct produces less dsRNA than previous constructs

The same RdRp sequence from YHV was cloned into the new p2xTRBL construct using golden gate and subsequently transformed into the TN72 (cw15 Δ*psbH*) strain using the glass bead method. Integration of the new cassette and RdRp specific sequence were confirmed by PCR using primers annealing to the flanking region on the chloroplast (Flank 1) and to the *psbH* at end of the p2xTRBL cassette (Rseq_mCRH1) yielding a band at ~2064 bp. Homoplasmic integration of the new gene can also be confirmed by PCR by using primers which amplify the *aadA* (Flank1 and rbcL.F) cassette, found on the chloroplast genome of TN72, which show a band at ~850 bp on the agarose gel, an absence of this band confirms the strain is homoplasmic. Figure 22 shows an agarose gel with the results of the PCR, the new strain shows a band a ~2064 bp with some other non-specific bands present. Despite the non-specific amplification these bands are not present in the parental strain TN72, although there does appear to be some non-specific amplification at ~3000 bp, it can be seen this is a different size to those bands seen in the dsRdRp strain. The agarose gel also confirms homoplasmic integration by absence of the ~850 bp band on the gel, this band is present in the TN72 strain which was used as a control. The sequence was also confirmed by extracting the band at 2064 bp in the dsRdRp lane which was checked by sequencing.

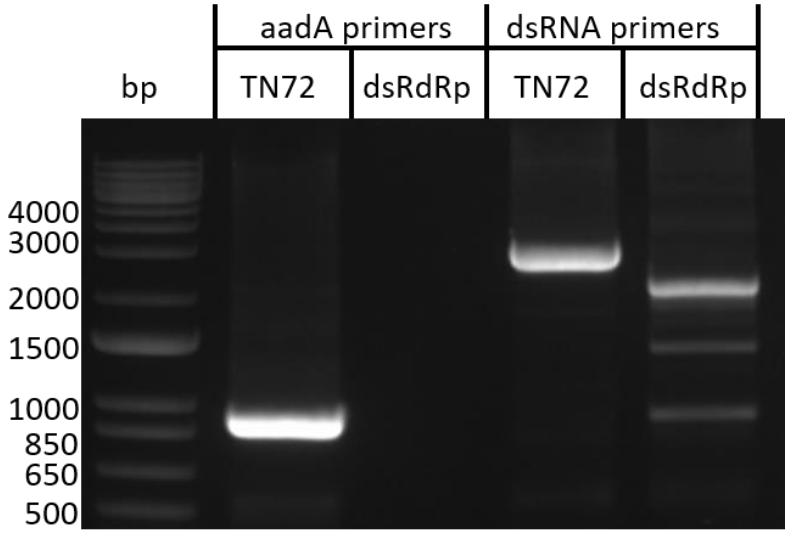


Figure 22. Agarose gel confirming homoplasmic integration of the p2xTRBL construct containing dsRdRp targeting YHV. Flank1 primer anneals on the chloroplast when combined with the rbcL.F primer which anneals to the end of the *aadA* cassette it produces an 850 bp product in strains carrying the *aadA* cassette. Rseq_mCRH1 binds downstream of the p2xTRBL and combines with the Flank1 primer, producing 2064 bp product in strains carrying the p2xTRBL cassette. The agarose gel shows integration of the p2xTRBL cassette gene in the new strain. A lack of band at 850 bp in the dsRdRp strain when using the primers for the *aadA* cassette indicate the strain is homoplasmic. The parental strain TN72 was also run as a control.

After confirmation of a homoplasmic strain the RNA extractions were carried out on the new dsRdRp strain and the PYP strain used in the previous study by Charoonnart *et al*, which was previously transformed and confirmed to be homoplasmic (Charoonnart *et al.*, 2019). For each strain two 1 L Erlenmeyer flasks containing 400 mL of TAP were inoculated using 10 mL of pre-culture and were incubated at 25°C under continuous light at 15-20 µmol photons m^{-2} s^{-1}. After 5 days of growth RNA extractions were carried out using Trizol and phenol:chloroform, with extractions being treated with DNase I and RNase A in presences of >300mM NaCl to remove DNA and ssRNA. The empty photosynthesis restored strain, CCK10, was also extracted and treated to serve as a negative control. After RNA extractions degradation of DNA was checked by running PCRs using primers specific to the RdRp sequence (YHV_RNA_F and YHV_RNA_R) which would yield a band at ~374 bp. Both strains carried the exact same sequence of RdRp allowing for use of the same primer set. Figure 23 shows a lack of band in both samples confirming DNA specific to the RdRp

sequence from the RNA extraction was no longer present. A positive control consisting of plasmid DNA was loaded and negative controls of both water (- control) and CCK10 strain were also loaded.

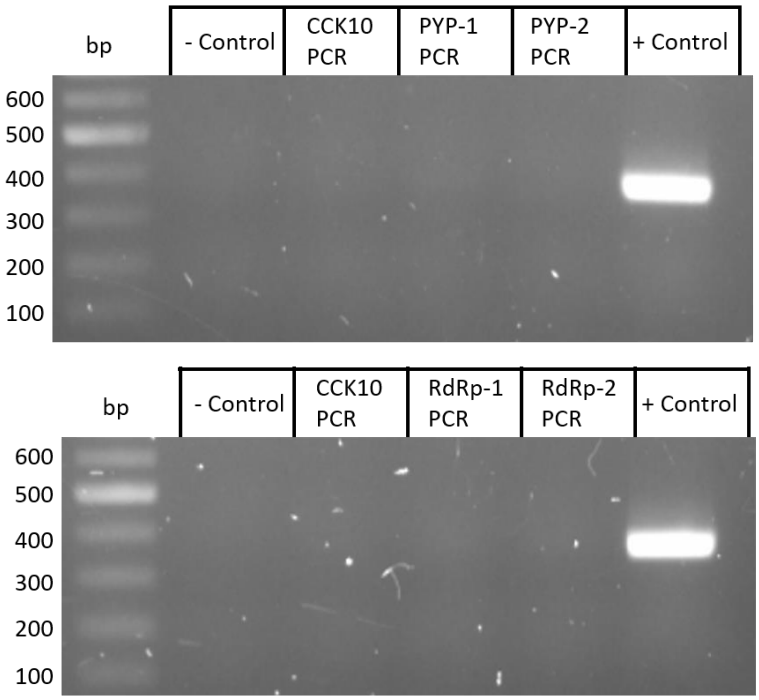


Figure 23. DNA check on original PYP strain and the new dsRdRp strain. Agarose gels of PCRs carried out of RNA extractions using YHV_RNA_F and YHV_RNA_R primers specific to RdRp sequence yielding a 374 bp band. The negative control of water in place of DNA, DNA from the CCK10 photosynthesis restored strain, a positive control was also loaded using plasmid DNA. PYP strain shown in the upper gel and the new dsRdRp strain in the lower gel.

Next the presence of dsRNA was to be established by the use of RT-PCR. Figure 24 shows an agarose gel of the results of the RT-PCR. The bands at 374 bp in the RNA extraction lanes in both the PYP and dsRdRp strains correspond to the sequence of RdRp from YHV. A band of the same size can be seen in the positive control, and no such band is present in CCK10.

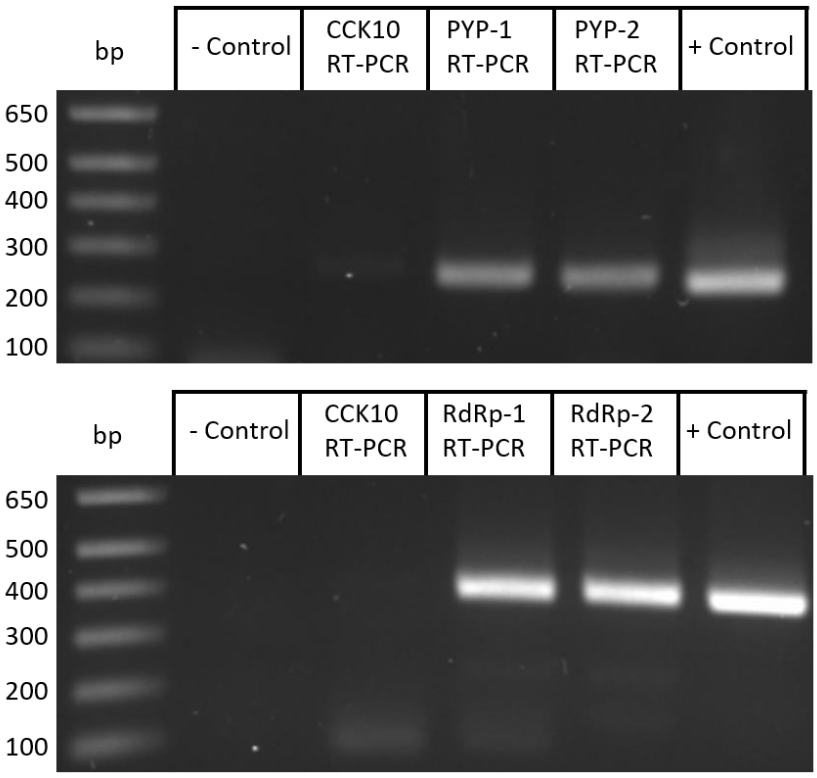


Figure 24. RT-PCR of RNA extracts of PYP strain and dsRdRp strain. Agarose gels of RT-PCR using YHV_RNA_F and YHV_RNA_R primers specific to RdRp sequence yielding a 374 bp band. The negative control of water in place of DNA, DNA from the CCK10 photosynthesis restored strain, a positive control was also loaded using plasmid DNA. PYP strain shown in the upper gel and the new dsRdRp strain in the lower gel.

The RT-PCR confirms the presence of dsRNA and although the extractions are treated with RNase A to degrade any ssRNA it is possible the amplification seen in figure 24 is from ssRNA. As a result, aliquots of the RNA extractions were treated with RNase III which specifically degrades dsRNA and the RT-PCR repeated to detect any remaining RNA. A control was also run which used an RNA extraction not treated with RNase III to ensure the dsRNA was not degraded or lost in the subsequent phenol:chloroform reaction or the precipitation required after the treatment. Figure 25 shows the result of the RT-PCRs carried out after the RNase III treatments. A lack of any amplification in the RNase III treated extractions suggests previous amplification (Fig. 24) was not due to the presence of ssRNA, this is supported by looking at the controls, which still show a band at the correct size of 374 bp, meaning RNA was not degraded or lost by the steps following the RNase III treatment.

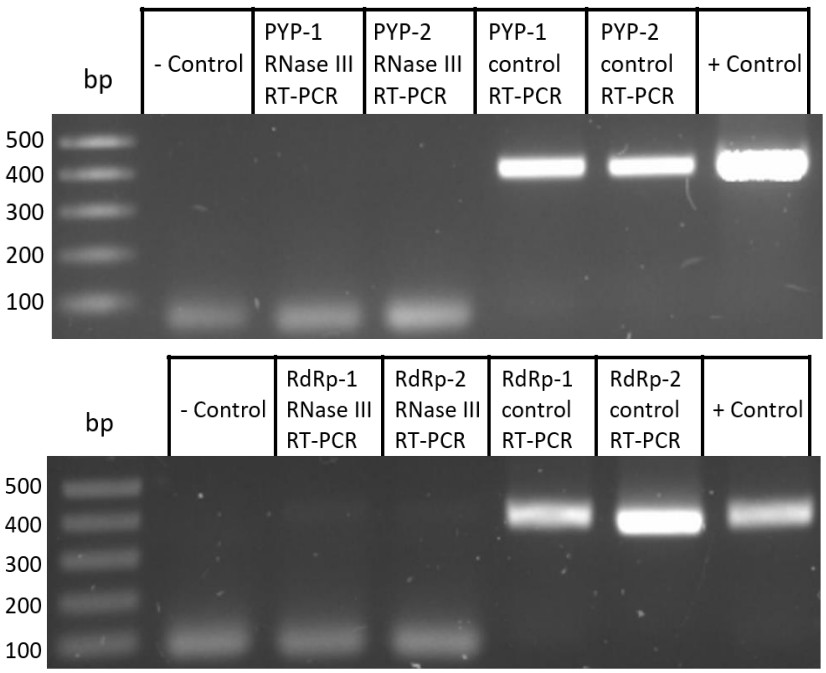


Figure 25. RT-PCR of RNase III digests of RNA extracts of PYP strain and dsRdRp strain. RNA extracts were treated with RNase III to specifically degrade dsRNA; controls were also run without the use of the RNase III enzyme. RT-PCR using YHV_RNA_F and YHV_RNA_R primers specific to RdRp sequence yielding a 374 bp band. The negative control of water in place of RNA, a positive control using plasmid DNA. PYP strain shown in the upper gel and the new dsRdRp strain in the lower gel.

After the RNA extractions were established to contain only dsRNA which appeared to be specific to the requisite RdRp sequence they were quantified using RT-qPCR. A standard curve was generated using dsRNA expressed in the RNase III deficient HT115 strain of *E. coli* and compared to the dsRNA extracted from the algal strains (Fig. 26).

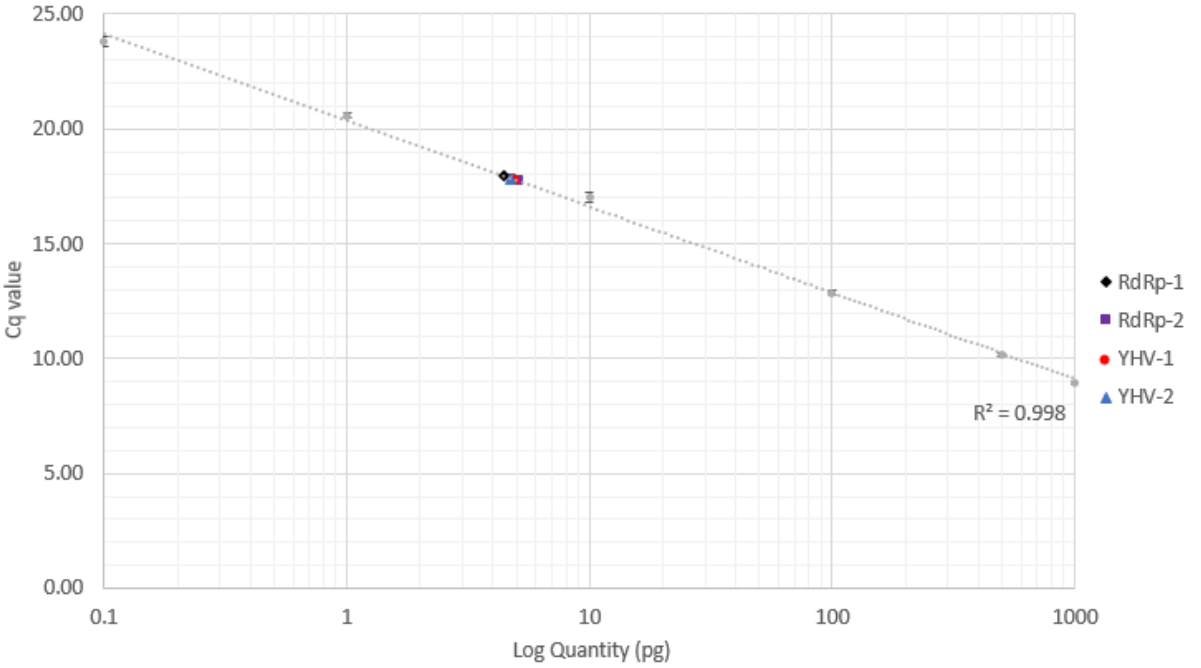


Figure 26. Standard curve of RT-qPCR data for dsRdRp. Standard curve of dsRdRp using dsRNA expressed in RNase III deficient HT115 *E. coli* cells for in a dilution series ranging from 1 ng to 0.1 pg of RNA. RNA from the PYP strain and dsRdRp strain are shown on the standard curve with 250 ng total of RNA loaded. The R^2 of the curve is displayed at 0.998.

