The development of polyester bead-based particulate subunit vaccine against Johne's disease

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Abstract

Johne's disease is the intestinal infection in ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). The disease is economically important in the dairy industry as infection of the calves or mature cattle can result in death. Current vaccination as a part of disease control is not only partially protective against MAP but also interferes with current diagnostic test for bovine tuberculosis. Therefore, more effective and defined vaccines are needed. In this study, vaccine candidates were developed by bioengineering *Escherichia coli* to produce polyhydroxyalkanoate (PHA) beads displaying selected vaccine candidate antigens as fusion proteins. The selected antigens were the MAP 85 antigen complex (Ag85A and Ag85B), Superoxide dismutase (SOD) and a recombinant fusion protein 74F, however, only the antigen-presenting beads with truncated Ag85A, Ag85B and SOD were successfully produced and purified. The fusion protein comprising the respective antigens was identified and confirmed to be associated with PHA beads. The PHA beads were partially purified for future characterisations such as binding of antigen specific antibodies on PHA beads *in vitro* and immunological properties in animal models.

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Abbreviation

°C	Degree Celsius
µm	Micrometers
BCA	Bicinchoninic acid used in protein assay
BSA	Bovine serum albumin
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
GC/MS	Gas chromatography mass spectrometry
HA_{SCL}	Short-chain-length hydroxyalkanoic acid
HA_{MCL}	Medium-chain-length hydroxyalkanoic acid
HRP	Horse radish peroxidise
IgG	Immunoglobulin G
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	Kilo Daltons
kbp	Kilo base pairs
MALDI-TOF MS	Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry
MAP	*Mycobacterium avium* subspecies *paratuberculosis*
MOPS	3-(N-Morpholino) propanesulfonic acid
Nile-Red	9-diethylamino-5-benzo[α]phenoxazinone
PB	Phosphate buffer saline
PBS	Phosphate buffer salt
PBST	Phosphate buffer salt tween-20
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutarate
PhaA	β-ketothiolase
PhaB	Acetoacetyl-CoA reductase
PhaC	PHA synthase
PhaCAB	PHA operon
PhaPs	Phasins
PhaR	Transcriptional regulator protein
PhaZ	PHA depolymerase
SDS-PAGE	Sodium dodecyl sulfate Polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
TFF	Tangential flow filtration
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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Chapter 1: Introduction

1.1 Polyhydroxyalkanoate (PHA)

Biopolyesters or polyhydroxyalkanoates (PHAs) are one of the common carbon storage forms being produced by various bacteria during nutrient starvation but carbon excess. These microorganisms are capable of accumulating these polyesters in the form of cytoplasmic inclusions that vary in size from 100-500 nm in diameter. The PHA granules are water insoluble and are proposed to be surrounded by phospholipids and surface associated proteins including PHA synthase (PhaC), depolymerase (PhaZ), transcriptional regulator protein (PhaR) and structural proteins or phasins (PhaPs) (Figure 1) (Rehm, 2007; Jendrossek, 2009).

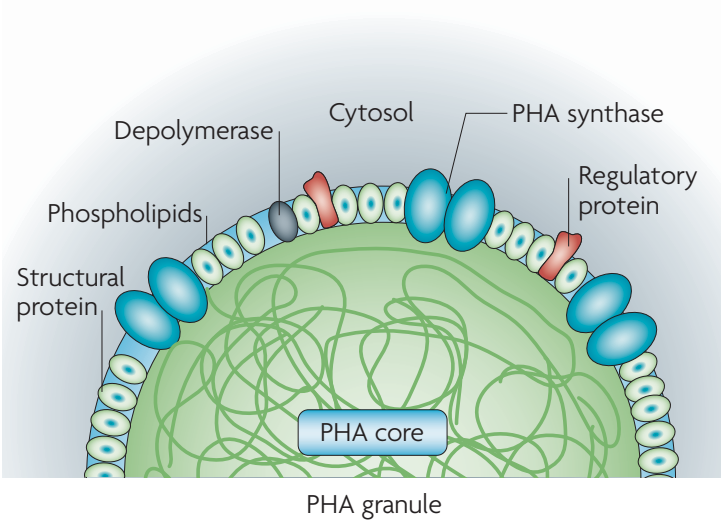

Figure 1. Model of PHA granule structure (Rehm, 2010).

There are a diversity of polyesters being synthesised depending on the composition. PHAs can be homopolymers or heteropolymers containing (R)-3-hydroxy fatty acids (3HAs) or (R)-4-hydroxy fatty acids(4HAs) with a range of the number of carbon atoms in these fatty acid chains. (Dennis *et al*., 1998; Rehm and Steinbuchel, 1999; Rehm, 2007). Rehm (2007), has categorised PHAs into 2 groups depending on the number of carbons in the fatty acid chains of substrates including short-chain-length (PHA_{SCL}) and medium-chain-length hydroxyalkanoic acids (PHA_{MCL}) which contain 3-5 and 6-14 carbon atoms, respectively. PHB, poly(3-hydroxybutyric acid) is one of PHAs that is produced by many bacteria such as *Bacillus megaterium* and *Ralstonia eutropha.*

1.1.1 PHA synthase

PHA synthases are the key enzymes in synthesis of PHAs and they vary in molecular weight, quaternary structure and substrate specificity. These enzymes catalyse the polymerisation of PHA monomers. PHA synthases have been catagorised into 4 classes, I to IV. Class I and II have only one subunit at 61 to 68 kDa. Class I (PhaC) is found in e.g. *R. eutropha* with substrate specificity for coenzyme A thioesters of $3HA_{SCL.}$ Class II synthase is found in *P. aeruginosa* in which coenzyme A thioester of $3HA_{MCL}$ are the substrates. Class III and class IV consist of 2 subunits at 40 kDa. Class III is expressed e.g. by *Chromatium vinosum* that has coenzyme A thioester of $3HA_{SCL}$ as substrates. Class IV is found in *B. megaterium* (Jendrossek, 2009). All PHA synthases share a conserved cysteine amino acid residue which is an active site that catalyses the polymerisation of specific substrates so this is the region that covalently attaches to the PHA polymer (Jendrossek, 2009). This cysteine is found to be conserved among PHA synthases.

There have been many studies that support that the PHA synthase is localised to the surface of PHA inclusions in the cytoplasm. The first evidence shows that the PHA synthase activity is associated with isolated PHA granules (Griebel and Merrick, 1971). Secondly, PHA synthase on PHA is detected by antibody raised against PHA synthase and electron microscopy (Figure 2A.) (Gerngross *et al.,* 1993). Thirdly, expression of GFP gene fusion with PhaC in *B. megaterium* show the localisation of PHA synthase on the surface of PHA inclusion (Figure 2B.) (McCool and Cannon, 1999).

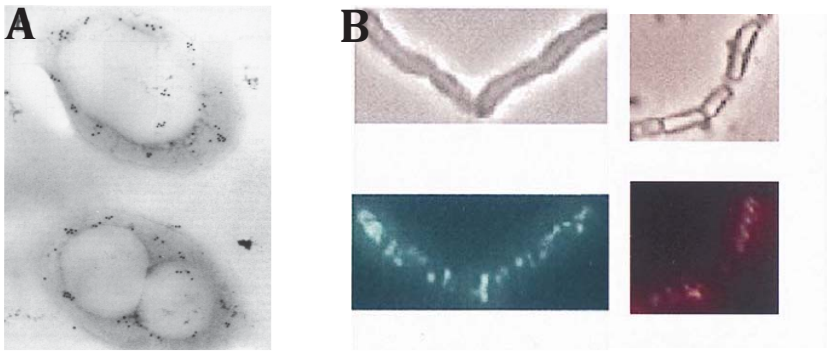

Figure 2. Electron microscopy images of *R. eutropha* A) accumulating PHA inclusion that is surrounded by PHA synthase (black dots) is labeled by specific antibodies. (Gerngross et al., 1993) B) (left) the picture of electron micrograph of *B. megaterium* with expression of gfp-PhaC (right) Nil-red dye of PHA inclusions (McCool and Cannon, 1999)

Apart from PHA synthase, there are the other PHA granule associated proteins that localise to the surface. For example, phasins (PhaPs), the protein that is present at high density on the surface of PHA inclusions in *R. eutropha*. The function and regulation has been studied by Wieczorek *et al.* (1996) in which a *phaP* deletion mutant showed significant reduction in the number of inclusions but the size of PHA inclusions increased, in contrast, overexpression of *phaP* increased the inclusion number while the size was reduced. This evidence suggested that phasins are involved in regulation of PHA granule formation. PhaR, the regulator of *phaP* is also located on the surface of PHA granules (Potter *et a*l., 2002). Another example is the PHA depolymerase (PhaZ1a), the enzyme that catalyses depolymerisation in *R. eutropha* (Saegusa *et. al*, 2001; Uchino, 2007; Uchino, 2008). Studies fusing GFP to PhaZ1a showed the location of this depolymerase on PHA inclusions in fluorescence microscopy. Also incubation of artificial PHB with PhaZa1 resulted in formation of 3HB-CoA which is the PHB monomer supporting that PhaZa1 is PHB depolymerase that catalyse a thiolysis reaction (Uchino, 2007). In addition, another experiment *in vivo*, using a *phaZa1* deletion mutant showed that less 3HB, the monomer for PHB was produced when compared to wild type (Uchino, 2007). This provided further support for the depolymerase function of PhaZa1.

1.1.2 Formation of PHA inclusions

One of PHA producing microorganisms that is the most studied is *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*). PHB is synthesised by a series of reactions catalysed by enzymes for which encoding genes have been identified. The enzymes include β-ketothiolase (PhaA), acetoacetyl-CoA reductase (PhaB), and PHA synthase (PhaC). The substrates are 3-5 carbon hydroxyl fatty acids. The biosynthesis pathway comprises that 2 molecules of acetyl-CoA are converted to one acetoacetyl-CoA by a condensation reaction catalysed by PhaA. The second step is the reduction reaction of Acetoacetyl-CoA to R-(-)-3-hydroxybutyryl-CoA catalysed by PhaB. Then the product of the second reaction, R-(-)-3-hydroxybutyryl-CoA is polymerised by PhaC. The genes responsible for expression of these enzymes are clustered as an operon (*PhaCAB*) in the bacterial genome (Slater *et al.*, 1998; Peoples and Sinskey, 1989).

Despite the fact that a lot of studies have been conducted on the synthesis of PHA and the proteins on the surface, it is still unclear how the PHA inclusion is initially formed. There are two models that have been proposed (Stubbe and Tian, 2003). One is a micelle model that PHA synthase is converted upon polymerisation of HA monomers into PHA into an amphipathic complex molecule, which self-assemble by hydrophobic interaction forming a micelle. Once the size of small micelles increase into large inclusions, the other surface associated proteins such as PhaR, PhaP and PhaZ bind to the inclusion later on (Figure 3A). The second model is the budding model which assumes that PHA synthase or newly synthesised PHA is initially associate with the cytoplasmic membrane by hydrophobic interaction. As a result, PHA is accumulated within the cell membrane from which the granule will be budding out with the other surface associated proteins forming the granules (Figure 3B). The evidence supporting these two models are contradictory. One study using transmission electron microscopy to analyse PHB granule formation in *R. eutropha* showed that early PHB granules are localised in the middle of cells which supports the micelle model (Tian *et al*., 2005). Another investigation using fluorescence microscopy shows that early formation of PHB is localised at the cell periphery or cell poles (Jendrossek, 2005; Peters and Rehm, 2005). This evidence supports budding model. In addition, the investigation of PHA synthase-GFP fusion and visualisation in living *R. eutropha* under fluorescence microscopy demonstrated that the early formation of PHB granules with PHA synthase on the surface is on the cell periphery or cell poles (Peters, Becher and Rehm., 2007). However, PHB granule is mobile so the localisation of PHB could be coincidental.

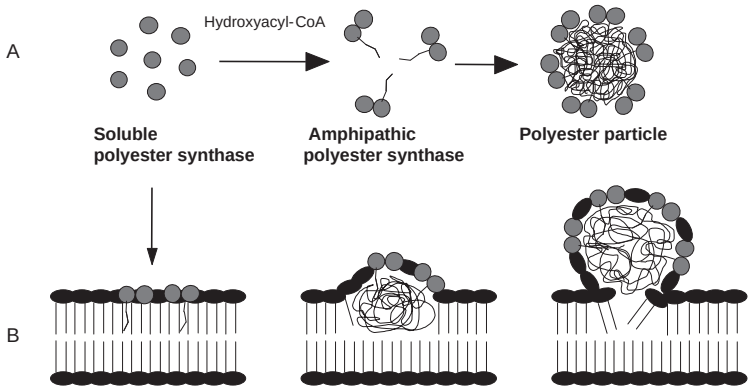

Figure 3. PHA synthesis. A) Micelle model. B) Budding model (Rehm 2007).

1.1.3 Current applications

Utilising the structure of PHA inclusions, spherical granules in which PHA synthase is covalently attached to the surface, and its stability outside the cells, had been realised by genetically engineering bacteria to produce nano/micro beads displaying proteins of interest (Jahns and Rehm, 2009). One of the examples is the fusion of the antigen of interest to PHA synthase (PhaC) to be displayed on PHA beads. Parlane *et al.* (2009) had demonstrated the potential use of PHA nanoparticles as a vaccine delivery agent by producing PHA displaying beads with *Mycobacterium tuberculosis* antigens. These PHA beads had been tested by immunising mice in which both humeral and cell mediated responses were induced. In addition, the fusion of antigens from *Mycobacterium bovis* and hepatitis C had been investigated. As a result, as with *M. tuberculosis*, a significant immune response was observed as well as no significant side effects (Parlane *et al*., 2011, 2012). These studies showed that PHA displaying beads are suitable for vaccination against bacterial and viral infection. The other applications apart from the use as particulate vaccine has been also developed. For example, fusion of proteins that specifically bind IgG, such as the ZZ domain fused to PhaC in order to produce functional beads for IgG purification (Lewis and Rehm, 2009). Also the immobilisation of the functional enzymes on PHA beads had been demonstrated by Peters and Rehm (2006). The immobilised enzymes could be recycled and showed enhanced stability when compared with their soluble counterparts.

1.2 Johne's disease

Johne's disease is a contagious intestinal infection in ruminants that is caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). The disease has been increasingly recognised as a serious economic problem in the dairy industry. The disease is widespread in Europe and most parts of the world including New Zealand (Salem *et al.* 2013). With regards to transmission, the most common route is post-natal infection via faeces, contaminated teats, udders and ingestion of contaminated food and water (Lombard, 2011).

1.2.1 Characteristics

MAP is a Gram-positive bacilli with a waxy cell wall that is acid-fast, approximately 0.5 x 1.5 µm in size, and is highly resistant to low pH and high temperature (Klanicova *et al.*, 2012). In addition, it is a slow growing microorganism that can survive under harsh environmental conditions. The generation time is over 20 hours under optimal aerobic conditions, at 37°C. The incubation period of the disease is 2-10 years and diagnosis determined by isolation and culture, as well as clinical symptoms, is difficult.

1.2.2 Pathogenesis

MAP is an intracellular pathogen. Once the host is infected, M cells of Peyer's patches transport the bacteria across the lumen of the intestine into intestinal mucosa. At this point, the bacteria will be non-specifically recognised by macrophages and phagocytosed. As MAP survives in macrophages, migration of this cell to lymph nodes facilitates dissemination of the organism. At the same time, inflammatory and cell-mediated immune responses are initiated (Salem *et al.*, 2013).

1.2.3 Vaccine antigen candidates

The 85 antigen complex including 85A(Ag85A), 85B(Ag85B) and 85C(Ag85C) are predominantly-secreted extracellular proteins in Mycobacterium species. These proteins have been found to be important in the adhesion of the bacteria to its hosts due to binding affinity to fibronectin (Patti *et.al*., 1995, Soto & Hultgren., 1999, Kuo *et al.* 2012). In MAP, the affinity of 85 antigen complex to fibroronectin has been studied as well as its binding motif. This study has shown that there is Ag85-Fn interaction that initiate infection of MAP and the host cells and the affinities of Ag85s are slightly different due to variations in amino acid sequences (Kuo *et al.* 2012). In addition, these proteins are homologous to the 85 antigens in *Mycobacterium tuberculosis* which have been shown to be effective at inducing protective immune responses against tuberculosis in humans (Dheenadhayalan *et al*, 2003). Cloning, sequencing and analysis of these proteins revealed 99 percent homology with *Mycobacterium avium* 85-complex protein sequences. Immunogenicity studies using these proteins have been done with individual and combined antigens. Antigen 85B, a 35 kDa protein was found to induce

significant immune responses (Mullard *et al*., 2003; Shin *et al,* 2005). The immunisation of mice with this antigen showed that the level of cytokine involved in cell mediated immunity such as interferon (IFN γ) and interleukin (IL-6 and IL-10) increased significantly compared to the control with no vaccination. In addition, *in vitro* immune response studies by Shin *et al.* (2005) showed significant lymphocyte proliferation and increases in the level of cytokine IFN-γ,IL-2, IL-12, and Tumor necrosis factor (TNF-α). The same result was obtained for each antigen 85A and antigen 85C.

Superoxide dismutase (SOD) is another vaccine antigen candidate that has been shown to induce significant immune responses (Mullerad *et al,* 2002a; Shin *et al,* 2005). SOD is a secreted protein produced by *Mycobacterium spp.* including MAP. Mice that were immunised with SOD in combination with adjuvants showed significant production of INF-γ, TNF-α and IL-6 and reduction in the IgG antibody. This result implies that immunisation with SOD induced both T helper cell type 1 and 2 responses (Mullerad *et al,* 2002b). However, in this study, the delayed-type hypersensitivity response in footpads of mice were observed. Another *in vitro* study using peripheral mono-nucleated cells from cattle showed an increase in the cytokine response, however, levels were lower when compared with antigen 85 (Shin *et al,* 2005).

A 35 kDa protein, the major membrane protein in MAP, is present during infection and was found to be one of the virulence factors (Bannantine *et al.*, 2003). It has been demonstrated that this antigen stimulated CD21+ B cells that act as part of the humoral response (Basagoudanavar, Goswami & Tiwari, 2006). In addition, many current developed vaccines that successfully induced a high level of immune response contained this antigen. So this 35kDa protein can be considered as one of the immunodominant antigens (Shin *et al,* 2005).

74F is a 98.2 kDa recombinant fusion protein that had been constructed by Chen *et al.* (2008). The hybrid gene encoding 74F was generated by linking ORFs of the approximately 17.6-kDa C-terminal fragment of Map3527 to the full-length ORF of Map1519, extended at the C-terminus with N-terminal portion (~14.6-kDa) of Map3527. Its ability to generate immune responses had been shown *in vivo*.

Immunisation of mice showed that the humoral immune response was significantly increased. Challenging immunised mice resulted in an increase of IFN-γ, the number of CD3+ and CD4+ cells as well as a reduction of MAP cells in liver, spleen, and mesenteric lymph nodes.

Hsp70, the 70kDa heat shock protein, is an antigenic protein that does not appear to interfere with *Mycobacterium bovis* diagnostic tests (Santema *et al.*, 2009). Immunisation studies in calves showed that proliferation of lymphocytes (especially T-cells) and the level of cytokines being produced by lymphocytes are significantly increased after being exposed to Hsp70 (Hoek *et al.*, 2010; Koets *et al.*, 2006). Furthermore, specific epitopes that activate T-cells in calves have been identified (Hoek *et al.*, 2010). Therefore, these antigens could be suitable for inclusion in subunit vaccines.

1.2.4 Vaccine development

A number of vaccines have been developed for prevention of MAP infections using different combinations of antigens. Attenuated S*almonella* expressing a truncated antigen 85A, 85B, SOD and 74F has been trialed. The effect of this vaccine was the induction of a Th1 response. The level of pro-inflammatory cytokines and Th2 cytokines varied at different times of immunisation. Challenging immunised mice resulted in reduction of acid-fast bacteria and improved histopathology in spleen and liver indicating that this vaccine induced a level of protection in the mouse model (Chandra *et al.*, 2012).

Recombinant protein vaccines have also been developed. Thakur *et al.* (2013) used antigen MAP0217, MAP1508, MAP3701c, MAP3783, and Ag85B as a protein cocktail vaccine with a cationic liposome adjuvant (CAF0). Immunisation of calves showed significant cell-mediated and humoral responses. In addition, it was demonstrated that the immunisation with this vaccine did not cross react with the diagnostic test for *M. bovis* while this antigen combination subunit vaccine has been shown to generate appropriate immune responses. However, challenge studies with MAP are needed to demonstrate the protectiveness of this vaccine.

A DNA vaccine cocktail has been developed by Park *et al.,* (2008) using the antigen 85A, 85B, and 85C, SOD and 35 kDa protein in combination. Immunisation and challenge in a mouse model demonstrated the effectiveness in the induction of both cell mediated and humoral response from the increase in production of Th1 response related cytokine (IFN-γ) as well as the development of CD4+ and CD+8 cells. Challenge of the immunised mice showed the decrease in MAP locating in the spleen and liver compared with unvaccinated controls. This type of vaccine has been shown to induce protective responses against MAP in a mouse model, however, large animal trials are still needed.

1.3 Conclusion

While the biosynthesis of activated precursors for PHA synthesis is well understood, the polymerisation mechanism of PHA and the self-assembly of PHA inclusions remain largely unknown. Nevertheless, molecular tools were successfully developed to bioengineer PHA inclusions for uses as antigen displaying particulate vaccines (Parlane et al., 2009, 2011, 2012). Johne's disease is still a problem in the dairy industry and the current vaccines have many disadvantages in regard to safety and efficacy. Previous studies have identified many antigens that are highly immunogenic and these are used in different combinations in different types of vaccine, which are currently being investigated for protective immunity in mice model. However, for these vaccines, further studies are required to illustrate the relevant information in immunogenicity and side effects. Given the possibility to decorate PHA inclusions with antigens of interest it might be also possible to develop a MAP vaccine comprising the various vaccine candidate antigen described above.

1.4 Aims

Aim 1: To design and construct a shuttle plasmid that contains the genes encoding four selected antigens fused to the PHA inclusion-forming PHA synthase.

This part of study focuses on molecular cloning which include 1) codon optimisation of genes encoding the antigens that has been selected based on demonstrated immunogenicity and protection in previous studies. 2) Construction of production plasmids that contain genes encoding respected antigens.

Aim 2: To demonstrate that the newly constructed plasmids mediate formation of antigen-displaying PHA beads in *Escherichia coli* and *B. megaterium*.

This part of study focuses on productions of antigen-presented PHA inclusions which include 1) transformation of production plasmids to the suitable production hosts. 2) Induction of PHA inclusion-forming PHA synthase and PHA detections. 3) PHA beads isolation and purification.

Aim 3: Molecular characterisation of the antigen-presenting PHA beads.
This part of study focuses on validating the presence of antigen-PhaC complex that are associated with PHA biobeads, purity assessment, and antigen quantifications. The aim is to ensure that the products are suitable for future *in vivo* study for immugenicity and protection against *M. paratuberculosis*.

Chapter 2: Materials and methods

2.1 Bacterial strains and plasmids

Table 1. Bacterial strains used in this study.

Bacterial strains	Phenotype characteristic	Reference
Escherichia coli		
XL1 (blue)	*recA1 endA1 gyrA96 thi-1 hsdR17*(rk-,mk+) *supE44 relA1 lac*[F′ *proAB lacI*^{q}*Z*ΔM15 Tn10 Tet^{R}]	Bullock *et al*. (1987)
Clearcoli BL21 (DE3)	Endotoxin free (*msbA148* Δ*gutQ* Δ*kdsD* Δ*lpxL* Δ*lpxM* Δ*pagP* Δ*lpxP* Δ*eptA*)	Lucigen
BL21 (DE3)	T7 polymerase	Invitrogen
Bacillus megaterium		
PHA05	PhaC knock out, T7 RNA pol, Amp^{R} and Cm^{R}	McCool & Cannon 2001

Table 2. Plasmids used in this study

Plasmids	Phenotype characteristic	Reference
pRARE2	*argU*, *argW*, *ileX*, *glyT*, *leuW*, *proL, metT, thrT, tyrU, and thrU, argX* Cm^R	Novagen
pGEM®-T Easy Vector	Amp^R, *lacZ*	Promega
pUC57 Frag1_N-terminal_Ag	Amp^R, the plasmid that host synthesised nucleotide sequences of truncated gene Ag85A, SOD, and Ag85B that was optimised for *B. megaterium*	IDT
pUC57 Frag2_C-terminal_Ag 2	Amp^R, the plasmid that host synthesised nucleotide sequences of 74F that was optimised for *B. megaterium*	IDT
pT7-RNAP	T7 RNA pol, PxylA, Amp^R, Cm^R	Mo Bi Tec
pPT7	*repU, ori B. megaterium, ori E. coli, T7 RNA polymerase promoter and terminator;* Amp^R Tet^R.	Mo Bi Tec
pPT7-PhaAB	Derivative of pPT7 with additional *phaA* and *phaB*,	Katrin Grage
pPT7-PhaCAB	Derivative of pPT7-PhaAB with additional *phaC*	Katrin Grage
pPT7-Ag85A, SOD, Ag85B linker_(A)PhaC	Derivative of pPT7-PhaCAB with additional truncated gene [Ag85A,	This study

	SOD, and Ag85B]	
pPT7-PhaC_linker 74F	Derivative of pPT7-PhaAB with additional 74F	This study
pPT7-Ag85A, SOD, Ag85B linker_(A)PhaC_linker 74F	Derivative of pPT7-PhaCAB with additional truncated gene [Ag85A, SOD, and Ag85B], 74F	This study

1. Phenotypic characteristics: Cm^R, chloramphenicol resistance; Amp^R, ampicillin resistance; Tet^R, tetracycline resistance

2.2 Primers

Table 3. Primers used in this study

Oligonucleotides	Sequences	Description	Reference
phaC(lac_op)_FWD	ACG GAA GGT GCT GTT GTT TTC GAA AAT G	Forward PCR primers for amplifying part of *phaC*	This study
phaC(stp-gly)_Rev	TGC ATG CAG ATC TTC CTG CTT TTG C	Forward PCR primers for amplifying part of *phaC* with the stop codon being modified to glycine	This study
PhaC(Stp-)_Rev	TCC TGC ATG CAG ATC TTG CTT TTG CTT TGA C	Forward PCR primers for amplifying part of *phaC* with the stop codon being removed	This study
pPT7 Fwd	AAG GCT TTT AAG CCG TCT G	For PCR amplifying 5' region of *phaC* (N-	Grage and Rehm unpublished
Rev3	TCC GTA ACA GCT ACA TTT CG	terminal insertion) and for sequencing	Grage and Rehm unpublished
Fwd6	TCG AAC ATC ATG GTT CAT GG	For PCR amplifying 3' region of *phaC* (C-	Grage and Rehm unpublished
Rev7	GTC CCA AAG ACC ATC AAC AA	terminal insertion)and for sequencing	Grage and Rehm unpublished
T7	TAA TAC GAC TCA CTA TAG GG	Sequencing primers	Allan Wilson Centre
SP6	ATT TAG GTG ACA CTA TAG		Allan Wilson Centre
Fwd74F	TGG TTG AAA TTT GCT CCA CCT	Sequencing primers for 74F	This study
Rev74F	ACC CAA AGC TCC AAG ACC TG		This study
Fwd frag1	TTA ATT AAC CAA	PCR primers for	This study

	GGA GGT GAA ATG	amplifying region on start codon of *phaC* to	
Rev F1(A)	GGT GTA CAT ACC ACC ACC ACT AC	add adenine nucleotide in front of TG	This study
Fwd74FPacI	TTA ATT AAC CAA GGA GGT GAA ATG CAT ATG GTA TTG GCA GTT GCT ATT GAT AAA AG	PCR primers for amplifying 74 F with additional restriction site, linkers, start codon and ribosome binding site	This study
Rev74FBsrGI	TGT ACA TAC CAC CAC CAC TAC CAG CAA CTT TAC CAG CAA CAG CAT TTG GAG		This study

2.3 Liquid media

E. coli

Luria-Bertani media (LB)

20 g LB

1 L RO water

According to the manufacture's instruction, LB was dissolved in water and autoclaved at 121 °C for 20 minutes.

