Evaluation of Primary Capture Procedures for Recombinant Antibodies Manufactured in *Pichia pastoris*: Method, Viability and Economics

By

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Declaration of Originality

I, Gaurav Maheshbhai Bhavsar, confirm that the work presented in this thesis is my own. I confirm that I have indicated where information has been obtained from other sources.

Gaurav Maheshbhai Bhavsar

Abstract

The eukaryotic yeast *Pichia pastoris* is an increasingly applied platform for heterologous protein production for therapeutic and diagnostic purposes. Recent advances in high production titre have increased pressure on downstream processes to reduce the overall cost of production. The work in this thesis investigates a means of reducing the cost of *P. pastoris* production by combining three steps (centrifugation or filtration, concentration and affinity purification) in one step and comparing the primary capture of recombinant antibodies between radial and expanded bed adsorption chromatography systems, using Immobilized Metal Affinity Chromatography (IMAC) as a primary capture step.

A hexa-histidine (His_6) tagged single-chain antibody shMFELL2cys was used for the study. The protein is secreted from *P. pastoris* strain X-33 and has an affinity towards the carcinoembryonic antigen (CEA), an onco-foetal tumour antigen overexpressed in cancer cells used for therapeutic as well as diagnostic purposes. A high cell density culture of *P. pastoris,* which had been induced to express shMFELL2cys was directly applied to (i) Cellthru™ resin in a radial bed and (ii) STREAMLINE™ chelating resin in expanded bed adsorption chromatography. Copper-mediated IMAC capture of the tagged protein occurred while cells and cell debris were allowed to flow through the matrix bed.

To conduct this study, first, the binding conditions were optimised at a small scale using 1 mL IDA chelating Cellthru™ and STREAMLINE™ chelating resins used in RBA and EBA chromatography columns, respectively. Second, a comparison of the static and dynamic binding capacity of the RBA and EBA resins was performed at a small scale using the purified His_6 tagged scFv antibody. Third, an investigation of the effect of the residence time on the recovery of the His_6 tagged scFv antibody using the RBA column was performed using a fermentation culture fluid.

The His_6 tagged scFv antibody shMFELL2cys was successfully expressed in the high cell density *P. pastoris* fermentation at a concentration of 680 to 600 mg/L, and captured using the RBA and EBA chromatography processes, respectively. The calculated step recovery for RBA and EBA chromatography was 30% and

47%, respectively. A total amount of 801 mg and 611 mg of the scFv antibody shMFELL2cys was purified from the EBA and RBA chromatography processes, respectively.

The scFv antibody shMFELL2cys purified using RBA and EBA chromatography has specificity for carcinoembryonic antigens (CEA). A single monomer peak of the purified scFv antibody was seen on the analytical SEC column and a scFv antibody band at ~ 27 kDa was present on the SDS-PAGE for the scFv which was purified using RBA and EBA chromatography. The HCP concentration in the final purified scFv antibody using EBA chromatography was 161.8 ng/mL, equivalent to 39 ng/mg of scFv antibody. The HCP concentration in the final purified scFv antibody using RBA chromatography was 83 ng/mL, equivalent to 22.3 ng/mg of scFv antibody. The high HCP concentration in the EBA eluted protein in comparison to the RBA eluted protein was also calculated.

Cost of goods analysis of the RBA and EBA chromatography processes were performed from 8L lab scale to 2000L large production scale using Excel-based BioSolve software®. At 2000L production scale, factors affecting the CoG/g and CoG/batch of the His_6 tagged scFv antibody were analysed using DoE software for the RBA and EBA chromatography processes. BioSolve software® cost comparisons between the capture steps revealed a significant decrease in the total CoG/g of the product manufactured at 2000L scale. At best-case scenario of 6 g/L fermentation yield, 40 mg/mL resin binding capacity, 60 resin CIP cycles and 60% step recovery, calculated CoG/g were £54 and £197, respectively for the RBA and EBA processes. In the worst-case scenario of 1 g/L fermentation yield, 20 mg/mL resin binding capacity, 20 resin CIP cycles and 20% step recovery, calculated CoG/g were £556 and £1400, respectively for the RBA and EBA processes. Primary chromatography process RBA out performed the EBA chromatography process, at both best-case and worst-case scenario levels.

Therefore, preference is given to introducing RBA chromatography as a primary capture step to purify the His_6 tagged scFv antibody from a high cell density *P.pastoris* fermentation run, following the evaluation of quality, quantity and cost of goods to manufacture the antibody.

Impact Statement

The economic evaluation of the primary capture procedures for the scFv antibody manufactured in high cell density *P.pastoris* fermenter and using RBA and EBA primary chromatography processes was successfully performed in this thesis. The model His_6 tagged scFv antibody shMFELL2cys was successfully expressed in the high cell density *P. pastoris* fermentation, and captured using the RBA and EBA chromatography processes.

A clear preference is given to introduce, primary capture via RBA chromatography process in the platform process to purify scFv antibody manufactured in high cell density *P.pastoris* fermentation run. High quality scFv antibody was purified through primary capture via RBA chromatography. A single monomer peak of the purified scFv antibody was seen on the analytical SEC column and a scFv antibody band at ~ 27 kDa was present on the SDS-PAGE. The HCP concentration in the final purified scFv antibody using RBA chromatography processes was, 22.3 ng/mg of scFv antibody.

BioSolve software® cost comparisons between the EBA and RBA capture steps also revealed a significant decrease in the total CoG/g of the scFv antibody manufactured at 2000L scale using RBA chromatography process in comparison to EBA process. Cost of the scFv antibody using primary chromatography process RBA out performed the EBA chromatography process while scaling up the process from lab scale to 2000L production scale.

The primary capture procedure using RBA chromatography to purify the scFv antibody from a high cell density *P.pastoris* fermentation run has a distinct advantage and potential to be introduced for the production of clinical and commercial grade products. Methods developed in this thesis, to purify the His_6 tagged scFv antibody using the RBA chromatography process as a primary capture step can be implemented to purify the whole antibody manufactured in high cell density *P.pastoris* fermentation runs.

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List of abbreviations

BMGY	Buffered Glycerol-complex medium
BMMY	Buffered Methanol-complex medium
CoG/batch	Cost of goods/batch
CoG/g	Cost of goods/gram
kDa	Kilodalton
DAB	3,3′-Diaminobenzidine tetrahydrochloride
DoE	Design of Experiemnt
dH_2O	deionised water
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EBA	Expanded bed adsorption
FPLC	Fast protein liquid chromatography
G	gram
GMP	Good manufacturing practice
H	hour
His_6	hexahistidine tag
HRP	horse radish peroxidase
RBA	Radial bed adsorption
IDA	Iminodiacetic acid
IMAC	Immobilised-metal affinity chromatography
L	Litre
mg	milligram
µg	microgram
mL	millilitre
µl	microlitre
mAu	Milli-Absorbance Units
mM	millimolar
µm	micron
Min	minute
M	Molar
MW	molecular weight
MWCO	molecular weight cut off
NaCl	sodium chloride
OD_{280}	optical density measured at 280 nm
OD_{600}	optical density measured at 600 nm
PBS	Phosphate Buffered Saline
PEG	polyethylene glycol
PES	Polyethersulfone
P. pastoris	*Pichia pastoris*
P. fluorescens	*Pseudomonas fluorescens*
PVDF	polyvinylidene difluoride
RT	room temperature
RPM	revolutions per minute
scFv	single-chain variable fragment

S	Second
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
S.cerevisiae	Saccharomyces cerevisiae
TFF	tangential flow filter
UK	United Kingdom
V	Voltage
V_L	variable light chain
V_H	variable heavy chain
v/v	percentage of volume per volume
w/v	percentage of weight to volume
YEPD	Yeast Extract Peptone Dextrose medium (primary culture)
YPD	Yeast Extract Peptone Dextrose
YPDS	Yeast Extract Peptone Dextrose Sorbitol

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Chapter 1 Introduction

1.1 Evolution of single chain antibody for therapeutic and diagnostic purpose

Antibodies protect humans from diseases such as cancer, viral infections and inflammation. A whole antibody IgG has two heavy chains and two light chains that are joined together with a series of disulphide bonds. Full-length monoclonal antibodies (~ 150 kDa) and scFv fragments (~ 27 kDa) can be manufactured using mammalian, insect, bacterial and yeast expression platforms. The scFv antibody comprises a complete antigen-binding site, which includes a variable region of the heavy chain (V_H) and a variable region of the light chain (V_L) domains joined together by covalent disulphide bonds and non-covalent bonds [1]. Recombinant antibody fragments, single-chain antibodies (scFv) derived from whole antibodies (IgG), can be used in both therapeutic and diagnostic applications [2]. scFv is the most popular recombinant antibody format due to its small size, short generation time and capacity to express in prokaryotic, yeast and mammalian cells [3-7]. Drugs, toxins, radionuclides for cancer treatment, viruses for gene therapy and biosensors for real-time detection of target molecules have been fused to the scFv antibody [8-14]. Figure 1.1 shows the structure of a typical whole antibody and the antibody fragment scFv.

The first recombinant IgG (Orthoclone OKT) was launched in 1986. Since then, over 100 monoclonal antibodies or antibody fragments have been commercialized and approved by the FDA for human therapy [15, 16]. The first scFv molecule was developed in 1988 and expressed in Escherichia coli [17, 18]. To isolate the scFv against specific targets, filamentous phage technology has been widely used [19]. The scFv antibody MFE-23 was produced directly using filamentous phage technology in 1994 [20]. The scFv antibody shMFE produced by yeast display was the humanised and stabilised version of MFE-23 [21]. The scFv antibody shMFELL2cys is a disulphide-stabilised version of shMFE, an approximately 27 kDa single-chain Fv antibody, reactive with the tumour marker carcinoembryonic antigen (CEA) [11, 22]. MFE-23 was the first scFv to enter clinical application [23]. The bacterially expressed scFv antibody MFE-23 was first purified using IMAC

resin in batch mode using the transition metal ion copper sulphate immobilized on an IDA chelating agent of agarose resin [24].

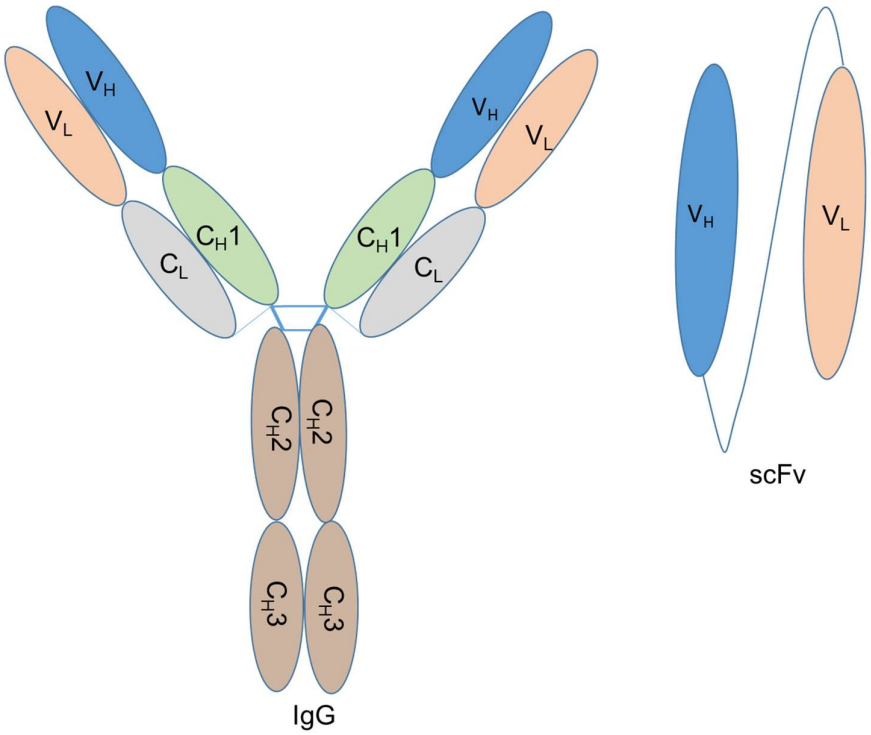

Figure 1.1 Schematic diagram of the antibody structure of the whole antibody immunoglobulins IgG (left) and antibody fragment scFv (right).
The schematic diagram of ~ 150 kDa size whole antibody structure shows it consists of two identical heavy and light chains joined together by a disulphide bridge. The heavy chain consists of four domains - V_H, C_H1, C_H2 and C_H3 - with a flexible hinge region separating the C_H1 and C_H2. The schematic diagram of ~ 27 kDa size single-chain antibody structure shows it consists of a variable region of heavy and light chains joined together by a disulphide bridge.

1.2 scFv antibody expression systems

Various expression platforms are available to produce the scFv antibody. The three main expression systems used in the production of the scFv antibody are *E.coli*, *P.pastoris* and *S.cerevisiae*. Selecting an antibody expression platform depends on the type of recombinant antibodies being produced, and the purity and quantity required of the final product. Expressed antibody fragments differ in their yield and activity because of several factors, including protein size, solubility, stability and the amino acid sequence [25]. Mammalian expression systems for monoclonal antibody production have high production costs and long cultivation times in comparison to bacterial and yeast expression systems [26]. The ability to perform glycosylation and post-translation modification of the monoclonal antibodies is the main advantage of the mammalian host system, which reduces the risk of an immunogenic response in patients [7, 27, 28].

An increase in the demand for full-length antibody and antibody fragments with lower production costs have led to the development of bacterial and yeast host systems as cost-effective expression platforms [29]. Prokaryotic *E.coli* was the first microbial organism employed for the production of human Fab protein that binds specifically to the human carcinoma cell line C3347 [30]. The *E.coli* K12 host had been used to manufacture FDA approved Fabs certolizumab pegol and ranibizumab [31]. In *E.coli*, the expression of the antibody fragment in the cytoplasm aggregates the antibody and affects the quality of the product. However, limited aggregation of the antibody expressed in the periplasm of *E.coli* was recorded [26]. The main advantage of a yeast expression system is that the protein is secreted into the culture supernatant, which can allow faster and easier purification. Progress in the development of a fermenter control strategy has resulted in the manufacture of functional antibodies using bacterial and yeast hosts [32]. In 2020 the FDA approved the first full-length antibody (Eptinezumab) manufactured in yeast *P. pastoris* for the treatment of migraine [15]. In this study, *P.pastoris* was used as the expression host to manufacture secreted His_6 tagged scFv antibody shMFELL2cys.

1.3 High cell density *P.pastoris* Fermentation

The ability of yeast species to utilize methanol as a sole source of carbon and energy was discovered in 1969 [33]. This resulted in the generation of yeast biomass as a high-protein animal feed because of the availability of methanol from natural gas methane [34]. In 1980 the Salk Institute Biotechnology/Industrial Association, Inc. (SIBIA) developed *P. pastoris* as a heterologous gene expression system. In 1993, Research Corporation Technologies (RCT) acquired the *P.pastoris* technology. Since then, *P. pastoris* has been widely used for the production of heterologous protein from large-scale fermentation processes. *P.pastoris* allows the cultivation of very high cell densities of 160 g/L cell dry weight on low-cost chemical define media [35]. *P.pastoris* holds a generally recognized as safe (GRAS) status.

Heterologous protein production in *P.pastoris* is regarded as being easier, faster and less expensive in comparison to insect and mammalian cell culture systems [34]. The production of secreted protein in a high cell density *P. pastoris* fermenter culture under the control of the methanol-inducible alcohol oxidase 1 (AOX1) promoter facilitates a high quality, quantity and clinical-grade product [29]. Disadvantages include high protease expression levels, high sensitivity to methanol levels, nutrient deficiency when grown on a defined medium, and health and safety concerns associated with the storage of large quantities of methanol [29]. In 1997, the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) constitutive promoter (pGAP) was isolated and is used to express many heterologous proteins using glucose or glycerol as a growth substrate. This eliminates the safety concerns with high volumes of methanol at large scale [36].

Low-cost heterologous protein production in eukaryotic *P. Pastoris* has become increasingly popular [37, 38]. *P. pastoris* expression of the His_6 tagged proteins in a chemically-defined fermenter medium may be induced through a methanol inducible AOX promoter. In this study, the wild-type *P.pastoris* strain X-33 was used for the production of the scFv in a chemically defined medium in a fermenter. The *P. pastoris* expressed shMFELL2cys, its parent MFE-23, and other derivatives have been used for pre-clinical and clinical imaging and therapy [9, 22, 39-41].

Antibodies are produced during the methanol induction phase. High cell density *P.pastoris* fermentation culture cultivation factors, such as pH, temperature (°C) and dissolved oxygen concentration (DO), are defined to control the protein production [42]. Antibody production during the fermentation is divided into three stages: a glycerol batch phase to build up biomass (Stage-1); a glycerol limited phase (stage-2), and a methanol induction phase (Stage-3) [37]. Temperatures as low as 15°C have been shown to enhance foreign protein expression without affecting the cell growth rate or decreasing protease activity [43, 44]. Extracellular protease activity and the stability of the protein between pH values 3 to 7 have been investigated. Decreasing the pH from 5 to 4 during the methanol induction stage increased the product concentration from 40% to 90% [45]. The dissolved oxygen level in the fermenter must be maintained as the fermentative by-products, such as ethanol, can impact the expression of the foreign protein [46]. Optimization of the *P.pastoris* fermentation condition exceeded the yield of 4 g/L of the humanized A33scFv antibody after 72 h induction with 0.5% (v/v) methanol and a low pH value of 3 [47]. Various scFv and Fab fragments are manufactured in *P. pastoris* [48].

In 1995, *P.pastoris* was used for the production of the first monoclonal rabbit scFv antibody fragment effective against the human leukaemia inhibitory factor (rhLIF) [49]. The highly efficient anti-HER2 scFv antibody variant scFv800E6 with a His_6 tag and C6.5 was manufactured in *P.pastoris* [50, 51]. The commercial IgG production of 1.6 g/L was achieved in a 1200 L fermenter using Glycoengineered *P.pastoris* [52]. The commercially available recombinant Hepatitis B vaccine Shanvac™ and Hepatitis C vaccine Shanferon™ (recombinant interferon-alpha 2b) are also manufactured in *P.pastoris.* The FDA approved Kalbitor®; a recombinant kallokrein inhibitor protein, is manufactured in *P.pastoris*. This is used for hereditary angioedema treatment. The recombinant human insulin Iinsugen®, used for diabetes therapy, is manufactured in *P.pastoris* by Biocon.

1.4 Affinity chromatography

Affinity chromatography has been used for many years in the isolation and purification of biologically active compounds. The main advantage is selective and

reversible binding to the chromatography support [53]. Chromatography support immobilised with affinity ligands purify the target compound from the complex mixture [54]. In 1910, Emil Starkenstein first used this approach to separate α-amylase by using insoluble starch as a stationary phase [55]. Agarose is the most widely used chromatography support from the various materials and matrices available. Other supports used as a stationary phase are cellulose, silica and glass. The main advantages of using agarose as a support for affinity chromatography are its low cost, large pore size, and low non-specific binding and stability over a broad pH range [54]. The support for affinity chromatography is mainly used in a packed column. In this study, an agarose-based support was used in the radial bed adsorption (RBA) and expanded bed adsorption (EBA) columns.

1.4.1 Immobilized Metal Affinity Chromatography (IMAC)

Immobilized Metal Affinity Chromatography (IMAC) can be used in axial, RBA and EBA chromatography to capture the tagged protein using metal ions bound to a chelating agent. The chelating agents immunodiacetic acid (IDA) and nitrilotriacetic acid (NTA) are used in IMAC. IDA is trivalent and NTA is a tetravalent ligand therefore the two ligands differ in the number of valencies with which they coordinate the metal ion. NTA ligand binds strongly to metal ion in comparison to IDA ligand because it has 4 valencies available to bind in comparison to 3 valencies of IDA ligand. In this study, an IDA chelating agent was used in the primary purification step in the RBA and EBA chromatography to compare the quantity and quality of the purified scFv antibody shMFELL2cys. IDA ligand was used previously to purify scFv antibody manufactured using *P.pastoris* for clinical trial using EBA column[40, 56]. Therefore, IDA ligand was used to compare EBA and RBA chromatography processes.

Various metal ions - copper (Cu), nickel (Ni), zinc (Zn), Iron (Fe) and cobalt (Co) - can be used with a chelating agent to capture the His tagged protein. Differences in the affinity of the His tagged protein towards the metal ion determines the quantity of the protein which can be purified using IMAC. The different metal ions have different binding efficiency with the chelating agents in terms of how strongly they bind. The His_6 tagged scFv antibody manufactured in *P.pastoris* was used in

this study. The clinical application of the His_6 tagged proteins has been proven, however its presence might reduce the potential immune response towards the target antigen [57]. Immunological impact of the presence of His tagged on the scFv or any other protein should be investigated before using for the clinical trial. Presence of His tagged on the scFv or any other product may reduce the potential immune response towards the target antigen. An illustration of the affinity capture of a His_6 tagged scFv antibody to an IDA chelating resin is shown in figure 1.2. Figure 1.3 shows a flowchart of the stages in the IMAC process.

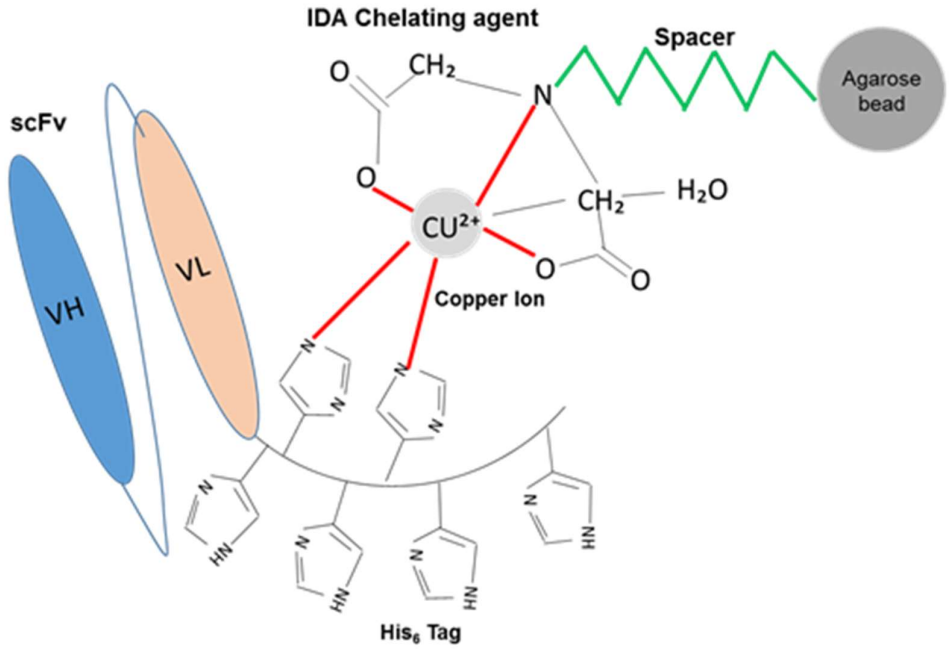

Figure 1.2 Illustration of affinity capture of a His_6 tagged scFv antibody to an IDA chelating resin.
Illustration of an IDA chelating agent attached to agarose resin and bound to the metal ion copper with three covalent bonds, leaving two valencies to bind to the His tag of the scFv antibody fragment.

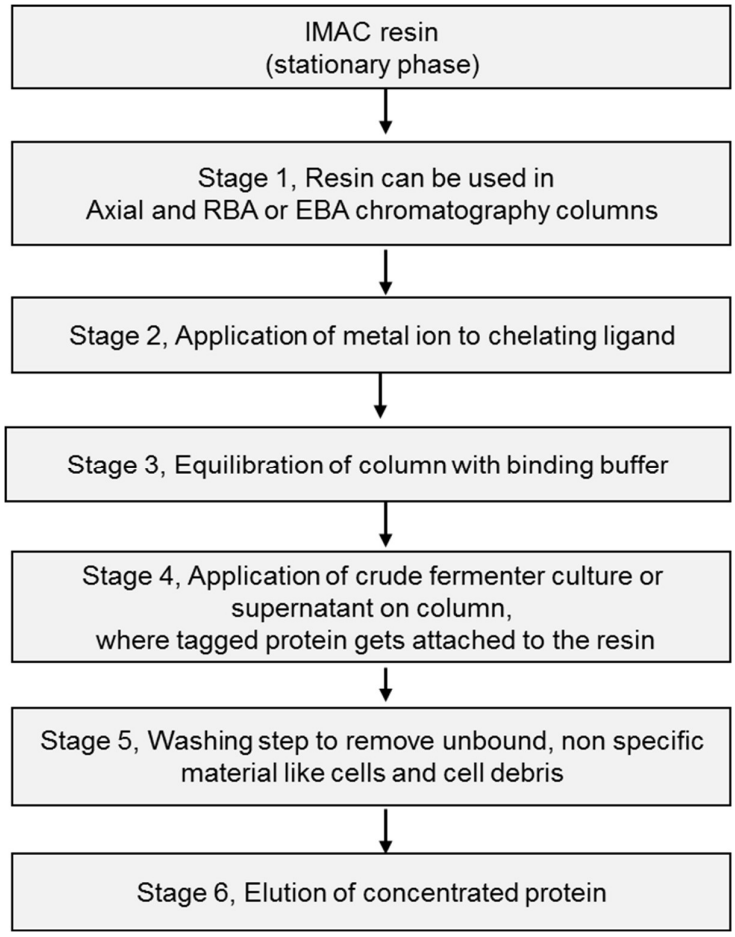

Figure 1.3 Flowchart of the IMAC process stages.
A flowchart illustrating the IMAC stages to purify the His tagged protein. An IMAC resin is used in the stationary phase and is packed into axial, RBA or EBA chromatography columns (stage 1). A metal ion is applied to a chelating agent (stage 2). Equilibration of the column with the binding buffer follows (stage 3). The crude fermenter culture or supernatant is applied to the column where the tagged protein gets attached to the resin (stage 4). Unbound and nonspecific materials like cells and cell debris are removed during the washing step (stage 5), and the bound protein is eluted in high concentrations (stage 6).

1.4.2 Direct capture Chromatography

The classic route to purifying protein expressed through *P.pastoris* fermentation includes fermentation culture clarification using centrifugation or depth filtration methods (step-1), the concentration of the clarified material using a tangential flow filtration system (step-2), and the affinity purification using affinity chromatography columns in axial flow. All three steps have high capital costs, material costs and operation costs. There is also product loss using the three purification steps, depending on the stability of the product and the product recovery from each step.

RBA or EBA primary purification processes combine all three steps into one step. A schematic comparison of traditional and RBA or EBA primary purification processes is described in Figure 1.4.

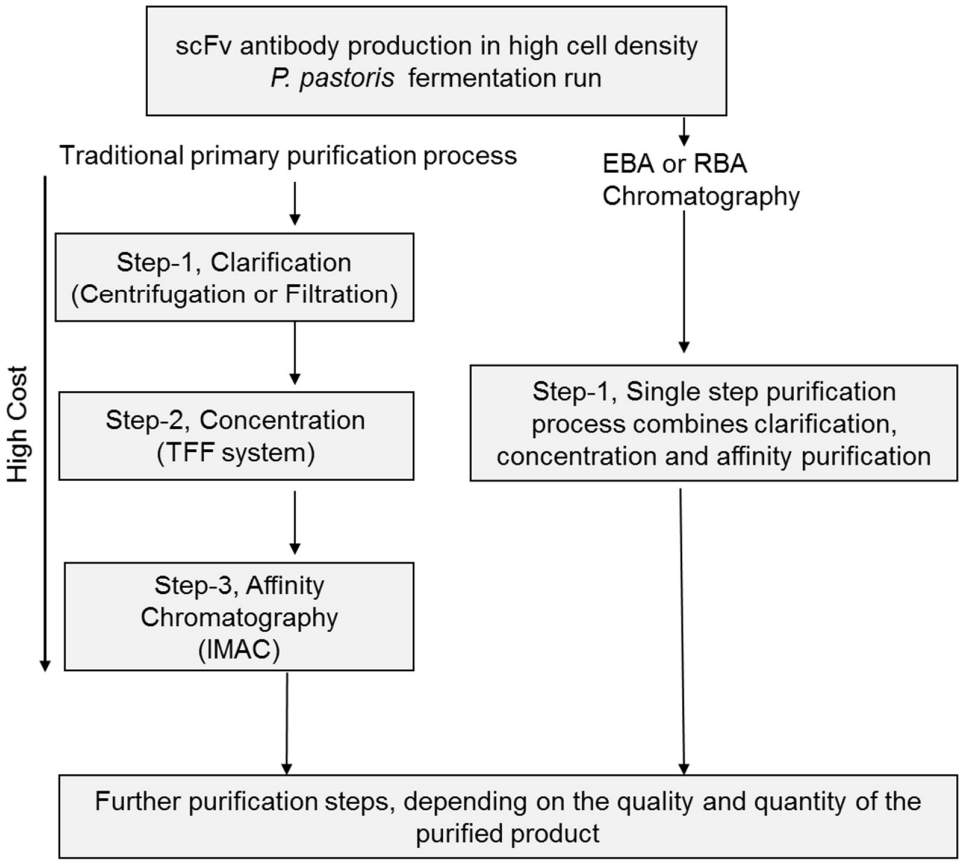

Figure 1.4 A schematic comparison of traditional and RBA or EBA primary purification processes.
A schematic comparison of the traditional and RBA or EBA primary purification processes. The traditional purification process includes clarification (centrifugation or filtration) in step 1, concentration of the product in step 2 and affinity chromatography in step 3. In comparison, RBA or EBA chromatography combines all three steps into one step.

1.4.2.1 Expanded bed adsorption chromatography

Expanded bed adsorption (EBA) chromatography combines the clarification, concentration and affinity purification of the product into one step. The EBA chromatography permits direct application of a crude fermenter culture or unclarified homogenate onto the bed. This facilitates chromatography resins packed inside the EBA column in capturing the product of interest through affinity

or ionic interaction, while solid materials, like cells and cell debris, pass through the column [58-61]. Hence, a cost-effective primary purification of the product is achieved without the clarification of the crude feedstock. The application of a liquid in an upward flow from the bottom of the EBA column expands the adsorbent beads, allowing the unclarified feedstock to pass through the beads while they capture the product.

The performance of the EBA column depends on its stable expansion while crude feedstock is applied in an upward direction [58, 62, 63]. The application of a high cell density viscous fermenter culture at a high velocity significantly affects the stability of the expanded bed during the operation. While applying biomass to the EBA column, the interaction between the adsorbent and the biomass can also affect the stability of the expanded bed [64]. Researchers have investigated the use of LED-based sensors to adjust the flow rate of the crude fermenter culture to maintain the bed height during the operation [58]. A particle-sized distribution study of the chromatography resins in the expanded bed was also performed to measure the stability of the EBA column [58]. The stable expansion of the EBA column was achieved by using a high-density adsorbent with a quartz or steel core [65].

In this project, a first-generation STREAMLINE™ 50 EBA column with 100-300 µm IDA chelating 6% cross-linked agarose beads containing a quartz core was used for comparison with the RBA system. In the EBA column, the expansion of the heavy IMAC beads allows the His_6 tagged antibody to bind to the metal ion while cells and debris pass through the column.

The EBA column with the STREAMLINE™ chelating resin has been successfully used for the manufacture of clinical-grade antibodies expressed in high cell density *P. pastoris* fermentation [41].

The viscosity and density of the crude feedstock affects the stable operation of the expanded bed; hence, it is necessary to scale up the column or reduce the velocity during application. It has been shown that the viscosity of the fermentation broth and degree of bed expansion during the capture step affects the adsorption performance of the expanded bed [66]. The biomass adsorbent interaction in the

expanded bed depends on the particle size and chromatographic method applied [67].

The variation in hydrodynamic and protein adsorption behaviour inside the EBA columns was investigated using an in-bed sampling procedure. It was discovered that the top region of the column had lower axial dispersion compared to the bottom region [68]. A reduction in dynamic binding capacity (DBC) was recorded with the axial dispersion inside the EBA column. The hydrodynamic, adsorption and separation performance of the EBA column was investigated using a scaled-down 1.9 mm ID EBA column, utilizing only a 5 mL sample volume compared with the 25 mm ID EBA column. The key parameters of the EBA columns - expansion, adsorption behaviour and hydrodynamics - were comparable [69]. The scalability and function of the lab-scale 25 mm ID to the production scale 600 mm ID EBA columns were tested using a yeast culture spiked with bovine serum albumin. The results showed that the production scale EBA column recovery was comparable to the lab-scale EBA column [70].

IMAC purification of the alcohol dehydrogenase (ADH) from a solution of homogenized packed baker's yeast using a STREAMLINE 25 EBA column packed with STREAMLINE™ chelating resin was successfully performed [71]. The direct capture of a His_6 tagged glutathione S-transferase (GST- (His_6)) from the unclarified *E.coli* homogenates using IMAC resin in the EBA column was successfully demonstrated [72].

Research was carried out by William et al., to investigate the experimental characterization of next- generation high particle density EBA adsorbents for the capture of the recombinant protein expressed in a high cell density yeast fermentation, which included FastLine® HSA (DSM), FastLine® MabDirect MM (DSM) and CM Hyper Z® adsorbents (Pall), and it demonstrated that the second generation adsorbents could be used for the pilot-scale EBA process [73]. An economically viable purification of chitosanase produced by Bacillus cereus through an EBA column packed with STREAMLIEN™ DEAE resin was also demonstrated [74]. Recently, *P.pastoris* has been used to express human apolipoprotein A-I (rhApoA1), which has been purified using mixed-mode

chromatography resin Capto MMC ligands using a STREAMLINE 25 EBA column, with a purity and recovery of 84% and 68%, respectively [75] .

Fluid distribution in the first generation of the EBA columns using a mesh and perforated plate led to a build-up of dead yeast cells at the edge on the upper side of the bottom distribution plate. Issues during the CIP of the EBA column to remove dead cells were overcome by applying a high flow rate backflush while scaling up the EBA column from the lab to the production scale [70]. The feedstock interaction between the cells and EBA resin can lead to a reduction in resin performance. The interaction between the resin and the cells, the cell debris, HCD and HCP can lead to bed instabilities and channelling; therefore, the composition of the fermenter culture becomes the limiting factor when seeking to maintain the steady state expansion of the EBA column [76]. Biotechflow Ltd Company manufacture large scale second generation EBA columns [77]. In comparison, a bench-scale EBA chromatography system with intuitive software is available from Xpure-systems [78].

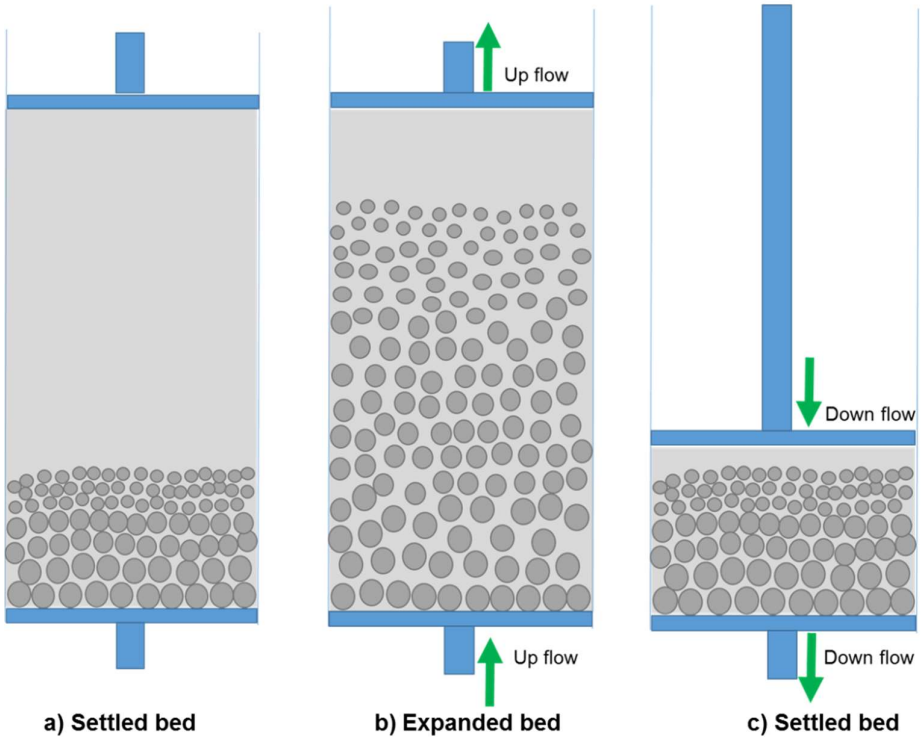

Figure 1.5 Schematic illustration of the EBA column Operation.
Figure 1.5 shows a schematic illustration of the EBA column. a) Shows the settled bed inside the EBA column when the top adapter is placed at the top of the column. b) Shows the expanded bed while applying equilibration and the wash buffer and crude fermenter feedstock in an upward direction. c) Shows the settled bed inside the EBA column while the bound protein is eluted in a downward flow when the top adapter is placed above the settled bed.

1.4.2.2 Radial bed adsorption chromatography

The design of the radial bed adsorption (RBA) chromatography columns utilises the outer and inner cylinder frits and resins are packed between them. The application of the product containing feed, wash buffer and elution buffer is from the outer cylinder to the inner cylinder in a radial flow [79, 80]. The radial flow operation of the column is illustrated in Figure 1.6. The bed height of the RBA column is the distance between the outer and inner cylinder. The chromatography resin-packing procedure for the RBA column is much simpler compared to an axial column. The RBA chromatography column is operated at much lower back pressure compared to axial flow columns [80]. The bed height of the RBA column

is kept constant during the scaling up of the column from laboratory to pilot scale; instead, the column height is increased vertically to increase the column's volume.

The RBA column's bed height for the lab scale is a minimum of 3cm and for the industrial scale is a maximum of 30cm. The outer cylinder has a high surface area compared to the inner cylinder; hence, the radial flow velocity at the inlet of the column is much higher in comparison to the outlet of the column. This difference in the flow velocity of the RBA column affects the protein mass transfer [81]. Therefore, the RBA columns are more suitable for adsorption chromatography, and protein adsorption at the inlet of the column is generally much higher than at the outlet. The higher surface area at the inlet of the RBA column compared to the outlet and the shorter bed height allow the RBA column to be operated at a high flow rate with low backpressure. The operation of the RBA column at a high flow rate speeds up the loading, washing and elution of the adsorption chromatography. Radial columns are not suitable for the separation of protein-based on their size because of the shorter bed height.

In an RBA column, the linear increase in the flow rate from the bench scale to the pilot scale can be easily achieved compared to an axial flow column. Proxcys and Sepragen are two major manufacturers of radial bed adsorption chromatography [82, 83]. No big increase in the footprint of the RBA column is measured while scaling up the column from the pilot to the larger production scale. The smaller footprint, the ability to operate at a large flow rate and the low back pressure involved make the RBA column operation economically viable.

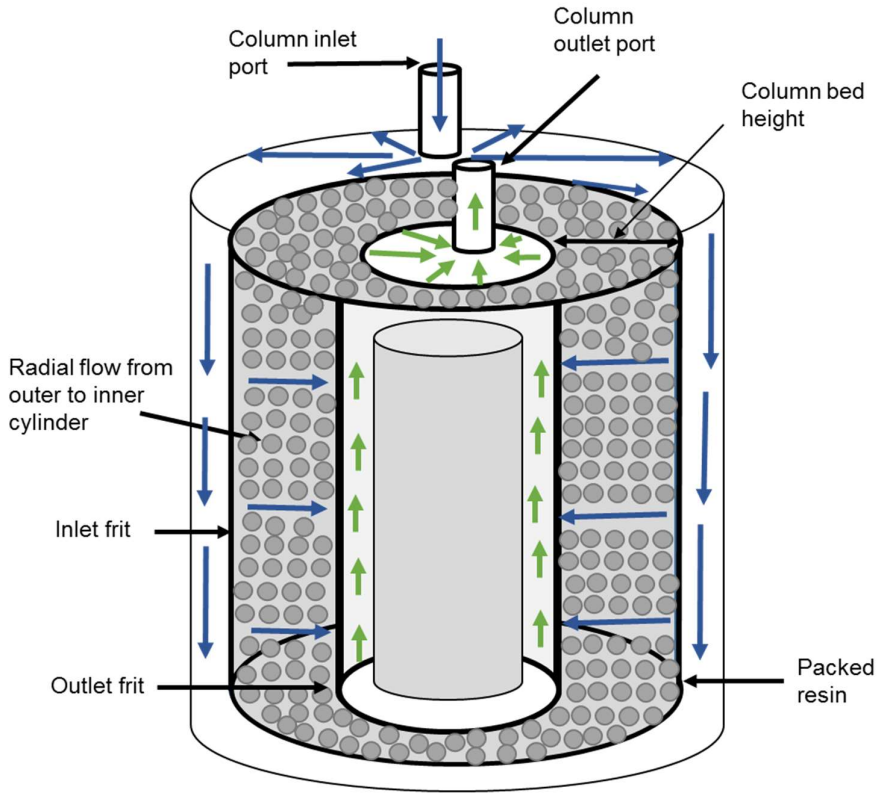

Figure 1.6 Schematic illustration of the RBA column Operation.
A schematic illustration of the RBA column shows the chromatography resin packed between the outer and inner cylinder, the application of fluid from the column inlet on top of the column, the radial flow of the fluid from the outer to inner cylinder and the fluid exit from the column outlet port. The column bed height is the distance between the outer and inner cylinder.

In this study, immunodiacetic acid chelating Cellthru™ BigBead with a particle size distribution of between 300 µm to 500 µm was used in a 250 mL RBA column to capture secreted His_6 tagged scFv antibody shMFELL2cys. One advantage of using Cellthru™ BigBead in an RBA column is that the beads allow cells and cell debris to pass through the column while applying the crude fermentation culture diluted with the buffer [56]. To apply the high cell density, *P.pastoris* yeast culture was diluted in the application buffer and applied onto the column; 40 µm frits were used at the inlet and outlet of the column. The His_6 tagged of the scFv antibody is only active at pH 7 and above and high viscous culture will reduce the DBC of the column. Hence, end of fermenter culture was diluted before applying onto the column. The small 250 mL wedge-shaped RBA column used in this study has the

same operational characteristics as the large cylindrical RBA column, which allows the operational parameters to be scalable from the pilot to large production-scale RBA columns. The performance of the RBA column was comparable to the axial column for affinity chromatography when the ratio between the outer and inner radius was around 2 [81]. In comparison to the axial column, a reduction in buffer consumption and total operation time and an increase in product recovery was achieved by the RBA column [84].

1.5 Economic analysis of the antibody production

Selecting an economically viable production process is essential for the production of pharmaceutical products [85]. A Cost of Goods (CoG) analysis of the pharmaceutical product during research and development can help to forecast the cost of the drug to the patient when launched. Evaluation of the cost-effective expression system must be performed to manufacture high quantity, high quality and low-cost pharmaceutical products. Since the first monoclonal antibody to be approved by the United States Food and Drug Administration (US FDA) in 1986, over 100 therapeutic monoclonal antibodies have been approved up to the end of 2020 [15, 16, 86, 87]. In 2018, the global therapeutic monoclonal antibody market was valued at approximately $115.2 billion. In 2019, it was expected to generate $150 billion, and by the end of 2025 it is expected to generate $300 billion [87].

In this study, a COG analysis of the scFv antibody production in *P.pastoris* using the radial and expanded bed adsorption chromatography as a primary purification step was performed using an Excel-based BioSolve software developed by Biopharma Services [88]. The economic viability and quality of the product are the two important criteria for the selection of the primary purification chromatography. BioSolve is a commercially available software package that can be used to perform a cost base analysis between different manufacturing processes. Various kinds of software have been developed to perform cost base analysis, including SimBiopharma, developed by UCL [89], SuperPro Designer [90], Aspen Batch Plus [91].

The economic analysis of the EBA technology was performed by comparing the conventional solid-liquid separation and the packed bed chromatography using a software tool based on a hierarchical framework and, a clear preference for the adoption of the EBA chromatography step was presented [92, 93]. Based on the data generated in previous study clear preference was given to adopt EBA chromatography step. In this thesis, no comparison was performed between traditional purification process and the EBA or RBA primary capture steps for the antibody production using high cell density *P.pastoris* fermentation. This study mainly focused on the cost-based comparison between the EBA and RBA primary chromatography processes and conclude which process can be implemented from pilot scale to up to 2000 L large scale manufacturing processes.

BioSolve has a cost database for the key materials, consumables and capital equipment used in different production and purification platforms. The cost model for a new process can be configured using the existing process library and unit database. BioSolve generates the total cost for each process based on user input data, such as the production yield of the fermenter, the product recovery at various steps in purification and the size of the fermenter and purification columns. The key factors that could impact the cost of the process at the large and pilot scale compared to the lab scale can be identified using BioSolve. A cost-based comparison of the different production titres in the expression of a system can also be performed using BioSolve software.

Direct and indirect costs associated with a manufacturing process can be calculated using BioSolve software. The indirect costs associated with the process include capital charges, insurance and taxes. The direct costs associated with the process include capital costs, material costs, consumable costs and labour. These are generated for the whole process. A comparison of each unit operation cost is also generated and divided into capital costs, material costs, consumable costs and labour. BioSolve has a facility to calculate the costs associated with the transfer of the process from one manufacturing facility to another. The cost of raw materials (chemicals), consumables (chromatography resin) and operations are compared between the radial and expanded beds primary capture steps.

The BioSolve software has been used for the comparison of the protein expression technology between *P.pastoris*, *E.coli*, mammalian and *P.fluorescens* [94], and to determine how the geographical location affected the CoG of the monoclonal antibody manufactured at a large scale in single-use bioreactors [95]. BioSolve software has also shown wide application in performing cost-based analysis of various bioprocess production platforms and technologies [96-103].

1.6 Research aims

Introducing RBA or EBA chromatography steps can achieve high downstream recovery of the product by combining three unit operations in one step. In recent years, increases in the upstream yield has challenged the downstream process steps; hence, a significant improvement in the cost-effectiveness of the downstream process is necessary. This thesis aims to develop and evaluate the cost of an effective EBA and RBA primary purification processes for the purification of the secreted His_6 tagged scFv antibody manufactured in a high cell density *P.pastoris* fermentation run. In this study, a comparison of the CoG/g and CoG/batch analysis of the EBA and RBA processes was performed using BioSolve software.

Below are the main objectives of this thesis and data generated to achieve below objectives in thesis are original.

1.0 An evaluation and comparison of the primary capture of the His_6 tagged scFv antibody between RBA and EBA chromatography (Chapter 3).

- Production of a His_6 tagged scFv antibody in a high cell density *P. pastoris* fermentation.
- Primary purification of the secreted His_6 tagged scFv antibody through EBA and RBA chromatography processes using IMAC resin.
- Qualitative analysis of the purified His_6 tagged scFv antibody to evaluate the quality of the antibody purified using EBA and RBA chromatography.

2.0 An experimental investigation of the His_6 tagged scFv antibody binding conditions for the RBA or EBA resins (Chapter 4). Data generated in this chapter using the RBA resin is novel.

- Measure the static binding capacity of the EBA and RBA resins to calculate the total amount of the His_6 tagged scFv antibody captured using 1 mL settled EBA and RBA resins in batch mode and to identify suitable pH condition for antibody purification.
- Measure the DBC of the EBA and RBA resins to investigate the total amount of the His_6 tagged scFv antibody that can be captured per 1 mL of resin.
- Investigate the effect of the pH and salt molarity on the binding efficiency of the His_6 tagged scFv antibody shMFELL2cys to the EBA and RBA resins.

3.0 An economic analysis of EBA and RBA chromatography using BioSolve software (Chapter 5). This is a novel objective as economic analysis of the EBA and RBA chromatography using BioSolve software is not performed previously or published.

- Perform cost base analysis of the His_6 tagged scFv antibody shMFELL2cys manufactured in a high cell density *P. pastoris* fermentation process and purified using EBA and RBA primary adsorption chromatography.
- Evaluate the effect of scale-up on the CoG analysis of the EBA and RBA primary purification processes.
- Evaluate the interaction of the factors; fermentation yield, EBA and RBA step recovery, EBA and RBA resin binding capacity and total number of CIP cycles on the CoG/g and CoG/batch of the EBA and RBA processes at 2000 L production scale using response surface design and employing the design of the experiment (DoE) software.

Chapter 2 Materials and methods

This chapter sets out all of the materials and experimental methods used in the pursuit of the doctoral studies. References to specific methods where they are applied are made in the subsequent chapter.

2.1 Materials and equipment

Materials used during the project along with their product code and supplier detail are listed in the table below.

Table 2.1 List of materials with product code and supplier detail.

No	Material name	Product code	Supplier
1	Sabouraud dextrose agar (SAB plate)	PO0410B	Fisher Scientific
2	Tryptone soya agar (TSA plate)	PO0821B	Fisher Scientific
3	250 mL Erlenmeyer flask	431407	Corning
4	2 L Erlenmeyer flask	431256	Corning
5	Bacto yeast extract	212750	Becton Dickinson difo-oxoid UK
6	Bacto agar	214010	Becton Dickinson difo-oxoid UK
7	Yeast nitrogen base without amino acids	233520	Becton Dickinson difo-oxoid UK
8	Biotin	B4639-100MG	Sigma-Aldrich
9	Phytone peptone	211906	Becton Dickinson difo-oxoid UK
10	D-Sorbitol	1.07758.1000	Merck (VWR)
11	D (+) Glucose	1.08337.1000	VWR Chemicals
12	Calcium sulphate ($CaSO_4$)	255548-100G	Sigma-Aldrich
13	Potassium sulphate (K_2SO_4)	P9458	Sigma-Aldrich
14	Magnesium sulphate heptahydrate	25167.298	VWR Chemicals
15	Ammonium sulphate	1.01816.1000	Millipore
16	Glycerol	24386.298	VWR Chemicals
17	Antifoam 204	A8311-50ML	Sigma-Aldrich

Table 2.1 (Continued)

No	Material name	Product code	Supplier
18	Sodium hexametaphosphate	14706	Alfa Aesar - Heysham, UK
19	Sodium hydroxide	28244.295	VWR Chemicals
20	PTM1 trace elements solution	J241-500ML	VWR Chemical
21	Ortho phosphoric acid 85%	20626.292	VWR Chemicals
22	Ammonium hydroxide solution	30501-1L-GL	Sigma-Aldrich
23	Methanol	1.06008.1000	Merck Millipore
24	Sodium chloride	27808.297	VWR Chemicals
25	Imidazole	8.14223.1000	Millipore
26	EDTA	1.08421.1000	G Biosciences
27	Ethanol	20905.296	VWR Chemicals
28	PBS	D5773-10L	Sigma-Aldrich
29	Biomax 5 filter	10686741	Fisher Scientific
30	Potassium phosphate dibasic (K_2HPO_4)	P2222-500G	Sigma-Aldrich
31	Potassium phosphate monobasic (KH_2PO_4)	P5655-500G	Sigma-Aldrich
32	IDA Chelating Cellthru™ BigBead. Particle size distribution is between 300 µm and 500 µm. (500 mL resin delivered in 20% ethanol preservative)	37311	Sterogene Bioseparations Inc, USA
33	*P. pastoris* HCP ELISA Kit	F140	Cygnus Technologies, Southport USA
34	Zeocin™	R25001	Invitrogen
35	PageRuler Prestained Protein Ladder	26617	Thermo Fisher Scientific
36	Superdex 75	17104402	GE Healthcare
37	Tetra His Antibody	34670	Qiagen
38	Gel Filtration Standard	1511901	Bio-Rad

Table 2.1 (Continued)

No	Material name	Product code	Supplier
39	STREAMLINE™ Chelating resin. Particle size distribution is between 100 µm and 300 µm. (300 mL resin delivered in 50 mM sodium phosphate and 20% ethanol preservative)	17-1280-01	GE Healthcare
40	ECL mouse IgG, HRP-linked F(ab)2 fragment	NA9310V	GE Healthcare
41	Phosphate-citrate buffer with sodium perborate (capsule)	P4922	Sigma-Aldrich
42	DC™ protein assay reagent A	500-0113	Bio-Rad
43	o-Phenylenediame dihydrochloride tablet, 10 mg substrate per tablet	P8287	Sigma-Aldrich
44	Carcinoembryonic antigen human	C4835	Sigma-Aldrich
45	Protein 80 kit	5067-1515	Agilent
46	DC™ protein assay reagent A	500-0113	Bio-Rad
47	DC™ protein assay reagent B	500-0114	Bio-Rad
48	Bovine serum albumin	A2153	Sigma-Aldrich
49	PageRuler™ Prestained Protein Ladder 10 to 180 kDa	26616	Thermo Fisher Scientific
50	Carcinoembryonic antigen human (CEA)	C4835	Sigma-Aldrich
51	Nunc MaxiSorp™ flat-bottom	44-2404-21	Thermo Scientific
52	Sealing tape for 96-well plates	15036	Thermo Scientific
53	pH probe	F-695-B420-DK	Broadley James

Table 2.1 (Continued)

No	Material name	Product code	Supplier
54	VESIFERM DO Probe	243666-251	Hamilton Sales and Service UK Ltd
55	Mini Dialysis Device, 20 kDa Molecular weight cut-off: 0.5 mL	88402	Thermo Fisher Scientific
56	Polyvinyliden fluoride (PVDF) membrane	IPVH00010	Merck Millipore
57	Whatman™ Protran™ nitrocellulose blotting membranes	10402452	GE Healthcare
58	1 mL FliQ FPLC Column	GEN-FliQ1	Generon

Equipment used during the project is listed in the table below.

Table 2.2 List of equipment used during the project.

No	Equipment	Manufacturer and address
1	New Brunswick BioFlo 3000 fermenter	New Brunswick Scientific Co PO Box 4005 44 Talmadge Road Edison New Jersey 08818-4005
2	AKTA Prime and software	Cytiva UK Limited Amersham Place Little Chalfont Buckinghamshire HP7 9NA UK
3	125 mL bed volume CRIO-MD 62 MK III radial bed method development column, 6 cm bed height, maximum operating pressure 300 kPa. Stainless steel frit porosity 40 µm, average surface area for linear velocity is 22.4 cm^2. Recommended flow rate is 15 to 200 mL/min.	Proxcys Downstream Biosystems Waanderweg 2 NL-7812 HZ Emmen The Netherlands

Table 2.2 (Continued)

No	Equipment	Manufacturer and address
4	250 mL bed volume CRIO-MD 63 MK III radial bed column, 6cm bed height, maximum operating pressure 350 kPa. Stainless steel frit porosity 40 µm, average surface area for linear velocity is 45 cm². Recommended flow rate is 35 to 400 mL/min.	Proxcys Downstream Biosystems Waanderweg 2 NL-7812 HZ Emmen The Netherlands
5	STREAMLINE 50 expanded bed column	Cytiva UK Limited Amersham Place Little Chalfont Buckinghamshire HP7 9NA UK
6	SkanIt microplate reader	Thermo Fisher Scientific 3 Fountain Drive Inchinnan Business Park Paisley PA4 9RF UK
7	Malvern Mastersizer 3000	Malvern Panalytical Ltd Enigma Business Park Grovewood Road Malvern WR14 1XZ United Kingdom
8	Malvern Kinexus rheometer	Malvern Panalytical Ltd Enigma Business Park Grovewood Road Malvern WR14 1XZ United Kingdom
9	New Brunswick™ Innova® shaking incubator	Eppendorf UK Limited Eppendorf House Gateway 1000 Arlington Business Park Stevenage SG1 2FP

Table 2.2 (Continued)

No	Equipment	Manufacturer and address
10	Biochrom WPA lightwave II UV/visible spectrophotometer	Biochrom Ltd Building 1020 Cambourne Business Park Cambourne Cambridge UK CB23 6DW
11	Biochrom WPA CO8000 cell density meter	Biochrom Ltd Building 1020 Cambourne Business Park Cambourne Cambridge UK CB23 6DW
12	Watson-Marlow 120U and 530 process pumps	Watson-Marlow Limited Bickland Water Road, Falmouth Cornwall United Kingdom TR11 4RU
13	Bioprene Loadsure TL element with PVDF connectors for maximum 2 bar pressure 9.6 mm OD with ¾" tri-clamp style sanitary clamp connectors	Watson-Marlow Limited Bickland Water Road, Falmouth Cornwall United Kingdom TR11 4RU
14	Labscale TFF System	Millipore (UK) Limited Suite 21, Building 6, Croxley green business Park Watford Hertfordshire WD18 8YH
15	Hanna pH Meter HI 112	Hanna Instrument Ltd Unit 28 /Pages Ind Pk/Eden way Leighton Buzzards LU7 4AD

Table 2.2 (Continued)

No	Equipment	Manufacturer and address
16	230L Power Door Priorclave autoclave	Priorclave Ltd 129-131 Nathan Way West Thamesmead Business Park London SE28 0AB
17	Eppendorf Centrifuge 5804 and 5418R	Eppendorf House Arlington Business Park Gateway 1000 Whittle way Stevenage SG1 2FP
18	XK 26/100 and XK 16/100 Superdex 75 prepacked column	GE Healthcare Life Science Amersham Place Little Chalfont Buckinghamshire HP7 9NA
19	Bio-Rad PowerPac 1000 electrophoresis power supply	Bio-Rad Laboratories Ltd The Junction Station Road Watford Hertfordshire WD17 1ET
20	Stuart™ orbital shaker	Cole-Parmer, Beacon Road Stone Staffordshire ST15 0SA
21	Sorvall WX 80 ultracentrifuge	Thermo Fisher Scientific 3 Fountain Drive Inchinnan Business Park Paisley PA4 9RF
22	Agilent 2100 Bioanalyzer	Agilent Technologies LDA UK Limited 5500 Lakeside Cheadle Royal Business Park Stockport, Cheshire SK8 3GR

Growth medium composition used during the project are listed in the table below.