Quantitative analysis resulted in a yield of the dsRNA in the original PYP strain of 61.43±3.57 ng of dsRNA per litre of culture (OD750 0.99-1.02), and the new dsRdRp strain contained 53.06±3.32 ng of dsRNA per litre of culture (OD750 1.27-1.33). Melt curve analysis revealed that the purified dsRNA from the bacterial expression system gave a melting temperature (Tm) of 82.65±0.12 °C while the dsRNA obtained from the PYP strain gave a Tm of 82.58±0.23 °C and the dsRdRp strain a Tm of 82.75±0.06 °C, suggesting qPCRs amplified the same target sequence. The new system seemed as though it was yielding a lower amount of dsRNA per litre of culture than the previous system despite the introduction of a more transcriptionally active promoter and addition of the extra terminator in the 3’ to 5’ direction.

5.2.2 Generation of new dsRNA strains targeting WSSV

Although the new construct design appeared to produce less dsRNA targeting YHV than the previous system it was decided to proceed with the new system to target WSSV due to the previously mentioned advantages such as it being able to be utilised in a pre-existing golden gate cloning system and the use of the 16s promoter in generating dsRNA in the bacterial system which could later be utilized in qPCR for quantifying the amount of dsRNA generated. Constructs were generated using the golden gate system and transformed into the TN72 strain as previously described. Strains were then screened for integration of the 2xTRBL cassette using the PCR primers Flank1 and Rseq_mCRH1. Once successful mutants had been identified, they were then repeatedly streaked on a minimal media containing no acetate until the strains became homoplasmic. DNA was extracted from the new strains and PCRs carried out using primers to confirm presence of the new dsRNA cassette (Flank1 and Rseq_mCRH1) and removal of the *aadA* cassette (Flank1 and rbcL.F). The images of the agarose gels in figure 27 show an absence of a band at 850 bp in both the dsVP9 and dsORF366 strain along with the band at 2064 bp. The sequence was also confirmed by extracting the bands at 2064 bp in the dsVP9 and dsORF366 lanes and sending for sequencing.

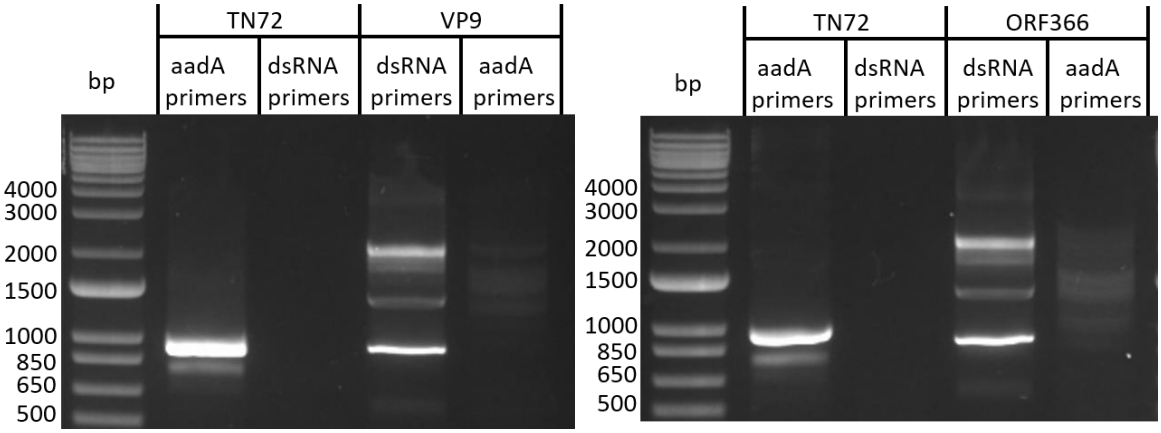


Figure 27. Agarose gels confirming homoplasmic Integration of the p2xTRBL construct containing dsVP9 and dsORF366. Flank1 primer anneals on the chloroplast when combined with the rbcL.F primer which anneals to the end of the *aadA* cassette it produces an 850 bp product in strains carrying the *aadA* cassette. Rseq_mCRH1 binds downstream of the p2xTRBL and combines with the Flank1 primer, producing 2064 bp product in strains carrying the p2xTRBL cassette. The agarose gels show integration of the p2xTRBL cassette gene in the new strains. A lack of band at 850 bp in the dsVP9 and dsORF366 strains when using the primers for the *aadA* cassette indicate that strains are homoplasmic. The parental strain TN72 was also run as a control. The left gel shows PCRs for dsVP9 and the right for dsORF366.

Once the homoplasmic strains were achieved the strains were grown in a 400 mL culture and RNA extracted after 5 days and samples treated with DNase I and RNase A to remove DNA and ssRNA. Degradation of DNA specific to the sequence being analysed was checked by PCR using sequence specific primers. The results of the PCR are shown in figure 28, samples from both strains and the negative controls (MqH_2O and CCK10) show no amplification whereas the positive control (plasmid DNA) show amplification at the correct sizes of 255 bp for VP9 and 246 bp for ORF366.

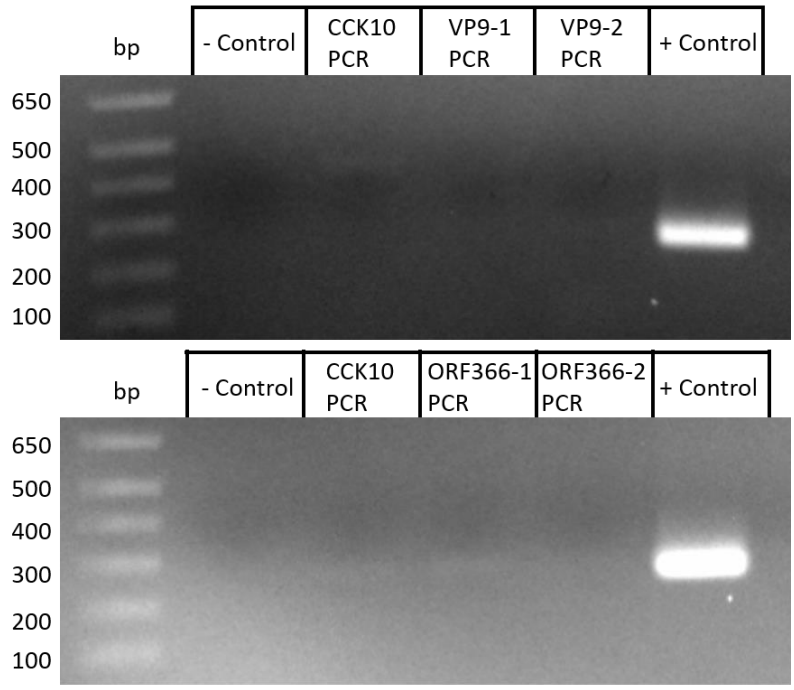


Figure 28. DNA check on dsVP9 and dsORF366 strains. Agarose gels of PCRs carried out on RNA extractions using sequence specific primers for VP9 (VP9_RNA_F and VP9_RNA_R) and ORF366 (ORF366_RNA_F and ORF366_RNA_R). The negative control of water in place of DNA, DNA from the CCK10 photosynthesis restored strain, a positive control was also loaded using plasmid DNA. The dsVP9 strain shown in the upper gel and the dsORF366 strain in the lower gel.

Next the presence of RNA was to be established by the use of RT-PCR, which was then later confirmed to be dsRNA by observing degradation using a dsRNA specific enzyme RNase III. Figure 29 shows results of the RT-PCR, the agarose gel shows bands in both the dsVP9 samples (255 bp) and the dsORF366 samples (246 bp), with an absence of bands in the negative controls. The results of degradation of the dsRNA can be seen when looking at the agarose gel in figure 30 which was loaded with RT-PCR samples using RNase III treated extractions as a template. The lack of bands in

RNase III treated samples in figure 30 show that no ssRNA is present in the RNA extractions which still show up in the control RNaseIII samples suggesting that the RNA present in figure 29 is dsRNA specific to the dsVP9 and dsORF366 sequence.

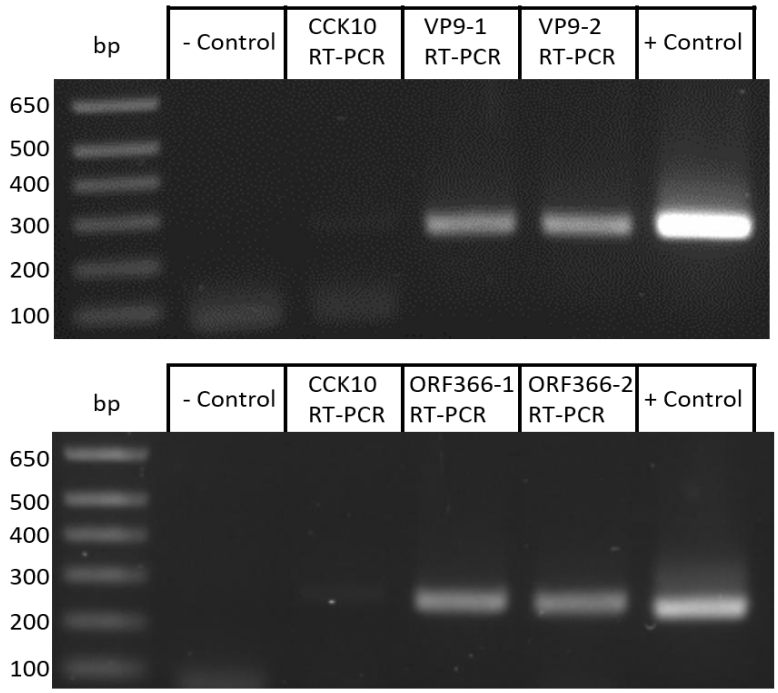


Figure 29. RT-PCR of RNA extracts of dsORF366 strain and dsVP9 strain. Agarose gels of RT-PCR using sequence specific primers for VP9 (VP9_RNA_F and VP9_RNA_R) and ORF366 (ORF366_RNA_F and ORF366_RNA_R). The negative control of water in place of DNA, DNA from the CCK10 photosynthesis restored strain, a positive control was also loaded using plasmid DNA. The dsVP9 strain shown in the upper gel and the dsORF366 strain in the lower gel.

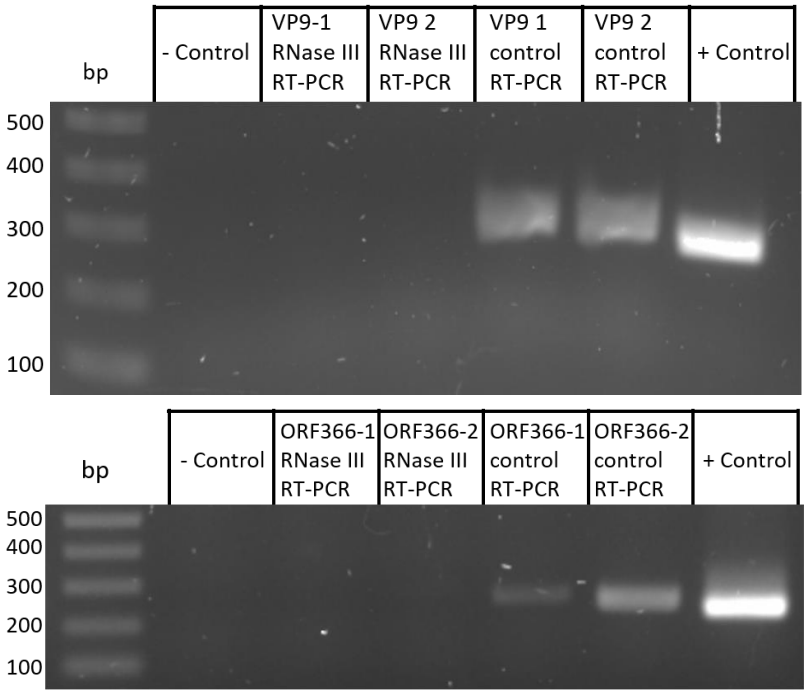


Figure 30. RT-PCR of RNase III digests of RNA extracts of dsORF366 strain and dsVP9 strain. RNA extracts were treated with RNase III to specifically degrade dsRNA; controls were also run without the use of the RNase III enzyme. RT-PCR using sequence specific primers for VP9 (VP9_RNA_F and VP9_RNA_R) and ORF366 (ORF366_RNA_F and ORF366_RNA_R). The negative control of water in place of DNA, DNA from the CCK10 photosynthesis restored strain, a positive control was also loaded using plasmid DNA. The dsVP9 strain shown in the upper gel and the dsORF366 strain in the lower gel.

5.2.3 Quantification of dsRNA in dsVP9 and dsORF366 strains

RNA extractions from both dsVP9 and dsORF366 had been proven to contain only dsRNA specific to the sequence which they were designed to produce. The next stage was to quantify the dsRNA present using RT-qPCR so that the yields from the cultures could be determined. As with the previous RT-qPCR a standard curve was generated using dsRNA expressed in the RNase III deficient HT115 strain of *E. coli* which could then allow for estimating the amount of dsRNA in the samples. Figures 31 and 32 show the standard curve of dsVP9 and dsORF366, respectively.

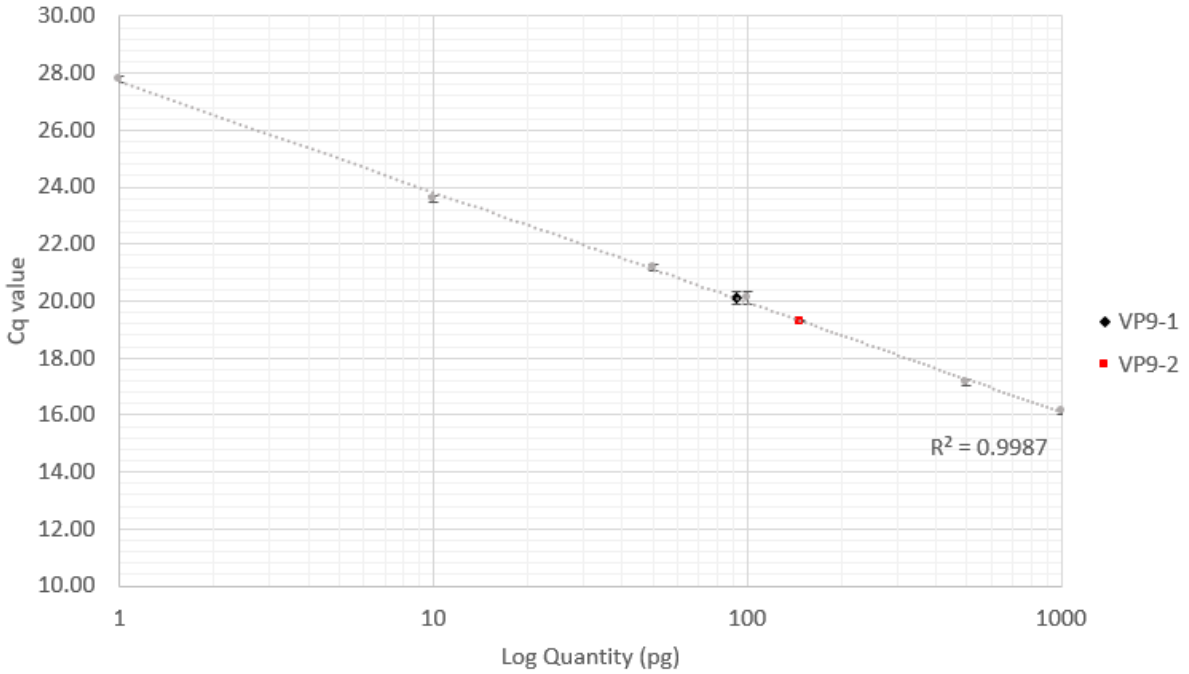


Figure 31. Standard curve of RT-qPCR data for dsVP9. Standard curve of dsVP9 using dsRNA expressed in RNase III deficient HT115 *E. coli* cells for in a dilution series ranging from 1 ng to 1 pg of RNA. RNA from the dsVP9 strain is shown on the standard curve with 50 ng total of RNA loaded. The R^2 of the curve is displayed at 0.9987.