LB miller media

20 g LB

5 g NaCl

1 L RO water

LB and NaCl were dissolved in water then autoclaved at 121 °C for 20 minutes.

B. megaterium

A5 media

1 g $(NH_4)_2SO_4$

1.76 g KH_2PO_4

2.84 g Na_2HPO_4

0.15 g $MgSO_4.7H_2O$

0.5 g yeast extract

449 mL RO water

Components were dissolved in water and autoclaved at 121 °C for 20 minutes.

Trace element solution

2 g $MnCl_2.4H_2O$

2.65 g $CaCl_2.2H_2O$

0.1 g $(NH_4)6Mo_7O_{24}.4H_2O$

0.1 g $CoCl_2.6H_2O$

40 mL RO water

Components were dissolved in water and filter-sterilised. 40 µl of trace element mix was added to 50 mL of A5 media

Iron solution

0.756 g Iron-II-Sulfate (Riedel des Haen 12353)

10 mL RO water

Components were solubilised in water and filter-sterilised. When required, 10 µl of this solution was supplemented to 50 mL of A5 medium.

2.4 Solid media

1.5% (w/v) of agar (Oxoid, UK) was added to the liquid media and autoclaved at 121 °C for 20 minutes.

2.5 Antibiotic stock solution and final concentration.

Table4. Antibiotics and concentration used in this study

Antibiotic	**Stock solution**	**Final concentration**
Ampicillin	100 mg/mL in MQ water	100 µg/mL
Chloramphenicol	50 mg/mL in absolute ethanol	34 µg/mL
Tetracycline	50 mg/mL in absolute ethanol	50 µg/mL

All the stocks were stored in -20 °C.

2.6 Cultivation condition

2.6.1 Pre-culture

E. coli

Liquid LB media was prepared in Erlenmeyer flasks in ratio 1:5 of media to volume of the flask with appropriate supplements according to bacterial strains. The culture was inoculated in the media then incubated at 37 °C with 200 rpm shaking for 16 h.

B. megaterium

Liquid A5 media was prepared in Erlenmeyer flasks ratio 1:10 of media to volume of the flask, with appropriate supplements. The culture was inoculated in the media then incubated at 37 °C with 200 rpm shaking for 6 h.

2.6.2 PHA accumulation condition

E. coli

Liquid LB media was prepared in Erlenmeyer flasks in ratio 1:5 with appropriate supplements (antibiotics and 1% glucose). 1% (v/v) of pre-culture was transferred to the medium then incubated at 37 °C for 4 hr (OD= 0.5). IPTG was added to the culture (1 mM in final concentration), and transferred to 25 °C incubation with 200 rpm shaking for 48 hours.

B. megaterium

B. megaterium strain harboring pT7-RNAP and pPT7-PhaCAB (or the derivatives) was used for PHA production. Liquid A5 media was prepared in Erlenmeyer flasks in ratio 1:10 of media to volume of the flask with appropriate supplements (antibiotics and 2% glucose). 2% (v/v) of pre-culture was transferred to the medium then incubated at 30 °C for 2 h (OD= 0.5). After adding xylose to the culture (to a final concentration of 1 mM), the culture was incubated at 25 °C for a further 16 to 18 h with shaking at 200 rpm.

2.7 Blue white selection

Blue white selection was the method used to select the successful ligation of a DNA fragment to pGEM®-T Easy Vector. After ligation, the reaction was transformed to *E. coli* XL1-Blue and plated on LB agar containing 0.1 mM IPTG, 40 µg/mL X-gal and 100 µg/mL ampicillin. After incubation at 37 °C overnight, successful transformants containing the pGEM®-T Easy Vector harboring the target gene of interest (insert) would appear white in colour because the insertion inactivates the β- galactosidase gene. Conversely, transformants containing the empty pGEM®-T Easy Vector would appear blue.

2.8 Long term storage of bacterial strains and revival

2.8.1 *E. coli*

For long-term storage of *E. coli* strains, 1 mL of overnight culture (2.6.1) was mixed with 70 µl of filter-steriled DMSO (final concentration 6.5% (v/v)) in a cryovial tube. The culture was stored at -80 °C.

2.8.2 *B. megaterium*

For long term storage of *B, megaterium* strains, 700 µl of culture that has been grown in A5 media to OD_{600}=0.5 was added to 300 µl of sterile glycerol (30% (v/v) glycerol), then stored at -80 °C.

2.8.3 Strain revival

Small amount of frosen stock was removed using a sterile loop and inoculated in LB or A5 media for *E. coli* or *B. megaterium* with appropriate antibiotic supplements and glucose.

2.9 Preparation of competent cells and protoplasts

2.9.1 *E. coli* competent cells

Pre-culture of *E. coli* was prepared overnight (2.6.1). 0.5 mL of an overnight culture (2.6.1) was used to inoculate 50 mL of LB medium in a 250 mL volume Erlenmeyer flask and incubated at 37 °C with shaking at 200 rpm for 3-4 hours until an OD_{600} of 0.3-0.4 was reached. Once this OD was met, the culture was placed on ice for 10-15 minutes and then cells were harvested by centrifugation at 8000 g for 15 minutes (Heraeus multifuge1 S-R, Sorvall). The cell pellet was resuspended in 16 mL of RF1 solution and incubated on ice for 30 minutes. After centrifugation, the pellet was resuspended in 4 ml of RF2 solution. 200 µl of cells were distributed in Eppendorf tubes and immediately stored in -80 °C.

RF1 solution

100 mM	RbCl
50 mM	$MnCl_2$
30 mM	Potassium acetate
10 mM	$CaCl_2.6H_2O$

Adjust to pH 5.8 using acetic acid

RF2 solution

10 mM	RbCl
10 mM	MOPS
75 mM	$CaCl_2.6H_2O$
15 % (v/v)	Glycerol

Adjust to pH 5.8 using NaOH

Solutions were sterilised by filtration (0.22 µm) and stored at -20 °C.

2.9.2 *B. megaterium* protoplasts

Pre-culture of PHA05 or recombinant PHA05 was prepared by inoculating from -80 °C culture stock in 50 mL of LB medium containing appropriate supplements in a 500 mL flask. This culture was incubated for 16 h at 37 °C with shaking at 100 rpm. One milliliter of the pre-culture was then transferred to 50 mL of LB medium in a 250 mL flask and incubated at 37 °C with shaking (200 rpm) until an OD_{600} of 1 was reached. Cells were harvested by centrifugation at 5000 rpm for 15 minutes (Heraeus multifuge1 S-R, Sorvall) and the cell was resuspended in 5 mL of SMMP with supplements (50 µl of 50 mg/mL lysozyme) then further incubated at 37 °C for 30 minutes. The formation of protoplast was checked under microscope. The protoplasts were harvested by centrifugation at 1300 g for 10 minutes at room temperature. The pellet was resuspended in 5 mL of SMMP with 750 µl of 87% (w/v) glycerol. 500 µl of the mixture was distributed in Eppendorf tubes and immediately stored at -80 °C.

Antibiotic Medium No. 3, Difco (2 times concentrated)

1 g of AB3 (Difco) was dissolved in 200 mL of deionised water then autoclaved at 121 °C for 20 minutes.

SMM (2 times concentrated)

0.04 g	malic acid
0.08 g	NaOH
0.04 g	$MgCl_2$ x $6H_2O$
1 g	sucrose

Above components were dissolved in 200 mL of deionised water, pH adjusted to 6.5 and solution was sterilised by filtering through 0.22 µm membrane filter.

SMMP

Mix the same proportion of AB3 and SMM together.

2.10 Transformation

2.10.1 *E. coli* transformation

200 µl aliquots of frozen *E. coli* competent cells were thawed on ice for 5 minutes. 1 µl of purified plasmid DNA or 10 µl ligation mix was added to the competent cells, followed by incubation on ice for an additional 20 minutes. The competent cells mixture was then heat-shocked at 42 °C for 90 seconds, followed by incubation on ice for 5 minutes. 800 µl of liquid LB media was then added to the cells and incubated at 37 °C for one hour so that cells were regenerated. For the selection and isolation of recombinant clones, 100 µl of cells was plated on solid LB-agar medium containing the appropriate antibiotics and incubated at 37 °C overnight.

2.10.2 Protoplast transformation

1-5 µg of DNA in water was added to 500 µl of protoplast. The protoplast mix was then added to 1.5 mL of PEG-P, mixed on a roller, incubated for 2 minutes at room temperature then added to 500 µl of SMMP, incubated for a further 45 minutes at 30 °C, and then a further 45 minutes at the same temperature with shaking at 300 rpm. The CR5 top agar was added to the protoplast. Immediately, the mixture was spread on selective LB agar plate and incubated overnight at 30 °C.

CR-5 top agar

Solution A

51.5 g	Sucrose
3.25 g	MOPS
0.3 g	NaOH

Above components were dissolved in MQ water to 250 mL. The pH was adjusted to 7.3 with NaOH. The solution was sterilised by filtering through 0.22 µm membrane filter.

Solution B

1 g agar

0.1 g casamino acids

5 g yeast extract

Above components were dissolved in MQ water to 142.5 mL then autoclaved at 121 °C for 20 minutes.

8x CR5-salts

1.25 g K_2SO_4

50 g $MgCl_2$ x $6H_2O$

0.25 g KH_2PO_4

11 g $CaCl_2$

Above components were dissolved in MQ water to 625 mL then autoclaved at 121 °C for 20 minutes.

12% proline

3 g of proline was dissolved in 25 mL of deionised water then the solution was sterilised by filtering through 0.22 μm membrane filter.

20% glucose

20 g glucose

Glucose was dissolved in MQ water to 100 mL then autoclaved at 121 °C for 20 minutes.

CR5 top agar portion mix

1.25 mL Solution A

0.713 mL Solution B

0.288 mL CR5-Salts

0.125 mL 12% proline

0.125 mL 20% glucose

Solutions were mixed together in a sterile container and used immediately.

PEG-P

20 g PEG-600

Dissolved in 1x SMM to 50 mL.

2.11 DNA manipulation

2.11.1 Plasmid isolation

Plasmids were isolated from *E. coli* using High Pure Plasmid isolation kit (Roche, USA) or Plasmid DNA Miniprep Kits (Invitrogen, USA) according to the manufacturer's instructions.

2.11.2 Polymerase chain reaction (PCR)

To amplify DNA fragments, various polymerases were used depending on the downstream application.

Platinum® Pfx DNA Polymerase (Invitrogen™, USA)
Pfx- DNA polymerase is a high fidelity polymerase, hence it was used to amplify DNA fragments for cloning.

Reaction mix (100 µl)

10 µl	10x buffer
5 µl	$MgSO_4$ (50 mM)
10 µl	forward primer (10 µM)
10 µl	reverse primer (10 µM)
2.5 µl	dNTPs (10 mM)
1µl	template (10 ng/µl)
10 µl	10x PCR enhancer
0.5 µl	Pfx-DNA-polymerase (2.5 U/µl)

Reactions were run in a Biometra personal thermocycler (Whatman Biometra, Germany) under the following conditions:

Initial denaturation	94 °C	2 minutes

35 cycle:

Denaturation	94 °C	15 seconds
Annealing	_ °C*	30 seconds (5 °C below T_m of the primers)
Polymerisation	68 °C	60 seconds per 1 kb
Final extension	68 °C	10 minutes
Hold	10 °C	

Platinum® *Taq* DNA High Fidelity Polymerase (Invitrogen™, USA)

Platinum® *Taq* DNA High Fidelity Polymerase is a high-fidelity polymerase which leaves an extra A at 3' end of PCR product. This enzyme was used to amplify DNA for cloning with the pGEM®-T Easy Vector.

Reaction mix (100 µl)

10 µl	10x High Fidelity PCR buffer
5 µl	$MgSO_4$ (50 mM)
10 µl	forward primer (10 µM)
10 µl	reverse primer (10 µM)
2.5 µl	dNTPs (10 mM)
1µl	template (10 ng/µl)
0.5 µl	Platinum® *Taq* DNA High Fidelity Polymerase (5 U/µl)

Reactions were performed in a Biometra personal thermocycler (Whatman Biometra, Germany) under the followings' conditions:

Initial-denature	94 °C	2 minutes

35 cycles:

Denaturation	94 °C	15 seconds
Annealing	_ °C*	30 seconds (5 °C below Tm of the primers)
Polymerisation	68 °C	60 seconds per 1 kb
Final extension	68 °C	10 minutes

Hold	10 °

Taq-polymerase (fisher biotec, Australia)

For analytical purposes *Taq*-polymerase was used.

Reaction mix (100 µl)

10 µl	10x Reaction buffer
6 µl	$MgCl_2$ (25 mM)
10 µl	forward primer (10 µM)
10 µl	reverse primer (10 µM)
2.5 µl	dNTPs (10 mM)
1µl	template (10 ng/µl)
0.5 µl	*Taq* polymerase (5.5 U/µl)

Reactions were conducted in a Biometra personal thermocycler (Whatman Biometra, Germany) under the followings' conditions:

Pre-denature	94 °C	2 minutes
35 cycle:		
Denaturation	94 °C	15 seconds
Annealing	_ °C*	30 seconds (5 °C below T_m of the primers)
Polymerisation	72 °C	60 seconds per 1 kb
Final extension	72 °C	10 minutes
Hold	10 °C	

2.11.3 Colony PCR

E. coli

To amplify DNA in intact cells, colony PCR was conducted using *Taq*-polymerase (fisher biotec, Australia) as described above with modified conditions as following:

pre-denature	94 °C	5 minutes
35 cycle:		
Denaturation	94 °C	15 seconds
Annealing	_ °C*	30 seconds (5 °C below T_m of the primers)
Polymerisation	72 °C	60 seconds per 1 kb
Final extension	72 °C	10 minutes
Hold	10 °C	

B. megaterium

To amplify DNA in *B. megaterium* transformants, the cells from selected colonies were suspended in 10 µl of sterile water and heated at 95 °C for 30 minutes. 1 µl of the suspension was added to PCR reaction mixture, then PCR reaction was proceeded according to the method in section 2.11.2.

2.11.4 Analysis of DNA concentration and purity

2.11.4.1 NanoDrop™ spectrometer

To analyse DNA concentration and purity, the NanoDrop™ spectrometer was used. According to manufacturer's instructions, 1 microliter of a sample was pipetted onto the measurement pedestal and an absorbance spectrum was obtained. 260/280 and 260/230 absorbance ratios between 1.8 to 2.0 and 1.88-2.2 indicated acceptable DNA purity.

2.11.4.2 Qubit fluorometer

The QubitTM fluorometer (Invitrogen, USA) and Quant-iT DNA BR assay kit (Invitrogen, USA) were used according to manufacturer's instructions. This method allowed accurate determination of DNA concentration between 2 to 1000 ng/µl.

2.11.5 DNA hydrolysis with restriction endonucleases

Restriction digestion was used for cloning and analysis of plasmid DNA according manufacturer's instructions. All restriction endonucleases were purchased from Invitrogen, Roche, or New England Biolabs.

2.11.6 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to separate DNA according to their molecular weight. One and 0.5% agarose gels were used to resolve DNA fragments of 500-2000 and 2000+, respectively. Gels were prepared in 1x TBE. DNA samples were mixed with an appropriate volume of 6x loadind dye before loading in the well. A suitable DNA ladder was also loaded. The gel electrophoresis was run in 1x TBE buffer at 80 V for small chamber and 120 V for a large chamber for 45 minutes to 1 hour. For analytical purposes, the gel was stained with ethidium bromide (5 µg/ml) for 20 minutes and destained in water for 5 minutes. The DNA on the gel was visualised by UV transilluminator at λ= 254 nm (Bio-RAD Gel Doc 2000). For cloning, *SYBR® Safe* gel

stain was added to the agarose gel, according to manufacturer's instructions. *SYBR® Safe* stained gels were visualised by blue light transilluminator (Invitrogen, USA).

10x TBE

500 mM	Tris-HCl
500 mM	Boric acid
25 mM	EDTA

Adjusted to pH 8.0 using HCl

6x loading dye

120 mM	Tris-HCl
120 mM	EDTA
60% (v/v)	Glycerol
0.2% (w/v)	Orange G
0.5% (w/v)	Xylene Cyanol FF

DNA molecular size standards

1. Lambda *Pst*I DNA standard
   λ-DNA (invitrogen, USA) was digested with *Pst*I-HIFI restriction endonuclease (2.11.5) overnight. loading dye was then added and stored at -20 °C. 10 µl was used in 5 mm well.

2. 1 Kb plus DNA Ladder
   This ladder was used according to manufacturer's instructions in 0.5% agarose gel when the expected DNA fragments were above 2.5 Kbp.

3. 1 kb sizer Gangnam DNA ladder
   This ladder was used as an alternative to Lambda *Pst*I DNA standard, according to manufacturer's instructions.

2.11.7 DNA fragment recovery

To extract DNA fragment from agarose gel (2.11.5), illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, UK) were used according to the manufacturer's instructions.

2.11.8 Dephosphorylation of 5' ends

To prevent recircularisation of plasmids digested with a single restriction enzyme Antarctic Phosphatase (New England Biolab, USA) was used according to manufacturer's instruction to remove 5' phosphate group.

2.11.9 DNA ligation

2.11.9.1 pGEM®-T Easy Vector Systems

When a DNA fragment was generated by PCR, it was ligated to an intermediate vector, pGEM®-T Easy Vector (Promega, USA). The DNA fragment was derived from PCR using Platinum® *Taq* DNA High Fidelity Polymerase (2.11.2) which the PCR product contains poly A-tail. The ligation reaction was performed according to manufacturer's instruction.

2.11.9.2 T4 DNA ligase

To ligate an insert to an appropriate expression vector in which both have compatible cut sites, T4 DNA ligase (Invitrogen, USA) was used in ligation reaction. The reaction was set up as below:

4 µl	5x buffer
X µl	Vector
Y µl	Insert
Z µl	Water
2 µl	Ligase (400 U/µl)

Total reaction volume 20 µl
Insert to vector ratio is 3:1
Total DNA was 50 ng

The reaction was incubated at 4 °C overnight.

2.11.10 DNA sequencing

To ensure the presence of the inserts in expression vector, the plasmid products were sequenced by Massey Genome Service (MGS) using a capillary ABI3730 Genetic Analyzer (Applied Biosystem Inc.) sample preparation for sequencing consist of 200 ng of plasmid DNA, 2 ng of primer and sterile water in total 20 µl in volume, in thin walled PCR tubes. The result was analysed by Geneious 6.1.6

2.10.11 DNA clean and concentration

The Clean and concentrator kit (Zymo Research, USA) was used to remove contaminants or concentrate plasmids and linear fragments of DNA required for cloning or DNA sequencing.

2.12 PHA detection, isolation and purification

2.12.1 Cell harvest

To isolate PHB beads from *E. coli,* the cells from induced cultures were harvested by centrifugation at 6000 g for 20 minutes.

2.12.2 Resuspension

2.12.2.1 Sonication

In appropriate solution, the biobeads were resuspended by using VirSonic 600 (Virtis, USA) with micro tip in 6 kHz for 20 seconds 6 times. Keep the tubes cold in the ice.

2.12.2.2 Homogeniser

In appropriate Solution, the biobeads or cell pellet were resuspended by homogenisation using MICCRA D-9 (ART Prozess- & Labortechnik GmbH & Co. KG, Germany) at lowest speed for 1 minute.

2.12.3 Nile-red detection

Nile-red stock solution (0.5 mg/mL in DMSO)

To observe the production of PHA inclusions in the induced cell culture, 1 mL of cell culture were centrifuge at 17,000 g for 1 minute. The pellet was resuspended with 1 mL of 50 mM potassium phosphate buffer (pH7.5) and centrifuged. 10 µl of nile-red stock solution was added and mixed with the cell sediment. 1 mL of 50 mM potassium phosphate buffer (pH7.5) was added and followed by incubation at room temperature for 15 minutes in the dark. The cells were harvested by centrifugation at 17,000 g for 1 minute and then resuspended in 1 mL of 50 mM potassium phosphate buffer (pH7.5). 1 µl was spotted onto the microscope slide with the cover slip and then examined by fluorescence microscope (Olympus, Japan) and Magnifier imaging software.

2.12.4 Cell disruption

2.12.4.1 Cell disruptor Sorvall WX Ultra 80 (Thermo Fisher Scientific, USA)

Different bacterial strains as production hosts were resuspended in the same buffer and supplements but lysed in different conditions due to their physiological differences in cell wall structure. To all strains used in this study, the harvested cells were suspended in 50 mM potassium phosphate buffer (pH 7.5) to 20% (w/v) of wet cell mass in solution and supplemented with EDTA-free protease inhibitor cocktail (Roche, USA) and 50 µg/mL DNase (Sigma-Aldrich, USA).

E. coli

7 mL of the suspension was loaded in the cell disrupter then disrupted at 20 kPsi (x2 passes).

B. megaterium

With additional 50 µg/mL lysozyme (Sigma-Aldrich, USA) in cell mixture, 7 mL of the mixture was loaded in the cell disrupter and disrupted at 35 kPsi (x3 passes).

2.12.4.2 French press

E. coli

30 mL of the suspension was loaded in the cell disrupter then disrupted at 4 kPsi 2 passes.

2.12.4.3 Microfluidiser

6-60 g of harvested *E. coli* Clearcoli BL21 (DE3) cells were suspended in 250 mL lysis buffer by homogeniser (2.12.2.2) the biomass were disrupted for 6 minutes with microfluidiser at 10 kPsi.

Lysis buffer (25 mM Tris, 5 mM EDTA, 0.08% (w/v) SDS, pH11)

3 g Tris base
1.9 g EDTA
0.4 g SDS
1 L MQ water
pH 11

All components were dissolved in 900 mL of water, adjust pH with 10 M NaOH . The volume was adjusted to 1 L and sterilised by 0.2 µm filter.

2.12.5 PHA purification

2.12.5.1 PBST wash

10x	PBS (pH7.4)
(0.2M	phosphate, 1.5 M NaCl)
2.28 g	NaH_2PO_4
11.5 g	Na_2HPO_4
43.84 g	NaCl

NaH_2PO_4 and Na_2HPO_4 were dissolved in MQ water and the pH were adjusted to 7.4 by adding HCl then the total volume was made up to 500 mL. The solution was filter sterilised and then diluted 10 fold to reach final concentration.

PBST
PBS with 0.05% Tween-20

0.5 g of biobeads was resuspended in 30 mL of PBST and later centrifuged at 6000 g for 20 minutes. The supernatant was discarded. The same process was repeated twice.

2.12.5.2 PBS wash

To remove either PBST, lysis buffer or caustic buffer, the 0.5 g of biobeads was resuspended in 30 mL of PBS and later centrifuged at 6000 g for 20 minutes. The supernatant was discarded.

2.12.5.3 Lysis buffer wash.

0.5 g of biobeads was resuspended in 30 mL of Lysis buffer by homogeniser (2.12.2.2) and later centrifuged at 6000 g for 20 minutes. The supernatant was discarded. This was done twice.

2.12.5.4 Caustic buffer wash

0.5 g of biobeads was resuspended in 30 mL of caustic buffer by homogeniser (2.12.2.2) and later centrifuged at 6000 g for 20 minutes. The supernatant was discarded. This was done twice.

Caustic buffer (0.05 M NaOH, 0.02% (w/v) SDS)

2 g	NaOH
0.2 g	SDS
1 L	MQ water

All the components were dissolved in water then the solution was sterilised by filter with 0.2 µm membrane.

2.12.5.5 Glycerol gradient

44% (v/v) glycerol in PBS

44 mL	glycerol
10 mL	10x PBS
46 mL	RO water

All the components were dissolved in water then autoclaved at 121 °C for 20 minutes.

88% glycerol in PBS

88 mL	glycerol
10 mL	10x PBS
2 mL	RO water

All components were dissolved in water then autoclaved at 121 °C for 20 minutes.

4.5 mL of 88% glycerol was loaded in the bottom of centrifuge tubes followed by 4.5 mL of 44% of glycerol on top. 2 mL of 20% (w/v) of hydrated biobeads were (prepared by resuspending in PBS) loaded on the top of glycerol. The tubes were centrifuge at 100,000 g for 2 hours (SW41 or Ti rotors). The biobeads on interface of glycerol was removed and suspended in 30 mL of PBS then centrifuged 6000 g for 30 minutes.

2.12.6 Gas chromatography mass spectrometry (GCMS)

2 mL of 15% methanolic sulfuric acid and 2 mL of Chloroform with 105 µg/mL undecane were mixed with 75 mg of freeze-dried samples (biomass or biobeads) in boiling tubes. The tubes were incubated in 100 °C oil bath for 5 hr, and later cooled to room temperature. 2 mL of MQ water was added to the tubes and votexed for 1 minute. The organic phase at the bottom layer was taken for Gas chromatography analysis (Plant and Food research)

15% Methanolic sulphuric acid

15 mL sulphuric acid

85 mL methanol

sulphuric acid was added to methanol drop-wise while on ice.

Chloroform with 105 µg/mL undecane

14.2 µl Undecane

100 mL Chloroform

add undecane to chloroform and mix well.