Table 2.3 *P. pastoris* growth medium and plates.

No	Medium	Composition
1	Yeast extract peptone dextrose sorbitol (YPDS) plates	• 10 g/L yeast extract • 182.2 g/L sorbitol • 20 g/L peptone • 20 g/L agar • Autoclave sterilise at 121°C for minimum of 20 min • 20% w/v glucose • Cool solution to ~60°C and add 100 µg/ mL Zeocin™ • Pour 25 mL of medium into 90 mm petri dish inside MSCII and store at 4°C in refrigerator
2	Yeast extract peptone dextrose (YPD) medium	• 10 g/L yeast extract • 20 g/L peptone • Autoclave sterilise at 121°C for minimum of 20 min • 20% w/v glucose
3	WCB storage medium	• 85% v/v YPD medium • 15% v/v 100% glycerol • Autoclave sterilise at 121°C for minimum of 20 min • 100 µg/mL Zeocin™
4	Buffered glycerol-complex medium (BMGY) with Zeocin™	Below composition is for 1 L final volume of medium • 10 g yeast extract • 20 g peptone • 600 mL dH_2O • Autoclave sterilise at 121°C for minimum of 20 min • 100 mL filter sterilised 1 M potassium phosphate buffer pH 6.0 • 100 mL filter sterilised 10% (w/v) yeast nitrogen base solution • 100 mL filter sterilised 10% (w/v) glycerol solution • 100 mL filter sterilised 10% (w/v) case amino acid solution • 600 µL filter sterilised Biotin solution • 100 µg/mL Zeocin™

Table 2.3 (Continued)

No	Medium	Composition
5	Buffered methanol-complex medium (BMMY) with Zeocin™	Below composition is for 1 L final volume of medium • 10 g yeast extract • 20 g peptone • 600 mL dH_2O • Autoclave sterilise at 121°C for minimum of 20 min • 100 mL filter sterilised 1 M potassium phosphate buffer pH 6.0 • 100 mL filter sterilised 10% (w/v) yeast nitrogen base solution • 100 mL filter sterilised 10% (v/v) methanol solution • 100 mL filter sterilised 10% (w/v) case amino acid solution • 600 µL filter sterilised Biotin solution • 100 µg/mL Zeocin™

SDS-PAGE buffer and gel and Western Blot buffer composition are given in the table below.

Table 2.4 SDS-PAGE electrophoresis and Western Blot transfer buffers.

No	Medium or buffer	Composition
1	4x reducing buffers	Below composition is for 50 mL final volume • 4 g SDS (8% w/v) • 20 mL glycerol (40% v/v) • 4 mg bromophenol blue (0.008% w/v) • 12.5 mL of 1 M Tris-HCl pH 6.8 • 2.5 mL (5% w/v) beta-mercaptoethanol 15 mL dH_2O
2	10x SDS-PAGE running buffer	Below composition is for 1 L final volume • 10 g (1% w/v) SDS • 30.3 g (3.03 % w/v) Tris-base • 144 g (14.4% w/v) glycine Add dH_2O to make final volume 1 L

Table 2.4 (Continued)

No	Medium or buffer	Composition
3	1x SDS-PAGE running buffer	Add 100 mL 10x SDS-PAGE running buffer into 900 mL dH_2O to make final concentration of 0.1 % (w/v) SDS, 192 mM glycine and 25 mM Tris
4	10x Western Blot transferred buffer	Below composition is for 1 L final volume • 30.3 g (3.03 % w/v) Tris-base • 144 g (14.4% w/v) glycine Add dH_2O to make final volume 1 L
5	1x Western Blot transferred buffer	Add 100 mL 10x Western Blot transferred buffer and 200 mL 100% methanol into 700 mL dH_2O to make final concentration of 192 mM glycine, 25 mM Tris and 20% (v/v) methanol
6	Western Blot analysis solution	Below composition is for 40 mL final volume • 10 mg 3,3′-diaminobenzidine tetrahydrochloride (DAB) hydrate 20 µL of 4 M hydrogen peroxide
7	Coomassie staining solution	Below composition is for 300 mL final volume • 1.25 g Coomassie brilliant blue (0.25% w/v) • 50 mL glacial acetic acid (10% v/v) • 150 mL methanol (30% v/v)
8	Coomassie destaining solution	Below composition is for 1 L final volume • 300 mL methanol (30% v/v) • 100 mL glacial acetic acid (10% v/v) • 600 mL dH_2O
9	10% resolving gel for SDS-PAGE	Below composition is for 16 mL final volume • 6.4 mL dH_2O • 5.34 mL 30% acrylamide (protogel) • 4 mL 1.5M Tris-HCL pH 8.8 • 160 µL 10% SDS • 160 µL 10% ammonium persulphate (APS) • 16 µL tetramethylethylenediamine (TEMED)

Table 2.4 (Continued)

No	Medium or buffer	Composition
10	4% stacking gel for SDS-PAGE	Below composition is for 8 mL final volume • 5.76 mL dH_2O • 1.07 mL 30% acrylamide (protogel) • 1.0 mL 1.5M Tris-HCL pH 6.8 • 80 µL 10% SDS • 80 µL 10% ammonium persulphate (APS) • 8 µL tetramethylethylenediamine (TEMED)

Fermentation medium compositions are detailed in the table below.

Table 2.5 Fermentation medium.

No	Medium name	Medium components and concentration
1	Yeast extract peptone dextrose medium (YEPD) (primary culture medium)	Below composition is for 230 mL final volume of medium • 2 g yeast extract • 4 g peptone • 3 g glucose • Autoclave sterilise at 121°C for minimum of 20 min • Add 100 µg/ mL Zeocin™ after autoclaving
2	Basic salt fermenter medium	Composition for 5.3 L final volume • 300 mL glycerol • 5.4 g $CaSO_4$ • 88 g K_2SO_4 • 70 g $MgSO_4.7H_2O$ • 54 g $(NH_4)2SO_4$ • 5 L dH_2O
3	Sodium hexametaphosphate	Dissolve 150 g sodium hexametaphosphate into 1 L dH_2O and filter sterilise
4	Glycerol limited feed	Below composition is for 600 mL final volume • 300 mL 100% glycerol • 300 mL dH_2O • Autoclave sterilise at 121°C for minimum of 20 min • After autoclaving add 7 mL filter sterilised trace elements

Table 2.5 (Continued)

No	Medium name	Medium components and concentration
5	Methanol limited feed	Add 24 mL trace elements into 2 L filter sterilised 100% methanol
6	Trace elements	Purchased from Amresco, below is the list of chemicals present in trace elements • 5.99 g $CuSO_4.(H_2O)_5$ • 0.08 g NaI • 3.0 g $MnSO_4.H_2O$ • 0.20 g $Na_2MoO_4.(H_2O)_2$ • 0.50 g $CoCl_2.(H_2O)_6$ • 20.04 g $ZnCl_2.(H_2O)_5$ • 65.05 g $FeSO_4.(H_2O)_7$ • 0.02 g H_3BO_3 • 19.2 mL 96.2% H_2SO_4 • 0.40 g D-biotin • Add dH_2O to make final volume 1 L

Expanded bed adsorption (EBA) and radial bed adsorption (RBA) buffer composition and usage are detailed in the table below.

Table 2.6 Buffer composition for RBA and EBA chromatography.

No	Buffer name	Buffer composition	Usage during primary purification step
1	RBA column storage buffer	0.1 M NaOH / 0. 5M NaCl	1 L
2	EBA column storage buffer	20% ethanol	2 L
3	RBA column packing buffer	0.5 M NaCl / 0.5x PBS	1 L
4	Copper sulphate	0.1 M $CuSO_4.(H_20)_5$	2 L
5	Equilibration buffer	0.5 M NaCl / 0.5x PBS / 10 mM imidazole	2 L
6	Biomass application buffer	2 M NaCl / 2x PBS / 40 mM imidazole	1:1 Dilution with crude harvest culture
7	Washing buffer	0.5 M NaCl / 0.5x PBS / 10 mM imidazole	15 L
8	Elution buffer	0.5 M NaCl / 0.5x PBS / 200 mM imidazole	2 L

Table 2.6 (Continued)

No	Buffer name	Buffer composition	Usage during primary purification step
9	EDTA solution	50 mM Na-EDTA	2 L
10	Cleaning solution	1 M NaCl / 1 M NaOH	2 L

2.2 Methods

A series of methods were either adopted or developed during the doctoral study. These are detailed in the following sections.

2.2.1 Working cell bank production from master cell bank

A master cell bank (MCB) of a cloned *P. pastoris* strain X-33 with scFv antibody shMFELL2cys sequence was manufactured in the research laboratory and was used to create a working cell bank (WCB) [104]. The MCB was stored in a -80°C freezer in 1 mL aliquots and in 1 mL cryovials. From 1 mL defrosted MCB of scFv antibody shMFELL2cys, 50 µL was spread onto an MCB YPDS plate and incubated at 30°C for a minimum of three days. A single clone was selected from the MCB YPDS plate after three days of incubation, diluted in 100 µL sterile water and 50 µL was spread onto a new YPDS plate. This WCB YPDS plate was incubated at 30°C for a minimum of three days. After three days of incubation, a single clone was selected to make the WCB. A single clone was grown in a 50 mL Falcon tube filled with 50 mL YPD medium containing 100 µg/mL final concentration of Zeocin™ at 30°C and 200 RPM for a minimum of 16–18 h. Once the culture absorbance reached between OD_{600} 10 and 20, the culture was centrifuged in a 50 mL Falcon tube at 300 RPM for a minimum of 5 min. The supernatant was discarded and the cells were resuspended in the WCB storage medium containing 100 µg/mL Zeocin™ (Table 2.3). The resuspended culture was

aliquoted into 1 mL cryovials and stored at -80 °C in a freezer. A single cryovial was used to perform subsequent fermentation runs (Method 2.2.4).

2.2.2 WCB and end of fermenter sample purity test

A purity test of the WCB and end of fermentation run sample was performed in a Class II cabinet. A serial dilution of the WCB and end of fermentation run samples were made by diluting 100 µL of concentrated WCB and end of fermentation run sample in sterile dH_2O from 10^{-1} to 10^{-7}. Then, 100 µL of serial diluted culture from 10^{-5} to 10^{-7} was spread on to TSA and SAB plates in duplicate. TSA is a non-selective medium that provides enough nutrients to grow a wide variety of microorganisms and was used in this experiment to detect microbial contamination in the WCB and end of fermentation run sample. SAB is a selective medium for growing fungus and yeast and was used in this experiment to detect fungus contamination in the WCB and end of fermentation run sample. One set of TSA and SAB plates spreaded with 100 µL of serial diluted culture was incubated at 22.5°C for five days to detect fungus contamination. A second set of TSA and SAB plates spreaded with 100 µL of serial diluted culture was incubated at 32.5°C for five days to detect microbial contamination. After five days of incubation, both sets of TSA and SAB pates were inspected for microbial and fungus contamination. Pictures of TSA and SAB plates spread with 100 µL of 10^{-5} serial diluted material and incubated at 22.5°C and 32.5°C for five days were taken to confirm no fungus or microbial contamination in the WCB and end of fermentation run sample. Typical growth of yeast *P. pastoris* colony was also confirmed in the same picture.

2.2.3 Test expression experiments

Small-scale test expression of the MCB and WCB colonies was performed in 50 mL BMMY medium in 250 mL baffled flasks for a maximum of 72h. The aim was to confirm that all clones from the MCB and WCB expressed the scFv antibody with the His tag. Three single clones from MCB and WCB YPDS plates were grown in 5 mL BMGY medium containing 100 µg/mL Zeocin™ in 50 mL Falcon tubes at

30°C and 200 RPM in a New Brunswick shaking incubator for 16–18 h. The cultures grown overnight in 50 mL Falcon tubes were centrifuged at 3000 RPM in a benchtop centrifuge for a minimum of 10 min and the supernatant was discarded. To induce protein expression, pellets were resuspended in 5 mL BMMY medium containing 10% methanol and transferred to 250 mL baffled flasks containing 45 mL BMMY medium and 100 µg/mL Zeocin™. All flasks were incubated for 72 h at 30°C and at 200 RPM in a New Brunswick shaking incubator. After 24 h and 48 h, flasks were induced again with 0.5% final volume of 100% methanol. Samples were taken at 24 h, 48 h and 72 h to perform a Western Blot analysis (Section 2.2.14.3) and to measure the OD_{600} using a spectrophotometer. Samples of 1 mL were taken to measure the OD_{600} using a spectrophotometer and a 1 mL sample was centrifuged in a benchtop centrifuge at 7000 RPM for 5 min. The supernatants were stored in a 4x reducing buffer at -80°C for Western Blot analysis**.**

2.2.4 High cell density fermentation of *P. pastoris* for the production of scFv antibody shMFELL2cys

Primary culture

One WCB vial of *P. pastoris* strain x-33 stored in a -80°C freezer was defrosted at room temperature and inoculated into a 2 L baffled flask containing 230 mL YPD medium and 100 µg/mL Zeocin™. All procedures were carried out in a sterile environment in a Class II hood. The primary culture flask was incubated at 30°C and at 220 RPM in a New Brunswick shaking incubator for 20 to 24 h until an OD_{600} was reached ≥ 12.

Secondary culture

Once the primary culture flask's OD_{600} was ≥ 12, then 1 mL of culture was used to inoculate a secondary culture flask. The secondary medium composition was the same as for the fermenter starting medium and was collected from the fermenter before autoclaving. Three hundred millilitres of medium prepared in the fermenter was filter sterilised and used in a 2 L baffled flask with 30 mL filter sterilised sodium hexametaphosphate solution. A solution of 1.2 mL trace elements and 100 µg/mL

Zeocin™ was also added to the secondary flask. A 1 mL aliquot of the overnight grown primary culture was used to inoculate the secondary culture flask. Once inoculated, the secondary culture flask was incubated at 30°C and at 220 RPM in a New Brunswick shaking incubator for a minimum of 15 to 17 h until absorbance had reached OD_{600} 5 to 6.

Fermenter setup

Fermentation runs were performed in benchtop 7.5 L maximum working volume New Brunswick glass fermenters (for fermenter details, see Table 2.2). Separate fermentation runs were performed for primary capture purification with RBA chromatography and EBA chromatography. Chemically defined medium compatible with a Good Manufacturing Practice (GMP) were used (for medium composition, see Table 2.5). The chemically defined medium was prepared in a clean glass bioreactor connected to the New Brunswick control system and mixed at 500 RPM agitation at 30°C with 10 L/min air flow-through the sparger. A Broadley James pH probe was calibrated using pH 4 and 7 calibration standard and was inserted inside the bioreactor to measure the pH during the fermentation run. A Hamilton optical dissolved oxygen sensor VisiFerm DO was calibrated at 30°C and 500 RPM inside the fermenter once all medium components had dissolved. This was used to measure dissolved oxygen concentration during the fermentation run. A 100% saturated dissolved oxygen calibration was performed first by adding 10 L/min air flow-through the sparger and once the dissolved oxygen concentration was saturated inside the fermenter. DO probe zero calibration was performed by switching the air flow to nitrogen gas through the sparger and by stabilising the DO reading to zero. The fermenter was autoclave sterilised at a minimum of 121°C for 15 min using the fluid cycle in the autoclave (Table 2.2) and the fermenter was allowed to cool down overnight at room temperature. After autoclaving, the fermentation medium was completed by adding filter sterilised 1 L sodium hexametaphosphate, 24 mL trace elements and 1 mL antifoam 204. After autoclaving, the 100% dissolved oxygen probe calibration was repeated before starting the fermentation run to check the functionality of the probe. pH was controlled at pH 5 +/- 0.1 in cascade mode during the run by adding 25% v/v

ammonium hydroxide base solution and 10% v/v Orth phosphoric acid solution as required.

High cell density *P. pastoris* growth phase to express secreted His_6 tagged scFv antibody shMFELL2cys

The fermenter was inoculated with 330 mL of secondary culture once the absorbance reached OD_{600} 5 to 6 in the secondary culture flask. Fermenter running conditions were as follows: temperature set to 30°C, dissolved oxygen set point of 30% controlled in cascade mode with an agitation starting speed of 500 RPM to a maximum of 1000 RPM and a continuous air flow rate of 10 L/min through the sparger. Pure oxygen was blended through the sparger in a cascade mode of control once the agitation speed reached 1000 RPM to control at 30% saturated dissolved oxygen concentration. pH set point was set to 5 +/- 0.1 for the initial growth phase stage. After 23 to 24 h of biomass build-up, the glycerol was depleted in the fermenter and a dissolved oxygen spike was observed. A limited feeding regime was started with 50% v/v sterile glycerol and 10 mL of methanol was used for induction after 1 h of glycerol feeding. The pH set point was changed from 5 to 6.5 once the dissolved oxygen spike had been observed. In total, around 400 mL of 50% v/v glycerol feed was added for a minimum of 4 h. The rate of glycerol feed addition was decreased from 150 mL/h for the first hour to 120 mL/h for the second hour, 100 mL/h for the third hour and 40 mL/h for the last hour. After 4 h of glycerol limited feeding, 100% methanol limited feeding was started for a further 40 h. For the first two hours 15 mL/h methanol limited feed was applied, for the second two hours 25 mL/h, and the third two hours 30 mL/h. After 6 h of culture, 45 mL/h 100% methanol feed was applied for the remaining 34 h.

During the fermentation runs, samples were taken to measure fermenter culture absorbance at OD_{600} and wet cell weight. The fermenter sample was centrifuged in benchtop centrifuge and supernatants were stored with 4x reducing buffer to perform sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot analysis. Samples were analysed withing 1-2 weeks post fermentation runs.

2.2.5 Biomass measurement using spectrophotometer

Ten to 15 mL of fermentation samples were taken aseptically for biomass measurement using a Biochrom WPA CO8000 cell density meter reading (Table 2.2) at an absorbance of OD_{600}. Serial dilution of fermenter samples was performed in duplicate and in 1mL Eppendorf tubes. First, 1 in 20 dilution was performed and from that diluted material, 1 in 25 dilution was performed. In total, a 1 in 500 dilution was performed before measuring the absorbance at OD_{600}.

2.2.6 Wet cell weight measurement

Wet cell weight (WCW) of the fermenter samples were measured by aliqouting 5 mL of mixed fermenter sample into pre-weighted 15 mL Falcon tubes in duplicate and centrifuging the sample at 3000 g for 10 min in a centrifuge (Table 2.2). After centrifugation, the supernatant was transferred into a clean 15 mL Falcon tube. WCW was calculated by subtracting the empty Falcon tube weight from the weight of the cells plus the 15 mL Falcon tube. A true representation of 1 L fermenter culture WCW was calculated by multiplying 5 mL WCW value by 200.

2.2.7 RBA column packing and setup

Radial bed adsorption (RBA) columns were packed manually through the packing port. The empty RBA column was first cleaned with dH_2O and assembled according to the RBA column manual [105]. The RBA column was assembled with 40 µm inlet and outlet frits, which allow yeast cells to pass through the column when diluted with a binding buffer. CRIO-MD/MP RBA columns have a bottom packing port so packing was performed by placing the column upside down, thus mimicking the top packing port procedure. In packing the column through the bottom packing port, there was a danger that the resin slurry would not be evenly distributed inside the column, leaving empty zones inside the column. A 30% resin slurry was prepared in the packing buffer and mixed gently and continuously on a

magnetic stirrer while packing the column for uniform suspension of the slurry in the packing buffer. The column was packed with IDA Chelating Cellthru™ BigBead resin in 0.5 M NaCl / 0.5x PBS solution (Table 2.6). The column was packed by adding 30% resin slurry inside the column using a peristaltic pump, and a pressure gauge was placed in line to measure the packing back pressure. First, the packing buffer was recirculated through the packing port and column inlet before switching the flow to the resin slurry. The column was packed at 250 mL/min flow rate, and this was decreased once the pressure started to increase inside the column. During the packing, high flow rate pulses were applied to remove the resin wall building up near the packing port, which can increase the back pressure. Column packing was stopped as soon as the pressure started to increase inside the column, even at a lower flow rate. Once packed, the column was rinsed with 10 CV dH_2O and stored in 0.1 M NaOH / 0.5 M NaCl solution. The quality of packing in the radial flow column was checked by applying 0.1 M copper sulphate solution at 250 mL/min and by verifying the radial flow of the copper sulphate solution from the inlet to the outlet of the column. Figure 2.1 shows the setup of the RBA column packing procedure and Figure 2.2 shows the resin distribution inside the RBA column.

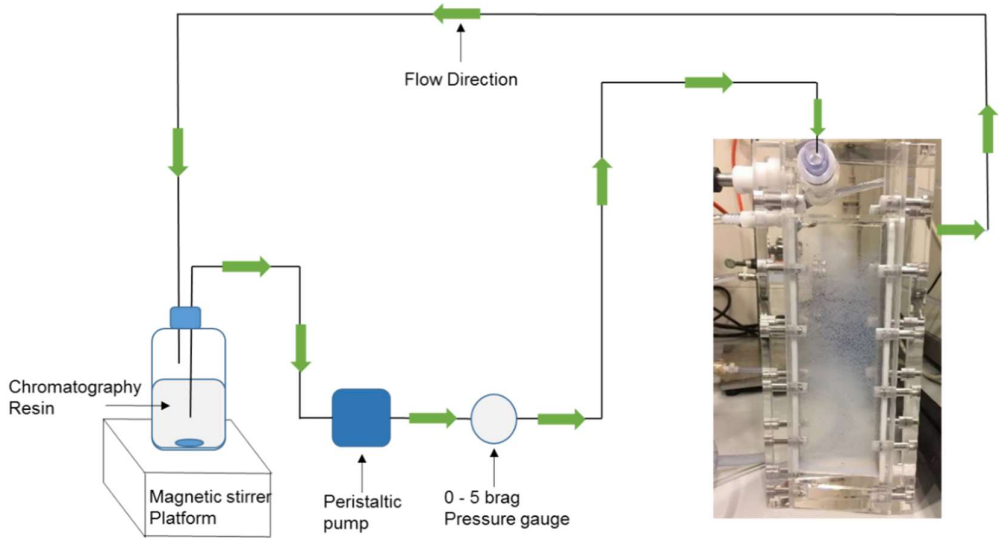

Figure 2.1 250 mL RBA column packing setup.
Figure 2.1 shows the setup of the RBA column packing procedure.

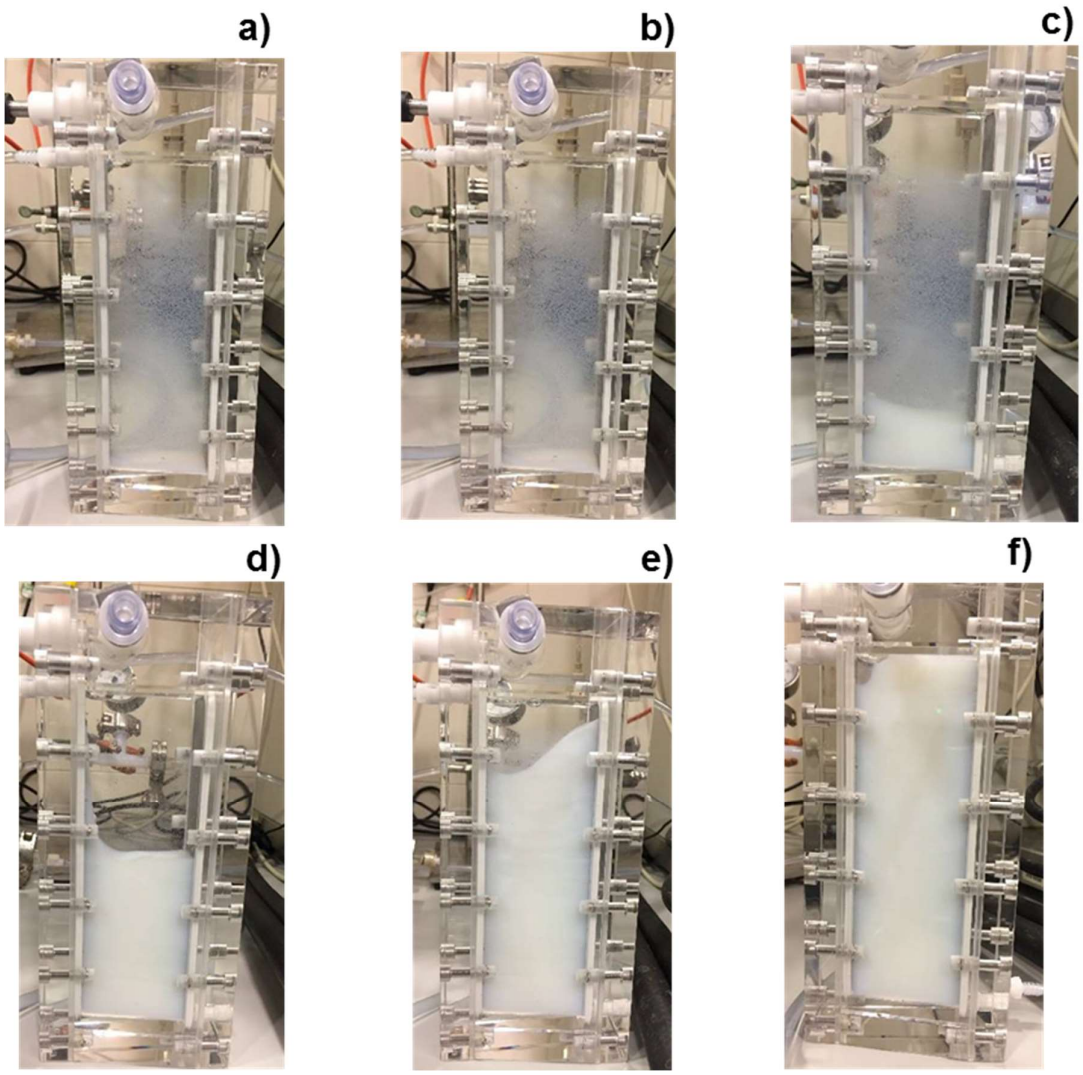

Figure 2.2 250 mL RBA column packing steps.
Pictures a) to f) illustrate the resin distribution during and at the end of the RBA column packing procedure.

2.2.8 Primary purification of the His_6 tagged scFv antibody using radial bed adsorption chromatography

Direct capture of the His_6 tagged scFv antibody was performed using a 250 mL RBA column. Feed from the end of a 72 h *P. pastoris* fermentation run was applied to the RBA column. The His_6 tagged scFv antibody was secreted into the culture medium. The RBA column was packed with 250 mL IDA Chelating Cellthru™ BigBead. The RBA column was operated at a high flow rate up to 70 mL/min and at very low pressures (< 0.1 bar (g)). The bed was stored in 0.1 M NaOH / 0.5 M NaCl solution between the two separate primary capture steps. Before use, the storage solution was exchanged with 10 L of purified water at 200 mL/min flow rate. High flow rate pulses were applied in both directions to remove air bubbles. The operation can be performed in both directions to remove air bubbles and cells during a washing step. 0.1 M Metal ion copper sulphate was applied to the column at 70 mL/min and equilibrated by applying 1 L of washing buffer (Table 2.6). Before applying cells, the bed was equilibrated with 1 L of equilibration buffer (Table 2.6). The fermenter harvest culture was diluted (1:1) with a biomass application buffer (Table 2.6) in a 2 L mixing vessel and applied at 70 mL/min (equivalent to a flow velocity of 93 cm/h) using a peristaltic pump. Before applying on to the column, the diluted fermenter culture and binding buffer pH were each adjusted to pH 7.5 by adding 25 mL K_2HPO_4 into each 2 L mix. The culture and binding buffer pH were each measured using a pH meter. The pH meter's temperature probe was used to measure the temperature.

While loading the culture on to the column, the fermenter methanol feed was decreased gradually after each 1 L of culture had been taken out of the fermenter. Fermenter temperature did not change during the harvest step. Flow rate was measured using a measuring cylinder and balance. Column inlet pressure was monitored through an inline pressure gauge. While loading the fermenter culture on to the column, flow-through was collected in a waste container and samples were taken for further analysis by SDS-PAGE gel and Western Blot and particle size distribution studies. In total, around 8 L fermenter culture was applied on to the column. Columns were washed with a washing buffer (Table 2.6) and in total, 11 L washing buffer was used to wash the bound cells and cell debris from the

column at 100 mL/min. During the wash stage, 3 L of washing buffer was applied in a reverse direction to remove cells and cell debris. Captured protein was eluted with 2 L elution buffer (Table 2.6) in 50 mL fractions at 70 mL/min flow rate. The OD_{280} of the eluted fractions were measured in a spectrophotometer, and fractions containing the protein of interest were pooled together and 0.2 µm filtered for further purification steps. Pooled fractions were concentrated to 75–125 mL using Biomax 5 filters on the Millipore Labscale TFF System (Table 2.2) before applying on a size exclusion chromatography column for further purification.

Cleaning in place

Post-elution, 2 L purified water was applied at 200 mL/min to remove leftover imidazole from the RBA column. Then, 50mM EDTA was applied at 70 mL/min to remove metal ion copper. Two litres of 0.5 M NaOH was used to clean and sanitise the column at 70 mL/min and the column was incubated for a minimum of 2h. After the two-hour incubation, 0.5 M NaOH was replaced by 0.1 M NaOH and the column then stored at room temperature.

2.2.9 Primary purification of antibody using expanded bed adsorption chromatography

EBA column setup

A first-generation STREAMLINE 50 expanded bed adsorption (EBA) column (Table 2.2) was used with 300 mL of STREAMLINE™ chelating resin to capture secreted His_6 tagged scFv antibody manufactured through *P. pastoris* fermentation (Method 2.2.4). Total column length was 100 cm. A manual STREAMLINE system with two-way valve system was used with two Watson-Marlow peristaltic pumps (Table 2.2). To minimise flow rate fluctuations, rigid tubing fixed to the Watson-Marlow pump was used. Operational flow rates were measured using a measuring cylinder and balance. The EBA flow distribution system includes a bottom net with a perforated metal plate. Three hundred millilitres of resin was poured inside the EBA column by opening the top adapter. The EBA column, tubing and resin were all rinsed with 10 L dH_2O to remove the 20% v/v ethanol used for storage. This was carried out in expanded mode. Five litres 0.5 M NaOH was used for each cleaning

in place (CIP) cycle and exchanged again using 10 L dH_2O in expanded mode. The EBA bed was stored in 20 % v/v ethanol after cleaning for long-term storage at room temperature. The bed was operated in upflow during CIP step and in downflow during the addition of ethanol for storage.

EBA column operation

STREAMLINE™ chelating resin was used to capture the secreted His_6 tagged scFv antibody. STREAMLINE™ chelating resin has a quartz core material that makes the resin heavier [106]. The resin has a quoted particle size distribution range of 100–300 µm. Five litres of 0.1 M copper sulphate, which binds to the IDA chelating agent, was applied on to the column at 100 mL/min. Unbound copper was removed by applying 5 L dH_2O in expanded mode. The settled bed height was 14 cm. The top adapter was raised to the maximum column length before applying the fermentation culture. The settled bed resin was equilibrated and then expanded with 4 L of equilibration buffer in upflow at 70 mL/min before applying the fermentation culture mix. During the equilibration step, the EBA bed was expanded to 35.5 cm in bed height. Once equilibrated, the inlet line of the EBA column was switched to prime fermenter harvest culture and binding buffer mix. The fermenter harvest culture was diluted (1:1) with binding buffer (Table 2.6) in a 2 L mixing vessel and applied at 70 mL/min (equivalent to a flow velocity of 214 cm/h) using a peristaltic pump. Before applying on to the column, the fermenter culture and binding buffer pH increased to pH 7.5 by adding 25 mL K_2HPO_4 into each 2 L batch. pH was measured using a pH meter (Table 2.2), and the pH meter temperature probe (Table 2.2) was used to measure the temperature. The fermenter culture and binding buffer were mixed continuously on a magnetic stirrer platform to prevent cell agglomeration.

Stable expansion of the bed was maintained at between 2.5 and 3 settled bed height. The outlet flow rate was measured after every 1 L had been applied to the column. Flow rate was slowly decreased after 7 L of loading culture with binding buffer had been applied on to the column to maintain the steady-state expansion of the column. At the end of the culture application, the flow rate was decreased to 60 mL/min (flow velocity 184 cm/ h). A total of 8 L of fermentation culture was

applied to the column. Flow velocity was maintained between 214 cm/h and 184 cm/h during the load step. A total of 15 L washing buffer (Table 2.6) was applied in expanded mode starting at 60 mL/min. During the washing step, expansion of the bed was maintained at 2.5 to 3 settled bed height. At the end of the washing step, flow rate was increased to 84 mL/min to maintain steady expansion of the bed. The column was operated in upflow for the cleaning, copper application, cell application, washing and regeneration steps with maximum adapter height. For elution, the expanded resin was allowed to settle. The top adapter was then lowered and placed 5 cm above the settled bed. Elution of the adsorbed His_6 tagged scFv antibody was performed in downflow at 70 mL/min with 1.5 L elution buffer (Table 2.6). Elution fractions of 30 x 50 mL were taken in 50 mL Falcon tubes. The OD_{280} of the eluted fractions were measured in a spectrophotometer, and fractions with higher OD_{280} were pooled together and 0.2 µm filtered for further purification steps. Pooled fractions were concentrated using Biomax 5 filters in a Millipore benchtop TFF system (Table 2.2) before applying on a size exclusion chromatography (SEC) column for further purification. Flow-through was collected in a waste container and samples taken at regular intervals for further analysis by SDS-PAGE gel and Western Blot to measure binding efficiency of the resin and availability of a His_6 tagged scFv antibody. Particle size distribution studies were performed on flow-through samples collected during the fermenter culture application and column washing steps to confirm that no resin particles were present in the flow-through samples.

EBA column post-use CIP cycle

After use, CIP of the EBA column and resin was performed using 0.5 M NaOH solution in expanded mode. The EBA column was rinsed with 10 L dH_2O in an upward direction followed by 10 L 0.5 M NaOH rinse at 70 mL/min flow rate. The expanded bed adapter was set to the maximum height during the CIP cycle. NaOH was removed by 10 L dH_2O in expanded mode. Finally, the column was stored in 20% v/v ethanol applied in downflow and with the adapter 5 cm above the settled bed height to apply five times the resin volume of the 20% v/v ethanol. Then, 20% v/v ethanol was applied in downflow, placing the adapter 5 cm above the resin to reduce the amount of ethanol required to store the column.

2.2.10 EBA column expansion experiment

Studies of the STREAMLINE 50 EBA column expansion were performed with an application buffer (Table 2.6) prepared with 25% v/v and 32% v/v glycerol concentrations so as to measure bed expansion in the presence of viscous material, such as fermentation broth. The column adapter was placed at maximum height and a calibrated Watson-Marlow pump with 9.6 mm OD tubing was used to apply feed. The application buffer was first applied in expansion mode at 50 cm/h, 100 cm/h, 150 cm/h, 200 cm/h and 300 cm/h flow velocity. Bed expansion height was recorded once the steady-state expansion was seen for a minimum of 5 min. The procedure was repeated with 25% v/v glycerol added to the application buffer at 50 cm/h, 100 cm/h, 150 cm/h and 200 cm/h linear velocities. The steady-state expansion of the settled bed was recorded. The column bed was not stable at or above 300 cm/h. Ten litres dH_2O was applied in an upward direction to remove the second expansion buffer. The column bed was then allowed to settle down and application buffer with 32% v/v glycerol was applied at 50 cm/h, 100 cm/h and 150 cm/h linear velocity. In this case the column bed was not stable at either 200 cm/h or 300 cm/h flow velocity. EBA column expansion ratio was measured and the data plotted so as to measure the effect of viscous material on the expansion of the settled bed as a function of linear velocity in the expanded bed.

2.2.11 Antibody concentration using tangential flow filtration (TFF) system

A Millipore Labscale® TFF system was used with Pellicon® XL Ultrafiltration Module Biomax 5 kDa 0.005 m² filters to concentrate the eluted protein from the RBA and EBA column. Three Biomax 5 filters were used together using a multi-filter connection assembly. The system reservoir, associated tubing and filters were all rinsed with dH_2O first and cleaned with 0.5 M NaOH solution and stored in 0.1 M NaOH. After cleaning, the system was rinsed with dH_2O and flushed with 200 mL elution buffer before starting the concentration step. Protein concentration was performed at room temperature, and both feed and retentate pressure during the concentration step were kept constant at 0.7 bar (g) by manually adjusting the

retentate back pressure valve and peristaltic pump speed. A magnetic stirrer was placed inside the reservoir to deliver continuous mixing of the protein. A setting of 3 from the TFF display panel was used. Transmembrane pressure (TMP) during the protein concentration step was maintained throughout at 0.7 bar (g). A Millipore Labscale® TFF system does not have pressure gauge connected to the permeate line. The eluted protein pH from both the RBA and EBA adsorption chromatography was pH 9.02. In total, 1000 mL of RBA eluted protein was concentrated to 108 mL in 2 h and 800 mL of EBA eluted protein was concentrated to 195 mL in 1 h 35 min. Protein concentration was continuously measured every 20 to 30 minutes by measuring the optical density at wavelength 280 nm. Once concentrated, the pH of the eluted protein was adjusted to pH 7.50 by adding 3 M K_2HPO_4 solution. Concentrated protein was stored in a fridge between 2°C and 8°C before being applied to the size exclusion column (Method 2.2.12).

2.2.12 Size exclusion chromatography

A 500 mL Superdex 75 gel filtration chromatography medium packed into an XK 26/100 column was used to separate the concentrated protein. The column was supplied prepacked by GE (Table 2.2). Pre-use, the column was cleaned with 0.5 M NaOH solution at a flow velocity of 23 cm/h, rinsed with dH_2O and equilibrated with PBS at a flow velocity of 45 cm/h. Molecular weight markers were run on the column before and after the fast protein liquid chromatography (FPLC) runs at a flow velocity of 45 cm/h. Concentrated protein was transferred to a clean super loop and connected to an AKTA Prime pump system (Table 2.2). The AKTA Prime was connected to a control computer so that the protein separation chromatogram could be seen on the AKTA Prime view software (Table 2.2). Twenty-five millilitres of concentrated protein was applied to the column at a maximum flow velocity of 45 cm/h and maximum running pressure of 0.3 MPa. Separated protein was collected in 5 mL fractions once the 280 UV absorbance value started to increase on the chromatogram. Monomer peak fractions of the chromatogram were selected based on the molecular weight marker protein separated on the column at the same volumetric flow velocity of 45 cm/h. The selected protein fractions were

pooled together, aliquoted in a 1 mL cryovial and stored in a -80°C freezer. Pooled samples were also stored in a reducing buffer for SDS-PAGE analysis (Method 2.2.14.2). In total, four FPLC runs were performed for protein concentrated from the RBA chromatography and five FPLC runs were performed for protein concentrated from the EBA chromatography.

2.2.13 Molecular weight marker on size exclusion columns

Gel filtration calibrated standards from Bio-Rad (Table 2.1) were run on the Superdex 75 gel filtration chromatography medium packed in XK 16/100 and XK 26/100 columns. This was done to ensure that the columns were properly packed and that samples were eluting properly. Molecular weight markers were run pre- and post-use of columns. Bio-Rad gel filtration standards contain a mixture of proteins from 1350 to 670000 Daltons, thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (13 kDa). Five hundred microliters of standards mixed with 1.5 mL PBS were applied on to the 500 mL Superdex 75 columns and run at 4 mL/min flow rate. Two hundred and fifty microliters of standards mixed with 1.75 mL PBS were applied on to the 125 mL Superdex 75 columns and run at 1.5 mL/min flow rate.

2.2.14 Protein tests

Samples taken during fermentation, primary purification, concentration and size exclusion chromatography were analysed by SDS-PAGE, Western Blot and analytical SEC. Total protein concentration was determined using Bradford assay and antibody quantification was determined using the Agilent Bioanalyzer. Enzyme-linked immunosorbent assay (ELISA) and host cell protein (HCP) concentration were performe to measure the quality of the antibody. Samples were also analysed to measure the particle size distribution using Mastersizer 3000.

2.2.14.1 Total protein concentration measurement using Bradford assay

A Bio-Rad DC™ protein assay kit (Table 2.1) was used to measure the total protein concentration of the samples. The Bio-Rad Protein Assay is a colorimetric assay that measures the formation of a blue copper complex colour between 650 and 750 nm absorbance. The colour development is mainly due to the reagent's interaction with amino acids tyrosine and tryptophan and, to a lesser extent, cystine, cysteine and histidine. Samples for analysis were stored in a -80°C freezer and analysed after defrosting at room temperature. Total protein concentration at the end of the fermentation run, the RBA and EBA elution, the concentrated and final purified protein samples were all measured. The total protein concentration of EBA and RBA resin screening samples were also measured. Serial dilution of the standard bovine serum albumin prepared at 10 mg/mL concentration in a 96-well plate was performed to form a calibration data set. Samples to be analysed were also diluted in PBS. Five microliters of serially diluted samples and standard were added to a 96-well plate using a multichannel pipette and 25 µL of reagent A (alkaline copper tartrate solution) from the protein assay kit was added to each sample and mixed two or three times using a multichannel pipette. Finally, 200 µL of reagent B (dilute Folin reagent) from the assay kit was added to each sample and mixed gently two or three times using a multichannel pipette and allowed to incubate at room temperature for a minimum of 15 min. Colour development was measured on a plate reader at 650 nm fixed wavelength. Protein concentration in the samples was determined by a standard graph of serially diluted protein.

2.2.14.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Bio-Rad PowerPac 1000 electrophoresis power supply (Table 2.2) was used to run the samples stored in a reducing buffer. A 10% resolving gel was prepared first using glass plates and a casting stand according to the recipe written in Table 2.4 and allowed to solidify. Adding isopropanol removed the air bubbles before the gel set. Once set, the 4% stacking gel was added on top of the 10% resolving gel and a 10-well comb was inserted and allowed to solidify to make 10 wells of SDS-PAGE. The 10% resolving gel was transferred to the gel apparatus and placed into the gel tank. SDS-PAGE running buffer was added into the tank before loading the samples. Samples stored in a 4x reducing buffer were defrosted at room

temperature and boiled at 95°C for 5 min to break the disulphide bond. Samples were loaded on to the gel along with a PageRuler Prestained Protein Ladder. The power supply was connected to the tank and gels were run at 150 V for 1 h. For SDS-PAGE analysis, gels were stained using Coomassie stain and destained using destaining solution. Protein bands were analysed by comparing them against molecular weight marker bands. Destaining solution (Table 2.4) was rinsed with dH_2O, and gels fixed using fixing buffer and dried overnight.

2.2.14.3 Western Blot analysis of the fermentation, RBA and EBA purified samples

A Bio-Rad PowerPac 1000 electrophoresis power supply was used at 100 V for 60 min to transfer the protein bands on to the polyvinylidene difluoride (PVDF) membrane after running samples on 10% SDS-PAGE gels (Method 2.2.14.2). Gels were placed between pre-wetted sponges and Whatman™ Protran™ nitrocellulose blotting membranes. Immobilon-P PVDF membrane was pre-activated in 100% methanol before being placed on to the gels and placed inside the Western Blot transfer cassette. The cassette was inserted into the transfer container and Western Blot transfer buffer was used to transfer the protein to the PVDF membrane using the Bio-Rad PowerPac. The PVDF membrane was blocked using 50 mL 5% milk for 1 h on a shaking platform or overnight in a fridge in the square container. After that, the blocking membrane was rinsed three times with 50 mL PBS / 0.1% Tween and then rinsed three times with 50 mL PBS. Twenty-five millilitres primary antibody (tetra His antibody/Mouse monoclonal) in 1% milk was applied at 1/1000 dilution to the membrane in the square container and placed on the shaking platform for 1 h. The membrane was rinsed three times with 50 mL PBS / 0.1% Tween and then rinsed three times with 50 mL PBS. Twenty-five millilitres secondary antibody (ECL mouse IgG, HRP-linked F(ab)2 fragment) in 1% milk was applied at 1/1000 dilution to the membrane in the square container and placed on the shaking platform for 1 h. The membrane was rinsed three times with 50 mL PBS. Finally, the membranes were developed with 40 mL dH_20 containing 20 µL hydrogen peroxide and 2 to 5 mg DAB (3, 3′-diaminobenzidine tetrahydrochloride hydrate). Once developed, the membranes

were rinsed with dH_2O and the developed solution was discarded in a hazardous waste container.

2.2.14.4 Analytical SEC

One hundred and twenty-five millilitres of Superdex 75 gel filtration chromatography medium packed in XK 16/100 column was used as an analytical SEC column to run the final pooled purified samples. Columns were supplied prepacked by GE Healthcare (Table 2.2). Before use, the columns were cleaned with 1 CV of 0.5 M NaOH solution and rinsed with 2 CV of dH_2O at 15 cm/h volumetric flow rate. The column was equilibrated with 1 CV of PBS at 45 cm/h volumetric flow rate. One millilitre freeze-thaw final purified protein samples from EBA and RBA columns were loaded on to a 2 mL loop connected to an AKTA Prime pump system. The AKTA Prime was connected to a control computer so that the protein separation chromatogram could be seen on the AKTA Prime view software (Table 2.2). Samples were applied to the column at a volumetric flow rate of 45 cm/h and maximum running pressure of 0.3 MPa. Separated protein peaks were analysed for antibody dimer and monomer by comparing the chromatogram with the molecular weight marker chromatogram. Fermenter harvest, EBA and RBA eluted protein and concentrated protein samples were also analysed on the analytical size exclusion column.

2.2.14.5 *P. pastoris* HCP ELISA

A Cygnus *P. pastoris* HCP ELISA kit (Table 1.1) was used to measure the *P. pastoris* HCP concentration in the fermenter harvest sample, EBA and RBA eluted protein samples and in the final purified protein samples. The standard assay protocol from Cygnus was used to measure the HCP concentration. The *P. pastoris* HCP kit contained microtiter strips pre-coated with antibodies against HCPs. Twenty-five microlitter of samples and standards supplied by Cygnus (0, 1, 4, 20, 75 and 250 ng/mL) in triplicate were added to the microtiter plate followed by 100 µl of *P. pastoris* HRP antibody. These standards are solubilized *P.pastoris* HCPs in a bovine serum albumin matrix with preservatives. All samples were dialysed against PBS overnight in a mini dialysis device (Table 1.1) before measuring HCP concentration. The dialysed fermenter harvest sample was serially diluted in PBS from 1/10 to 1/10000. Serially diluted 1/1000 and 1/10000 fermenter samples were

analysed. EBA and RBA purified protein was 1/50 diluted and applied to measure HCP concentration. Microtiters were covered with a sealing film during the incubation on the shaker for 3 h. Next, the contents from the well were discarded by inverting the plate and washing the microtiters with 1 x diluted wash buffer using a squirt bottle. The step was repeated a total of four times. Between each rinse, the wash buffer was dumped by inverting the microtiter plate and tapping it on the tissue paper. Next, 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate was placed by pipette into each microtiter well and incubated at room temperature without shaking for 30 min. After 30 min, 100 µl stop solution was added into each microtiter well and colour development was measured using a plate reader at 450 nm fixed wavelength. The mean of standards value was calculated and a graph was plotted to measure the HCP concentration of the samples. For a standard graph, the absorbance value for 0 standard was subtracted from other standards before calculating the mean and plotting the graph.

2.2.14.6 CEA ELISA of the scFv antibody shMFELL2cys

The His_6 tagged scFv antibody shMFELL2cys, which has an affinity for carcinoembryonic antigen (CEA), was used for the study. CEA ELISA was performed to check the affinity of the scFv antibody manufactured in *P. pastoris* and purified using RBA and EBA chromatography. A 96-well Nunc MaxiSorp™ flat-bottom plate was first coated with 100 µL commercially available CEA (Table 1.1) at 1 µg/mL concentration using a pipette and incubated at room temperature for a minimum of 1 h. To measure the background effect of the samples, 100 µL of the PBS was also added using the pipette and incubated for a minimum of 1 h in separate wells in the same 96-well plate. The plate was covered with a sealing film during the incubation times. After coating, all 96 wells were rinsed two times with the PBS / 0.1% Tween twenty wash buffer and then rinsed two times with the PBS buffer. Between each rinse, the wash buffer was dumped by inverting the microtiter plate and tapping it on the tissue paper. After the rinse, 100 µL of the low fat 5% Milk prepared in PBS was added to the coated wells for blocking and the plate was incubated for 1 h on a shaker platform (Table 2.2) or overnight in a fridge. After blocking, the plate was rinsed two times with the PBS / 0.1% Tween buffer and then rinsed two times with the PBS buffer. Between each rinse, the excess fluid

was removed by inverting the plate and tapping it on the tissue paper. All of the samples to be analysed were diluted in the PBS to a starting concentration of 5 µg/mL in 1.5 mL Eppendorf tubes. From 5 µg/mL starting concentration, samples were serially diluted from 10^{-1} to 10^{-3} in a second 96-well plate in PBS. One hundred microliters of 5 µg/mL starting concentration samples and serially diluted samples from 10^{-1} to 10^{-3} were added to a 96-well plate coated with CEA using the multichannel pipette. All of the samples were added in duplicate and incubated for 1 h on the shaker platform. After 1 h, the incubation plate was rinsed two times with the PBS / 0.1 % Tween buffer and two times with the PBS buffer. Between each rinse, the excess fluid was removed by inverting the plate and tapping it on the tissue paper. One hundred microliters of 1 µg/mL primary antibody (tetra His antibody/Mouse monoclonal) prepared in the 1% milk was added to each of the wells using the multichannel pipette and incubated at room temperature on a shaker platform for 1 h. After 1 h incubation, the plate was rinsed with PBS / 0.1% Tween buffer and twice with PBS buffer. One hundred microliters of 1 µg/mL secondary antibody (mouse IgG, HRP-linked F(ab)2 fragment) prepared in 1% milk was added to each of the wells using the multichannel pipette and incubated at room temperature on a shaker platform for 1 h. Finally, the plate was rinsed three times with PBS buffer. ELISA development solution was prepared by dissolving one capsule of phosphate-citrate in 100 mL dH_2O first and then adding one OPD tablet into 25 mL phosphate-citrate solution. One hundred microliters of development solution was added to each well using the multichannel pipette and a yellow colour change was noticed due to the enzymatic reaction. Once the yellow colour developed, the reaction was stopped by adding 50 µL 4 M HCl solution per well using the multichannel pipette and colour development was measured using a plate reader (Table 2.2) at 450 nm fixed wavelength. The mean absorbance reading of all samples was analysed by preparing a graph in an Excel sheet to measure scFv antibody affinity to the CEA.

2.2.14.7 Particle size analysis using Malvern Mastersizer 3000 laser diffraction particle size analyser

Malvern Mastersizer 3000 (Table 2.2) was used to measure the particles in samples collected during the primary capture step using RBA and EBA

chromatography. Samples taken during the primary capture step were stored at -80°C. To measure particle size distribution, samples were first defrosted at room temperature. Samples were diluted in dH_2O while analysing the samples in a HydroSV 7 mL max volume dispersion system. The cuvette in the HydroSV unit was cleaned thoroughly and rinsed with dH_2O first. A small stirrer (15 x 1.5 mm (L x D)) was placed inside the cuvette and the cuvette was placed inside the HydroSV cell. The stirrer speed was kept at 1000 RPM during sample loading and analysis. To analyse the samples, spherical particle type was selected in the Mastersizer 3000 software. dH_2O was selected as the dispersant material as all samples were analysed in dH_2O. Before measuring the samples, the instrument was initialised and the background was measured first. All samples were added using a pipette after measuring the background. The measurement obscuration for wet dispersion was maintained between 5% lower limit and 25% upper limit. While analysing the samples, five measurements per sample were taken and an average of five measurements were analysed. All samples were analysed one after another and the HydroSV chamber was cleaned thoroughly using dH_2O on a cleaning stand. Results were stored in a computer and analysed separately. The overlay graphs of the particle size distribution of the RBA and EBA chromatography samples were prepared and are discussed in detail in Chapter 3.

2.2.14.8 Protein quantification using Agilent 2100 Bioanalyzer

An Agilent protein 80 kit was used to measure the His_6 tagged scFv antibody concentration in samples taken at the end of the fermentation run, eluted protein from the RBA and EBA capture step and final concentrated samples. All samples to be analysed were stored at -80°C in a freezer. Samples were defrosted at room temperature and equilibrated at room temperature for a minimum of 30 min before analysing. The Agilent protein 80 kit contains gel, dye, sample buffer and protein 80 ladder. The gel-dye mix was prepared by centrifuging 650 µL of gel at 2500 g for 15 min in the Agilent protein 80 Gel-Matrix spin filter vial supplied by Agilent in a benchtop centrifuge. Once filtered, 25 µL of the dye was added to 650 µL of gel and stored at -20°C until further use. To analyse the sample, 4 µL of the sample was mixed with 2 µL of the sample buffer containing 1 M dithiothreitol (DTT) in a 0.5 mL Eppendorf vial. The sample mix and 6 µL aliquot of protein 80 ladder were

boiled in the heat block at 95°C for 5 min. Once the sample had cooled down at room temperature, 84 µL of deionised water was added into the sample mix and protein ladder vials. Six microliters of sample and protein ladder along with 12 µL of gel-dye-mix and destaining solution were loaded on to the protein chip according to the manual's loading instructions [107]. In total, 10 samples can be analysed using one protein 80 chip. The chip was analysed within 5 min of loading the samples and ladder on the Agilent 2100 Bioanalyzer. The His_6 tag scFv antibody at 1 mg/mL and 2 mg/mL concentrations were added on to the protein 80 chip to quantify the unknown concentration of scFv antibody in the samples. The Agilent 2100 Bioanalyzer calculates the concentration values of unknown samples using calibration values of known protein concentration samples. All samples were loaded in duplicate and average concentration values of the samples were calculated. The His_6 tagged scFv antibody concentration of the samples is discussed in detail in Chapter 3.

2.2.15 Static binding capacity experiment of the EBA and RBA resins

The His_6 tagged scFv antibody shMFELL2cys purified from RBA and EBA chromatography was used to perform the static binding capacity experiment using 1 mL settled resin volumes. IDA Chelating Cellthru™ BigBead and STREAMLINE™ chelating resin were used in the RBA and EBA chromatography, respectively. A sediment resin volume of 1 mL in the 1.5 mL Eppendorf tube was used to perform the static binding capacity experiment. The resins were allowed to settle down first for a minimum of 1 h in the Eppendorf tube. After 1 h, 500 µL of dH_2O was added to each Eppendorf tube to resuspend the resins and transferred to 15 mL Falcon tubes using a plastic pasture pipette. A plastic pasteur pipette was used to transfer and resuspend the resins because it was difficult to decant the resin with 1 mL pipette tips due to the larger size of the resins. To remove the 20% ethanol storage solution, the resins were washed three times with 10 mL dH_2O in the 15 mL Falcon tubes. The 15 mL Falcon tubes were securely placed on the Falcon tube stand and the resins were gently washed by turning the Falcon tubes upside down three to four times. After each wash, the resins were allowed to settle

down for 5 min before removing the supernatant using a 10 mL pipette. The same procedure was repeated to remove unbound copper and equilibrate buffer from the resins. Next, 100 mM of 10 mL copper sulphate solution was added to each resin tube and mixed as before. The resins were allowed to settle down before removing the unbound copper supernatant with a 10 mL pipette. A further 10 mL of dH_2O was added to the Falcon tube and washed three to four times to remove unbound copper. Both resins were equilibrated with 10 mL of 0.5 M NaCl / 0.5x PBS/ 10 mM imidazole and mixed for a minimum of three to four times, then transferred into 250 mL cleaned and autoclaved conical flasks. The purified His_6 tagged scFv antibody shMFELL2cys in a final buffer concentration of 1 M NaCl / 1x PBS / 10 mM Imidazole was added to each flask. A total of 70 mL and 80 mL protein buffer mix at 0.70 mg/mL concentration was added to the RBA and EBA resin flasks, respectively. Flasks were placed inside the shaker incubator (Table 2.2) at 25°C and 120 RPM for a minimum of 20 h. Binding of the His_6 tagged antibody to the metal ion copper was measured by reading the optical density of the supernatant sample using a spectrophotometer at wavelength 280 nm. Once the OD_{280} reading was stable and had not decreased after an extra hour of mixing, the static binding experiment was stopped. Resins were allowed to settle down and the supernatant was removed.