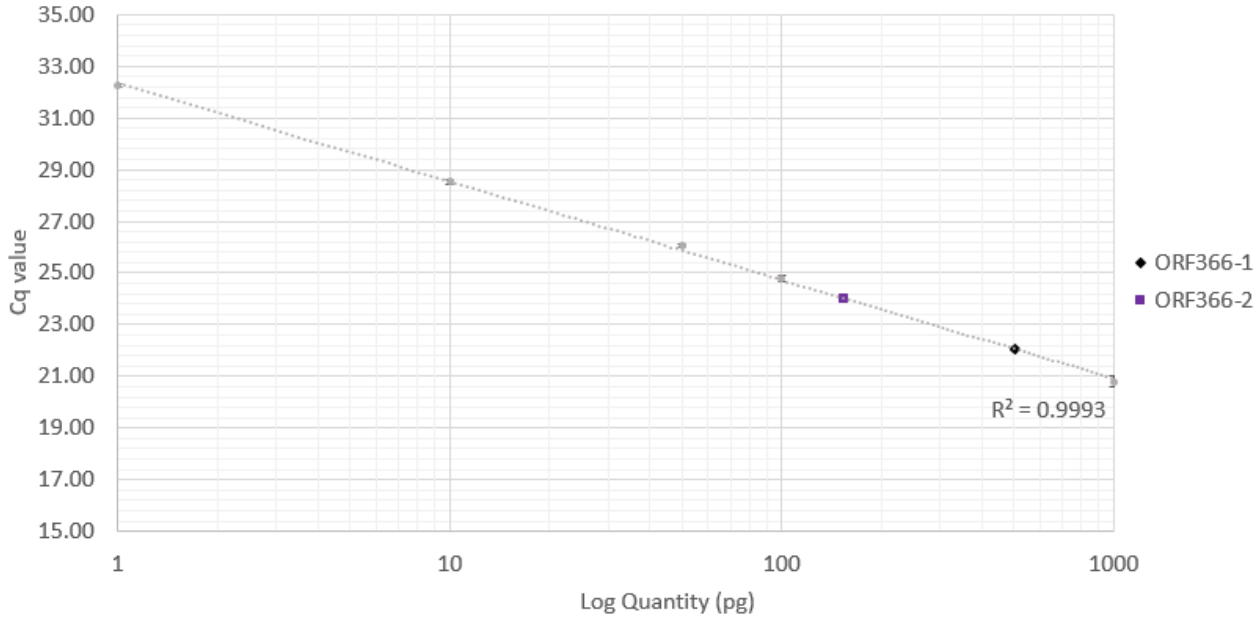


Figure 32. Standard curve of RT-qPCR data for dsORF366. Standard curve of dsORF366 using dsRNA expressed in RNase III deficient HT115 *E. coli* cells for in a dilution series ranging from 1 ng to 1 pg of RNA. RNA from the dsORF366 strain is shown on the standard curve with 50 ng total of RNA loaded. The R^2 of the curve is displayed at 0.9993.

Calculating the yield of dsVP9 from the qPCR data determined that the dsVP9 stain expressed approximately 3.86±1.07 µg of dsRNA per litre of culture (OD750 0.94-0.96). Analysis of the melt

curve data showed that the purified dsRNA from the bacterial expression system gave a melting temperature (Tm) of 81.75±0.16 °C while the dsRNA obtained from the microalgae strain gave a Tm of 81.76±0.15 °C, suggesting qPCRs amplified the same target sequence. The same data analysis was applied to the dsORF366 strain of microalgae giving a yield of 11.63±5.84 µg dsRNA per litre of culture (OD750 1.11) and a Tm of 81.23±0.11 °C from the bacterial expressed dsRNA and a Tm of 81.23±0.09 °C from the microalgae strain. Both strains expressed a significantly higher amount of dsRNA than the previous RdRp strains being over 50-fold higher.

5.2.4 Challenge trials using dsVP9 and dsORF366

To test the new strains expressing dsVP9 and dsORF366 a challenge trial was conducted. The shrimp challenge trial was performed under the supervision of Dr Patai Charoonnart at the Centex Shrimp, Mahidol University, Thailand. The microalgae strains were generated at the University of Kent and grown for challenge trials at Mahidol University. Post larvae (PL) specific pathogen free shrimp (*P. vannamei*) ~0.03g body weight were acclimatised for 3 days prior to challenge experiment and observed for signs of infection and mortality. PL shrimps were then divided into 5 groups, positive (no treatment), negative (no treatment), dsORF366 (treated with dsORF366 algal cells), dsVP9 (treated with dsVP9 algal cells) and wild type/WT (treated with algal cells containing a dsRFP cassette). Shrimp were fed twice daily with commercial feed at 10% body weight. Treated shrimp were given an additional daily feed with biomass of the stated strain of algae equating to ~1 x 10^9 cells/tank. All groups, with exception of the negative control were challenged with WSSV infected shrimp cells at 50% of the body weight of the shrimp at day 4 of the experiment and observations made daily for mortality. Results are reported as a % of mortality. Results from the challenge trial can be in figure 33.

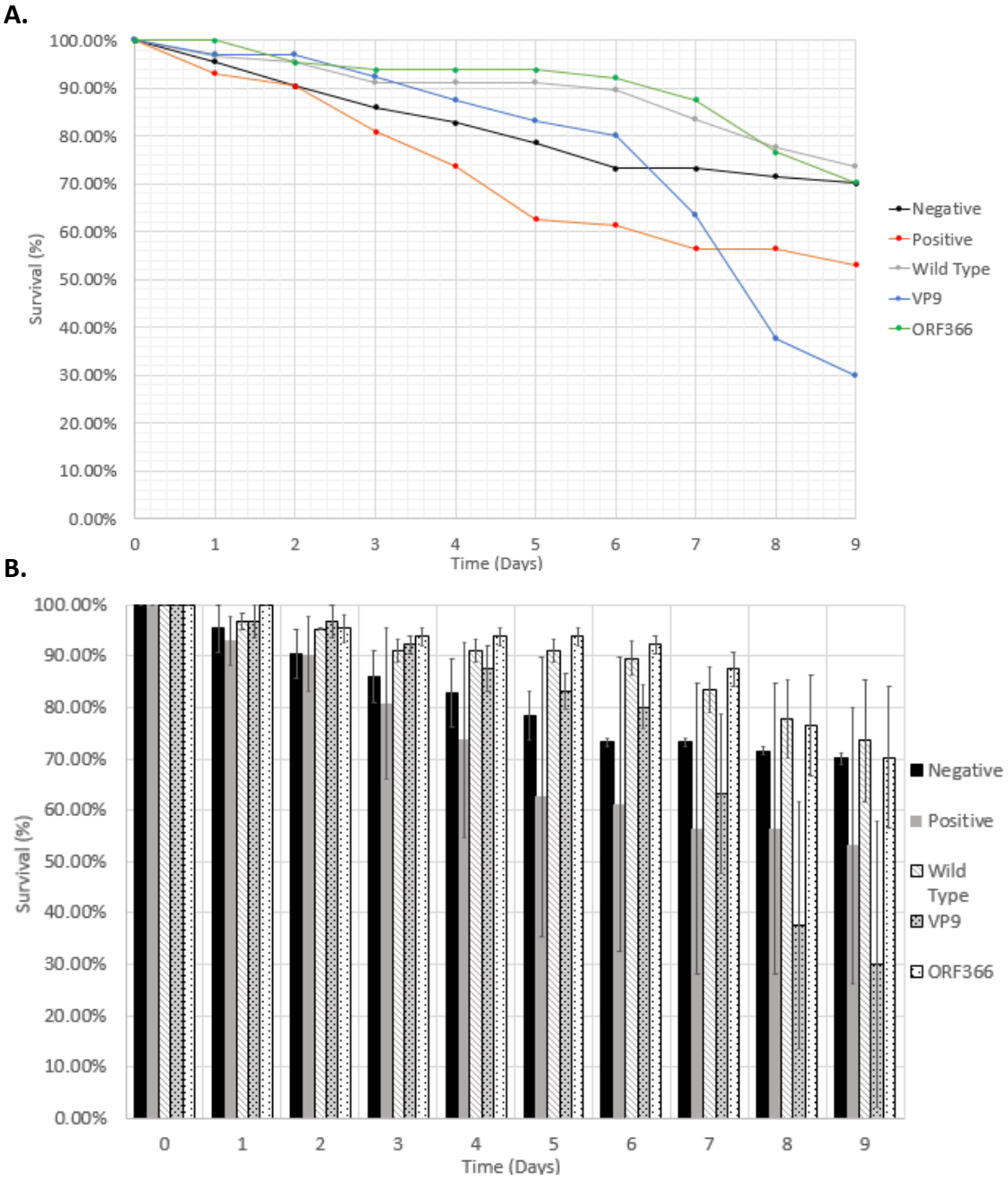


Figure 33. Percentage of survival of groups challenged with WSSV. Each group was made up of 20-25 shrimp in triplicate. The control groups had no treatment and were fed as normal. In addition to normal feed ORF366, VP9 and Wild Type groups were fed each day with ~1 x 10^9 cells/tank of microalgae of the stated strain. The shrimp in all groups were challenged on day 4 with infected WSSV shrimp at 50% body weight. Shrimp monitored daily and mortality reported as a % of survival. **A.** Line graph representing mean % survival. **B.** Bar graph representing mean % survival with error bars using standard error.

Figure 33A shows the survival of shrimp over the 9-day period. The ORF366 treated group ended with an average survival higher than that of the positive group being 70% and 53% respectively,

this was comparable to the negative control which had also experienced some mortality also having an average survival of 70%. Mortality in shrimp seemed to be common in all groups even before the challenge on day 4, demonstrating the delicate nature of PL shrimp. The wild type shrimp outperformed all groups ending with an average survival 73.5%. Surprisingly the VP9 strain performed the worst of all the groups ending with an average survival under 30% even worse than the untreated group at 53%. Figure 33B shows the standard error on the bar graph showing high variability between sample groups.

5.3 Conclusions

This study set out to improve and expand upon previous work expressing dsRNA in the freshwater microalgae *C. reinhardtii*. The purpose made dsRNA construct used the transcriptionally stronger 16S promoter without the unnecessary 5' UTR and incorporated a terminator in the 3' to 5' antisense strand. However, a strain expressing the same dsRdRp sequence using the new system expressed less dsRNA when compared to the original PYP strain at 53.06±3.32 ng/L (dsRdRp) comparing to 61.43±3.57 ng/L (PYP). Despite this difference the new construct proposed other benefits over the old one, such as being able to fit into a pre-existing golden gate system which made generation of new targets more streamlined and the ability of the 16S promoter to produce dsRNA in HT115 *E. coli* cells proving convenient when later quantifying dsRNA by qPCR.

Two new targets were also generated targeting the WSSV using the sequence of the VP9 protein and ORF366. After establishing that the dsRNA was being produced this was again quantified using qPCR. These two strains yielded significantly more dsRNA than either of the RdRp containing strains having over 50x more dsRNA, at 3.86±1.07 µg/L for VP9 and 11.63±5.84 µg/L for ORF366. The large difference between the accumulation of each of these dsRNA targets could be as a result of their length. One study demonstrated that longer dsRNA expressed in the chloroplasts of potato plants resulted in a lower accumulation of dsRNA than that of a shorter dsRNA (He *et al.*, 2020). This finding would help to explain the results found here as the RdRp sequence was 374 bp and the VP9

and ORF366 dsRNA were 255 bp and 246 bp, respectively. However, the variability between samples of the same target is more likely to be related to the nature of processing RNA which is well known to be susceptible to degradation.

A challenge trial was carried out to try to determine if the strains would be effective at protecting shrimp from WSSV infection. The challenge trial showed that the negative groups, ORF366 and Wild Type group all had comparable survival rates between 70-75%. The equally comparable survival in the ORF366 strain and Wild Type strain may be due to a protective effect from the microalgae as opposed to any impact from the dsRNA within the cells. The Wild Type strain may also have helped protect the shrimp from infection from the nonspecific dsRNA of the RFP inserted within the chloroplast. Non-specific dsRNA can upregulate RNAi machinery within shrimp and dsGFP has been demonstrated to increase survival of shrimp challenged with WSSV (Maralit *et al.*, 2015). Nevertheless, this does not explain the poor performance of the VP9 treated shrimp, especially given that dsVP9 has shown to be effective at protecting shrimp from WSSV infection when delivered by intra-muscular injection (Alenton *et al.*, 2016a). Overall, the results from the challenge trial are unreliable, figure 33B shows a high variability between the samples within the same groups, this may be explained by the inconsistency in dosage of both the microalgae and infected shrimp meat ingested by each shrimp, which is one of the issues of delivering treatments orally.

Chapter 6: Alpha-2-macroglobulin as a disease resistant marker in *Epinephelus fuscoguttatus*

6.1 Introduction

Brown-marbled grouper (*Epinephelus fuscoguttatus*) is a favoured species in Asia due to its desirable taste, high growth rate, and resistance to stress (Sun *et al.*, 2016). Culturing of grouper has increased over the years with production reaching over 160,000 tons in China in 2018 (Yang *et al.*, 2020). Currently brown-marbled grouper farming relies on wild caught fish for stocking, which is not only unsustainable but also causes issues with variability in the animal's resistance to disease.

Vibriosis is one of the most common diseases within aquaculture, infections results can result in reduced growth and ultimately contributes to high mortality rates (Ina-Salwany *et al.*, 2019). Vibriosis is incredibly prevalent among grouper, samples of grouper from Malaysia were shown to have a high level of *Vibrio spp,* with detection in around 72% of the sampled fish. *V. communis* and *V. parahaemolyticus* were the most prevalent infections in those fish sampled making up 28% and 25% of infections respectively (Amalina *et al.*, 2019). Currently infections are treated with antibiotics, the problems associated with the use and misuse of antibiotics to control infections has already been covered in previous chapters. Although vaccination is a more desirable and a successful technique for controlling infections availability, cost, and practicality in administering vaccination are all major hurdles which still need to be overcome. One such problem with vaccination against vibriosis is the large diversity of *Vibrio* strains meaning creating vaccines to protect across different strains and their serotypes is difficult (Li *et al.*, 2010).

An alternative strategy to the prevention of disease is to identify strains of the animal which are already resistant to infection. However, in contrast to terrestrial animal farming the production

from selectively bred stocks in aquaculture sits at <10% (Gjedrem, Robinson and Rye, 2012). Selective breeding programs have successfully been established in aquaculture such as for improving the growth rates of Nile tilapia (Bentsen *et al.*, 1998) and for disease resistance in Penaeid shrimp (Cock *et al.*, 2009)**.** However, traditionally selective breeding programs are slow and imprecise relying on phenotypic traits of the animal. These animals are used in mass selection breeding and can generate animals with genetically desirable traits. In aquaculture this can be carried out relatively cheaply due to the low value of fish. Despite this, mass selection does not always work, one study found that after mass selection of Nile Tilapia no increase in growth rates were seen between the parental group and the selected group after 2 generations (Hulata, Wohlfarth and Halevy, 1986). Another study found that selectively breeding hybrid groupers are still susceptible to infections from *Vibrio spp.* (Shen *et al.*, 2017). A quicker and more reliable strategy for breeding involves using biomolecular tools which allow for the identification of biomarkers which correlate to a resistance to disease, increased growth rates or a resistance to stress. Once these biomarkers are identified, individuals with these biomarkers can be easily selected and bred to develop a stock which is more resistant to infection, decreasing the need and use of antibiotics and vaccines.

Alpha-2-macroglobulin (A2M) is an extracellular protein which acts as a broad-spectrum protease inhibitor, which uses a specific “bait” region which is a string of amino acids presenting as a good region for proteolysis. Once the proteases cleave this bait region, A2M then collapses and traps proteases in a cage like structure removing the protease’s ability to interact with large substrates (figure 34) (Vandooren and Itoh, 2021). A2M is well known to be associated with the innate immune system through regulation of endogenous and exogenous proteases. A2M assists in the immune response by clearing proteases produced by pathogens which aid in their invasion and proliferation in the host organism (Armstrong and Quigley, 1999).

