Optimising host cell physiology and stress avoidance for the production of recombinant human tumour necrosis factor in Escherichia coli

Selas Castineiras, Tania; Williams, Steven; Hitchcock, Antony ; Cole, Jeffrey; Smith, Daniel; Overton, Timothy

DOI:
10.1099/mic.0.000622

Document Version
Early version, also known as pre-print

Citation for published version (Harvard):
Selas Castineiras, T, Williams, S, Hitchcock, A, Cole, J, Smith, D & Overton, T 2018, 'Optimising host cell physiology and stress avoidance for the production of recombinant human tumour necrosis factor in Escherichia coli', Microbiology, vol. 164, pp. 440-452. https://doi.org/10.1099/mic.0.000622

PREPRINT

Optimising host cell physiology and stress avoidance for the production of recombinant human tumour necrosis factor α in *Escherichia coli*

Tania Selas Castiñeiras[1,2,3], Steven G Williams[1], Antony Hitchcock[1], Jeffrey A Cole[3,4], Daniel C Smith[1], Tim W Overton[2,3*].

[1]Cobra Biologics, Stephenson Building, The Science Park, Keele ST5 5SP, UK.

[2]School of Chemical Engineering, [3]Institute of Microbiology & Infection, and [4]School of Biosciences, The University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

* To whom correspondence should be sent: t.w.overton@bham.ac.uk +44 (0) 121 414 5306

Keywords: Heterologous protein; High Cell Density Culture; Fed-batch fermentation; Protein solubility; Biopharmaceutical.

Subject category: Biotechnology

Word count: 4958

ABSTRACT

As high-level recombinant protein production (RPP) exerts a massive stress on the production host, an extensive literature on RPP optimisation focuses on separating the growth phase from RPP production once sufficient biomass has been obtained. The aim of the current investigation was to optimise benefits of the relatively neglected alternative strategy to achieve high level RPP during growth by minimizing stress on the host. High yields of the biopharmaceutical recombinant human Tumour Necrosis Factor alpha (rhTNFα) were obtained by fed-batch fermentation relevant to industrial production based upon parameters that most severely affected RPP in preliminary laboratory scale batch cultures. Decreasing the inducer concentration and growth temperature but

increasing the production period were far more effective for increasing RPP yields than changing the growth phase at which production was induced. High yields of up to 5 $g \cdot L^{-1}$ of rhTNFα were obtained with minimal plasmid loss even in synthetic media that lack animal-derived components and are therefore fully compliant with regulatory requirements. Most of the product was soluble and biologically active. In summary, stress minimisation was shown to be an effective way to optimise production of rhTNFα. Data generated in shake-flask experiments allowed design of intensified bioreactor cultures in which RPP and growth could be balanced, leading to higher yield of both rhTNFα and biomass than previous fermentations. Additional benefits of this approach include avoidance of lysis during harvesting and downstream processing and the ability to adjust the process to minimize the need for long periods of staff supervision.

INTRODUCTION

Recombinant proteins (RP) for use as human biopharmaceuticals represent a commercially important product group for the pharmaceutical industry, with 40 % of pharmaceutical sales accounted for by biopharmaceutical drugs. Seven of the top-selling 10 drug products in 2013 were protein biologics [1]. Bacteria such as *Escherichia coli* are favoured as the production host when the product is relatively simple and does not require glycosylation or other extensive posttranslational modification for function. *E. coli* remains an important host for biopharmaceutical production due to its relative simplicity when compared to mammalian cell systems such as CHO, and its ease of use [2].

An extensive literature documents two major routes for RP production in *E. coli*: generation of RPs in insoluble inclusion bodies, which although easy to harvest need first to be denatured then refolded *in vitro* to a functional soluble form [3]; or generation of soluble functional RP *in vivo* [4]. Although there are industrial examples of both routes, the former pathway relies upon successful refolding following inclusion body denaturation, which for many RPs can be very inefficient, complex and costly. Therefore, production of soluble RP in *E. coli* remains an important objective of process development.