The protein bound to the resin was eluted by adding 15 mL of 0.5 M NaCl / 0.5x PBS/ 200 mM imidazole buffer to each resin flask and mixing gently for 2 to 5 min. The protein concentration in the supernatant was measured using a spectrophotometer at wavelength 280nm. RBA or EBA shake flask supernatant and eluted protein samples were stored in a -80°C freezer for analysis by SDS-PAGE gel. The binding capacity of the resins was calculated based on the total amount of antibody bound to the resin. The static binding capacity experiment results are discussed in detail in Chapter 4.

2.2.15.1 Static binding capacity experiment of the EBA and RBA resins in the presence of 20% (v/v) glycerol

Static binding capacity experiment of the 1 mL settled EBA and RBA resin was performed using the purified His_6 tagged scFv antibody shMFELL2cys in binding buffer (1 M NaCl / 1x PBS / 10 mM Imidazole) and supplemented with 20% (v/v)

glycerol. One millilitre of the EBA and RBA resins were aliquoted in duplicate in the 1.5 mL Falcon tubes. Preparation of the settled resin, washing and equilibration of resin with copper was performed according to Method 2.15. A 20% (v/v) final glycerol concentration was added to one of the EBA and RBA resin flasks. Protein was applied with 1 M NaCl / 1x PBS / 10 mM Imidazole and pH was adjusted to pH 7.5 using 3 M potassium phosphate dibasic and 3 M potassium phosphate monobasic buffers. The total volume was 100 mL into each flask at a protein concentration of between 0.36 and 0.39 mg/mL. All four flasks were placed into the shaking incubator at 25°C at 150 RPM. Resin binding capacity was measured by measuring the optical density at wavelength 280 nm in a spectrophotometer. The results of this experiment are discussed in detail in Chapter 4.

2.2.15.2 Static binding capacity experiment of the EBA and RBA resin at four different pH

The static binding capacity experiment was repeated with 1 mL of the RBA and EBA resins at four different pH using the purified His_6 tagged scFv antibody shMFELL2cys in binding buffer (1 M NaCl / 1x PBS / 10 mM Imidazole). Four times 1 mL of settled EBA and RBA resins were aliquoted in the 1.5 mL Falcon tubes. Preparation of the settled resins, washing and equilibration of resin with copper was performed according to the method detailed in 2.15. Protein was applied with 1 M Nacl / 1x PBS / 10 mM Imidazole buffer and the pH was adjusted to pH 7.0, 7.5, 8.0 and 8.5 using 3 M potassium phosphate dibasic and 3 M potassium phosphate monobasic buffers. The final total volume was 100 mL into each flask at scFv antibody concentration of 0.38 mg/mL. All eight flasks were placed inside the shaking incubator at 25°C at 150 RPM for a minimum of 20 h. Resin binding capacity was measured by measuring the optical density at wavelength 280 nm in a spectrophotometer. The results of this experiment are discussed in detail in Chapter 4.

2.2.16 Dynamic binding capacity experiment of the RBA and EBA resins

The dynamic binding capacity (DBC) experiment of the RBA and EBA resins was performed using 1 mL settled EBA and RBA resins packed into 1 mL FliQ column (Table 2.1) in downflow. One millilitre settled resin was measured in a 1.5 mL

Eppendorf tube. The column was packed manually using the Watson-Marlow 120U pump (Table 2.2) and small diameter tubing connected to the inlet and outlet adapter of the FliQ column. The resin was added into the column using a Pasteur pipette and the pressure gauge was connected in line to measure the pressure during the packing procedure. After adding 1 mL of the resin inside the column, the top adapter of the column was pushed down to remove the liquid from above the resin and the column was rinsed with 20 mL of dH_2O. The column was stored in 20% (v/v) ethanol and in the fridge. Before use, the column was connected to the AKTA Prime and was cleaned with 0.5 M NaOH at 2 mL/min. The storage solution 20% (v/v) ethanol was removed from the column using dH_2O at 2 mL/min flow rate before performing the DBC experiment. Steps including the application of 100 mM copper sulphate, removal of unbound copper and equilibration of the column were performed while the column was connected to the AKTA Prime at 2 mL/min. The column was equilibrated using 0.5 M NaCl / 0.5x PBS and 10 mM imidazole at pH 8.29 at 2 mL/min flow rate. The His_6 tagged scFv antibody shMFELL2cys purified from fermentation runs was used to perform the DBC experiment on the EBA and RBA resins. An antibody concentration of 0.1 mg/mL was applied in 1 M NaCl / 1x PBS / 10 mM imidazole buffer. The application of the antibody was performed at a flow velocity of 60 cm/h (equivalent to a flow rate of 0.3 mL/min for the 1 mL FliQ column). The calculated residence time for 1 mL FliQ column at 60 cm/ h flow velocity was 3.5 min. The 100 % breakthrough curve was generated without connecting the column to the AKTA Prime and by applying the antibody at a 0.3 mL/min flow rate and until a stable UV 280 reading in mAu was recorded. DBC of the 1 mL resin was calculated at 10% breakthrough. Once the DBC was measured, the column was rinsed with 50 mL wash buffer 0.5 M NaCl / 0.5 M PBS and the bound scFv antibody was eluted using 0.5 M NaCl / 0.5 M PBS / 200 mM imidazole concentration at 2 mL/min flow rate. The column was rinsed with dH_2O at 2 mL/min flow rate to remove the elution buffer. The column was regenerated by applying 50 mM EDTA solution to remove copper first and washing with dH_2O at 2 mL/min flow rate. The column was cleaned with 0.5 M NaOH at 2 mL/min flow rate and incubated for a minimum of 2 h before storing in the 20% (v/v) ethanol. The DBC chromatograms were saved in the AKTA Prime software downloaded to the control computer. The results of this experiment are discussed in detail in Chapter 4.

2.2.16.1 Dynamic binding capacity experiment of the EBA and RBA resins in the presence of 20% (v/v) glycerol

The DBC experiment of the 1 mL settled EBA and RBA resin was performed using the purified His_6 tagged scFv antibody shMFELL2cys in a binding buffer (1 M NaCl / 1x PBS / 10 mM imidazole) and supplemented with 20% (v/v) glycerol. The 1 mL EBA and RBA columns packed in Section 2.16 were used to perform the DBC experiment. The scFv antibody concentration applied to the RBA and EBA columns was 0.12 mg/mL at 0.3 mL/min flow rate. The 100% breakthrough curve was generated without connecting the column to the AKTA Prime and by applying the antibody at 0.3 mL/min and generating a stable UV 280 reading in mAu. DBC of the 1 mL resin with 20% glycerol was calculated at 10% breakthrough. The DBC chromatograms were saved in the AKTA Prime software downloaded to the control computer. The results of this experiment are discussed in detail in Chapter 4.

2.2.17 EBA and RBA resins CIP experiments with 0.5 M NaOH

To investigate the effect of the cleaning agent on the binding capacity of the RBA and EBA resins, the DBC of the resins was measured before and after 10 CIP cycles using 0.5 M NaOH solution. By measuring the DBC before and after 10 CIP cycles using 0.5 M NaOH stability of the IDA chelating agent to agarose resin was investigated. The 1 mL FliQ column was packed with the RBA and EBA resins as described in Method 2.16 and used to perform the CIP cycle experiments. The 1 mL EBA and RBA columns were connected to the AKTA Prime system and 10 consecutive CIP cycles were performed to clean the resins. The chromatograms were stored in the control computer connected to the AKTA Prime system. The columns were rinsed with dH_2O first to remove the 20% (v/v) ethanol and cleaned with 10 CV 0.5 M NaOH at room temperature and incubated for a minimum of 2 h. The application of dH_2O, 0.5 M NaOH and 20% (v/v) ethanol was performed at 2 mL/min flow rate. After 2 h incubation, the 0.5 M NaOH was removed by applying 10 CV dH_2O at 2 mL/min flow rate and the column was stored in 20% (v/v) ethanol. To measure the effect of the cleaning agent (0.5 M NaOH) on the binding capacity of the resins, a DBC experiment of the EBA and RBA resins was performed before

and after the CIP cycle experiments (Method 2.16). The results of this experiment are discussed in detail in Chapter 4.

2.2.18 EBA and RBA resin screening experiment

The EBA and RBA resin screening experiments were performed to investigate a suitable binding condition for the His_6 tagged scFv antibody shMFELL2cys to the RBA and EBA resin. The high cell density *P. pastoris* fermentation run was performed (Method 2.2.4) to generate a culture supernatant containing the secreted His_6 tagged scFv antibody. The end of fermentation culture was centrifuged first in 250 mL centrifuge buckets at 3000 RPM for 45 min in a Sorvall ultracentrifuge (Table 2.2) to separate the cells and supernatant. The centrifuged supernatant was then filtered using 0.2 µm bottle top filters, aliquoted in 50 mL Falcon tubes and stored in a -80°C freezer. The screening experiments were performed using the 1 mL settled RBA and EBA resin volume. Preparation of the RBA and EBA resins including 1 mL settled resin volume measurement, transfer of the resins inside the flask, removal of the storage solution, application of copper sulphate and equilibration of resins were performed according to the static binding capacity experiment, Section 2.15. The 50 mL freeze-thaw fermenter culture supernatant was mixed with a binding buffer before being applied to the 1 mL EBA and RBA resins inside the 250 mL flask. The EBA and RBA resin screening experiments were performed at three different salt concentrations of (i) 0.5 M NaCl, (ii) 0.75 M NaCl and (iii) 1 M NaCl and at three pH values of pH 7.5, 8.0 and 8.5 at each salt concentration inside a 250 mL conical flask. The RBA and EBA screening experiments were performed using 50 mL and 100 mL freeze-thaw fermenter supernatants mixed with the binding buffer, respectively. The pH was adjusted using 3 M potassium phosphate monobasic and 3 M potassium phosphate dibasic buffers. The flasks were placed inside the incubator at 25°C at 120 RPM for a minimum of 24 h. After 24 h incubation, the supernatant samples were taken to measure the total protein concentration. The total protein concentration of the supernatant samples was measured using Bradford assay and the results were analysed to find the suitable binding condition for the His_6 tagged scFv antibody

shMFELL2cys to the EBA and RBA resins. The results of this experiment are discussed in detail in Chapter 4.

2.2.19 Effect of residence time on primary purification of the His_6 tagged scFv antibody using RBA and EBA chromatography

The high cell density *P. pastoris* fermentation run was performed to investigate the His_6 tagged antibody-mediated purification at three separate residence times on three separate RBA columns. The chelating agent copper sulphate was used in all three columns. The high cell density *P. pastoris* fermentation run was performed (Method 2.2.4) to express the His_6 tagged scFv antibody shMFELL2cys into the fermenter culture. At the end of the fermentation run, 1 L of each fermenter culture was applied to three separate RBA columns. The 1 L end of fermentation culture was applied (mixed with 1 L buffer, 2 M NaCl, 2 x PBS and 20 mm imidazole) at (i) 200 mL/min on the first 125 mL RBA column, (ii) 40 mL/min on the second 125 mL RBA column and (iii) 15 mL /min on the third 250 mL RBA column. The residence times of 40 s at 200 mL/min, 3.5 min at 40 mL/min and 18 min at 15 mL/min were calculated. After applying the 1 L fermenter culture, all three columns were washed with 10 L of wash buffer 0.5 M NaCl / 0.5x PBS / 10 mM imidazole at 200 mL/min flow rate. The His_6 tagged antibody bound on the RBA column was eluted using elution buffer 0.5 M NaCl / 0.5x PBS / 200 mM imidazole at 200 mL/min in 50 mL fractions in Falcon tubes. The scFv antibody concentration in fractions was measured using a spectrophotometer at OD_{280} nm and fractions with the highest OD_{280} values were pooled together and sterile filtered using 0.2 µm filter. The eluted proteins from three RBA columns were pooled separately and concentrated using a Labscale TFF system (Method 2.2.11). The concentrated scFv antibody was further purified using the 500 mL Superdex 75 size exclusion column (Table 2.2). The flow-through samples were taken during the culture application and wash steps on RBA columns to measure the presence of the His_6 tagged scFv antibody in the flow samples. The samples were analysed using SDS-PAGE and the presence of a His_6 tagged antibody on the samples was confirmed by Western Blot analysis. The pooled scFv antibody concentration was measured using the Agilent

2100 Bioanalyzer (Method 2.2.14.8). The results of this experiment are discussed in Chapter 4.

2.2.20 Cost base analysis of the EBA and RBA chromatography processes using BioSolve software

The cost base analysis of the His_6 tagged scFv antibody production in the high cell density *P. pastoris* fermentation and purified using EBA or RBA chromatography was performed using BioSolve software developed by Biopharm Services Ltd. Complete new yeast typical RBA and EBA worksheets with unit operations were generated using a microbial worksheet template. The total final working volume of the 8 L production fermenter size was added to both RBA and EBA worksheets. For primary purification using RBA chromatography, a 250 mL column size was used and for EBA chromatography, a STREAMLINE 50 column was selected with 300 mL settled bed resin volume. The costs of columns, resins, chemicals and materials were added into the BioSolve software if not available on the worksheet template. The process flow and operational time, operator numbers and operation flow rates of the RBA and EBA column were also added to the worksheet to perform a cost base analysis of clinical-grade material manufacturing in a clean room environment. The experimental data of the total concentration of the His_6 tagged scFv antibody shMFELL2cys expressed in the 8 L fermenter and captured using the RBA and EBA primary recovery steps were also added into the software. Antibody recovery from subsequent purification steps; TFF, SEC and sterile filtration were also added into the software. In total, seven unit operations were added for both the RBA and EBA worksheets for a cost comparison with several operation steps per unit operation. A cost base comparison between the RBA and EBA primary purification processes was performed based on the primary recovery of the target scFv antibody purified using the RBA and EBA chromatography process flowsheets. The cost of goods/batch (CoG/batch) and cost of goods/gram (CoG/g) of the scFv antibody production using the EBA and RBA primary chromatography steps were generated. Analysis of the data generated using BioSolve software is discussed in detail in Chapter 5.

Chapter 3 Evaluation and comparison of primary capture of the His_6 tagged scFv antibody between radial bed and expanded bed chromatography

3.1 Abstract

The work in this chapter describes the primary capture of the secreted recombinant His_6 tagged scFv antibody shMFELL2cys using the RBA and EBA chromatography and manufactured in a high cell density *P. pastoris* fermentation. The investigation was performed to evaluate and compare primary capture of scFv antibody using EBA and RBA chromatography. RBA and EBA chromatography each combine three traditional purification steps in one step: (i) centrifugation or depth filtration, (ii) concentration and (iii) affinity purification of target proteins. A commercially available immobilised metal ion affinity chromatography (IMAC) resin with IDA chelating ligand was used in both the RBA and EBA column formats (Table 2.1). A suitable binding condition for the His_6 tagged scFv antibody shMFELL2cys to the IDA chelating agent is identified in Chapter 4. Quantitative and qualitative analysis of the purified scFv antibody was performed using analytical SEC, ELISA and HCP ELISA.

3.2 Introduction

In RBA chromatography, the affinity resin was packed between the inlet and outlet frits and the application culture passed through the column in a radial direction. RBA columns are cylindrical and the flow direction is from the outer layer to the centre of the column. A small-scale, 250 mL RBA column with 40 µm frits was used for the experiment, which was a small segmented piece of the pilot-scale radial bed column and had the same hydrodynamics as a full-scale radial bed column. The particle size range of the resin used was quoted as being 300–500 µm. IDA Chelating Cellthru™ BigBead packed in the RBA column allowed cells and cell debris to pass through the column while capturing the secreted His_6 tagged scFv antibody shMFELL2cys.

In EBA chromatography, STREAMLINE™ chelating resin was used with a particle size range of 100–300 µm. The particles have an inert quartz core and an approximate mean particle density of 1.2 g/mL. This was used for affinity purification of the secreted His_6 tagged scFv antibody shMFELL2cys. High cell density *P. pastoris* culture diluted with binding buffer was applied to the STREAMLINE™ 50 EBA column in expanded bed mode to affinity purify the His_6 tagged scFv antibody. A stable control level of expansion of the expanded bed was maintained. Particle size distributions of both the application culture and the flow-through samples were performed.

Antibody purified using either EBA or RBA was further purified by concentrating it first in a Labscale TFF system followed by SEC and 0.2 µm final sterile filtration. The quantity of the His_6 tagged scFv antibody shMFELL2cys was measured with an Agilent 2100 Bioanalyzer and a spectrophotometer at UV 280 nm wavelength (Chapter 2).

The quality of the His_6 tagged scFv antibody shMFELL2cys was determined by analysing the *P. pastoris* HCP concentration, CEA ELISA and total protein concentration using Bio-Rad DC Bradford assay (Chapter 2).

EBA and RBA chromatography purification process materials, methods, operation time and scFv antibody recovery and final concentration data were subsequently used in Chapter 5 to perform a cost base analysis between EBA and RBA chromatography.

The main objectives of this chapter are listed below.

Objectives:

- Production of a His_6 tagged scFv antibody in a high cell density *P. pastoris* fermentation.
- Primary purification of the secreted His_6 tagged scFv antibody through RBA and EBA chromatography processes using IMAC resin.
- Qualitative analysis of the purified His_6 tagged scFv antibody to evaluate the quality of the antibody purified using EBA and RBA chromatography.

- Quantitative analysis of the purified His_6 tagged scFv antibody to perform cost base analysis of the EBA and RBA processes using BioSolve software (Chapter 5).

3.3 High cell density *P. pastoris* fermentation runs

Four fermentation runs were successfully performed with a chemically defined medium for a maximum of 72 h. Figures 3.3 and 3.4 show the *P. pastoris* fermentation growth profile of OD_{600} and WCW. During all four fermentation runs, OD_{600} nm was measured between 360 and 390. For two of the fermentation runs, WCW was measured between 298 and 302 g/L. After 23 to 24 h of inoculation, the dissolved oxygen concentration inside the fermenter was increased to around 60% as 6% (v/v) of glycerol was completely consumed by cells during the batch phase (Stage 1 of Figure 3.2). In Stage 2, a total of 460 mL of 50% glycerol was added during the glycerol limited fed batch stage over 4 h and 100% methanol limited feed was started at 20 mL/hr and increased to 45 mL/hr after 6 h (Stage 2 of Figure 3.2). A total of 1.6 L of 100% methanol was added at around 45 mL/hr flow rate during the methanol induction stage (Stage 3 of Figure 3.2). Small scale test expression study of the WCB in shake flask was successfully performed before commencing the fermentation runs (Appendix 3). The secreted His_6 tagged scFv antibody shMFELL2cys was expressed successfully in all four fermentation runs (Chapter 2, Section 2.2.4). Previously published parameters were used to run the fermenter [37]. The concentration of scFv antibody production was measured using the Agilent protein 80 kit (Chapter 2, Section 2.2.14) at 600–680 mg/L. The 27 kDa protein band can be seen on the SDS-PAGE gel analysis of different time points of fermenter samples in Figure 3.8 and 3.15. Figure 3.1 shows the increase in the cell density during the *P. pastoris* fermentation run. Figure 3.1 a) is a picture of the fermenter at the start of the fermentation run and Figure 3.1 b) is a picture of the high cell density culture of *P. pastoris* at the end of the fermentation run. Figure 3.2 shows the temperature, pH, dissolved oxygen and agitation profile during a typical 72 h fermentation run of *P. pastoris.* Figure 3.2 represent the temperature, pH, dissolved oxygen and agitation profile for all four fermentation runs. Temperature was successfully maintained at 30°C (+/- 1°C) for a total of 72

h. pH was maintained at pH 5 (+/- 0.1) for the first 24 h till the dissolved oxygen spike was seen inside the fermenter (Figure 3.2) and then increased to pH 6.5 during the scFv antibody expression stage and maintained at 6.5 (+/- 0.1) using orthophosphoric acid and ammonium sulphate solution in pH cascade mode. A Hamilton optical dissolved oxygen probe (Chapter 2, Table 2.1) was used to measure the dissolved oxygen concentration during the fermentation runs. The saturated dissolved oxygen concentration was maintained at 30% inside the fermenter by increasing the stirrer speed to 1000 RPM from 500 RPM starting speed and by adding sterile air at 10 L/min and pure oxygen using a mass flow controller in cascade mode.

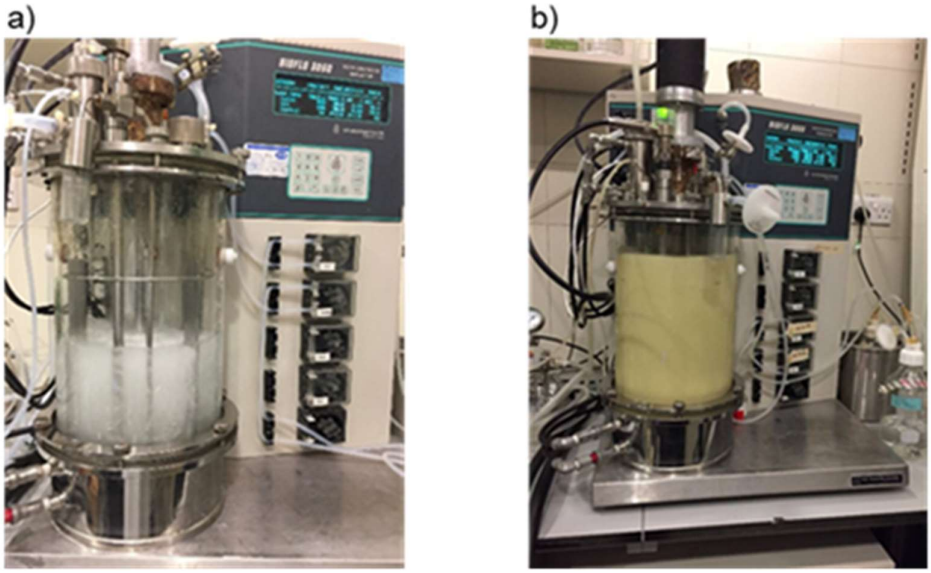

Figure 3.1 High cell density *P. pastoris* fermentation culture pictures.
Comparison of the increase in the cell density during the *P. pastoris* fermentation run. Figure 3.1 a) is a picture of the fermenter at the start of the fermentation run and Figure 3.1 b) is a picture of the high cell density culture of *P. pastoris* at the end of the fermentation run.

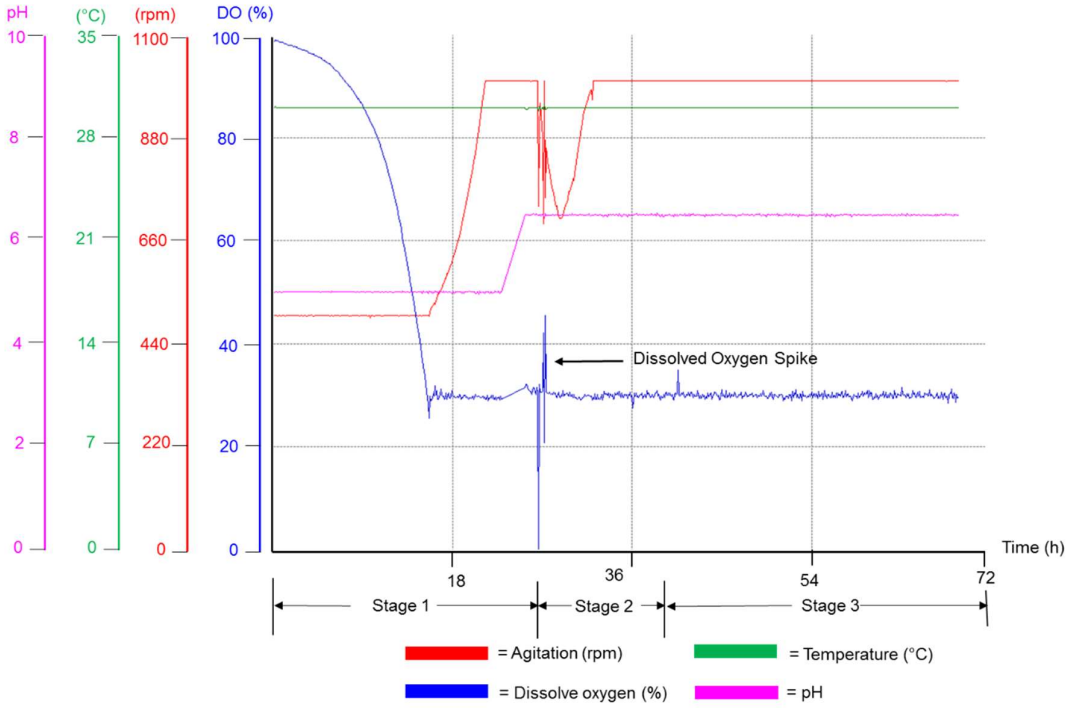

Figure 3.2 Dissolved oxygen, agitation, temperature and pH profile during the typical 72 h fermentation run in a New Brunswick benchtop glass fermenter.

Figure 3.2 shows the typical profile of the dissolved oxygen, agitation, temperature and pH during the 72 h of the high cell density *P. pastoris* fermentation run. Stage 1 is a glycerol depletion phase, Stage 2 is a limited glycerol and methanol feed and Stage 3 is a 100% methanol feed phase.

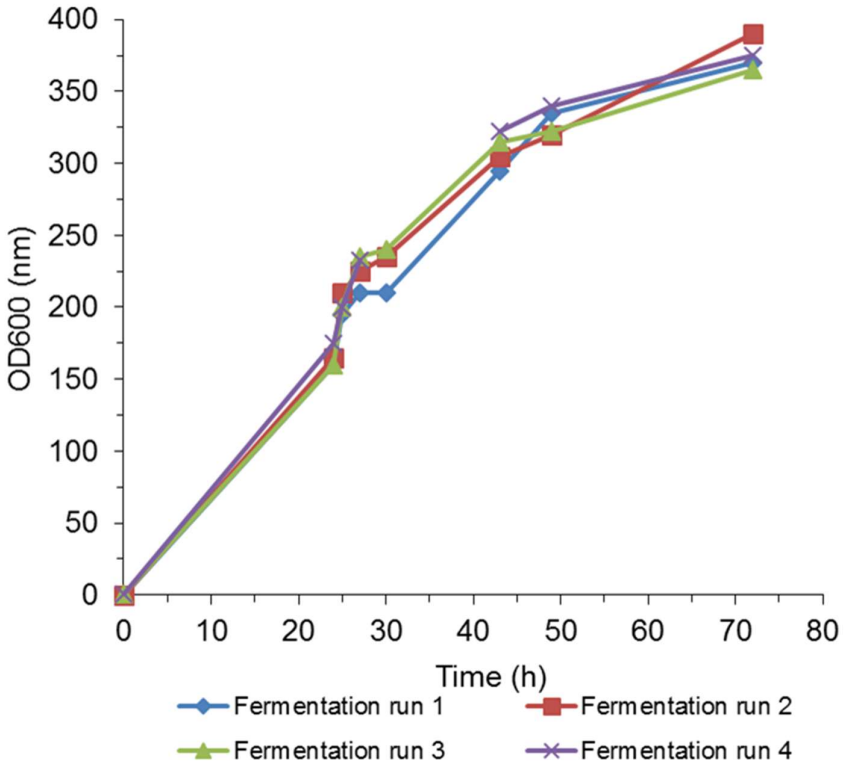

Figure 3.3 Growth profile of four fermentation runs.
Data shows a high level of reproducibility between the four fermentation batches. Final OD_{600} of 390 achieved after 72 h of high cell density *P. pastoris* fermentation runs.

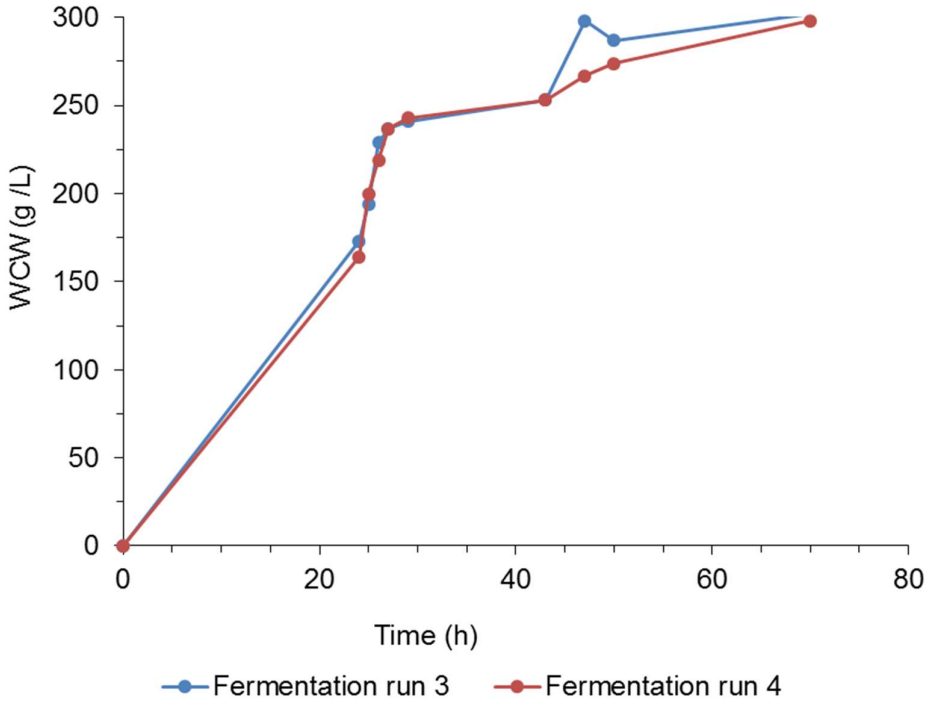

Figure 3.4 *P. pastoris* X-33 strain WCW profile in fermenter.
Data shows a high level of reproducibility between two fermentation batches. Final WCW of 300 g/L recorded after 72 h of total *P. pastoris* fermentation run.

3.4 Primary capture of the His_6 tagged scFv antibody using RBA affinity chromatography

High cell density *P. pastoris* fermentation culture was diluted (1:1) with mixing buffer 2 M NaCl /2x PBS/ 20 mM imidazole and successfully applied to a 250 mL RBA column at 70 mL/min using a Watson-Marlow peristaltic pump (equivalent to volumetric flow rate of 93 cm/h). Mixing buffer was selected after performing small scale buffer selection experiments using 1mL of RBA resin (Chapter4). Dilution of the high cell density fermenter culture was required to increase the pH of the culture media and to reduce the viscosity of the fermenter culture to allow the secreted His_6 tagged scFv antibody shMFELL2cys to bind to IMAC resin (Chapter 4, section 4.6). The radial flow of the liquid was verified by applying the copper sulphate solution on to the column at 70 mL/min. Small scale experiments were performed to select the buffer composition and operational flow rate (Chapter 4). Figure 3.5 illustrates the radial flow of metal ion copper when applied on to 250 mL RBA column at 70 mL/min. Small-scale experiments were performed to identify the suitable pH and salt concentration for maximum recovery of the secreted His_6 tagged scFv antibody shMFELL2cys from the fermenter culture, which is discussed in Chapter 4. A total of 1.7 g of the scFv antibody shMFELL2cys was captured using the RBA column (Section 3.12), which resulted in a 30% recovery.

Figure 3.6 shows the RBA column at the start of primary capture and the end of the primary capture step after applying the fermenter culture. Figure 3.8 a) shows the scFv antibody expression during fermentation. A single protein band at 27 kDa can also be seen at the radial bed pool, concentrated and final purified protein pool sample. Figure 3.8 b) Western Blot analysis of the fermenter and purified protein samples shows the availability of a His_6 tag on the scFv antibody.

Flow-through samples from the RBA column were taken during the primary culture application steps to analyse the binding efficiency of the His_6 tagged scFv antibody to the RBA column packed with IDA Chelating Cellthru™ BigBead. In Figure 3.9 a), a scFv antibody band is seen at 27 kDa in flow-through samples on the SDS-

PAGE after applying fermenter culture mix on to the RBA column. Antibody band thickness is seen increasing from 4.5 L to 16 L in the flow-through samples. Western Blot analysis of the flow-through samples confirmed that a His_6 tag was present on the scFv antibody and bands were seen at 27 kDa.

Analysis of the SDS-PAGE and Western Blot (Figure 3.9 a) and b)) confirmed that primary capture of the His_6 tagged scFv antibody using RBA column did not capture all of the antibody. SDS-PAGE and Western Blot results in Figures 3.10 a) and b), respectively, show that the His_6 tagged scFv antibody was leaching through the column from samples taken after 1 L of the wash buffer was applied to the column. Western Blot analysis of the wash samples confirmed that a His_6 tag was present on the scFv antibody and bands were seen at 27 kDa (Figure 3.10 b)). The amount of scFv antibody flowing through the RBA column was reduced during the washing step from the start to end as the scFv antibody band thickness decreased from 1 L wash to 9 L wash samples. These can be due to the reduction in the residual material contained in the column or scFv antibody leaching through the RBA column. Application of washing buffer supplemented with imidazole removed cells and cell debris from the RBA column and non-specifically bound HCP [108]. The RBA column washing step was performed at 100 mL/min to remove cells, cell debris and non-specifically bound protein from the column, resulting in a smaller amount of the His_6 tagged scFv antibody leaching through the column.

During the fermenter culture application on the column, *P. pastoris* cells accumulated at the bottom of the 250 mL RBA column frits. Extended washing in the reverse direction was applied to remove accumulated cells and cell debris. Accumulation of the cells and cell debris at the RBA column inlet frits can be seen in Figure 3.7 a). Figure 3.7 b) shows the extended and reverse direction washing of the RBA column performed to remove the accumulated cells and cell debris from the RBA column inlet frits. There was no pressure during operation of the RBA column while applying fermenter culture, washing the column and eluting the scFv antibody from the RBA column.

The captured protein was eluted using the elution buffer containing 200 mM imidazole at pH 9.5 in 50 mL fractions at 70 mL/min. The fractions with the highest OD_{280} values were pooled together and filtered through 0.2 µm filter. Figure 3.11 shows the graph of the scFv antibody elution profile. In total, 30 fractions were collected and fractions 5 to 24 were pooled together for further purification steps.

The captured protein pool was concentrated from 1000 mL to 100 mL in 1 h 50 min by manually maintaining transmembrane pressure at 10 psi. Once concentrated, the scFv antibody pH was adjusted to pH 7.50 using 3 M H_2KO_4P. The concentrated protein was further purified on a size exclusion Superdex75 500 mL column.

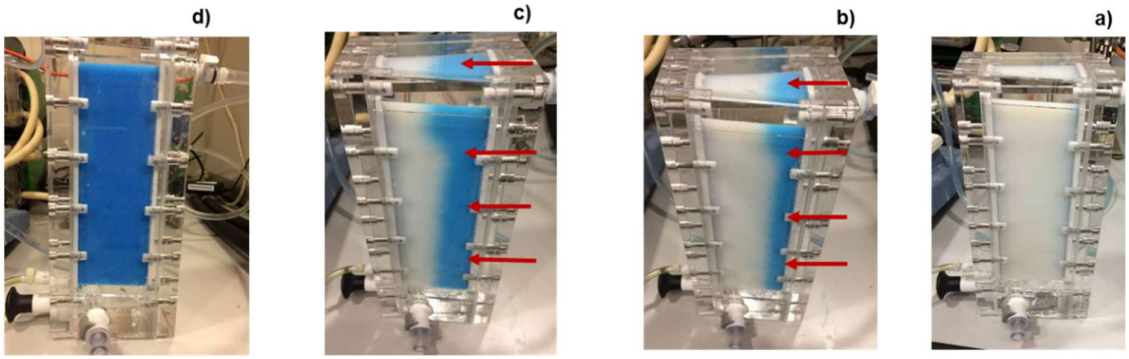

Figure 3.5 Represent the different images taken while loading the copper in 250 mL radial bed column from a) to b).

Radial flow on the 250 mL RBA column was verified by applying 100 mM copper sulphate buffer. In Figure 3.5 a), 250 mL RBA column was seen packed with IDA Chelating Cellthru™ BigBead resin, Figures b) and c) represent the radial flow of the copper on the column and Figure d) shows the column completely equilibrated with copper.

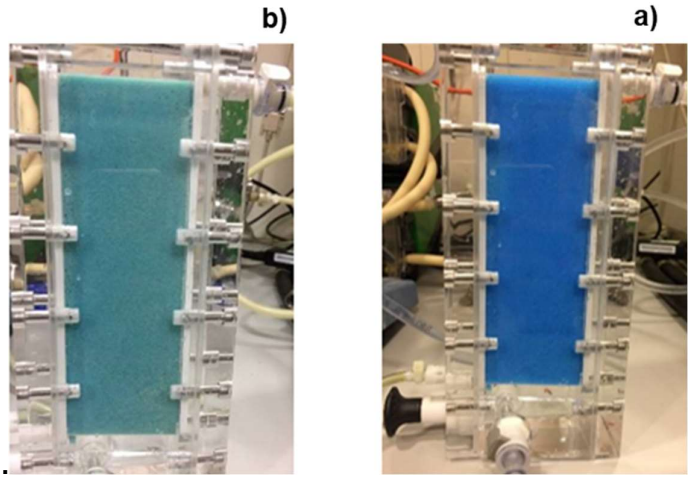

Figure 3.6 Represent the pictures of 250 mL RBA column equilibrated with copper a) and after application of total *P. pastoris* fermenter culture b).
Figure 3.6 shows a comparison of the 250 mL RBA column completely equilibrated with copper sulphate (a) and after application of *P. pastoris* fermenter culture (b).

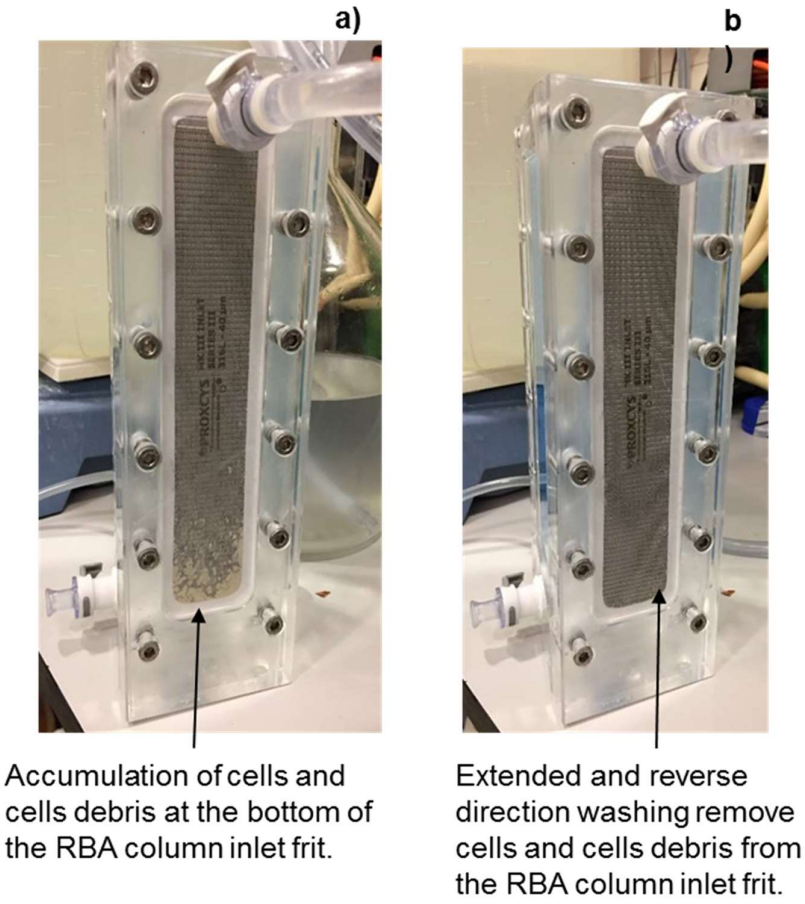

Figure 3.7 Picture of 250 mL RBA column inlet frits during washing step.

Comparison of the 250 mL RBA column inlet frit while washing the column with wash buffer. In Figure a), accumulation of the cells and cell debris can be seen at the bottom of the inlet frit of the 250 mL RBA column and Figure b) shows 250 mL RBA column inlet frits without cells and cell debris after extended and reverse direction washing.

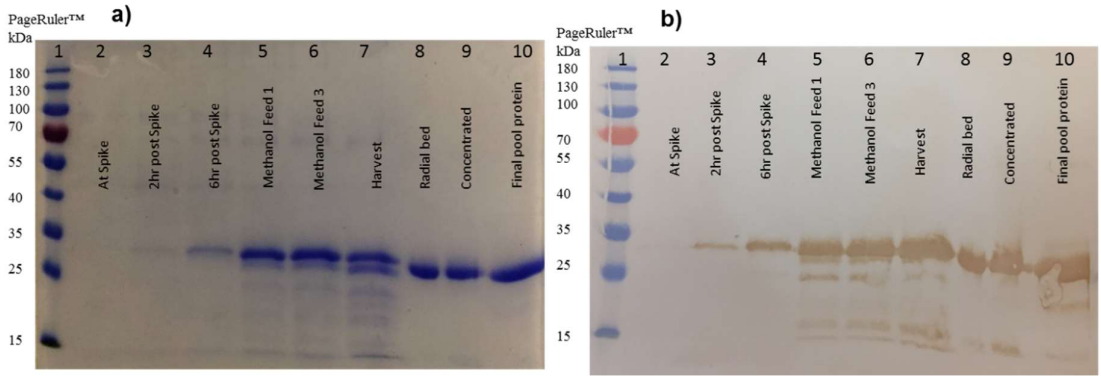

Figure 3.8 SDS-PAGE (a) and Western Blot (b) analysis of the samples taken during the fermentation run and capture of the His_6 scFv antibody using the RBA primary chromatography step.

Analysis of the His_6 tag scFv antibody expressed in the fermenter and captured through RBA chromatography was performed using SDS-PAGE (a) and Western Blot (b). The antibody band was seen at 27 kDa in the fermenter samples and RBA purified samples on the SDS-PAGE (a) and availability of the His_6 tagged on the antibody was confirmed with Western Blot (b) analysis. Sample details are described in the table below. Identical samples were run on SDS-PAGE (a) and on Western Blot (b).

Sample lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sample volume	10 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL	2 µL	2 µL	2 µL
Sample detail	Molecular weight marker	Fermenter sample at the glycerol spike	Fermenter sample 2 h post glycerol spike	Fermenter sample 6 h post glycerol spike	Fermenter sample 24 h post glycerol spike (Methanol feed 1)	Fermenter sample 30 h post glycerol spike (Methanol feed 2)	Fermenter harvest sample	scFv antibody eluted from RBA column	Concentrated scFv antibody from RBA capture	Final pooled purified scFv antibody from RBA capture

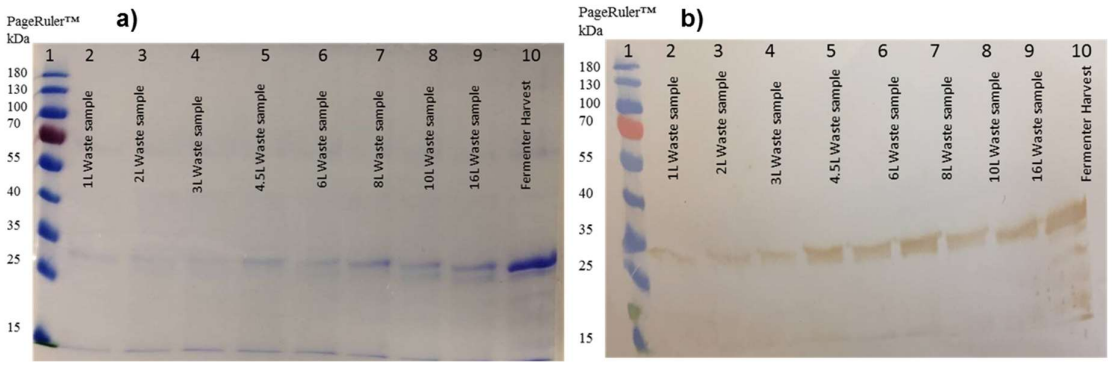

Figure 3.9 SDS-PAGE (a) and Western Blot (b) analysis of the samples taken during direct capture of the His_6 scFv antibody from the fermenter culture through the 250 mL RBA column.

Analysis of the His_6 tag scFv antibody in the RBA harvest flow-through samples was performed using SDS-PAGE (a) and Western Blot (b). The antibody band was seen at 27 kDa in the flow-through samples on the SDS-PAGE (a) and availability of a His_6 tagged on the antibody was confirmed with Western Blot (b) analysis. Antibody band thickness was seen increasing from 4.5 L to 16 L in the flow-through samples on the SDS-PAGE. Sample details are described in the table below. Identical samples were run on SDS-PAGE (a) and on Western Blot (b).

Sample lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sample volume	10 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL
Sample detail	Molecular weight marker	1 L flow-through sample from RBA column	2 L flow-through sample from RBA column	3 L flow-through sample from RBA column	4.5 L flow-through sample from RBA column	6 L flow-through sample from RBA column	8 L flow-through sample from RBA column	10 L flow-through sample from RBA column	16 L flow-through sample from RBA column	Fermenter harvest sample

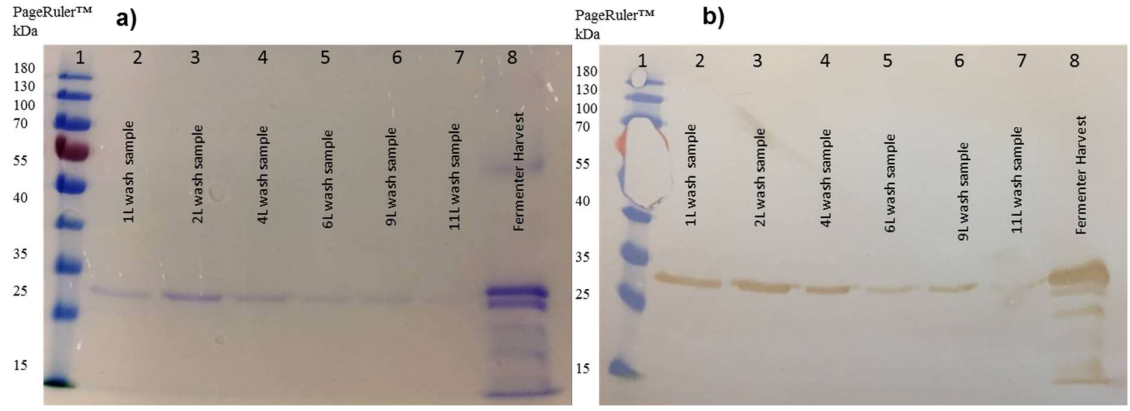

Figure 3.10 SDS-PAGE (a) and Western Blot (b) analysis of the samples taken during the washing step of the 250 mL RBA column.

Analysis of the His_6 tag scFv antibody in the RBA wash samples was performed using SDS-PAGE (a) and Western Blot (b). Antibody band was seen at 27 kDa in the wash samples on the SDS-PAGE (a) and availability of a His_6 tagged on the antibody was confirmed with Western Blot (b) analysis. Antibody band thickness decreased from 1 L to 9 L in the wash samples on the SDS-PAGE. Sample details are described in the table below. Identical samples were run on SDS-PAGE (a) and on Western Blot (b).

Sample lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Sample volume	10 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL
Sample detail	Molecular weight marker	1 L wash sample of the RBA column	2 L wash sample of the RBA column	4 L wash sample of the RBA column	6 L wash sample of the RBA column	9 L wash sample of the RBA column	11 L wash sample of the RBA column	Fermenter harvest sample

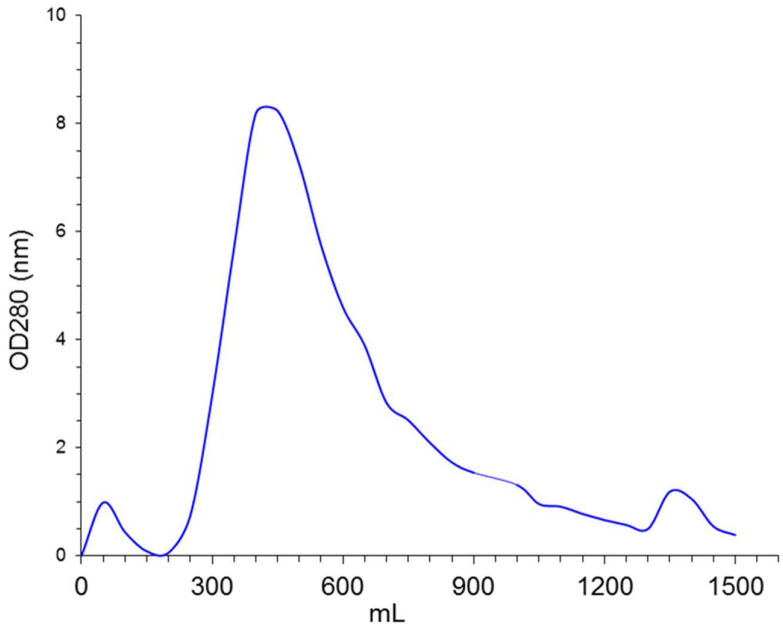

Figure 3.11 RBA eluted protein fractions profile.
Figure 3.11 shows the elution profile of the scFv antibody eluted from the 250 mL RBA column. The scFv fractions from 200 mL to 1200 mL were pooled together and further purified.

3.5 Primary capture of the His_6 tagged scFv antibody using EBA affinity chromatography

High cell density *P. pastoris* fermentation culture was diluted (1:1) with mixing buffer 2 M NaCl / 2x PBS / 20 mM imidazole and successfully applied to the first generation STREAMLINE 50 EBA column packed with STREAMLINE™ chelating resin. Small-scale experiments were performed to identify the most suitable pH and salt concentration for maximum recovery of the secreted His_6 tagged scFv antibody shMFELL2cys from the fermenter culture. This is discussed in Chapter 4. Mixing buffer was selected after performing small scale buffer selection experiments with 1mL of EBA resin (Chapter4). Dilution of the high cell density fermenter culture was required to increase the pH of the culture media and to reduce the viscosity of the fermenter culture to allow the secreted His_6 tagged scFv antibody shMFELL2cys to bind to IMAC resin (Chapter 4, section 4.6). The residual dead fluid volume in the pipework was replaced with dH_2O during the EBA column setup step. The equilibration of the column and culture application was performed in an upward flow direction using a Watson-Marlow peristaltic pump and the column adapter was placed at the maximum height. Stable expansion of the EBA column was maintained by manually adjusting the flow velocity between 214 cm/h and 184 cm/h. In order to maintain the steady state expansion of the EBA column flow velocity were maintained manually by increasing and decreasing the pump flow rate and by measuring the flow rate using measuring cylinder and balance (Figure 3.12). EBA column expansion was maintained between 2.5 and 3 settled bed height during the capture step. A total of 2.3 g of the scFv antibody shMFELL2cys was captured using EBA column (Section 3.12), which resulted in a 47% recovery.

The EBA column STREAMLINE™ 50 setup during the operation is shown in Figure 3.12. Figure 3.13 shows pictures of the STREAMLINE™ chelating resin expansion during the pre-use cleaning (Figure 3.13 a)), application of copper sulphate (Figure 3.13 b)) and equilibration (Figure 3.13 c)) steps at 70 mL/min in the upward flow direction. Figure 3.14 shows the EBA column pictures at the start of the fermenter culture application (Figure 3.14 d)), at the end of fermenter culture application

(Figure 3.14 e)), during the washing step (Figures 3.14 f) and g)) and protein elution step (Figure 3.14 e)).

Figure 3.15 a) shows the scFv antibody expression during fermentation. A single protein band at 27 kDa can also be seen at the EBA pooled protein, concentrated and final purified protein pool sample. Figure 3.15 b) Western Blot analysis of the fermenter and purified protein samples shows the availability of a His_6 tag on the scFv antibody.

Flow-through samples from the EBA column were taken during the primary culture application step to analyse the binding efficiency of the His_6 tagged scFv antibody to the EBA column. Analysis of the SDS-PAGE gel and Western Blot Figures 3.16 a) and b), respectively, confirm that no scFv antibody bands were seen in the flow-through samples. Therefore, a high binding capacity of the His_6 tagged scFv antibody was achieved.

The EBA column was washed with 0.5 M Nacl / 0.5x PBS / 10 mM imidazole buffer in expanded bed mode starting at 60 mL/min (flow velocity of 184 cm/h) to remove cells, cell debris and HCP. Application of washing buffer supplemented with imidazole removed cells and cell debris from the EBA column and non-specifically bound HCP. During the washing step, stable expansion of the expanded bed was maintained by maintaining the bed expansion between 2.5 and 3 settled bed height. Flow rate was increased to 84 mL/min at the end of the washing step. In total, 15 L of washing buffer was applied in expanded bed mode.

SDS-PAGE and Western Blot results in Figures 3.17 a) and b), respectively, show that a very small amount of the His_6 tagged scFv antibody was leaching through the column from flow-through samples taken after 1.5 L to 10 L of the wash buffer was applied to the column. Western Blot analysis of the wash samples confirmed that a His_6 tag was present on the scFv antibody and bands were seen at 27 kDa (Figure 3.17 b)). The amount of scFv antibody leaching through the EBA column was reduced during the washing step from the start to end as the scFv antibody band thickness decreased from 1.5 L wash to 10 L wash samples. A particle size distribution study was performed on the flow-through samples from the EBA column and the results are discussed in detail in Section 3.15.

Captured protein was eluted using the elution buffer containing 200 mm imidazole at pH 9.6 in 50 mL fractions at 70 mL/min in downflow. The fractions with the highest OD_{280} values were pooled together and filtered through 0.2 µm filter. Figure 3.18 shows the graph of the scFv antibody elution profile. In total, 30 fractions were collected and fractions 10 to 24 were pooled together for further purification steps.

The captured protein pool was concentrated from 800 mL to 150 mL in 1 h 40 min by manually maintaining the transmembrane pressure at 10 psi. The concentrated scFv antibody pH was changed to pH 7.50 using 3 M H_2KO_4P. The concentrated protein was further purified on a size exclusion Superdex75 500 mL column.

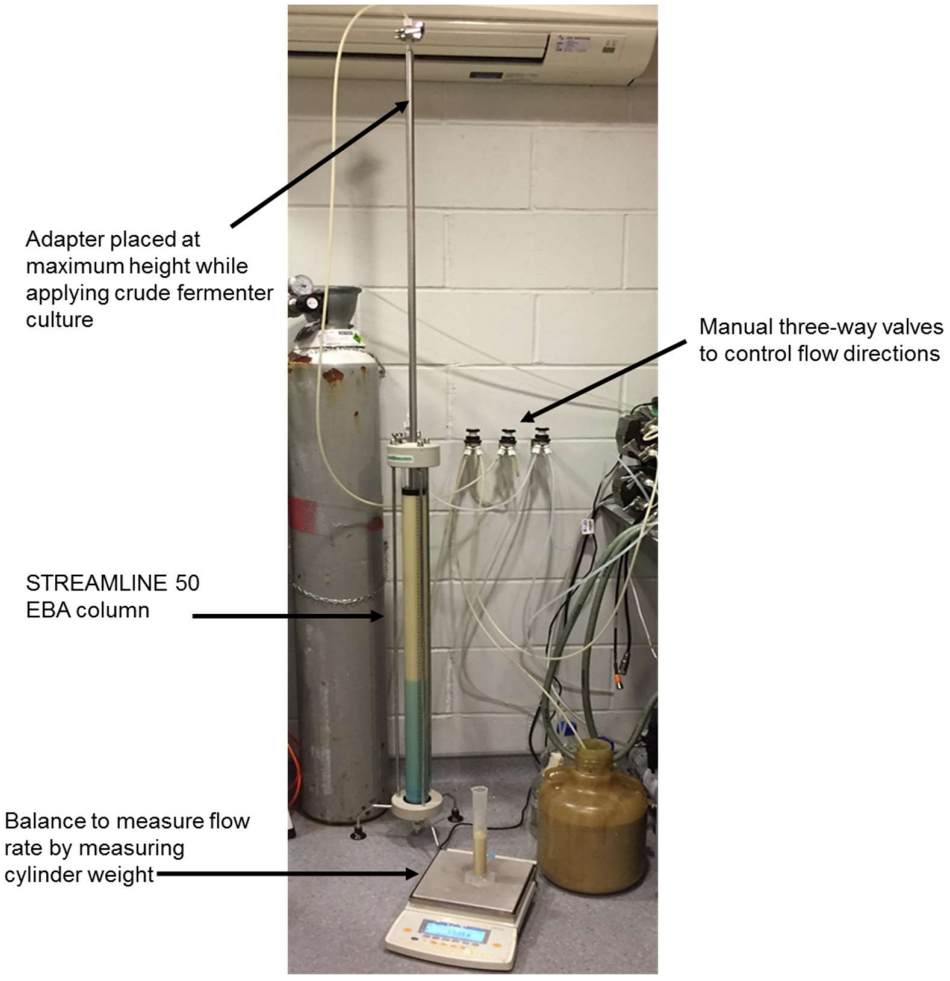

Figure 3.12 EBA column STREAMLINE™ 50 setup during operation.
Figure 3.12 is a picture of the STREAMLINE™ 50 EBA column setup during the operation.