2.13 Protein analysis

2.13.1 SDS-PAGE

Discontinuous polyacrylamide gel electrophoresis under denaturing condition in the presence of sodium dodecyl sulfate (SDS-PAGE) is the method used to determine the level of expression of fusion protein and purity of antigen-presenting beads is this study. The proteins that have associated to biobeads, were extracted by mixing with SDS sample buffer to 1x and heat treated at 95 °C for 10 minutes. The samples were later centrifuged at 10,000 g for 2 minutes. The supernatant was loaded in appropriate bis-acrylamide gel. The protein samples in which the fusion protein was expected to be ~150-180 kDa were resolved in 6% bis-acrylamide gel and the samples in which the protein were expected to be 60-100 were resolved 8% bis-acrylamide. the running condition was 90 V for 45 minutes in electrode buffer.

SDS sample buffer (6x)

7 mL 4x Tris .HCl/SDS, pH 6.8
3 mL glycerol
1 g SDS
0.93 g DTT
2 mg bromophenol blue
Add all the components together then add water to 10 ml (final volume), store in 0.5 ml aliquots at -70 °C.

Bis-acrylamide gels consist of two layers including upper stacking gel layer (4% (w/v) PH 6.8) and resolving gel (6-8%). Both gels were prepared between two glass plated (16 x 19.5 cm) separated by 0.8 mm gap (bio-rad, USA). The resolving gel was prepared by adding polymerising agent N, N, N', N'-tetramethylethyl-endiamin (TEMED) and ammonium persulfate (APS) (40 %, w/v) to bis-acrylamide in bis-tris buffer (6-8%). The solution was filled in the glass plates and immediately layered with isopropanol. After 40 minutes, the isopronol was removed and the space was filled with stacking gel and 9 mm comb. The gel was left to stand for 30 minutes to set.

6% acrylamide gel

2.9 mL 3.5x bis-tris buffer
2 mL 30% bis-acrylamide
5.06 mL water
17 µl APS
19 µl TEMED

8% acrylamide gel

2.9 mL 3.5x bis-tris buffer
2.67 mL 30% bis- acrylamide gel
4.39 mL water
17 µl APS
19 µl TEMED

Stacking gel

3.12 mL 3.5 x bis-tris buffer
1.88 mL 30% bis-acrylamide
7.5 mL water
8 µl APS
9 µl TEMED

Bis-Tris buffer (3.5x)

1.25 M Bis-Tris
Adjust pH with HCl till its pH 6.5-6.8, store at room temperature

Electrode buffer
100 mL 5x running buffer
2.5 mL 200x reducing agent
97.5 mL water

Running buffer (5x)
250 mM MOPS
250 mM Tris-buffer
5 mM EDTA
0.5% SDS
Do not adjust pH.

200x reducing agent
(1 M sodium bisulfite)
5.305 g Sodium bisulfite
50 mL water
After the gel finish running, the acrylamide gel was removed from the plates and stained with comassie blue for 1 hour or over night. Then the gel was removed from comassie blue stain and the excess stain was washed by distilled water. The stain was later

removed by soaking in destaining solution for 3 hours or the background colour was removed.

Coomassie blue staining solution

1 g Comassie blue R-250
450 mL Ethanol
90 mL Acetic acid
460 mL Distilled water

Destaining solution

660 mL Ethanol
200 mL acetic acid
1140 mL distilled H2O

2.13.2 western blot

The protein band was transferred from the polyacrylamide gel to nitrocellulose membrane using the iBlot® 7-minute blotting system (thermoscientific, USA). The membrane was blocked by block solution for 1 hour. The membrane was washed 5 minutes with PBST three times. Later, the membrane was incubated with PhaC monoclonal antibody dilution 1:20,000 in PBST with 1% BSA for 1 hour follow by the washing for 15 minutes with PBST, repeated 2 more times. The membrane was then incubated with polyclonal secondary antibody, HRP-conjugated antibody. The wash step was repeated as described previsaw2ously. The membrane was then incubated for 5 minutes with supersignal west pico Lumino/Enhancer solution and Peroxide solution (Thermoscientific, USA). In a dark room, the membrane was exposed to an X-ray film (Kodak, USA) for 20 seconds. The signal on the film was developed by an automated developer.

Block solution

5% (w/v) skim milk
1% BSA
In PBS buffer

2.13.3 Bicinchoninic acid assay (BCA)

Bicinchoninic acid assay (BCA) is the method used to quantify the total amount of protein that's associated with the biobeads. Pierce™ BCA Protein Assay Kit (Thermo fisher scientific, USA) was used in this study according to manufacturer's instruction. As biobeads could interfere the signal reading, the protein concentration can not be accurately determined. Therefore, the method was modified by an additional step to the sample treatment. For instance, the sample of the biobeads was diluted to a range of concentration from 10, 5, 2.5 to 1.25% (w/v) with PBS. BCA working solution was prepared by adding 1 part of Reagent B to 50 parts of reagent A. In Eppendorf tubes, 60 µl of bead sample was added to 480 µl of working solution. All the reactions were incubated in 37 °C for 30 minutes with rotator (Pipette, USA). After incubation, the samples were centrifuged at 12000 rpm for 2 minutes. 200 µl of supernatants were transferred to microtitre plate (Greiner bio-one, Germany). The signal was detected at 563 nm by ELx808 IU Ultra Microplate reader. (BIO-TEK instruments, USA)

2.13.4 Densitometry

Densitometry is the method used to quantify the amount of fusion protein being present on the biobeads. The quantification was done by running SDS-PAGE with the known amount of BSA standards from 500 ng to 50 ng at the same time as the fusion protein from biobeads (2.13.1). The amount of proteins were analysed by Image-Lab 5.2.1.

2.13.5 MALDI-TOF MS

The protein bands corresponding to protein of interest were cut and removed on SDS-PAGE acrylamide gel for peptide fingerprinting by Matrix-assisted laser desorption/ionisation Time-of flight mass spectrometry (Maldi-TOF MS). All the process was conducted by The Centre for Protein Research (Otago university).

Chapter 3: Results

3.1 Plasmid construction for the antigen-presenting beads production

Plasmids were designed and constructed to immobilise selected antigens on PHA beads. The four antigens used in this study were a truncated version of Ag85A, superoxide dismutase and Ag85B (Chandra *et al.,* 2012) and full-length antigen 74F (Chen *et al*., 2008). The target host for production was initially *B. megaterium*, the plasmid pPT7-PhaCAB (previously developed by Grage and Rehm, IFS, Massey University) was used to generate the following production plasmid pPT7-Ag85A, SOD, Ag85B linker_PhaC_linker 74F.

The aim of this study was to demonstrate that the antigen-presenting beads can be designed and manufactured by *E. coli* and *B. megaterium.* Therefore, the plasmids were constructed for introduction into both of the production hosts. *Lactococcus lactis* would also be a suitable host in case the above production hosts fail to produce the desired products.

3.1.1 Codon optimisation and gene synthesis of Ag85A, SOD, Ag85B and 74F

The amino acid sequence of truncated Ag85A, SOD, Ag85B (Chandra *et al.,* 2012) with linker on the N-terminal and 74F with SG linker on the C-terminal (Jahn & Rehm, 2009), were sent for codon optimisation for *B. megaterium* and *L. lactis.* The nucleotide sequences including the restriction sites were chemically synthesised by Genscript and provided on the plasmid pUC57.

3.1.2 Generation of DNA fragment encoding PhaC without stop codon (TAA)

pPT7-PhaCAB was used as an expression vector, and gene fragments were inserted at 5’ and 3’ of *phaC*. However, to ensure proper expression of the recombinant proteins (containing C-terminal fusions), the *phaC* stop codon (TAA) had to be removed (Figure 4). To accomplish this, site-directed mutagenesis was conducted by PCR using pPT7-PhaCAB as a template and high fidelity *taq* DNA polymerase using specific primers (2.11.2). The DNA fragment was successfully generated by PCR using of using phaC (stp-)_Rev and phaC(lac_op)_FWD primers (data not shown). The PCR product was

extracted from agarose gel (2.11.7) and ligated to pGEM®-T Easy Vector as an intermediate vector (2.11.9), and subsequently transformed into *E. coli* XL1 blue competent cells (2.10.1). After transformation, the cells were subjected to blue white selection using LB agar media for containing 100 µg/mL of ampicillin. The white colonies were screened by PCR for the presence of the inserted PCR product (data not shown). Based on PCR result, four colonies were selected to grow overnight for plasmid extraction (2.11.1). To confirm the presence of the insert in the intermediate vector, the plasmid was digested by *Bgl*II. Gel electrophoresis of restriction digest product show the presence of 4 kb band indicating the presence of *phaC* fragment in pGEM®-T Easy Vector (Figure 5). The absence of stop codon and correct DNA sequence was confirmed by DNA sequencing (data not shown).

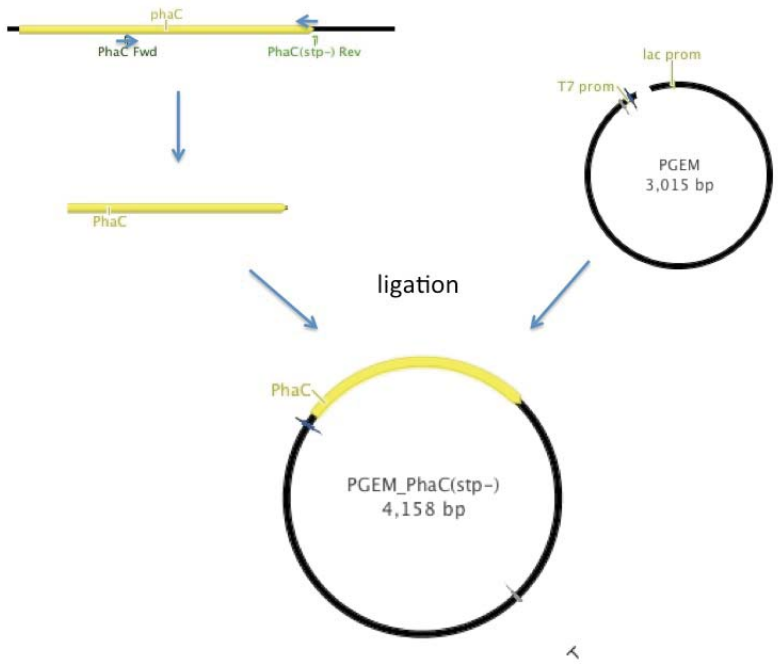

Figure 4. The strategy for removing the *phaC* stop codon from pPT7-PhaCAB. The product was cloned into intermediate vector pGEM®-T Easy Vector.

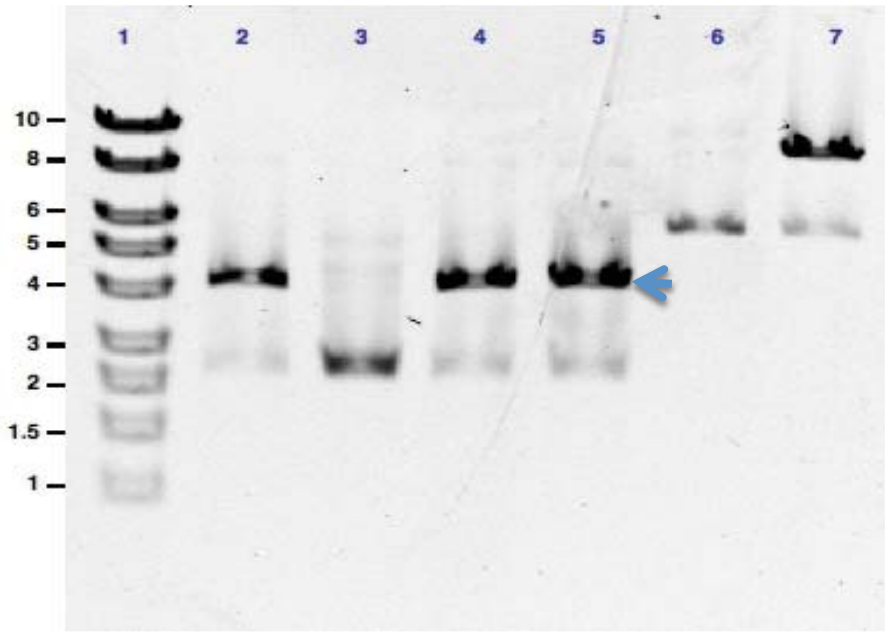

Figure 5. Restriction fragment analysis for confirmation of pGEM®-T-PhaC^{stp-}. The plasmids were hydrolysed with *Bgl*II to confirm the presence of PCR fragment. Lanes 1, 1 kb plus DNA ladder (kbp); Lanes 2-5, hydrolysis products of plasmids isolated from selected transformants; Lane 6, pPT7-PhaCAB hydrolysis positive control. Lane 7, pPT7-PhaCAB hydrolysis negative control (no *Bgl*II). Expected size of the digested product is 4 kbp.

3.1.3 Construction of pPT7-Ag85A, SOD, Ag85B linker_PhaC_linker 74F

Genes encoding truncated versions of Ag85A, SOD and Ag85B, and full-length 74F were synthesised chemically as described in 3.1.1 and the DNA fragments were delivered in pUC57. The cloning strategy involved three steps as outlined in Figure 6A-C. In short, both pUC57 Frag1_N-terminal_Ag and pPT7-PhaCAB were hydrolysed with *Pac*I and subsequently *Bsr*GI (2.11.5). The digested products were separated by gel electrophoresis using 1% agarose gel containing *SYBR® Safe* (2.11.6). The expected products for Frag1 and pPT7-PhaCAB were 1.2 and 8.9 kbp. These fragments were cut from agarose gel for DNA isolation (2.11.7) (GE healthcare). After DNA extraction, the vector and insert were used for ligation reaction according to the company's protocol (2.11.9). After ligation, the reaction was transformed into *E. coli* XL1 blue (2.10.1) and grew on selective agar media containing ampicillin 100 µg/mL. The colonies that grew in selective environment were screened by PCR (2.11.3) (Figure

7) using pPT7Fwd and Rev3 primers. The expected product was at 1.9 kbp indicating the presence of the pPT7-Ag85A, SOD, Ag85B linker_PhaC, in comparison to pPT7-PhaCAB in which the product was expected to be 0.7 kb. The positive clones were selected to grow overnight in liquid media for plasmid extraction (2.11.1). The presence of Ag85A_SOD_Ag85B sequence was confirmed by sequencing (Massey Genome Service).

To remove the stop codon from *phaC* in pPT7-Ag85A, SOD, Ag85B linker_PhaC, pGEM®-T-PhaC^{stp-} (from the result 3.1.2) was digested with *Bst*BI and *Bgl*II (2.11.5) as well as pPT7-Ag85A, SOD, Ag85B linker_PhaC. The DNA products were purified from agarose gel (2.11.7) and ligated together (2.11.9). The ligation mixture was transformed to *E. coli* XL1 blue and plated on selective agar media containing ampicillin 100 µg/mL. The colonies that grew on selective media were screened by PCR (2.11.3) using PhaC fwd and Rev PhaC^{stp-} primers. The PCR product at 1.1 kb indicate the presence of PhaC^{stp-} fragment in the vector (Figure 8). PCR using pPT7 fwd and Rev3 was run in parallel to confirm the presence of the 5' insert (Figure 9). The absence of stop codon was confirmed by DNA sequencing (data not shown).

To clone 74F to C-terminal of PhaC, 74F gene sequence was chemically synthesised as previously mentioned. pUC57 Frag2_C-terminal_Ag 2 was digested with *Bgl*II (2.11.5). The expected products for pUC57 Frag2_C-terminal_Ag 2 were 2.7 and 2.4 kb. From agarose gel electrophoresis containing *SYBR® Safe*, 2.4 kb product were isolated, purified (2.11.7) and ligated to pPT7-Ag85A, SOD, Ag85B linker_PhaC^{stp-} that has been linearised with *Bgl*II (2.11.9). The ligation reaction was then transformed to *E. coli* XL1 blue and plated on selective media containing 100 µg/mL of ampicillin. The transformants were screened by colony PCR using Fwd6 and Rev7 primers (Figure 10). The PCR product at 2.9 kb indicates the presence of 74F on the C-terminal of *PhaC*. To confirm the 5' insert of *phaC* in this vector, colony PCR with pPT7 Fwd and Rev3 were run in parallel. The product at 1.9 kb confirms the 5' insertion (Figure 11). In addition, the presence of 74F gene was confirmed by restriction digest with *Sma*I and *Bam*HI (Figure 12) and DNA sequencing (data not shown).

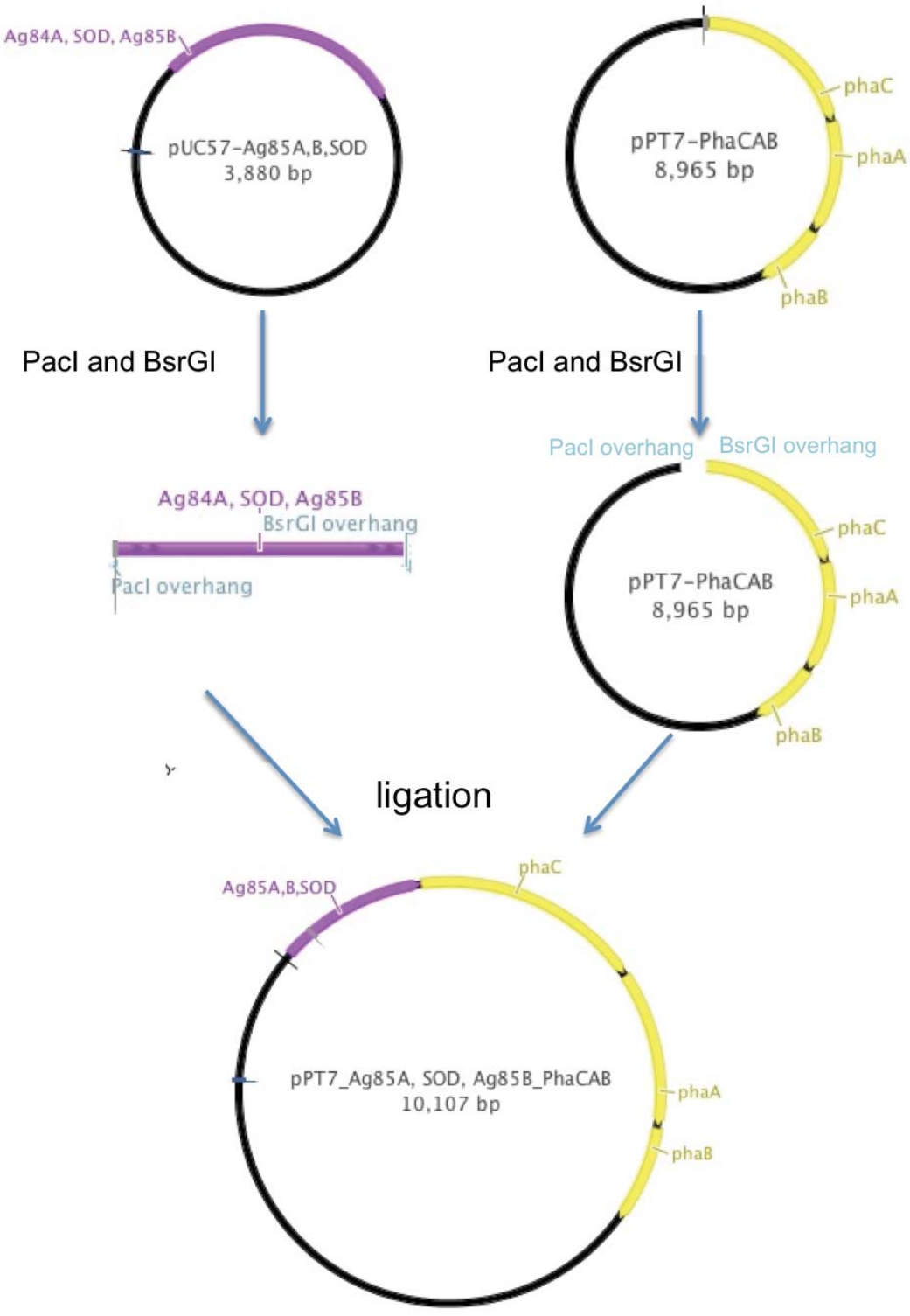

Figure 6A. Cloning diagram step 1, Fragment1 (Ag85A, SOD, Ag85B) was inserted to encode for antigens N-terminally fused to PhaC.

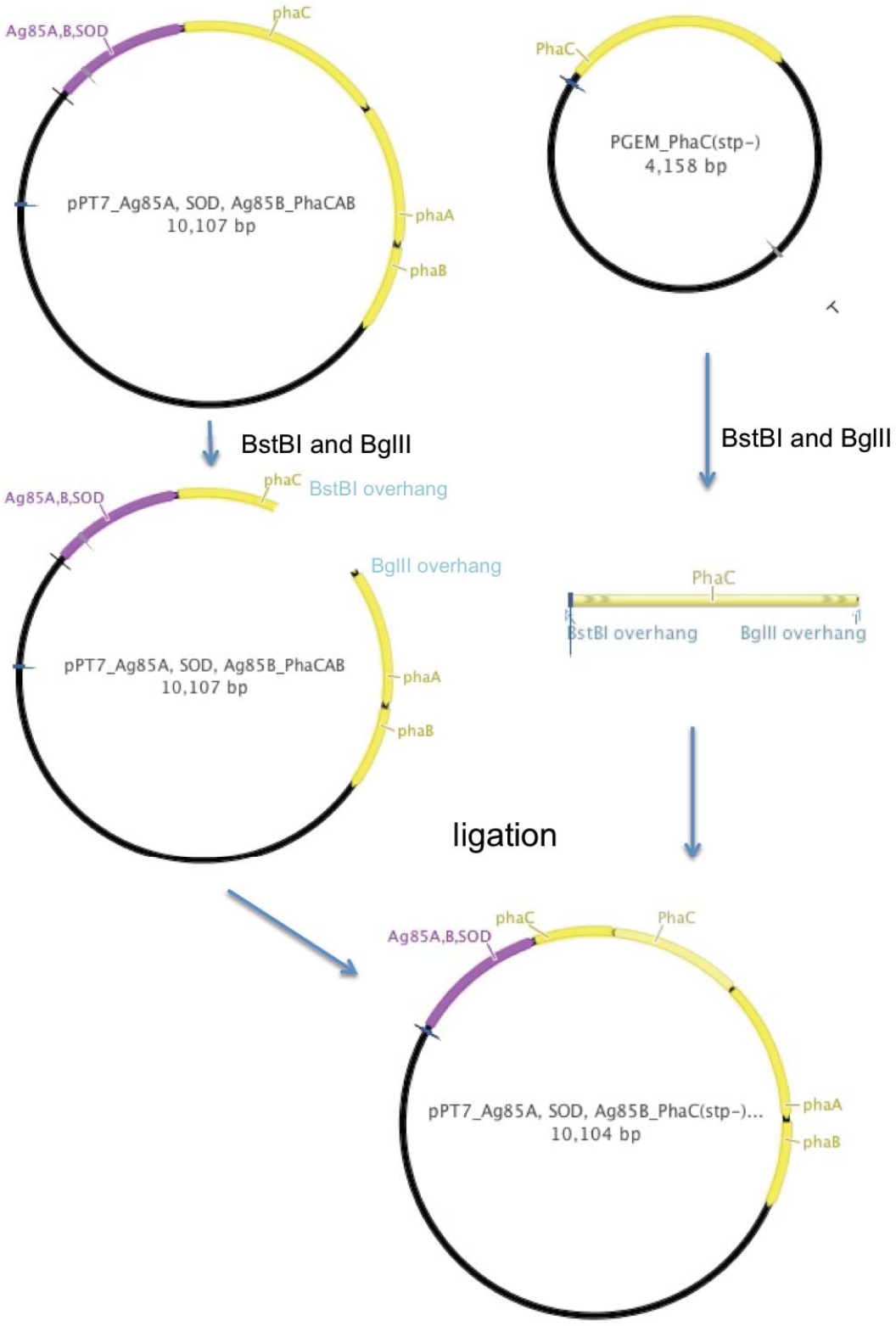

Figure 6B. Cloning diagram step 2, stop codon was removed from *phaC.*

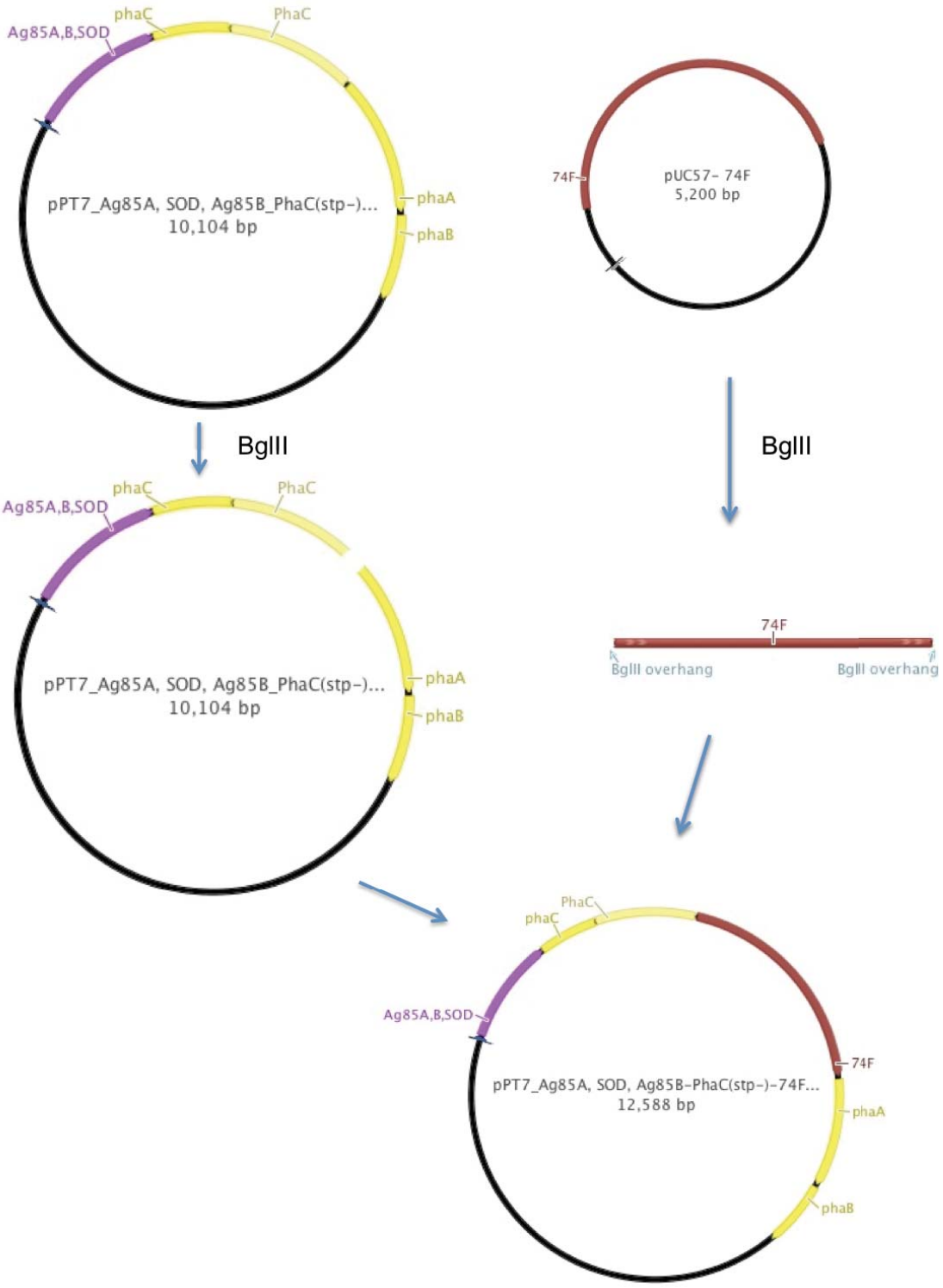

Figure 6C. Cloning diagram step 3, 74F was inserted to encode for 74F fused to the C-terminus of PhaC.