Industrially relevant fermentation processes are designed to generate large quantities of RP along with high biomass yields. These conflicting objectives result in severe physiological stress on the bacterial host due to competition between the two processes for metabolic resources such as amino acids and other metabolites, aminoacylated tRNAs, energy and reducing power. Many successful fermentations avoid this conflict by separating the growth and RP production phases, thereby minimizing the selection of unproductive, plasmid-free bacteria or selection of mutants defective in RP accumulation. In the current study we have exploited a less studied approach involving concomitant growth and RP production under conditions that decrease the stress on the host bacteria [5,6,7]. Previous studies have shown that by growing bacteria at a lower temperature and inducing production at a lower level by use of weaker promoters or lower inducer concentrations, RP production can be more easily balanced with biomass accumulation, allowing higher biomass concentrations to be achieved. As RP is generated more slowly, successful folding is enhanced, thus increasing soluble protein production. However, only a very few of the previous studies using this approach have reported the effects of all of the key variables such as the effects of medium composition, temperature, inducer concentration, the structure of the recombinant plasmid and then reported process development into fed batch cultivation. In many cases the target was green fluorescent protein rather than an industrially important product, and the medium components used would not meet current GMP (Good Manufacturing Practice) requirements.

Human Tumour Necrosis factor (TNFα) is a cell signalling protein involved in systemic inflammation and its primary role is the regulation of immune cells. TNFα is first synthesised in humans as a 26 kDa transmembrane precursor protein, which is processed into an active, soluble 17 kDa protein that associates into homotrimers [8]. Recombinant human TNFα (rhTNFα) is currently on the market under the international non-proprietary name tasonermin. It is expressed as the soluble 17 kDa monomer in *E. coli*. It was approved by the European Medicines Agency in 1999 for the treatment of soft-tissue sarcoma and commercially produced by Boehringer Ingelheim under the trade name of Beromun®. TNFα was selected as a model RP in this study due to its commercial relevance, because it has been used for other studies as a model protein for cytoplasmic RP production in *E. coli* [9] and since reference material can be commercially obtained. Our first aim was to define parameters that

are most significant for the production of soluble rhTNFα in *E. coli* shake-flask cultures. Data from these initial studies were used to direct development of high cell density fed-batch bioreactor cultures that lack components of animal origin. The results demonstrate that stress minimisation can be successfully applied to generate soluble rhTNFα production in an industrially relevant process.

METHODS

Bacterial strain and plasmids

E. coli BL21-T7 (F- *ompT lon hsdS*$_B$*(r*$_B^-$ *m*$_B^-$*) gal dcm araBAD::T7RNAP*) sourced from Cobra Biologics (Keele, UK) was used for the production of rhTNFα. The gene coding for hTNFα was synthesised and cloned into the pLT72 vector (Cobra Biologics, Keele, UK), under the transcriptional control of the T7 promoter. Addition of arabinose to *E. coli* BL21-T7 induces production of the T7 RNA polymerase, inducing expression from pLT72. Additionally, three different vectors were generated containing: (i) the sequence encoding the hTNFα gene and the T7 terminator sequence downstream from the multiple cloning site (pLT72-T7t-TNFα); (ii) the T7 terminator sequence and the kanamycin gene in reverse orientation (pLT72-T7tKan-TNFα); and (iii) the T7 and T2 terminator sequences flanking the kanamycin resistance gene (pLT72-T7tKanT2t-TNFα). Plasmid vectors pLT72 and the pLT72-TNFα were kindly generated by Bruce Humphrey at Cobra Biologics.

Shake-flask growth experiments

In initial experiments, biomass and rhTNFα production in induced and non-induced conditions in shake-flasks were compared using two commonly used media; Luria Bertani broth (LB) and Terrific broth (TB). Luria Bertani (LB) agar contained 10 g·L^{-1} BBL™ phytone peptone (BD), 5 g·L^{-1} Bacto™ yeast extract (BD), 5 g·L^{-1} NaCl and 15 g·L^{-} extra-pure agar (Merck Millipore) in deionised water. Luria Bertani (LB) broth contained 10 g·L^{-1} BBL™ phytone peptone, 5 g·L^{-1} Bacto™ yeast extract and 5 g·L^{-1} NaCl in deionised water. Terrific broth (Life technologies) contained 47 g·L^{-1} of premade terrific broth powder (equivalent to 11.8 g·L^{-1} SELECT peptone 140, 23.6 g·L^{-1} yeast extract, 9.4 g·L^{-1} K_2HPO_4 and 2.2 g·L^{-1} KH_2PO_4) and 4 mL·L^{-1} of glycerol in deionised water. Starter cultures were grown overnight at 30 °C and 200 rpm from a single colony of bacteria in 10 mL of LB broth with 50

μg·mL^{-1} kanamycin in a 20 mL bottle. Cultures were grown in 50 mL of LB or TB supplemented with 50 μg·mL^{-1} kanamycin in 250 mL baffled shake-flasks. Sufficient inoculum was added to achieve a starting OD_{600} of 0.1. Upon induction, casamino acids were added to cultures to a final concentration of 2 %, as it has been reported that the addition of casamino acids has a beneficial effect on rhTNFα production [10].