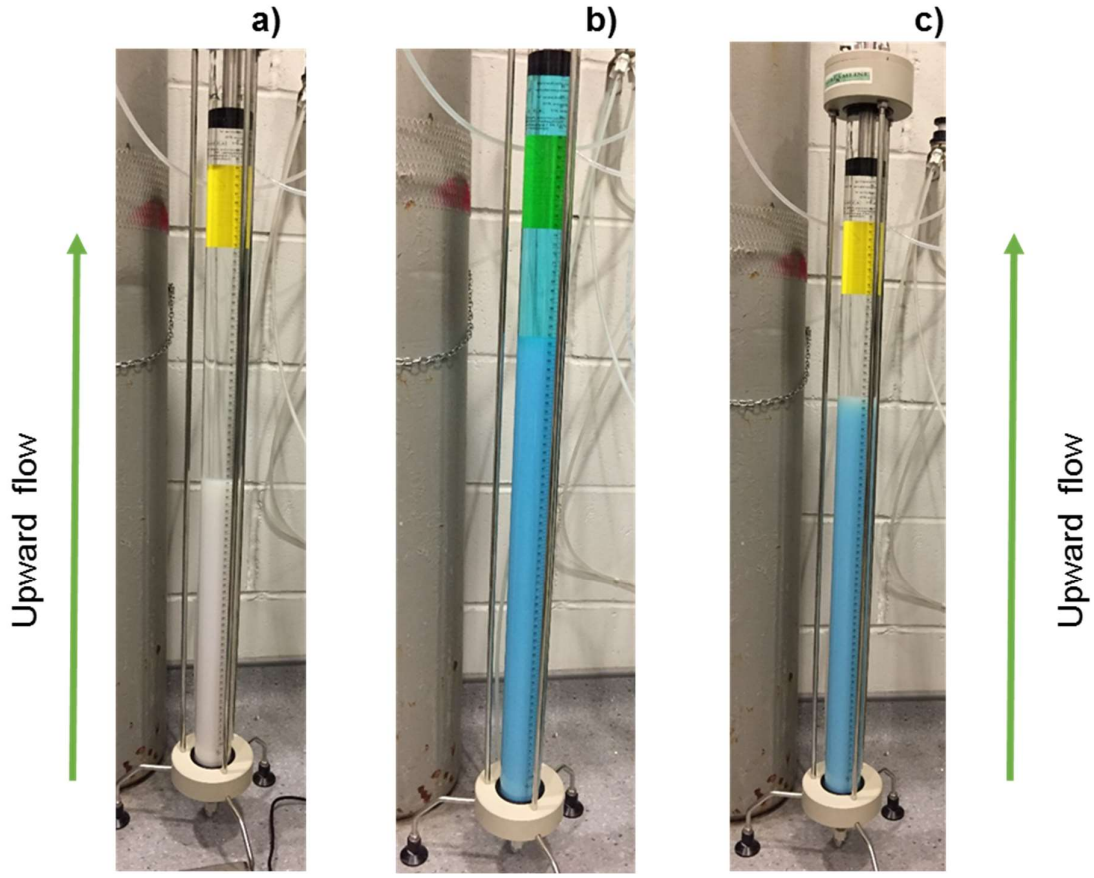

Figure 3.13 EBA column operation steps in upward direction.

Figure 3.13 shows pictures of the STREAMLINE 50 EBA column during the pre-use cleaning step (a), application of copper sulphate step (b) and application of equilibration buffer step (c).

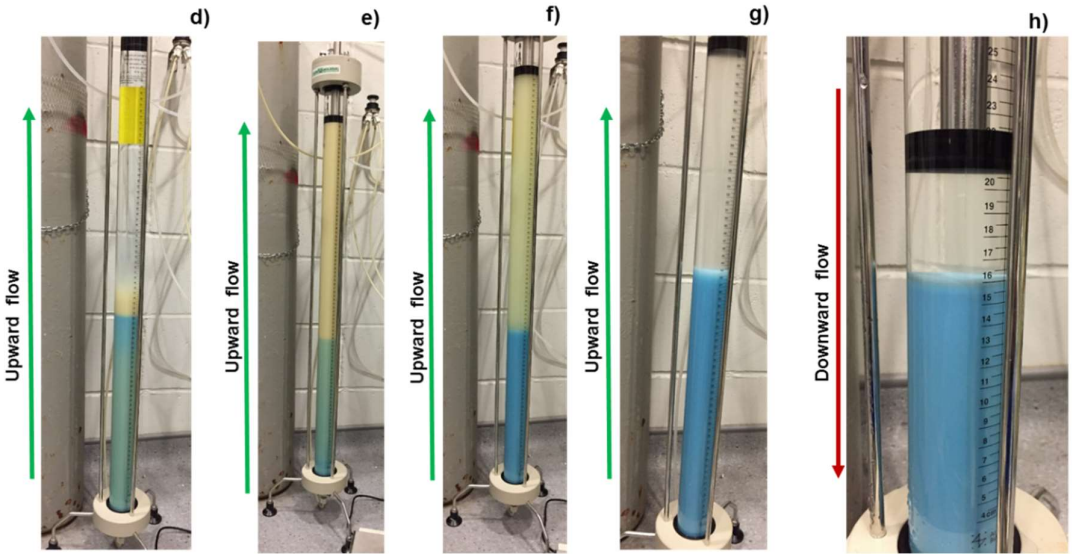

Figure 3.14 EBA column operation steps in an upward and downward direction.

Figure 3.14 shows pictures of the STREAMLINE 50 EBA column during the application of fermenter culture (d), at the end of fermenter culture application (e), during the column washing step (f), at the end of column washing step (g) and the scFv antibody elution in downflow (h).

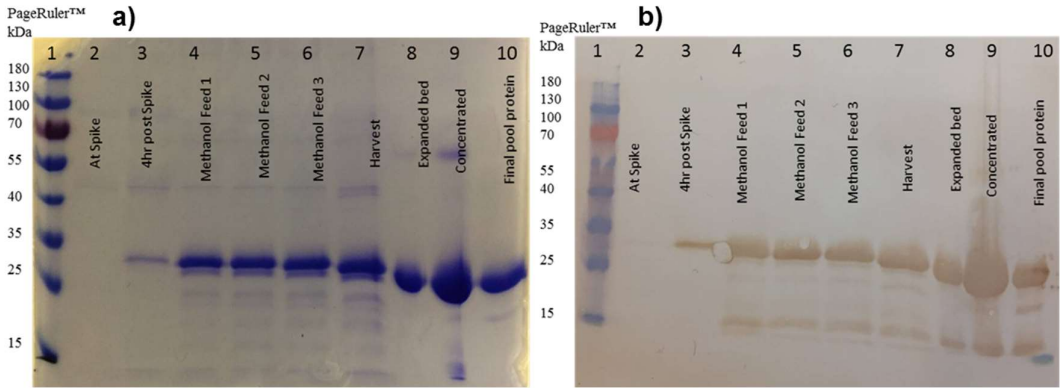

Figure 3.15 SDS-PAGE (a) and Western Blot (b) analysis of the samples taken during the fermentation run and capture of the His_6 tagged scFv antibody using the EBA primary chromatography step.

Analysis of the His_6 tagged scFv antibody expressed in the fermenter and captured through EBA chromatography was performed using SDS-PAGE (a) and Western Blot (b). Antibody band was seen at 27 kDa in the fermenter and EBA purified samples on the SDS-PAGE (a) and availability of the His_6 tagged on the antibody was confirmed with Western Blot (b) analysis. Sample details are described in the table below. Identical samples were run on SDS-PAGE (a) and Western Blot (b).

Sample lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sample volume	10 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL	1 µL	1 µL	1 µL
Sample detail	Molecular weight marker	Fermenter sample at the glycerol spike	Fermenter sample 4 h post glycerol spike	Fermenter sample 24 h post glycerol spike (Methanol feed 1)	Fermenter sample 27 h post glycerol spike (Methanol feed 2)	Fermenter sample 30 h post glycerol spike (Methanol feed 3)	Fermenter harvest sample	scFv antibody eluted from EBA column	Concentrated scFv antibody from EBA capture	Final pooled purified scFv antibody from EBA capture

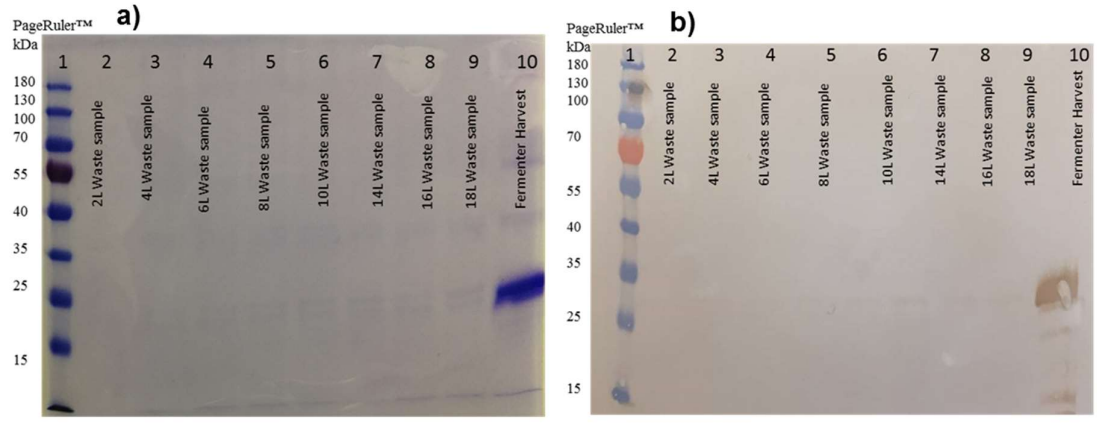

Figure 3.16 SDS-PAGE (a) and Western Blot (b) analysis of the samples taken during direct capture of the His_6 tagged scFv antibody from the fermenter culture through the STREAMLINE 50 EBA column.

Analysis of the His_6 tagged scFv antibody in the EBA harvest flow-through samples was performed using SDS-PAGE (a) and Western Blot (b). Antibody band was not seen in the EBA flow-through samples on the SDS-PAGE (a), and a thin antibody band was seen during the Western Blot (b) analysis in the flow-through samples from 2 L to 14 L. Sample details are described in the table below. Identical samples were run on SDS-PAGE (a) and on Western Blot (b).

Sample lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sample volume	10 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL
Sample detail	Molecular weight marker	2 L flow-through sample from EBA column	4 L flow-through sample from EBA column	6 L flow-through sample from EBA column	8 L flow-through sample from EBA column	10 L flow-through sample from EBA column	14 L flow-through sample from EBA column	16 L flow-through sample from EBA column	18 L flow-through sample from EBA column	Fermenter harvest sample

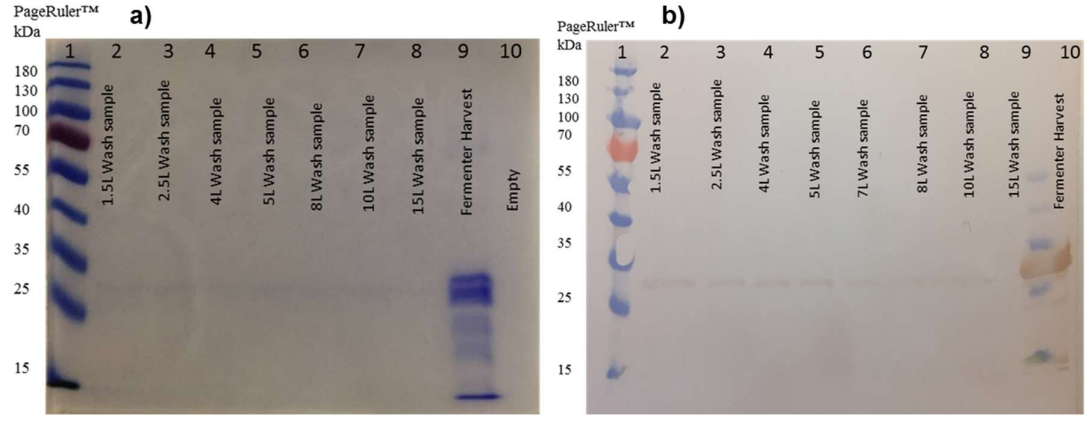

Figure 3.17 SDS-PAGE (a) and Western Blot (b) analysis of the samples taken during the washing step of the STREAMLINE 50 EBA column.
Analysis of the His_6 tagged scFv antibody in the EBA wash samples was performed using SDS-PAGE (a) and Western Blot (b). A light antibody band was seen at 27 kDa in the wash samples from 1.5 L to 5 L wash on the SDS-PAGE (a) and availability of a His_6 tagged on the antibody was confirmed with Western Blot (b) analysis. Sample details are described in the table below. Identical samples were run on SDS-PAGE (a) and on Western Blot (b).

Sample lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sample volume	10 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL
Sample detail	Molecular weight marker	1.5 L wash sample of the EBA column	2.5 L wash sample of the EBA column	4 L wash sample of the EBA column	5 L wash sample of the EBA column	7 L wash sample of the EBA column	8 L wash sample of the EBA column	10 L wash sample of the EBA column	15 L wash sample of the EBA column	Fermenter harvest sample

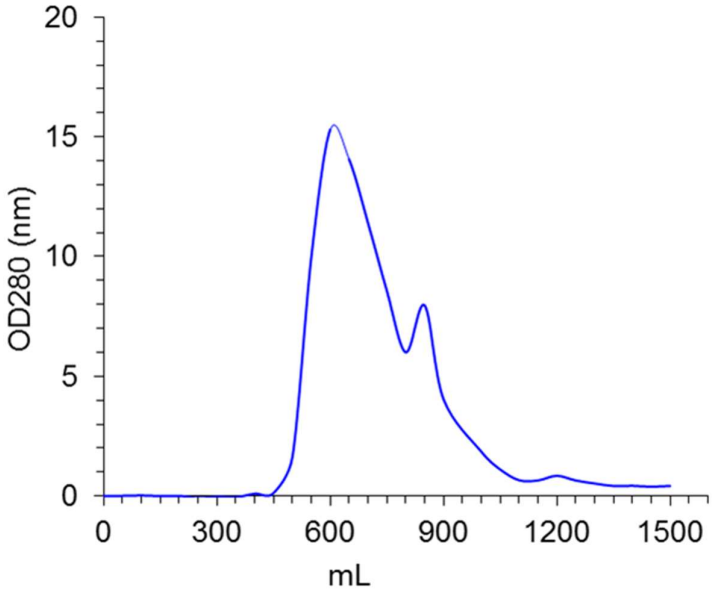

Figure 3.18 Expanded bed eluted protein fractions profile.
Figure 3.18 shows the elution profile of the scFv antibody eluted from the EBA column. The scFv fractions from 500 mL to 2250 mL were pooled together and further purified.

3.6 SEC of the His_6 tagged scFv antibody shMFELL2cys purified using RBA chromatography

RBA chromatography eluted His_6 tagged scFv antibody shMFELL2cys was concentrated and run on the 500 mL Superdex 75 SEC column to separate the dimer and monomer of scFv antibody and other impurities. The Superdex 75 500 mL column was purchased prepacked from GE Healthcare, and Bio-Rad gel filtration standard, which is a mixture of 1350 to 670000 Daltons molecular weight marker, was run to ensure that the column was properly packed and the markers were separating evenly (Appendix 1). Based on separation profile of the molecular weight markers containing thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (13 kDa) on Superdex 75 500 mL column monomer of the His_6 tagged scFv antibody fractions were selected and pooled together.

The concentrated protein concentration of 16 mg/mL was measured using a UV spectrophotometer at OD 280 nm and a total of 400 mg (25 mL of 16 mg/mL)

protein was applied to the Superdex 75 500 mL column for gel filtration purification. In Figure 3.19 a), a broad protein peak is seen from 170 mL to 300 mL CV, during which it was collected in 5 mL fractions. A total of four SEC FPLC runs were performed. The full chromatogram of the first FPLC run and overlay chromatogram of four FPLC runs can be seen in Figure 3.19 a) and Figure 3.19 b), respectively. Figures 3.20 a) and b) show the zoomed chromatogram of the first FPLC run and zoomed overlay chromatogram of all four FPLC runs, respectively.

For FPLC run 1, fractions 19 to 24 inclusive were pooled together and the pooled protein was run on the analytical 125 mL Superdex 75 SEC column to confirm that only the monomer of the scFv antibody was present in the pooled protein. From the first FPLC run, a total of 114 mg of scFv antibody was purified. From FPLC runs 2, 3 and 4, the pooled scFv antibody concentrations were 164 mg, 189 mg and 167 mg in total, respectively. All four FLPC run chromatograms were reproducible and monomer of the scFv antibody was purified successfully.

A green pigmented by-product of *P. pastoris* that was co-purified during the RBA chromatography process increased the protein measurement when measured using a spectrophotometer. Two types of pigments are generated in the fermentation induction process of pichia pastoris, one type is caused by alcohol oxidase (Aox1p), which is green and dark green. It is only produced after methanol induction, and is fermented to the later stage, and released into culture medium along with cell lysis. Another type is pigments in the fermentation medium that appear yellow, brown, or even reddish brown [109, 110]. The physical appearance of the eluted RBA protein can be seen in Figure 3.28. To verify that the monomer of the scFv antibody was separated from the dimer pooled antibody from four FPLC runs, the protein was run on to the 125 mL Superdex 75 analytical column. The pooled scFv antibody from four SEC FPLC runs was sterile filtered using 0.2 µm bottle top filter and stored in a freezer at -80°C.

All four pooled SEC runs were run on to SDS-PAGE gel and a single band of the His_6 tagged scFv antibody can be seen at 27 kDa molecular weight (Figure 3.26). A total of 613 mg of the His_6 tagged scFv antibody shMFELL2cys at 3.7 mg/mL concentration was purified using RBA chromatography.

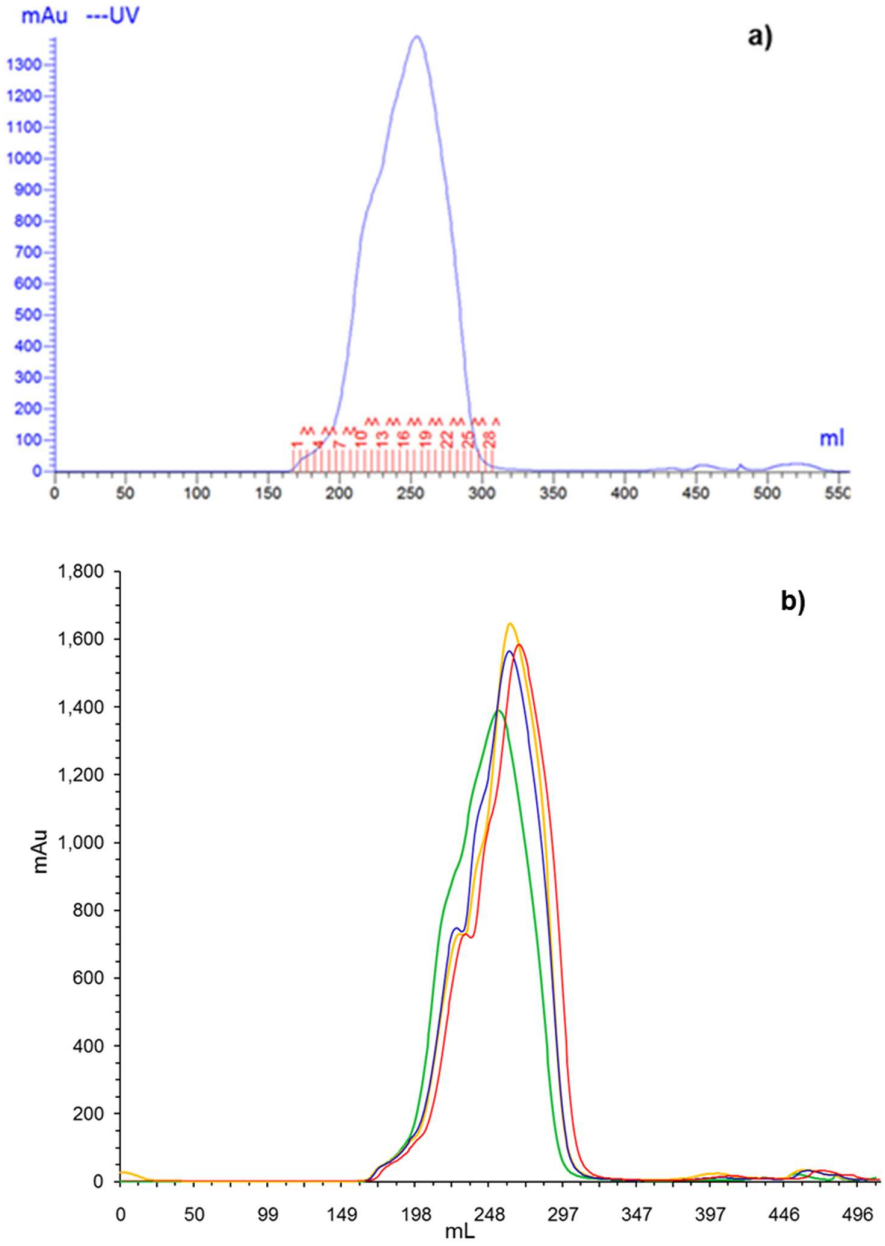

Figure 3.19 Chromatogram of the SEC FPLC run of the scFv antibody eluted from the RBA column.

Figures 3.19 a) and b) show the full chromatogram of the first SEC FPLC run and overlay chromatogram of all four FLPC runs, respectively. In Figure 3.19 b), the overlay chromatogram of the four SEC FPLC runs shows the broad chromatography peak where the dimer and monomer of the scFv antibody were not separated.

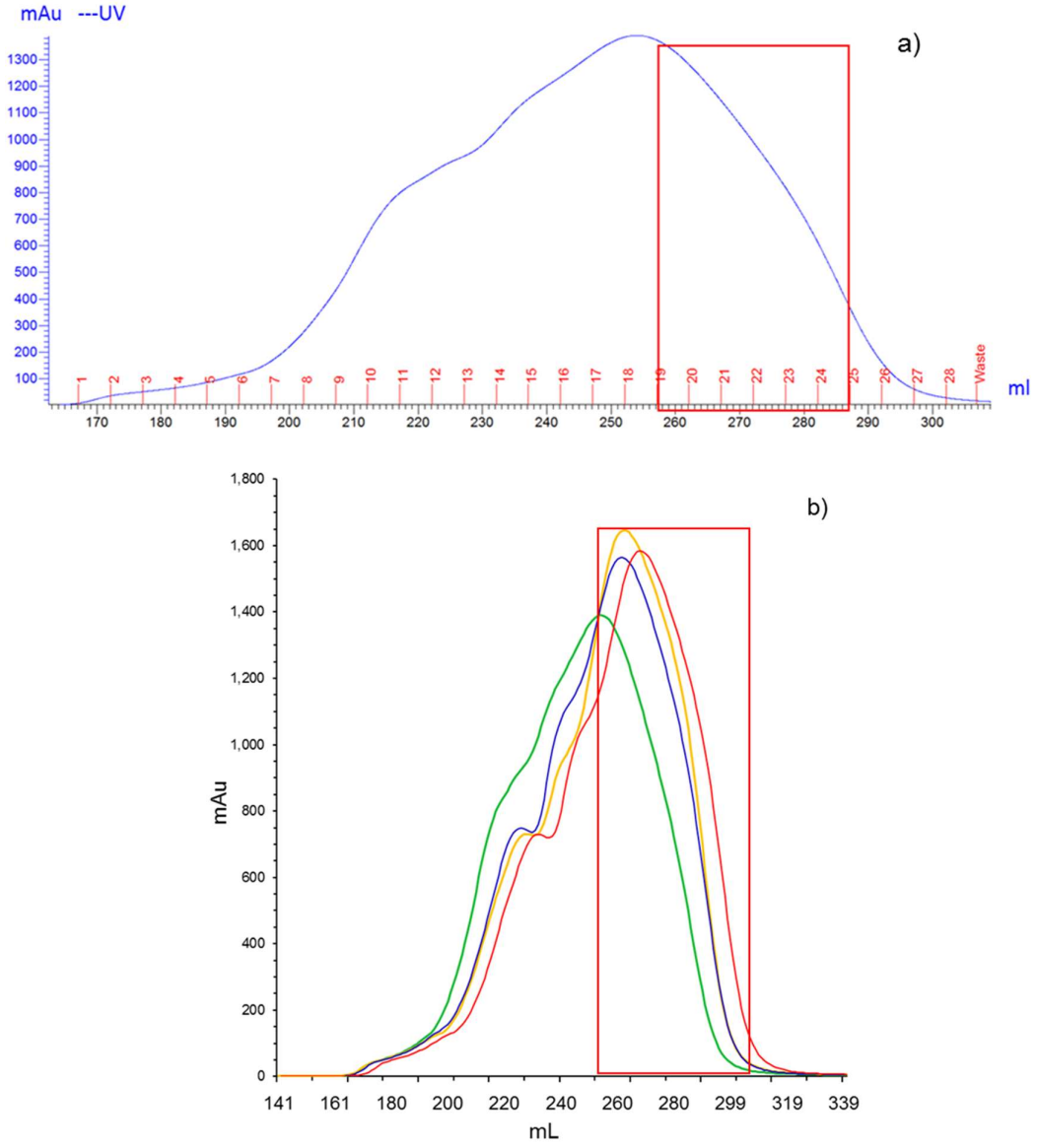

Figure 3.20 Zoomed chromatogram of SEC FPLC run of the scFv antibody eluted from the RBA column.

Figures 3.20 a) and b) show the zoomed chromatogram of the first SEC FPLC run and zoomed overlay chromatogram of the four SEC FPLC runs, respectively. All four chromatograms show a broad peak containing the dimer and monomer of the scFv in one peak. Fractions from the highlighted red rectangle area were selected and pooled together to separate the monomer from the dimer.

3.7 SEC of the His_6 tagged scFv antibody shMFELL2cys purified using EBA chromatography

The EBA chromatography eluted His_6 tagged scFv antibody shMFELL2cys was concentrated and run on the 500 mL Superdex 75 SEC column to separate the dimer and monomer of the scFv antibody and other impurities. Between 25 mL and 30 mL of concentrated scFv antibody was applied in each SEC run and a total of five SEC FPLC runs performed. The concentrated protein concentration of 19 mg/mL was measured using a UV spectrophotometer at OD 280 nm and a total of 475 mg (25 mL of 19 mg/mL) protein was applied on to the Superdex 75 500 mL column for gel filtration purification. In Figure 3.21 a), a broad protein peak is seen from 170 mL to 300 mL CV, which was collected in 5 mL fractions. A total of five SEC FPLC runs were performed. The full chromatogram of the first FPLC run and overlay chromatogram of five SEC FLPC runs can be seen in Figure 3.21 a) and Figure 3.21 b), respectively. Figures 3.22 a) and b) show the zoomed chromatogram of the first FPLC run and zoomed overlay chromatogram of all fiver FPLC runs, respectively.

For FPLC run 1, fractions 21 to 28 inclusive were pooled together and the pooled protein was run on the analytical 125 mL Superdex 75 SEC column to confirm that monomer scFv antibody was present in the pooled protein. From the first FPLC run, a total of 149 mg of scFv antibody was purified. From FPLC runs 2, 3, 4 and 5, the pooled scFv antibody concentrations were 136 mg, 176 mg, 154 mg and 186 mg in total, respectively. The physical appearance of the eluted expanded bed protein can be seen in Figure 3.29. All five FLPC run chromatograms were reproducible and monomer of the scFv antibody was purified successfully.

To verify that the monomer of the scFv antibody was separated from the dimer, the pooled antibody from five FPLC runs was run on the 125 mL Superdex 75 analytical column. The pooled scFv antibody from four SEC FLPC runs was sterile filtered using 0.2 µm bottle top filter and stored in a freezer at -80°C. All five pooled SEC runs were run on SDS-PAGE gel, and a single band of the His_6 tagged scFv antibody can be seen at 27 kDa molecular weight (Figure 3.27).

A total of 801 mg of the His_6 tagged scFv antibody shMFELL2cys at 4.1 mg/mL concentration was purified using EBA chromatography.

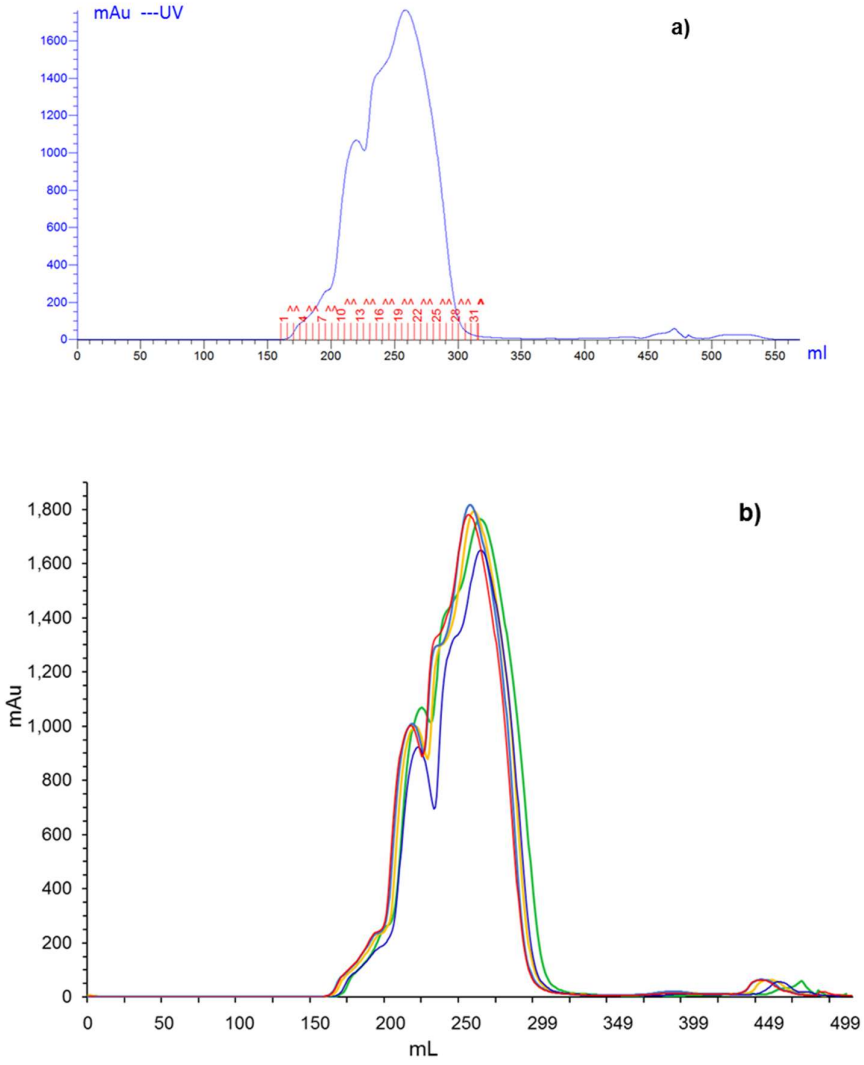

Figure 3.21 Chromatogram of the SEC FPLC run of the scFv antibody eluted from the EBA column.

Figures 3.21 a) and b) show the full chromatogram of the first SEC FPLC run and overlay chromatogram of all five FLPC runs, respectively. In Figure 3.21 b), the overlay chromatogram of the five SEC FPLC runs shows the broad chromatography peak where the dimer and monomer of the scFv antibody were not separated.

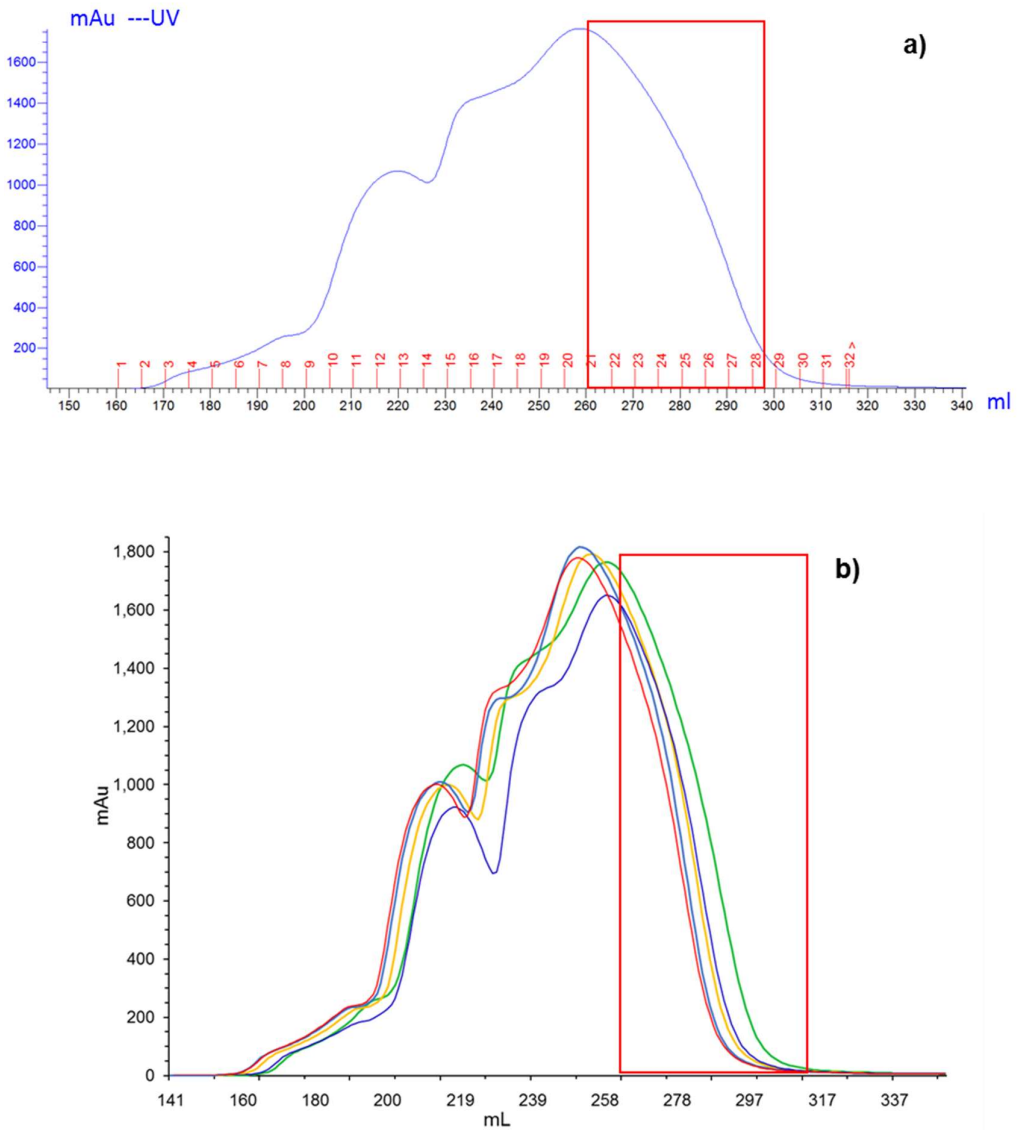

Figure 3.22 Zoomed chromatogram of SEC FPLC run of the scFv antibody eluted from the EBA column.

Figures 3.22 a) and b) show the zoomed chromatogram of the first SEC FPLC run and zoomed overlay chromatogram of the five SEC FPLC runs, respectively. All five chromatograms show a broad peak containing the dimer and monomer of the scFv in one peak, and fractions from the highlighted red rectangle area were selected and pooled together to separate the monomer from the dimer.

3.8 Analytical SEC FPLC runs of the samples taken during RBA and EBA chromatography

Analytical Superdex 75 125 mL column was used to run the samples during RBA and EBA chromatography to analyse the scFv antibody aggregation, degradation and dimer formation. Superdex 75 125 mL column was purchased prepacked from GE Healthcare, and Bio-Rad gel filtration standard, which is a mixture of 1350 to 670000 Daltons molecular weight marker, was run to ensure that the column was properly packed and markers were separating evenly before running the samples (Appendix 1).

The dimer and monomer peak of the scFv antibody was seen on the RBA and EBA eluted scFv antibody chromatogram (Figures 3.23 a) and b)). The monomer peak height of the EBA eluted scFv antibody was 110 mAu compared to the RBA eluted scFv antibody monomer peak height of 55 mAu. Therefore, double the amount of scFv antibody was purified using EBA compared to RBA chromatography.

Figures 3.24 a) and b) show the chromatogram of the RBA and EBA eluted and concentrated scFv antibody, respectively. The RBA concentrated scFv antibody chromatogram was seen with one broad peak combining three peaks together compared to two peaks of the EBA concentrated scFv antibody. The scFv antibody concentration in the EBA sample was higher than the RBA sample as the peak height was around 400 mAu for EBA compared to around 200 mAu for the RBA sample.

A single monomer peak of the final pooled scFv antibody was seen on the RBA and EBA chromatogram (Figure 3.25 a) and b)). After the three-step downstream purification process, monomer scFv antibody shMFELL2cys was successfully purified.

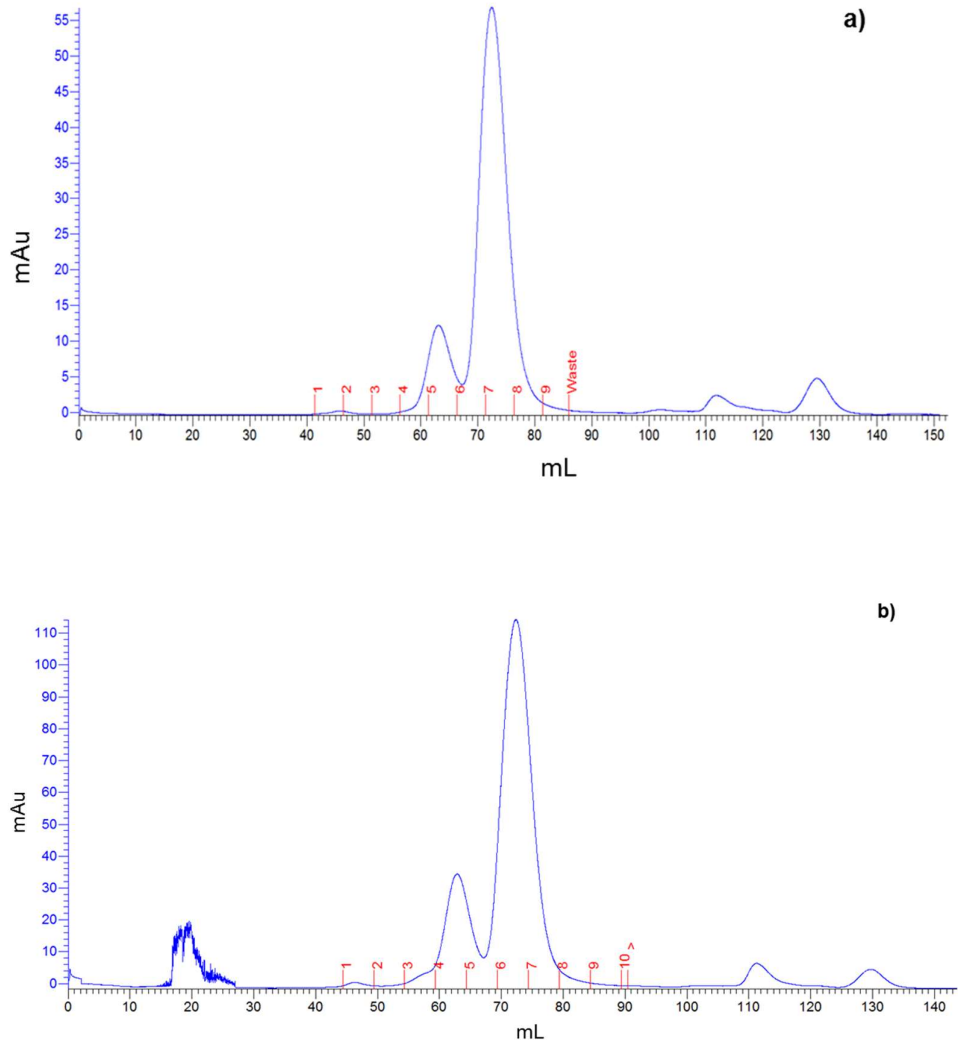

Figure 3.23 Chromatogram of analytical SEC FPLC run of the RBA and EBA eluted scFv antibody. (Figure a) RBA eluted scFv antibody sample and Figure b) EBA eluted scFv antibody sample).

Comparison of the RBA and EBA eluted protein chromatogram. a) Dimer scFv antibody peak was present at 65 mL CV and monomer scFv antibody peak was present at 70 mL CV in RBA eluted protein chromatogram. b) Dimer scFv antibody peak was present at 65 mL CV and monomer scFv antibody peak was present at 70 mL CV in EBA eluted protein chromatogram. Monomer scFv antibody concentration was higher in the EBA eluted protein sample compared to the RBA eluted protein sample.

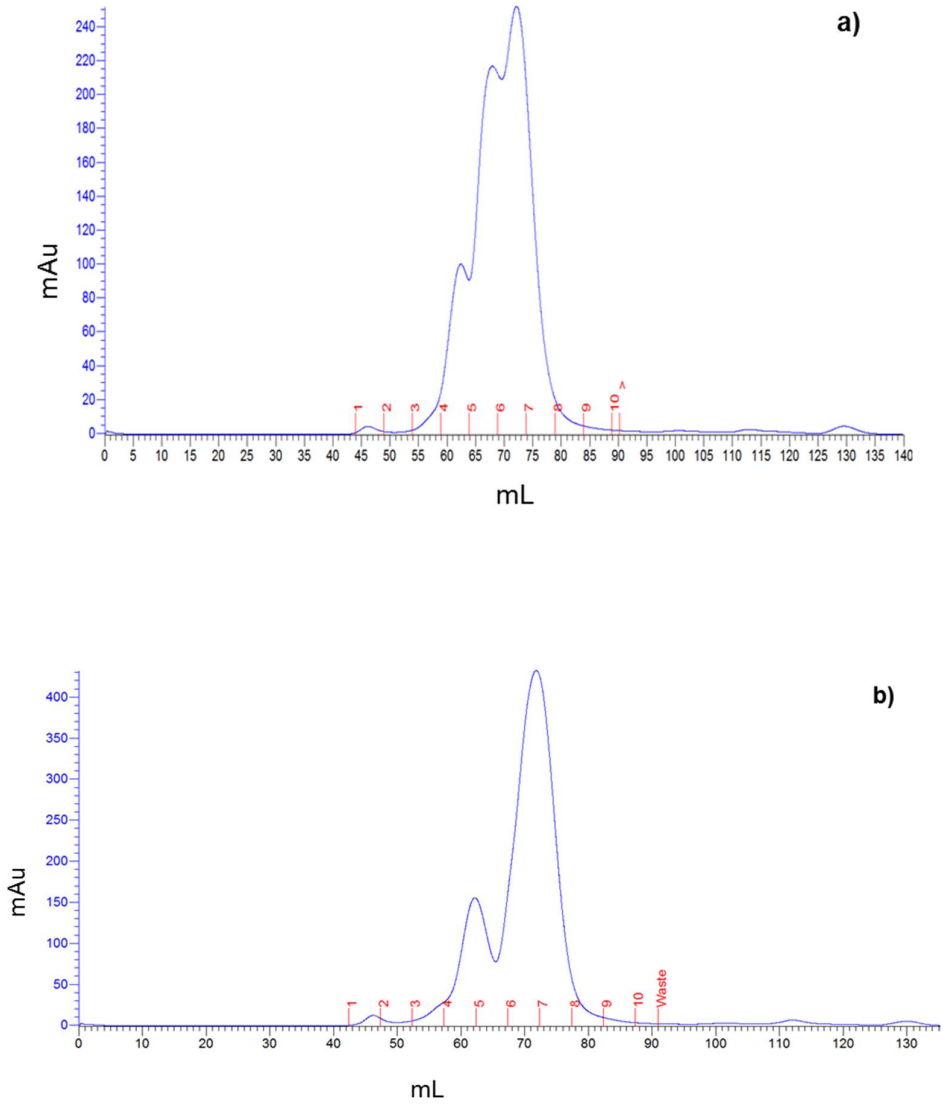

Figure 3.24 Chromatogram of analytical SEC FPLC run of the RBA and EBA eluted and concentrated scFv antibody. (Figure a) RBA eluted and concentrated scFv antibody sample and Figure b) EBA eluted and concentrated scFv antibody sample).

Comparison of the RBA and EBA eluted and concentrated protein chromatogram. a) Broad peak of scFv antibody included dimer and monomer scFv antibody was present in RBA eluted and concentrated protein chromatogram. b) Dimer scFv antibody peak was present at 65 mL CV and monomer scFv antibody was present at 70 mL CV in EBA eluted and concentrated protein chromatogram. Monomer scFv antibody concentration was higher in the EBA sample compared to the RBA sample.

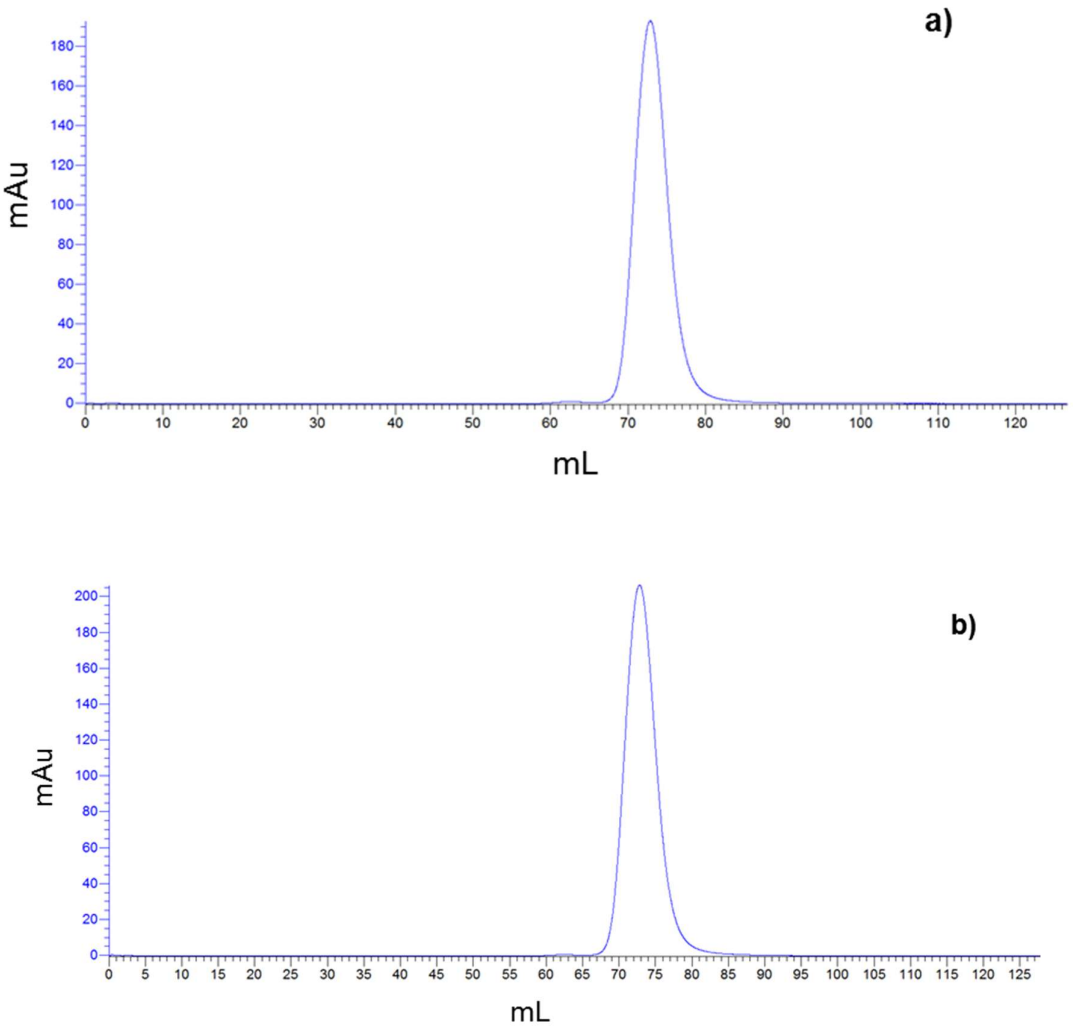

Figure 3. 25 Chromatogram of analytical SEC FPLC run of the final pooled scFv antibody purified using RBA and EBA chromatography. (Figure a) RBA final purified scFv antibody sample and Figure b) EBA final purified scFv antibody sample).

Comparison of the RBA and EBA final purified scFv antibody chromatograms. a) A single monomer peak of scFv antibody was present at 75 mL CV in the final purified scFv antibody from RBA chromatography. b) A single monomer peak of scFv antibody was present at 75 mL CV in the final purified scFv antibody from EBA chromatography.

3.9 SDS-PAGE of the His_6 tagged scFv antibody shMFELL2cys purified using RBA and EBA chromatography

SDS-PAGE analysis of the scFv antibody purified using RBA and EBA chromatography was performed to confirm the molecular weight of the scFv antibody. The molecular weight of the scFv antibody was 27 kDa [111]. A single band of the scFv antibody at 27 kDa was seen for the pooled scFv antibody from each SEC FPLC run and final pooled protein for RBA and EBA chromatography. Figure 3.26 show the SDS-PAGE gel picture of the scFv antibody purified using RBA chromatography. Figure 3.27 show the SDS-PAGE gel picture of the scFv antibody purified using EBA chromatography.

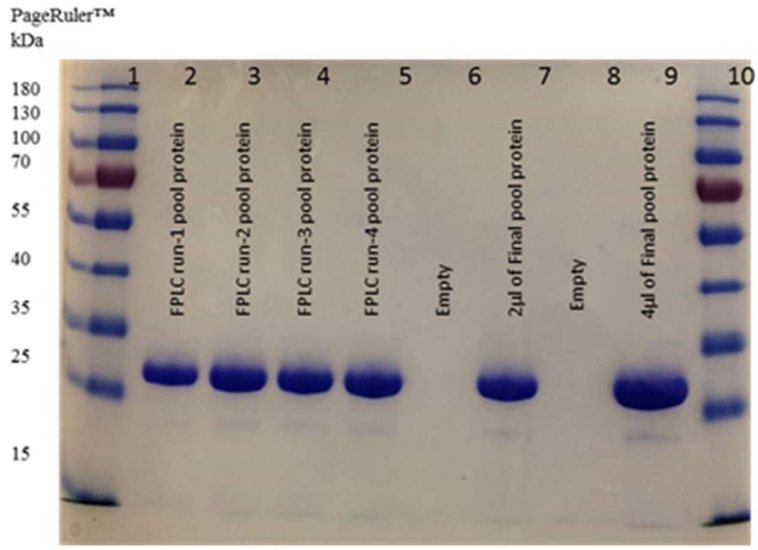

Figure 3.26 SDS-PAGE of the scFv antibody shMFELL2cys purified using RBA chromatography.

Analysis of the His_6 tagged scFv antibody purified using RBA chromatography was performed using SDS-PAGE. A single scFv antibody band was seen at 27 kDa in the SEC FPLC run samples and final pooled sample. Sample details are described in the table below.

Sample lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sample volume	10 µL	2 µL	2 µL	2 µL	2 µL	N/A	2 µL	N/A	4 µL	10 µL
Sample detail	Molecular weight marker	Pool protein from SEC FLPC run 1	Pool protein from SEC FLPC run 2	Pool protein from SEC FLPC run 3	Pool protein from SEC FLPC run 4	Empty lane	Final pool protein from SEC FLPC run 1, 2, 3 and 4	Empty Lane	Final pool protein from SEC FLPC run 1, 2, 3 and 4	Molecular weight marker

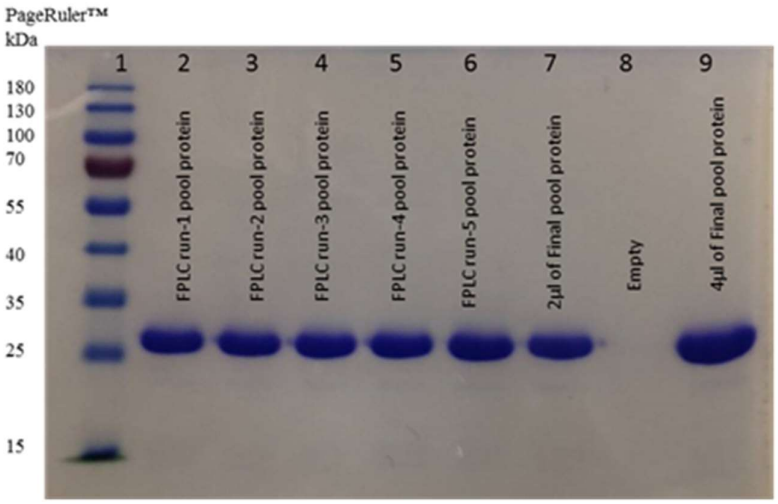

Figure 3.27 SDS-PAGE of the scFv antibody shMFELL2cys purified using EBA chromatography.

Analysis of the His_6 tagged scFv antibody purified using EBA chromatography was performed using SDS-PAGE. A single scFv antibody band was seen at 27 kDa in the SEC FPLC run samples and final pooled sample. Sample details are described in the table below.

Sample lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Sample volume	10 µL	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL	N/A	4 µL
Sample detail	Molecular weight marker	Pool protein from SEC FLPC run 1	Pool protein from SEC FLPC run 2	Pool protein from SEC FLPC run 3	Pool protein from SEC FLPC run 4	Pool protein from SEC FLPC run 5	Final pool protein from SEC FLPC run 1, 2, 3, 4 and 5	Empty lane	Final pool protein from SEC FLPC run 1, 2, 3, 4 and 5

3.10 Colour appearance of the eluted protein from RBA and EBA chromatography

A comparison of the colour appearance of the eluted scFv antibody using RBA and EBA chromatography was performed to compare the nonspecific binding of *P. pastoris* pigmented material produced during the fermentation process that was not completely washed out during the washing step. The *P. pastoris* pigmented material was co-purified; hence, the appearance of the eluted protein was light green. RBA eluted scFv antibody was light green compared to the dark green appearance of the EBA eluted protein, which suggests that a higher amount of pigmented material was co-purified when using EBA compared to RBA chromatography. The appearance of the final pooled scFv antibody after SEC was colourless and transparent for both EBA and RBA purified scFv antibody, which confirms that pigmented material was removed during the SEC step.

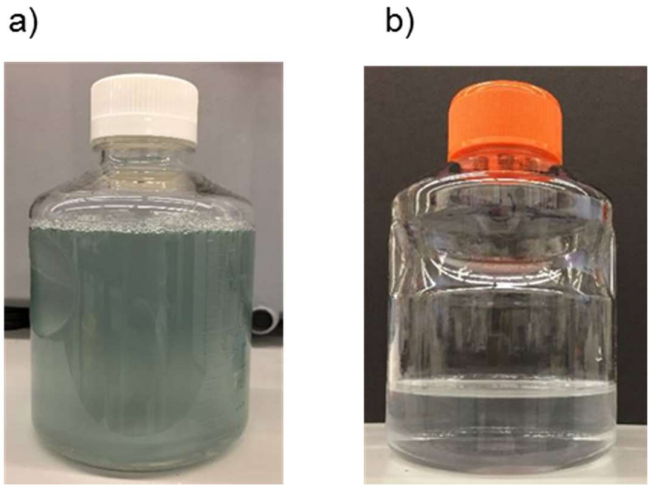

Figure 3.28 Pictures of the scFv antibody appearance after being eluted from the RBA column and the final pooled purified protein after the SEC runs. Comparison of the scFv antibody appearance after being eluted from the RBA column and the final pooled purified protein after the SEC runs. Figure a) picture of the RBA eluted protein and b) picture of the final pooled protein.

Figure 3.28 compares the colour appearance of the scFv antibody eluted by RBA chromatography (figure 3.28, a) and the final pooled scFv antibody after SEC (figure 3.28, b). Figure 3.29 compares the colour appearance of the scFv antibody eluted by EBA chromatography (figure 3.29, a) and the final pooled scFv antibody after SEC (figure 3.29, b). Colour appearance of the EBA eluted protein was dark green (figure 3.29, a) compared to the light green colour of the RBA eluted protein (figure 3.28, a). This suggests that a higher amount of the pigmented material was co-purified when using EBA compared to RBA chromatography.