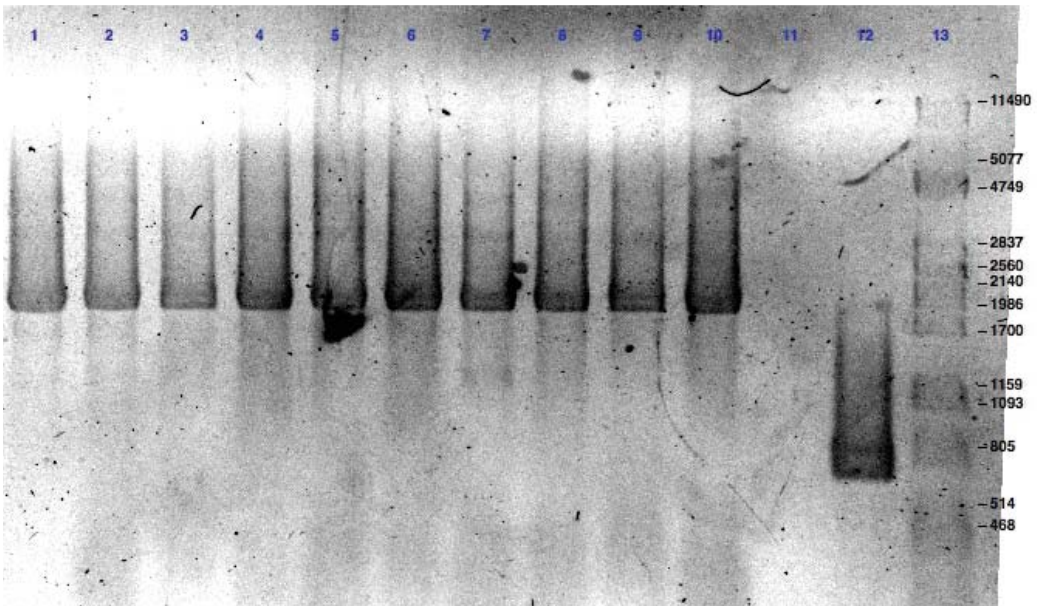

Figure 7. **Colony PCR screening the colonies harbouring pPT7-Ag85A, SOD, Ag85B linker_PhaC**. Lanes 1-10, Selected transformants from ligation reaction of pPT7-PhaCAB *Pac*I *BsrG*I with pUC57 Frag1_N-terminal_Ag *Pac*I *BsrG*I; Lane 11, water as a negative control; lane 12, *E. coli* XL1 harboring pPT7-PhaCAB as a positive control.

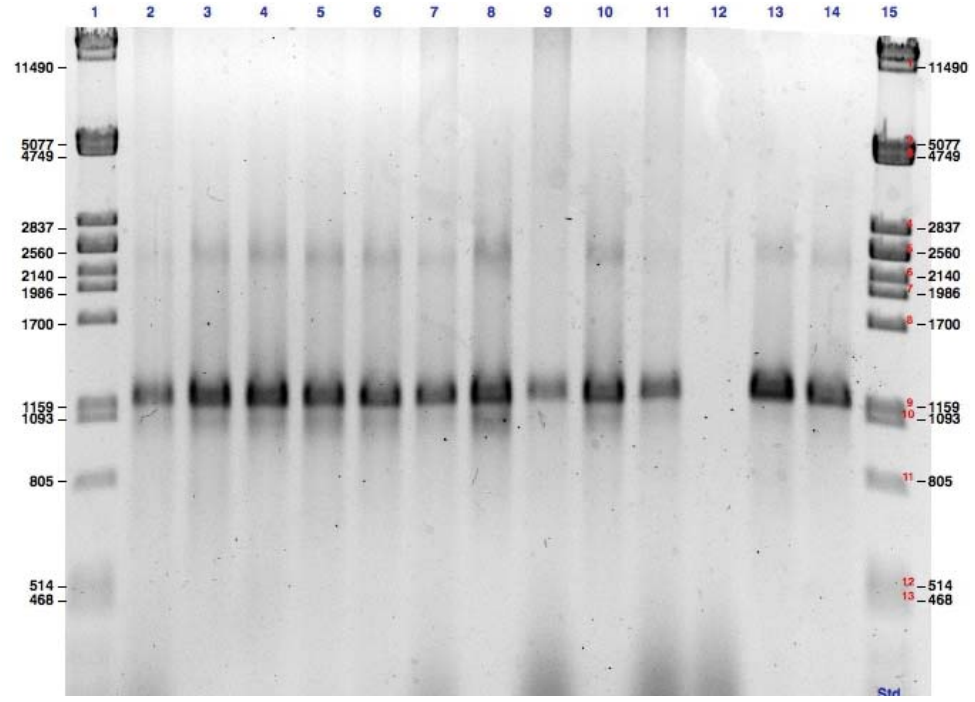

Figure 8. **Colony PCR on the transformants from ligation reaction of pPT7-Ag85A, SOD, Ag85B linker_PhaC digest *BstB*I *Bgl*II with pGEM®-T-PhaC^{stp-} digest *BstB*I and *Bgl*II.** Lanes 1 and 15, lambda phage *Pst*I ladder; lanes 2-11, selected transformants, lane 12, water; lane 13, *E. coli* XL1 blue harboring pPT7-PhaCAB lane 14, pGEM®-T-PhaC^{stp-}

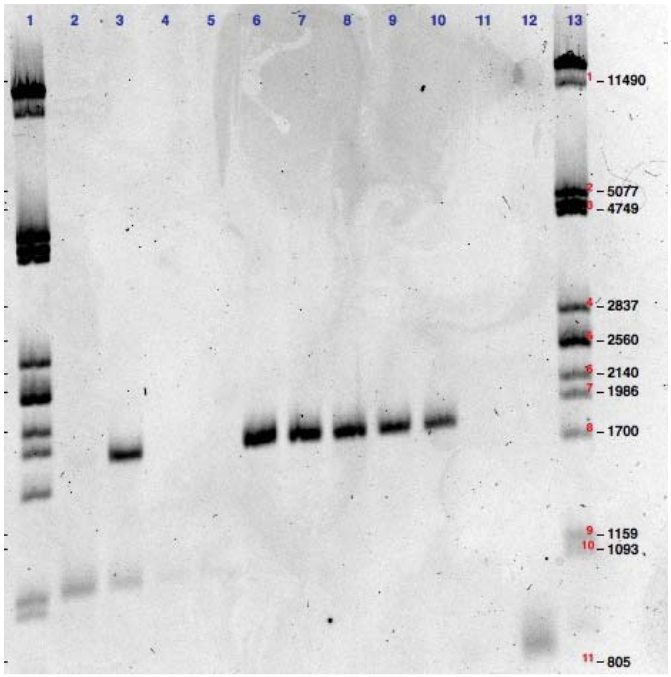

Figure 9. **Colony PCR that detect the N-terminal cloning to ensure the transformants have the 5' insertion of *phaC* retained**. Lanes 1 and 13, lambda phage *Pst*I ladder; lanes 2-10, transformants; lane 11, water; lane 12, pPT7-PhaCAB plasmid. The expected size is 1.1 kb.

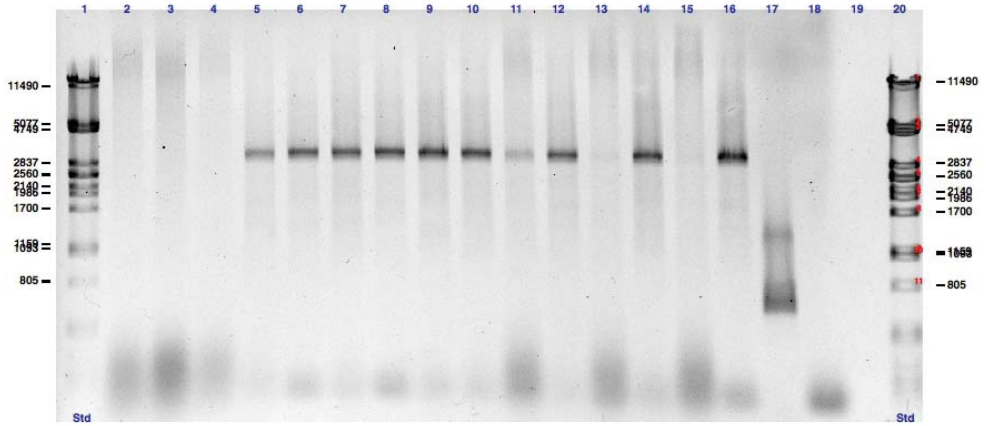

Figure 10. **Colony PCR for the presence of 74F on 3' of *phaC***. Lanes 1 and 20, Lambda phage *Pst*I ladder (bp); lanes 2-16, Selected transformants; lane 17, pPT7-PhaCAB control; lane 18, *E. coli* XL1 blue; lane 19, water.

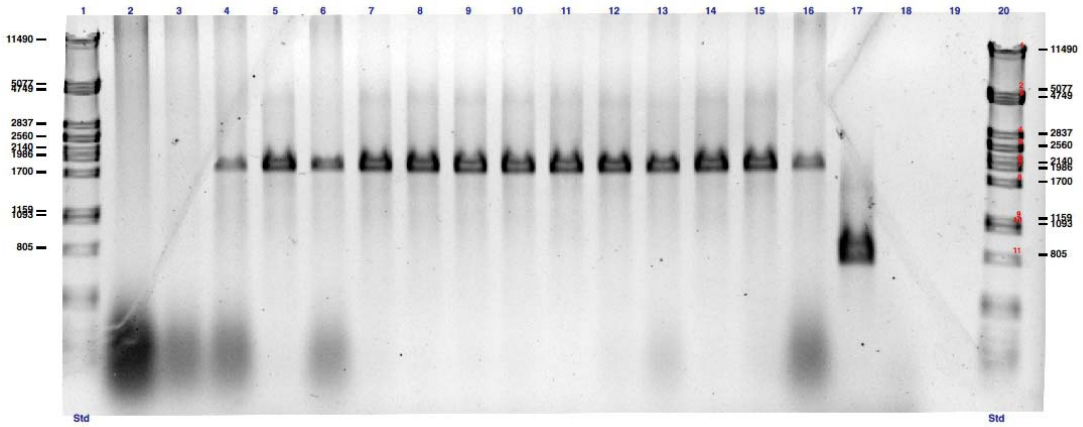

Figure 11. Colony PCR for the presence of 5' of *phaC*. Lanes 1 and 20, Lambda phage *Pst*I ladder (bp); lanes2-16, Selected transformants; lane 17, pPT7-PhaCAB control; lane 18, *E. coli* XL1 blue; lane 19, water.

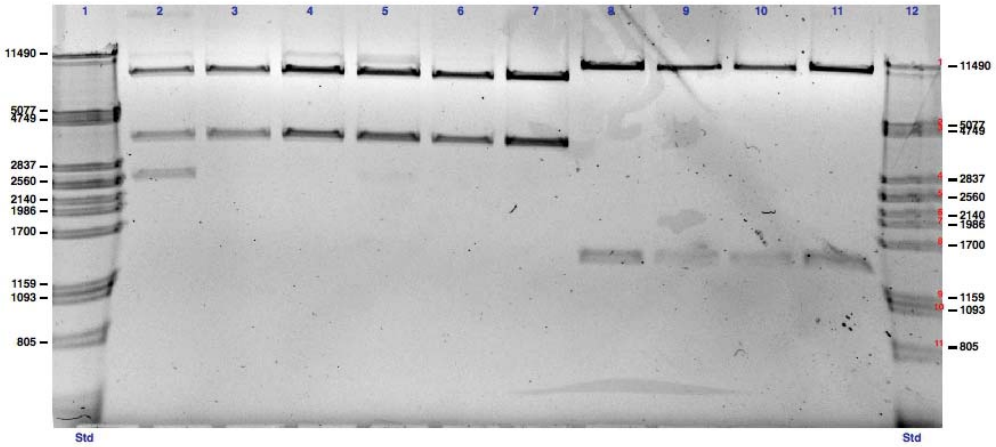

Figure 12. Restriction confirmation for the presence of 74F on 3' of *phaC*. Lanes1 and 12, Lambda phage ladder *Pst*I; lane 2-11, Plasmids from 10 different colonies digested with *Sma*I and *Bam*HI.

3.2 Antigen-presenting beads production in *E. coli* BL21 (DE3)

3.2.1 Transformation of production plasmids into *E. coli* BL21 (DE3)

Plasmids pPT7-Ag85A, SOD, Ag85B linker_PhaC_linker 74F and pPT7-PhaCAB were transformed into *E. coli* BL21 (DE3) (2.10.1) for production of antigen-presenting beads and wildtype beads, respectively. The cells were plated on solid media containing 100 µg/mL of ampicillin. The colonies that grew were screened by PCR (2.11.3) to confirm the presence of transformed plasmids. Colonies passing the PCR test were inoculated overnight in liquid media for plasmid isolation (2.11.1) and subject to restriction digest confirmation (2.11.5). To confirm the presence of pPT7-Ag85A, SOD, Ag85B linker_PhaC_linker 74F, the isolated plasmids were hydrolysed with *Sma*I and *Xba*I (Figure 13). The presence of 9 and 3.6 kbp products indicated the presence of correct plasmids.

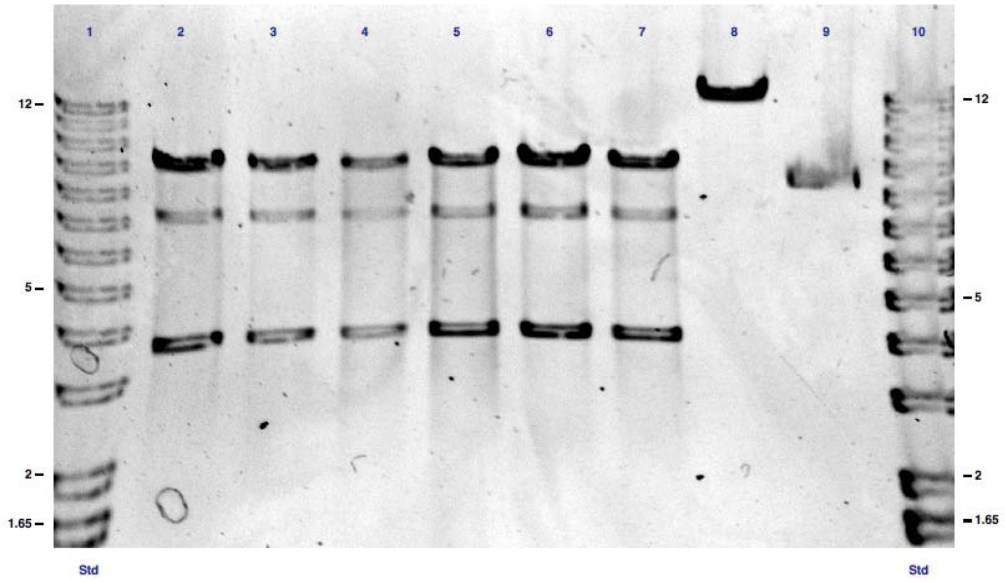

Figure 13. The plasmids isolated from *E.coli* BL21 (DE3) transformants were digested with *Sma*I and *Xba*I to confirm the presence of pPT7-Ag85A, SOD, Ag85B linker_PhaC_ linker 74F. Lanes 1 & 10, 1 kb plus ladder (kbp); lanes 2-6, plasmids from selected transformants digested with *Sma*I and *Xba*I; Lane 7, pPT7-Ag85A, SOD, Ag85B linker_PhaC_ linker 74F digested *Sma*I and *Xba*I as a positive control; Lane 8, pPT7-Ag85A, SOD, Ag85B linker_PhaC_ linker 74F digested with *Sma*I, and lane 9, pPT7-Ag85A, SOD, Ag85B linker_PhaC_linker 74F with no enzyme.

3.2.2 PHA production in *E. coli* BL21 (DE3)

Three colonies of *E. coli* BL21 (DE3) harbouring pPT7-Ag85A, SOD, Ag85B linker_PhaC_ linker 74F were selected and grown under PHA accumulating condition (2.6.2). To observe PHA inclusions, the cells were stained with nile-red and visualised by fluorescence microscopy. The cells hosting pPT7-Ag85A, SOD, Ag85B linker_PhaC_ linker 74F had higher fluorescence intensity compared to the negative control (Data not shown). This result indicated that the bacteria form intracellular PHA inclusions.

To confirm the presence of the antigen fusion protein in association with PHA inclusions, the cells were harvested (2.12.1), disrupted (2.12.4.1), purified with PBST (2.12.5.1) and glycerol gradient (2.12.5.5). Samples were denatured and run on SDS-PAGE (2.13.1). Protein profiles show the band representing the antigen fusion at lower molecular weight than the theoretical one. The band appeared on the gel was approximately 140 kDa while the theoretical molecular weight was expected to be 180 kDa. The theoretical molecular weight of the antigen fusion protein was calculated based on amino acid sequence by the ProtParam tool from ExPASy proteomic server (http://www.expasy.org/). In comparison to wildtype PhaC, the band is at the expected molecular weight (60 kDa). It is also show that the level of expression for both wildtype PhaC and the antigen fusion proteins are low as the bands corresponding to these proteins are not dominant (Figure 14A). In addition, western blot analysis using monoclonal antibodies against PhaC confirmed the band correspond to antigen fusion proteins. It also showed that there is potential degradation. (Figure 14B) SDS-PAGE also showed low purity of PHA beads from isolation and purification process as mentioned previously.

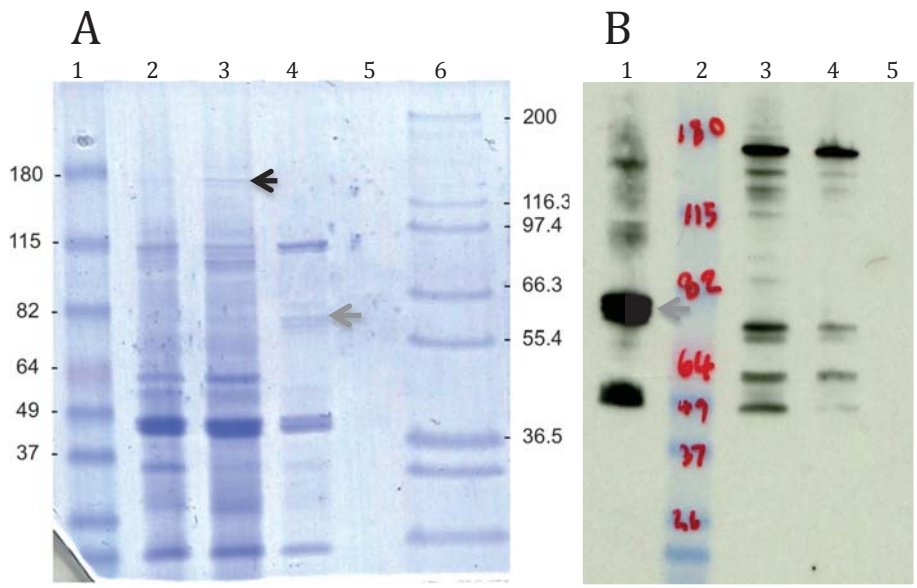

Figure 14. **(A) SDS-PAGE of fusion proteins associated with PHA beads**. Lane 1, Benchmark protein ladder (kDa) [1]; Lanes 2 and 3, Antigen-presenting beads (Ag85A, SOD, Ag85B-PhaC-74F) [2]; Lane 4, Wildtype PhaC [3]; Lane 5, empty lane; Lane 6, Mark (12 kDa). **(B) Western blot analysis to confirm the presence of antigen fusion protein that is associated with PHA beads.** Lane 1, Wildtype PhaC beads as a positive control[3] Lane 2, Benchmark protein ladder (kDa), Lane 3-4. Antigen presenting beads (Ag85A, SOD, Ag85B-PhaC-74F).

1 Benchmark protein ladder (kDa) was incompatible with bis- acrylamide gel (12.3.1) therefore the positions of protein reference are not accurate. This was used only as a reference to proteins shown on western blot. Accurate protein standards are Mark 12 (lane 6), not visible on Western blot.

2 the black arrow indicates the antigen complex from antigen-presenting PHA beads.

3 the grey arrow indicates PhaC.

3.3 Antigen-presenting beads production in *B. megaterium*

3.3.1 *B. megaterium* PHA05 production strain development

3.3.1.1 Transformation of pT7-RNAP to B. megaterium PHA05

Since pPT7 vector system is a T7 RNA polymerase dependent promoter, and *B. megaterium* PHA05 is not a producer, therefore pT7-RNAP was introduced to this strain to produce this polymerase. In the process, 1 µg of pT7-RNAP was transformed into *B. megaterium* PHA05 protoplasts (2.10.2). The transformants harboring the plasmid was selected by agar media containing 4.5 µg/mL of chloramphenicol. The colonies were screened by gradient PCR to detect the presence of the plasmid (2.11.3). The product at 0.9 kb signified the presence of pT7-RNAP (Figure 15). This colony with positive PCR result was selected for making long-term frozen stock for subsequent experiments (2.8.1).

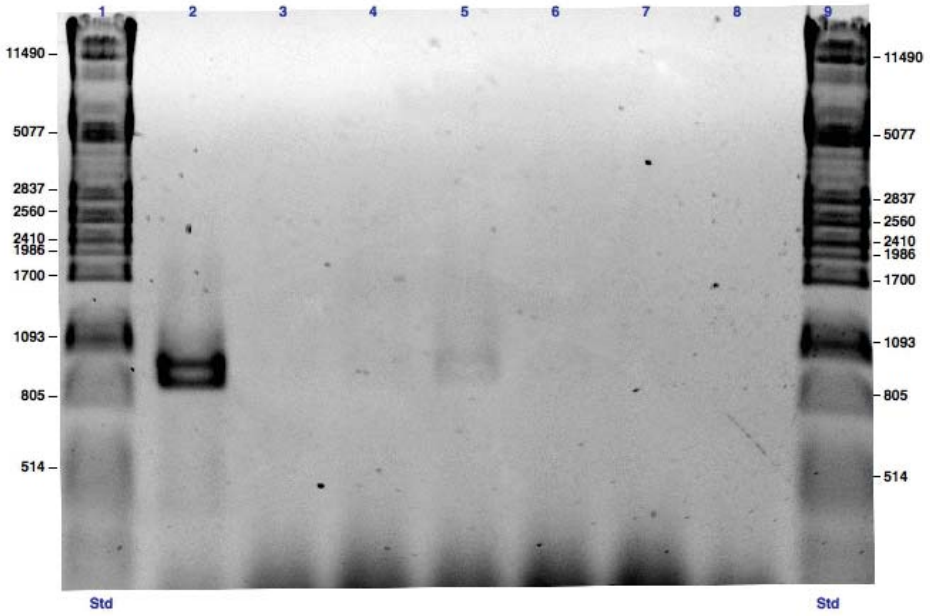

Figure 15. Gradient colony PCR of a transformant to detect the presence of pT7-RNAP using T7seq1 and T7seq2R (table 2.1). Lanes 1 and 9, Lambda phage *Pst*I ladder (bp). Lanes 2-7, a selected transformant at annealing temperature 53, 55, 57, 59, 61 and 63 °C. Lane 8, *B. megaterium* PHA05 as a negative control.

3.3.1.2 Transformation of pPT7-Ag85A, SOD, Ag85B linker_PhaC_linker 74F.

B. megaterium PHA05 harboring pT7-RNAP was used for making protoplast, for transformation with pPT7-PhaCAB or pPT7-Ag85A, SOD, Ag85B linker_PhaC_linker 74F (2.9.2). These plasmids were transformed into protoplasts (2.10.2) and grown on selective media containing 10 µg/mL tetracycline and 4.5 µg/mL chloramphenicol. The transformants were screened by colony PCR (2.11.3) to confirm the presence of pPT7 plasmids. To generate the strain producing wildtype PhaC beads, pPT7-PhaCAB was introduced. PCR screening of colonies after transformation confirmed that pPT7-PhaCAB was present (750 bp product) (Figure 16). To generate the strain for producing antigen-presenting beads, pPT7-Ag85A, SOD, Ag85B linker_PhaC_linker 74F was introduced into protoplasts and selected with the same process with PCR (yielding a product of 1.9 kb (Figure 17). Three colonies were selected for production of antigen-presenting beads.

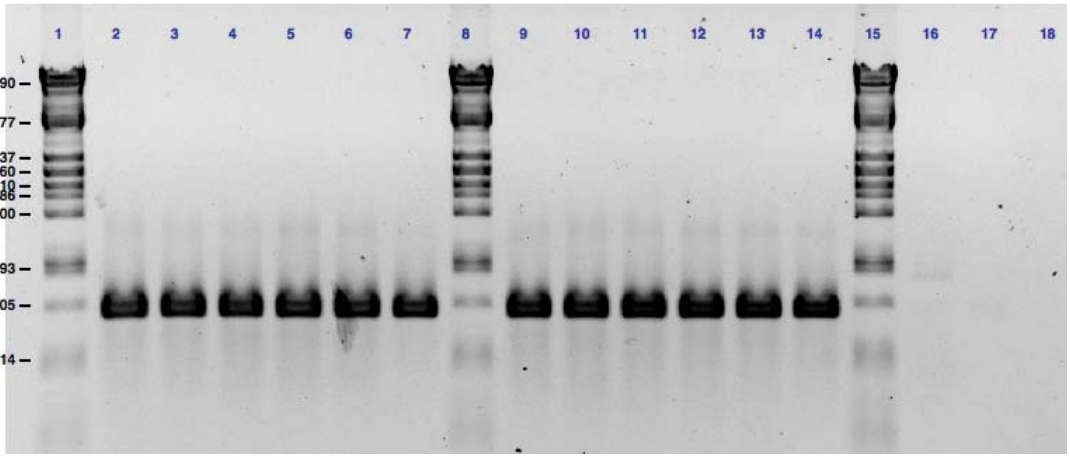

Figure 16. Gradient colony PCR to detect the presence of pPT7-PhaCAB in *B. megaterium*. Lanes 1, 8 and 15, Lambda phage *pst*I DNA ladder (bp). Lanes2-7, Colony1 at annealing temperature 53, 55, 57, 59, 61, and 63 °C respectively. Lanes 9-14, Colony2 at annealing temperature 53, 55, 57, 59, 61, and 63 °C respectively. Lane

16 *B. megaterium* PHA05 containing pT7-RNAP. Lane 17, Water.