Fed-batch fermentation methods are described in Supplemental information.

For harvest of cell pellets for purification of rhTNFα, the culture was centrifuged at 3,500 *g* at 4 °C for 30 min (Sorvall RC3B Plus, rotor Sorvall HLR6/H6000A/HBBC). Pellets were resuspended in phosphate buffered saline (PBS; Gibco, Life Technologies) and homogenised using a Dounce homogeniser. The homogenised cell paste was centrifuged at 7,500 *g* at 4 °C for 30 min (Sorvall RC53 Plus, rotor Sorvall SS-34). Cell paste was stored at -20 °C.

Analysis techniques

The optical density of cultures at 600 nm (OD_{600}) was measured using an Amersham Pharmacia Ultrospec 1100 Pro UV/ Visible Spectrophotometer. Culture samples were also serially diluted in PBS and plated onto LB agar for determination of CFU. For plasmid retention analysis, LB agar plates were incubated at 37 °C overnight; colonies were transferred by replica plating to LB agar and LB agar supplemented with 50 mg·L^{-1} kanamycin and incubated overnight at 37 °C.

Subcellular fractionation

For separation of soluble and insoluble protein fractions, a volume of culture equivalent to 1 mL at an OD_{600} of 1 was centrifuged at 12,000 *g* for 10 min. Pellets were re-suspended in 250 μL of 50 mM Tris-HCl pH 8, 10 mM $MgCl_2$ and 1 μL of benzonase nuclease (Merck Millipore) and incubated on ice. Lysozyme (3 μL of 10 mg·mL^{-1}; Sigma-Aldrich) was added and samples incubated on ice for 30 mins. Cells were lysed using freeze / thaw cycles; a minimum of 3 cycles of freeze (ethanol/dry ice bath) and thaw (37 °C) were carried out for each sample. Samples were centrifuged at 12000 *g* for 30 min, to separate the soluble (supernatant) and insoluble (pellet) protein fractions. Pellets were re-

suspended in 250 µL of 50 mM Tris-HCl pH 8, 10 mM $MgCl_2$, constituting the insoluble protein fraction.

SDS-PAGE

SDS-PAGE and Western blotting were performed according to standard methods as detailed in the supplemental information.

Purification of rhTNFα

The purification of rhTNFα obtained from fermentation studies was carried out by Nicola Barison at Cobra Biologics. A proprietary purification protocol was used for the purification of rhTNFα and only a summary of the process will be described here. The cell paste obtained from fermentation studies was resuspended and cells were disrupted by the use of a high-pressure cell disruption system (Constant systems). The soluble protein fraction was obtained by centrifugation and clarified. rhTNFα was purified by a process comprising an ammonium sulphate precipitation and several chromatography steps including anion exchange and heparin affinity chromatography. The final product presented a purity greater than 95 %, as quantified by densitometry from SDS-PAGE gels (Supplemental Fig. S1).

TNFα cytotoxicity assay

The C3H mouse fibrosarcoma cell line L929, a cell line sensitive to the activity of TNFα, was used to evaluate the activity of rhTNFα produced by fed-batch fermentation [11]. L929 cells were grown in T225 flasks with Eagle's minimum essential medium (EMEM) supplemented with 10 % (v/v) fetal bovine serum (FBS), 2 mM glutamine and 0.1 % $NaHCO_3$. The cell culture was incubated at 37 °C and 5 % CO_2 for three days. Once they reached confluency, cell cultures were passaged to a new T225 flask by removing the culture medium, washing the cells with PBS, trypsinisation to detach cells, resuspension in fresh EMEM and transfer to new T225 flasks with fresh EMEM.