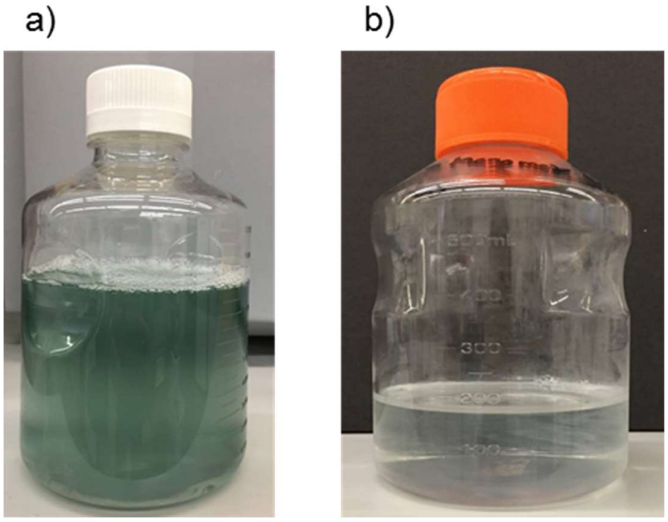

Figure 3.29 Picture of the scFv antibody appearance after being eluted from the EBA column and the final pooled purified protein after the SEC runs. Comparison of the scFv antibody appearance after being eluted from the EBA column and the final pooled purified protein after the SEC runs. Figure a) picture of the EBA eluted protein and b) picture of the final pooled protein.

3.11 Total protein analysis of the EBA and RBA eluted protein using Bio-Rad DC protein assay

Total protein analysis was performed to measure the nonspecific binding of the HCPs during RBA and EBA chromatography. Samples were taken at the end of the fermentation runs, during the EBA and RBA chromatography step, from concentrated scFv antibody and 0.2 µm sterile filtered EBA and RBA scFv antibody. The total protein concentration of samples was measured using Bio-Rad DC protein assay (Chapter 2). Total protein concentration in the fermenter harvest sample purified using EBA chromatography was 30% higher than in the sample taken from the fermenter harvest sample purified using RBA chromatography. Protein eluted from the EBA chromatography capture step had 60% higher total protein concentration compared to RBA chromatography. This result indicates that there might be high nonspecific binding of the HCP during EBA chromatography in comparison to RBA chromatography and that it was not removed during the washing step. EBA and RBA columns were rinsed with washing buffer supplemented with 10 mm imidazole concentration in washing buffer to remove nonspecific binding of HCP. The EBA column was washed with 15 L of washing buffer compared to 11 L for RBA column. However, the total protein concentration of the protein eluted from the EBA column was still higher than that from the RBA column. Application of the end of fermenter culture to the EBA column was performed at 70 mL/min (equivalent to a flow velocity of 214 cm/h) with residence time of around 24 min compare to 70 mL/min (equivalent to a flow velocity of 93 cm/h) for the RBA column with residence time of 3.5 min, which resulted in higher nonspecific binding of the HCP to the EBA column compared to the RBA column. Higher dynamic binding capacity (DBC) of the EBA resin in comparison to RBA resin could have led to higher total protein concentration in the EBA eluted protein in comparison to RBA eluted protein (Chapter 4).

The concentration step of the RBA and EBA eluted protein using Biomax 5 kDa membrane also concentrated non-specifically bound HCP along with scFv antibody. At the same time, during the concentration step protein impurities below 5 kDa in size were removed from the concentrated protein. Total protein

concentrations in the concentrated protein sample of the EBA and RBA protein and final purified EBA and RBA pooled protein samples were very similar. SDS-PAGE analysis of the concentrated sample taken during the RBA and EBA chromatography processes can be seen in figure 3.8 and figure 3.15, respectively. Concentrated scFv antibody band thickness at 27 kDa was higher for EBA process in comparison to RBA process. These results indicate that a higher number of impurities in the EBA eluted protein was removed during the protein concentration and SEC FPLC runs. Results of the total protein concentration in the samples taken during EBA and RBA chromatography are given in Table 3.1 below. No big difference in the total protein concentration in the EBA and RBA concentration protein samples were measured. Possible degradation of the HCP during concentration step led to reduction in the total protein concentration in EBA concentrated protein.

Table 3.1 Total protein concentration of samples taken from EBA and RBA harvest using Bio-Rad DC Bradford assay.

Sample identification	Total protein concentration (mg/mL)	
	EBA chromatography	RBA chromatography
Fermenter harvest	2.7	1.9
Eluted protein	6.4	2.7
Concentrated protein	13.0	13.3
Final purified protein	7.0	7.0

3.12 Quantitative analysis of the scFv antibody samples using Agilent protein 80 chip

Quantification of the His_6 tagged scFv antibody shMFELL2cys expressed and purified using RBA and EBA chromatography was performed using Agilent 80 protein quantification chip (Chapter 2, Section 2.2.14). Final purified 1 mg/mL and 2 mg/mL scFv antibody shMFELL2cys was used as a control standard for quantification of the scFv expressed in the fermenter and captured using EBA and RBA chromatography.

Fermentation yield of 600 mg/L and 680 mg/L for the scFv antibody shMFELL2cys was measured for EBA and RBA chromatography, respectively. During the EBA chromatography step, recovery of 47% was measured, and a total of 2.3 g scFv antibody was captured. During the RBA chromatography step, recovery of 30% was measured, and a total of 1.6 g scFv antibody was captured. Tables 3.2 and 3.3 detail the measured scFv antibody concentrations for the EBA and RBA chromatography steps.

Table 3.2 The scFv antibody results for the EBA chromatography samples.

Sample identification	Agilent protein 80 chip result (ng/µL)	Volume (mL)	scFv concentration (mg/mL)	scFv amount (mg)	% recovery from the previous step	Overall yield (%)
Harvest	579	8000	0.60	4800	100	16
EBA eluted protein	2870	800	2.9	2288	47	
Concentrated	9336	150	9.3	1395	60	
Final pooled protein	See Note	195	4.1	801	57	

Note: EBA purified final pooled protein concentration could not be measured using Agilent protein 80 chip. The final pooled scFv antibody concentration was measured using a spectrophotometer at OD_{280} nm.

Table 3.3 The scFv antibody results for the RBA chromatography samples.

Sample identification	Agilent protein 80 chip result (ng/µL)	Volume (mL)	scFv concentration (mg/mL)	scFv amount (mg)	% recovery from the previous step	Overall yield (%)
Harvest	689	8000	0.68	5440	100	11
RBA eluted protein	1632	1000	1.6	1650	30	
Concentrated	27.9	100	15.8	1579	95	
Final pooled purified	6.6	165	3.7	611	38	

Note: RBA concentrated scFv antibody sample could not be measured using Agilent protein 80 chip. The concentrated scFv antibody concentration was measured using a spectrophotometer at OD_{280} nm.

3.13 Analysis of the *P. pastoris* HCP in the EBA and RBA captured scFv antibody

The *P. pastoris* host cell protein contamination in the scFv antibody purified using EBA and RBA chromatography and SEC was measured using Cygnus Technologies immunoenzymatic assay. *P. pastoris* HCP concentration was measured at 450 nm wavelength in a plate reader and by generating a standard graph using 0 ng/mL, 4 ng/mL, 20 ng/mL, 75 ng/mL and 250 ng/mL standards in triplicate. Samples were applied in duplicate and mean absorbance values were converted into HCP concentration using standard graph values. HCP concentration was measured at 450 nm wavelength because change in blue colour due to the enzymatic reaction can only be at 450 nm instead of OD280 nm wavelength. HCP concentrations measured in the two end of fermentation run samples were 800 µg/mL and 990 µg/mL.

HCP concentration in the scFv antibody purified using EBA chromatography was 13.2 µg/mL compared to 10.2 µg/mL for the scFv antibody purified using RBA chromatography. HCP concentration in the final purified scFv antibody using EBA

chromatography was 161.8 ng/mL, equivalent to 39 ng/mg of scFv antibody. HCP concentration in the final purified scFv antibody using RBA chromatography was 83 ng/mL, equivalent to 22.3 ng/mg of scFv antibody.

HCP concentration in scFv antibody purified using EBA chromatography was twice as much as in the antibody purified using RBA chromatography but within the traditional practice of setting the target of 100 ng/mg therapeutic protein [112].

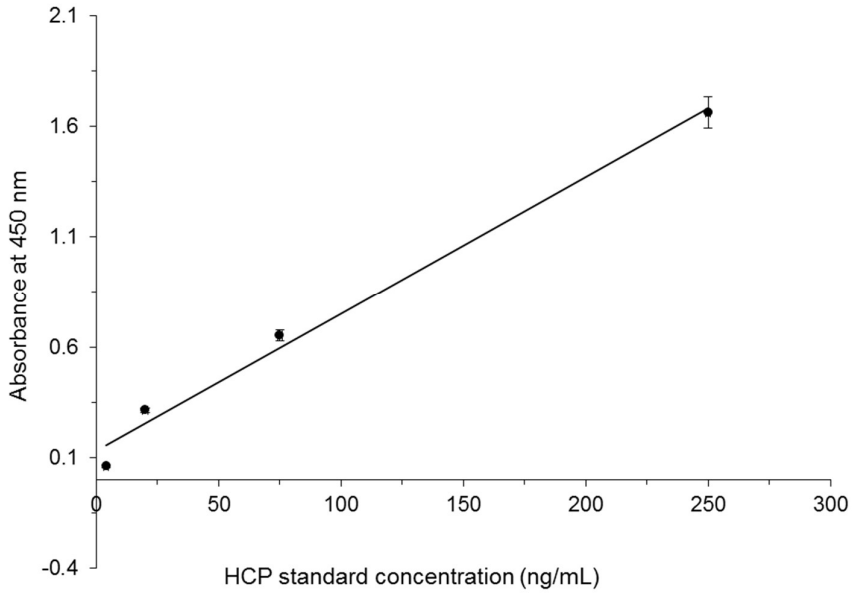

Figure 3.30 HCP calculation standard graph.
Figure 3.30 shows a graph of the HCP standards against absorbance at 450 nm. Data points were the mean of triplicate values. Data shown in the graph was the mean absorbance values of standards and error bars indicate standard deviation (n=3).

Table 3. 4 HCP concentrations of the EBA and RBA chromatography samples.

Sample identification	HCP concentration (µg/mL)	
	EBA	RBA
End of fermentation run	800.04	989.91
Eluted protein	13.2	10.2
Final protein	0.16	0.08

3.14 Measurement of the scFv antibody shMFELL2cys affinity to CEA using ELISA test

The scFv antibody shMFELL2csy used in this study has specificity and affinity for carcinoembryonic antigen (CEA), which is expressed on cancer cells [113]. CEA ELISA of the purified scFv antibody shMFELL2cys was performed to measure the specificity and affinity of the purified scFv antibody shMFELL2cys for CEA (Chapter 2, Section 2.2.14). The results in Figures 3.31 and 3.32 show that the scFv antibody shMFELL2cys expressed in the fermenter and purified using EBA and RBA chromatography has affinity for CEA. Affinity for CEA was measured for the scFv antibody samples taken after elution from the RBA and EBA columns, concentrating the scFv and purified scFv antibody from SEC (Figures 3.31 and 3.32). The scFv antibody manufactured using EBA and RBA chromatography processes has specificity and affinity for carcinoembryonic antigen (CEA), which is expressed on cancer cells. There is no big difference in the affinity towards the CEA between the EBA and RBA samples were measured.

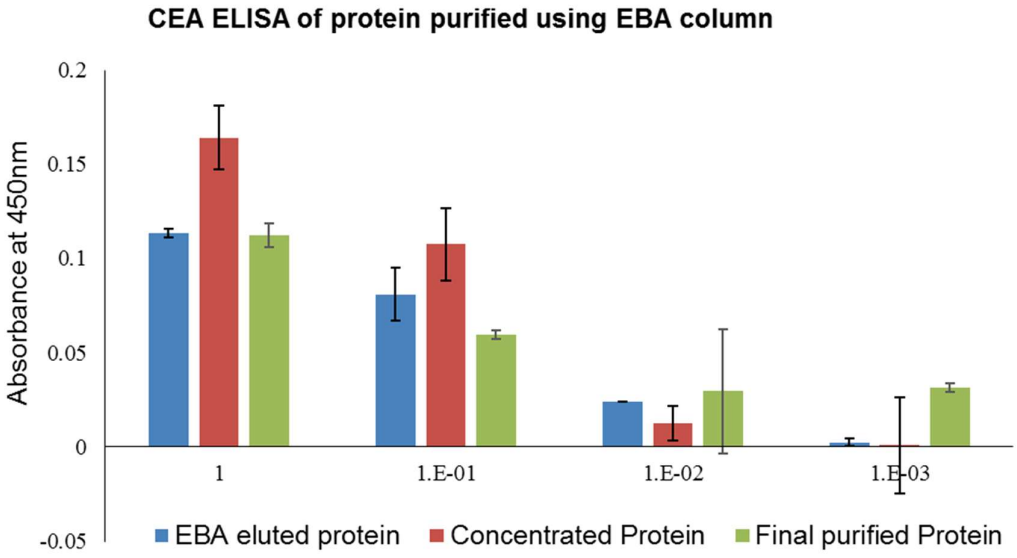

Figure 3.31 CEA ELISA of the His_6 tagged scFv antibody shMFELL2cys purified using EBA chromatography.

Figure 3.31 shows a comparison of the CEA ELISA results of scFv antibody samples taken from EBA elution, concentrated and final purified scFv at two dilution factors. Data shown in the graph was the mean binding efficiency of the scFv antibody shMFELL2cys to CEA and error bars indicate standard deviation (n=2).

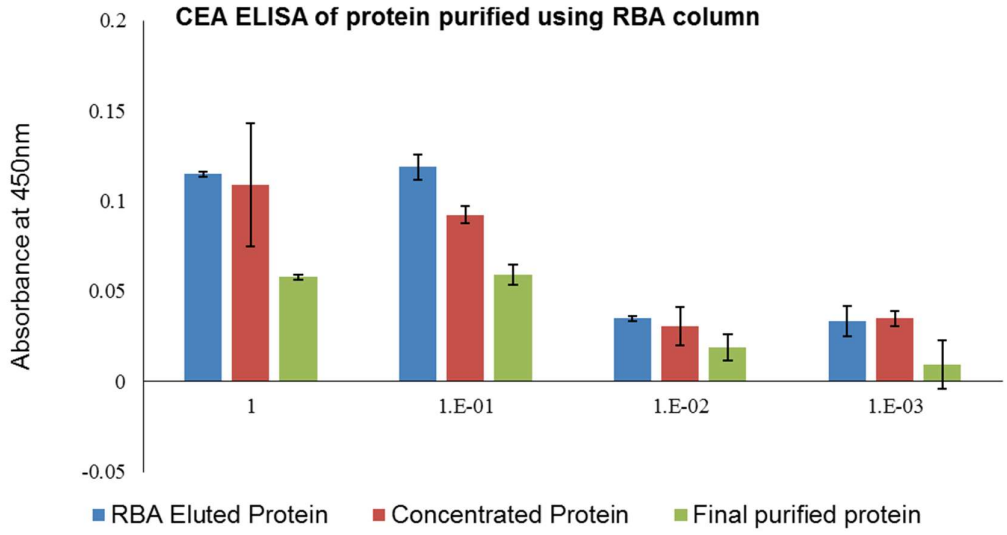

Figure 3.32 CEA ELISA of the His_6 tagged scFv antibody shMFELL2cys purified using RBA chromatography.

Figure 3.32 shows a comparison of the CEA ELISA results of scFv antibody samples taken from RBA elution, concentrated and final purified scFv at two dilution factors. Data shown in the graph was the mean binding efficiency of the scFv antibody shMFELL2cys to CEA and error bars indicate standard deviation (n=2).

3.15 Particle size distribution study of the samples taken during EBA and RBA chromatography

A particle size distribution study was performed to measure the size of particles present in the flow-through samples taken during EBA and RBA chromatography while applying crude fermenter culture. A Mastersizer 3000 was used to analyse the samples. This uses laser diffraction of the dispersed particulate sample and angular variation in intensity of the scattered light was measured and calculate the size of the particles. Mastersizer 3000 can measure particles between 10 nm and 3.5 mm. A Mastersizer 3000 measures the Dv10, Dv50 and Dv90 values of the samples. The Dv10 value of the sample represents 10% of the particles are below the measured Dv10 value of the sample. The Dv50 and Dv90 values of the samples represents 50% and 90% of the particles are below the measured Dv50 and Dv90 value of the sample, respectively.

The Dv10, Dv50 and Dv90 values from the particle size distribution of the RBA chromatography samples taken during culture application and column washing were very close at the end of the fermenter harvest sample, which indicates that particles from the end of fermenter culture flow-through the RBA column. The RBA resin's particle size distribution was recorded between 258 µm and 464 µm. The particle size distribution result of the RBA eluted scFv antibody sample recorded the presence of yeast cells as the Dv10, Dv50 and Dv90 were recorded close to the end of fermenter sample values. The measured Dv10, Dv50 and Dv90 values for the RBA eluted protein sample is 1.7 µm, 2.5 µm and 4.0 µm, respectively. And the typical size of the yeast cells is between 3 to 4 µm [114]. This indicates that yeast cells were present in the scFv antibody eluted from the RBA column. An overlay graph of the particle size distribution of the RBA samples is presented in Figure 3.33. Particle size distribution results of the RBA samples are detailed in Table 3.5.

The EBA resin's particle size distribution was recorded between 140 µm and 252 µm. The Dv10, Dv50 and Dv90 values from the particle size distribution of the EBA chromatography samples taken during culture application and column washing

were very close at the end of the fermenter harvest sample, except the results of the flow-through sample taken after 15 L wash. The recorded Dv90 value of the 15 L wash sample was 296 µm, which was very close to the EBA resin Dv90 value. For the EBA column at the end of the washing step, flow rate was increased to 84 mL/min, which could have resulted in the loss of EBA resin from the column. Apart from the 15 L wash sample, all other flow-through samples did not record any loss of EBA resin from the column during culture application and column washing, which indicates that stable expansion of the EBA column was maintained by manually adjusting the flow rate.

The particle size distribution results of the EBA eluted scFv antibody showed the presence of clumps of yeast cells as the measured Dv90 value was 25.4 µm, which is greater than the Dv90 value of the end of the fermenter run sample and less than the Dv10 value of the EBA resin. This indicates that washing of the EBA column in the downflow direction before eluting the absorbed scFv antibody should be introduced to wash out the accumulated yeast cells at the bottom net of the EBA column. An overlay graph of the particle size distribution of the EBA samples is presented in Figure 3.34. Particle size distribution results of the EBA samples are detailed in Table 3.6.

Table 3.5 Raw data of the particle size distribution study of the samples taken during RBA chromatography.

Sample name	Dv10 (µm)	Dv50 (µm)	Dv90 (µm)	D (3,2) Surface weighted mean (µm)	D (4,3) Volume weighted mean (µm)	Specific surface area (m^2/kg)	Laser obscuration (%)
RBA resin	258	347	464	338	355	17.73	7.81
Fermenter harvest sample	2.1	3.1	4.7	3.26	3.08	1918	19.67
2 L waste sample	1.0	3.0	4.5	3.1	2.9	2007	9.5
5 L waste sample	2.0	3.0	4.5	3.2	2.9	1966	11.4
10 L waste sample	1.9	2.9	4.3	3.0	2.8	2074	9.1
6 L wash sample	2.0	3.4	7.3	16.1	3.3	1733	15.8
11 L wash sample	1.7	2.5	4.0	2.7	2.4	2348	6.7
RBA eluted protein	1.7	2.5	4.0	2.7	2.4	2358	11.0

Table 3.6 Raw data of the particle size distribution study of the samples taken during EBA chromatography.

Sample name	Dv10 (µm)	Dv50 (µm)	Dv90 (µm)	D (3,2) Surface weighted mean (µm)	D (4,3) Volume weighted mean (µm)	Specific surface area (m^2/kg)	Laser obscuration (%)
EBA resin	140	188	252	183	192	32.8	16.4
Fermenter harvest sample	2.2	3.3	4.9	3.1	3.4	1815	13.9
3 L waste	1.9	3.0	4.9	2.9	3.2	1992	10.3
6 L waste	2.0	3.0	4.8	2.9	3.2	1968	10.7
8 L waste	2.0	3.0	4.8	2.9	3.3	1944	18.2
10 L waste	2.0	2.9	4.7	2.8	3.1	2051	12.8
14 L waste	2.0	3.0	4.9	2.9	3.3	1950	10.0
5 L wash	1.9	2.9	4.8	2.8	3.1	2068	14.7
7 L wash	1.7	2.7	4.2	2.5	2.8	2248	5.6
10 L wash	1.8	2.8	4.4	2.6	3.0	2154	5.1
15 L wash	1.9	3.5	296	3.9	86.7	1473	7.3
EBA eluted protein	1.8	2.9	25.4	2.9	9.1	1950	3.7

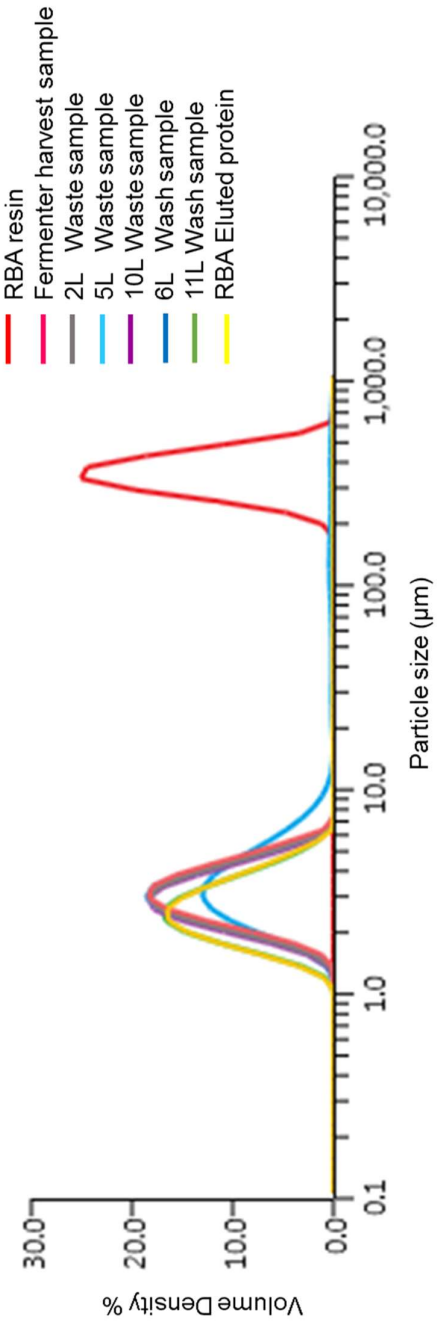

Figure 3.33 Overlay graph of the particle size distribution of the samples taken during the RBA chromatography step.

Comparison of the particle size of the samples taken during RBA chromatography is presented in the overlay graph in Figure 3.33.

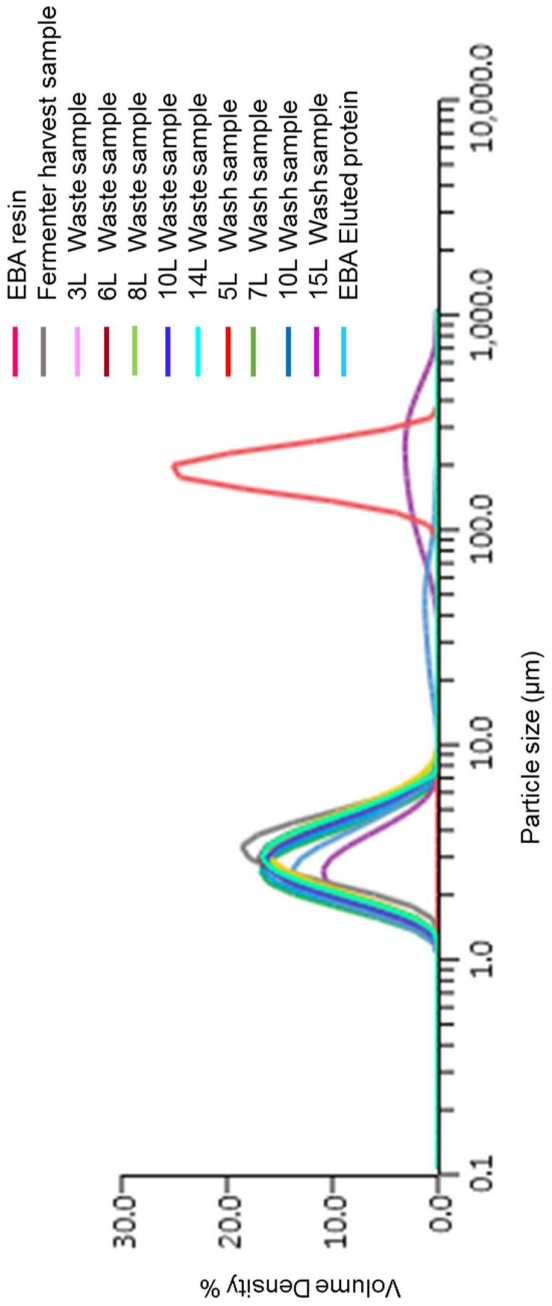

Figure 3.34 Overlay graph of the particle size distribution of the samples taken during the EBA chromatography step.

Comparison of the particle size of the samples taken during EBA chromatography is presented in the overlay graph in Figure 3.34.

3.16 Conclusions

The His_6 tagged scFv antibody shMFELL2cys was successfully expressed in the high cell density *P. pastoris* fermentation at a concentration of 680 to 600 mg/L and captured using the RBA and EBA chromatography processes, respectively. The calculated step recovery for RBA and EBA chromatography was 30% and 47%, respectively. A total amount of 801 mg and 611 mg of the scFv antibody shMFELL2cys was purified from the EBA and RBA chromatography processes, respectively. Data generated in this chapter was used to perform the cost base analysis between the RBA and EBA chromatography processes.

Flow-through samples taken during the RBA chromatography step and analysed using SDS-PAGE show that not all of the secreted scFv antibody was captured. This result indicates that increase in the column size will increase the step recovery of the RBA chromatography process. The presence of the His_6 tagged scFv antibody in the RBA column wash samples indicated that washing buffer supplemented with 10 mM imidazole to remove cells, cell debris and HCP also leached some of the bound scFv antibody from the IDA chelating resin. The washing step of the RBA column can be investigated with a suitable operation flow rate and imidazole concentration.

The His_6 tagged scFv antibody was not seen in the flow-through samples taken during EBA chromatography. A very small amount of the His_6 tagged scFv antibody was leached from the IDA chelating resin during the EBA column washing step. This indicated that EBA column washing with buffer supplemented with 10 mm imidazole can be investigated further to increase the step yield. For EBA column, an increase in the operational flow rate is not possible as that will have an impact on the steady-state condition of the EBA column and more resin will be lost from the column.

The particle size distribution study results show the presence of yeast cells with the scFv antibody captured using RBA chromatography. This result suggests that extended washing of the RBA column in the reverse direction will improve the efficiency of removal of cell and cell debris attached to the resin and frits. The particle size distribution study on the samples taken during EBA chromatography

shows that resin particles were not present in the waste samples while applying the fermenter culture on the column and that steady-state expansion of the EBA was maintained by manually adjusting the culture application flow rate. The wash sample taken after application of the 15 L wash buffer shows the presence of EBA resin particles because the application flow rate was increased at the end of the washing step. This result indicates that careful adjustment of the flow rate is necessary to maintain the steady-state expansion of the EBA column. The particle size distribution study results show the presence of yeast cell clumps with the scFv antibody captured using EBA chromatography. This result suggests that washing the EBA column in downflow before eluting the product is required to wash out accumulated yeast cells at the bottom net.

The scFv antibody shMFELL2csy purified using EBA and RBA has specificity and affinity for CEA, which is expressed on cancer cells. While comparing HCP concentration higher nonspecific binding of the HCP was measured in the EBA eluted scFv antibody sample compared to the RBA eluted scFv antibody. There was also a higher HCP concentration in the final purified scFv antibody when using the EBA column compared to the RBA column. The HCP of the RBA and EBA eluted protein is withing the limit to be used for therapeutic and diagnostic purposes. The HCP contamination in the scFv antibody purified using RBA chromatography was 0.08 µg/mL. The HCP contamination in the scFv antibody purified using EBA chromatography was 0.16 µg/mL which is twice as much as in the scFv antibody purified using RBA chromatography. Therefore, preference is given to use scFv antibody shMFELL2cys purified using RBA chromatography process to be used for therapeutic and diagnostic purposes.

Chapter 4 An experimental investigation of the His_6 tagged scFv antibody binding conditions on the expanded and radial bed resins

4.1 Abstract

An investigation of the conditions for binding capacity of the His_6 tagged scFv antibody shMFELL2cys to the IMAC resin used in both EBA and RBA chromatography was performed on a small scale (1 mL). Application of the crude viscous fermentation culture directly on to the resin reduced both the static and dynamic binding capacity of the resin. Experiments were performed to measure the effect of loading such a viscous material on the static and dynamic binding capacity of the EBA and RBA resins by adding 20% (v/v) glycerol to the purified His_6 tagged scFv antibody on a small scale (1 mL). Aim of the experiments were to investigate the effect of viscous material on the static and dynamic binding capacity of the EBA and RBA resin at small scale (1 mL) therefore experiments were performed using purified His_6 tagged scFv antibody supplemented with 20% (v/v) glycerol and without yeast cells. A method scouting study was also performed using fermenter supernatant to measure the effect of pH and salt molarity on the binding capacity of the EBA and RBA resins. The effect of residence time on the binding capacity of the His_6 tagged scFv antibody shMFELL2cys to the IDA Chelating Cellthru™ resin packed in the RBA column was also studied by applying end of fermenter culture directly on three separate RBA columns.

4.2 Introduction

Metal ion copper was immobilised on the EBA and RBA resins to coordinate bond formation with the His_6 tag of the scFv antibody shMFELL2cys. Metal ion copper and STREAMLINE™ chelating resin have been used previously for the purification of clinical-grade scFv antibody MFECP expressed in high cell density *P. pastoris* fermentation and using a STREAMLINE 50 expanded bed column [37]. Bacterially expressed scFv MFE-23 has also been purified using metal ion copper and IDA chelating resin packed in axial column for a clinical trial [24]. Variation in salt

concentration in the binding buffer changes the electrostatic interaction of the protein with the stationary phase and has a direct impact on the product yield [115]. pH of the buffer controls protonation of histidine, and imidazole, a histidine analog, competevely reacts with metal ions and effects the binding efficiency of the His tagged protein [108, 116]. The impact of salt concentration on the binding capacity of the His_6 tagged scFv antibody was studied on a small scale (1 mL) using EBA and RBA resins in batch mode. Affinity of the His_6 tag to metal ion copper sulphate immobilised on to the EBA and RBA resin at different pH was also investigated in a small-scale experiment.

The 20% (v/v) glycerol was supplemented with scFv antibody to stimulate viscous fermentation culture while measuring the static and DBC of the EBA and RBA resins. Glycerol was used to mimic the viscous fermenter culture as it was recommended to reduce the nonspecific binding of the protein and increase the solubility and stability of the protein [117, 118]. Calculation of the DBC of the resins pre- and post-CIP cycles was recommended to study the ageing of the resin in biopharmaceutical industries [119]. The DBC of the EBA and RBA resins was calculated pre- and post-CIP cycles to investigate the effect of cleaning agent on the amount of scFv antibody that can be purified at the operational flow rate. The static and dynamic binding capacity of the IDA Chelating Cellthru™ and STREAMLINE™ Chelating resin was calculated as per the method detailed in Sections 2.15 and 2.16 of Chapter 2. The effect of pH on the static binding capacity of the His_6 tagged scFv antibody to the resin in batch mode was also calculated according to the method detailed in Section 2.15.2 of Chapter 2.

4.3 Study objectives

The main objectives for this chapter are listed below.

- Measure the static binding capacity of the EBA and RBA resins to calculate the total amount of the His_6 tagged scFv antibody captured using 1 mL settled EBA and RBA resins in batch mode and to identify suitable pH condition for antibody purification.

- Measure the DBC of the EBA and RBA resins to investigate the total amount of the His_6 tagged scFv antibody that can be captured per 1 mL of resin packed in axial column at operational flow rate.
- Measure the effect of loading viscous material on the static and DBC of the EBA and RBA resin to measure the total amount of the His_6 tagged scFv antibody purified by stimulating the viscous fermenter feed.
- Investigate the effect of the pH and salt molarity on the binding efficiency of the His_6 tagged scFv antibody shMFELL2cys to the EBA and RBA resins.
- Investigate the effect of residence time on the binding capacity of the IDA Chelating Cellthru™ resin packed in the RBA column to purify the His_6 tagged scFv antibody shMFELL2cys directly from the fermenter culture.

4.4 Static binding studies

Static binding capacity studies of the EBA and RBA resins were performed by applying purified His_6 tagged scFv antibody shMFELL2cys at pH 8.02 in a binding buffer (Section 2.15, Chapter 2). Static binding capacities of 35 mg/mL and 31 mg/mL were measured for EBA and RBA resins, respectively (Table 4.1), after a total 27 mg and 20 mg scFv antibody was eluted from the EBA and RBA resins, respectively. Antibody elution was performed to calculate the total amount His_6 tagged scFv antibody shMFELL2cys that can be recovered after measuring static binding capacity. Figure 4.1 shows a single band of the His_6 tagged scFv antibody shMFELL2cys at 27 kDa from the samples collected during EBA and RBA static binding condition experiments.

Table 4.1 EBA and RBA static binding results

Resin type	Total His_6 tagged scFv antibody shMFELL2cys applied (mg/mL)	Static binding capacity (mg/mL)	Eluted His_6 tagged scFv antibody shMFELL2cys (mg/mL)
EBA	56	35	27
RBA	49	31	20

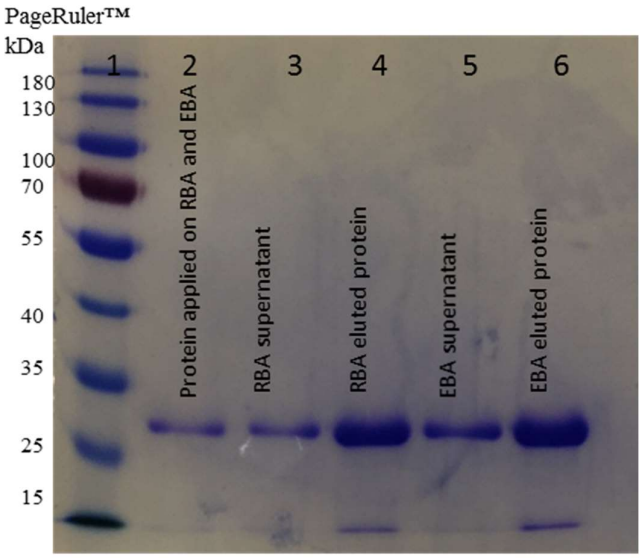

Figure 4.1 SDS-PAGE analysis of eluted protein from static binding samples.

SDS-PAGE analysis of the samples collected during static binding experiments. SDS-PAGE gel was stained with Coomassie blue. A single band of the His_6 tagged scFv antibody can be seen at 27 kDa for all samples. All samples were mixed with 4x SDS-PAGE reducing buffer and run on a 10% SDS-PAGE gel. Sample details are described in the table below.

Sample lane	**1**	**2**	**3**	**4**	**5**	**6**
Sample volume	10 µL	5 µL	5 µL	5 µL	5 µL	5 µL
Sample detail	Molecular weight marker	Protein applied on EBA and RBA resins	RBA shake flask supernatant	Eluted protein from RBA resin	EBA shake flask supernatant	Eluted protein from EBA resin

4.4.1 Static binding experiments of the EBA and RBA resins at different pH values

Static binding capacities of the EBA and RBA resins were examined by applying purified His_6 tagged scFv antibody shMFELL2cys to the binding buffer at pH 7.0, 7.5, 8.0 and 8.5 (Section 2.15.2, Chapter 2). In total, 38 mg of the His_6 tagged scFv antibody shMFELL2cys was applied at each condition. Figure 4.2 represents a comparison graph of the static binding capacities of the EBA and RBA resins at four different pH values and lists the static binding capacity results at different pH values. No significant trends in static binding capacity of the EBA resin with change in pH was observed. However, maximum static binding capacity of the RBA resin was measured at pH 8.0 and decreased at pH 8.5. A 20% increase in the static binding capacity of the RBA resin was observed from pH 7.0 to pH 8.0. A 50% decrease in the static binding capacity of the RBA resin was observed at pH 8.5 compared to pH 8.0. Combination of buffer composition and pH had affected the binding of His_6 tagged scFv to RBA resin. For the EBA resin at the various pH set points with the same buffer composition His_6 tagged of the scFv antibody may have been deprotonated and bound to metal ion copper or it could be because of the high amount of metal ion copper bound to the EBA resin. That was not the case for the RBA resin.

In this study, an IDA chelating agent was used in the primary purification step in the RBA and EBA chromatography. The chelating agents IDA and NTA are mainly used in IMAC. NTA ligand binds strongly to metal ion in comparison to IDA ligand because it has 4 valencies available to bind in comparison to 3 valencies of IDA ligand. Leaching of the copper ion from IDA chelating agent and low amount of metal ion bound to the RBA resin could have resulted in reduction of static binding capacity of the RBA resin with increase in the pH value from pH 8.0 to pH 8.5. With increase in the pH value from pH 8.0 to pH 8.5 50% decrease in the static binding capacity of the RBA resin was measured which could be because of the amount of copper ion leached from the IDA chelating agent.

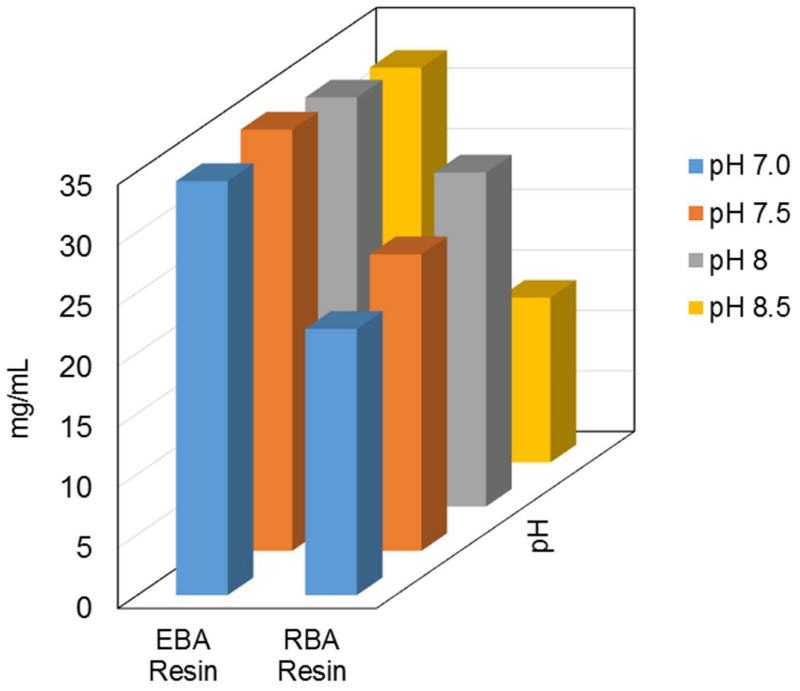

Figure 4.2 Comparison of static binding capacity of EBA and RBA resins at pH 7.0, 7.5, 8.0 and 8.5.

The graph represents a comparison of the static binding capacities of the EBA and RBA resins at different pH values. The results are detailed in the below table 4.2. Static binding capacity of the EBA resin had no significant change with change in pH. Static binding capacity of the RBA resin increased with an increase in pH from pH 7.0 to 8.0 and decreased at pH 8.5.

Table 4.2 EBA and RBA static binding results at pH 7.0, 7.5, 8.0 and 8.5.

	Resin type	pH			
		7.0	**7.5**	**8.0**	**8.5**
Static binding capacity (mg/mL)	**EBA**	34	35	34	33
	RBA	22	25	28	14

4.4.2 Static binding experiments of the EBA and RBA resins supplemented with 20% glycerol to simulate viscous fermenter feed

Static binding capacities of the EBA and RBA resins were investigated by applying purified His_6 tagged scFv antibody shMFELL2cys at pH 7.5 in binding buffer supplemented with 20% (v/v) glycerol to simulate the viscous fermenter feed (Section 2.15.1, Chapter 2). Table 4.3 lists the static binding capacity results for the EBA and RBA resins with and without 20% glycerol in binding buffer at pH 7.5.

Figure 4.3 shows the graphical representation of the static binding capacity result with and without the presence of 20% glycerol. Results show that the static binding capacities of EBA and RBA resins decreased by 32% and 27% in the presence of 20% glycerol, respectively. Adding 20% glycerol to the binding buffer in the presence of the His_6 tagged scFv antibody represented a viscous fermenter culture while performing the static binding capacity of the resin. The results show that applying a crude viscous feed directly on to the EBA and RBA resins in STREAMLINE 50 EBA and 250 mL RBA column reduces the DBC.

Table 4.3 EBA and RBA static binding results with and without 20% glycerol.

No.	Resin type	Protein applied (mg)	Static binding capacity (mg/mL)	Eluted His_6 tagged scFv antibody shMFELL2cys (mg)	% decrease in static binding capacity in comparison to without glycerol experiment
1	RBA without glycerol	39	35	32	0
2	RBA with 20% glycerol	38	26	20	27
3	EBA without glycerol	39	37	28	0
4	EBA with 20% glycerol	37	26	26	32

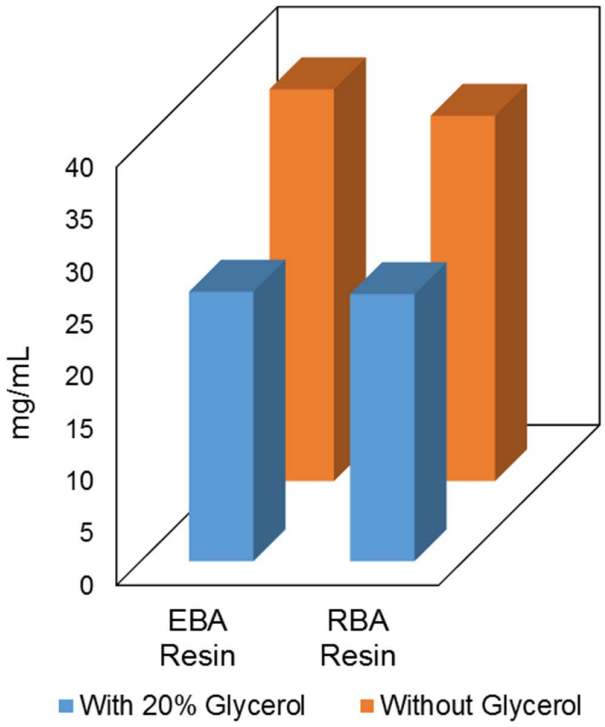

Figure 4.3 Comparison of static binding capacity of EBA and RBA resin with and without 20% glycerol.
Static binding capacities of 35 mg/mL and 37 mg/mL were measured for the EBA and RBA resins without glycerol, respectively. A static binding capacity of 26 mg/mL was measured for both the EBA and RBA resins supplemented with 20% (v/v) glycerol in the binding buffer.

4.5 Dynamic binding capacity of the EBA and RBA resins using His_6 tagged scFv antibody shMFELL2cys

DBC of the EBA and RBA resins was studied using 1 mL settled resin packed in 1 mL FliQ column in axial flow (Section 2.16, Chapter 2) to measure the total amount of scFv antibody purified at operational flow rate. Purified His_6 tagged scFv antibody shMFELL2cys was applied at 0.3 mL/min, equivalent to flow velocity of 60 cm/h in axial flow. The effect of a viscous material on the DBC of the EBA and RBA resin was also measured by adding final concentration of 20% (v/v) glycerol to the His_6 tagged scFv antibody. EBA and RBA resin DBC was measured pre- and post-10 CIP cycles with 0.5 M NaOH. This study was performed to investigate the effect of cleaning agent on the EBA and RBA resins. The effect of the cleaning agent on the chelating agent was calculated by measuring the DBC of the resins before and after the CIP cycles.

4.5.1 RBA resin DBC

The DBC of the RBA resin packed in 1mL FliQ column was measured at 10% breakthrough pre- and post-CIP cycles with 0.5 M NaOH. The DBC calculated at 10% breakthrough was 5.8 mg/mL and 6 mg/mL pre- and post-CIP cycles, respectively.

Figures 4.4 show the section of the RBA DBC experiment chromatogram pre- and post-CIP cycles. Figure 4.5 shows the full chromatogram of the RBA DBC experiment.

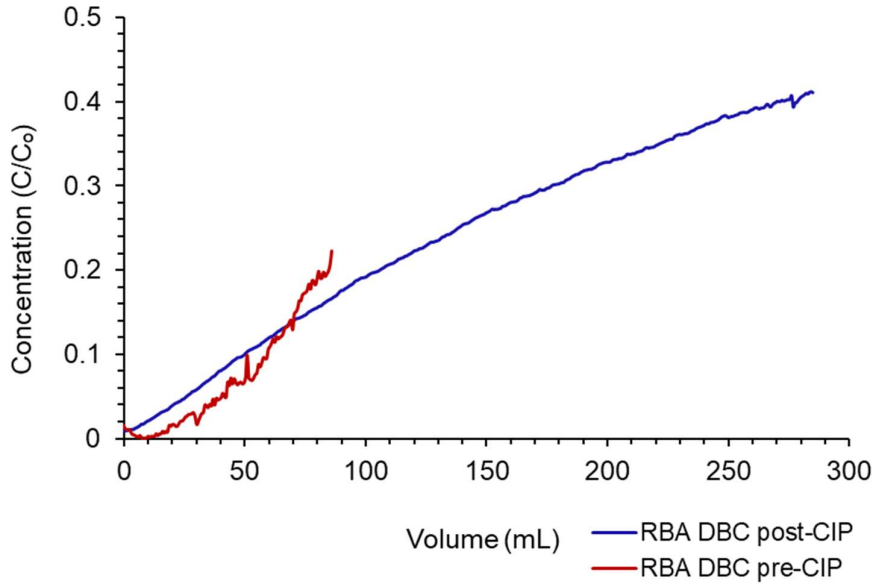

Figure 4.4 His_6 tagged scFv antibody breakthrough curve on 1 mL RBA resin packed in axial column at 0.3 mL/min flow rate performed pre- and post-CIP cycles.

The graph represents the His_6 tagged scFv antibody breakthrough curve against the volume applied. The RBA resin DBC calculated at 10% breakthrough was 5.8 mg/mL and 6.0 mg/mL for pre- and post-CIP cycles, respectively. Antibody concentration of 0.08 mg/mL and 0.1 mg/mL was applied at 0.3 mL/min flow rate for pre- and post-CIP cycles, respectively.

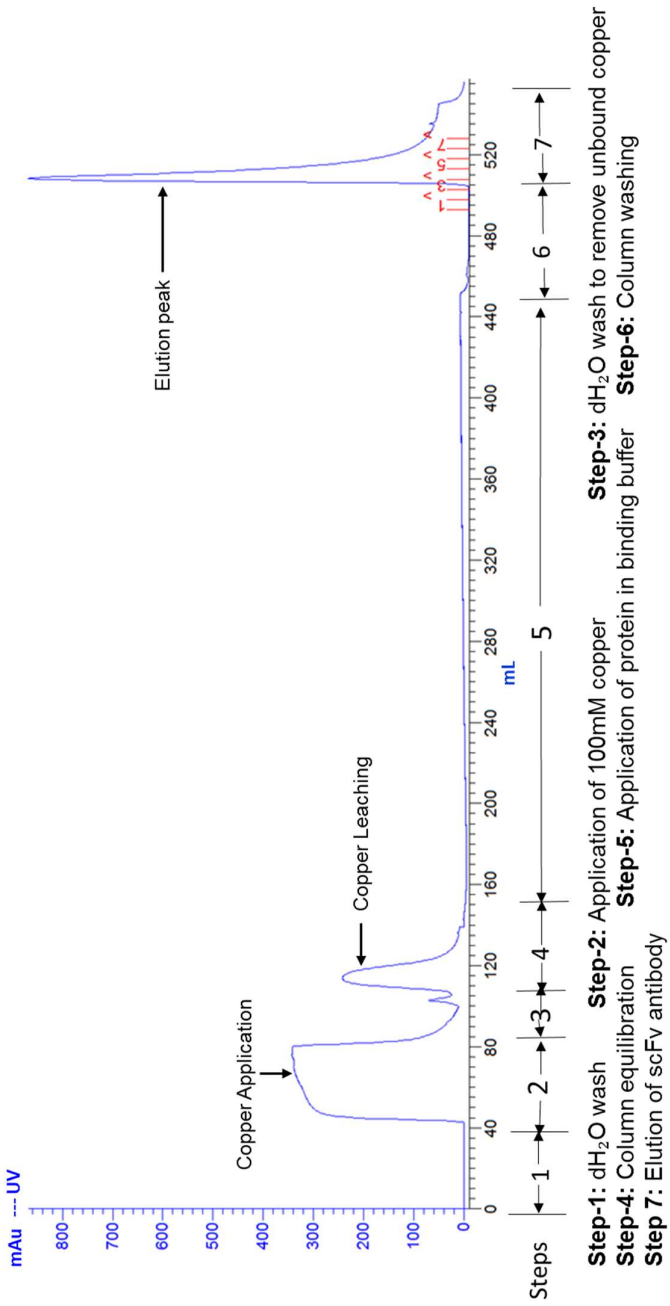

Figure 4.5 Full chromatogram of RBA DBC experiment post-CIP cycles in 1 mL FliQ column.

The full chromatogram of the RBA DBC experiment represents column wash (Step 1), copper application (Step 2), washing of unbound copper (Step 3), column equilibration (Step 4), protein application for DBC measurement (Step 5), column washing (Step 6) and scFv antibody elution (Step 7) steps.

4.5.2 EBA resin DBC

The DBC of the EBA resin packed in a 1 mL FliQ column was measured at 10% breakthrough pre- and post-CIP cycles with 0.5 M NaOH. The DBC calculated at 10% breakthrough was 30 mg/mL and 29 mg/mL pre- and post-CIP cycles, respectively. Figure 4.6 show the section of the EBA DBC experiment chromatogram pre- and post-CIP cycles. Figure 4.7 shows the full chromatogram of the EBA DBC experiment.

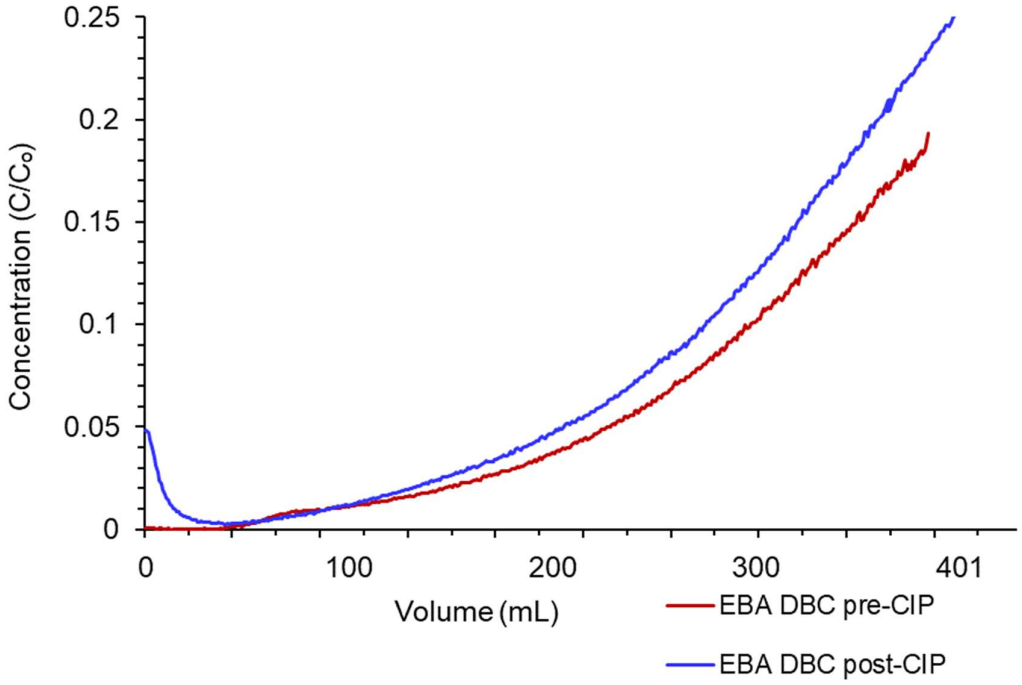

Figure 4.6 His_6 tagged scFv antibody breakthrough curve on 1 mL EBA resin packed in axial column at 0.3 mL/min flow rate performed pre- and post-CIP cycles.

The graph represents the His_6 tagged scFv antibody breakthrough curve against the volume applied. The RBA resin DBC calculated at 10% breakthrough was 28 mg/mL and 30 mg/mL for pre- and post-CIP cycles, respectively. Antibody concentration of 0.1 mg/mL was applied at 0.3 mL/min flow rate for pre- and post-CIP cycles.

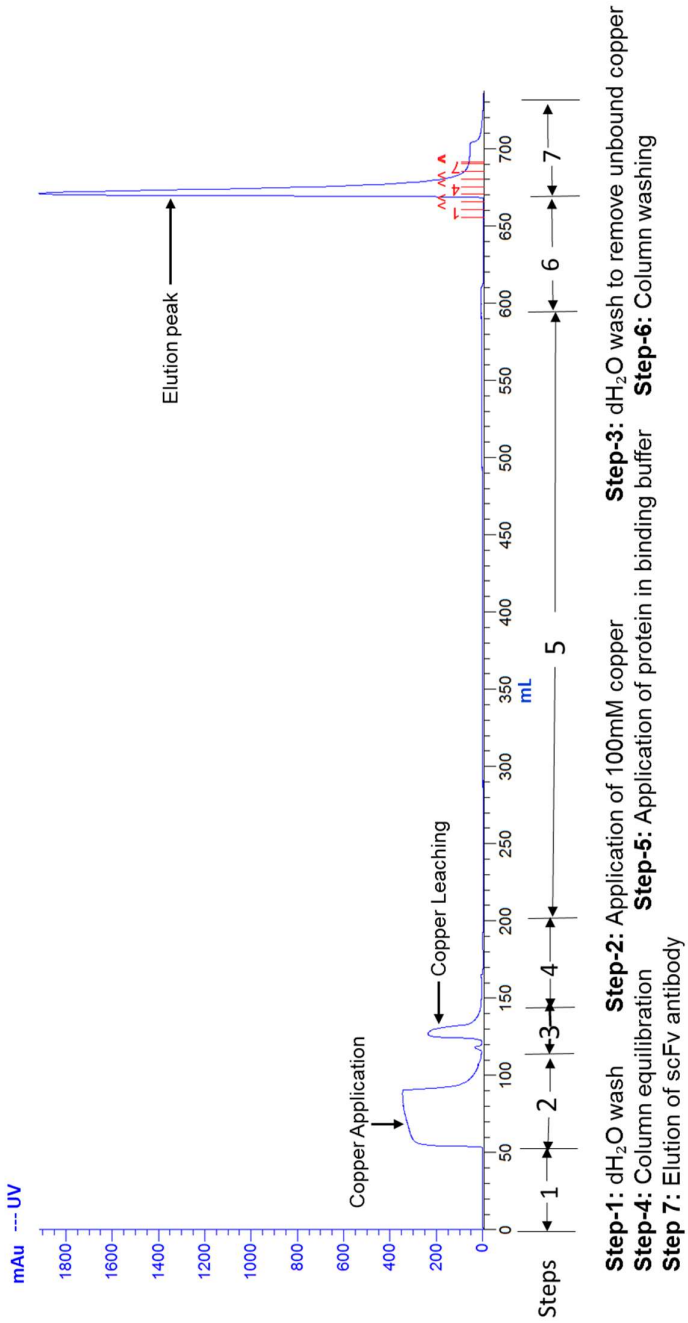

Figure 4.7 Full chromatogram of EBA DBC experiment post-CIP cycles in 1 mL FliQ column.

The full chromatogram of the EBA DBC experiment represents column wash (Step 1), copper application (Step 2), washing of unbound copper (Step 3), column equilibration (Step 4), protein application for DBC measurement (Step 5), column washing (Step 6) and scFv antibody elution (Step 7) steps.

4.5.3 EBA resin DBC experiment in presence of 20% (v/v) glycerol

The DBC of the EBA resin in the presence of 20% (v/v) glycerol packed in 1 mL FliQ column was measured at 10% breakthrough. The 20% (v/v) glycerol was added into the binding buffer together with the His_6 tagged scFv antibody to create a viscous fluid that mimics a high-density fermentation broth. A DBC of 16 mg/mL was measured at 10% breakthrough. A 53% decrease in the DBC of the EBA resin was observed in the presence of 20% glycerol (v/v). Figure 4.8 shows the section of the EBA and RBA DBC experiment chromatogram in the presence of 20% (v/v) glycerol.