Expression of A2M, among other proteins, has been postulated to be a potential biomarker for disease resistance in the brown-marbled grouper *Epinephelus fuscoguttatus* (Low *et al.*, 2015). Here A2M from *Epinephelus fuscoguttatus* was expressed in *E. coli* and solubilised and a specific antibody generated for detection of A2M expression in brown marbled grouper. The antibody generated in these experiments contributed to a publication which identified A2M as a potential biomarker for disease resistance in the brown-marbled grouper *Epinephelus fuscoguttatus* (Ibrahim *et al.*, 2022).

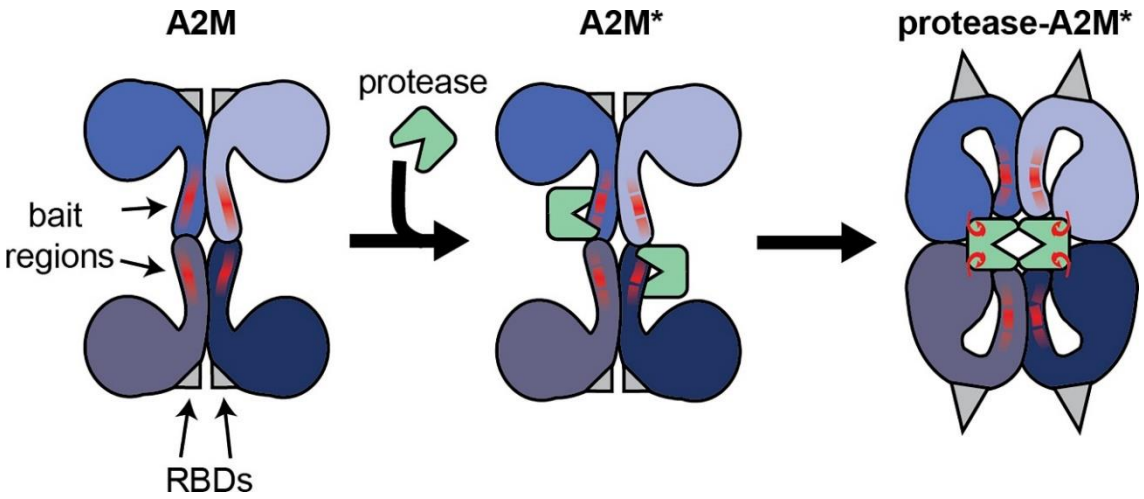


Figure 34. Mechanism of A2M trapping of proteases. A2M presents a bait region, a string of amino acids which are susceptible to proteolysis by proteases. Upon cleavage of these amino acids A2M undergoes conformational changes which inactivate the protease by trapping it in the structure. Image from (Vandooren and Itoh, 2021).

6.2 Results

6.2.1 Expression of Alpha-2-macroglobulin

A2M was first codon optimised for expression in *E. coli* and was cloned into a pET23/ptac vector using Gibson assembly, with a 6xHis tag at the C-terminus for detection and purification generating pCLW2. To assess expression of the protein pCLW2 was expressed in both W3110 and BL21 cells and grown at varying temperatures in LB media. Cells were grown to mid log phase and induced with 10 µM IPTG and were harvested 3 hours post induction and after expression overnight. The cells were then processed into their soluble and insoluble fractions and visualised by SDS PAGE and immunoblotting. The protein was expressed as an insoluble protein at all temperatures and in both cell lines (Fig. 35A and 35B). The protein also appeared to be degraded at higher temperatures, evidenced by the smaller bands seen in the immunoblots. The degradation more which is more obvious in figure 35B may account for why A2M to not be visible in all immunoblots at its predicted size of 53.9 kDa. Small amounts of soluble protein did appear to be present at 30°C in W3110 (Fig. 35B). Soluble protein was necessary for purification and generation of a specific antibody which would be used in subsequent experiments.

Due to the majority of the protein appearing as insoluble it was decided to clone the A2M gene into the single copy PEXT22 vector to help alleviate this issue**.** A2M in PEXT22 (pCLW14) was expressed in W3110 since it appeared to be the better cell line in previous experiments and at both 30°C and 25°C. After expression again the protein appeared in the insoluble fraction (Fig. 35C).

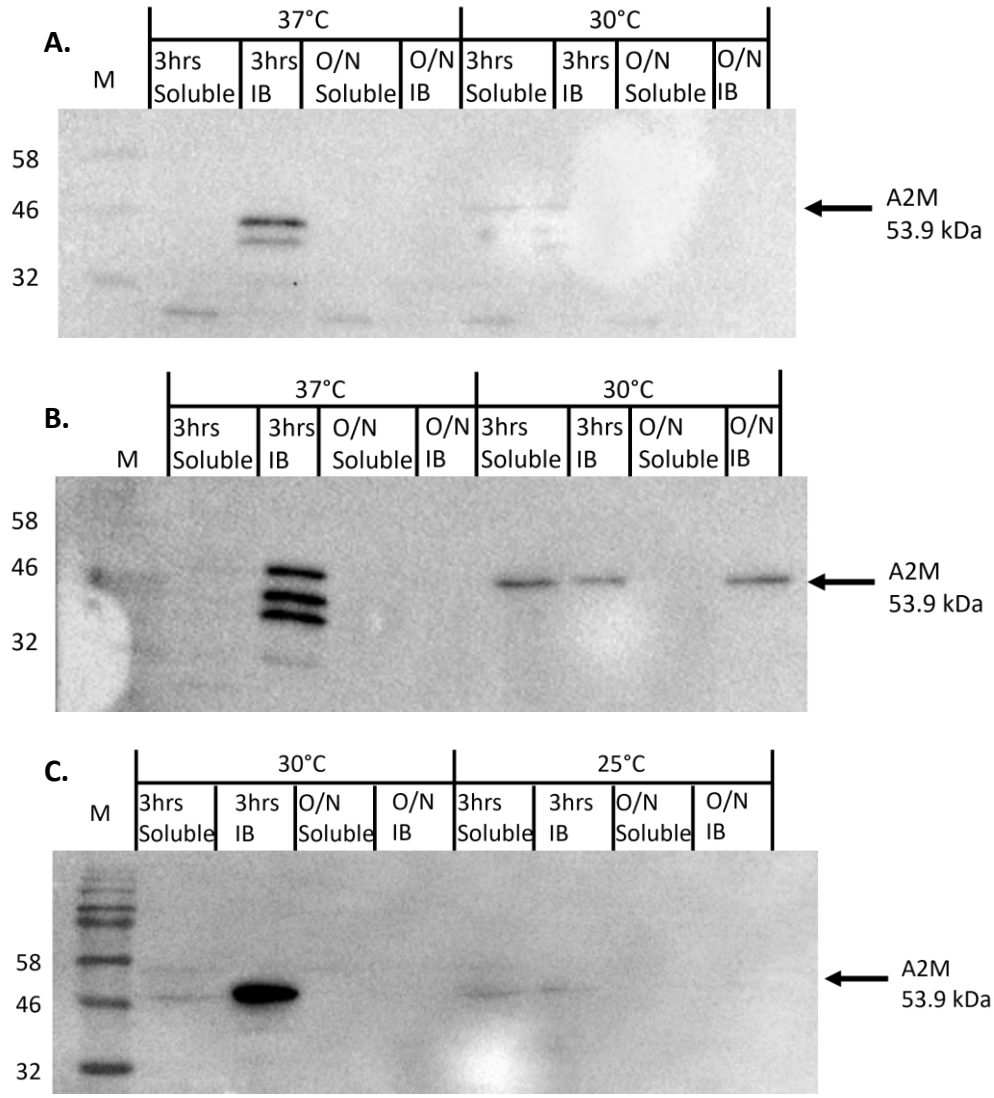


Figure 35. Shake flask culture of W3110 and BL21 cells expressing A2M constructs at different temperatures. Cultures were grown and expressed at either 37°C, 30°C or 25°C in LB medium. Cells were harvested 3 hours post induction or after overnight expression then processed into both insoluble and soluble fractions. Following extraction proteins were separated by SDS PAGE and visualise by western blot **A.** Anti-HIS Western Blot of BL21 cells expressing A2M in a pET23/ptac vector **B.** Anti-HIS western blot of W3110 cells expressing A2M in a pET23/ptac vector **C.** Anti-HIS western blot of W3110 cells expressing A2M in a pEXT22 vector. The predicted size of A2M with HIS tag is indicated at 53.9 kDa. IB: Insoluble protein.

Terrific Broth (TB) medium has been formulated for increased yield and solubility of proteins (Sahdev, Khattar and Saini, 2008). Therefore, the next experiments compared expression and solubility of the A2M protein in LB and TB media. Figure 36 shows that TB media doesn't appear to increase either the yield or solubility of A2M. Managing to get insoluble proteins to correctly fold and be soluble is a widely researched area and is well known to be a challenging task. In addition to

this A2M contains disulfide bonds which, due to the reducing environment of the cytoplasm of *E. coli* cells, often causes protein to not fold correctly (Prinz *et al.*, 1997). As such, because a large amount of insoluble A2M is clearly present in cultures grown at 37°C and only enough purified soluble protein is required for generation of an antibody it was decided that solubilising the protein *in vitro* would be a better strategy.

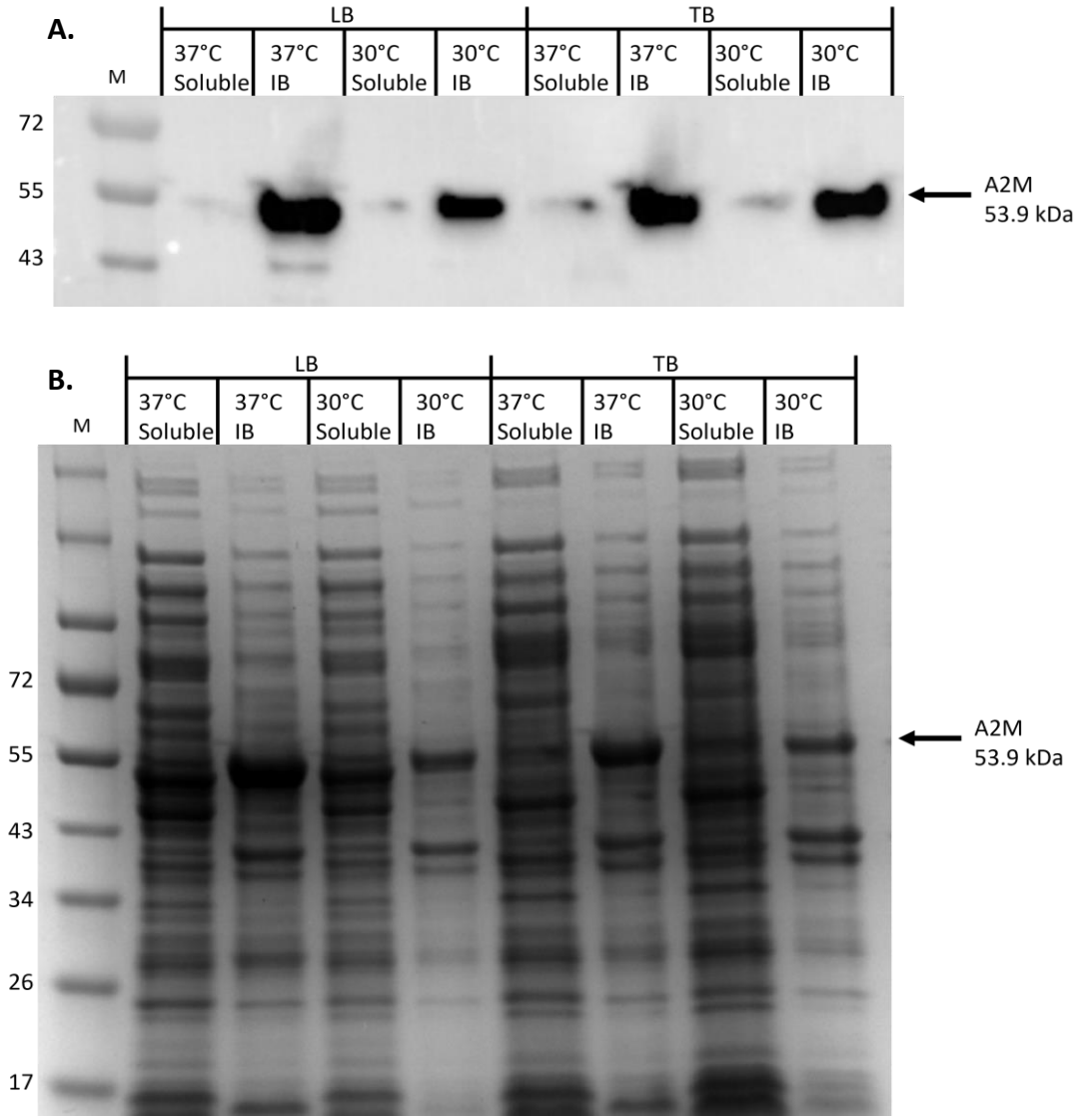


Figure 36. Shake flask culture of W3110 expressing PEXT22 A2M construct at different temperatures. Cultures were grown and expressed at either 37°C or 30°C in LB and TB medium. Cells were harvested 3 hours post induction then processed into both insoluble and soluble fractions. Following extraction proteins were separated by SDS PAGE and visualised by **A.** Anti-HIS western blot and **B.** Coomassie stained gel. The predicted size of A2M with HIS tag is indicated at 53.9 kDa. IB: Insoluble protein.

6.2.2 Solubilisation of inclusion bodies

To solubilise the A2M inclusion bodies a culture of 200 mL TB was grown at 37°C and left to express overnight. From this culture 16 samples standardised to 10 OD_{600} units were harvested, and the insoluble fraction taken. Insoluble protein was washed and then resuspended in a range of different buffers (see Table 12) and left stirring overnight at room temperature. Buffer composition contained increasing amounts of urea (2-8 M) and pH ranging from 6-12. In high concentrations urea unfolds proteins secondary structure by disrupting hydrogen bonds and hydrophobic interactions facilitating insoluble proteins into solution. The upper limit of urea was chosen based on the limitations of the purification column and guidelines for rabbit immunisation. The pH range was chosen due to the proteins predicted isoelectric point (pI) which is 6.47, since proteins are less stable in buffers which are within 1 pH unit of their pI. Small concentrations of DTT were also added but concentrations above 5 mM could not be used since this can interfere with purification which was required prior to immunisation for generation of the antibody. Samples were centrifuged after being left overnight stirring in their buffer and the soluble and insoluble fractions loaded and visualised by immunoblot. From the immunoblots (Fig. 37) it appears as though the ideal buffer composition appears to be with 4 M urea at pH 12. Most of the other buffer conditions resulted in partial or no solubilisation of the protein.