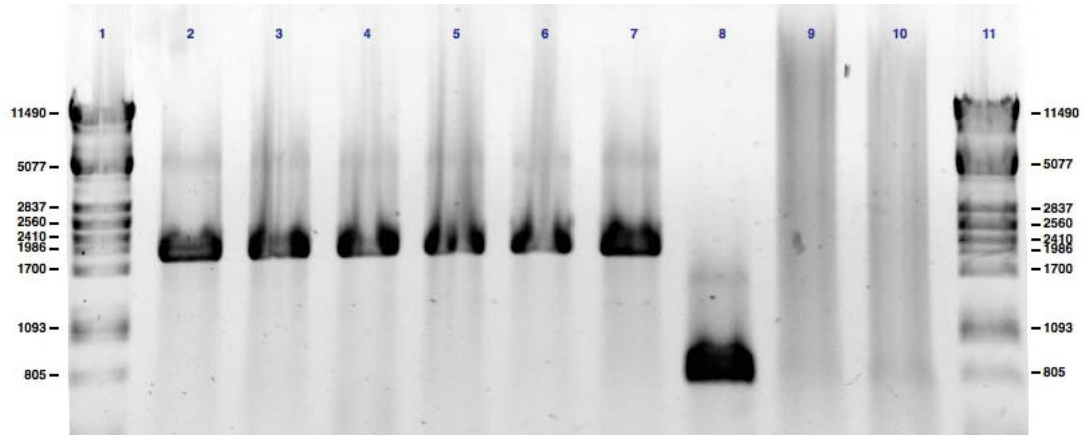

Figure 17. Gradient colony PCR to detect pPT7-Ag85A, SOD, Ag85B linker_PhaC_linker 74F in transformants. Lanes 1 & 11, Lambda phage *pst*I DNA ladder (bp). Lanes 2-7, selected colonies after transformation. Lane 8, *B. megaterium* PHA05 containing pT7-RNAP. Lanes 9 and 10, water.

3.3.2 Production of PHA beads in *B. megaterium* PHA05

B. megaterium PHA05 harbouring both pT7-RNAP and pPT7-Ag85A, SOD, Ag85B linker_PhaC_ linker 74F or pPT7-PhaCAB were inoculated under PHA accumulating conditions (2.6.2). The indication of PHA production was visualised by fluorescence microscopy of nile-red stained bacterial cells (2.12.3). Figure 18A-C showed higher fluorescence intensity in the strains that harbour pPT7-Ag85A, SOD, Ag85B linker_PhaC_linker 74F as well as pPT7-PhaCAB in comparison to the cell that harbour only pT7-RNAP that has low fluorescence intensity. This observation indicates that intracellular PHA inclusions were produced.

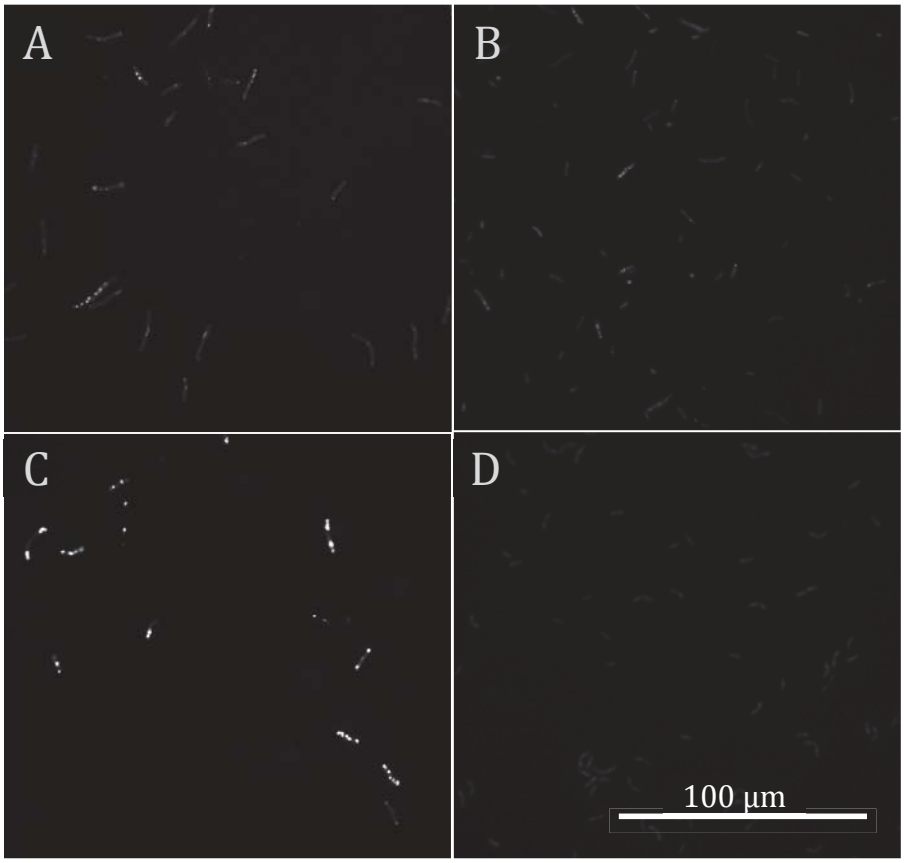

Figure 18. Fluorescence microscopy of *B. megaterium* PHA 05 cells under PHA accumulating condition stained with nile-red. A and B) pT7-RNAP and pPT7-Ag85A, SOD, Ag85B linker_PhaC_linker 74F 3) pT7-RNAP and pPT7-PhaCAB 4) pT7-RNAP. The fluorescence signal indicates the presence of PHA inclusions.

3.3.3 Detection of antigen fusion protein in association with PHA beads

To test whether the antigen fusion protein is present in association with PHA beads, SDS-PAGE (2.13.1) with Coomassie Blue stain was used to analyse the protein profile and western blot (2.13.2) was used to confirm the presence of antigen fusion proteins. The theoretical molecular weight of the antigen fusion protein was calculated based on amino acid sequence by the ProtParam tool from ExPASy proteomic server (http://www.expasy.org/). The molecular weights of the antigen fusion protein and PhaC were predicted to be 180 and 63 kDa, respectively.

SDS-PAGE showed no obvious bands corresponding to the expected weights of PhaC (63 kDa) or antigen fusion protein (180 kDa) (Figure 19). Subsequent western blot analysis using primary antibodies against PhaC confirmed the presence of PhaC at the

expected molecular weight (~63 kDa) (Figure 20). In contrast, the antigen fusion protein was detected at a lower than expected molecular weight of around 120 kDa. In addition, there was another non-specific band at ~90 kDa. Isolated PHA beads were further purified by glycerol gradient. However, SDS-PAGE analysis did not show further improvement (data not shown).

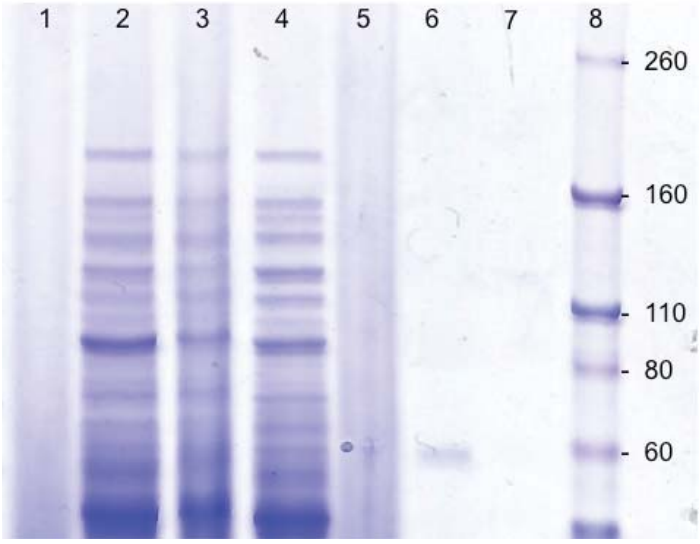

Figure 19. Protein profiles of isolated PHA beads. Lanes 1-3, *B. megaterium* PHA05 harbouring pT7-RNAP and pPT7-Ag85A, SOD, Ag85B linker_PhaC_ linker 74F. Lane 4, whole cell *B. megaterium* PHA05 with no plasmid as a negative control. Lane 5, *B. megaterium* PHA05 harbouring pT7-RNAP and pPT7-PhaCAB. Lane 6 &7, recombinant *E. coli* at 0.4% (w/v) and 0.04% (w/v) (David Hooks, IFS). Lane 8, Novex® Sharp Pre-stained Protein Standard (kDa).

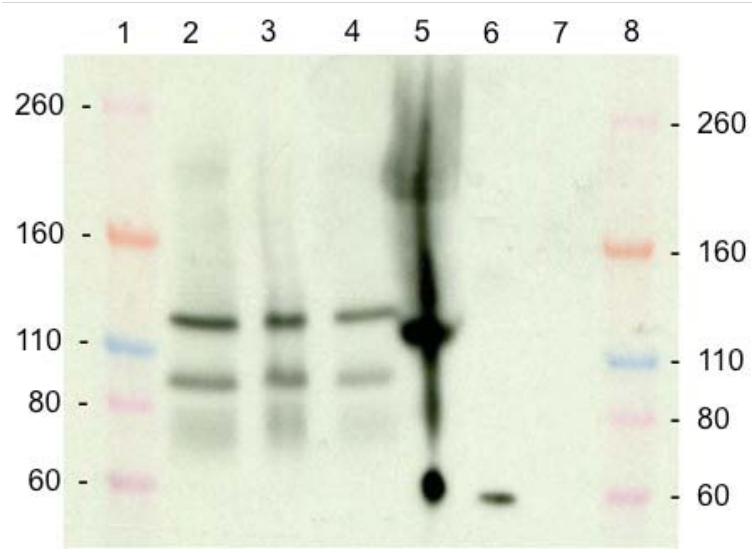

Figure 20. Western blot analysis of isolated PHA beads. Lanes 1 & 8, Novex® Sharp Pre-stained Protein Standard (kDa). Lanes 2-4, *B. megaterium* PHA05 harbouring pT7-RNAP and pPT7-Ag85A, SOD, Ag85B linker_PhaC_linker 74F**.** Lane 5, *B. megaterium* PHA05 harbouring pT7-RNAP and pPT7-PhaCAB. Lane 6, recombinant *E. coli* 0.04% (w/v) (David Hooks, IFS). Lane 7, *B. megaterium* PHA05 with no plasmid.

3.4 Analysis of expression vector pPT7-Ag85A, SOD, Ag85B linker_PhaC_linker 74F

Analysis of the open reading frame (Geneious 6.1.6) showed that a single adenine nucleotide (A) was missing from the start codon of *phaC* (ATG). Therefore, the nucleotide sequence of the entire fusion protein was partially in the incorrect frame resulting in the fusion protein complex not being translated continuously giving the products at 46 and 147 kDa (Figure 21). DNA sequencing also showed the absence of adenine (Figure 22). Therefore, pPT7-Ag85A, SOD, Ag85B linker_PhaC_linker 74F had to be modified by adding a single adenine nucleotide in the sequence at the start codon of *phaC*.

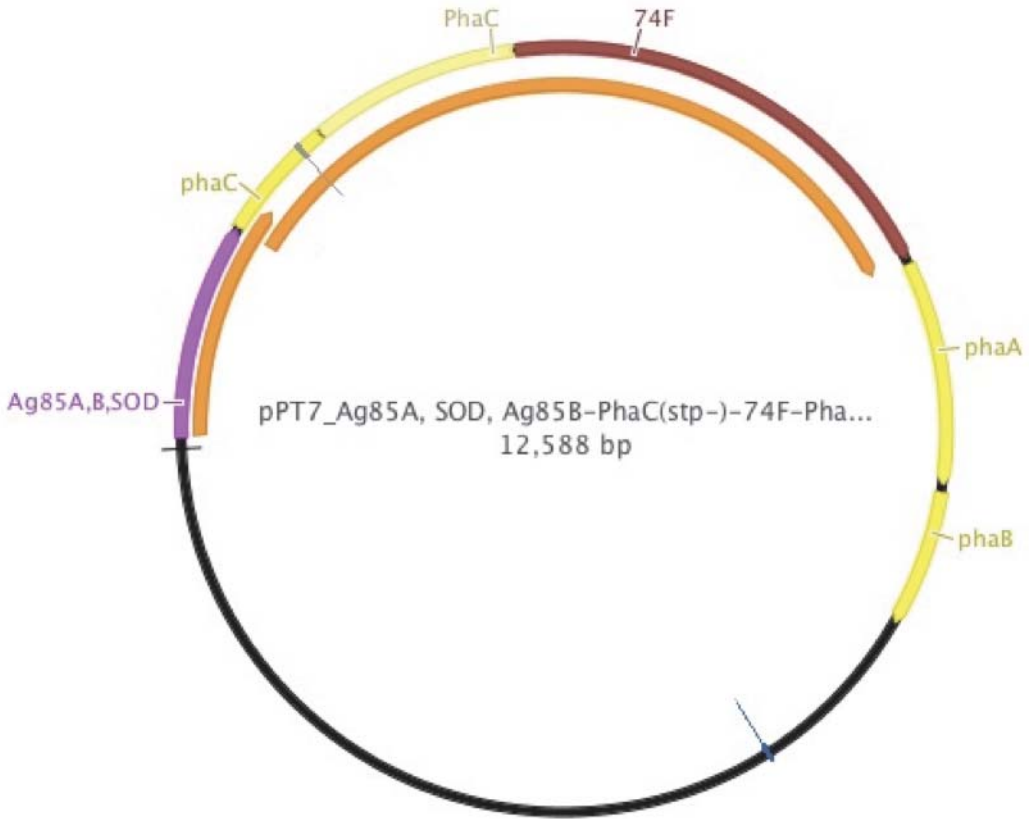

Figure 21. Analysis of open reading frames in pPT7-Ag85A, SOD, Ag85B linker_PhaC_linker 74F.

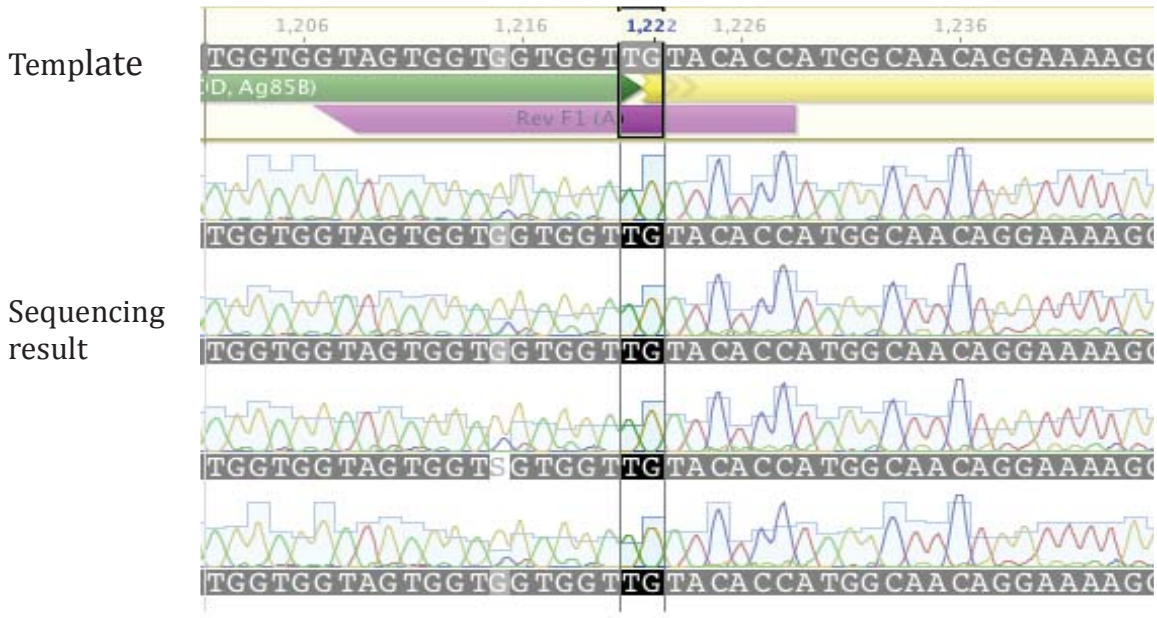

Figure 22. Nucleotide sequencing of pPT7-Ag85A, SOD, Ag85B linker-PhaC confirm the absence of adenine in the start codon of *phaC*.

To generate the DNA fragment at which adenine is introduced at the start codon of *phaC*, a pair of primers were ordered to conduct PCR for mutagenesis. PCR was conducted using pUC57 Frag1_N-terminal_Ag as a template and HIFI *taq* as polymerase so that the PCR product contained poly-A tails (2.11.2). The PCR product was analysed by agarose gel electrophoresis and verify the presence of product at 1.2 kbp (2.11.6). This was isolated from agarose gel (2.11.7) and ligated into pGEM®-T Easy Vector (2.11.9) according to manufacturer's instructions. The ligation reaction was subsequently transformed into *E. coli* XL1 blue (2.10.1) and plated on selective LB media for blue-white selection (2.7). White colonies were selected for screening by colony PCR and PCR products were analysed by gel electrophoresis (2.11.3). The correct product size was obtained (1.2 kbp) (Figure 23). Colonies yielding correct PCR products were inoculated overnight in LB media (2.6.1) for plasmid isolation and restriction endonuclease confirmation with *Nco*I and *Bsr*GI. The presence of insert was confirmed by a product at 1.2 kb (Figure 24). The plasmids pGEM®-T-Ag85A, SOD, Ag85B (A) were sent for nucleotide sequencing to confirm the whole gene as well as insertion of an additional adenine for ATG start codon (Figure 25).

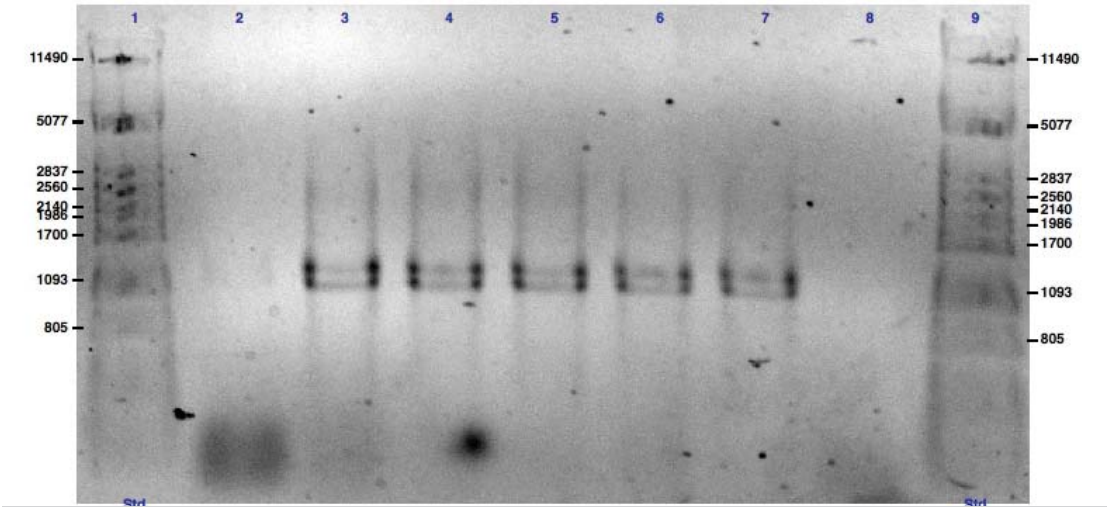

Figure 23. **Colony PCR screening for pGEM®-T Easy Vector with DNA fragment from PCR for mutagenesis**. Lanes 1 & 9, Lambda *Pst*I ladder (bp); Lanes 2-6, selected white colonies; Lane 7, pUC57 Frag1_N-terminal_Ag; Lane 8, Water.

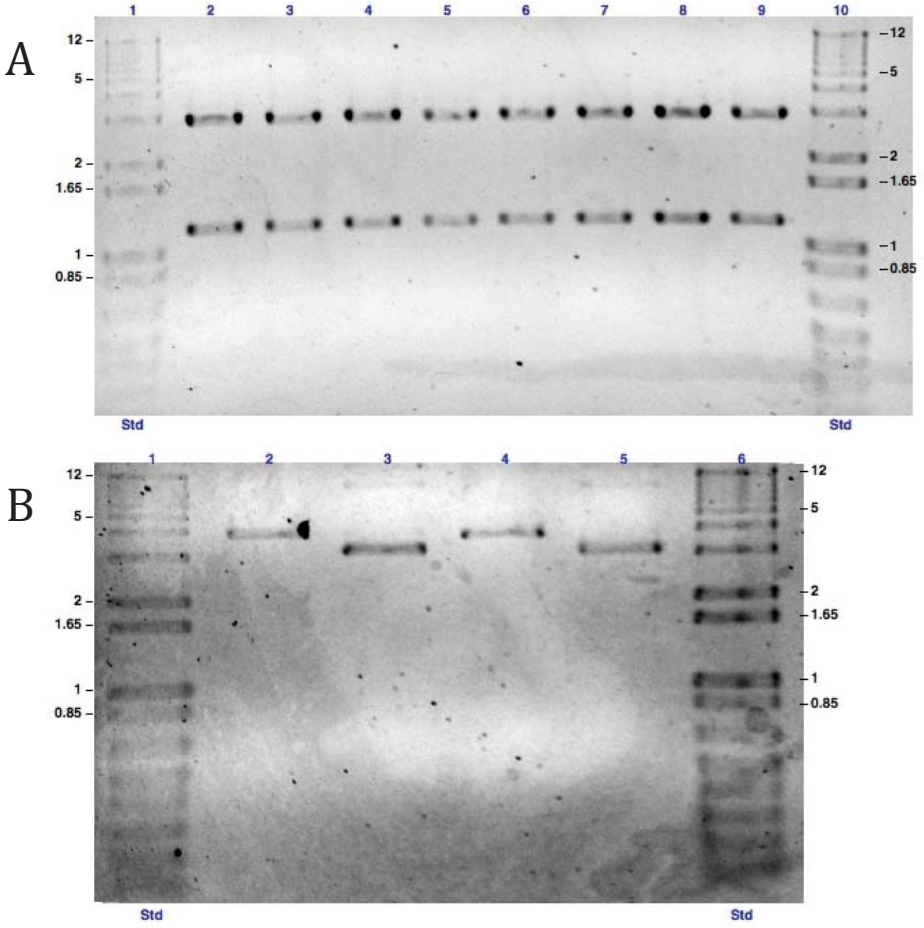

Figure 24. Restriction endonuclease confirmation for the presence of DNA fragment from PCR for mutagenesis in pGEM®-T Easy Vector (A) Lane 1 & 10, 1 kb plus ladder (kbp). Lanes 2-9, plasmids isolated from selected colonies digest with *Nco*I and *Bsr*GI. (B) Lanes 1 & 6, 1 kb plus ladder (kbp). Lane 2, pUC57 Frag1_N-terminal_Ag digest with *Bsr*GI and *Nco*I. Lanes 3 & 4, pUC57 Frag1_N-terminal_Ag digest with *Bsr*GI and *Nco*I, respectively. Lane 5, undigested pUC57 Frag1_N-terminal_Ag plasmid.

To modify pPT7-Ag85A, SOD, Ag85B linker_PhaC_linker 74F, this vector was hydrolysed by restriction digest with *Pac*I and *Bsr*GI as well as pGEM®-T- Ag85A, SOD, Ag85B (A). Gel electrophoresis (2.11.6) showed the expected fragments at 12 kb and 1.2 kb for the vector and DNA insert, respectively. Both products were isolated from agarose gel (2.11.7) and ligated together (2.11.9). The ligation mixture was transformed into *E. coli* XL1 blue and grown on selective LB agar containing 100 μg/mL ampicillin. Transformants were inoculated in LB media overnight for plasmid extraction (2.11.1). The presence of introduced nucleotide as part of start

codon was confirmed by DNA sequencing.

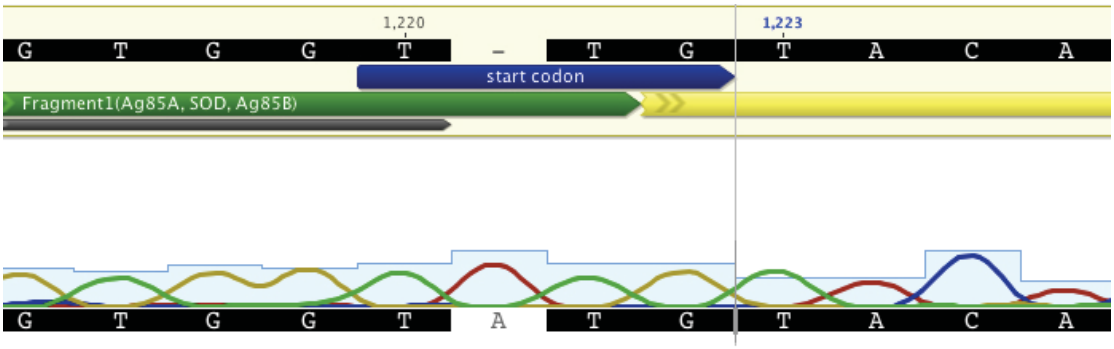

Figure 25. Nucleotide sequence analysis of pGEM®-T-Ag85A, SOD, Ag85B (A) confirms the presence of additional adenine (A) at start codon of *phaC*.