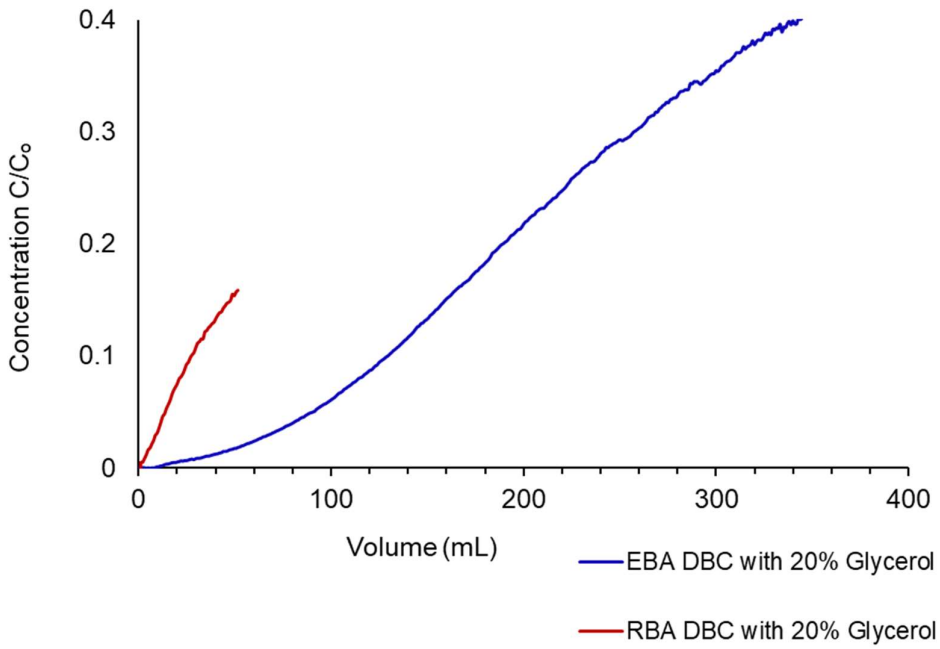

Figure 4.8 His_6 tagged scFv antibody breakthrough curve on 1 mL RBA and EBA resin packed in axial column at 0.3 mL/min flow rate supplemented with 20% (v/v) glycerol.

The graph represents the His_6 tagged scFv antibody breakthrough curve against the volume applied. The EBA resin DBC calculated at 10% breakthrough was 16 mg/mL and the RBA resin DBC calculated at 10% breakthrough was 3 mg/mL. Antibody concentration of 0.12 mg/mL supplemented with 20% (v/v) glycerol was applied at 0.3 mL/min flow rate for both EBA and RBA DBC experiment.

4.5.4 RBA resin DBC experiment in presence of 20% (v/v) glycerol

The DBC of the RBA resin in the presence of 20% (v/v) glycerol packed in 1 mL FliQ column was measured at 10% breakthrough. Glycerol was added into the binding buffer together with a His_6 tagged scFv antibody to create a viscous fluid. A DBC of 3 mg/mL was measured at 10% breakthrough. A 53% decrease in the DBC of the RBA resin was observed in the presence of 20% glycerol compared to DBC without glycerol. Figure 4.8 shows the section of the RBA DBC experiment chromatogram in the presence of 20% (v/v) glycerol.

4.6 Small-scale EBA and RBA screening experiments

Harvest fluid contains a relatively low concentration of salt ions, which are not enough to block electrostatic interaction during the load step. The conductivity of the fermenter feed may play an important part in blocking the electrostatic interaction during the primary capture step. Screening experiments were performed to measure the optimum level of buffer components for the His_6 tagged scFv antibody shMFELL2cys to bind to the EBA or RBA IMAC resin. One millilitre of resin was used in batch mode for screening experiments with the end of fermenter culture stored in a -80°C freezer post-centrifugation and 0.2 µm filtration step (Chapter 2). Two key IMAC variables, pH and salt concentration, were investigated in batch mode of binding. Experiments were performed at three pH values of 7.5, 8.0 and 8.5 with a combination of three different salt (NaCl) concentrations — 0.5 M, 0.75 M and 1 M.

In total, 100 mL and 50 mL filtered supernatants were applied to the 1 mL aliquots of EBA and RBA resins, respectively, diluted in binding buffer (Section 2.16.2, Chapter 2). A total protein concentration of 1.61 mg/mL was measured in the filtered fermenter supernatant (Chapter 3). The concentration of the His_6 tagged scFv antibody in the filtered fermenter supernatant mentioned above was 0.46 mg/mL (Chapter 3).

4.6.1 Results of EBA screening experiment

The binding capacity of the EBA resin was calculated based on the total protein concentration remaining in the supernatant. A decrease in the unbound protein concentration represented an increase in the binding capacity of the resin.

Figure 4.9 shows a graphical representation of the unbound protein concentration at three pH values of 7.5, 8.0 and 8.5 with a combination of three different salt (NaCl) concentrations — 0.5 M, 0.75 M and 1 M. The binding capacity of the EBA resin was found to decrease with increase in pH (pH 7.5 to 8.5) at all three salt concentrations. An increase in salt concentration from 0.5 M to 1.0 M at pH 7.5 and 8.0 did not affect the binding capacity of the His_6 tagged antibody to the EBA resin. At pH 8.5, the binding capacity decreased as the salt concentration increased from 0.5 M to 1.0 M. While performing screening experiments, a flask with 0.75 M salt concentration and pH 8.5 came off the flask holder inside the incubator and the supernatant was spilled inside the incubator. Hence, an increase in the binding capacity at salt concentration of 0.75 M from 0.5 M at pH 8.5 was recorded. The experiment with 0.75 M salt and pH 8.5 was not repeated because a decrease in the binding capacity from 0.5 M to 1.0 M salt was recorded. Therefore, it was assumed that at 0.75 M salt concentration and at pH 8.5, binding capacity would decrease in comparison to 0.5 M salt concentration at the same pH.

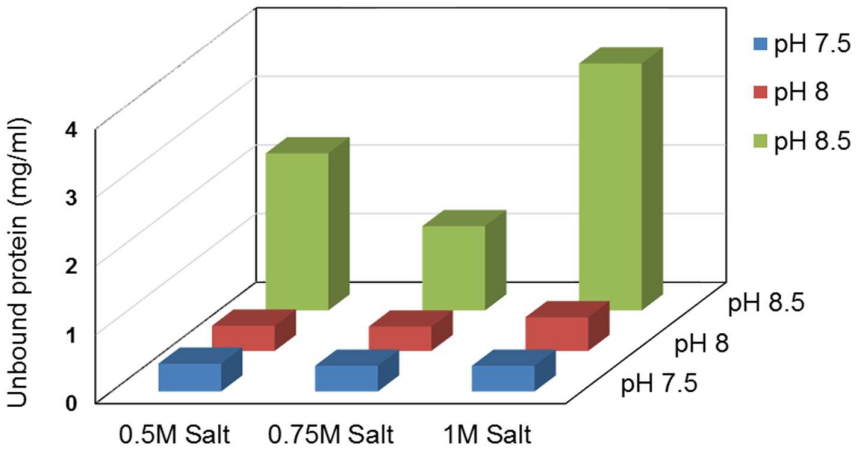

Figure 4.9 EBA resin screening experiment results graph.
The graph represents the binding capacity of the His_6 tagged scFv antibody at pH 7.5, 8.0 and 8.5 with a combination of three salt (NaCl) concentrations — 0.5 M, 0.75 M and 1 M. A decrease in the unbound protein concentration represents an increase in the binding capacity of the resin. An increase in salt concentration at pH 7.5 and pH 8.0 did not affect the binding capacity of the resin. At pH 8.5, the binding capacity decreased with an increase in the salt concentration from 0.5 M to 1.0 M. Data is presented in the below table 4.4.

Table 4.4 EBA resin screening experiment result at pH 7.5, 8.0 and 8.5 with a combination of three salt (NaCl) concentrations — 0.5 M, 0.75 M and 1 M.

	Unbound protein concentration in supernatant (mg/mL)		
	0.5 M (NaCl)	**0.75 M (NaCl)**	**1.0 M (NaCl)**
pH 7.5	0.41	0.38	0.38
pH 8.0	0.379	0.367	0.49
pH 8.5	2.29	1.22	3.60

4.6.2 Results of RBA screening experiment

It was noted that with an increase in the salt concentration at pH 7.5, the binding capacity of the His_6 tagged scFv antibody to the RBA resin increased. However, at pH 8.0 and pH 8.5 it was observed that as the salt concentration increased from 0.5 M to 1.0 M, the binding capacity of the RBA resin decreased. At a fixed high salt concentration of 1.0 M, a decrease in the binding capacity of RBA resin from pH 7.5 to 8.5 was measured. The optimal binding capacity of RBA resin was achieved at a high salt concentration of 1.0 M and pH 7.5; hence, these parameters were selected to capture the His_6 tagged scFv antibody shMFELL2cys directly on

the RBA resin. There is a clear correlation between the salt concentration and pH and the binding capacity of the RBA resin. The results are summarised in Figure 4.10.

Statistically this result is not significant. Experiment was performed once, and each condition were tested in duplicate. Total protein concentration in the supernatant was measured to measure the effect of pH and salt concentration on the binding capacity of EBA or RBA resin. Static binding capacity experiment result had concluded that at pH 8.5 scFv binding capacity to the RBA resin decreases, although that experiment was performed at fixed salt concentration of 1M (step 4.4.1). Experimentally similar result was seen at pH 8.5 with various salt concentration while using fermenter supernatant to screen the suitable binding condition. High salt concentration decreases the nonspecific binding of the protein to the resin. Therefore, scientifically combination of salt concentration and pH had affected the binding of His_6 tagged scFv to RBA resin. Although buffer composition was same for both the RBA and EBA resin screening experiment, the RBA resin had significant changes in the binding capacity in comparison to EBA resin. Buffer salt concentration is an important factor in IMAC chromatography. Variation in binding capacity with salt concentration indicates that nonspecific interaction is important [115].

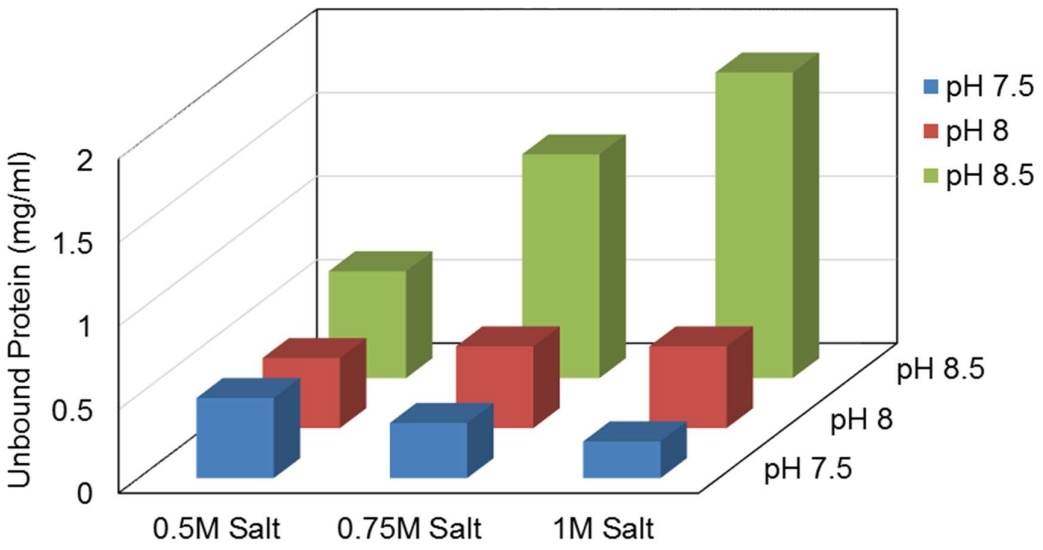

Figure 4.10 RBA resin screening experiment results graph.
This graph represents the unbound protein concentration (mg/mL) at pH 7.5, 8.0 and 8.5 with a combination of three salt (NaCl) concentrations — 0.5 M, 0.75 M and 1 M. A decrease in the unbound protein concentration represents an increase in the binding capacity of the resin. An increase in salt concentration at pH 7.5 increased the binding capacity of the His_6 tagged scFv antibody to the resin. At pH 8.0 and 8.5, binding capacity decreased with an increase in the salt concentration. Data is presented in the below table 4.5.

Table 4.5 RBA resin screening experiment result at pH 7.5, 8.0 and 8.5 with a combination of three salt (NaCl) concentrations — 0.5 M, 0.75 M and 1 M.

	Unbound protein concentration in supernatant (mg/mL)		
	0.5 M (NaCl)	**0.5 M (NaCl)**	**0.5 M (NaCl)**
pH 7.5	0.48	0.33	0.22
pH 8.0	0.42	0.49	0.49
pH 8.5	0.64	1.34	1.83

4.7 Effect of residence time on the direct capture of the His_6 tagged scFv antibody via RBA chromatography

A fermentation run was performed to investigate the effect of residence time on the direct capture of the His_6 tagged scFv antibody simultaneously on three RBA columns. Chelating agent copper sulphate was used in all three columns. One litre of the end of fermentation culture was applied (mixed with 1 L buffer, 2 M NaCl, 2 x PBS and 20 mM Imidazole) at (i) 200 mL/min on the first 125 mL RBA column (ii) 40 mL/min on the second 125 mL RBA column and (iii) 15 mL/min on the third 250 mL RBA column side by side. Residence times of 40 s at 200 mL/min, 3.5 min at 40 mL/min and 18 min at 15 mL/min were calculated. The total pooled eluted protein concentrations of the radial beds were (i) 197 mg for residence time of 40 s on 125 mL RBA column, (ii) 437 mg for residence time of 3.5 min on 125 mL RBA column and (iii) 436 mg for residence time of 18 min on 250 mL RBA column. The total concentrations of the His_6 tag scFv antibody shMFELL2cys were 105 mg, 187 mg and 192 mg, respectively, for columns (i), (ii) and (iii). A two-fold increase in protein concentration was achieved by increasing the residence time from 40 s to 3.5 min. No further increase in protein concentration was achieved by increasing the residence time from 3.5 min to 18 min. It was also noted that the His_6 tagged antibody concentration in flow-through samples fell with increasing residence time.

Figure 4.11 shows the scFv antibody expression levels in the fermenter samples. A single protein band at 27 kDa was seen in the fermenter harvest sample at the end of fermentation harvest and in the harvest supernatant sample. Flow-through samples were taken during the primary culture application steps to analyse the binding efficiency of the column. In Figure 4.12 a), the SDS-PAGE result shows the protein to be flowing through the column at 200 mL/min flow rate and in Figure 4.12 b), the Western Blot analysis result confirms the presence of the His_6 tag on the scFv antibody. In Figures 4.13 a) and b), SDS-PAGE and Western Blot results show a decrease in the amount of protein flowing through the RBA column at 40 mL/min compared to 200 mL/min; availability of a His_6 tag was also confirmed by Western Blot analysis. In Figures 4.14 a) and b), SDS-PAGE and Western Blot results do not show any protein flowing through the RBA column at 18 mL/min

harvest compared to 200 mL/min and 40 mL/min harvest. Therefore, the optimum binding condition of the His_6 tagged scFv antibody using RBA column can be achieved at a lower operating flow rate.

SEC of the 1 mL eluted protein from three RBA columns was run using 125 mL analytical Superdex 75 FPLC column to compare the protein peaks (Figure 4.15). For all three columns, eluted protein (scFv antibody shMFELL2cys) had a dimer peak at 59 mL of the column volume and monomer peak at 69 mL of the column volume. The protein peak for RBA column (ii) was higher than for columns (i) and (iii). The band of protein eluted from RBA column (ii) is thicker than protein eluted from RBA columns (i) and (iii), which can be seen on the SDS-PAGE result (Figure 4.16). Therefore, column (ii) operation flow rate of 40 mL/min was used for the direct capture of the His_6 tagged scFv antibody shMFELL2cys expressed in high cell density *P. pastoris* fermentation.

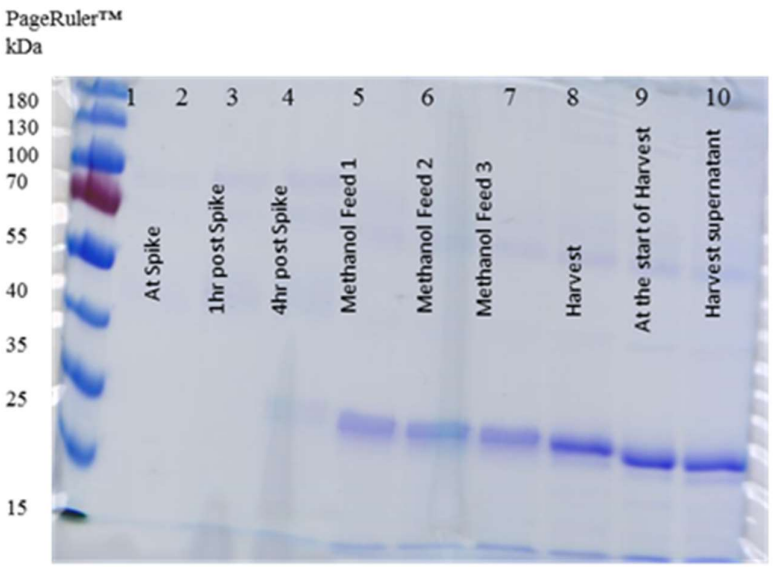

Figure 4.11 SDS-PAGE result of fermentation run samples.
Analysis of the His_6 tagged scFv antibody expression in fermenter and harvest supernatant samples. SDS-PAGE gel stained with Coomassie blue. All samples were mixed with 4x SDS-PAGE reducing buffer and run on a 10% SDS PAGE gel. Sample details are described in the table below. A band of the scFv antibody shMFELL2cys expressed in the fermenter can be seen on the samples loaded from lane 5 to lane 10.

Sample lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sample volume	10 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL
Sample detail	Molecular weight marker	Fermenter sample taken at glycerol spike	Fermenter sample post 1 h spike	Fermenter sample post 4 h spike	Methanol feed 1 (fermenter sample 24 h post spike)	Methanol feed 2 (fermenter sample 48 h post spike)	Methanol feed 3 (fermenter sample 51 h post spike)	Fermenter sample at the start of harvest	Fermenter sample at the end of harvest	Fermenter harvest supernatant sample

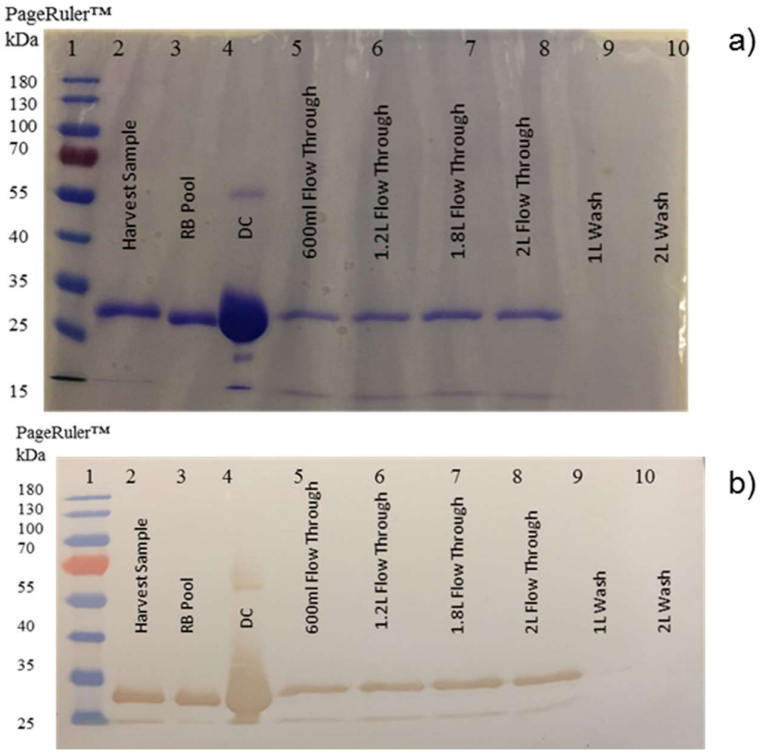

Figure 4.12 SDS-PAGE (a) and Western Blot (b) results of the samples taken during direct application of the fermenter culture on the 125 mL radial bed column at 200 mL/min flow rate.

Analysis of the His_6 tagged scFv antibody in radial bed harvest flow-through and wash samples using SDS-PAGE (a) and Western Blot (b). Antibody bands were seen in the flow-through samples on the SDS-PAGE (a) and the availability of a His_6 tag on the antibody was confirmed with Western Blot (b) analysis. Sample details are described in the table below. Identical samples were run on SDS-PAGE (a) and Western Blot (b).

Sample lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sample volume	10 µL	10 µL	10 µL	10 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL
Sample detail	Molecular weight marker	Fermenter sample at the harvest	Radial bed pool sample	Concentration sample	600mL flow-through sample from radial bed column	1.2 L flow-through sample from radial bed column	1.8 L flow-through sample from radial bed column	2 L flow-through sample from radial bed column	1 L flow-through wash sample from radial bed column	2 L flow-through wash sample from radial bed column

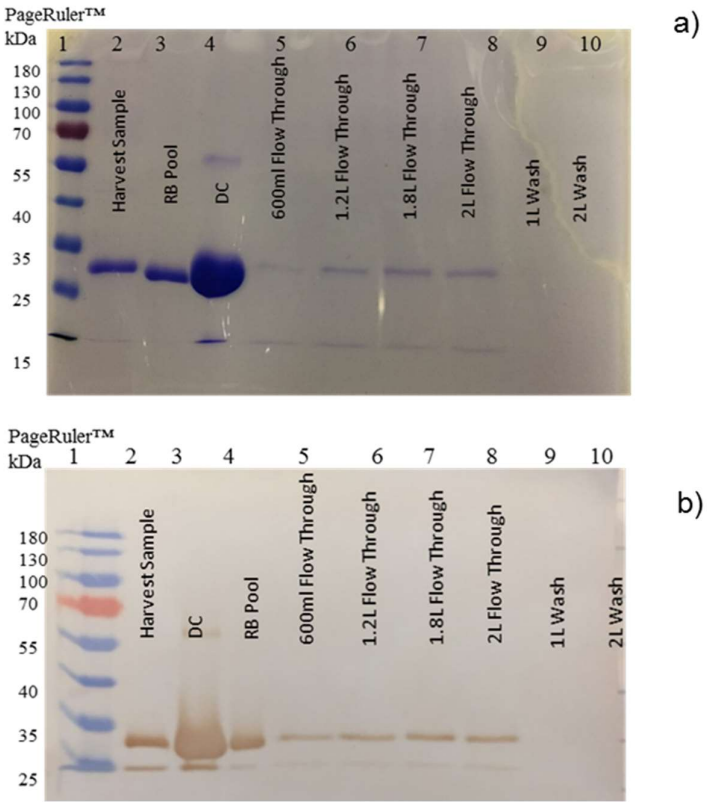

Figure 4.13 SDS-PAGE (a) and Western Blot (b) results of the samples taken during direct application of the fermenter culture on the 125 mL radial bed column at 40 mL/min flow rate.

Analysis of the His_6 tagged scFv antibody in radial bed harvest flow-through and wash samples using SDS-PAGE (a) and Western Blot (b). Thinner antibody bands were seen in the flow-through samples on the SDS-PAGE (a) and availability of a His_6 tagged on the antibody was confirmed with Western Blot (b) analysis. Sample details are described in the table below. Identical samples were run on SDS-PAGE (a) and Western Blot (b).

Sample lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sample volume	10 µL	10 µL	10 µL	10 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL
Sample detail	Molecular weight marker	Fermenter sample at the harvest	Radial bed pool sample	Concentration sample	600 mL flow-through sample from radial bed column	1.2 L flow-through sample from radial bed	1.8 L flow-through sample from radial bed column	2 L flow-through sample from radial bed column	1 L flow-through wash sample from radial bed column	2 L flow-through wash sample from radial bed column

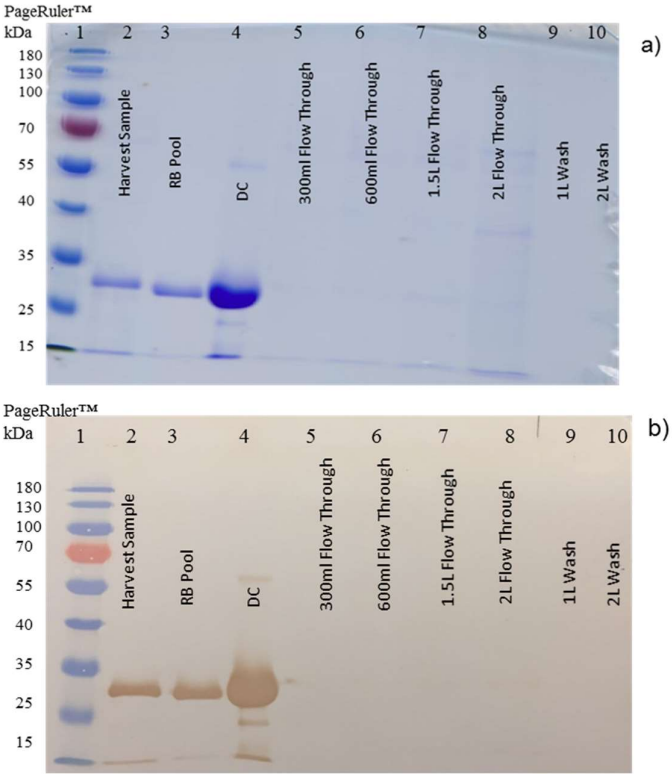

Figure 4.14 SDS-PAGE (a) and Western Blot (b) results of the samples taken during direct application of the fermenter culture on the 250 mL radial bed column at 15 mL/min flow rate.

Analysis of the His_6 tagged scFv antibody in radial bed harvest flow-through and wash samples using SDS-PAGE (a) and Western Blot (b). Antibody bands were not seen in the flow-through samples on the SDS-PAGE (a) and availability of a His_6 tagged of the antibody was confirmed with Western Blot (b) analysis. Sample details are described in the table below. Identical samples were run on SDS-PAGE (a) and Western Blot (b).

Sample lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sample volume	10 µL	10 µL	10 µL	10 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL
Sample detail	Molecular weight marker	Fermenter sample at the harvest	Radial bed pool sample	Concentration sample	300 mL flow-through sample from radial bed column	600 mL flow-through sample from radial bed column	1.5 L flow-through sample from radial bed column	2 L flow-through sample from radial bed column	1 L flow-through wash sample from radial bed column	2 L flow-through wash sample from radial bed column

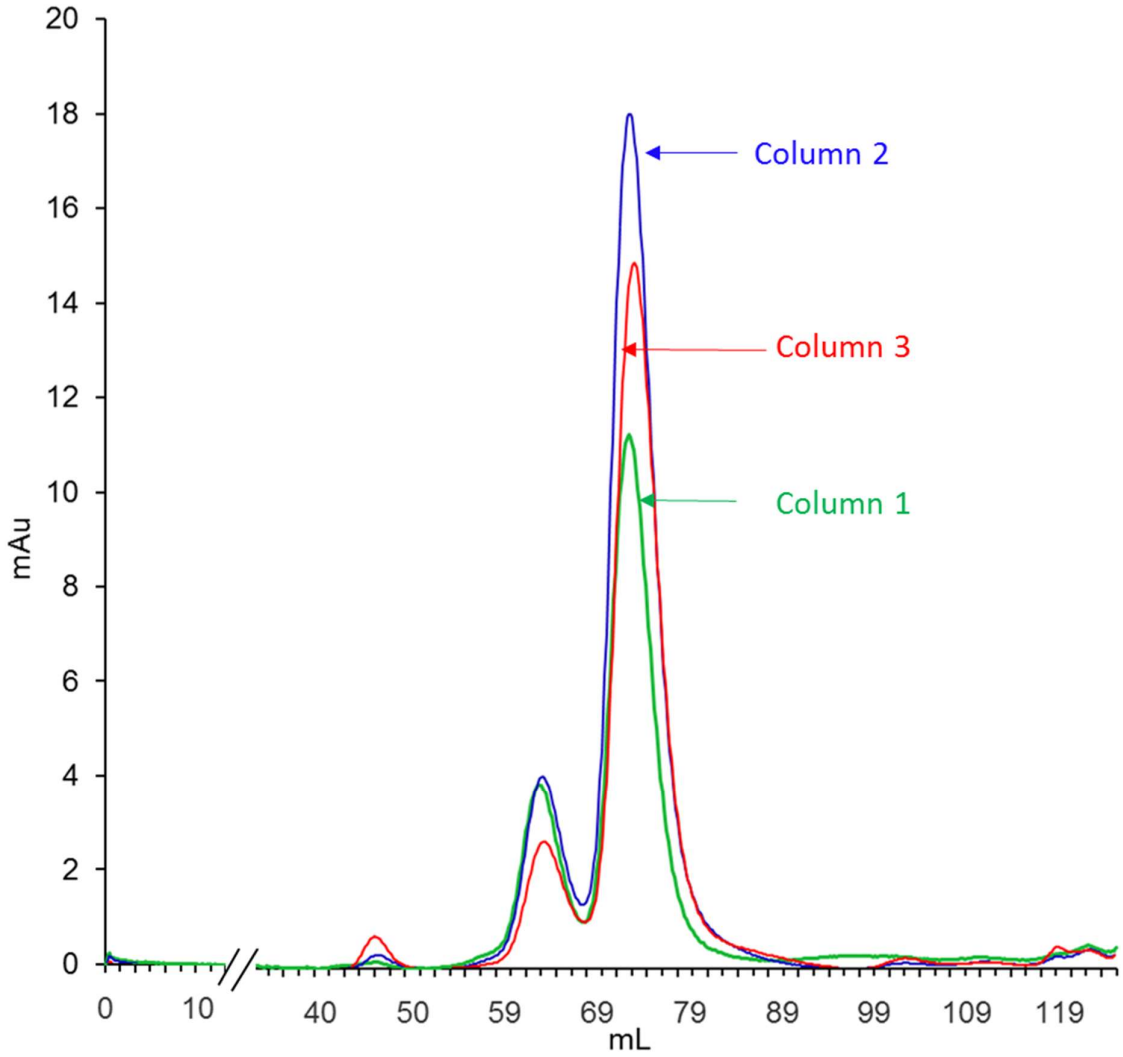

Figure 4.15 Overlay chromatogram of three radial bed eluted pooled proteins run on the 125 mL analytical Superdex 75 FPLC column.
SEC of the 1 mL eluted protein from three radial bed columns were run using 125 mL analytical Superdex 75 FPLC column. An overlay chromatogram of the eluted protein was created to compare the protein peaks and size. All three column eluted proteins (scFv antibody shMFELL2cys) had a dimer peak at 59 mL of the column volume and monomer peak at 69 mL of the column volume. Column 2 had the highest monomer peak in comparison to columns 1 and 3.

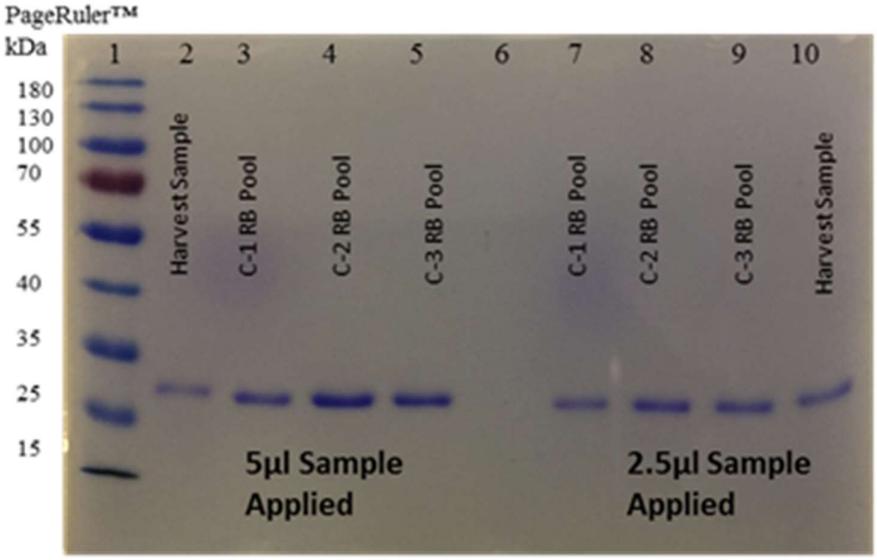

Figure 4.16 SDS-PAGE result of three RBA purified pooled protein samples. Analysis of the His_6 tagged scFv antibody in three radial bed harvest purified pooled protein samples. SDS-PAGE gel stained with Coomassie blue. All samples were mixed with 4x SDS-PAGE reducing buffer and run on a 10% SDS PAGE gel. The band of the protein eluted from radial bed column (ii) is thicker than protein eluted from radial beds (i) and (iii).

4.8 Conclusions

Results from this chapter show that the static and dynamic binding capacities of the EBA resin were higher than those of the RBA resin. The pH had no significant impact on the static binding capacity of the EBA resin. By comparison, for the RBA resin, a 20% increase in static binding capacity was seen when the pH was increased from pH 7.0 to 8.0, which then decreased by 50% if the pH was further increased to pH 8.5. This shows that the static binding capacity of the RBA resin is affected by pH. At pH 8.0 and 8.5 static binding capacity of the RBA resin decreased as reduction in His_6 tagged of the scFv antibody binding to metal ion copper chelated to IDA chelating agent was recorded. This shows that deprotonation of the His_6 tagged of the scFv antibody occurred which then decreased the static binding capacity of the RBA resin. Decrease in the static binding capacity could be because of the amount of copper ion leaching from the IDA chelating agent of the RBA resin. In contrary this was not the case for the EBA resin. Hence this result was not statistically significant.

Introducing a viscous fermenter culture in the presence of 20% v/v glycerol decreased the static binding capacity by 32% and 27%, respectively, for the EBA and RBA resins. A 53% decrease in the DBC of both the EBA and RBA resins was also measured in the presence of 20% v/v glycerol. Hence, viscosity of the end of fermenter culture has a significant impact on the direct capture of the His_6 tagged scFv antibody using EBA and RBA chromatography.

Results from the EBA and RBA screening experiments concluded that 1 M NaCl concentration and pH 7.5 provided the best conditions for the direct capture of the His_6 tagged scFv antibody shMFELL2cys expressed in high cell density *P. pastoris* fermentation using EBA and RBA chromatography. This chapter also demonstrated the significance of the residence time on the direct capture of the His_6 tagged scFv antibody using RBA chromatography. A two-fold increase in the scFv antibody concentration was achieved by increasing the residence time from 40 s to 3.5 min for RBA chromatography. Preliminary experiment with the EBA column shows even at high flow rate of 300 cm/h most of the His_6 tagged scFv antibody bound to the EBA resin therefore binding capacity experiment at lab scale

EBA column was not performed. In comparison while performing preliminary experiment with the RBA column His tagged antibody was leaching in flow through and wash samples Therefore, this experiment was only performed using the RBA column. Preliminary data is not presented in this thesis.

As detailed in chapter 3, results generated in this chapter were used to improve the conditions necessary to perform direct capture of the His_6 tagged scFv antibody shMFELL2cys using EBA and RBA chromatography.

Chapter 5 Economic analysis of EBA and RBA chromatography using BioSolve software

5.1 Abstract

Economic analysis of the EBA and RBA primary chromatography step was performed with the help of the BioSolve software version 7.6 [120]. BioSolve is an Excel-based software developed by Biopharma Services Limited to perform CoG analysis of biopharmaceutical processes [120]. CoG analysis was applied to the putative flowsheet for the recovery and purification of the His_6 tagged scFv antibody shMFELL2cys expressed in a high cell density *P. pastoris* fermentation. The protein was purified using two different primary capture chromatography techniques: EBA and RBA chromatography. CoG analysis was performed using actual data obtained in Chapter 3 to populate the models. EBA and RBA chromatography techniques seek to combine three unit operations: clarification (centrifugation or filtration), concentration and affinity purification, thus aiming to reduce the total cost of production. First, total CoG/g analysis was performed for the manufacture of the scFv antibody based on an 8 L final fermenter volume, purified using either EBA or RBA primary chromatography. Next, the EBA and RBA unit operation CoG were analysed to compare the capital and operational costs for each mode to identify the more cost-effective primary chromatography step.

The BioSolve software can be applied to:

- Generate and compare CoG/g data of the product to be manufactured in a good manufacturing (GMP) environment for use in clinical trials and large-scale fermenter volumes using experimental data generated on a small scale for both the EBA and RBA chromatography steps.
- Aid decision-making based on cost of capital expenditures from small- to large-scale processes, including the cost of building a new facility or refurbishing an existing facility. Evaluation of the operation cost, which is mainly divided into the cost of labour, materials and consumables. There are also some indirect costs associated with running each unit operation.

For example, costs for the safe disposal of waste, maintenance of equipment and cost of running the utilities.

The main objectives for this chapter are listed below.

5.2 Objectives

- Generate typical EBA and RBA worksheets for yeast process using BioSolve software.
- Perform cost base analysis of the His_6 tagged scFv antibody shMFELL2cys manufactured in a high cell density *P. pastoris* fermentation process and purified using EBA and RBA primary adsorption chromatography.
- Perform comparison analysis between the unit operations of EBA and RBA primary chromatography with the above process.
- Evaluate the effect of scale-up on the CoG analysis of the EBA and RBA primary purification processes.
- Perform scenario analysis of the EBA and RBA primary purification processes to compare the CoG/g at different expression levels and fermenter volumes.
- Evaluate the interaction of the factors—fermentation yield, EBA and RBA step recovery, EBA and RBA resin binding capacity and total number of CIP cycles—on the CoG/g and CoG/batch of the EBA and RBA processes at 2000 L production scale using response surface design and employing the design of the experiment software.

5.3 Results

5.3.1 Creation of typical EBA and RBA worksheet based on scFv antibody production in the high cell density *P. pastoris* fermentation process

Typical EBA and RBA process worksheets were created in BioSolve process software version 7.6 using a microbial process worksheet saved in the process library. Some common process inputs, for example 8 L user-defined bioreactor volume, medium and buffer preparation per batch and use of the single-use buffer preparation system, were selected for both the EBA and RBA processes on the software dashboard. Expression levels of 0.60 g/L and 0.68 g/L for the His_6 tagged scFv antibody in the 8 L final fermenter volume were selected for the EBA and RBA worksheets, respectively (Chapter 3). These were the practical data generated at 8L fermenter scale while purifying His_6 tagged scFv antibody using EBA and RBA primary purification processes. Therefore, two separate expression level were selected for the EBA and RBA processes. While performing cost base analysis at various large scale volume same expression levels were selected for both the EBA and RBA processes. Target capacity utilisation time for production activity was set to 80% to project the number of batches that can be manufactured per year using either the EBA or RBA process and to carry out validation and maintenance work. All of the parameters selected on the dashboard for the typical EBA and RBA worksheets are listed in Table 5.1.

In total, seven unit operations were configured from the process library in the same flowsheet. These unit operations were: primary culture flask, (2) secondary culture flask, (3) fed batch production culture, (4) EBA or RBA primary capture step, (5) product concentration, (6) SEC and (7) 0.2 µm sterile filtration. Once all of the operational parameters had been added into the worksheet for each unit operation, product concentration (g/L), total product volume (L), mass (g), operation yield (%) and duration (h) were calculated using the BioSolve software. Cost data, including the materials, consumables, equipment and associated costs, were sourced from individual suppliers and were selected to create a worksheet. Some of the process-specific equipment, such as the STREAMLINE 50 EBA (Chapter 2) and the 250

mL CRIO-MD 63 MK III RBA (Chapter 2) columns, were added into the cost data worksheet. Costs of the EBA and RBA resins were also received from the manufacturer and added into the cost data worksheet. The cost of 300 mL RBA resin was £875 from Sterogene Bioseparations Inc. (Chapter 2) and the cost of 500 mL EBA resin was £1960 from GE Healthcare (Chapter 2).

Table 5.1 BioSolve dashboard parameter settings for base case worksheet.

Input	
Process	
Product titre or expression level	0.6 (g/L)
User-defined production bioreactor working volume	8.0 (L)
Target capacity utilisation	80%
Total number of production bioreactors installed in facility	1
Number of bioreactors pooled per batch	1
Solution preparation	
Medium make-up basis	per batch
Buffer make-up basis	per batch
Single-use systems	
Medium preparation (up to 2000L)	Yes
Buffer preparation (up to 2000L)	Yes
Type of buffer preparation system	Disposable bag liner
Medium and buffer hold (up to 3000L)	Yes
Intermediate product hold (up to 2000L)	Yes
Use stainless steel above threshold	Yes
Threshold volume	1000

To perform CoG/g and CoG/batch analysis, a 12-month production campaign was selected. Bought in purified water (PW) and water for injection (WFI) were selected to perform CIP of the bioreactor and chromatography columns and to prepare medium and buffers, respectively, for the small-scale CoG/g analysis. These costs were then calculated as a material cost per batch rather than as a fixed utility cost to generate PW and WFI. To perform CoG/g analysis on a pilot and large manufacturing scale, onsite PW and WFI generation was selected. The total capital cost for the equipment and to run the facility was also added into the CoG analysis. For both EBA and RBA processes, the total capital cost duration was five years. The capital cost for CoG/g and CoG/batch was calculated based on the number of batches that can be manufactured per year. The equipment selected from the

facility support equipment worksheet consisted of freezer, refrigerator, incubator, microbiological safety cabinet, autoclave, clean steam generator, compressed air generator, CIP skid, waste collection tank and neutralisation skid. These equipment costs were included in the total capital costs.

User-defined operational parameters were added to each unit operation, which included the product mass and volume applied and recovered from each unit operation. The total duration of the unit operations was calculated from the sub-unit operation steps. The duration for the sub-unit operation steps were calculated based on the flow rate applied to operate the chromatography column and time taken during bioreactor growth and feeding phases to manufacture the product. For the EBA and RBA primary capture unit operation step, the column size was selected from the equipment drop-down list once added into the equipment library. In terms of personnel resources, two GMP operators were selected for each sub-unit operation step to calculate the labour cost for both the EBA and RBA processes. Resin cost per batch was calculated based on the size of the column, resin capacity (g/L), resin target cycle and maximum reuses of the resin. For STREAMLINE™ chelating resin, a binding capacity of 36 g/L (Chapter 4), 10 target cycles were used to define the EBA primary capture step. IDA Chelating Cellthru™ BigBead resin binding capacity of 32.0 g/L (Chapter 4), 10 target cycles were used to define the RBA primary capture step (Appendix 5 lists the detailed operating parameters of each of the unit operations for the EBA and RBA worksheets). Operational parameters for the unit operations of primary flask, secondary flask and production in fermenter were the same for both the EBA and RBA primary purification processes.

5.3.2 CoG analysis of EBA and RBA process

Once all of the operational parameters, list of materials and consumables, list of equipment and recovery of the scFv antibody concentration and volume from Chapter 3 had been added into the BioSolve software, the total CoG/g and CoG/batch were calculated. Tables 5.2 and 5.3 detail the mass balance and time data for each process step, the scFv antibody concentration at each process step, the total mass and volume at each step and step recovery of the scFv antibody.

Step yield for the EBA and RBA chromatography unit operations were input as 47.0% and 30.3% (Chapter 3), respectively. The RBA concentrated scFv antibody sample could not be measured using Agilent protein 80 chip and was measured using a spectrophotometer at OD_{280} nm therefore there is major variation between UF/DF step recovery between the EBA and RBA primary purification processes. Protein measurement using Agilent protein 80 chip is more accurate in comparison to the measurement using spectrophotometer at OD_{280}. For the UF/DF step purification of the green pigmented by-product with scFv antibody had affected the measurement of scFv antibody. Same is the case for the SEC step as the co purification of the green pigmented by-product with scFv antibody had affected separation of the scFv antibody while purifying using the SEC.

A total downstream process yield of 16% and 11% were calculated for EBA and RBA chromatography, respectively.

Table 5.2 Details of typical EBA process sequence including scFv concentration (g/L), volume (L), mass (g), yield (%) and duration (h) at each process sequence.

No	Process sequence	Concentration (g/L)	Vol (L)	Mass (g)	Yield (%)	Duration (h)
1	Primary flask	0.0	0.001	N/A	0%	24
2	Secondary flask	0.0	0.231	N/A	0%	24
3	Production	0.6	8	4.8	100%	90
4	EBA	2.8	0.801	2.3	47%	23
5	UF/DF	9.0	0.151	1.4	60%	6
6	SEC	5.1	0.151	0.8	57%	16
7	Filtration (0.2um)	4.8	0.151	0.8	95%	3

Table 5. 3 Details of typical RBA process sequence including scFv concentration (g/L), volume (L), mass (g), yield (%) and duration (h) at each process sequence.

No	Process sequence	Concentration (g/L)	Vol (L)	Mass (g)	Yield (%)	Duration (h)
1	Primary flask	0.0	0.001	N/A	0%	24
2	Secondary flask	0.0	0.231	N/A	0%	24
3	Production	0.7	8	5.4	100%	90
4	RBA	1.7	0.965	1.7	30%	17
5	UF/DF	16.2	0.097	1.6	95%	6
6	SEC	6.2	0.097	0.6	38%	16
7	Filtration (0.2um)	5.9	0.097	0.6	95%	3

Total production time to manufacture the His_6 tagged scFv antibody using the EBA and RBA as a primary purification step (including setup, operation and pre- and post-use cleaning of the equipment) were 184 h and 178 h, respectively. Compared to the RBA primary purification process, the primary purification step using the EBA twenty-four hours longer. The main factors contributing to the increase in time for the EBA primary purification process steps were the flow rate for the pre- and post-use cleaning and operation flow rate to maintain the steady-state condition of the column compared to the RBA chromatography step (Chapter 3).

Shift parameter was selected on BioSolve software for production, EBA and RBA primary capture and downstream steps in the parameters worksheet, which adjusts the total duration of the process stage if the operation time exceeds the shift duration. For production, 24 h/day shift duration, and for the primary recovery and downstream steps, 8 h/day shift duration were selected in the parameter worksheet. The adjusted duration included additional time if the operation time for the unit operation exceeded the shift duration. These adjusted durations for the processing stages are listed in Tables 5.4 and 5.5 for the production step, EBA and RBA step and SEC step, including shift parameter. The BioSolve software incorporates the adjusted time during different stages to calculate the total number of production batches/year in the facility.

Table 5.4 Details of typical EBA process sequence operating hours including shifts parameter, setup, process and post-use cleaning time.

Step no.	Process stage	Description	Duration (d)	Shifts	Adjusted duration (h)	Start (d)	Setup (d)	Process (d)	Cleaning (d)
1	Upstream	Primary flask	1	1	24	0.0	0.10	0.87	0.00
2	Upstream	Secondary flask	1	1	24	0.87	0.10	0.87	0.00
3	Upstream	Production	3.7	4	90	1.64	0.29	3.04	0.41
4	Purification	EBA (Gram)	0.95	3	55	4.84	0.05	2.02	0.20
5	Purification	UF/DF	0.25	1	6	6.80	0.12	0.04	0.06
6	Purification	SEC	0.7	1	16	6.76	0.21	0.11	0.32
7	Purification	Filtration (0.2um)	0.12	1	3	7.04	0.04	0.052	0.02

Table 5.5 Details of typical RBA process sequence operating hours including shifts parameter, setup, process and post-use cleaning time.

No.	Process stage	Description	Duration (d)	Shifts	Adjusted duration (h)	Start (d)	Setup (d)	Process (d)	Cleaning (d)
1	Upstream	Primary flask	1	1	24	0.00	0.10	0.87	0.00
2	Upstream	Secondary flask	1	1	24	0.87	0.10	0.87	0.00
3	Upstream	Production	3.7	4	90	1.56	0.29	3.04	0.41
4	Purification	RBA	0.67	2	32	4.83	0.06	1.22	0.07
5	Purification	UF/DF	0.2	1	6	5.98	0.12	0.05	0.06
6	Purification	SEC	0.7	1	16	5.95	0.21	0.23	0.20
7	Purification	Filtration (0.2um)	0.1	1	3	6.34	0.04	0.052	0.04

The BioSolve software also generates a Gantt chart for the configured process. Figures 5.1 and 5.2 show the Gantt chart for the typical EBA and RBA processes, respectively, where each process stage was divided into three process steps: setup, process and cleaning. The software calculated that 69 batches per year can be manufactured for both EBA and RBA processes by installing one fermenter and using 80% of the facility capacity. The bottleneck unit operation for both the EBA and RBA processes was the production stage in the fermenter with a total calculated operation time of 90 h, which included 24 h/day shift.

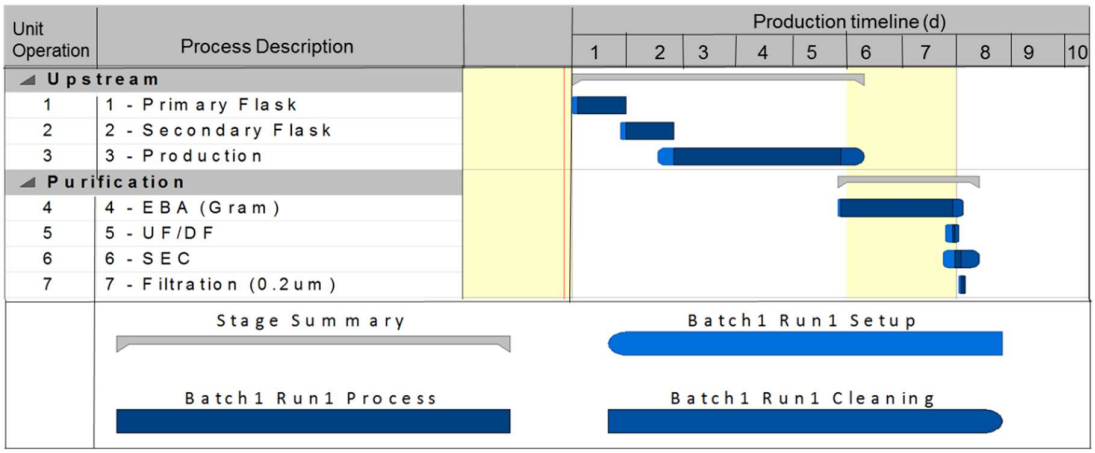

Figure 5.1 Yeast typical EBA process Gantt chart detailing total production days.

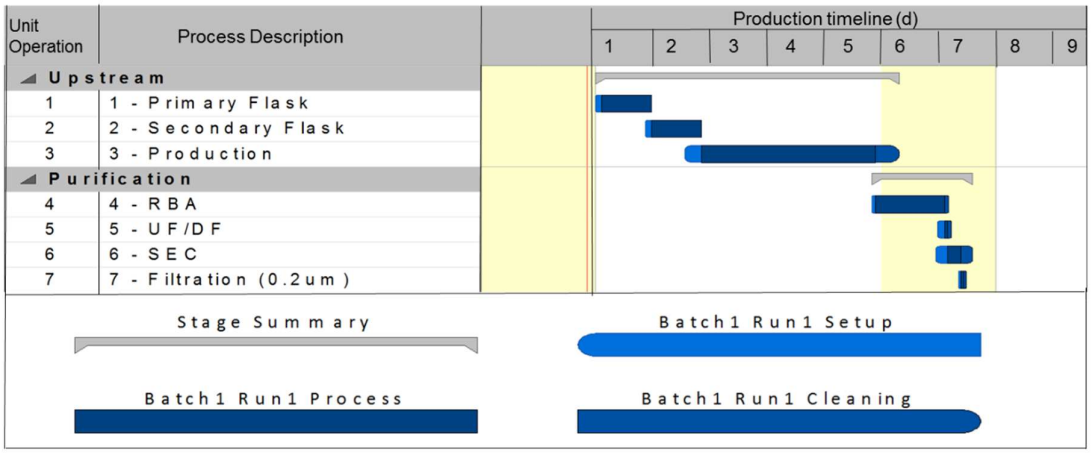

Figure 5.2 Yeast typical RBA process Gantt chart detailing total production days.

The total CoG/g to manufacture the His_6 tagged scFv antibody shMFELL2cys in the high cell density *P. pastoris* fermentation using EBA and RBA primary chromatography was £72K and £92K, respectively. Total CoG/batch to manufacture the His_6 tagged scFv antibody shMFELL2cys in the high cell density *P. pastoris* fermentation purified using EBA and RBA primary chromatography was £52K for each process. Total CoG includes capital, materials, consumables, labour and other costs (maintenance, utilities, waste management and insurance). Capital costs were estimated based on the equipment used during the process and size of the facility. For production cost of the fermenter and for SEC unit operation cost for

the chromatography skid plus columns were calculated. These costs were calculated for each unit operation. Figures 5.3 and 5.4 show the yeast typical EBA and RBA process cost breakdown for each unit operation and each unit operation breakdown of the capital, materials, consumables, labour and other costs.

Total CoG/g and CoG/batch were broken down into the capital, materials, consumables, labour and other indirect costs. The pie chart in Figure 5.5 illustrates the percentage of cost breakdown for each category for the EBA and RBA per batch. For both the EBA and RBA processes, the cost breakdown was identical for each category.

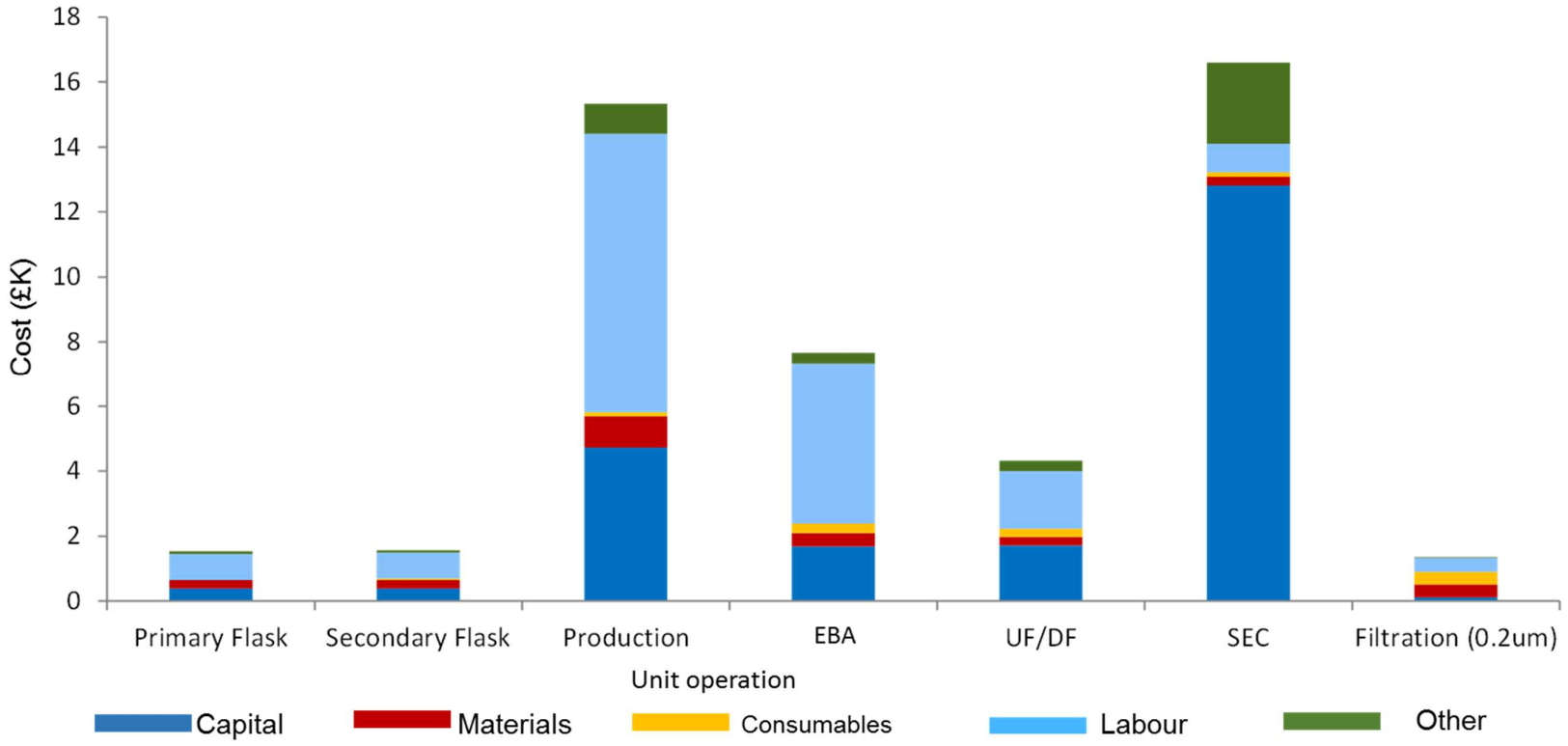

Figure 5.3 EBA process: unit operation cost breakdown graph.