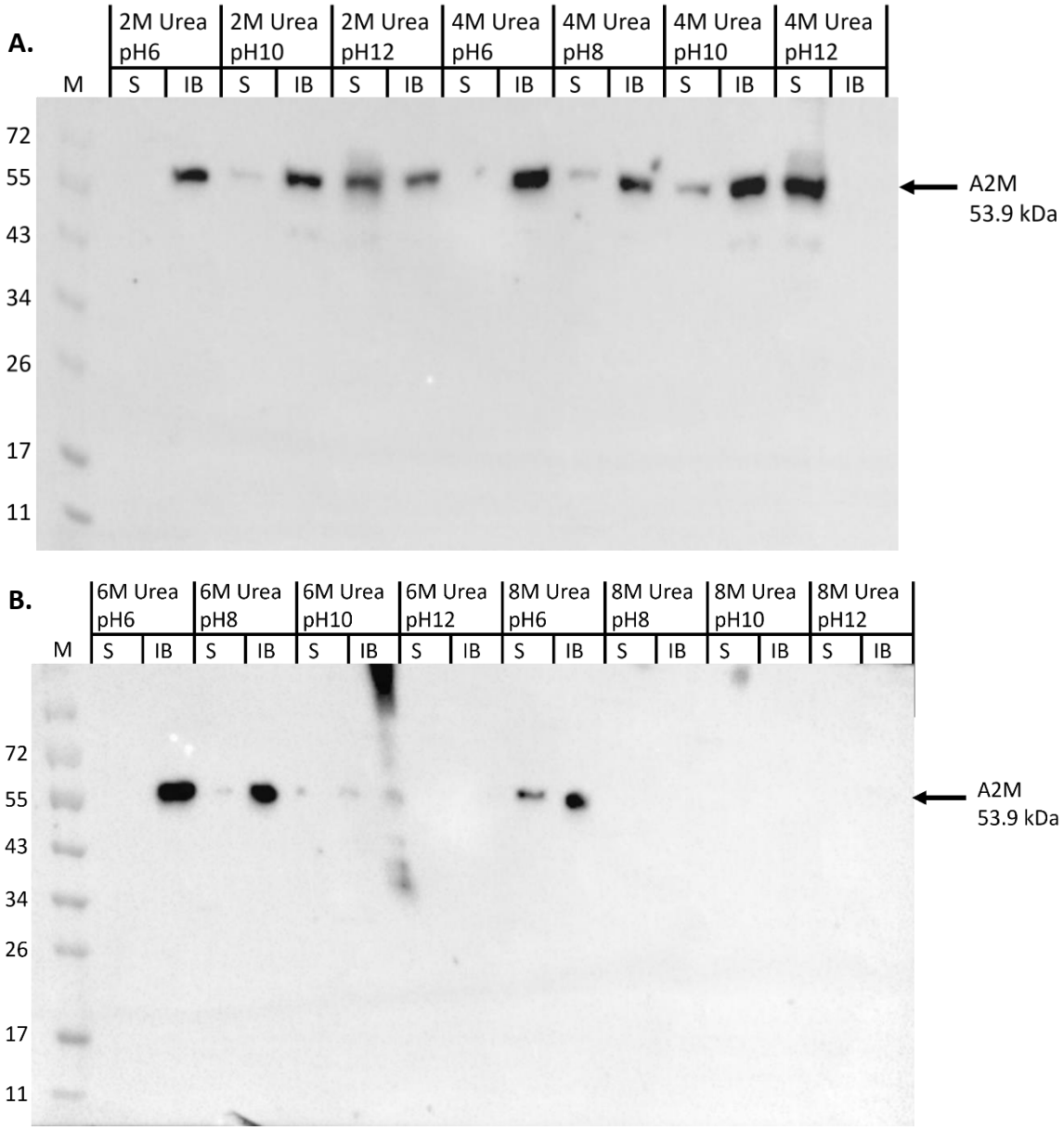


Figure 37. Small scale resuspension of insoluble A2M. Cultures expressing PEXT22 A2M were grown and expressed at 37°C in TB medium. Cells were harvested 3 hours post induction with the insoluble fraction being taken. Insoluble fractions were washed and resuspended in a range of buffers containing 2-8 M urea, 1 M NaCl, 5 mM DTT, at pH 6-12. Details of the buffers used can be seen in Table 12. Samples were left overnight stirring at room temperature. The following morning samples were centrifuged, and the pellets were resuspended in the same buffer used to solubilise the inclusion bodies. Samples were then separated by SDS PAGE and visualised by anti-HIS western blot **A.** Buffers ranging from 2-4 M Urea, pH 6-12 **B.** Buffers ranging from 6-8 M Urea, pH 6-12. The predicted size of A2M with HIS tag is indicated at 53.9 kDa. S: Soluble protein. IB: Insoluble protein.

6.2.3 Purification of soluble Alpha-2-macroglobulin

After the right buffer conditions (4 M urea at pH 12) were identified the purification of A2M could be carried out by IMAC. IMAC was chosen due to the presence of the C-terminal His6 tag and the

high levels of purity which can be achieved. A 400 mL culture of A2M expressing *E. coli* was first grown in TB and induced. The following morning the cells were harvested, lysed, and processed into insoluble and soluble protein. The insoluble protein was resuspended in the solubilisation buffer identified in chapter 6.2.2 and left overnight. The following morning the sample was centrifuged, and the soluble protein purified by IMAC. The immunoblot in figure 38A shows that protein is produced, and the majority is solubilised as no protein at the correct size is left in the inclusion body (IB) fraction. Most of the protein appears to bind to the column and is not washed off with no protein at the correct size being detectable. The protein is eluted off in all fractions with more being present in elution 2 and 3 (around 75-500 mM imidazole). Figure 38B shows that the fractions contain some contaminating bands of different sizes however, proteins of different sizes are also detected on the immunoblot (Fig. 38A) suggesting these may be degradation products although the band intensity on the immunoblot is much lower than that of the Coomassie stained gel. The wash fractions show little, or no non-specifically bound protein being washed off and increased imidazole concentrations could be used in the wash buffers to reduce the number of contaminating bands in the elutions.

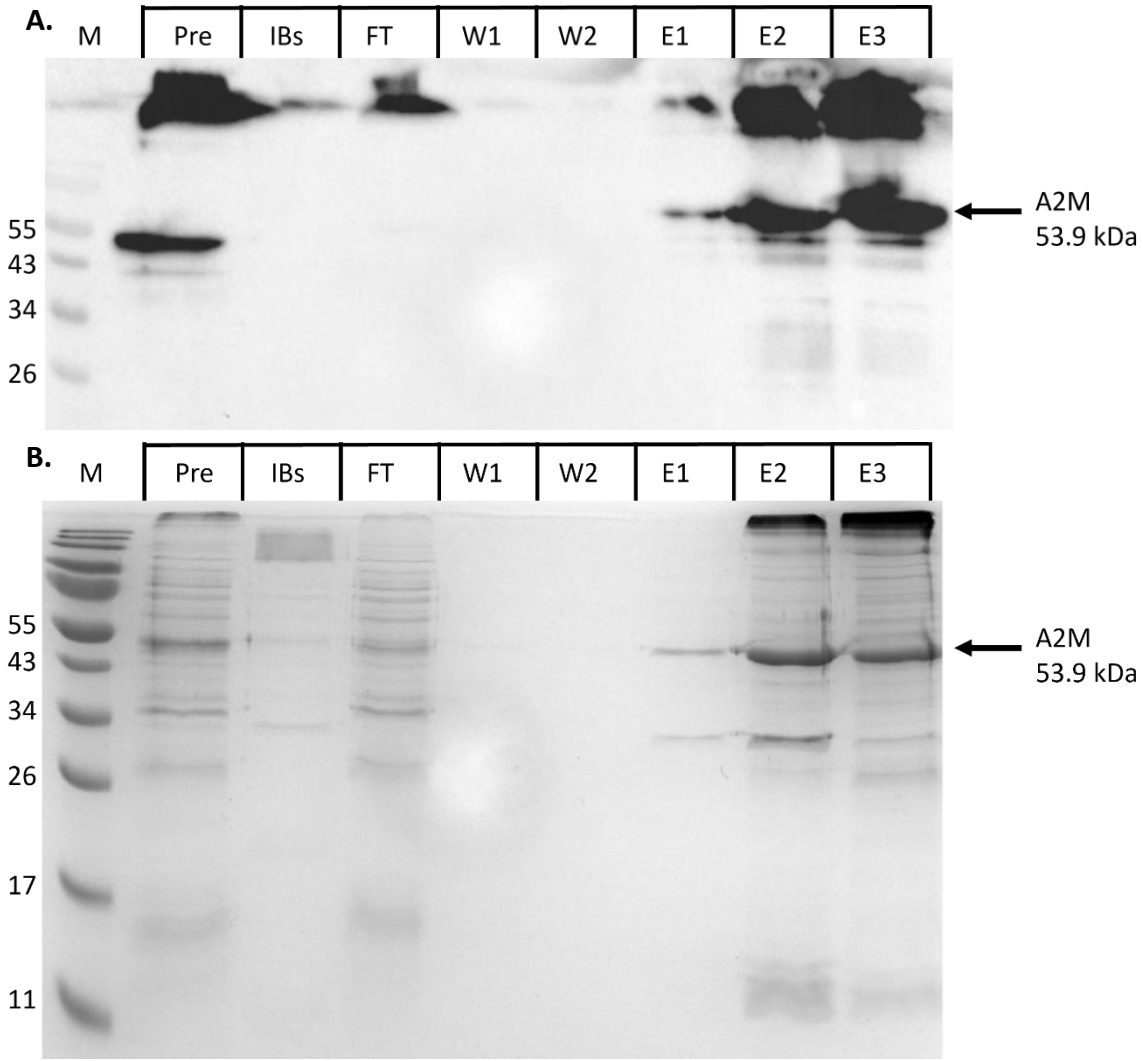


Figure 38. Purification of solubilised A2M protein. Solubilised protein was purified by IMAC using an AKTA automated purification system fitted with a GE LifeSciences 5 mL HisTrap HP pre-packed column. The sample was run through the column and the flow through collected proceeded by wash steps carried out using 40 mM imidazole. Protein eluted stepwise using 3 different concentrations of imidazole being 75 mM, 100 mM, and 500 mM respectively. Following purification proteins were separated by SDS PAGE and visualised by **A.** Anti-His western blot & **B.** Coomassie staining. The insoluble protein (IB) from the preceding solubilisation step was also loaded. The predicted size of A2M is indicated at 53.9 kDa. Pre: Soluble pre-purification cell extract. IB: Insoluble protein pre-purification. FT: Column flow through. W: Wash. E: Elution.

Figure 38 shows a protein at a much higher molecular weight which was not seen in previous experiments. This protein could be an aggregate of the A2M protein since it is still detectable with the His6 tag. Regardless the presence of this larger protein was considered not ideal, and it was decided that removal of this might be achieved by size exclusion chromatography (SEC). Prior to SEC the samples pH was adjusted to between 7-7.5. A 500 µL purified protein sample was loaded

onto a Superdex 200 Increase 10/300 column. The sample was then eluted into 16 1 mL fractions and visualised by SDS PAGE and staining with Coomassie blue to determine purity. Figure 39 shows the A2M protein was eluted in fractions 6-10, with fractions 6-8 showing that the large aggregate appears to still be present. It is likely that the aggregate is an artifact of the protein being produced in *E. coli* and is unlikely to be present in the plasma samples from the animal. Given this and the fact that the fractions from the SEC appeared to have fewer contaminating bands, these samples were used for generation of an antibody to A2M.

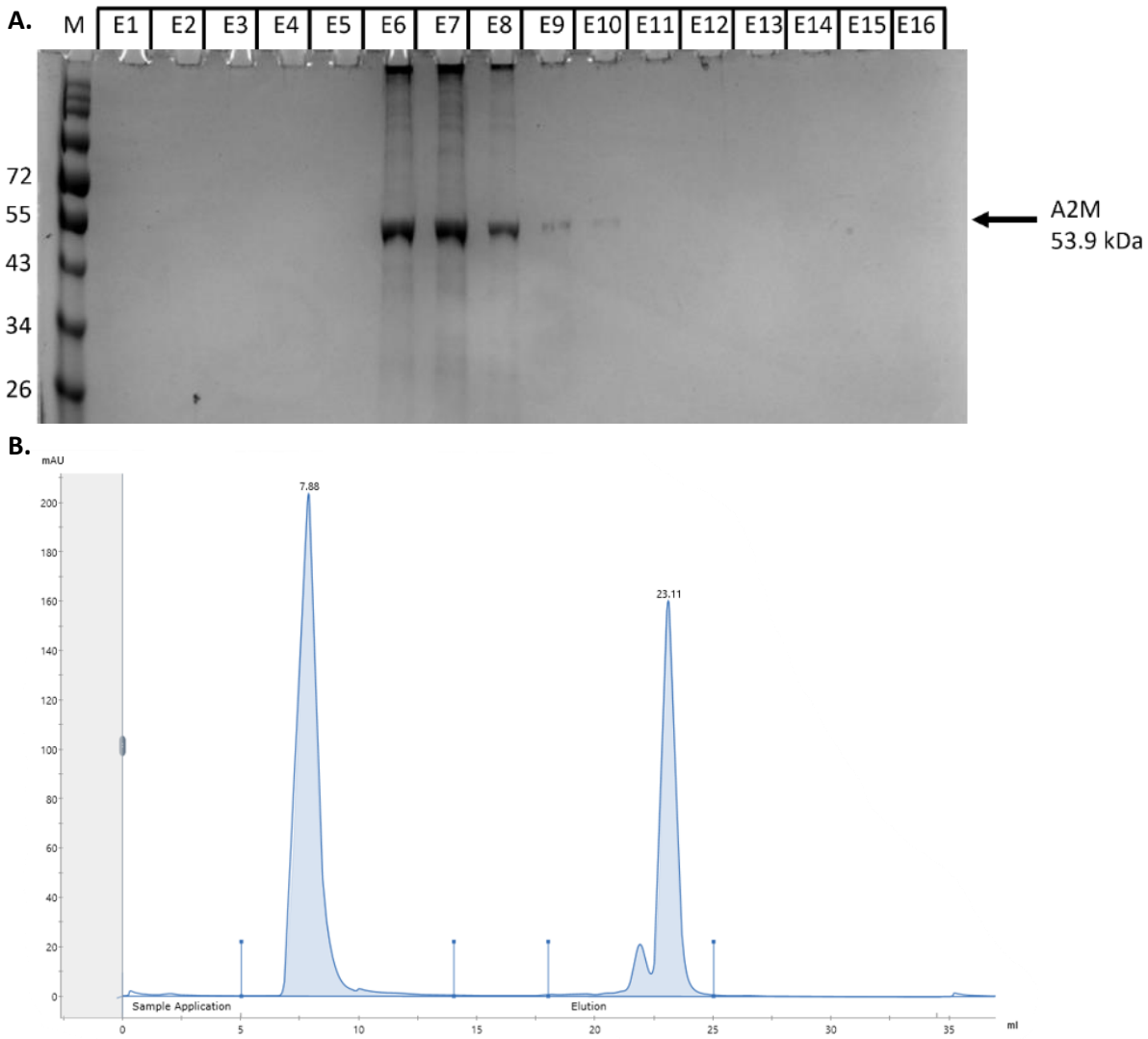


Figure 39. Size exclusion chromatography of purified A2M samples. 500 µL of purified protein was loaded onto a GE LifeSciences Superdex 200 Increase 10/300 column. The sample was then eluted into 16 1 mL fractions in the resuspension buffer. Following purification proteins were separated by SDS PAGE and visualised Coomassie staining. The predicted size of A2M is indicated at 53.9 kDa. E: Elution. **A.** Coomassie-stained gel of elution sample. **B.** SEC profile.

6.2.4 Generation and testing of an Alpha-2-macroglobulin antibody

For generation of a specific antibody to A2M purified protein samples were transferred to Cambridge Research Biochemicals. Rabbit immunisation was carried out on two rabbits, L66 and L67, 0.2 mg of the purified protein was used to immunise each rabbit on weeks 0, 2, 4, 6 and 8. Serum samples were taken on weeks 0 (pre-immune control), 3 (test bleed 1), 5 (test bleed 2), 7 with the final harvest bleed being taken on week 10 (production bleed). Samples of test bleeds were transferred for testing of antibody specificity by immunoblot. Serum samples were made in a dilution of 1:5700 and tested with lysate from *E. coli* cells producing A2M. A control immunoblot was also run with Anti-His6 antibody to confirm presence of the protein. A control of hGH-His6 was also loaded to test for any specificity generated to the His6 tag on the C-terminus of the protein. Figure 40 shows the results from the serum samples of L67. Protein was shown to be present in the Anti-His6 immunoblot (Fig. 40A). Pre-immune serum (Fig. 40B) showed no detection and appears to have no non-specific binding to the A2M protein. Binding to the A2M protein increased over the subsequent test bleeds, test bleed 2 (Fig. 40D) showed binding to both the A2M at the given size of 53.9 kDa and the larger aggregate. The final production bleed seemed to have little specificity to the larger aggregate but binding to the correct size A2M remained. Lack of binding to the hGH protein in any of the test or production bleed demonstrated that the antibody was specific to A2M and showed no affinity to the His6 tag on the C-terminus of the protein. Successful generation of an antibody specific to the A2M protein from *Epinephelus fuscoguttatus* allowed for further experiments to be carried out to determine if A2M protein could be used as a disease resistant biomarker.