3.5 Antigen-presenting beads production in *E. coli* (Clearcoli BL21 (DE3) harboring pRARE2)

To correct the protein expression problem due to the presence of rare codons for *E. coli* BL21 (DE3), this corrected production plasmid was aimed be introduced to *E. coli* BL21 Rosetta. Which contains pRare encoding for *E. coli* tRNAs for rare codons. However, as this produce will be used in animal experments in the future, it is better to use *E. coli* Clearcoli BL21 (DE3) that have no LPS. Hence, ClearColi, an *E .coli* strain harboring pRARE2 was developed Rubio Reyes and Rehm, unpublished.

3.5.1 Transformation of modified expression vector into *E. coli* Clearcoli BL21 (DE3)

Three plasmids were independently introduced into competent Clearcoli cells (2.10.1): pPT7-PhaCAB (for producing wildtype beads) pPT7-PhaAB (as a negative control), and pPT7-Ag85A, SOD, Ag85B linker_(A)PhaC_linker 74F, (for producing antigen displaying beads). Transformants were selected for on media containing 34 µg/mL chloramphenicol and 100 µg/mL ampicillin. Colonies were screened by PCR (2.11.3) for the presence of the correct insert in plasmids.

The correct PCR products for pPT7-PhaCAB (Fwd6 ,Rev 7 primers), pPT7-PhaAB using (Fwd 8, Rev 9), and pPT7-Ag85A, SOD, Ag85B linker_(A)PhaC_linker 74F (pPT7Fwd and Rev3) were 0.7, 1.2 (data not shown) and 1.9 kbp (Figure 26), respectively (data not shown). PCR verified transformants were grown overnight in liquid media and kept at -80 °C as stock for later biobeads production experiments.

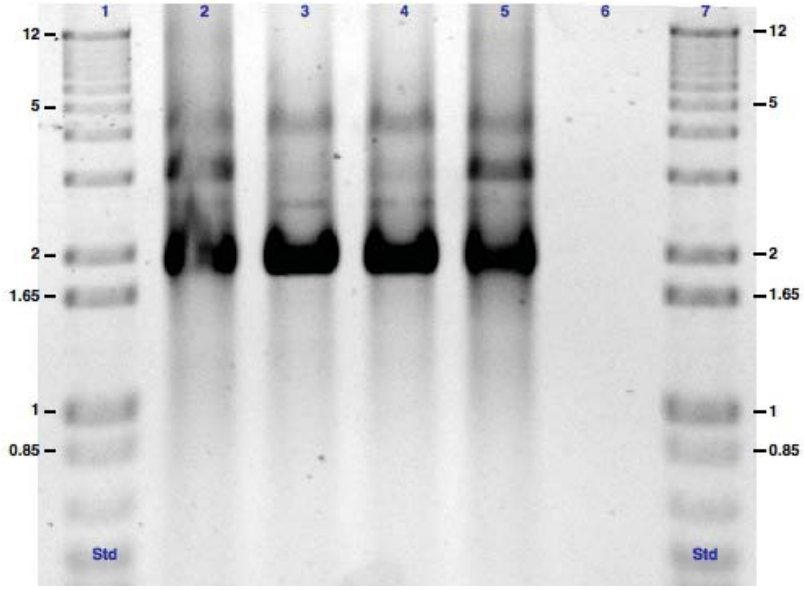

Figure 26. **Colony PCR screening for the presence of pPT7-Ag85A, SOD, Ag85B linker_(A)PhaC_linker 74F using pPT7Fwd and Rev3 primers**. Lanes 1 & 7, 1 kb plus ladder (kb). Lanes 2-4, candidate colonies transformed with this plasmid; Lane 5, plasmid pPT7-Ag85A, SOD, Ag85B linker_(A)PhaC_linker 74F; Lane 6, water.

3.5.2 PHA beads production in *E. coli* Clearcoli BL21 (DE3)

E. coli Clearcoli BL21 (DE3) harbouring pPT7-Ag85A, SOD, Ag85B linker_(A)PhaC_linker74F, pPT7-PhaCAB, and pPT7-PhaAB were inoculated under PHA accumulation condition. To detect the formation of PHA inclusions, whole cells were stained with nile-red and observed under fluorescence microscope. In Figure 27 fluorescence was weak for the negative control (Clearcoli harboring pPT7-PhaAB). In contrast, other Clearcoli strains containing plasmids pPT7-Ag85A, SOD, Ag85B linker_(A)PhaC_linker74F or pPT7-PhaCAB displayed intense fluorescence, indicating the formation of PHA inclusions. The presence of fusion protein being produced in this stain [Clearcoli + pPT7-Ag85A, SOD, Ag85B linker_(A)PhaC_linker 74F] was detected by SDS-PAGE and western blot analysis. Figure 28A shows that the fusion protein Ag85A_SOD_Ag85B-PhaC-74F was present at expected molecular weight (180 kDa). Western blot analysis using PhaC monoclonal antibody (Figure 28B) confirmed that the protein band at 180 kDa was the antigen fusion protein. Western blot also show the other lower molecular weight bands which indicated either incomplete translation or degradation of the fusion protein. To assess the level of fusion protein expression in relation to PHA beads, the cells were disrupted and the beads were purified and analysed by SDS-PAGE. Our results showed that the yield and purity of the fusion protein was low (Figure 29).

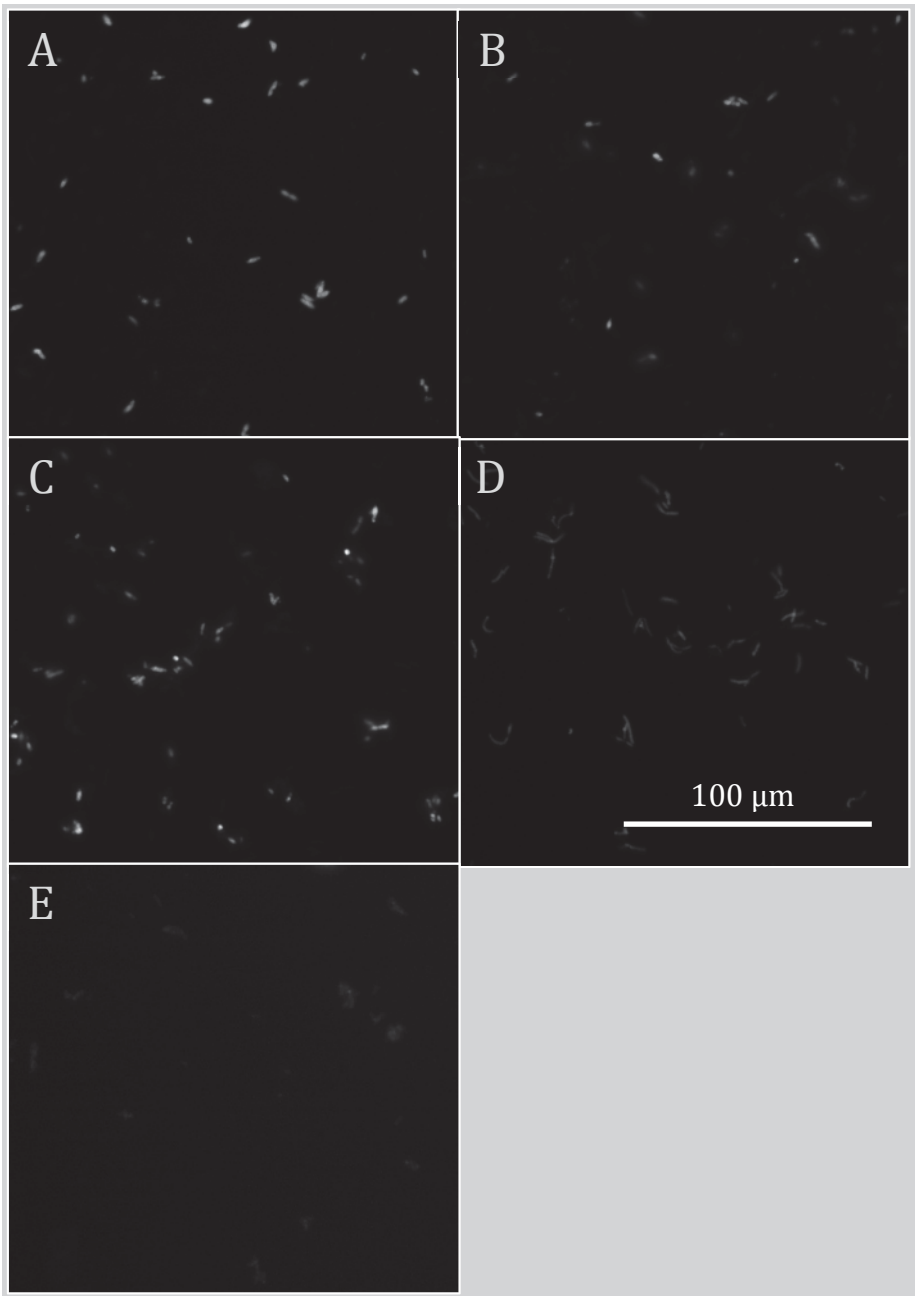

Figure 27. Fluorescent picture of recombinant *E. coli* Clearcoli BL21 (DE3) harboring pPT7-Ag85A, SOD, Ag85B linker_(A)PhaC_linker 74F (picture A-C), pPT7-PhaCAB (picture D), pPT7-PhaAB (picture E*).

*The brightness +40%, contrast -20% for visualisation.

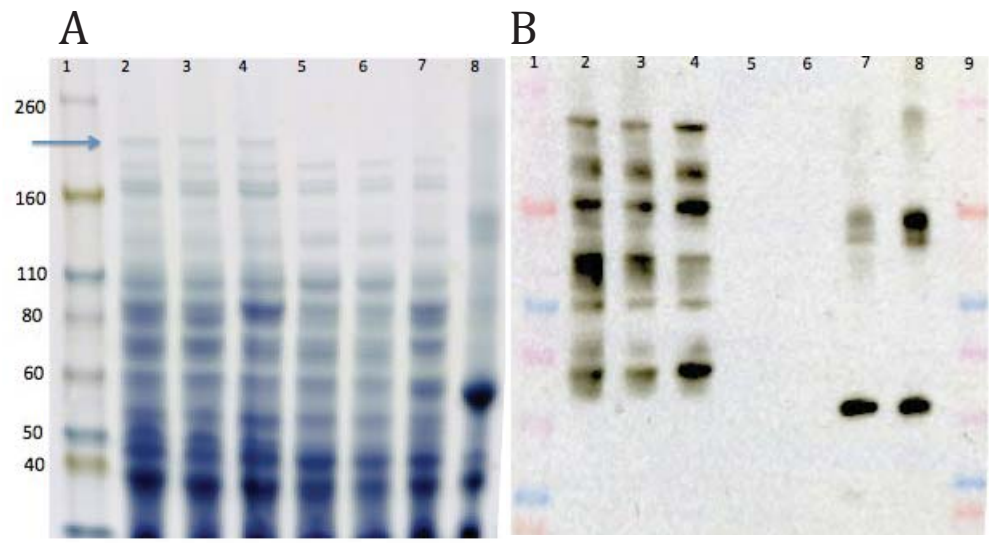

Figure 28. (A) SDS-PAGE and (B) Western blot of recombinant *E. coli* Clearcoli BL21 (DE3) whole cell samples. Lane 1 and 9, Novex® Sharp Pre-stained Protein Standard (kDa); Lanes 2-4, pPT7-Ag85A, SOD, Ag85B linker_(A)PhaC_linker74F; Lanes 5-6, pPT7-PhaAB; Lane 7, pPT7-PhaCAB; Lane 8, Wildtype PhaC beads control. The arrow shows the antigen fusion at expected molecular weight.

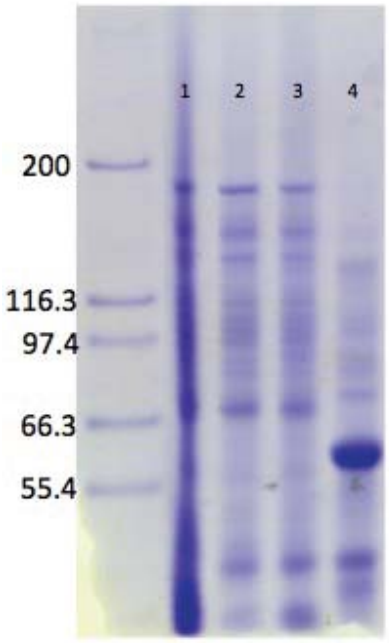

Figure 29. SDS-PAGE of isolated beads from recombinant *E. coli* Clearcoli BL21 (DE3). Mark 12 ladder (kDa). Lanes 1-3, pPT7-Ag85A, SOD, Ag85B linker_(A)PhaC_linker 74F; Lane 4. pPT7-PhaCAB

3.6 Construction of plasmids for antigen-presenting beads containing the antigens fused to one terminus of the PHA synthase.

3.6.1 Construction of pPT7-Ag85A, SOD, Ag85B linker_(A)PhaC (N-terminal antigens)

pGEM®-T-Ag85A, SOD, Ag85B(A) and pPT7-PhaCAB were hydrolysed with restriction enzymes *Pac*I and *Bsr*GI. Both products from pGEM®-T-Ag85A, SOD, Ag85B(A) were subjected to agarose gel electrophoresis (2.11.6) to separate the DNA fragments encoding the truncated antigens from the rest of the plasmid. The targeted DNA fragment was then isolated from agarose gel (2.11.7). This DNA fragment was then ligated (2.11.9) to the vector pPT7-PhaCAB that was previously digested and purified (with same restriction enzymes). The ligation reaction was transformed to *E. coli* XL1 blue (2.10.1) and plated on selective media containing 100 µg/mL of ampicillin. The positive colonies were screened by colony PCR (2.11.3). The DNA fragment of 1.2 kbp that was detected by gel electrophoresis (2.11.6) indicated the presence of the vector pPT7-Ag85A, SOD, Ag85B linker_(A)PhaC (Figure 30). The positive colonies based on this PCR, were selected for overnight inoculation for plasmid extraction (2.11.1). To confirm the presence of Ag85A, SOD, Ag85B(A) fragment in the plasmid, the extracted plasmids were digested by *Pst*I and *Xho*I. The product at 4 and 1.3 kbp confirmed the presence of Ag85A, SOD, Ag85B(A) (Figure 31). The presence of Ag85A, SOD, Ag85B(A) fragment in the plasmid was further confirmed by DNA sequencing (data not shown).

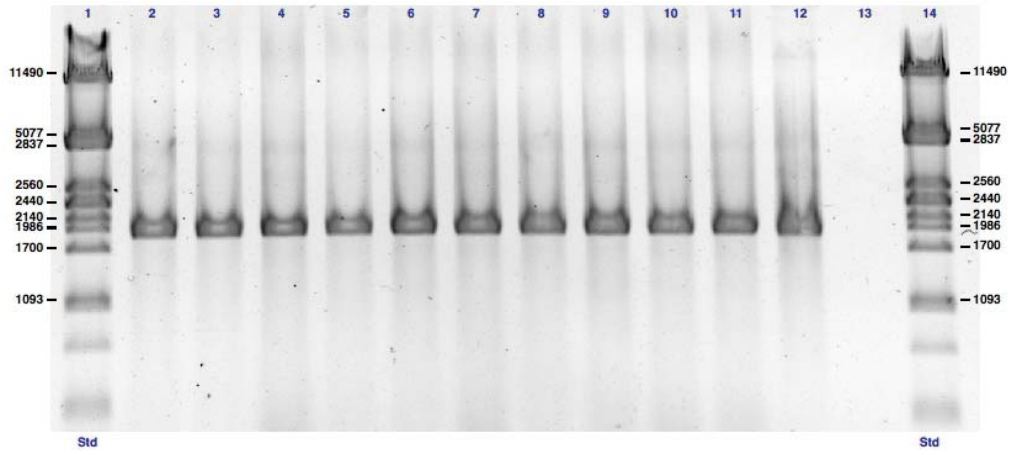

Figure 30. Colony PCR detecting the presence of pPT7-Ag85A, SOD, Ag85B-linker_(A)PhaC in *E. coli* XL1 blue transformants. Lane 1, Lambda phage *Pst*I ladder. Lanes 2-11, selected colonies from plasmid transformation. Lane 13, water.

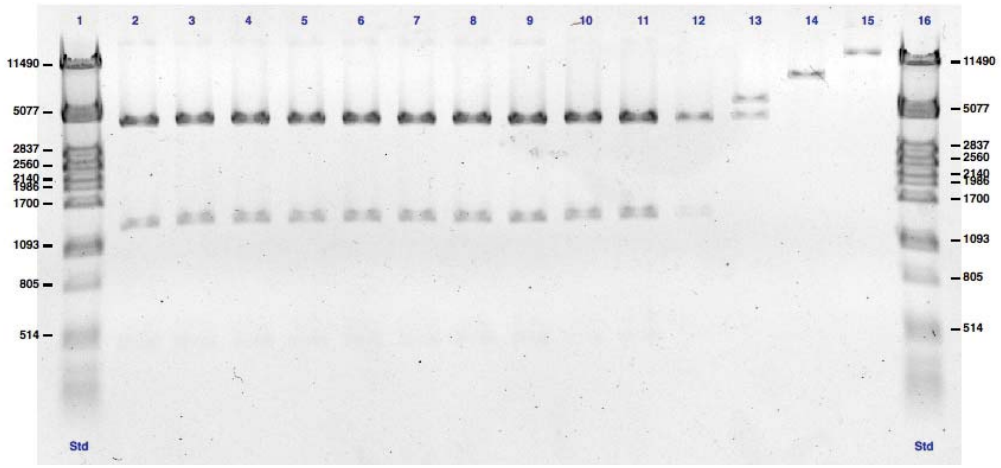

Figure 31. **Confirmation of *E. coli* XL1 transformation with pPT7-Ag85A, SOD, Ag85B linker_(A)PhaC by restriction digest.** Lanes 1 and 16, Lambda phage *Pst*I ladder (bp). Lanes 2-10, restriction digest of isolated plasmids from selected transformants with *Pst*I and *Xho*I. Lane 11, (positive control) pPT7-Ag85A, SOD, Ag85B linker_PhaC digested with *Pst*I and *Xho*I; Lane 13, (negative control) undigested pPT7-Ag85A, SOD, Ag85B linker_PhaC. Lanes 14 & 15, pPT7-Ag85A, SOD, Ag85B linker_PhaC digested with only *Pst*I and *Xho*I, respectively.

3.6.2 Construction of pPT7-PhaC_linker 74F

The strategy to construct pPT7-PhaC_linker 74F included removing the stop codon from *phaC* and then cloning the 74F gene into its 3' end. To remove the stop codon from *phaC,* both pPT7-PhaCAB and pGEM®-T-PhaC^{stp-} were hydrolysed by restriction enzymes *Bst*BI and *Bgl*II. Both reactions were subjected to agarose gel electrophoresis (2.11.6) and the DNA fragments of interest were extracted (2.11.7). The PhaC^{stp-} fragment was then ligated with the pGEM®-T Easy Vector (2.11.9) and transformed into *E. coli* XL1 blue (2.10.1). After transformation, the cells were plated on LB agar containing 100 µg/mL ampicillin. The colonies that grew were screened by PCR (2.11.3) to confirm the presence of the plasmid. Agarose gel electrophoresis of PCR reaction visualised the DNA product at 1.1 kbp indicating that the transformants contain pPT7-PhaCAB potentially have the absence of stop codon (Figure 32). The absence of stop codon at the end of *PhaC* was confirmed by gene sequencing (2.11.10).

To insert 74F encoding gene into 3' end of *phaC*, pPT7-PhaCAB^{stp-} and pUC57 Frag2_C-terminal_Ag 2 were hydrolysed with *Bgl*II (2.11.5). The 5' Phosphate group of the linearlised vector was removed by phosphatase treatment (2.11.8). The product from pUC57 Frag2_C-terminal_Ag 2 digest was subject to agarose gel electrophoresis (2.11.6) and the DNA fragment (1.1 kbp) was purified from the agarose gel (2.11.7). This fragment was cloned to the vector backbone (pPT7-PhaCAB^{stp-} hydrolysed with *Bgl*II) (2.11.9) then transformed into *E. coli* XLI blue (2.9.1). The cells were plated on LB agar containing 100 µg/mL ampicillin. Colonies were screened by colony PCR for the presence of the plasmid (2.11.3). Figure 33 showed that the product at 1.1 kbp indicate the presence of the ligation product in the selected colonies. The positive colonies were then inoculated in liquid media for plasmid extraction (2.11.1). The isolated plasmids were hydrolysed with *Xho*I to confirm the presence of the insert at the correct orientation (2.11.5). Plasmids containing insert in the correct orientation were expected to yield three products (9.8, 1.2 and 0.4 kb) after hydrolysis, while plasmids with the insert oriented in the opposite direction would produce two fragments (8.2 and 2.8 kbp) (Figure 34). Plasmids yielding correct digestion product pattern were further verified by sequencing (2.11.10).

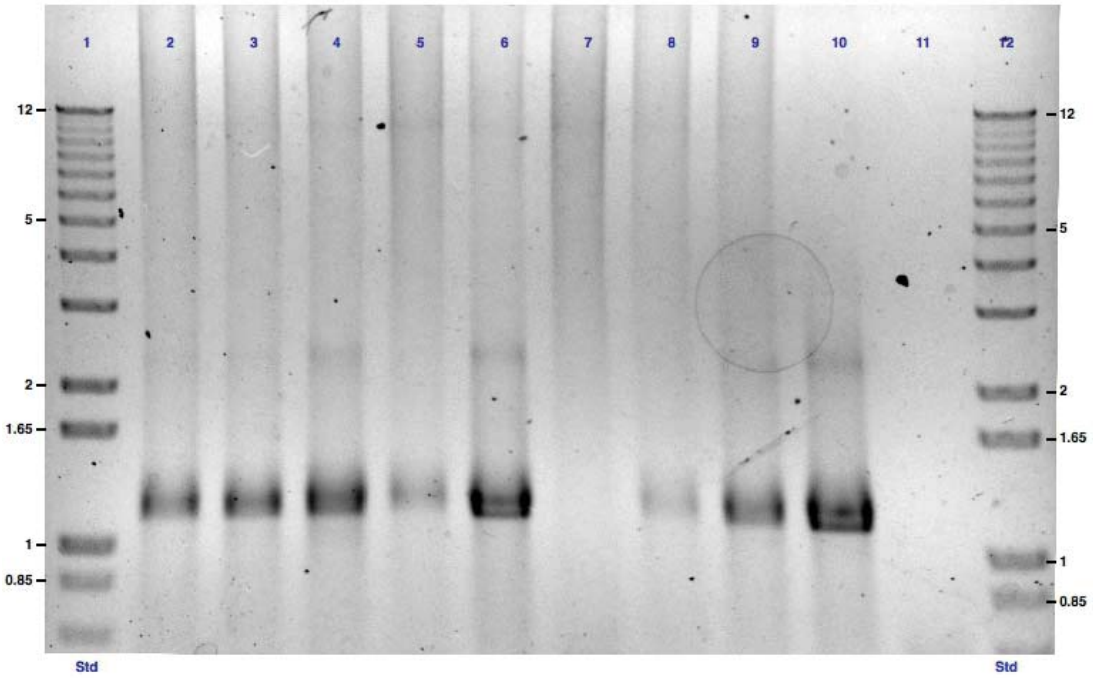

Figure 32. **Colony PCR detecting the presence of pPT7-PhaCAB $^{stp-}$ in *E. coli XL1* blue transformants.** Lanes 1 & 12, 1 kb plus ladder. Lanes 2-9, selected colonies of transformants. Lane 10, pPT7-PhaCAB (positive control). Lane 11, water (negative control).

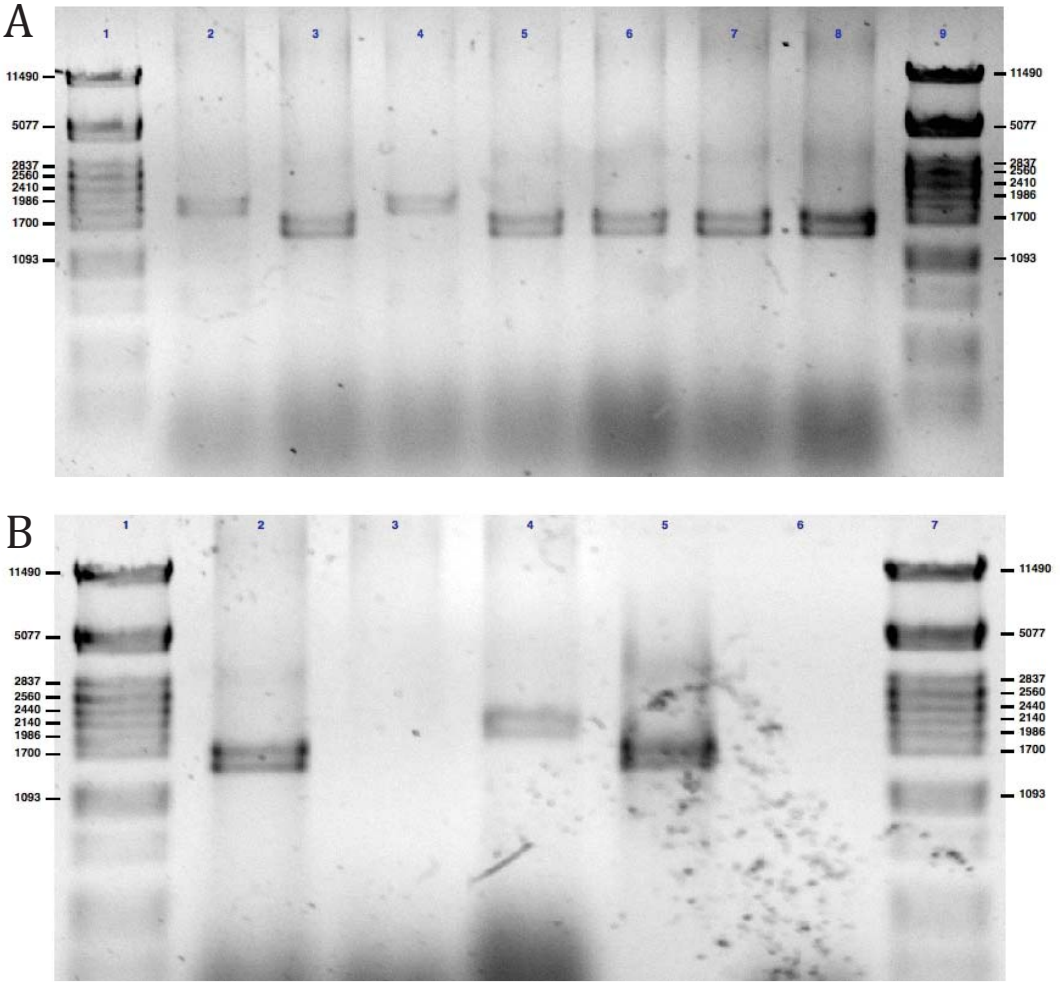

Figure 33. **Colony PCR for the presence of 74F on 3' of *phaC* in correct orientation (1.5 kb).** (A) Lanes 1& 9, Lambda phage *Pst*I ladder (bp). Lanes 2-8, selected colonies. (B) Lanes 1 & 7, Lambda phage *Pst*I ladder (bp). Lanes 2-4, selected colonies. Lane 5, (positive control) pPT7-Ag85A, SOD, Ag85B linker_PhaC_linker 74F plasmid as PCR template. Lane 6, (negative control) water.