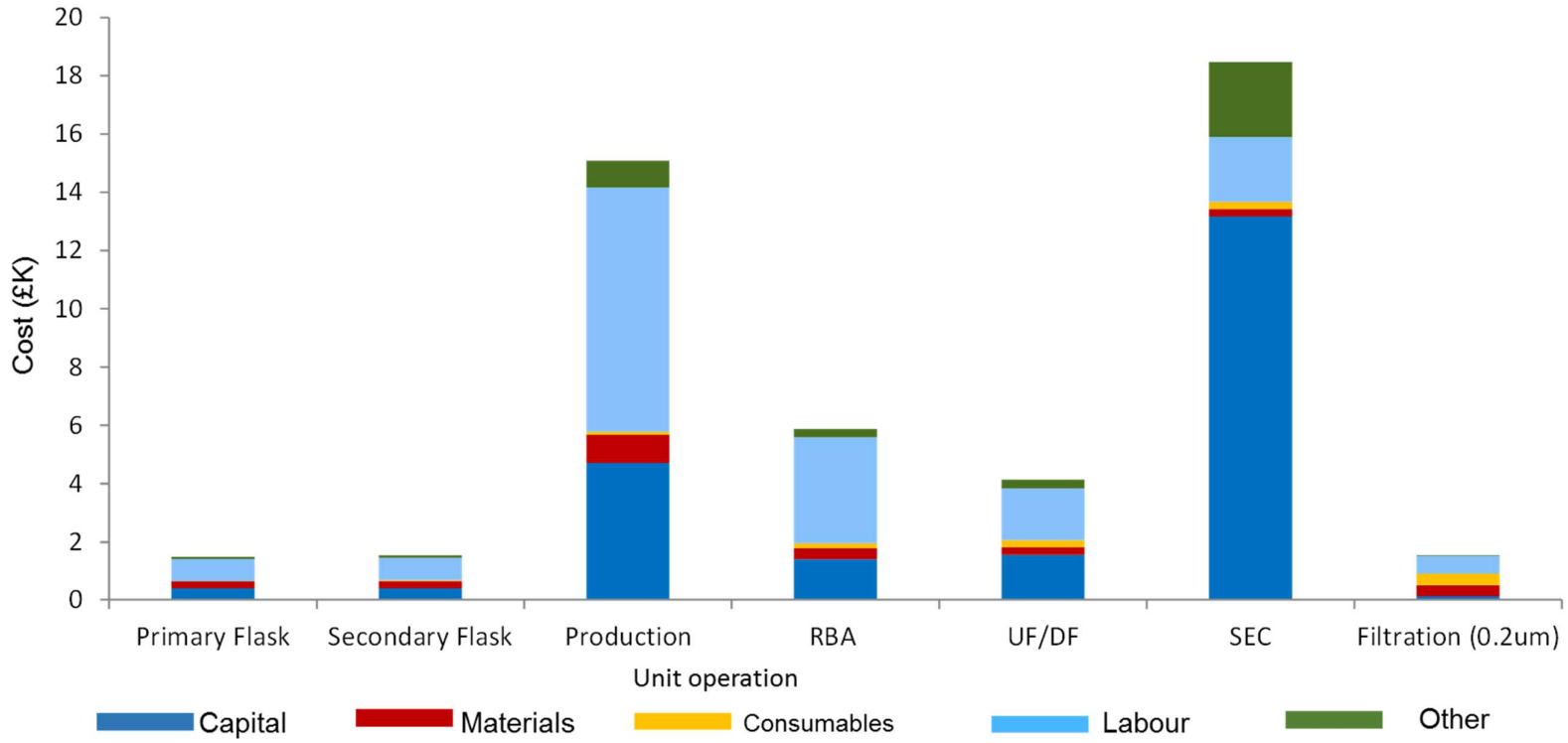

Figure 5.4 RBA process: unit operation cost breakdown graph.

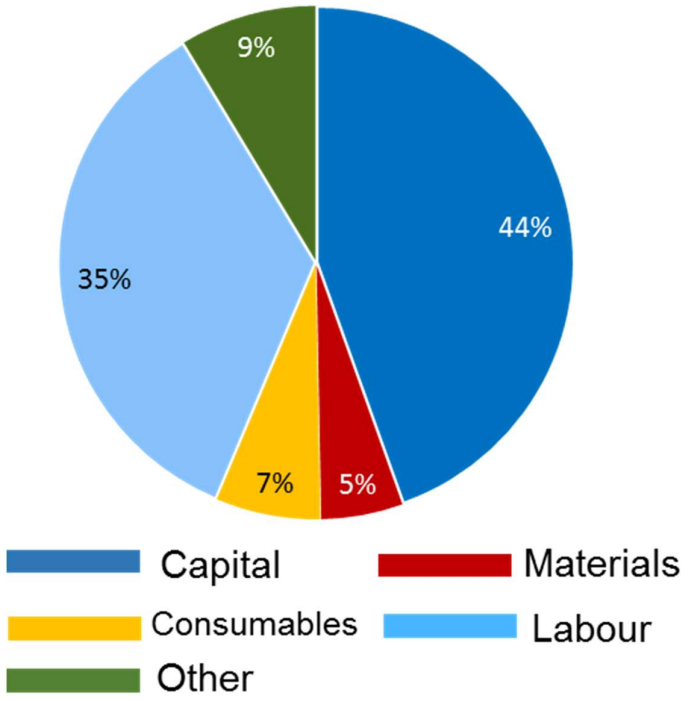

Figure 5.5 Pie chart illustrating the proportion of the cost categories for both EBA and RBA processes per batch at 8L fermenter volume.
CoG/batch for both the EBA and RBA processes was £52K at 8L fermenter volume and the calculated cost categories breakdown were same in percentage for both processes.

CoG/batch for the scFv antibody purified using the EBA and RBA processes showed no significant differences. However, on a CoG/g basis, a notable difference is seen. A 23% increase in the CoG/g for the scFv antibody purified using the RBA primary purification process was calculated in comparison to the EBA primary purification process. Table 5.6 details the cost breakdown for the EBA and RBA processes.

Although the resin cost is different for both the RBA and EBA processes, percentage breakdown for each category does not change at 8L fermenter volume (figure 5.5). Total CoG/Batch for both processes was calculate same £52K at 8L fermenter volume therefore there is no big difference in percentage breakdown for

each category between RBA and EBA processes was recorded by BioSolve software.

Table 5.6 CoG table for the EBA and RBA processes showing total CoG/g and CoG/batch and cost breakdown for capital, materials, consumables, labour and other costs.

	EBA		RBA	
	CoG/gram (£K)	CoG/batch (£K)	CoG/gram (£K)	CoG/batch (£k)
Capital	32	23	41	23
Materials	4	3	5	3
Consumables	5	3	6	3
Labour	25	18	32	18
Other	6	5	8	5
Total (£K)	72	52	92	52

Table 5.7 details the cost breakdown of each category, including subcategories for the EBA and RBA processes. Material costs include the costs for medium, buffers, direct raw materials, cost of WFI and PW and material used for CIP cycles and QC tests. Consumable costs include costs for resin, filters and bags. Total cost for labour includes labour cost for the operators involved in the process, for QA and QC and the logistic and engineering departments. Labour costs for the operators are calculated by taking into count hours for direct operation, solution preparation, cleaning and supervising these activities for each unit operation. Facility and utilities maintenance costs, waste management costs and insurance cost were included in the 'Other' cost category. Capital cost is estimated based on all equipment used during the process and facility size. For production cost of Bioreactor and for SEC cost for chromatography skid plus columns. Facility operation cost are also included for each unit operation.

Table 5.7 Detailed cost breakdown of each category of yeast typical EBA and RBA processes.

		EBA		RBA	
		Gram (£K)	Batch (£K)	Gram (£K)	Batch (£K)
Capital charge		32	23	41	23
Materials	Total cost	4.44	2.80	4.76	2.70
	Medium	0.14	0.10	0.18	0.10
	Buffer	0.68	0.05	0.08	0.05
	Bought WFI & PW	0.32	0.24	0.38	0.21
	CIP	0.00	0.00	0.00	0.00
	QC tests	3.30	2.41	4.12	2.33
Consumables	Total cost	4.70	3.42	7.12	3.46
	Resins/MA	0.19	0.14	0.14	0.08
	Bags	3.70	2.70	5.00	2.80
	Filters	0.74	0.54	0.96	0.54
	Other	0.06	0.04	0.08	0.04
Labour	Total cost	24.8	18.21	32.19	18.21
	Process	11	8.10	14.32	8.10
	Quality	9.60	7.03	12.43	7.03
	Indirect	4.20	3.08	5.44	3.08
Other	Total cost	6.15	4.51	8.00	4.53
	Insurance & other	1.31	0.96	1.70	0.96
	Waste management	0.02	0.01	0.02	0.01
	Maintenance	1.58	1.16	2.05	1.16
	Utilities	3.24	2.38	4.20	2.40
TOTAL (£K)		72	52	92	52

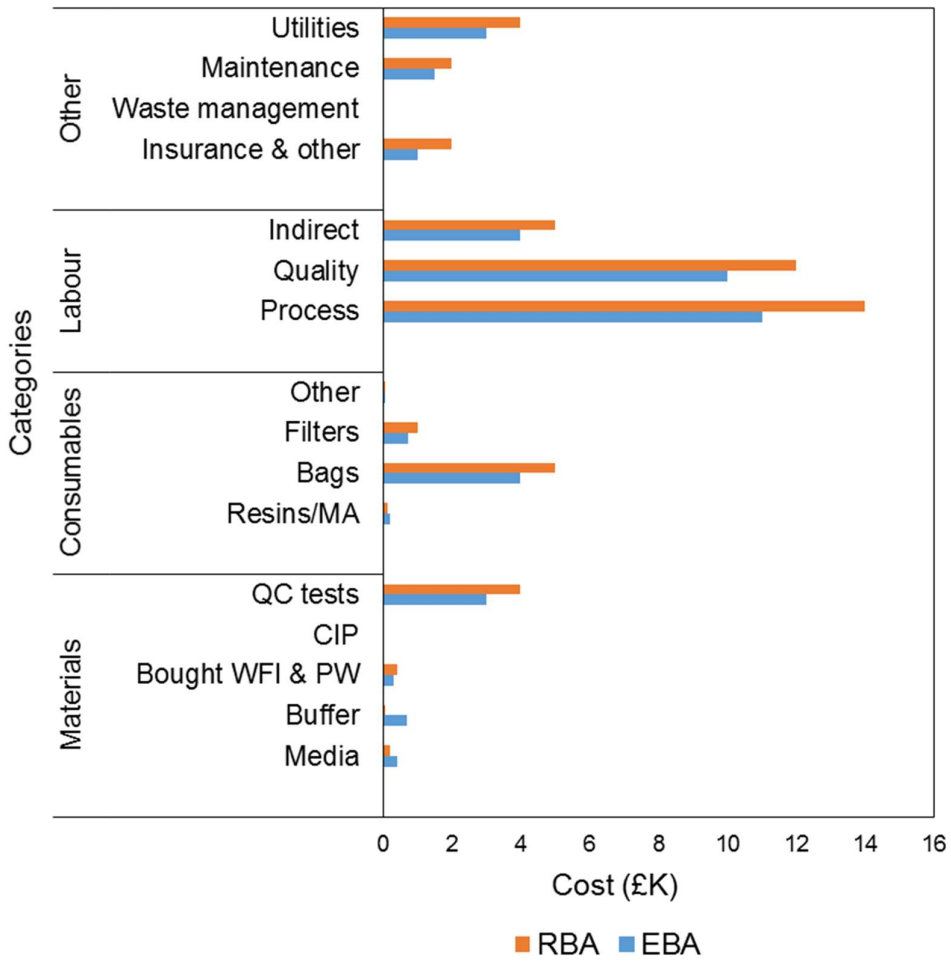

Figure 5.6 Comparison of CoG/g breakdown of each category including subcategories for EBA and RBA processes.

On an 8 L production scale, the calculated costs of the labour and consumables for the RBA process were higher than for the EBA process. Figure 5.6 shows the side-by-side cost comparison of each subcategory for the EBA and RBA processes. The total labour cost for CoG/g for the RBA process was 22% higher than for the EBA process. This increase was due to the process labour cost for the RBA process being 22% higher than for the EBA process. On the other hand, in comparing the CoG/batch there was no significant difference in the total labour cost between the EBA and RBA processes. Total scFv antibody recovery for the RBA process was 11% in comparison to the 17% for the EBA process per batch.

BioSolve software® converts the COG/batch into COG/gram therefore higher labour cost for the RBA process was calculated when compared in CoG/gram.

The BioSolve software was used to generate the detailed CoG/batch breakdown for each unit operation. Total costs of the EBA and RBA primary purification steps are listed in Table 5.8. Total CoG/batch and CoG/g for the EBA unit operation were £9.8K and £13.4K, respectively. Total CoG/batch and CoG/g for the RBA unit operation were £8.2K and £14.5K, respectively. Labour costs for the EBA unit operation was calculated to be 38% higher than the RBA unit operation on a per batch basis. Hence, the labour costs for the EBA unit operation was higher than for the RBA unit operation when comparing on a total CoG/batch basis.

The BioSolve software calculated the resin cost for the chromatography step based on the operation parameters included in the software per batch as £98 and £36, respectively, for the EBA and RBA unit operation.

Table 5.8 Detailed CoG breakdown for the EBA and RBA unit operation per batch.

		EBA unit operation cost (£K)	RBA unit operation cost (£K)
Capital charge		3	3
Materials	Total cost	0.4	0.4
	Medium	N/A	N/A
	Buffer	0.10	0.04
	Bought WFI & PW	0.10	0.08
	CIP	N/A	N/A
	QC tests	0.26	0.25
Consumables	Total cost	0.77	0.70
	Resins/MA	0.10	0.04
	Bags	0.70	0.70
	Filters	N/A	N/A
	Packages	N/A	N/A
	Other	N/A	N/A
Labour	Total cost	5	3.6
	Process	2.2	1.6
	Quality	2.0	1.4
	Indirect	0.83	0.61
Other	Total cost	0.6	0.6
	Insurance & other	0.14	0.12
	Waste management	0.003	0.003
	Maintenance	0.16	0.15
	Utilities	0.32	0.3
Total unit operation cost per batch (£K)		9.8	8.2
Normalised total unit operation cost per gram (£K)		13.4	14.5

Table 5.9 List of medium and buffers used for EBA process.

Unit op	Solution	Type	Volume required (L)	Cost (£)
1	YPD medium	Medium	0.23	0.2
2	Yeast fermentation (chemical define medium)	Medium	0.37	1.6
3	Yeast fermentation (chemical define medium)	Medium	5.2	23.2
3	50% glycerol	Medium	0.56	12
3	100% methanol	Medium	1.6	69
4	100 mM copper sulphate	Buffer	1.05	1.9
4	0.5 M NaCl, 0.5 M PBS, 200 mM imidazole	Buffer	0.70	1.2
4	100 mM EDTA	Buffer	2.01	2.4
4	20% ethanol	Buffer	1.5	6.6
4	2 M NaCl, 2 M PBS, 20 mM imidazole	Buffer	2.01	1.8
4	0.5 M NaCl, 0.5 M PBS, 10 mM imidazole	Buffer	14.8	12.1
4	0.5 M NaOH	Buffer	11	2
5	0.1 M NaOH	Buffer	0.5	N/A
5	0.5 M NaOH	Buffer	0.5	0.11
6	0.5 M NaOH	Buffer	0.8	0.16
6	20% ethanol	Buffer	1	2.4
6	PBS	Buffer	0.5	0.05

BioSolve calculated the cost for medium and buffers used for each unit operation. Tables 5.9 and 5.10 list the medium and buffers used during the EBA and RBA processes with detailed information on the medium and buffer costs and volumes required per batch. The total material costs to prepare medium and buffers per batch for EBA and RBA processes was £3K.

Table 5.10 List of medium and buffers used for the RBA process.

Unit op	Solution	Type	Volume required (L)	Cost (£)
1	YPD medium	Medium	0.23	0.2
2	Yeast fermentation (chemical define medium)	Medium	0.37	1.6
3	Yeast fermentation (chemical defined medium)	Medium	5.2	23
3	50% glycerol	Medium	0.6	12
3	100% methanol	Medium	1.7	69
4	100 mM copper sulphate	Buffer	2	5.1
4	0.5 M NaCl, 0.5 M PBS, 200 mM Imidazole	Buffer	2	3.4
4	100 mM EDTA	Buffer	2	2.4
4	2 M NaCl, 2 M PBS, 20 mM Imidazole	Buffer	8	16
4	0.5 M NaCl, 0.5 M PBS, 10 mM Imidazole	Buffer	14	16
4	0.5 M NaOH	Buffer	2	0.4
4	0.1 M NaOH	Buffer	2.5	0.01
5	0.5 M NaOH	Buffer	0.5	0.11
5	0.1 M NaOH	Buffer	0.5	0.02
6	0.5 M NaOH	Buffer	1.62	0.32
6	20% ethanol	Buffer	0.5	2.4
6	PBS	Buffer	0.54	0.05

CoG/batch also includes the cost for QC tests performed to release the batch. The BioSolve software has flexibility to select the QC tests required for each unit operation and also for the final purified product. Table 5.11 lists the cost for QC tests selected for the production step and for the final purified product.

Table 5.11 QC test cost for the EBA and RBA processes.

Unit operation	Test name	No of tests	Cost per batch (£)
Production	Sterility	1	609
Batch QC test	Bioburden	2	348
Batch QC test	Concentration (A280nm)	1	87
Batch QC test	Conductivity	1	78
Batch QC test	pH	1	78
Batch QC test	Purity (SDS-PAGE)	1	261
Batch QC test	Purity (SEC-HPLC)	1	261
Batch QC test	Sterility	1	609

5.3.3 CoG analysis of the EBA and RBA processes from lab to large scale

The BioSolve model was used to estimate the CoG/g of the scFv antibody manufactured in pilot- and large-scale fermenters and using the EBA and RBA primary purification processes. Final fermentation volume was changed from 8 L to 2000 L in the EBA and RBA worksheet and CoG analysis was performed to evaluate the effect of the scale-up on the CoG/g. Cost model was set up for 1 Bioreactor per facility, operational parameters were scaled up linearly and facility was set to produce in-house WFI and PW. Because of the operation and scale-up limitation of the SEC step on a large scale, this step was replaced with a bind and elute ion exchange chromatography step with 80% recovery for both EBA and RBA processes (Tables containing the small- to large-scale EBA and RBA column information with resin volume, cost and flow velocity details are in Appendix 6). The BioSolve model was updated to calculate the RBA column volume and average flow velocity for the pilot- and large-scale columns. Appendix 7 details the formula for calculating the RBA column volume and average flow velocity.

Figure 5.7 represents the increase in the CoG/batch for both the EBA and RBA processes from 8 L lab scale to 2000 L large-scale production processes. Production bioreactor and column size increased from 8L to 20L scale which increases the CoG/Batch. Capital (Bioreactor size and facility operation), material and resin cost increases from 500L to 1000L scale hence there is a big jump in CoG/batch from 8L to 2000L scale. Calculated CoG/batch for the EBA process

increased from £52K at 8 L to £153K at 2000 L scale. For the RBA process, the CoG/batch increased from £52K at 8 L scale to £133K at 2000 L scale. The increased CoG/batch was mainly due to the increase in the proportion of materials and consumables required to run the process at 2000 L fermenter volume for both processes. While scaling up the process from 8L to 2000L scale, right column size and operation parameters were in selected in BioSolve software to make sure the model calculate the accurate CoG/Batch. At 2000 L scale BioSolve has calculated higher Capital, Material and consumables cost for both the EBA and RBA processes. Higher capital (facility and process equipment) and consumable cost (resin and buffers bags) were calculated for EBA process were higher in comparison to RBA process at 2000L scale. Therefore, CoG/batch for the EBA process is higher than RBA process.

Cost model was set up for one bioreactor per facility, operational parameters were scaled up linearly and facility was set to produce in-house WFI and PW which increased the capital cost at large scale. Increase in the production bioreactor and column size has increased COG/Batch from 8 L to 20 L scale. CoG/Batch from 20 L to 100 L production scale has also increased. Big jump in the CoG/batch from 500 L to 1000 L scale was calculated by BioSolve mainly because of increase in the size of bioreactor and cost of facility operation, material and resin.

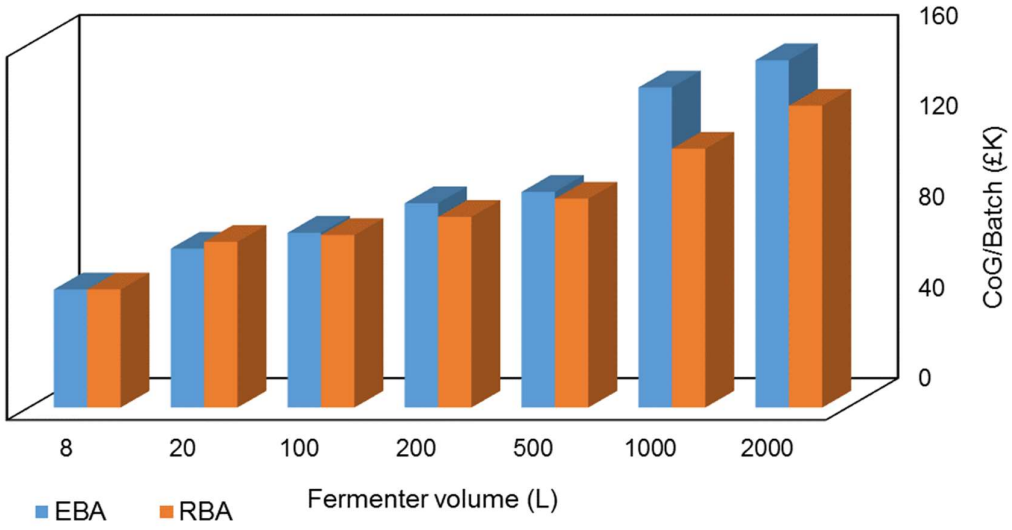

Figure 5.7 Comparison of the CoG/batch for the EBA and RBA processes from 8 L to 2000 L fermenter volume.
The CoG/batch for the EBA and RBA processes increased with a rise in fermentation scale from 8 L to 2000 L. Calculated CoG/batch for the EBA process increased from £52K at 8 L to £153K at 2000 L scale. For the RBA process, the CoG/batch increased from £52K at 8 L scale to £133K at 2000 L scale. A significant £20K higher CoG/batch for the EBA process was calculated in comparison to the RBA process at 2000L scale. Capital and material costs are the two main factors increases the CoG/Batch from 8L to 2000L scale both EBA and RBA processes.

Figure 5.8 represents the increase in the production yield of the His_6 tagged scFv antibody from the 8 L to 2000 L scale for the EBA and RBA processes. The production yield for the EBA process increased from 0.8 g/batch at the 8 L scale to 244 g/batch at the 2000 L scale. The production yield for the RBA process increased from 0.6 g/batch at the 8 L scale to 297 g/batch at the 2000 L scale.

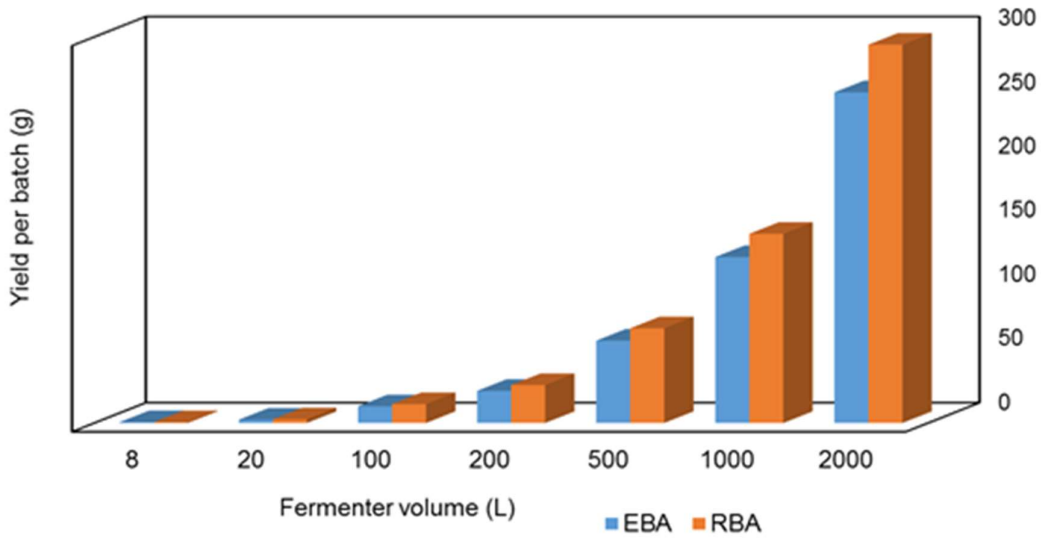

Figure 5.8 Comparison of the scFv antibody yield per batch for the EBA and RBA processes from 8 L to 2000 L fermenter volume.
The scFv antibody yield per batch increased from the 8 L to the 2000 L fermentation scale. For the RBA process at the 2000 L scale, the scFv antibody yield was 37 g more in comparison to the EBA process.

Figure 5.9 represents the total annual throughput for the EBA and RBA processes to manufacture scFv antibody from the 8 L to 2000 L fermenter scale. Total throughput per year for the EBA process increased from 0.05 Kg at the 8 L scale to 17 Kg at the 2000 L scale. Total throughput per year for the RBA process increased from 0.04 Kg at the 8L scale to 21 Kg at the 2000 L scale. The downstream processing yields for the EBA and RBA processes at the 2000 L scale were 20% and 22%, respectively. With an increase in throughput per year, the CoG/g for the EBA process decreased from £72K at the 8 L scale to £600 at the 2000 L scale. In comparison, an increase in throughput for the RBA process showed a decrease from £92K at the 8 L scale to £452 at the 2000 L fermenter volume. Reductions in the CoG/g for the EBA and RBA processes was mainly due to the increase in the total mass of the scFv antibody purified using the EBA or RBA primary chromatography step. In comparison to the EBA process, a 25% reduction in the CoG/g for the RBA process was calculated at the 2000 L scale because of the higher throughput per year and the CoG/batch cost was 13% lower for the RBA process.

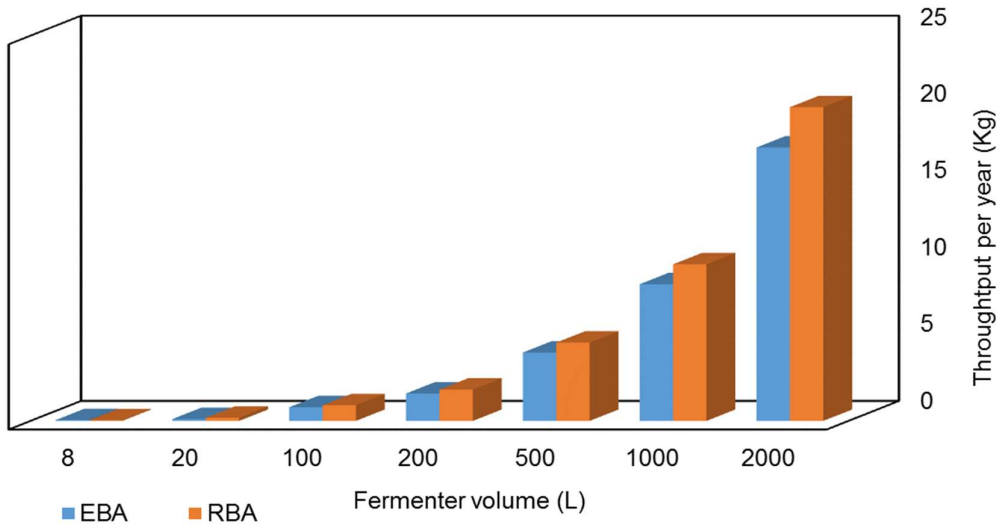

Figure 5.9 Comparison of the scFv antibody throughput per year for the EBA and RBA processes from the 8 L and 2000 L scale.
The calculated RBA process scFv antibody throughput per year at 1000 L and 2000 L was 10 Kg and 20 Kg, respectively. For the EBA process, the calculated throughput per year was 8.8 Kg and 17.7 Kg, respectively, at the 1000 L and 2000 L fermentation scale. The RBA process throughput was 12% higher compared to the EBA process.

To investigate the increase in the CoG/batch for the EBA and RBA processes while scaling up the process from 8 L to 2000 L fermenter volume, analysis of the cost of buffers, labour and resins was performed at 20 L, 100 L, 200 L, 500 L, 1000 L and 2000 L scales. Figures 5.10 and 5.11 present the cost of buffers, labour and resins at 20 L, 100 L, 200 L, 500 L, 1000 L and 2000 L scales.

At the 200 L pilot scale, the calculated labour cost for the EBA process was 40% higher than for the RBA process at the same scale. A 53% increase in the resin cost and a 50% increase in the buffer costs for the EBA process compared to the RBA process was calculated at the 200 L pilot scale. Increase in the EBA column size while scaling up the process from 8L to 2000L scale has increased the cost of resin. increase in the EBA labour cost was due to the longer operation time for the EBA process compared to the RBA process. The increase in the EBA buffer cost was due to the increase in the EBA column volume in expanded bed mode. In expanded bed mode, the buffer consumption to equilibrate, wash, clean and regenerate the column was much higher compared to the RBA process. Due to the

higher EBA resin purchase cost compared to the RBA resin, a 50% increase in the EBA resin cost was calculated. Increase in the EBA column size in comparison to the RBA column for large scale operation has increased the cost of resin.

For large-scale EBA and RBA operations, there was a significant 50% increase in the EBA resin cost from the 500 L to 2000 L scale compared to the RBA resin cost. A 30% increase in the EBA buffer cost was calculated compared to the RBA buffer cost at 2000 L. The model calculated a higher CoG/batch for the EBA process at a 2000 L scale of operation because of the increased cost of resin, buffers and labour. The reduced CoG/g for the RBA process compared to the EBA process while scaling up the process from 8 L to 2000 L production scale was calculated.

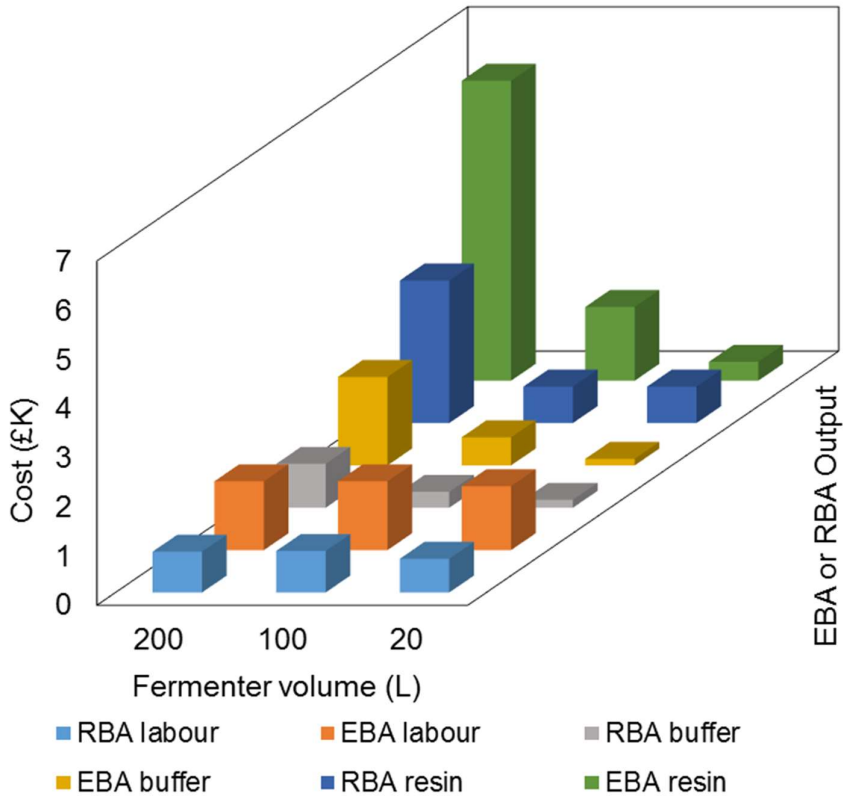

Figure 5.10 Comparison of the EBA and RBA processes' buffer, resin and labour cost per batch at 20 L, 100 L and 200 L scale.
On scaling up the EBA and RBA processes from 20 L to 200 L, there was an increase in the buffer, resin and labour cost per batch. The EBA process buffer, resin and labour cost per batch was calculated as higher in comparison to that of the RBA process.

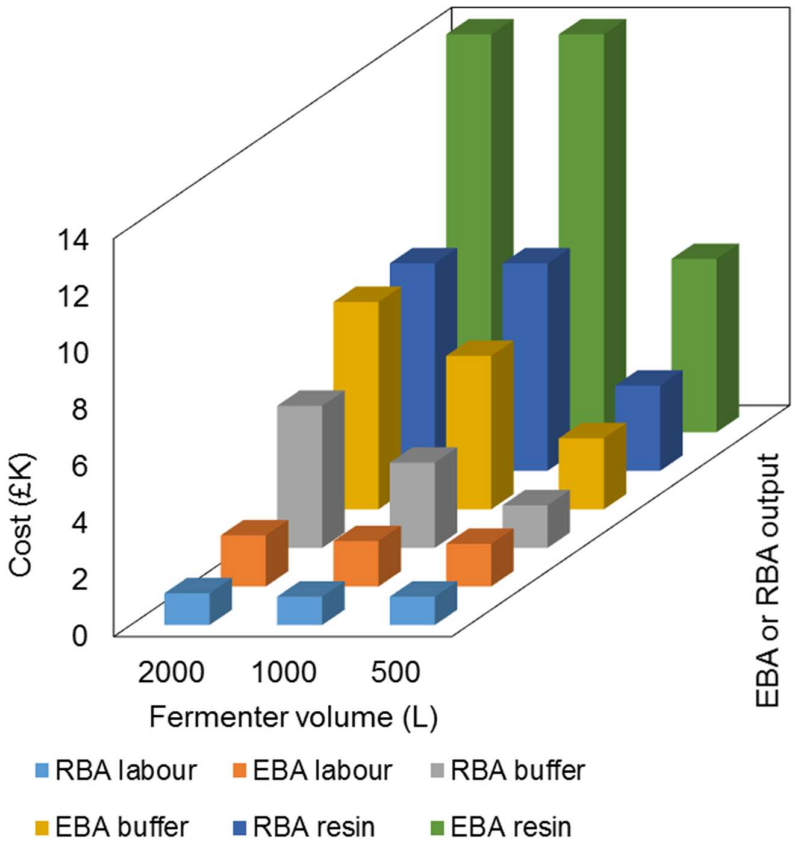

Figure 5.11 Comparison of the EBA and RBA processes' buffer, resin and labour cost per batch at 500 L, 1000 L and 2000 L scale.
On scaling up the EBA and RBA process from 500 L to 2000 L, there was an increase in the buffer, resin and labour cost per batch. The EBA process buffer, resin and labour cost per batch was calculated as higher in comparison to that of the RBA process.

Figure 5.12 represents a comparison of the buffer usage between the EBA and RBA process from 8 L to 2000 L fermentation scale. The buffer consumption for the EBA process increased gradually in comparison with the RBA process from 8 L to 2000 L scale. A 33% increase in the buffer usage for the EBA process compared to the RBA process at 2000 L scale was calculated.

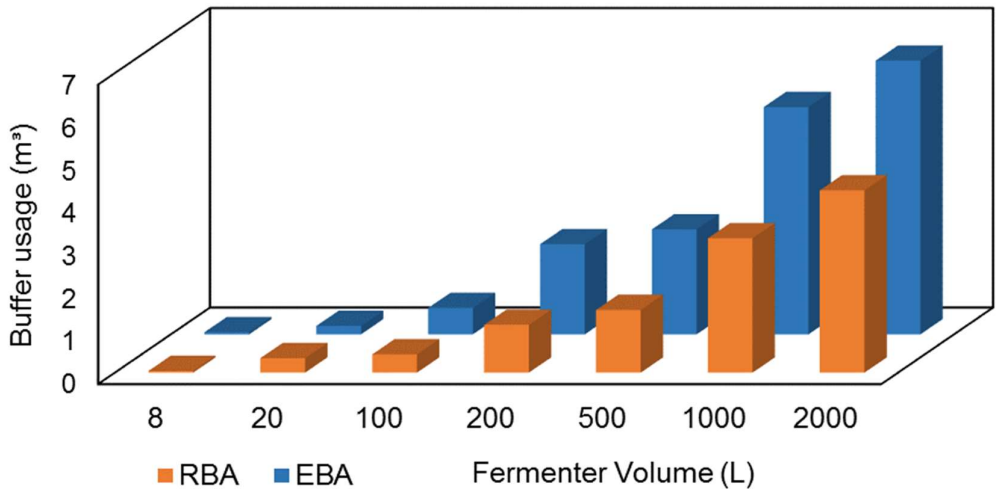

Figure 5.12 Comparison of the buffer usage between the EBA and RBA processes from 8 L to 2000 L scale.
For the EBA process at 1000 L and 2000 L scale, the buffer requirement was calculated as 40% and 33% higher, respectively, in comparison to that of the RBA process.

The capital cost for the EBA and RBA processes was £40K and £39K per batch at the 100 L scale, respectively. At the 2000 L scale, a more significant increase in the capital cost for the EBA process was calculated. The capital cost per batch for the EBA process was £65K compared to £48K for the RBA process. Cost of large scale EBA column and facility operation has increased the capital cost per batch for EBA process.

5.3.4 Scenario analysis of EBA and RBA chromatography processes

Scenario analysis of the EBA and RBA primary purification processes was performed to calculate the total CoG/g at three expression levels in the 8 L to 2000 L final fermentation volume range. The scenario analysis was performed at three expression levels of 0.6, 3 and 6 g/L in the fermenter at pilot and large-scale fermenter volumes (for the RBA process, the lowest expression level was 0.7 g/L instead of 0.6 g/L). Figures 5.13 and 5.14 show the CoG/g for the EBA and RBA processes from 8 L to 2000 L production scale volume.

By increasing the His_6 tagged scFv antibody expression level from 0.6 g/L to 6 g/L in the 8 L fermenter volume, a 75% and 74% decrease in the total CoG/g was calculated for the EBA and RBA processes, respectively. Similarly, an 88% decrease in the total CoG/g was calculated for both the EBA and RBA processes by increasing the expression level from 0.6 to 6 g/L in the 20 L fermenter volume.

At the 100 L production scale, with an increase in the expression level from 0.6 to 6 g/L, the CoG/g for the scFv antibody decreased from £6K to £1K for the EBA process and from £5.2K to £650 for the RBA process. This was a significant 84% and 87% decrease in the CoG/g for the EBA and RBA processes, respectively.

Similarly, at the 2000 L production scale, increases in the expression level from 0.6 to 6 g/L resulted in the CoG/g for the scFv antibody decreasing from £600 to £261 for the EBA process and from £452 to £163 for the RBA process. This was a significant 57% and 64% decrease in the CoG/g for the EBA and RBA processes, respectively.

At the 2000 L scale, scFv antibody production throughput increased from 18 Kg/year to 44 Kg/year with an increase in the production titter from 0.6 to 6 g/L for the EBA process and a 57% decrease in the CoG/g. Similarly, for the RBA process at the 2000 L scale, scFv antibody production throughput increased from 20 Kg/year to 81 Kg/year with an increase in the production titter from 0.7 to 6 g/L and a 64% decrease in the CoG/g.

Therefore, as expected an increase in the expression level clearly decreased the total CoG/g for both the EBA and RBA processes at a pilot scale of operation. In comparison to the EBA process significant decrease in the CoG/g for the RBA process was predicted.

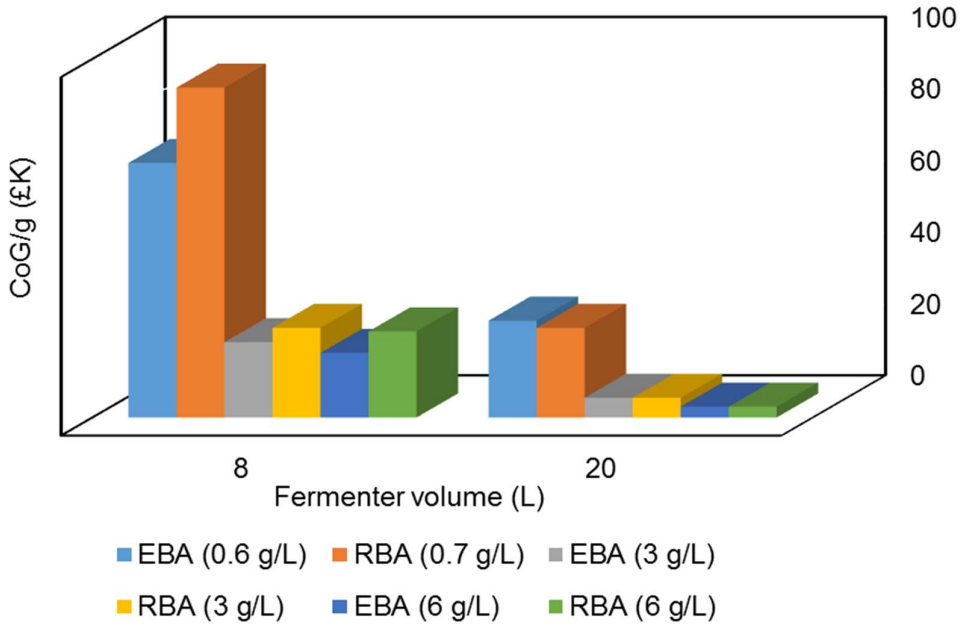

Figure 5.13 Comparison of the scFv antibody CoG/g using the EBA and RBA processes between the 8 L and 20 L scale at three different fermentation yields.

With an increase in the fermentation yield from 0.6 to 6 g/L at 8 L fermenter volume, the calculated RBA process CoG/g decreased from £92K to £24K, and for the EBA process CoG/g decreased from £71K to £16K. With an increase in the fermentation yield from 0.6 to 6 g/L at 20 L fermenter volume, the calculated RBA process CoG/g decreased from £25K to £3K, and the EBA process CoG/g decreased from £27K to £3K.

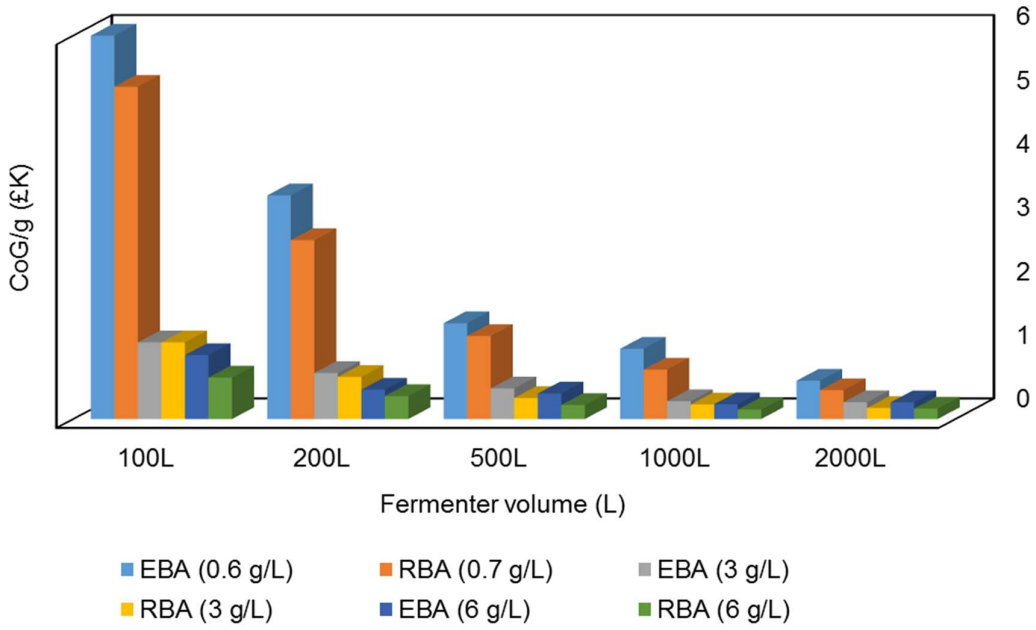

Figure 5.14 Comparison of the scFv antibody CoG/g using the EBA and RBA processes from 100 L to 2000 L scale at three different fermentation yields. With an increase in the fermentation yield from 0.6 to 6 g/L at a 100 L fermenter volume, the calculated RBA and EBA process CoG/g decreased by 88% and 84%, respectively. With an increase in the fermentation yield from 0.6 to 6 g/L at a 2000 L fermenter volume, the calculated RBA and EBA process CoG/g decreased by 64% and 57%, respectively.

5.3.5 CoG analysis using design of experiment response surface design

The CoG analysis for the EBA and RBA processes was performed using design of experiment (DOE) response surface design in JMP software [121]. The DOE was used to systematically determine the relationship between the factors affecting the COG of the EBA and RBA processes. The CoG analysis was performed at the 2000 L production scale to investigate the effects of four major factors: (i) fermentation yield, (ii) EBA and RBA step recovery, (iii) DBC of the EBA and RBA resin and (iv) resin CIP cycles. A central composite design was used to investigate the effects of factors on the CoG analysis of the scFv antibody. A central composite design in combination with two-level fractional design, including centre and axial points, was used for the analysis. Response surface designs are useful for modelling response surface with curvature where a minimum or maximum value of the response curve occurs.

Responses for CoG/g, CoG/batch and cost of resin, labour and buffers were analysed after modelling the data using BioSolve software. Once the experimental table was created, the set points for the factors were added into the BioSolve software to generate the values for the response curve. The model was then analysed to identify the significant factors for the CoG analysis. The summary report shows the Log Worth and P value for each effect in the model for the EBA and RBA processes. A Log Worth that exceeded 2 was significant at the 0.01 P value. All of the factors for the EBA and RBA process modelled in the central composite design were significant. Data generated here not obtained from practical experiments therefore there is a limitation in accepting them in real scenario. Without the practical data CoG analysis using DoE were generated in conjunction with BioSolve software. Generation of experimental data at 2000L scale was not possible due to various factors for example the cost associated with running the process at 2000L scale. Therefore, simulated data generated using BioSolve software were used to compare the interaction between different factors.

Figures 5.15 and 5.16 show the surface response curve for the CoG/g for the RBA and EBA processes against the fermenter yield and resin binding capacity, respectively. Figures 5.17 and 5.18 show the surface response curve for the

CoG/batch for the RBA and EBA processes against the fermenter yield and resin binding capacity, respectively. In comparison to the EBA process, the RBA process CoG/g and CoG/batch were significantly lower in the best and worst-case scenarios. For the best-case scenario of the RBA process, CoG/g was £54 and CoG/batch was £326K compared to the EBA process for which the CoG/g was £197 and CoG/batch was £635K. With an increase in the fermentation yield, the downstream purification cost also increased and therefore a higher CoG/batch was calculated. For the worst-case scenario of the RBA process, the calcu2lated CoG/g was £556 and CoG/batch was £175K compared to the EBA process for which the CoG/g was £1400 and CoG/batch was £295K.

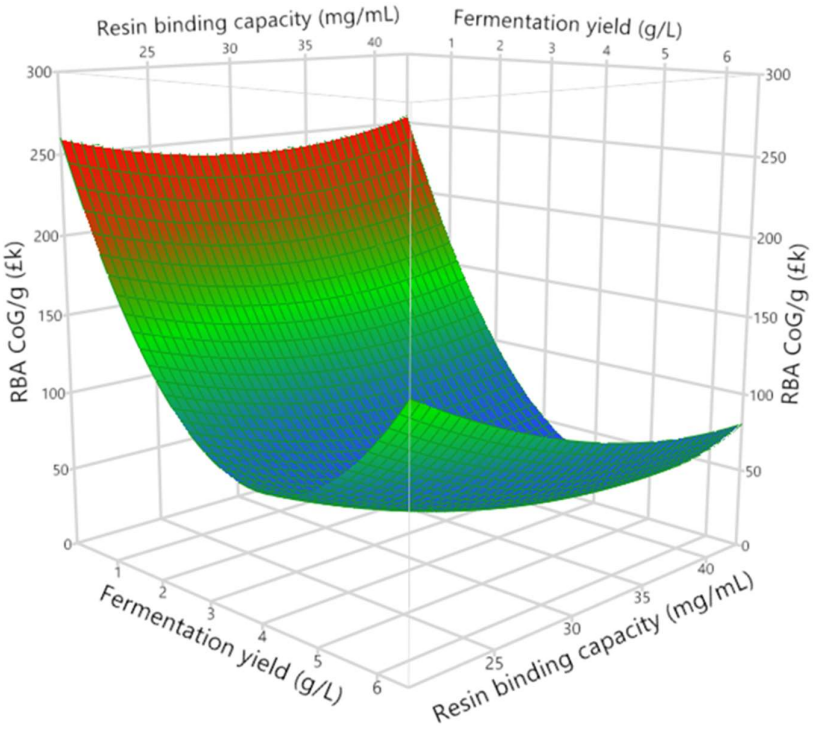

Figure 5.15 Surface response curve for the CoG/g of the scFv antibody production using the RBA process against the fermentation yield and the resin binding capacity.
In the best-case scenario of £54 CoG/g at 6 g/L fermentation yield and 40 mg/mL resin binding capacity, 60 resin CIP cycles and 60% step recovery were calculated.

In the worst-case scenario of £556 CoG/g at 1 g/L fermentation yield and 20 mg/mL resin binding capacity, 20 resin CIP cycles and 20% step recovery were calculated.

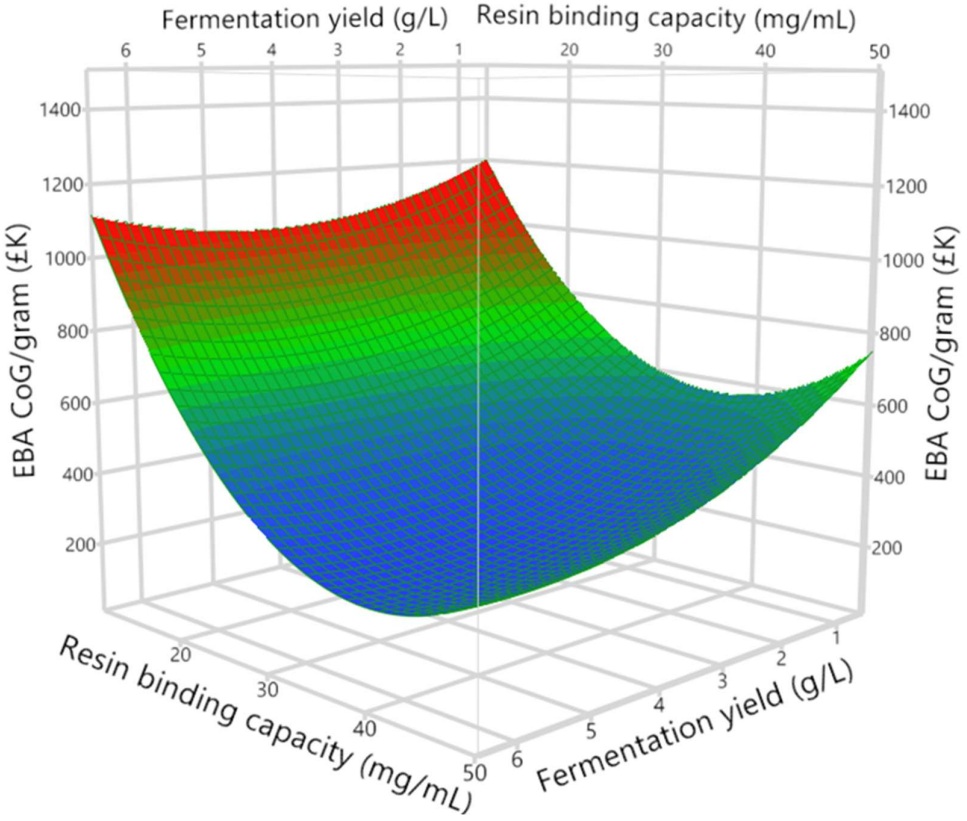

Figure 5.16 Surface response curve for the CoG/g of the scFv antibody production using the EBA process against the fermentation yield and the resin binding capacity.

In the best-case scenario of £197 CoG/g at 6 g/L fermentation yield and 40 mg/mL resin binding capacity, 60 resin CIP cycles and 60% step recovery were calculated.

In the worst-case scenario of £1400 CoG/g at 1 g/L fermentation yield and 20 mg/mL resin binding capacity, 20 resin CIP cycles and 20% step recovery were calculated.

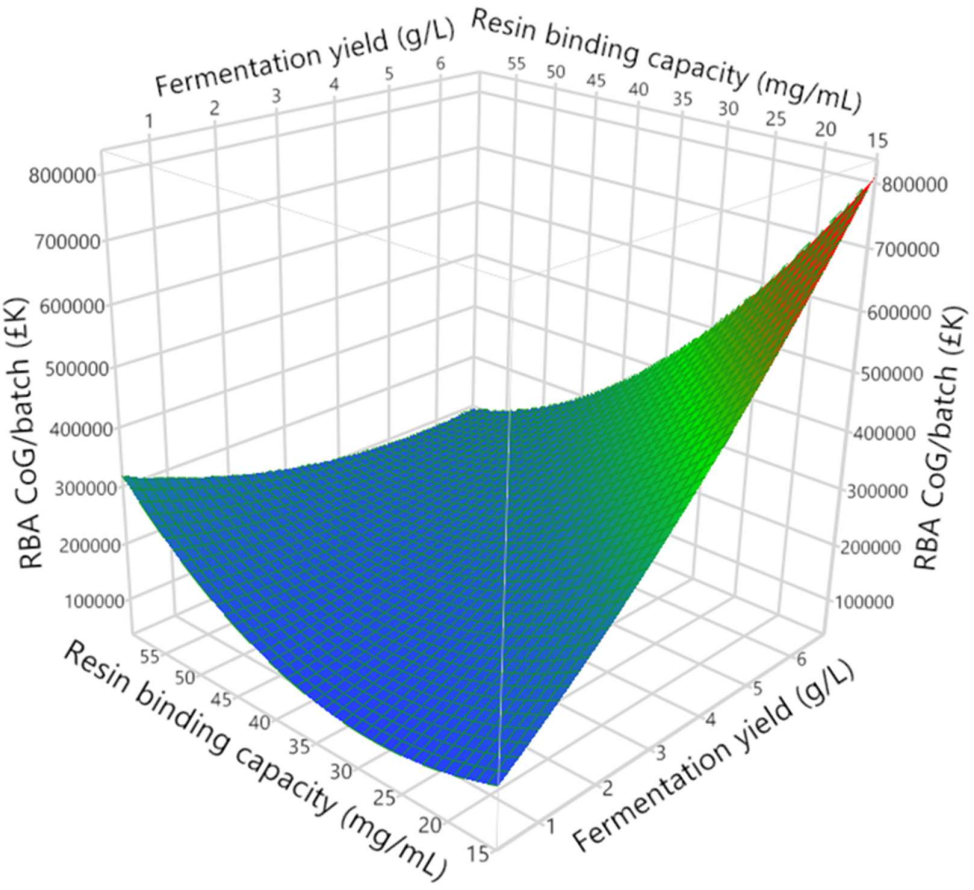

Figure 5.17 Surface response curve for the RBA process CoG/batch against fermentation yield and resin binding capacity.

In the best-case scenario of £326K CoG/batch at 6 g/L fermentation yield and 40 mg/mL resin binding capacity, 60 resin CIP cycles and 60% step recovery were calculated. The calculated CoG/g for the scFv antibody was £54.

In the worst-case scenario of £175K CoG/batch at 1 g/L fermentation yield and 20 mg/mL resin binding capacity, 20 resin CIP cycles and 20% step recovery were calculated. The calculated CoG/g for the scFv antibody was £556.

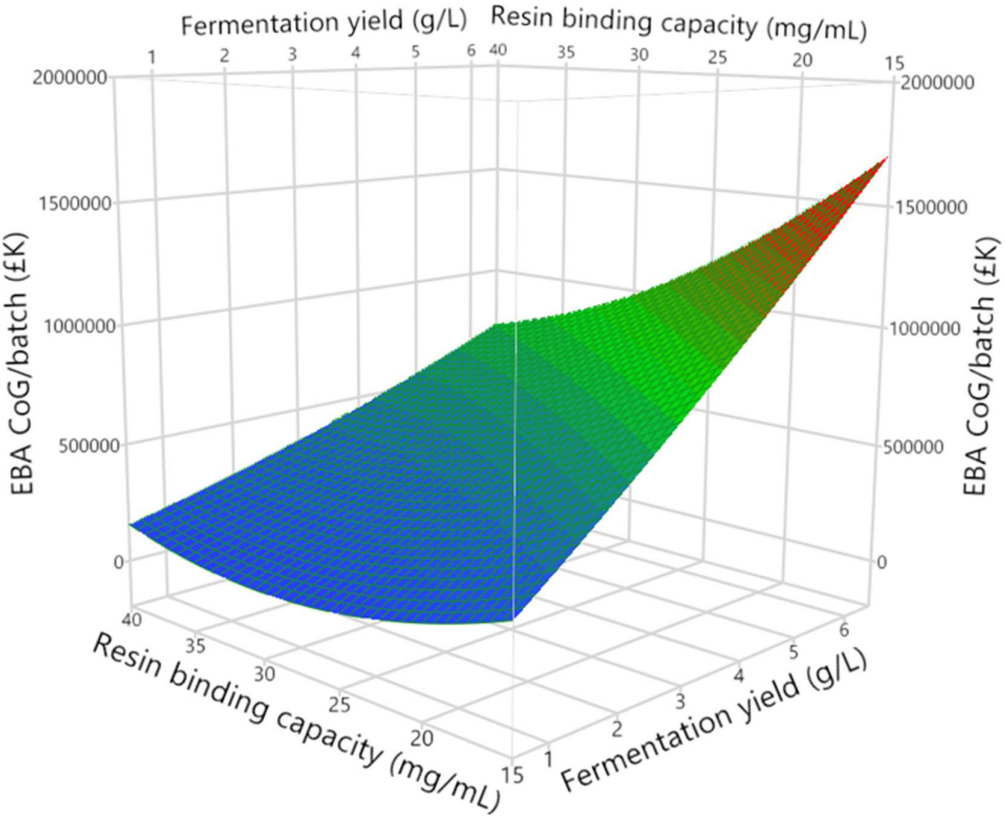

Figure 5.18 Surface response curve for the EBA process CoG/batch against fermentation yield and resin binding capacity.
In the best-case scenario of £635K CoG/batch at 6 g/L fermentation yield and 40 mg/mL resin binding capacity, 60 resin CIP cycles and 60% step recovery were calculated. The calculated CoG/g for the scFv antibody was £197.