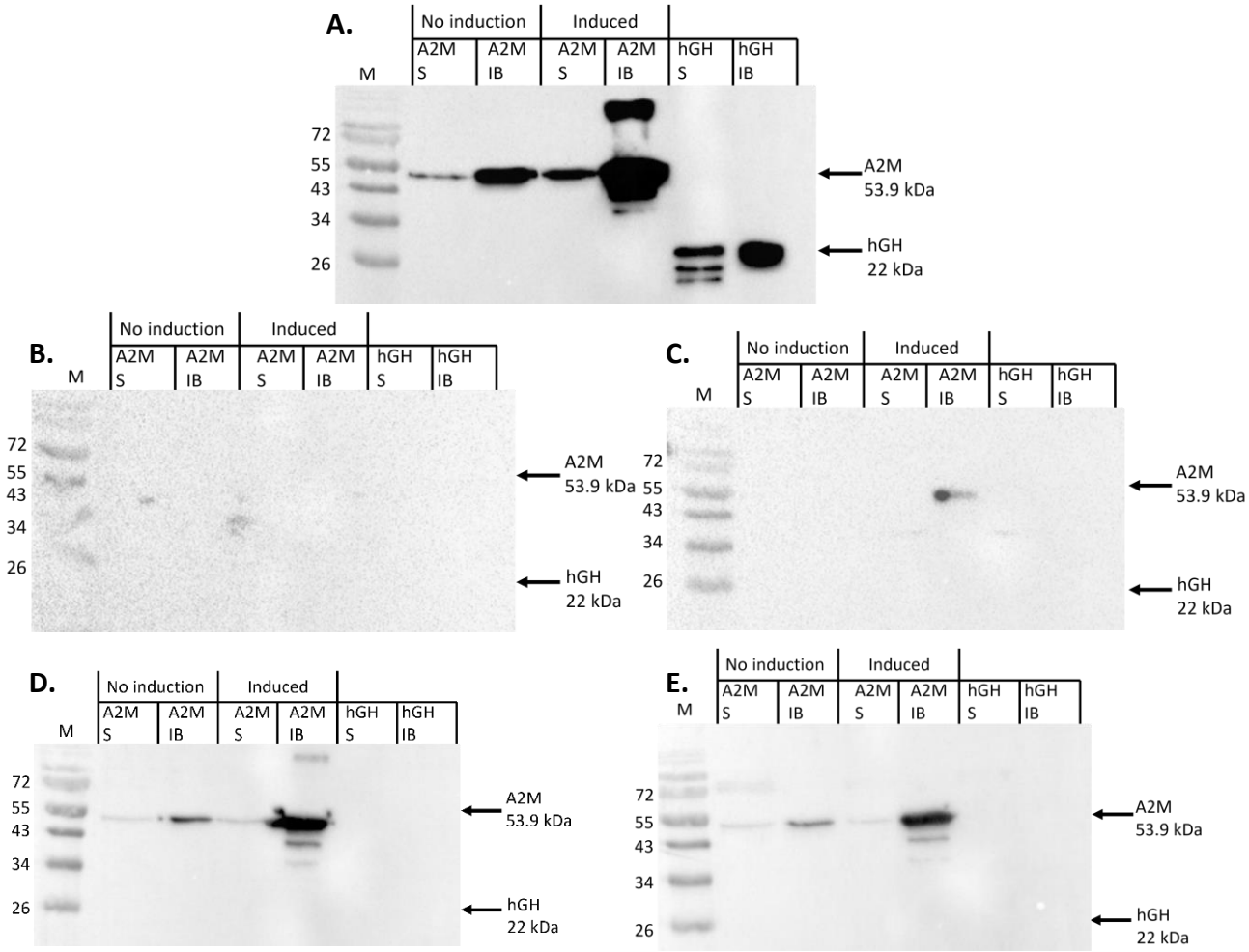


Figure 40. Immunoblots using serum from L67 rabbit immunised with A2M protein. Samples of uninduced A2M, induced A2M and induced hGH were fractionated into soluble and insoluble fractions and loaded. Each sample was normalised to 10 OD600 units of protein from *E. coli* lysate. Immunoblots were blocked with 5 % milk in PBS-Tween and incubated with the stated antibody at a dilution of 1:5700. After incubation blots incubated with a secondary antibody. **A.** Anti-His6 antibody **B.** Pre-immune bleed **C.** Bleed 1 **D.** Bleed 2 **E.** Production Bleed. The size of A2M and hGH are indicated at 53.9 kDa and 22 kDa respectively. S: Soluble protein. IB: Insoluble protein.

6.2.5 Determination of Alpha-2-macroglobulin as a disease resistant biomarker in *Epinephelus fuscoguttatus*

After generation and testing of an A2M specific antibody this was transferred to the collaborators in Malaysia where it was used to determine if expression of A2M was higher in serum of infection resistant fish. Fish were infected with *V. parahaemolyticus at* a concentration of 5.0×10^{11} CFU/g fish. Survival rates were used as a measure of disease resistance as it was decided it was the best overall indication of host-pathogen interaction. After initial infection and characterisation of disease by clinical signs and mortality, serum samples were taken, and a Jess assay was carried out

by a private lab (Biomed Global, Malaysia) and statistical analysis was carried out using software by collaborators Universiti Putra Malaysia. The jess assay is an automated system which separates and probes proteins with a specific antibody in a capillary system and works using the same principles as traditional SDS PAGE and immunoblot. Samples for the Jess assay were standardised by total protein. The results of the Jess assay can be seen in Figure 41. Binding of the antibody at approximately 58 kDa was determined to be the A2M protein confirmation of which was shown by presence of the positive control, purified A2M protein derived from *E. coli* (Fig. 41A and 41C). The band intensity of the infection susceptible fish (Fig. 41B) appeared to be lower than that of the band intensity in infection-resistant fish (Fig. 41C). The difference in band intensity is directly correlated to the amount of A2M protein present in the samples, suggesting that expression of A2M is higher in fish which are more resistant to infections of *V. parahaemolyticus.* This difference in expression can be more clearly seen in the representative samples taken (Fig. 41A), where samples from infection susceptible fish from lanes 10, 11 and 12 show a much lower level of protein compared to the samples from infection resistant fish, from lanes 15, 20 and 23.

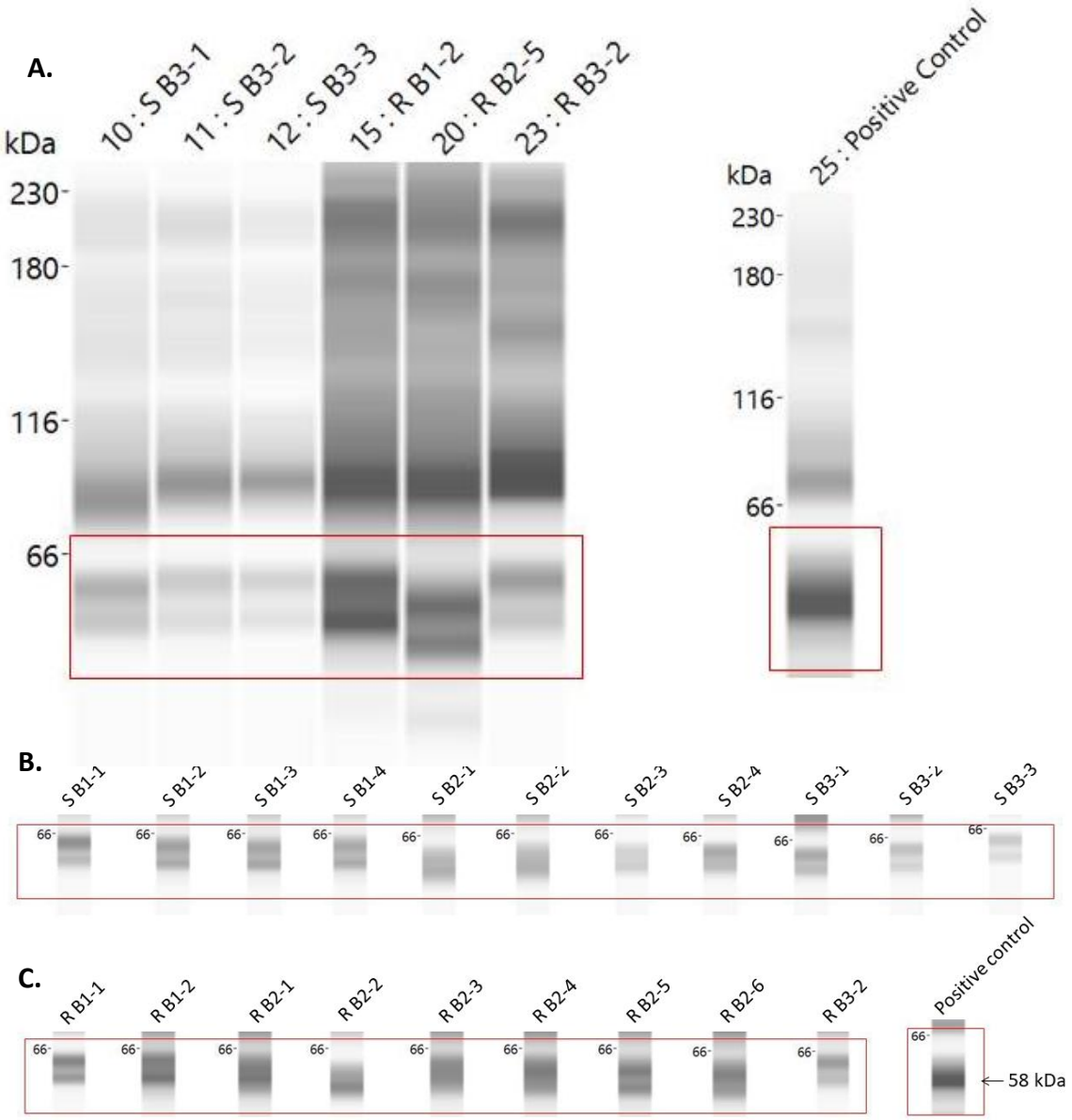


Figure 41. Jess assay images demonstrating A2M expressed in infection-resistant and infection susceptible fish. Jess assay was carried out using the specific antibody for A2M generated in Chapter 6.2.4. Plasma samples tested were from infection-resistant (N = 9) and infection susceptible (N = 11) fish. **A.** Representative samples of infection-resistant fish and infection susceptible fish. **B.** All infection susceptible samples tested. **C.** All infection-resistant samples tested. Positive control used was the A2M derived from *E. coli.* Size of A2M: around 58 kDa. R: Infection-resistant fish. S: Infection susceptible fish.

6.3 Conclusions

After cloning the codon optimised A2M protein from *Epinephelus fuscoguttatus* into a pET23/ptac vector the protein was found to be expressed insolubly. Since soluble protein is required for purification and generation of an antibody, different temperature and strains of *E. coli* were used in an attempt to express soluble protein. Finally, after cloning the gene into a single copy vector and trialling different growth medium it was decided to solubilise the protein in vitro using different buffer conditions. The buffer pH range was chosen based on the theoretical pI of the protein, along with an increasing concentration of urea. The ideal buffer conditions were found to be 4 M urea at pH 12, partial solublisation was also seen at 2 M urea, pH 12, interestingly increasing amounts of urea showed no protein on the immunoblot suggesting degradation of the protein.

Once conditions for solubilising the protein were determined the protein was able to be purified by IMAC. Purification revealed a band at a high molecular weight which was not seen in previous expression of the protein, this protein was thought to be some kind of aggregate as it is still visible on the immunoblot. SEC was carried out to remove the protein leaving only the purified A2M, however, the high molecular weight band was still visible even after SEC. Despite the lack of success, the SEC fractions did appear to contain fewer contaminating bands than purification by IMAC alone. As such, SEC fractions were chosen to be carried forward for generation of the antibody.

The serum samples received from Cambridge Research Biochemicals were tested by immunoblot, with an uninduced A2M control, hGH-His6 control and the A2M induced samples. The controls showed no specificity of the antibody to endogenous *E. coli* proteins which may be present in the samples transferred as well as no specificity to the C-terminal His6 tag on the protein. Bleed 1, 2 and production bleed did show specificity to the A2M protein along with the high molecular weight aggregate with increasing intensity as subsequent immunisations were carried out.

Finally, the specific A2M antibody was transferred to collaborators at the Universiti Putra Malaysia where it was used in a Jess assay with infection sensitive and infection-resistant fish serum. A2M expression was seen to be correlated with disease resistance in brown marbled grouper and as such could be used as a biomarker for disease resistance.

Breeding programs can produce fish which are more resistant to pathogens reducing the dependence on antibiotics and vaccination and ultimately increase productivity. Production of the specific antibody seen in this work could allow for quick detection of disease resistant fish and could easily be applied in a more practical and high throughput method by using ELISAs.

Chapter 7: Discussion

The introduction outlined the substantial growth that has been occurring in the aquaculture sector over the past decades and its importance for providing a nutritious food source as well as the economic value of the sector, especially in SE Asian countries who make up some of the largest producers of aquaculture products. The growth of the aquaculture sector is, however, restricted by constant disease outbreaks which are becoming more difficult to manage due to the prevalence of certain pathogens and the trend of increased antimicrobial resistance found in certain pathogens.

This research aimed to develop new approaches to vaccination including administration of vaccines orally as a solution to more traditional delivery methods such as injection or immersion. Chapter 3 looked at producing both purified soluble and insoluble GapA protein using *E. coli* as a host. Growth of cells and expression of protein was trialled in small scale fed-batch fermentation to see if this process would be viable using techniques which are commercially relevant. Later both forms of protein were administered orally to fish which were challenged with *S. agalactiae* to determine its effectiveness as a vaccine candidate when used in this way. Chapter 4 looked to build further on the previous chapter's work by expressing the same GapA in the chloroplast of *C. reinhardtii* cells. Optimisation of protein expression was carried out to find the best growth conditions for protein production. Cells were grown at a larger scale and freeze dried for use in any subsequent challenge trials that might occur at a later date. Freeze dried cells containing the protein were stored at different temperatures and regularly checked for presence of the protein to gain some insight into the stability of the protein in dried biomass over time.

Chapter 5 aimed to expand on work in dsRNA expression in *C. reinhardtii* chloroplasts to treat viral infections in shrimp by utilising RNAi. The new dsRNA construct was compared directly with the previously analysed (and published) construct with growth, extraction, and quantification of dsRNA of the same target being carried out in the same conditions. Two new strains were generated which

targeted two different genes in the WSSV virus and produced more dsRNA than the previous target (RdRp). The new strains were tested in a challenge trial where shrimp were fed microalgae which were then subjected to a viral challenge. Finally, chapter 6 looked at an alternative strategy for the use of biomarkers for potential improvements in selective breeding. Alpha-2-macroglobulin, a potential resistance marker to *V. parahaemolyticus* infections in *Epinephelus fuscoguttatus*, was expressed in *E. coli*, solubilised, and purified. After purification an antibody was generated and used to determine levels of A2M in the serum of both infection susceptible and infection resistant fish.