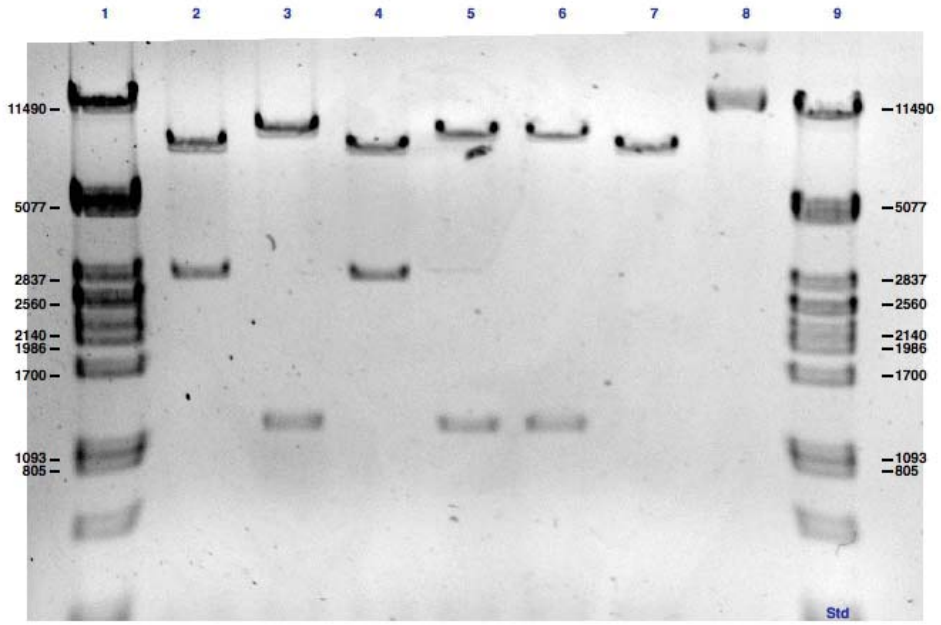

Figure 34. Restriction digest by *Xho*I confirm the presence of 74F on 3' of *phaC* (pPT7-PhaC $^{stp-}$_linker 74F) Lanes 1&9, Lambda phage *Pst*I ladder (bp); Lanes 2-6, selected plasmids digest with *Xho*I; Lanes 7 & 8, (positive and negative controls) plasmid pPT7-PhaCAB lacking 74F, digested with and without *Xho*I, respectively.

3.7 Single-fusion antigen-presenting bead production in *E. coli* Clearcoli BL21 (DE3)

3.7.1 Transformation of pPT7-Ag85A, SOD, Ag85B linker_(A)PhaC and pPT7-PhaC_linker 74F to *E. coli* Clearcoli BL21 (DE3)

The respective plasmids were transformed into Clearcoli competent cells (2.10.1) and grown on selective LB agar containing 34 µg/mL chloramphenicol and 100 µg/mL ampicillin. The colonies that grew on selective media were screened by PCR (2.11.3) for the presence of the transformed plasmid. Specific products at 1.9 and 3.1 kbp indicated presence of pPT7-Ag85A, SOD, Ag85B linker_(A)PhaC and pPT7-PhaC_linker 74F respectively (Figure 35). Positive colonies were selected for plasmid expression to produce antigen-presenting PHA beads.

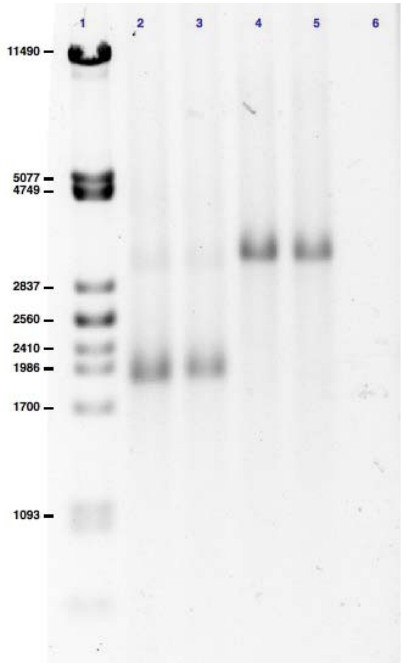

Figure 35. Transformation of pPT7 plasmid into *E. coli* Clearcoli BL21 (DE3). Lane 1, Lambda phage *Pst*I ladder (bp); Lanes 2-3, Selected colonies from pPT7-Ag85A, SOD, Ag85B linker_(A)PhaC transformation; Lanes 4-5, Selected colonies from pPT7-PhaC_linker 74F transformation. Lane 6, water.

3.7.2 Single-fusion antigen displaying PHA bead production

E. coli Clearcoli BL21 (DE3) harbouring pPT7-Ag85A, SOD, Ag85B linker_(A)PhaC and pPT7-phaC_linker 74F were cultured under PHA accumulating conditions for production of antigen-presenting beads (2.6.2). Fluorescence microscopy (2.12.3) of harvested cells that were stained with nile-red showed that both strains produced intracellular PHA inclusions (data not shown).

SDS-PAGE analysis (2.13.1) of isolated and purified PHA beads displaying (1) truncated Ag85A_SOD_Ag85B (N-terminal) and (2) 74F (C-terminal) showed the presence of fusion proteins at 100 kDa and 150 kDa, respectively (Figure 36). However, other protein bands at lower molecular weights were also detected suggesting the presence of co-purified protein contaminants. In addition, western blot analysis confirmed that the bands as previously identified at the molecular weight 100 kDa and 150 kDa, were the antigen fusion proteins truncated Ag85A_SOD_ Ag85B-PhaC and PhaC-74F, respectively. However, there were other extra bands present at lower molecular weight as previously mentioned. For the Ag85A, SOD, Ag85B antigen-presenting beads, there were small bands present at 60 kDa and 40 kDa. For 74F antigen-presenting beads, western blot show multiple unspecific bands at lower molecular weight (Figure 37). These could be due to proteolytic degradation.
To find out whether the non-specific bands were from degradation due to processing or intracellular degradation, whole cell samples of the strain producing both antigen-presenting beads after growth under PHA accumulating condition were analysed by western blot (2.13.2). We showed that non-specific bands were present prior to processing (Figure 38), indicating that the unspecific protein bands were most likely a result of intracellular processes -either degradation and/or incomplete translation.

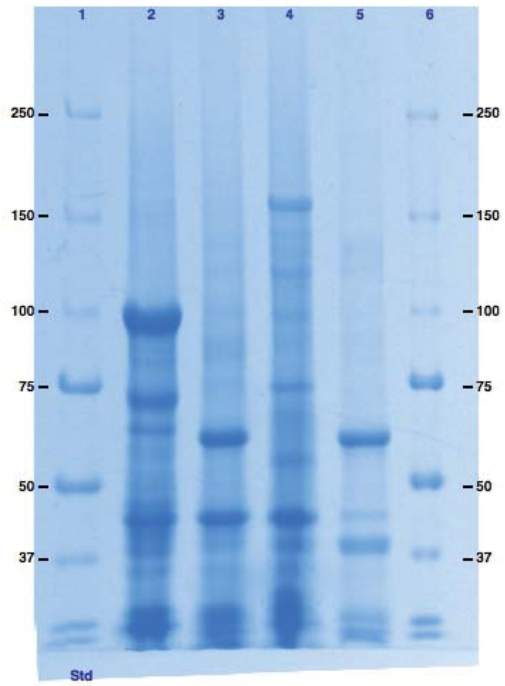

Figure 36. SDS-PAGE of isolated beads from recombinant *E. coli* Clearcoli BL21 (DE3). Lanes 1 and 6, Precision Plus Protein™ standard (kDa); Lane 2, truncated Ag85A, SOD, Ag85B-presenting beads (N-terminal); Lane 3, PhaC beads (WT); Lane 4, 74F-presenting beads (C-terminal); Lane 5, PhaC beads (David Hooks, IFS).

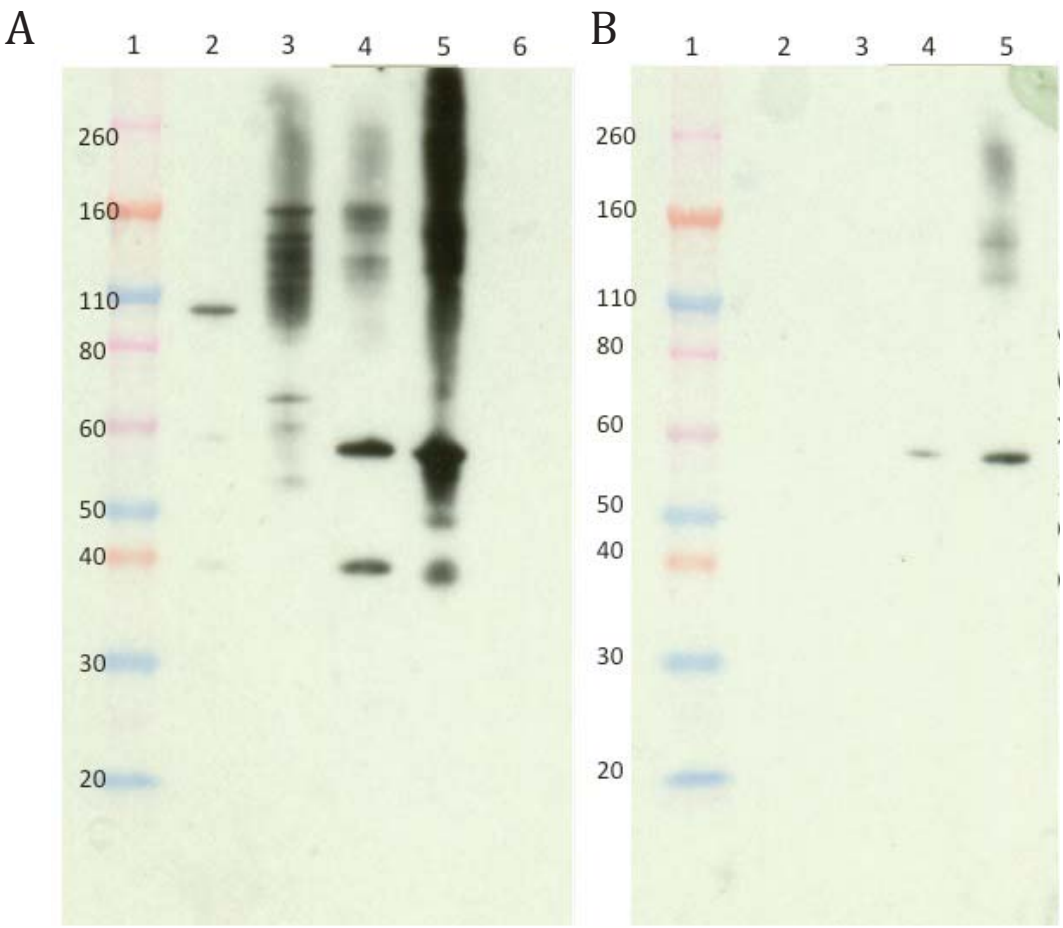

Figure 37. Western blot analysis of isolated beads from *E. coli* Clearcoli BL21 (DE3) producing single terminal antigen-presenting beads using monoclonal antibody against PhaC. (A) the amount of the samples were 0.4% (w/v) of wet beads; Lane 1, Precision Plus Protein™ standard; Lane 2, truncated Ag85A, SOD, Ag85B-presenting beads (N-terminal); Lane 3, 74F-presenting beads (C-terminal); Lane 4, PhaC beads (WT); Lane 5, PhaC beads (David Hooks, IFS); Lane 6, PhaAB cells; (B) the amount of the samples were 0.04% (w/v) of wet beads samples; Lane 1, Precision Plus Protein™ standard (kDa); Lane 2, truncated Ag85A, SOD, Ag85B-presenting beads (N-terminal); Lane 3, 74F-presenting beads (C-terminal); Lane 4, PhaC beads (WT); Lane 5, PhaC beads (David Hooks, IFS);

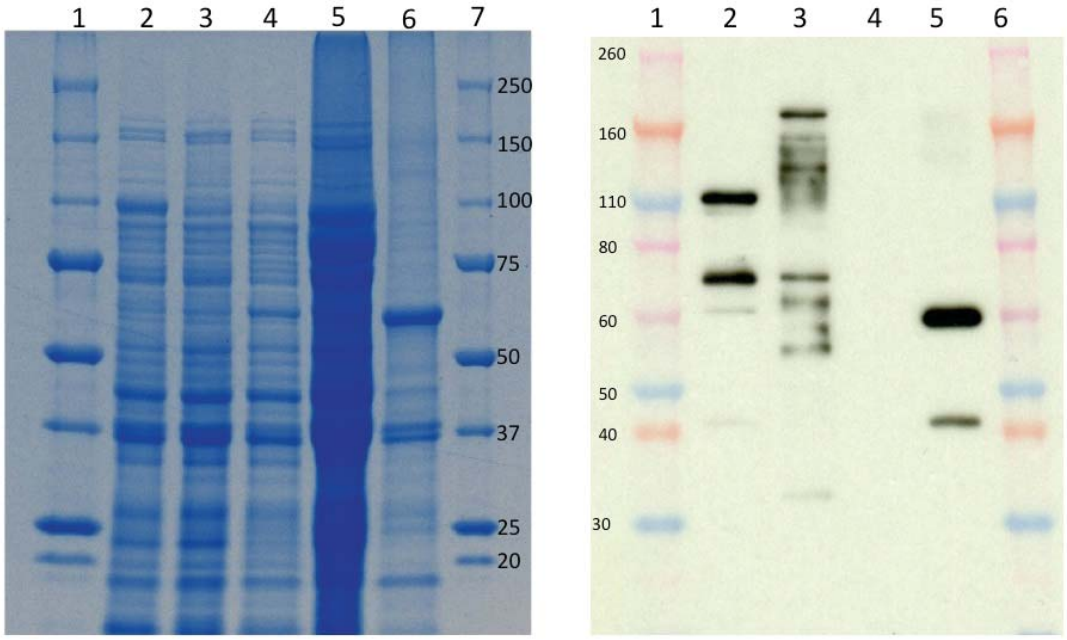

Figure 38. Antigen fusion protein analysis from whole cell samples of *E. coli* Clearcoli BL21 (DE3) after PHA accumulating condition. (A) Protein profile. Lanes 1 and 7, Precision Plus Protein™ standard (kDa); Lane 2, pPT7-Ag85A, SOD, Ag85B linker_(A)PhaC; Lane 3, pPT7-PhaC_linker 74F; Lane 4, pPT7-PhaCAB; Lane 5, pPT7-PhaAB; Lane 6, PhaC beads (David Hooks, IFS). and (B) western blot analysis. Lane 1 and 6, Precision Plus Protein™ standard (kDa); Lane 2, pPT7-Ag85A, SOD, Ag85B linker_(A)PhaC; Lane 3, pPT7-PhaC_linker 74F; Lane 4, pPT7-PhaAB; Lane 5, pPT7-PhaCAB.

3.7.3 Purification analysis of Ag85A, SOD, Ag85B-PhaC beads and wildtype PhaC beads

E. coli Clearcoli BL21 (DE3) harboring pPT7-Ag85A, SOD, Ag85B linker-(A)PhaC and pPT7-PhaCAB were inoculated under PHA accumulating condition in large scale of 6 liters. The cells were harvested and resuspended in lysis buffer to 20% (w/v) of biomass, then disrupted by microfluidiser (2.12.4.3). The PHA biobeads were isolated by centrifugation, which resulted in 7 g of the antigen-presenting beads and 10 g wildtype PhaC beads. These biobeads were then purified according to standard operating procedure (Patent application WO_2012_077080_A1). In short, the biobeads were suspended in lysis buffer to 20% (w/v) by homogeniser (2.12.2.2) then the

biobeads were harvested by centrifugation. This step was repeated for the second round with lysis buffer then followed by using caustic buffer for the next wash step (2.12.5.4). Additionally, the biobeads were washed by PBS twice to remove caustic buffer. After each process, the samples were taken for analysis by SDS-PAGE. For wildtype PhaC beads, the PhaC protein was more abundant while the other proteins were reduced after lysis buffer wash and caustic wash. For antigen-presenting beads, the antigen fusion protein was more abundant after lysis wash, however caustic wash significantly reduced the amount of antigen fusion present in association with the biobeads (Figure 39).

Western blot analysis of the whole cell sample, intermediate product after lysis buffer treatment, and the end product after caustic buffer treatment showed that the treatment by caustic buffer removed unexpected protein bands below the correct molecular weight of 150 kDa (Figure 40B). However, SDS-PAGE showed that the band corresponding to the antigen fusion protein was significantly reduced by caustic buffer washes (Figure 40A). Quantitatively, densitometry analysis showed that the total amount of fusion protein present on the beads was 280 ng of fusion/mg of wet beads. Taking into account that the antigen is 42% of fusion protein; therefore, each mg of beads is composed of 117.7 ng of antigen. Analysis of antigen fusion protein by BCA also showed consistent result. According to the previous immunisation study of tuberculosis antigen-presenting beads, the maximum volume that can be inoculated at a concentration of 20% (w/v) was 200 µl (unpublished data). In addition, the amount of the antigen required in immunisation was 30 µg, which the current antigen-presenting beads could not achieve. Therefore, the entire production process was repeated with modified purification process by excluding caustic buffer wash steps in order to achieve lower reduction of fusion protein present. SDS-PAGE analysis of the product showed that the antigen fusion protein remained abundant (Figure 41). Lysis buffer removed some protein contaminants and PBS did not reduce the amount of fusion protein. Quantification of antigen fusion protein by densitometry analysis and BCA of the final product showed that there was 605 ng of antigen/mg of beads.

As for wildtype PhaC, the PHA beads were produced, processed and analysed in the same manner. The presence of wildtype PhaC was dominant after purification with the presence of many other bands at lower molecular weights (Figure 41). Western blot analysis confirmed the presence of PhaC at 60 kDa. There was also the band at below

40 kDa which corresponds to a degradation product. The amount of PhaC present was 1.79 µg of PhaC/mg of beads according to quantification by densitometry.

To find out whether the additional lysis wash step would increase the purity of antigen-presenting beads, 0.5 g of each sample was taken and washed with lysis buffer and analysed by SDS-PAGE. Figure 42 showed that additional washing did not improve the purity of PHA beads and it also reduced the amount of the fusion protein that's associated to the beads. Based on this result, the biobeads were processed as demonstrated in Figure 42. The product was then sterilesed with 70% ethanol, resuspended in 30% glycerol and stored in -80 °C for future *in vivo* study.

Fusion protein analysis of Ag85A, SOD, Ag85B-PhaC and wildtype PhaC
MALDI-TOF MS analysis showed identity of the antigen fusion protein had correct amino acid sequence as expected. The sequence coverage was 11% (Appendix).

To further assess the purity of PHA beads, the samples were analysed by GC/MS; the analysis showed that dry samples of Ag85A, SOD, Ag85B and wildtype PhaC beads contributed approximately 44.8% and 54%. PHA content of PHA-accumulating cells was assessed by GC/MS. The analysis showed that PHA contributed to approximately 43% and 54% of cellular dry weight for Ag85A, SOD, Ag85B-PhaC and wildtype PhaC, respectively (data not shown).

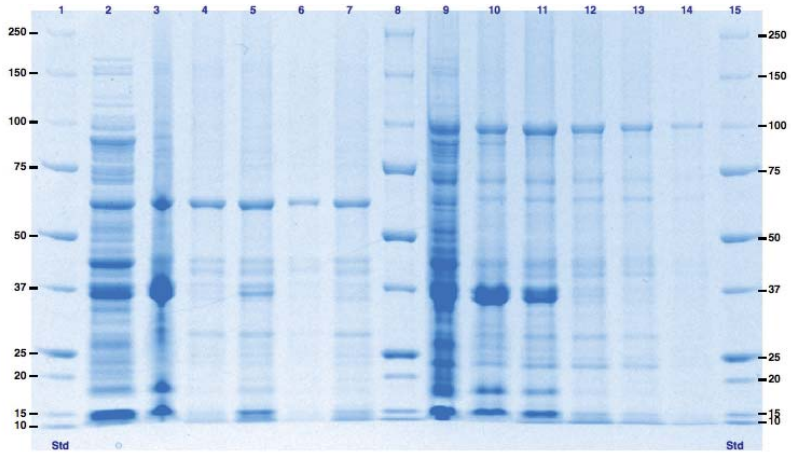

Figure 39. SDS-PAGE analysis of isolated biobeads after each purification steps. Lanes 1, 8 and 16, Precision Plus Protein™ standard (kDa) (Lane 2-7 are wildtype PhaC) Lane 2, Whole cell PhaC; Lane 3, Isolated beads after cell lysis; Lane 4, Lysis buffer wash II; Lane 5, Lysis buffer wash I; Lane 6, PBS; Lane 7, Caustic buffer. (Lanes 9-14 are Ag85A, SOD, Ag85B-linker-PhaC beads sample) Lane 9, Whole cell; Lane 10, Isolated beads after cell lysis; Lane 11, Lysis buffer wash I; Lane 12, Lysis buffer wash II; Lane 13, Caustic buffer; Lane 14, PBS.

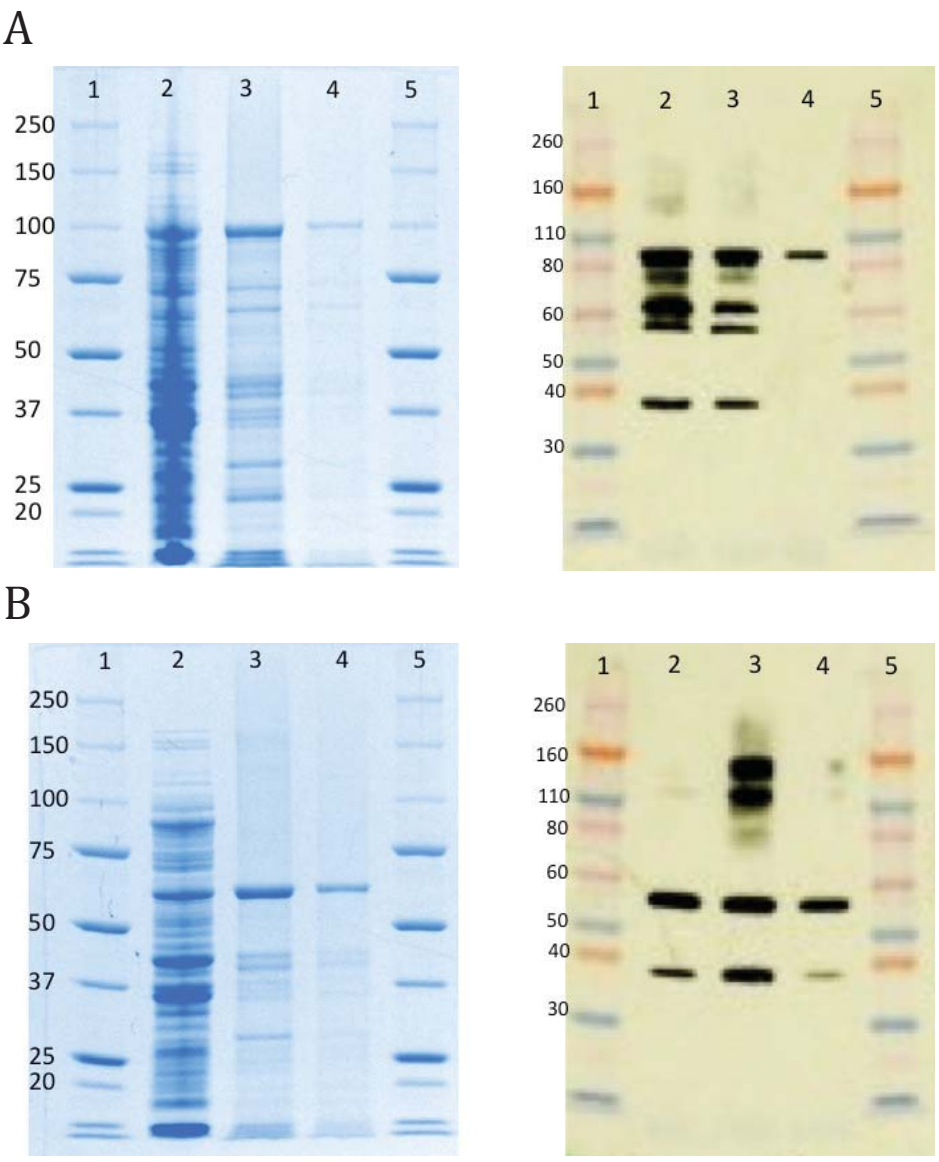

Figure 40. Analysis of proteins that were associated to biobeads after each step of purification by SDS-PAGE (left) and western blot analysis (right). (A) the antigen-presenting beads (Ag85A_SOD_AG85B_PhaC) and (B) wildtype PhaC. Lanes 1 and 5, Precision Plus Protein™ standard (A) Novex® Sharp Pre-stained Protein Standard (B) (kDa); Lane 2, Whole cell; Lane 3, Intermediate product after lysis buffer wash; Lane 4, End product after caustic buffer wash

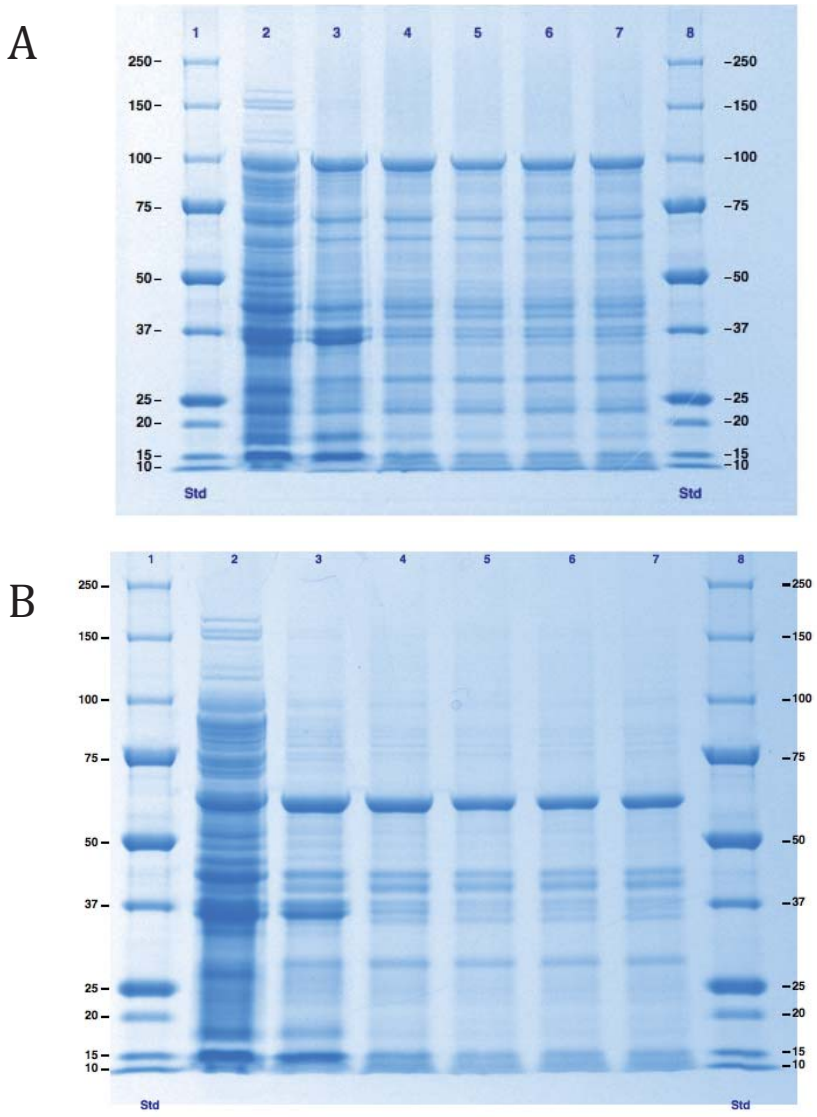

Figure 41. Protein profiles of the antigen presenting beads (Ag85A_SOD_AG85B_PhaC) (A) and wildtype PhaC beads (B) after each purification step for final product. Lane 1 and 8, Precision Plus Protein™ standard (kDa); Lane 2, Whole cell; Lane 3, Cell lysate; Lane 4, Lysis buffer wash I; Lane 5, Lysis buffer wash II; Lane 6, PBS wash; Lane 7, The final product that was stored in 25% glycerol.