In the worst-case scenario of £295K CoG/batch at 1 g/L fermentation yield and 20 mg/mL resin binding capacity, 20 resin CIP cycles and 20% step recovery were calculated. The calculated CoG/g for the scFv antibody was £1400.

The prediction profiler for the EBA and RBA processes shows the cross-section of the model in Figure 5.19 and Figure 5.20, respectively. The prediction profiler was used to analyse the interaction between the four factors to perform analysis of the CoG/g, CoG/batch and cost of resin, buffer and labour per batch. The EBA step recovery, resin binding capacity and fermentation yield each had a significant impact on the CoG/g of the EBA process in comparison to the number of CIP cycles of the resin. The EBA resin binding capacity and fermentation yield had a significant impact on the CoG/batch and cost of resin, buffer and labour per batch

of the EBA process in comparison to the number of CIP cycles of the resin. A similar interaction was also seen in the RBA process.

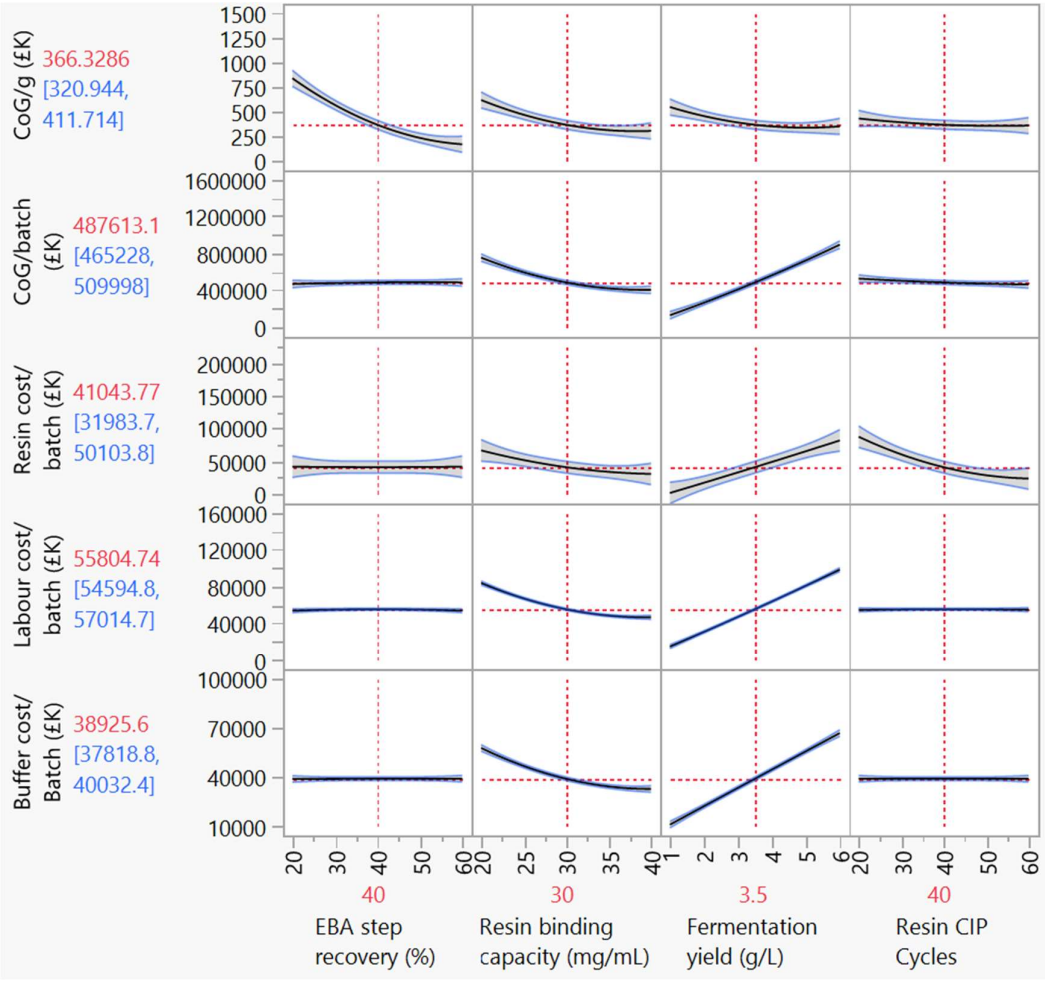

Figure 5.19Prediction profiler for the EBA process.
The prediction profiler for the EBA process shows the interaction between fermentation yields, EBA step recovery, resin binding capacity and resin CIP cycles on the CoG/g, CoG/batch and cost of resin, labour and buffer per batch at the 2000 L production scale. Significant interactions between the EBA step recovery, fermentation yields and resin binding capacity and the CoG/g for the EBA process were recorded. The EBA resin binding capacity and fermentation yield had a significant impact on the CoG/batch and cost of resin, buffer and labour per batch of the EBA process in comparison to the number of CIP cycles of the resin.

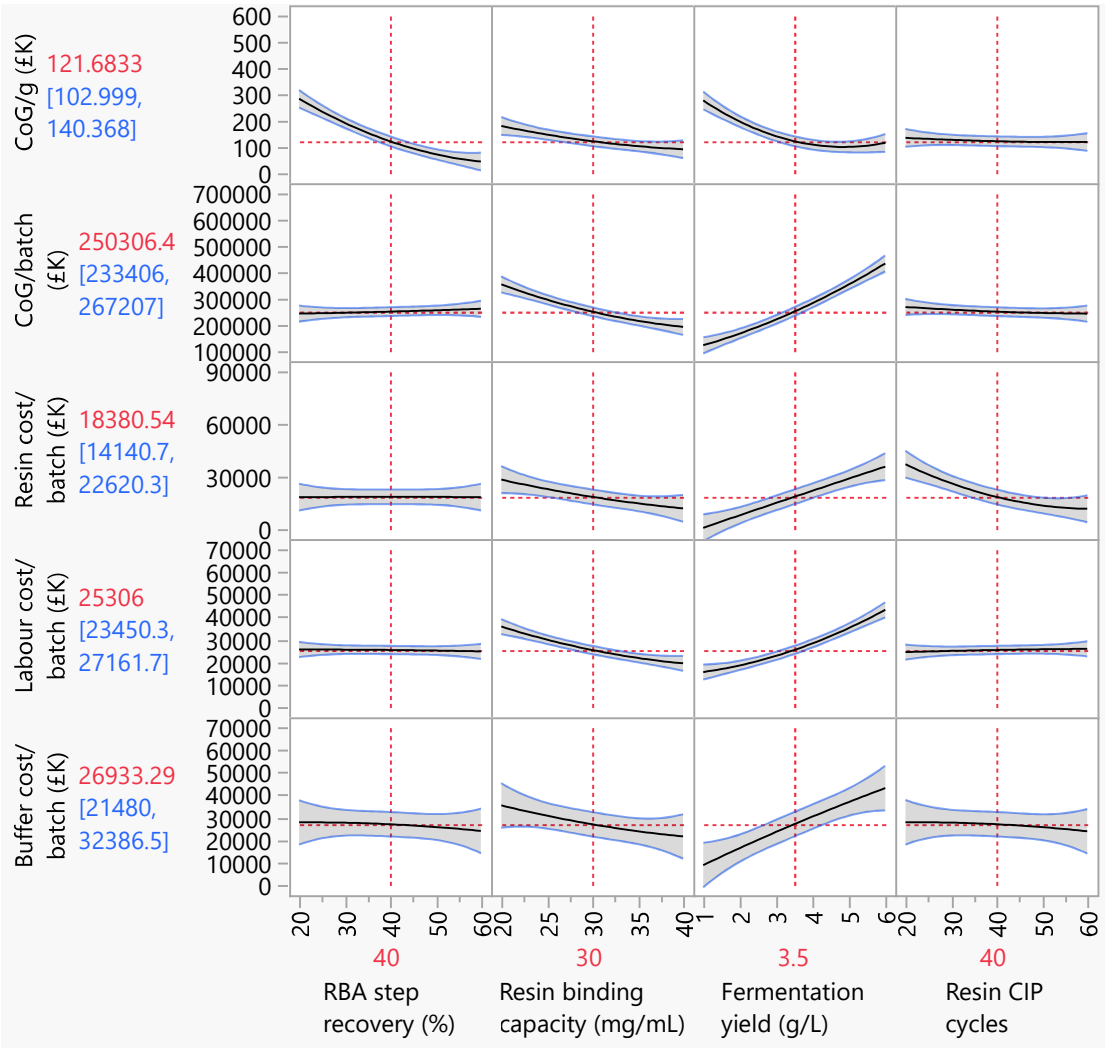

Figure 5.20 Prediction profiler for the RBA process.
The prediction profiler for the RBA process shows the interaction between fermentation yields, RBA step recovery, resin binding capacity and resin CIP cycles on the CoG/g, CoG/batch and cost of resin, labour and buffer per batch at the 2000 L production scale. Significant interactions between the RBA step recovery, fermentation yields and resin binding capacity and the CoG/g of the RBA process were recorded. The RBA resin binding capacity and fermentation yield had a significant impact on the CoG/batch and cost of resin, buffer and labour per batch of the RBA process in comparison to the number of CIP cycles of the resin.

5.4 Conclusions

In this chapter, the total CoG/g and CoG/batch for the His_6 tagged scFv antibody shMFELL2cys, manufactured in a high cell density *P. pastoris* fermentation and purified using EBA or RBA as a primary affinity purification step, were calculated using the BioSolve software. Economic evaluation of the EBA and RBA primary purification chromatography steps was completed to purify the His_6 tagged scFv antibody shMFELL2cys using EBA and RBA as a primary purification process.

A higher CoG/g for the EBA process compared to the RBA process when scaling up the process from a lab to a large manufacturing scale was calculated.

Comparing the cost of labour, buffer and resin per batch from a lab to pilot scale, higher costs for the EBA process compared to the RBA process were estimated. An increase in the CoG/batch increased the cost of the scFv antibody production in the EBA process compared to the RBA process.

A significant decrease in the CoG/g of the EBA and RBA processes was calculated when performing a scenario analysis at three different expression levels and from lab scale to pilot scale. In comparison to the EBA process, the RBA process showed a greater percentage of CoG/g decrease at the 2000 L scale.

The analysis was extended to investigate the interaction of various factors using response surface design in a DOE model on a large scale (2000 L). The DOE model calculated a lower CoG/g for the RBA process compared to the EBA process in both the best and worst-case scenario conditions.

A clear preference to introduce an RBA primary chromatography step with lower CoG/g was evident at all scales from small- to large-scale manufacturing processes to capture the His_6 tagged scFv antibody shMFELL2cys directly from the high cell density *P. pastoris* fermentation.

Chapter 6 Conclusions and future work

6.1 Conclusions

The aim of this study was to perform a cost-based analysis of the RBA and EBA chromatography processes for the direct capture of the His_6 tagged scFv antibody manufactured using high cell density *P.pastoris* fermentation. The aim was achieved by performing a CoG analysis of the RBA and EBA processes using BioSolve software, by optimising the RBA process and by generating practical data at 8L fermenter volume for the direct capture using the RBA and EBA processes.

The His_6 tagged scFv antibody shMFELL2cys was successfully expressed in the high cell density *P. pastoris* fermentation at a concentration of 680 to 600 mg/L and captured using the RBA and EBA chromatography processes. The calculated step recovery for RBA and EBA chromatography was 30% and 47%, respectively. A total amount of 801 mg and 611 mg of the scFv antibody shMFELL2cys was purified from the EBA and RBA chromatography processes, respectively.

The presence of the His_6 tagged scFv antibody in the flow-through samples taken during the RBA chromatography step indicates that a further reduction in the flow rate or dilution of the crude fermenter culture and an increase in the column size will facilitate a higher step recovery of the RBA process. In comparison to RBA chromatography, the His_6 tagged scFv antibody was not seen in the flow-through sample taken from the EBA chromatography, and a very small amount of antibody was seen in the wash samples.

The particle size distribution study results show the presence of yeast cells with the scFv antibody captured when using both RBA and EBA chromatography. This result suggests that extended washing of the RBA column in the reverse direction and the EBA column in downflow might improve the efficiency in washing out accumulated yeast cells. The particle size distribution study of the samples taken during EBA chromatography shows that resin particles were present in the wash sample taken after the application of the 15 L wash buffer because the application flow rate increased at the end of the washing step. While applying fermenter culture

to the column, steady-state expansion of the EBA column was maintained as resin particles were not seen in the flow-through samples.

The scFv antibody shMFELL2csy purified by using EBA and RBA has specificity and affinity for CEA, which is expressed on cancer cells. The HCP contamination in 1mg of scFv antibody purified using EBA chromatography was found to be twice as much as in the scFv antibody purified using RBA chromatography but within the traditional practice of setting the target of 100 ng/mg of the therapeutics protein. Therefore, the scFv antibody shMFELL2cys purified using the RBA and EBA chromatography processes can potentially be used for therapeutic and diagnostic purposes.

Results from the EBA and RBA screening experiments confirmed that 1 M NaCl concentration and pH 7.5 provided the best conditions for the direct capture of the His_6 tagged scFv antibody shMFELL2cys expressed in high cell density *P. pastoris* fermentation using EBA and RBA chromatography.

The static and dynamic binding capacities of the EBA resin were higher than those of the RBA resin. The pH had a significant impact on the static binding capacity of the RBA resin in comparison to the EBA resin. A 20% increase in the static binding capacity of the RBA resin was seen when the pH was increased from 7.0 to 8.0, which then decreased by 50% if the pH was further increased to pH 8.5.

The viscosity of the end of fermenter culture had a significant impact on the direct capture of the His_6 tagged scFv antibody using EBA and RBA chromatography. Resembling a viscous fermenter culture in the presence of 20% v/v glycerol decreased the static binding capacity by 32% and 27% respectively for the EBA and RBA resins. A 53% decrease in the DBC of both the EBA and RBA resins was also measured in the presence of 20% v/v glycerol.

A two-fold increase in the scFv antibody concentration was achieved by increasing the residence time from 40 s to 3.5 min for the RBA chromatography. This demonstrated the significance of the residence time on the direct capture of the His_6 tagged scFv antibody using RBA chromatography.

The economic evaluation of the EBA and RBA primary purification chromatography step was completed after purifying the His_6 tagged scFv antibody shMFELL2cys

using EBA and RBA as a primary purification process. A higher CoG/batch for the EBA process compared to the RBA process when scaling up the process from a lab to a large manufacturing scale was calculated.

When comparing the cost of labour, the buffer and resin per batch from the lab to pilot scale, higher costs for the EBA process compared to the RBA process were estimated. An increase in the CoG/batch increased the cost of the scFv antibody production in the EBA process compared to the RBA process.

A significant decrease in the CoG/g of the EBA and RBA processes was calculated when performing a scenario analysis at three different expression levels and from lab scale to pilot scale. In comparison to the EBA process, the RBA process showed a greater percentage of CoG/g decrease at the 2000 L scale. The analysis was extended to investigate the interaction of various factors using a response to surface design in a DOE model on a large scale (2000 L). The DOE model calculated a lower CoG/g for the RBA process compared to the EBA process in both the best and worst-case scenario conditions. While performing CoG/batch and CoG/gram analysis using DOE model and scenario analysis for the EBA and RBA processes at 2000L scale there was a limitation of the availability of practical data. Analysis was performed purely based on theoretical assumption. Output from the BioSolve software and DOE model had given preference to introduce RBA process. Use of EBA process at 2000L large scale manufacturing process has some practical and economical limitations. Higher cost of resin, limitation in maximum operational flow rate and validation of CIP procedure and commercial availability of large scale EBA column had direct impact on the introduction of EBA process at large scale. RBA column operation is comparatively less affected by changes in operational flow rate. It is recommended that the process is optimised from lab to large scale to ensure that the DBC is not adversely affected by operating the RBA column at higher flow rate. Availability of the large-scale RBA column and lower cost of resin and operation are in favour of introducing RBA process for the large scale manufacturing. In conclusion, this work shows that preferential introduction of an RBA primary chromatography step yields a lower CoG/g, from the small- to large-scale manufacturing processes, when capturing the His_6 tagged

scFv antibody shMFELL2cys directly from the high cell density *P. pastoris* fermentation.

6.2 Future work

Methods developed in this study should be repeated to perform small scale optimisation work with EBA and RBA resin using 1 mL to 5 mL RBA and EBA columns. In this study the small-scale experiments were performed using axial columns and shake flasks in batch mode. Experiments in this study were performed once and data generated using 8 L fermenter culture were used to perform CoG/batch and CoG/gram analysis for the EBA and RBA processes. Repeating the experiments to perform the CoG analysis will increase the confidence to introduce RBA as a primary chromatography step for the large-scale manufacturing process.Future work on the CoG analysis using BioSolve software and DOE model should be performed using practical data generated at large scale RBA or EBA processes. The cost of running the process can be a limiting factor for the amount of data that can be generated. This can be overcome by collaborative work between Biotech companies to investigate this process at large scale. An increase in the residence time of the application of a crude fermenter culture to the RBA or EBA chromatography facilitates a high level of non-specific binding of the HCP, HCD and cell debris to the column. Future work shall be performed to optimise the washing buffer composition with a range of imidazole concentrations to wash out the non-specifically bound contaminants. To reduce the amount of HCP and HCD contaminants in the eluted product , further investigation can be performed by introducing a step involving the elution of the bound His_6 tagged antibody with varying concentrations of imidazole in the elution buffer and a qualitative analysis of the elution faction for non-specifically bound contaminants. Optimisation of the washing buffer composition and investigation of the elution step of the bound proteins can reduce the burden on the subsequent polishing step by removing non-specifically bound contaminants.

The linear flow velocity of the RBA column increases from the inlet to the outlet of the column as the outer surface area is higher in comparison to the inner surface area. Therefore, average linear flow velocity should be calculated, and effect of linear flow rate should be considered while optimising the washing and elution strategy, it is necessary to consider the decrease contact time of the washing buffer from the inlet to the outlet of the RBA column. Further investigation can be performed on the application of the elution buffer in a reverse direction from the inner surface area to the outer surface area of the RBA column to reduce the concentration effect of the imidazole present in the elution buffer; this can remove the non-specifically bound contaminants from the column.

Direct capture of the untagged antibody expressed in the fermenter culture of high cell density *P.pastoris* using ion-exchange resin can be investigated using RBA chromatography. Similarly, an investigation can be performed into the direct capture of the antibody manufactured in *E.coli*, Bacillus subtilis and Saccharomyces cerevisiae using RBA or EBA chromatography.

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Appendix 1 Molecular weight markers run on 500 mL and 125 mL size exclusion column

Superdex 75 500 mL column used for SEC to purify EBA and RBA eluted scFv antibody. Gel filtration calibrated standards from Bio-Rad were run on the Superdex 75 column to ensure that columns were packed properly and samples eluting properly. Bio-Rad's gel filtration standard contains a mixture of proteins from 1350 to 670000 Daltons. Molecular weight markers are separated at the same column volume in pre- and post-use molecular weight marker runs, which ensures that the column was packed properly, and samples were separated evenly while applying RBA and EBA eluted protein after the concentration step. The pre- and post-use chromatogram of molecular weight marker run on Superdex 75 500 mL column can be seen in Figure 7.1.

Superdex 75 125 mL column was used for analytical SEC to analyse final pooled scFv antibody purified using EBA and RBA chromatography. Gel filtration calibrated standards were also run on analytical SEC column. Molecular weight markers are separated at the same column volume in pre- and post-use run, and so there is no impact on the column's performance in analysing the final purified scFv antibody purified using RBA and EBA chromatography. The pre- and post-use chromatogram of molecular weight marker run on Superdex 75 125 mL column can be seen in Figure 7.2.

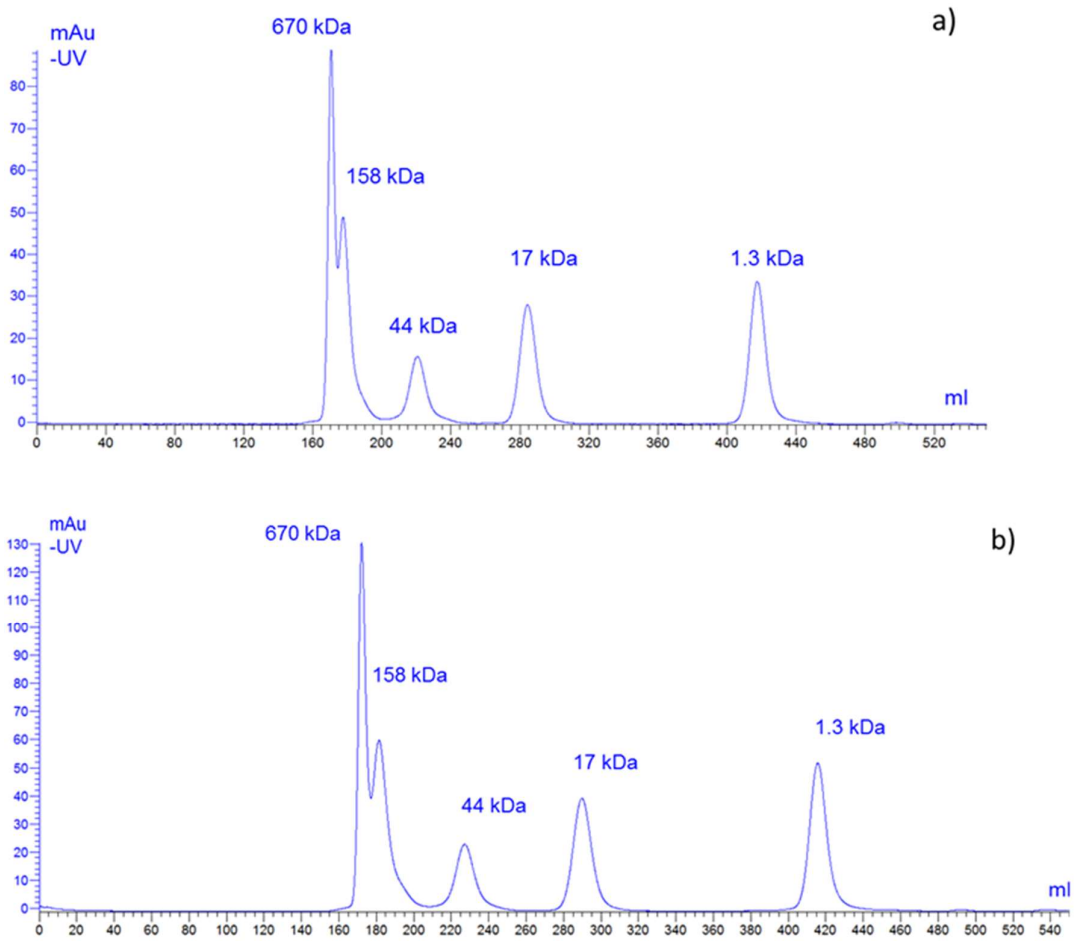


Figure 7.1 Chromatogram of molecular weight marker run on Superdex 75 500 mL column.

Molecular weight marker run on Superdex 75 500 mL column. a) Pre-use SEC run molecular weight marker run (500 µL of gel filtration standard applied with 1.5 mL of PBS at 4mL/min), b) Post-use SEC run molecular weight marker run (450 µL gel filtration standard applied with 1.5 mL PBS at 4 mL/min).

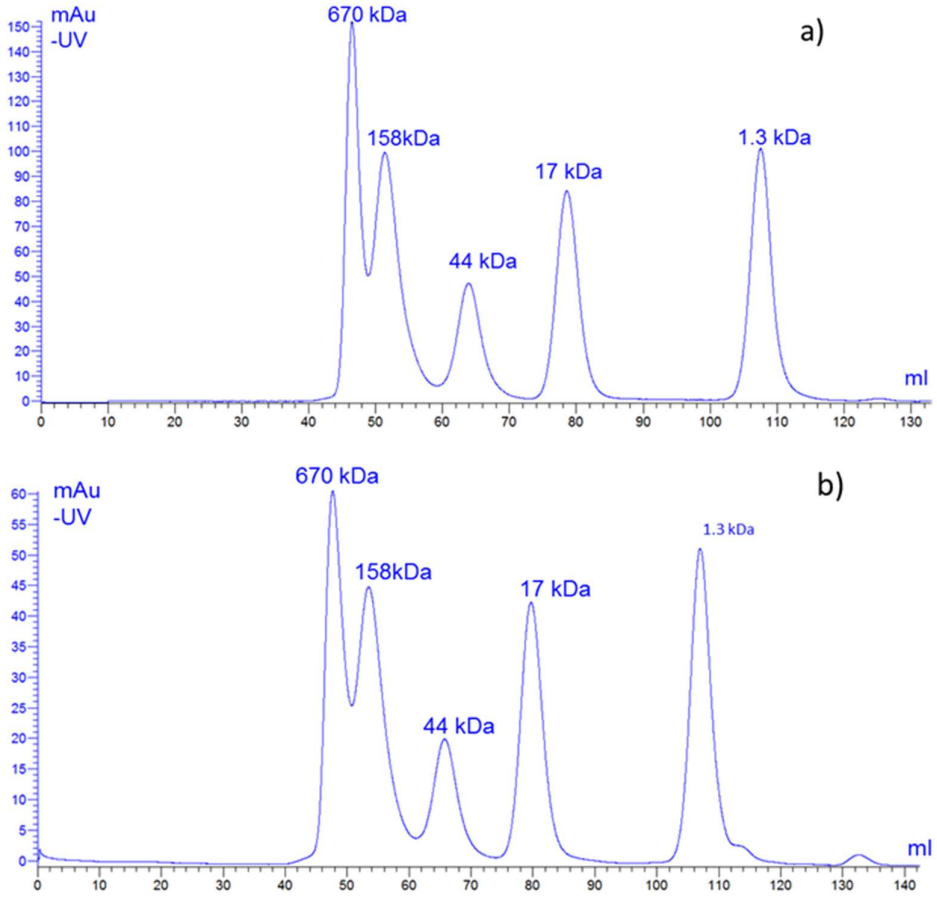


Figure 7.2 Chromatogram of molecular weight marker run on Superdex 75 125 mL column.

Molecular weight marker run on Superdex 75 125 mL column. a) Pre-use SEC run molecular weight marker run (250 µL gel filtration standard applied in 1 mL PBS at 1.5 mL/min), b) Post-use SEC run molecular weight marker run (250 µL gel filtration standard applied with 1.75 mL PBS at 1.5 mL/min).

Appendix 2 WCB production

WCB of *P. pastoris* strain X-33 expressing His_6 tagged scFv antibody shMFELL2cys was successfully manufactured from a single colony grown on YPDS WCB plate and stored in a 1 mL cryovial in a -80°C freezer. One single cryovial was thawed at room temperature for the production of the secreted His_6 tagged scFv antibody in a high cell density *P. pastoris* fermentation run. Figure 7.3 shows pictures of MCB and WCB colonies on the YPDS plate.

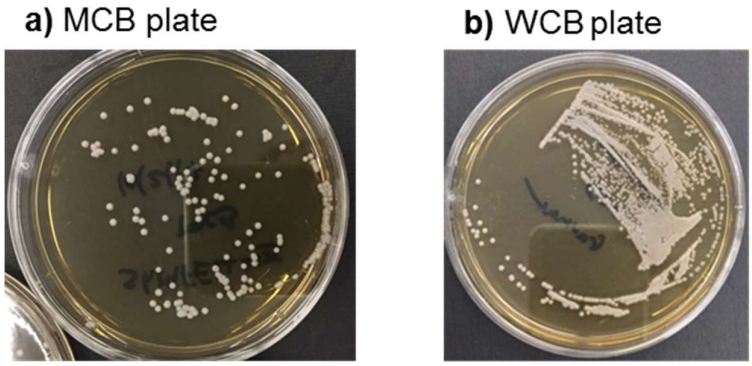


Figure 7.3 a) MCB colonies on YPDS Plate, b) WCB colonies on YPDS Plate.
A single colony of the MCB and WCB clones can be seen on the MCB YPDS plate (Figure a) and on the WCB plate (Figure b).

Appendix 3 Test expression

All three clones of the MCB and WCB selected from the YPDS plate grew to an average OD_{600} of 62 in 50 mL of BMMY medium after 72 h of incubation time. Figure 7.4 shows the growth profile of the MCB and WCB clones. Samples taken at 24, 48 and 72 h methanol induction points were analysed by Western Blot. All clones expressed the His_6 tagged 27kDa scFv antibody shMFELL2cys and protein bands can be seen on the Western Blot results in Figure 7.5.

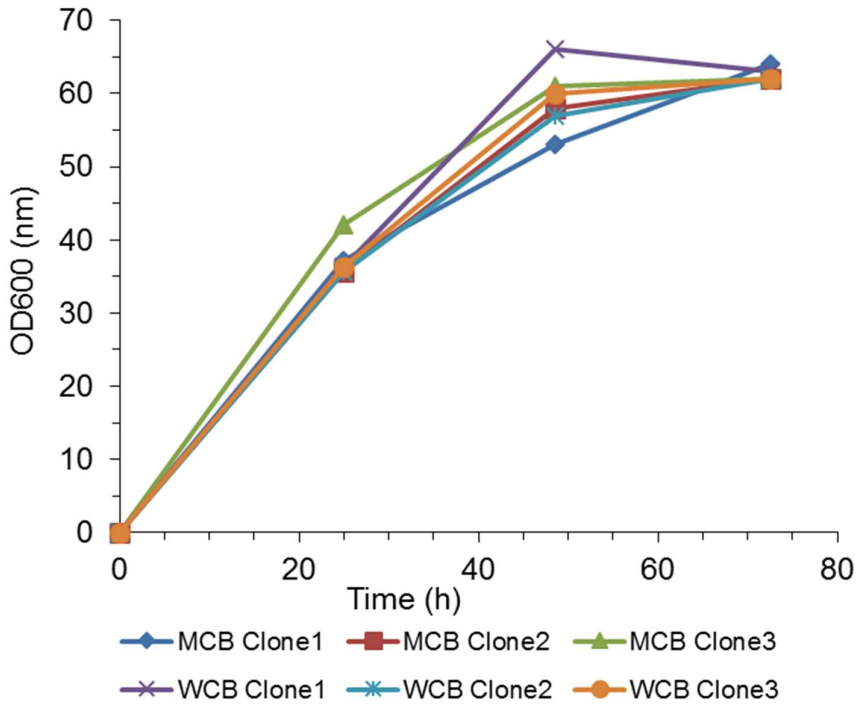


Figure 7.4 Growth profile of MCB and WCB clones in 250 mL baffled flasks. Compatibility of the growth kinetics of the MCB and WCB clones from the samples taken at 24 h, 48 h and 72 h from 50 mL final culture volume in a 250 mL baffled flask at 30°C and 200 RPM. Average OD_{600} of 62 was calculated for both MCB and WCB clones.

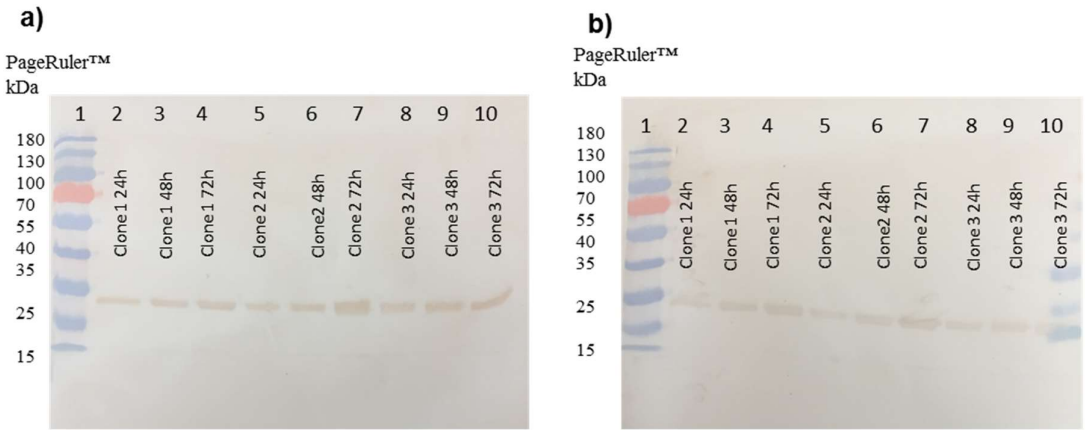


Figure 7.5 Western Blot results of the MCB and WCB test expression of three clones each.

Detection of the His_6 tagged scFv antibody fragment performed by Western Blot analysis of MCB and WCB clones test expression samples. Figure a) Western Blot analysis results of the MCB test expression clones expressing the His_6 tagged scFv antibody fragment at 24 h, 48 h and 72 h and Figure b) Western Blot result of WCB test expression clones expressing His_6 tagged scFv antibody fragment at 24 h, 48 h and 72 h. All three clones from MCB and WCB expressed 27 kDa His_6 tagged scFv antibody shMFELL2cys.

Appendix 4 Purity test of WCB and end of fermentation run samples

A purity test was run on the WCB and the end of fermentation run samples. No contaminant was found on TSA or SAB plates incubated for a minimum of five days at 22.5 °C and 32.5°C. Figures 7.6 and 7.7 confirm the result; only yeast colonies can be seen on the plates. This experiment confirms that only yeast *P. pastoris* X-33 strain was applied on the RBA and EBA columns during the primary purification step.

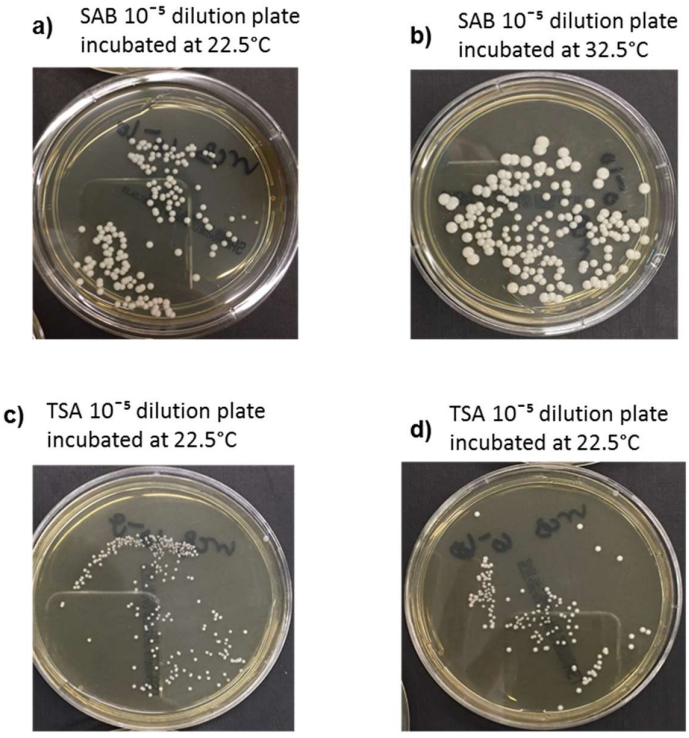


Figure 7.6 WCB purity test colonies on TSA and SAB plate after a minimum of five days' incubation.

A purity test result picture shows that only yeast colonies grew on the TSA and SAB plates incubated at 22.5°C and 32.5°C. Figures 6 a) and 6 b) show the colonies grown on the SAB plates after serial diluted at 10^{-5} WCB culture spread on the SAB plate and incubated at 22.5°C and 32.5°C. Figures 6 c) and 6 d) show the colonies grown on the TSA plate after serial dilution at 10^{-5} WCB culture spread on the TSA plate and incubated at 22.5°C and 32.5°C.

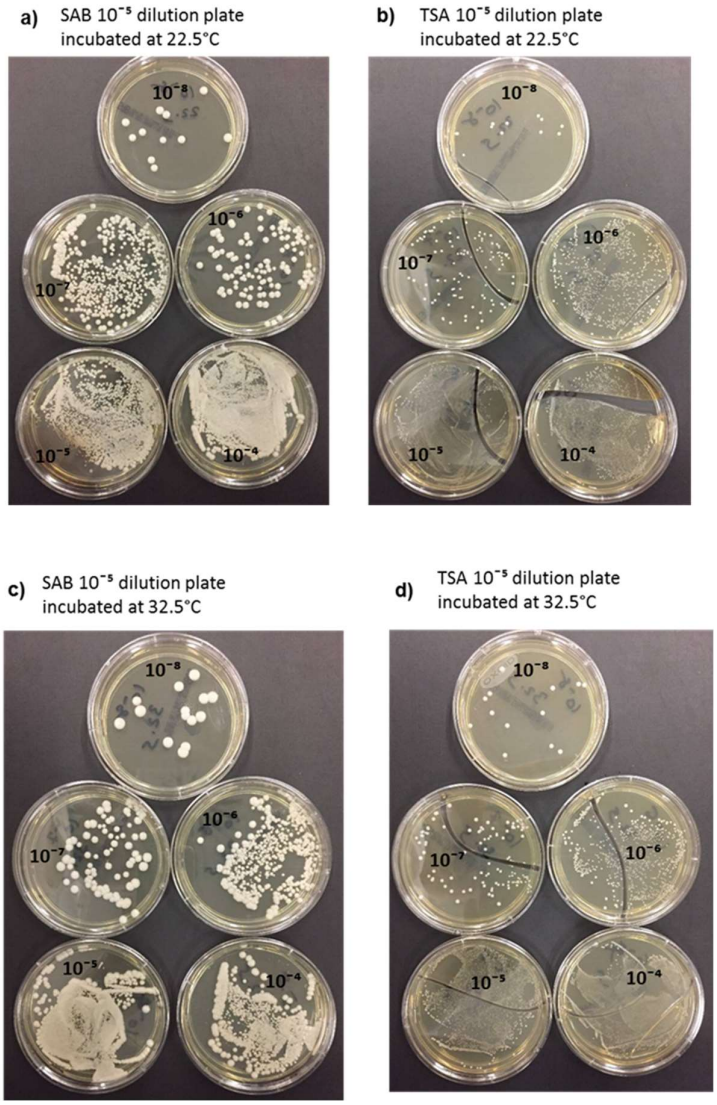
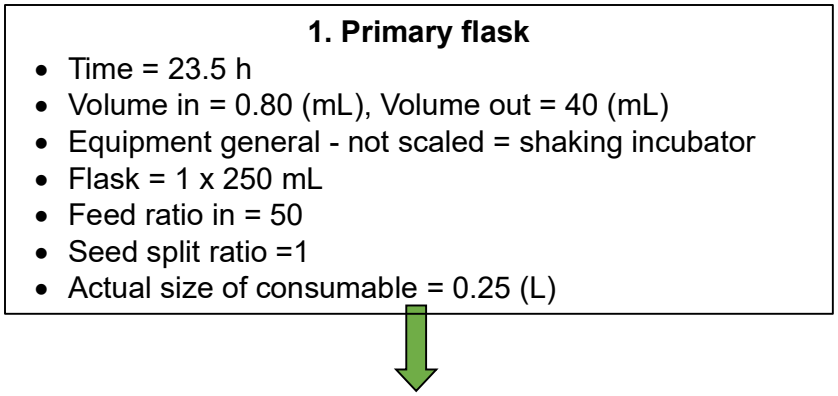


Figure 7.7 End of the fermenter culture purity test on TSA and SAB plates after five days' minimum incubation.

A purity test result picture shows that only yeast colonies grew on the TSA and SAB plates incubated at 22.5°C and 32.5°C. Figures 7 a) and 7 c) show the colonies grown on the TSA and SAB plates after serial dilution at 10^{-5} end of fermenter culture spread on the SAB plate and incubated at 22.5°C and 32.5°C. Figures 7 b) and d) show the colonies grown on the TSA and SAB plates after serial dilution at 10^{-5} end of fermenter culture spread on the TSA plate and incubated at 22.5°C and 32.5°C.

Appendix 5 Schematic diagram of detailed parameters for each unit operation added into the BioSolve software



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3. Production

- Time = 90.0 h
- Yield = 100%
- Volume in = 0.53 (L), Volume out = 8.0 (L)
- Product concentration = 0.60 (g/L)
- Total product mass out = 4.80 (g)
- Bioreactor = 1 x 20 L
- Actual fermenter volume (L) = 8
- Feed ratio in = 15.15
- Bioreactors pooled = 1
- Bioreactor type = stainless steel
- Target size equipment 1 (L): 8

4. EBA

- Total time = 23.1 h
- Yield = 47.0%

	In	Out
Total product mass (g)=	4.80	2.26
Total product volume (L)=	8.00	0.80
Product concentration (g/L)=	0.60	2.82

- EBA column size = 1 x 5 cm
- EBA column resin volume = 0.3 L
- Resin capacity (g/L) = 36
- Column bed height = 15
- Target Resin Cycles = 20
- Column Bed Expansion Ratio = 3.50
- Maximum Resin reuses = 10

4. RBA

- Total time = 16.5 h
- Yield = 30.3%

	In	Out
Total product mass (g)=	5.44	1.65
Total product volume (L)=	8.00	0.97
Product concentration (g/L)=	0.68	1.71

- RBA column size = 1 x 6 cm
- RBA column resin volume = 0.25L
- Resin capacity (g/L) = 32
- Column bed height = 6
- Target Resin Cycles = 20
- Column Bed Expansion Ratio = 0
- Maximum Resin reuses = 10

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5. UF/DF (EBA)

- Total time = 5.80 h
- Yield = 60.0%

	In	Out
• Total product mass (g) =	2.25	1.35
• Total product volume (L) =	0.80	0.15
• Product concentration (g/L) =	2.82	9.0

- UF skid size =0.015 m²
- Filter size = 0.015 m²
- Operation flux (LMH) = 80.0
- Duration = 1.0 h
- Concentration factor = 5.3
- Maximum filter reuses = 10
- Total membrane area (m²) = 0.015

5. UF/DF (RBA)

- Total time = 5.98 h
- Yield = 95.0 %

	In	Out
• Total product mass (g) =	1.65	1.57
• Total product volume (L) =	0.97	0.09
• Product concentration (g/L)=	1.7	16.2

- UF skid size =0.015 m²
- Filter size = 0.015 m²
- Operation flux (LMH) = 80.0
- Duration = 2.0 h
- Concentration factor = 10
- Maximum filter reuses = 10
- Total membrane area (m²) = 0.015

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6. SEC (EBA)

- Time = 15.5 h
- Yield = 57.0%

	In	Out
• Total product mass (g) =	1.35	0.77
• Total product volume (L) =	0.15	0.15
• Product concentration (g/L) =	9.0	5.11

- SEC column size = 2.6 cm
- Chromatography skid = 3 L/min
- SEC column resin volume = 1 x 0.59 L
- Column bed height = 100 cm
- Target cycles = 5.0
- Total concentrated cycles = 5.0
- Maximum flow rate = 57.0 cm/h
- Maximum re-uses = 50
- Working diameter = 2.60 cm

6. SEC (RBA)

- Time = 15.6 h
- Yield = 38.0%

	In	Out
• Total product mass (g) =	1.57	0.60
• Total product volume (L) =	0.09	0.09
• Product concentration (g/L) =	16.2	6.2

- SEC column size = 2.6 cm
- Chromatography skid = 3 L/min
- SEC column resin volume = 1 x 0.59 L
- Column bed height = 100 cm
- Target cycles = 4.0
- Total concentrated cycles = 4.0
- Maximum flow rate = 57.0 cm/h
- Maximum re-uses = 50
- Working diameter = 2.60 cm

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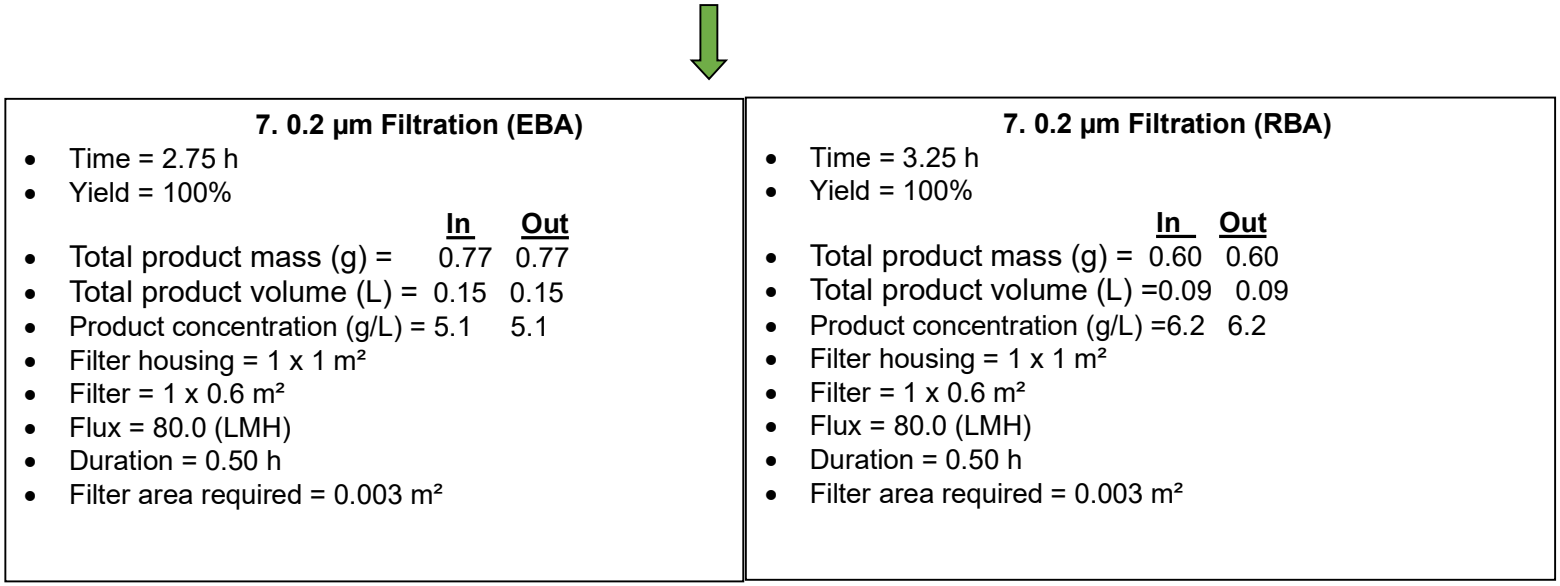

Figure 7.8 Schematic diagram of detailed parameters for each unit operation added into the BioSolve software.

Appendix 6 Table 7.1 and 7.2 listing RBA and EBA column setup for the CoG analysis at pilot and large scale

Table 6. 1 EBA yeast typical process work sheet setup

Fermenter volume (L)	EBA column	Column diameter	Cost (£K)	Resin volume at 15 cm settled bed height (L)	Expansion ratio	Expanded bed volume at 3.5 expansion	Flow velocity (cm/h)	Flow rate (L/min)	Volumetric flow rate (L/hr)	Average residence time (min)
8	STREAMLINE 50	5	3.5	0.300	3.5	1	213	0.07	4.2	14.8
20	STREAMLINE 100	10	7	1.180	3.5	4.12	213	0.28	16.7	14.8
100	STREAMLINE 200	20	11	4.7	3.5	16.45	213	1.1	66.9	14.8
200	STREAMLINE 400	40	11	18.8	3.5	66	213	4.5	268	14.8
500	STREAMLINE 400	40	35	18.8	3.5	66	213	4.5	268	14.8
1000	STREAMLINE 400	60	89	42.4	3.5	148	213	10	602	14.8
2000	STREAMLINE 600	60	89	42.4	3.5	148	213	10	602	14.8

Table 6.2 RBA yeast typical process work sheet setup.

Fermenter volume	RBA column volume (L)	Cost (£K)	Bed height	Height	r1 (outer cylinder radius)	r2 (inner cylinder radius)	Average flow velocity (cm/h)	Volumetric flow rate (L/min)	Flow rate (L/hr)	Residence time (min)
8	0.250	3	6	N/A	N/A	N/A	93	0.070	4.2	3.5
20	5	40	6	15	12	6	100	1.46	87.6	3.6
100	5	40	6	15	12	6	100	1.46	87.6	3.6
200	20	62	6	60	12	6	100	5.4	324	3.6
500	20	62	6	60	12	6	100	5.4	324	3.6
1000	50	82	6	63.2	24	18	100	13.2	792	3.6
2000	50	82	6	63.2	24	18	100	13.2	792	3.6

Note 1: RBA 250 mL column is a small section of the pilot-scale RBA column. A total cross-section area of 45 cm² was calculated by the supplier; therefore, height, r1 and r2 values were not applicable (N/A).

Note 2: RBA column bed heights and volumetric flow rate at 100 cm/h data was received from Proxcys Downstream Biosystems. Approximate values of RBA column cylinder height and outer and inner cylinder radius were used to calculate the RBA column volume (Appendix 7).

Appendix 7 Formula to configure the yeast typical RBA worksheet to calculate RBA column volume and average flow velocity

- **The formula below was configured in the BioSolve software to calculate radial bed column volume:**

Column volume (V)

$$V = \pi(r1^2 - r2^2) \times \mathrm{L}$$

Where,

L = Radial bed cylinder height

r1 = Outer cylinder radius

r2= Inner cylinder radius

User-defined parameters were added into the yeast typical RBA worksheet to calculate the large-scale column volume in the BioSolve software.

The formula below was updated in the yeast typical RBA worksheet:

=ROUNDUP(PI()*(F72^2-F74^2)*F56/4000,2)

=ROUNDUP(PI()*(**Outer cylinder radius**^2-**Inner cylinder radius**^2)***Radial column cylinder height**/4000,2)

- **The formula below was configured in the BioSolve software to calculate radial bed average velocity:**

Calculation of the average velocity ū (cm/h)

$$\bar{\mathrm{U}} = \frac{F}{2\pi L} \times \frac{ln\left(\frac{d1}{d2}\right)}{H}$$

Where,

L = Radial bed cylinder height

F = Volumetric flow rate (L/min)

d1 = Outer cylinder diameter (d1)

d2 = Inner cylinder diameter (d2)

H = Radial bed height

- User-defined parameters were added into the yeast typical RBA worksheet to calculate the sub-operation step time based on the average flow velocity.

  The formula below was updated in the yeast typical RBA worksheet.

=((((**flow velocity cm/h**)*2*PI()***Radial column cylinder height**)*(**redial bed resin height**/(ln(**Outer cylinder diameter/Inner cylinder diameter**))))/60)/1000

Figure 7.9 shows the schematic representation of the RBA column (L= radial bed cylinder height, r1 = outer cylinder radius, r2 = inner cylinder radius and H = radial bed height).

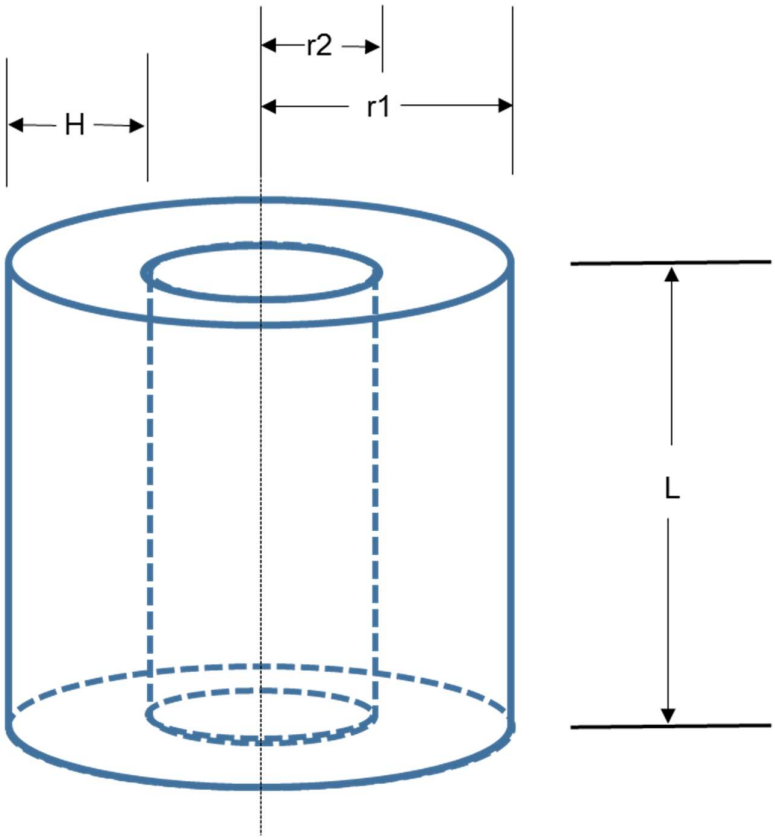

Figure 7.9 Pilot-scale radial bed column.
The figure above shows the schematic representation of the RBA column (L= radial bed cylinder height, r1 = outer cylinder radius, r2 = inner cylinder radius and H = radial bed height).

Appendix 8 Detail of annual salary, labour cost and list of consumables used during EBA and RBA processes

To manufacture the product in a GMP facility, eight production staff, one production supervisor, four quality assurance staff and three quality control staff were selected for both EBA and RBA processes in BioSolve software.

Table 6.3 details the annual salaries, overhead costs and hourly rates for each category.

Table 6.3 Detail of annual salaries of different function staff involved in manufacturing activity.

Type	Annual salary (£K)	Overhead
Production operator	41	52%
Production supervisor	58	52%
QA	35	52%
QC	50	52%

Tables 6.4 and 6.5 list the detailed labour costs for each subcategory of each unit operation per batch calculated using BioSolve software at 8L working volume. High production operation labour costs for the EBA unit operation increase the labour cost per batch for the EBA process.

Table 6.4 Detailed labour cost breakdown of each unit operation for yeast typical RBA process.

Labour hours					Labour cost per batch (£)					
Unit op	Direct hours	Solution prep	Cleaning	Total man hours	Production operator	Production supervisor	QA	QC	Indirect	Total cost
1	9.4	0.0	0.0	9.4	260	36	154	104	112	668
2	9.4	0.0	0.0	9.4	260	36	154	104	112	668
3	48.9	0.0	52.0	100.9	2782	389	1645	1115	1205	7137
4	36.1	7.7	0.0	43.8	1207	169	714	484	523	3098
5	12.2	5.1	4.0	21.3	586	82	346	235	253	1503
6	16.3	10.6	0.0	26.9	740	103	437	297	320	1900
7	3.6	0.0	4.0	7.6	209	29	124	84	90	539

Table 6.5 Detailed labour cost breakdown of each unit operation for yeast typical EBA process.

Labour hours					Labour cost per batch (£)					
Unit op	Direct hours	Solution prep	Cleaning	Total man hours	Production operator	Production supervisor	QA	QC	Indirect	Total cost
1	9.4	0.0	0.0	9.4	260	36	154	104	112	668
2	9.4	0.0	0.0	9.4	260	36	154	104	112	668
3	48.9	0.0	52.0	100.9	2782	389	1645	1115	1205	7137
4	50.8	7.3	0.0	58.2	1604	224	948	643	694	4116
5	11.8	4.9	4.0	20.6	568	79	336	228	246	1459
6	3.9	6.5	0.0	10.4	285	40	168	114	123	733
7	3.1	0.0	2.0	5.1	139	19	82	55	60	358

BioSolve can generate the list of consumables used during the production to plan the bill of materials required per batch. Table 6.6 lists the consumables required for the EBA and RBA processes. These quantities were calculated based on 10 maximum re-use cycles defined for both EBA and RBA resins. In total, 69 production batches per year were calculated for both EBA and RBA processes.

Table 6.6 List of consumables used for the EBA and RBA processes.

No	Type	Description	Size	Units	Per batch
1	Column resin	EBA	0.300	L	0.030
2	Column resin	RBA	0.250	L	0.025
3	Column resin	SEC	0.590	L	0.0590
4	Filter	Sterile filter	0.600	m^2	1
5	Filter	UF filter	0.015	m^2	1
6	Flask	250 ml	0.250	L	1
7	Flask	2L	2	L	2
8	Hold bag	Hold bag	5	L	4
10	Hold bag	Hold bag	10	L	1
11	Solution mixer liner	Prep bag	20	L	1
12	Solution mixer liner	Prep bag	50	L	4