7.1 Expression of GapA in *E. coli*

The GapA protein was initially cloned into a pET23/pTac vector which expressed in W3110 and BL21 cells at different temperatures. Protein expression was visualised using immunoblots which indicated that the optimal conditions for soluble protein were at 37°C in W3110 cells. Shake flask experiments were followed by experiments using fed-batch fermentation. Fed-batch fermentation had two different aims, to evaluate the growth and expression of the protein in a commercially relevant system and to provide enough soluble protein which could be later used in challenge trials. Results showed that the cells grew well over a three-day period reaching a final OD_{600} of 134, with soluble protein being detected over the course of the experiment. The soluble protein was also able to be purified by IMAC, with little contaminating proteins being observed. IMAC was chosen due to its specificity in purification, with it being possible to provide 95% purity in samples (Burden and Whitney, 1995)**.** However, IMAC can be an expensive purification method and a more cost effective solution such as ion exchange (IEX) which enables for a higher throughput with a relatively pure product**,** with purities of >90% being able to be achieved (Kuo *et al.*, 2016), should be considered for any future applications.

Insoluble protein/inclusion bodies were formed through the use of a high expression T7 promoter, along with high cell densities achieved in the baffled flasks and rich TB media. Although there are other methods to induce the formation of inclusion bodies this seemed convenient as

overexpression of the protein would likely produce higher overall protein yields than soluble protein. Large scale production of inclusion bodies for subsequent challenge trials was carried out using shake flask expression. Later it was discovered that inclusion bodies were readily formed in fed-batch fermentation by adjusting the growth and expression temperature to 37°C when using the new T7 construct, providing a much quicker and convenient method for producing large quantities of the protein. Finally challenge trials were carried out by feeding both the soluble and insoluble forms of the GapA protein along with the animal's regular diet before later being challenged by immersion. These results showed that both forms of the protein were able to increase survival rates of the fish 7 days post challenge when compared to the control group. The insoluble form of the protein appeared to be effective at protecting the animal with survival being 75.6% compared to 51.1% in the group fed soluble protein. This may be due to the fact that insoluble proteins are more stable than their soluble counterpart, which makes them less susceptible to degradation in the aqueous environment and the harsh conditions of the gut. Experiments to test this could be carried out by simulating the same conditions of a fish stomach *in vitro* and comparing the degradation of both forms of the protein side by side. Usually inclusion bodies are seen as undesirable by-products of recombinant protein production and there is a large amount of research carried out in preventing the formation of inclusion bodies (Bhatwa *et al.*, 2021). However, there is a growing interest in the potential value of using inclusion bodies as therapeutic agents (Rinas *et al.*, 2017). One study investigated formation of cytokines for inclusion bodies to combat their instability and short half-life in their soluble form (Torrealba *et al.*, 2016). In this study it was found that when expressed as inclusion bodies the protein was able to resist harsh pH and temperature conditions and stimulated an immune response when delivered by injection. It does appear as though inclusion bodies can release functional proteins that can be utilised once uptake has occurred (Liovic *et al.*, 2012).

Inclusion body fractions were less pure than the soluble protein and showed contaminating bands (Fig. 6). The proteins in this fraction will be made up of other insoluble proteins including

components such as lipopolysaccharides (LPS) which can lead to endotoxin immune responses within humans. The presence of the LPS could, however, lead to stimulating the immune response and effectively act as an adjuvant, especially in fish which are more resistant to endotoxin shock (Swain *et al.*, 2008). The long-term effects of using inclusion bodies as an oral therapeutic would require further research, if proven to be a problem there are alternative methods for production of these proteins. There are already commercially available strains of *E. coli* which do not produce LPS (Mamat *et al.*, 2013), alternatively Gram-positive strains could be investigated as production platforms (Ilk *et al.*, 2011; Jørgensen, Vrang and Madsen, 2014) or even yeast production platforms such as *Saccharomyces cerevisiae* or *Pichia pastoris* which are GRAS (Kulagina *et al.*, 2021).

A wide range of techniques have been employed for vaccination against *S. agalactiae* in Tilapia with varying success. Injectable vaccines are the most common, with a commercial vaccine being available "AQUAVAC Strep *Sa*" consisting of inactivated *S. agalactiae* which provides a relative percent survival (RPS) of 90% 7 days post challenge and 85% 14 days post challenge (MSD, 2012). The results in the study conducted in chapter 3 gave an RPS of ~51.1% when using the soluble antigen and ~75.6% when using inclusion bodies 7 days post challenge. It is difficult to compare the protective effects of different vaccines without carrying out challenges trials directly comparing the methods side-by-side. The age of the animals, whether they are juvenile, or adult can influence their susceptibility to infection, along with both the method and bacterial load of the challenge. For instance, the challenge trial carried out in chapter 3 used an immersion method for challenging the animals, whereas other studies choose to challenge via intraperitoneal injection. Immersion exposes the fish to the pathogen in a way which they are more likely to encounter the pathogen on a farm, whereas injection offers a more consistent bacterial load to be administered. With that being said, the oral vaccine provided a high level of protection to the animal, which offers a simplified and ultimately cheaper way to protect animals from infection in aquaculture. The protective effects of the oral vaccine may be improved with optimisation of the dose and dosage time which are important factors to consider (Dubey *et al.*, 2016). A prolonged trial should be

carried out to see if the protective effects last in the animal and to observe any other effects on the animal, though in AQUAVAC's trial mortality in the vaccinated group only increased by 5% between days 7-14 post challenge. Overall, a method for production of an oral vaccine which provides high levels of protection, requires very little downstream processing, and is convenient to administer, has been demonstrated here.

7.2 Expression of GapA in *C. reinhardtii*

This chapter aimed to explore microalgae as a method for generation of antigens which could then use the intact cells containing the protein as a delivery vector to the animals for vaccination against disease. The use of intact cells to deliver the antigen would reduce downstream processing costs and microalgae could potentially give an additional immune boost to the animal. The same GapA protein was chosen allowing for comparison between the system in bacteria and microalgae. GapA was first codon optimised for production in the chloroplast of *C. reinhardtii* and transformed into the cells using restoration of photosynthesis as a selection marker, omitting the need for antibiotic resistance genes which are seen as undesirable. Due to the polyploid nature of the chloroplast in *C. reinhardtii* homoplasmic integration of the gene was first confirmed through rounds of streaking on minimal media to force the cells to grow autotrophically, which was confirmed by PCR. Once a homoplasmic strain was generated, expression of GapA was visualised by immunoblot and the protein was found to be expressed largely as a soluble protein. Protein expression was quantified by densitometry and found to be ~18 mg/L of culture. Optimisation of growth conditions revealed that protein production favoured low light level at 20 µmol photons m^{-2} s^{-1}, although unsurprisingly higher growth was seen in the higher light level conditions 50 and 145 µmol photons m^{-2} s^{-1}. Growth of the new GapA strain and a photosynthetically restored strain (CCK10) were then assessed at a larger scale using hanging bags. Production of GapA did not appear to impede growth of the culture with a relatively similar OD_{750} being seen throughout the growth, although the GapA-producing strain did produce slightly less biomass at 29.72 g compared to strain 32.58 g in CCK10. Large scale

cultures were centrifuged and lyophilised to preserve the biomass for use in later experiments. The lyophilised biomass was subjected to immunoblot to confirm presence of GapA. This raised the question of how long GapA was able to be preserved in the biomass before it could be used, as such a GapA stability trial was carried out to assess the shelf life of the product. Lyophilised biomass was stored at room temperature, 4°C and -20°C for up to 54 days and cells were periodically checked for presence of GapA by immunoblot; GapA was still detectable in all samples up to 54 days. Some degradation may have taken place because although the exact concentrations of protein were not measured, there is a reduced signal in the immunoblot after 14 days of storage. Freeze drying works effectively in smaller scale experiments however for larger scale alternative methods of drying the biomass may be required. One such technique is spray drying, a method which atomises slurry which can be obtained from centrifugation and rapidly dries the solution in hot air. Spray drying has been shown to be a viable technique for the drying of microalgae which have produced an antigen able to vaccinate fish (Vilatte *et al.*, 2023). This study showed that the drying method resulted in no significant loss of the protein. However, when stored for more than 27 months at 4°C and room temperature, degradation of the protein was observed resulting in losses of 50% and 92% respectively. Although it is likely that dried algal feeds would be used more rapidly, cold-chain transport and storage of the product may be necessary to ensure proteins remain intact, especially when considering the use in South East Asian countries where ambient temperatures are often higher.

The application of microalgae has previously attracted attention as a vaccine production and delivery system for use in terrestrial animals and human diseases. Microalgae have shown potential applications in vaccination against the foot-and-mouth disease virus (FMDV), the classical swine fever virus (CSFV) along with infections of *S. aureus* (Sun *et al.*, 2003; He *et al.*, 2007; Dreesen, Hamri and Fussenegger, 2010). However, there is also scope for production of other recombinant proteins for use in aquaculture. For instance, a fish growth hormone has been successfully expressed in the microalgae *Nannochloropsis oculata*. The transgenic microalgae were first incubated with artemia

to allow for digestion of the cells. After incubation with the microalgae for 6 hours the artemia were fed to red-tilapia larvae over a 4-week period. The artemia which were incubated with the microalgae producing the fish hormone had a weight gain of 316% compared to the control's weight gain of 104% (Chen *et al.*, 2008). These studies demonstrate ways in which microalgae could be a useful tool within the aquaculture industry.

Microalgae share many of the advantages of plant systems, including being able to generate biomass from light and relatively cheap resources but they also have unique advantages such as not being restricted to seasonal growth, can be contained in large bioreactors and are more easily processed. Many green microalgae are 'Generally Regarded As Safe' (GRAS) and as such can be used directly in feed without any further purification or other processing. In addition to this, *C. reinhardtii* contains a large chloroplast which occupies around half the volume of the cell (Engel *et al.*, 2015) compared to higher plants which have hundreds of chloroplasts, therefore generating stable homoplasmic strains of *C. reinhardtii* is much easier. However, even though work is being carried out to improve protein yields, the low yields of protein are a major hurdle for implementation microalgae (Taunt, Stoffels and Purton, 2018). Thus, it is important to assess its effectiveness in comparison to more widely used protein production platforms such as bacteria, since the protein may be degraded less when encapsulated in the cell and still be able to deliver an effective dose. Consequently, challenge trials which compare the performance of the GapA producing *C. reinhardtii* would need to be carried out in the same way as chapter 4 to determine the effectiveness of this strain at protecting the animals from infections of *S. agalactiae*. It would be important to include a wild type *C. reinhardtii* strain to control for any beneficial health effects of the microalgae itself, as has been previously discussed, some strains of microalgae could function as an immune stimulant. Here, it has been demonstrated that microalgae are capable of recombinant protein production which can be cultured at a large scale without the need for expensive equipment, they are able to be easily harvested and dried preserving at least some of the protein for up to 54 days at room temperature. The methods developed and used in this study,

if effective, could significantly reduce costs associated with vaccine production, storage, and administration.

7.3 dsRNA expression in *C. reinhardtii*

The aim of this chapter was to improve upon work which involves the use of RNAi as a tool to prevent viral infections in shrimp. Previous work had demonstrated that dsRNA can be produced and accumulate in the chloroplast of *C. reinhardtii*. A new purpose-built dsRNA construct was used that contained promoters which have higher transcriptional levels than the previous promoters. The yield of dsRNA from each construct was tested directly against each other, using the same target sequence (YHV RdRp), to assess whether there were improvements in the dsRNA levels. Quantification using qPCR revealed that dsRNA levels were lower than that of the previous construct being 53.06 ng/L and 61.43 ng/L, respectively. Despite this, the new system did provide a more convenient method for generation of new constructs and so was implemented in the generation of two new strains which produced dsRNA targeting VP9 and a fragment of ORF366 from WSSV. These strains were able to produce much larger amounts of dsRNA than the other strains with dsVP9 producing 3.86 µg/L and dsORF366 producing 11.63 µg/L. Yields of dsRNA were variable and appeared to be target dependent. As previously mentioned, this could be due to the length of the dsRNA, with it being previously found that shorter dsRNA could accumulate in higher amounts that longer dsRNA (He *et al.*, 2020). There may also be other factors which influence dsRNA production aside from length, the accumulation of dsRNA could be sequence dependent with GC contents also playing a role in overall stability of the dsRNA. Another issue to consider would be any secondary structures formed by the ssRNA which would impair its ability to bind to its complementary strand and therefore be unable to produce dsRNA. Further investigations into the nature of dsRNA formation would be required so the more appropriate sequences can be chosen for production.

The challenge trials provided results which were conflicting with results seen in other publications. The dsORF366 strain and the "wild type" strain performed similarly giving a survival of 70% and 73.5%, respectively, 14 days' post challenge. In the "wild type" strain containing a dummy dsRFP cassette, the dummy dsRFP may have upregulated RNAi machinery in the animals which then aided the ability of the shrimp to use this mechanism to knockdown viral genes. Upregulation of RNAi machinery by non-specific dsRNA has been previously observed. For instance shrimp injected with dsGFP were found to have upregulation in proteins related to the immune response including those involved in the RNAi pathway for instance Argonaute 2 and Dicer 2 (Maralit *et al.*, 2015). Shrimps injected with dsGFP also exhibited an increased survival when challenged with WSSV, compared to those injected with PBS as a control. Because of the upregulation of RNAi from non-specific dsRNA it would be expected that dsORF366 would not only upregulate RNAi proteins but also contribute to viral gene knockdown in a sequence specific manner. However, little difference could be seen between the wild type and dsORF366 treated groups. The dsVP9 strain performed the worst of all strains with only 30% survival 14 days' post challenge being lower than that of the positive control which had a final mortality of 53%. It is unclear why dsVP9 from microalgae would elicit an increased mortality in shrimp when previous experiments using the same sequence of purified dsVP9 show a protective effect in shrimp (Alenton *et al.*, 2016a). The use of oral treatments and challenges could be sources of significant error. Shrimp will feed at different rates and therefore take up different amounts of both the microalgae and infected shrimp tissue giving an unequal distribution of both virus and treatment to each animal. Unsurprisingly it has been shown that a significantly higher dose is required when challenging shrimp orally with WSSV compared to challenges using an immersion or injection method (Satoh, Nishizawa and Yoshimizu, 2008). Given that a challenge through injection in post-larvae *P. vannamei* would be impractical due to their small size it may be better to use an immersion method for challenge which may allow for less variability in viral load infecting each animal. The experiment used liquid algal feed which was prepared in advance of the trial and left at 4°C until used. The effects of storing the algal solution

at 4°C on dsRNA concentration were not carried out prior to the challenge trial and could have negatively impacted the amount of dsRNA within the cells. Overall, the data is inconsistent with previous experiments involving purified dsRNA and oral application of dsRNA produced in microalgae. The large error bars indicate a lack of precision in the experiment which could be explained by the methods of challenging. A repeat of the experiment should be carried out with stricter control over the preparation of algal feeds to ensure dsRNA is not degraded before being used in addition to this, a more controlled way of challenging the animal should be considered for more reliable data to be collected.

Most of the studies using dsRNA to protect shrimp from viral infections use injection of purified dsRNA, which is effective but ultimately impractical. Some cases of oral administration of dsRNA have been found to be effective using an attenuated strain of *E. coli* HT115 or nanoparticles to deliver (Sarathi *et al.*, 2008). The effects of feeding shrimp *E. coli* have yet to be investigated and is often undesirable and the plasmids used to produce dsRNA, contain antibiotic resistance cassette which could lead to horizontal gene transfer. In addition to this, oral administration of dsRNA usually does give a lower protection when compared to injection. It may be that dsRNA is somewhat degraded when given orally and higher doses might need to be given. Dosing of dsRNA is a key consideration and should be investigated more extensively for the best protection. In some studies, increasing dsRNA dose does provide better protection (Thammasorn *et al.*, 2015) however, another study did suggest that lower doses are more effective (Parenrengi *et al.*, 2021). Identification of the targeted genes should also warrant more research, the effect of dsRNA on preventing mortality in shrimp seems to be highly dependent on the specific viral gene targeted (Mejía-Ruíz *et al.*, 2011). This appears to be the case in chapter 5 which could explain the large difference between dsVP9 and dsORF366.