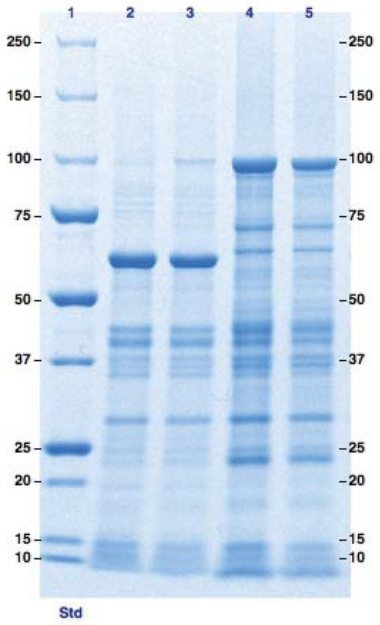

Figure 42. PHA beads-associated protein profile before and after the third wash with lysis buffer. Lane 1, Precision Plus Protein™ standard (kDa); Lane 2, PhaC beads after second lysis wash; Lane 3, PhaC beads after third lysis wash; Lane 4, Antigen-presenting beads after second lysis wash; Lane 5, Antigen-presenting beads after third lysis wash.

Chapter 4: Discussion

4.1 Development of subunit vaccine against Johne's disease using the antigens Ag85A, superoxide dismultase, Ag85B and 74F

Johne's disease remains a problem in the dairy industry despite the effort for disease control. Disease control program for this disease is challenging due to many factors including; 1) long latent phase of Johne's disease for up to 3 years (Klanicova *et al.*, 2012), 2) resistance of MAP under harsh environmental condition (Klanicova *et al.*, 2012), 3) the ability to infect both ruminants and non-ruminants including deer, rodents, bison though non-ruminants animals do not develop symptoms (Meng *et al*. 2015, Krzysiak *et al*., 2015) 4) low specificity of current diagnostic test for MAP in animals (Santema *et al.*, 2009; Knust *et al*., 2013). Even though these factors do not all directly impact on the development of the disease in ruminants which is important for dairy industry, all these factors could increase risk of transmission of MAP and difficulties in disease control. The current Johne's disease control program include, test and cull and vaccination. Test and cull alone is not effective in reducing disease prevalence (Knust *et al*., 2013; Bastida & Juste., 2001), heavily rely on MAP diagnostic test that is low in sensitivity (Bastida & Juste., 2001), and not economically effective therefore vaccination is important as part of disease control.

Vaccination is one of the current disease control methods, which aim to prevent the disease symptoms as well as new infections. However, current whole killed vaccine on the market has a major disadvantage because it interferes with currently used diagnostic tests for bovine tuberculosis (Dunn *et al*., 2005; Larsen, Moyle & Himes., 1978). In addition, the results from different studies on the efficiency of this vaccine has been contradictory (Sweeney *et al*., 2009; Muskens *et al*., 2002). Current vaccine is also another problem as it is only prevent the development of symptoms but not reducing the transmission (Knust *et al*., 2013). Therefore, a more efficient and reliable vaccine is needed. Previous studies showed that TB antigen-coated PHA beads stimulated a mixed Th1, Th2 and Th17 immune response which mediated protective immunity against *Mycobacterium bovis* in a murine model (Parlane et al., 2012).
In this study, we developed particulate subunit vaccine candidate for this disease by selecting highly immunogenic antigens to be expressed on the surface of PHA beads.

These densely MAP antigen coated PHA beads could be more safe and effective in disease control.
Based on recent vaccine development by Chandra *et al.* 2012, it has been demonstrated that the truncated version of Ag85A, superoxide dismutase, Ag85B and 74F is protective against MAP in a murine model. In addition, Parlane *et al.* 2011 had demonstrated that PHA beads could be used as a vaccine delivery system by presenting selected antigens on the surface. This study showed the adjuvant effect of PHA beads while the beads alone were not immunogenic. Our study combines these MAP vaccine antigen candidates with the PHA bead display technology to prove the concept that PHA beads can be engineered to serve as a vaccine delivery system for Johne's disease.

To produce the antigen-presenting beads, the production vector that contains a hybrid gene encoding fusion proteins Ag85A, SOD, Ag85B-PhaC-74F was made. However, this vector was unsuccessful as it did not enable overproduction of the antigen-containing fusion protein in *E. coli* BL21 (DE3), *B. megaterium* PHA05 and *E. coli* Clearcoli BL21 (DE3).

4.1.1 Production of the antigen-presenting beads in *E. coli* BL21 (DE3)

The production vector was successfully transformed into *E. coli* BL21 (DE3) mediating formation of polyester beads as shown by fluorescent microscopy. The fusion protein associated with the surface of polyester beads was present as it was detected by SDS-PAGE and western blot analysis using monoclonal anti-PhaC antibodies (Figure 14). The expression of this fusion protein was weak as no visible band at 160 kDa was observed on SDS-PAGE stained with coomassie blue. In comparison with wildtype PhaC beads, the molecular weight of the PhaC band is 60 kDa as expected with a low level of expression (Figure 14A). However, a protein with an apparent molecular weight of 160 kDa could be detected by immunoblotting, which deviated from the theoretical molecular weight of the fusion protein of 180 kDa. The most likely reason for low expression of the fusion protein could be the presence of rare codons in the genes encoding the selected antigens that was optimised for *L. lactis*. It is compatible with *B. megaterium* but less with the codon usage of *E. coli* BL21 (DE3) (data not shown). In addition, the genes encoding PhaCAB were optimised for *B. megaterium* so it contained rare codons for *E. coli* BL21 (DE3). Codon analysis of the antigens sequence and *phaC*

showed that these genes contain rare codons for *E. coli* (DE3) particularly AGA (rare), AGG (rare) and CGA (low). Furthermore, the antigen fusion proteins were likely degraded and/or incompletely translated as shown by SDS-PAGE and immunoblotting for both whole cell and beads alone. Therefore, *E. coli* BL21 (DE3) may not be a suitable host for production of antigen-presenting PHA beads based on constructed plasmids used. The plasmid was designed for use in endotoxin production host *B. megaterium* or *L. lactis*.

4.1.2 *B. megaterium* PHA05

B. megaterium PHA05 was considered to be used as a main production host because of the absence of endotoxin which is a main advantage for the product which will be used for *in vivo* studies. This strain was developed by McCool and Cannon 2001, as a mutant with *phaC* and *phaP* being disrupted so it is unable to naturally accumulate PHA in order for the strain to produce only the recombinant antigen-presenting beads.

In this study, the transformation of the production vector into *B. megaterium* PHA05 to develop the strain producing the fusion antigen-presenting beads was successful. However, expression of these antigen presenting beads (along with naked PHA beads) was also challenging in *B. megaterium* PHA05. The fusion protein was present at 160 kDa which is lower than expected and the antigen fusion protein can only be detected by western blot. Likewise, wildtype PhaC had molecular weight of 60 kDa as expected and it can only be detected by western blot. So both antigen presenting beads and wildtype PhaC had very low level of expression which was not suitable for vaccine production in this study. Despite the fact that all the genes encoding selected antigens and *PhaCAB* were optimised for this host, this study found that the expression of both PhaC and the antigen presenting beads was still low. This result contradicts previous findings that showed a high level of expression using pPT7 system in this host (Grage and Rehm unpublished). One explanation is the possibility that there was a mutation in the pT7-RNAP promoter region as was previously observed by Grage and Rehm (unpublished).

Analysis of the production vector revealed a nucleotide that was missing (Figure 28), causing a translational frame shift resulting in a protein that is predicted to be

approximately 147 kDa (Figure 27). This molecular weight is consistent with the fusion protein band observed from the antigen-presenting beads when produced in *E. coli* BL21 (DE3) and *B. megaterium* PHA05. To correct this frame shift, a nucleotide was successfully inserted by site-directed mutagenesis (Figure 31).

The modified production vector was transformed into *E. coli* Clearcoli BL21 (DE3). This strain was developed from ClearColi^{TM} BL21 (DE3) (Lucigen) by introducing pRare2 encoding the tRNAs for *E. coli* rare codons (Rubio Reyes and Rehm, unpublished). The purpose of this modification was to solve the translation problem due to lack of codons as mentioned previously. ClearColi^{TM} BL21 (DE3) is a mutant *E. coli* BL21 (DE3) with lipopolysaccharide (LPS), being modified by deletion of the oligosaccharide chain, and two of the six acyl chains were removed. This modification eliminated production of endotoxins making this strain an attractive host for production of endotoxin-free antigen presenting beads for vaccine development. In the current study, PHA beads that were produced from this strain containing corrected production vectors manufactured PHA beads displaying correctly sized antigen-fusion protein and wildtype PhaC (180 and 60 kDa) (Figure 34). In addition, PhaC expression was substantially increased. The presence of the antigen fusion protein was visible in SDS-PAGE (Figure 36). However, the problem with intracellular degradation or incomplete translation remained an issue (Figure 34). To solve this problem, different production plasmids were constructed for producing single-terminal antigen fusion protein-presenting beads such as 1)truncated Ag85A, SOD and Ag85B on the N-terminus of phaC, 2)74F on the C-terminus of PhaC and 3) 74F on the N-terminus of PhaC which arrangement could influence the level of expressions (Jahns & Rehm, 2009).

4.2 Single terminal antigens-presenting beads

4.2.1 N-terminal antigen fusions

The plasmid (pPT7-Ag85A, SOD, Ag85B linker_(A)PhaC) was successfully transformed into *E. coli* Clearcoli BL21 (DE3) mediating intracellular PHA production as demonstrated by fluorescent microscopy and PHA quantification using GC-MS. Expression of fusion antigens associated with PHA beads was dominant at 100 kDa

consistent with the predicted theoretical molecular weight. The expression was confirmed by SDS-PAGE and western blot analysis (figure 38). Purification of N-terminal antigen fusion protein coated beads by lysis buffer effectively removed protein contaminants and enhanced the presence of fusion the protein (Figure 41). However, bands indicating intracellular degradation or incomplete translation were still observed in western blots. Nevertheless, treatment with lysis buffer and caustic buffer partially removed this problem (Figure 41). In fact, caustic buffer removed the improperly translated or degraded fusion protein but the amount of correctly sized fusion protein was also significantly reduced. Therefore, it was not practical to use the antigen-presenting beads after caustic buffer treatment for future *vivo* studies towards immune response analysis. As the amount of the antigen may not be sufficient to induce significant immune response given that the maximum volume that can be injected in mice is 200 µl at about 5% concentration. In addition, it was observed that the beads clump after caustic buffer treatment which could potentially cause blockage of injection needles and the antigen exposure to the immune system could be limited. Therefore, it was decided that the product to be used in animal study, was only treated by lysis buffer.

Analysis of final bead product showed the presence of fusion protein was at the expected molecular weight. In addition, the amino acid sequence was confirmed by MALDI-TOF MS. Sterilisation of the beads was achieved by using 70% ethanol. The fusion protein quantification was done by densitometry and BCA, both suggesting that the antigen presenting beads was 0.6 µg of antigen per 1 mg of beads. Wildtype PhaC beads were also produced for use as a control. The amount of PhaC detected by densitometry was 1.8 µg of PhaC per 1 mg of beads. While the protein was detected to be 4 µg of PhaC/mg of beads when using a BCA assay method. This disagreement could be due to not all the protein being removed from the beads for densitometry analysis; a possibility that should be examined in future experiments. This discrepancy may also indicate that the amount of fusion protein could be underestimated.

4.2.2 C-terminal antigen fusions

To produce the antigen-presenting beads with 74F antigen fused to the C-terminus of PhaC, another production plasmid was designed. The plasmid was successfully

constructed and transformed into *E. coli* Clearcoli BL21 (DE3) mediating PHA production. The fusion-protein associated with PHA-beads had an apparent molecular weight of 140 kDa consistent with the theoretical molecular weight. However, the fusion protein was intracellularly degraded in combination with low amounts of fusion protein being present. Therefore, it was decided to produce the antigen-presenting beads which placed the 74F antigen at the N-terminus of PhaC. The cloning was attempted to make the vector construct for this expression, however, 74F fragment was not successfully ligated to the vector backbone (data not shown). Therefore consideration should be given to truncation of 74F antigen which could potentially improve the level of expression and reduce degradation. However, it was suggested by Faisal *et al.* (2013) that 74F in the truncated form does not have significant contribution to immune response and protectiveness against *MAP* in the murine model. As a result, it was decided that only fusion antigen Ag85A, SOD, Ag85B on the N-terminus of PhaC should be processed further as final product for *in vivo* studies. Only this N-terminal fusion was produced at larger scale for *in vivo* studies.

4.3 Purification

Initially, the PHA bead purification protocol was adapted from previous studies that use PBST, PBS and glycerol gradient to remove the protein contaminants from PHA beads (Jahns *et al.* 2008; Jahns *et al*., 2013). However, for the particular antigen-presenting beads being produced in this study, this method did not effectively remove the protein contaminants (Figure 14). This could be due to different properties of our antigen-presenting beads in regard to the bead surface properties mediated by the particular antigens. The purification protocol by glycerol density gradient was originally developed based on the principle of separating materials that have different densities and sedimentation velocity (Jahns et al., 2009 and 2013). PHA has greater sedimentation coefficient in comparison to the other cell components (Wieczorek *et al*., 1996) Therefore, these beads remain in the interphase between the 44 and 88% glycerol layers after centrifugation, while other components sediment. However, the expression of additional/different proteins (antigens) on our PHA beads may have altered their density and/or interact with other co-purifying proteins.

Another protocol used for purification of the antigen-presenting beads was according to

a patent application (WO_2012_077080_A1) involving washes with lysis and caustic buffers. While washes with lysis buffer effectively removed protein contaminants, caustic buffer did not. In fact, the latter buffer significantly reduced the amount of fusion protein associated to the beads (figure 48). This problem may be solved by optimising conditions for our antigen-presenting bead such as pH, molar concentration of each component in the buffers. Also for purification, adjusting the concentration of SDS, and EDTA in addition to varying pH could improve purification efficiency. It is worthwhile to note that protein contaminants in purified beads were visible only by Western blot detection (figure 40). This is because Western blot using chemiluminescent detection has high sensitivity in detecting protein (minimum quantity in pictograms) whereas Comassie blue staining visualise proteins in the minimal quantity of nanograms (Thermofisher Scientific).

Additionally, there is a potential purification method using tangential flow filtration (TFF). TFF is a method for separation of biomolecules based on their size through membranes containing micro/nanopores at selected diameter size. The filtration processes involves passing the sample across a membrane, which allows contaminants smaller than the size cut off to be removed, while retaining larger particles. Using this method, protein contaminants can be effectively removed from beads (WO_2012_077080_A1); this is an alternative purification strategy that can be used in the future.

4.4 Evaluation of antigens selected for this study

The antigens used in this study were initially 3 truncated antigens consisting of Ag85A, SOD and Ag85B, and the full-length 74F. However, the final product consisted of only these truncated antigens without the 74F and the antigen fusion protein are expressed as N-terminal fusion to PhaC. The 74F antigen was considered as vaccine candidate not only because of its immunogenicity but also because of its lack of interference with the current bovine tuberculosis diagnostic test.

The Ag85 family proteins are conserved among *Mycobacterium* species (Kuo *et al.*, 2011). Despite the chance for cross reaction with the bovine tuberculosis diagnostic test that detect the response to *Mycobasterium bovis*, the fact that, this study uses truncated

version of Ag85A and Ag85B of non-conserved regions, could potentially reduce the chance for interference with tuberculosis control tests.

For superoxide dismutase, on the other hand, the protein region used in this study was also present in superoxide dismutase of *M. bovis*. SOD is a secreted enzyme which neutralises superoxide radicals (Chan *et al.* 1989, Liu *et al.* 2001). Additionally, the current TB skin test is based on identifying skin reaction to purified protein derivatives (PPD). As PPD is injected into the skin of cattle, the result of thickening at the site of injection due to delayed-type hypersensitivity (DTP) immune response indicates the previous exposure to *Mycobacterium* species (Pollock & Andersen, 1997). However, since SOD is present in *M. bovis* and also potentially in PPD, it is possible that this antigen could interfere with currently available TB skin test.

To understand the interference to bovine tuberculosis diagnostic test as a result of vaccination with this version of antigen-presenting beads, the experiment on cross-reaction to bovine skin test should be conducted. For example, comparing the animals' responses to bovine tuberculosis diagnostic test in groups of animals that are 1) vaccinated with this antigen-presenting beads, 2) infected with *M. bovis*, 3) healthy with no vaccination.

4.5 Conclusion.

E. coli BL21 (DE3) and *B. megaterium* PHA05 are not suitable hosts for antigen-presenting beads as both produce very low level of expression of the antigens on beads as well as PhaC. Although PhaC was still functionally produced as shown by mediating PHA inclusion formation, the expression levels were found to be low. The level of expression was improved by changing the production host to *E. coli* Clearcoli (pRARE2) and rearranging the hybrid gene in plasmid pPT7-Ag85A, SOD, Ag85B linker_(A)*PhaC*_ linker 74F to pPT7-Ag85A, SOD, Ag85B linker_(A)PhaC and pPT7-PhaC_linker 74F. An attempt in cloning pPT7-74F linker_PhaC was not successful and truncated version would not significantly contribute to the immune response. pPT7-Ag85A,SOD, Ag85B linker_(A)PhaC give the highest level of expression compared to the other constructs while there's some degradation. The antigen is 0.6 µg/mg of beads.

The identity of the antigen fusion protein (Ag85A_SOD_Ag85B_PhaC) was confirmed by SDS-PAGE and western blot. The amino acid sequence was further confirmed by MALDI-TOF MS. The purity of the antigen presenting beads was achieved to a level deemed suitable for vaccination experiments. The sterility was achieved by ethanol treatment. To understand the immune response which could be induced by this vaccine an *in vivo* mouse trial will need to be conducted in future.

4.6 Future direction.

Johne's disease is one of the ongoing problems in the dairy industry worldwide. It is challenging to develop a vaccine that is highly protective and limit disease transmission. In this study, vaccine candidates were developed by combining Ag85A, SOD and Ag85B displayed on PHA beads. This study demonstrated that the antigen-presenting beads can be produced by *E. coli* Clearcoli (pRARE2). The presence of hybrid antigens was confirmed to be associated with PHA beads.

Although a purification protocol for the beads was developed, there is still scope to optimise the process to further improve purity of the antigen-presenting beads. In addition, the upstream process for growth of the production host could further improved to enhance the yield of beads as well as fusion protein content. Finally, immunological studies will need to be conducted to understand which mode and level of immune response can be induced in animal models.

Appendix

MALDI-TOF MS (analysis performed by The Centre for Protein Research, Otago University)

Protein View: 4175_Seq1 (Seq1_14175

(Seq1_14175) AG85A_SOD_Ag85B_PhaC

Database: UserTemp
Score: 459
Nominal mass (M_r): 104798
Calculated pI: 5.65

Sequence similarity is available as an NCBI BLAST search of 4175_Seq1 (Seq1_14175 against nr.

Search parameters

MS data file: 14175_B1.txt

Enzyme: Trypsin: cuts C-term side of KR unless next residue is P.

Variable modifications: Carbamidomethyl (C), Deamidated (NQ), Oxidation (M)

Protein sequence coverage: 11%

Matched peptides shown in ***bold***

```
  1 MGPSLIGLAM GDAGGYKAAD MWGPKEDPAW ARNDPSLQVG KLVANNTRIW
 51 VYCGNGKPSD LGGDNLPAKF LEGFVRTSNL KFQDAYNGAG GHNAVWNFDA
101 NGTHDWPYWG AQLQAMKPDL QSVLGATPGA GPATAAATNA GNGQGTMAEY
151 TLPDLDWDYA ALEPHISGQI NEIHHTKHHA TYVKGVNDAL AKLEEARANE
201 DHAAIFLNEK NLAFHLGGMI LAVNHPDQFI YAGSLSALLD PSQGMGPSLI
251 GLAMGDAGGY KADAMWGPSS DPAWQRNDPS LHIPELVGHN TRLWVYCGNG
301 TPSELGGANM PAEFLENFVR SSNLKFQDAY NGAGGHNAVF NFNANGTHSW
351 EYWGAQLNAM KPDLQGTLGA SPGGGGGSGG GMYTMATGKG AAASTQEGKS
401 QPFKVTPGPF DPATWLEWSR QWQGTEGNGH AAASGIPGLD ALAGVKIAPA
451 QLGDIQQRYM KDFSALWQAM AEGKAEATGP LHDRRFAGDA WRTNLPYRFA
501 AAFYLLNARA LTELADAVEA DAKTRQRIRF AISQWVDAMS PANFLATNPE
551 AQRLLIESGG ESLRAGVRNM MEDLTRGKIS QTDESAFEVG RNVAVTEGAV
601 VFENEYFQLL QYKPLTDKVH ARPLLMVPPC INKYYILDLQ PESSLVRHVV
651 EQGHTVFLVS WRNPDASMAG STWDDYIEHA AIRAIEVARD ISGQDKINVL
701 GFCVGGTIVS TALAVLAARG EHPAASVTLL TTLLDFADTG ILDVFVDEGH
751 VQLREATLGG GAGAPCALLR GLELANTFSF LRPNDLVWNY VVDNYLKGNT
801 PVPFDLLFWN GDATNLPGPW YCWYLRHTYL QNELKVPGKL TVCGVPVDLA
851 SIDVPTYIYG SREDHIVPWT AAYASTALLA NKLRFVLGAS GHIAGVINPP
901 AKNKRSHWTN DALPESPQQW LAGAIEHHGS WWPDWTAWLA GQAGAKRAAP
951 ANYGNARYRA IEPAPGRYVK AKA
```

Unformatted sequence string: 973 residues (for pasting into other applications).

Sort peptides by ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Show predicted peptides also

Query	Start – End	Observed	Mr(expt)	Mr(calc)	ppm	M	Score	Expect	Rank	U	Peptide
13	70 – 76	867.4845	866.4772	866.4650	14.1	0	33	0.00056	1Score **> 13** indicates **identity**	U	K.FLEGFVR.T
27	185 – 197	1385.7618	1384.7545	1384.7310	17.0	1	24	0.004	1Score **> 13** indicates **identity**	U	K.GVNDALAKLEEAR.A
25	447 – 458	1309.7362	1308.7289	1308.7150	10.7	0	98	4.8e-10	1Score **> 18** indicates **identity**	U	K.IAPAQLGDIQQR.Y
9	486 – 492	822.4038	821.3965	821.3820	17.7	0	39	0.00044	1Score **> 18** indicates **identity**	U	R.FAGDAWR.T
24	499 – 509	1256.6871	1255.6798	1255.6713	6.78	0	34	0.0013	1Score **> 17** indicates **identity**	U	R.FAAAFYLLNAR.A
33	510 – 525	1673.8707	1672.8634	1672.8631	0.17	1	37	0.00068	1Score **> 18** indicates **identity**	U	R.ALTELADAVEADAKTR.Q
20	554 – 564	1173.6591	1172.6518	1172.6401	10.0	0	94	1.5e-09	1Score **> 18** indicates **identity**	U	R.LLIESGGESLR.A
31	577 – 591	1623.7980	1622.7907	1622.7900	0.44	1	56	8.3e-06	1Score **> 18** indicates **identity**	U	R.GKISQTDESAFEVGR.N
29	579 – 591	1438.6917	1437.6844	1437.6736	7.54	0	120	3.8e-12	1Score **> 18** indicates **identity**	U	K.ISQTDESAFEVGR.N
19	947 – 957	1160.6006	1159.5933	1159.5846	7.51	1	23	0.018	1Score **> 18** indicates **identity**	U	K.RAAPANYGNAR.Y
16	948 – 957	1004.4987	1003.4914	1003.4835	7.89	0	45	9.8e-05	1Score **> 17** indicates **identity**	U	R.AAPANYGNAR.Y
18	958 – 967	1129.6307	1128.6234	1128.6040	17.2	1	27	0.0065	1Score **> 18** indicates **identity**	U	R.YRAIEPAPGR.Y
30	958 – 970	1519.8403	1518.8330	1518.8307	1.56	2	30	0.0033	1Score **> 18** indicates **identity**	U	R.YRAIEPAPGRYVK.A
22	960 – 970	1200.6864	1199.6791	1199.6662	10.7	1	23	0.02	1Score **> 18** indicates **identity**	U	R.AIEPAPGRYVK.A

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