The reasoning behind using RNAi as a tool to treat viral infections in shrimp relies on the assumption that they lack a traditional adaptive immunity seen in vertebrates. Recently though, there is

increasing evidence that suggests an alternative adaptive immune system might exist in some invertebrates (Chou *et al.*, 2009; Chang *et al.*, 2018). This is backed up by examples of shrimp being administered inactivated virus, inactivated bacteria and recombinant viral antigens exhibiting some level resistance to infection when challenged with the pathogen (Witteveldt, Vlak and Van Hulten, 2004; Singh *et al.*, 2005; Patil *et al.*, 2014). In fact, *C. reinhardtii* has been able to produce the VP28 protein from WSSV, the cells containing the protein were then fed to shrimp and was successful at protecting them from the virus when challenged with the virus (Lanh *et al.*, 2021). The results from this study gave an impressive survival rating of 70% compared to 100% mortality of untreated shrimp 14 days' post challenge. The results also showed that the wild type strain also marginally decreased mortality, being 87.3%, which helps to verify the claim that microalgae are able to act as immunostimulants (Ma *et al.*, 2020). This showcases the potential of microalgae as a production and delivery platform for therapeutics in shrimp. Given that more molecular tools are available for protein production in *C. reinhardtii* and the ability for recombinant protein to offer protection to shrimp from certain pathogens, it may be more beneficial for further research to be directed towards vaccination rather than RNAi.

7.4 Alpha-2-macroglobulin as a disease resistant marker in *Epinephelus fuscoguttatus*

Selective breeding for desirable characteristics of plants and terrestrial animals has been conducted for generations, although the process is often long and tedious. With the onset of a wide range of molecular tools now available it is possible to pinpoint genetic biomarkers which are advantageous to the organism and as a result can correctly direct and increase the benefit of breeding programs. Traditional selective breeding programs in aquaculture have been hit and miss in generating populations of fish with the desirable trait. Although there have clearly been successful examples of selective breeding programs being able to generate stock with a number of beneficial traits (Embody and Hayford, 1925; Hussain *et al.*, 2002), there have also been several examples where

efforts have yielded no benefits (Hulata, Wohlfarth and Halevy, 1986; Teichert-Coddington and Smitherman, 1988).

The aim of this work was to investigate a disease resistant biomarker for brown-marbled grouper, which could be used as a tool for breeding programs to generate more robust stocks of fish. Previous literature had indicated that Alpha-2-macroglobulin could be used as a potential biomarker for resistance to *V. parahaemolyticus*. To be able to determine levels of A2M present in serum samples, an antibody to the protein was first generated. Expression of the protein was carried out in *E. coli* where it was found to be produced as an insoluble protein. Soluble protein is required for purification by IMAC. Several attempts were made to produce soluble A2M, by reducing temperature, changing growth media, and cloning of the gene into a single copy plasmid, none of which were successful. Since only enough soluble protein was required for generation of the antibody it was decided to solubilise the protein *in vitro*, and this was carried out by testing a range of buffers which had varying pHs and concentrations of urea. Once the correct solublisation buffer was identified the expression and solublisation of the protein could be carried out at a larger scale, after which purification by IMAC could be carried out. Post purification a protein at a large molecular weight became apparent on Coomassie stained SDS gels and anti-his immunoblots. Attempts to remove this band by SEC were unsuccessful, however generation of the antibody was still performed. The A2M antibody was tested and found to specifically bind to A2M expressed in *E. coli*. Once an antibody was generated and tested serum samples from disease-resistant fish and those susceptible to infection were evaluated by Jess assay, upon which it appeared that A2M expression was significantly higher in disease resistant animals, seemingly confirming A2M as a successful biomarker for resistance to *V. parahaemolyticus* in *Epinephelus fuscoguttatus*.

Generation and purification of the protein to be tested as a biomarker used well established methods allowing for a quick and convenient method to then produce the antibody, even when considering the requirement to first solubilise the protein. Once the antibody has been developed

and determined to be an appropriate biomarker for the desirable trait this method could easily be scaled up by using ELISA which could be carried out in 96 well plates. The use of a high throughput method and more directed method of determining if animals have the desirable trait required for selective breeding could decrease the time required for selective breeding programs while also increasing the effectiveness of such programs.

7.4 Future perspectives

It has been outlined here that the aquaculture sector is constantly threatened by infectious diseases that cause major losses in the industry. Currently the sector is developing slowly in comparison to farmed terrestrial animals which is partly due to the low value of the aquaculture products. As the sector expands it is critical to find cost effective solutions to control the impact of infectious disease especially in developing countries, where the industry is a key contributor to both economic and food security. This thesis has explored several different approaches which have shown some promise in increasing the survival rates of aquatic animals utilising tools from biotechnology. The use of bacteria as a recombinant protein production platform allows for production of low-cost proteins which can be applied orally, negating the requirement for skilled workers to inject each fish individually which, although effective, is expensive and labour intensive. The discovery that inclusion bodies may increase the effectiveness of the oral vaccine while simultaneously reducing the need for purification could be valuable in the future and should be further explored considering oral application of vaccines are highly sought after.

The use of RNAi to protect shrimp from viral infections shows some promise in a range of studies (Yodmuang *et al.*, 2006; Sarathi *et al.*, 2008; Alenton *et al.*, 2016b), however, the experiments carried out here exhibited mixed results. The discovery that shrimp may have some form of alternative adaptive immunity (Chou *et al.*, 2009) and that microalgae producing viral proteins may be able to vaccinate shrimp (Lanh *et al.*, 2021), could lead to new routes of protecting shrimp from viral infections. Using microalgae to produce proteins and using the dried biomass as an oral

administration method is an interesting concept and should be further explored. More work needs to be done on increasing protein yields since they are much lower than those of other cell factories. In addition to this the challenge trials using microalgae producing GapA should be carried out to see how effective this could be for any future use.

Selective breeding of aquatic animals has been undertaken for many years and is set to continue, with breeding efforts being directed at disease resistance aswell as increased growth rates. It is therefore very likely that more biomolecular tools will be implemented more to direct and speed up these breeding programs making them more efficient.

Overall, the data presented here has made some steps towards developing cost effective solutions to reducing the impact of infectious disease in aquaculture.

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Supplementary

DNA sequences

ORF366

AGGAAAATGACCTCTATGAAGAAGAACAAGAAAGGAGGAGACGTCGCTCATCAAAGATGGGGAAGATCC
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VP9

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RdRp

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GapA (*E. coli*)

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GGTCCGCATCGTGGTGGTGATCTGCGTCGTGCACGTGCCGGTGCAGCCAATATTGTTCCGAATAGTACCGG

TGCCGCAAAAGCAATTGGTCTGGTTATTCCGGAACTGAATGGTAAACTGGATGGTGCAGCACAGCGTGTT
CCGGTTCCGACCGGTAGCGTTACCGAACTGGTTGCGACCCTGGAAAAAGATGTTACCGTGGAAGAGGTTA
ATGCAGCAATGAAAGCAGCAGCAAATGATAGCTATGGTTATACCGAAGATCCGATTGTTAGCAGCGATATT
GTTGGTATTAGTTATGGCAGCCTGTTTGATGCAACCCAGACCAAAGTTCAGACCGTTGATGGTAATCAGCT
GGTTAAAGTTGTGAGCTGGTATGATAACGAAATGAGCTATACCAGCCAGCTGGTTCGTACACTGGAATATT
TTGCCAAAATCGCCAAACATCATCACCATCATCATTAA

GapA (*C. reinhardtii*)

ATGGTTGTAAAAGTAGGTATTAACGGTTTCGGTCGTATTGGTCGTTTAGCTTTCCGTCGTATTCAAAACGTT
GAAGGTGTTGAAGTAACTCGTATTAACGATTTAACTGACCCAAACATGTTAGCTCACTTATTAAAATATGAT
ACTACACAAGGTCGTTTCGATGGTACTGTAGAAGTTAAAGAAGGTGGTTTCGAAGTTAACGGTCAATTCGT
TAAAGTTTCTGCTGAACGTGAACCTGCTAACATTGATTGGGCTACTGATGGTGTTGAAATTGTTTTAGAAGC
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TTACAGCTCCAGGTGGTAACGATGTTAAAACAGTTGTTTTTAACACTAACCACGACATTTTAGATGGTACTG
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TTGGTGTTAAACAAGGTTTAATGACAACTATTCACGCTTACACAGGTGATCAAATGATTTTAGATGGTCCAC
ACCGTGGTGGTGACTTACGTCGTGCTCGTGCTGGTGCTGCTAACATTGTTCCAAACTCAACTGGTGCTGCTA
AAGCTATTGGTTTAGTTATTCCAGAATTAAACGGTAAATTAGATGGTGCTGCTCAACGTGTACCAGTTCCAA
CAGGTTCAGTAACAGAATTAGTTGCTACTTTAGAAAAAGATGTTACTGTAGAAGAAGTAAACGCTGCTATG
AAAGCTGCTGCTAACGATTCTTACGGTTACACAGAAGACCCTATTGTATCTTCTGATATTGTAGGTATTTCTT
ACGGTTCATTATTCGATGCTACTCAAACAAAAGTTCAAACAGTAGACGGTAACCAATTAGTAAAAGTAGTA
TCTTGGTATGATAACGAAATGAGTTATACAAGTCAATTAGTACGTACATTAGAATATTTCGCTAAAATTGCT
AAATACCCATACGACGTACCAGATTACGCTTAA

A2M (*E. coli*)

ATGAAAAATCTGGACGGCCTGCTGAAAATGCCGTATGGTTGTGGTGAACAGAATATGGCACTGCTGGCAC
CGAATATCTATATTCTGCAGTATCTGCGTAATACCCAGCAGCTGACACCGGCAATTAAAGAAAAAGCAACC
AATTTCATGACCAGCGGTTATCAGCGTCAGCTGAACTATAAACATGATAGCGGTGCATATAGCACCTTTGG
TAGCGGTCCGGGTAATACCTGGCTGACCGCATTTGTTGTTCGTACCTTTGCAAAAGCACAGAGCTTCATTTA
TATCGATCCGGCAAAAATTGGCGAAAGCATGAATTGGTTAAAAATGCGCCAGCGTAAAAACGGTTGTTTTG
AACGTAGCGGTAACCTGTTTAATAACCGTATGAAAGGTGGTGTGAGTGATGAAGTTACCCTGAGCGCATAT
ATTACCGCAGCATTTCTGGAAATGAATACCAGCACCGATGAACTTGAGATGATGAAATCACTGAGCTGTCT
GCGTGAAAGCATTGGTGATCTGAATAATACCTATACCACAGCGCTGCTGGCCTATGTTTTCACCCTGGCAG
GCGATATGGAAACCCGTGCATATCTGCTGAAACATCTGGATACCGTTGCAATTCAAGAAGGTGGTTTTCTG
TATTGGAGCCAGACCGCAACCGAAACCAGCGCAAGCCTGAGCGTTGAAATTAGCAGCTATGTTCTGCTGG

CAAAACTGAGCGCGAGCCCGAGTACCGAAGAACTGGGTTATGCAAGCCGTATTGTTCGTTGGCTGACAGG
TCAGCAGAATTATTACGGTGGTTTTAGCAGCACCCAGGATACCGTGGTTGCACTGCAGGCACTGGCACTGT
ATAGCACCCTGGTTTTTAGCGCAGAAGGTAGCAGTACCGTTACCGTTCAGAGCCCGAGCGGTCAGCTGGC
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GCCTGGCAGTTGAAGGCACCGCATGTGCAAGCATGCAGATTAGCGTTCATTATAACGTTCCGACACCGACC
AAAGTTTCAGCCTTTAGCGTGAAAGTTAAAAGCGAAGCAGATTGTAAAAGCAGCCGTCCGAAACTGACCCT
GAAACTGCGTAGCCTGTATACCGGTAAAGAAACCACCACCAATATGGTGATTCTGGACATTAAAATGCTGA
GCGGTTTTCTGCCTGATCAAGAAAGCCTGAAACGTCTGAAAGGTGCCCTGCTGGTTGATCGTGTTGAACAG
AGTGATGATCATGTGCTGGTTTATATTGAAGCCCTGACCAAAGATATCCCGATTAATCATAGCCTGGATCTG
ATTCAGCAGGTTCCGGTTAAAAACCTGAAACCGGCAGTTGTGAAAATCTATGATTATTATCAGCCGAGCGA
TCACGATGATACCGAGTATACCTATCCGTGTGTTGAAGGTCATCATCACCATCATCATTAA

Protein sequences

GapA (*E. coli*)

MVVKVGINGFGRIGRLAFRRIQNVEGVEVTRINDLTDPNMLAHLLKYDTTQGRFDGTVEVKEGGFEVNGQFVK
VSAEREPANIDWATDGVEIVLEATGFFASKEKAEQHIHENGAKKVVITAPGGNDVKTVVFNTNHDILDGTETVIS
GASCTTNCLAPMAKALQDNFGVKQGLMTTIHAYTGDQMILDGPHRGGDLRRARAGAANIVPNSTGAAKAIG
LVIPELNGKLDGAAQRVPVPTGSVTELVATLEKDVTVEEVNAAMKAAANDSYGYTEDPIVSSDIVGISYGSLFDA
TQTKVQTVDGNQLVKVVSWYDNEMSYTSQLVRTLEYFAKIAKHHHHHH*

GapA (*C. reinhardtii*)

MVVKVGINGFGRIGRLAFRRIQNVEGVEVTRINDLTDPNMLAHLLKYDTTQGRFDGTVEVKEGGFEVNGQFVK
VSAEREPANIDWATDGVEIVLEATGFFASKEKAEQHIHENGAKKVVITAPGGNDVKTVVFNTNHDILDGTETVIS
GASCTTNCLAPMAKALQDNFGVKQGLMTTIHAYTGDQMILDGPHRGGDLRRARAGAANIVPNSTGAAKAIG
LVIPELNGKLDGAAQRVPVPTGSVTELVATLEKDVTVEEVNAAMKAAANDSYGYTEDPIVSSDIVGISYGSLFDA
TQTKVQTVDGNQLVKVVSWYDNEMSYTSQLVRTLEYFAKIAKYPYDVPDYA*

A2M (*E. coli*)

MKNLDGLLKMPYGCGEQNMALLAPNIYILQYLRNTQQLTPAIKEKATNFMTSGYQRQLNYKHDSGAYSTFGSG
PGNTWLTAFVVRTFAKAQSFIYIDPAKIGESMNWLKMRQRKNGCFERSGNLFNNRMKGGVSDEVTLSAYITA
AFLEMNTSTDELEMMKSLSCLRESIGDLNNTYTTALLAYVFTLAGDMETRAYLLKHLDTVAIQEGGFLYWSQTA
TETSASLSVEISSYVLLAKLSASPSTEELGYASRIVRWLTGQQNYYGGFSSTQDTVVALQALALYSTLVFSAEGSST
VTVQSPSGQLAFSVNQNNKLLYQEEVLKDATGKYSLAVEGTACASMQISVHYNVPTPTKVSAFSVKVKSEADCK
SSRPKLTLKLRSLYTGKETTTNMVILDIKMLSGFLPDQESLKRLKGALLVDRVEQSDDHVLVYIEALTKDIPINHSLD
LIQQVPVKNLKPAVVKIYDYYQPSDHDDTEYTYPCVEGHHHHHH*

Tables and figures

Table S1. Concentration of Elutions from fermentation purification. Concentration is shown in mg/mL and were worked out using the standard curve shown in figure 8. Concentrations were adjusted based on dilution and total elution volume. Elution 9 and 10 were estimated using densitometry of the SDS gel in figure 7A.

Elution	OD_{595} Average	Concentration (mg/mL)	Adjusted concentration (mg/mL)	Total Concentration in 1.8 mL elution (mg)
E11 1:10	0.4225	0.428	4.28228	7.708
E12 1:10	0.2265	0.176	1.764072	3.175
E13	0.4835	0.507	0.507	0.912
E14	0.221	0.169	0.169	0.305
E15	0.1735	0.108	0.108	0.195
E9			2.355	4.239
E10			3.854	6.937
			Total concentration (mg)	23.472