For the cytotoxic bioassay, 6×10^4 cells suspended in EMEM medium were added to each well of a 96 well plate and incubated at 37 °C and 5 % CO_2 for 18 hours. Once confluent, the medium was

exchanged for fresh EMEM medium containing 2 $\mu g \cdot mL^{-1}$ actinomycin D, a cell growth inhibitor preventing cell proliferation and sensitising the cells to the activity of rhTNFα, leading to apoptosis [11]. Different concentrations of rhTNFα reference material (Life Technologies), rhTNFα purified from cell paste generated in fermentation 1 or buffer were added to the plates and incubated for 12 - 18 hours at 37 °C and 5 % CO_2. After incubation, the culture medium was discarded and 200 µL staining solution (0.5 % (v/v) crystal violet in 20 % (v/v) methanol) was added to each well for 10 minutes. The staining solution was discarded by inversion of the plate, and excess staining solution removed by the addition of deionised water. The waste was removed and the L929 cells were solubilised by the addition of 100 µL of 1 % (w/v) sodium dodecyl sulphate solution. The plate was incubated for 1 hour on a rotary shaker at 180 rpm. The OD_{580} of each well was measured using a FLUOstar Omega Microplate Reader (BMG LABTECH). The mean absorbance for each triplicate set of standards or samples assayed were calculated. Using the mean absorbance data, the percentage of cytotoxicity was calculated using equation 2:

$$\% \text{ cytotoxicity} = \left[1 - \frac{\text{absorbance of sample}}{\text{absorbance of zero TNF}\alpha \text{ control}}\right] \times 100 \qquad (2)$$

The concentration of purified rhTNFα giving rise to a cytotoxicity value of 50 % (LD_{50}) was determined. A hTNFα standard curve was constructed by plotting the percentage cytotoxicity values for the hTNFα standards against their concentration using GraphPad Prism® software. The standard curve was used to calculate the specific activity of the purified rhTNFα. A concentration of 1 unit (U) of specific activity per mL is defined as that which gives rise to 50 % cytotoxicity in the bioassay (ie the LD_{50}).

RESULTS AND DISCUSSION

Selection of culture medium for the production of rhTNFα

Expression of rhTNFα was driven from an arabinose-induced T7 expression system, similar to the widely-used DE3 / pET system [12]. *E. coli* strain BL21-T7 was used as a host, carrying a chromosomal T7 RNA polymerase gene under the control of an arabinose-inducible promoter. The gene encoding rhTNFα was cloned into pLT72 under the control of a T7-dependent promoter. In

initial experiments, *E. coli* BL21-T7 transformed with either pLT72-TNFα or the empty vector (pLT72) were grown with aeration in Luria Bertani broth (LB) or Terrific Broth (TB). At an OD_{600} of 1, half of the cultures were induced with a final concentration of 0.2 % (w/v) arabinose and casamino acids were added [10]. Biomass accumulation, culturability (colony forming units), plasmid retention and protein production were analysed (Figs. 1 & 2). Growth of cultures transformed with either the control plasmid or the production plasmid stopped soon after induction. This was expected because production of T7 RNA polymerase, even without production of an RP, induces stress responses in *E. coli* [13]. The final biomass concentration in non-induced cultures in LB was higher than in TB. For each medium, non-induced cultures containing the empty vector and the vector encoding rhTNFα grew similarly. However, for induced cultures, TB cultures grew faster and reached a higher OD_{600} than LB cultures. After either 2 h or 24 h post-induction, induced cultures expressing rhTNFα also had higher culturability in TB than in LB.

SDS-PAGE analysis revealed that rhTNFα accumulated gradually after induction, reaching a maximum of 20 % of the total cell protein after 24 hours of growth for both TB and LB (Fig. 2). Very little rhTNFα was present in cells before induction or in non-induced cells after 24 hours growth, revealing that this expression system offers tight regulation of RPP. Bacterial pellets harvested after 24 hours growth were also fractionated into soluble and insoluble fractions. SDS-PAGE revealed that around 55 % of the rhTNFα was present in the soluble fraction for both media. As a result of its buffering capacity and the slight improvements noted in growth and culturability, TB was selected for use in further experiments.

SDS-PAGE analysis identified an unexpected protein band (~31 kDa) in induced cultures of cells carrying both the empty vector and the vector encoding rhTNFα. The concentration of this unknown protein band accounted for up to 10 % of the total cell protein (in samples from induced cultures carrying the empty vector). It was hypothesised that this unknown protein could be the product of the kanamycin resistance gene, aminoglycoside 3' phosphotransferase (APH), which has a comparable molecular weight. The lack of terminator sequences downstream of the multiple cloning site on the backbone of vector pLT72 may have allowed read-through by the T7 RNA polymerase, leading to the overproduction of APH.

Effect of inducer concentration and induction point on rhTNFα production

Addition of 0.2 % (w/v) arabinose as an inducer in the previous experiment resulted in stress as evidenced by growth arrest, a decrease in viability and plasmid loss (potentially due to a decrease in culturability of plasmid-containing, productive, bacteria), even in cultures containing the empty vector. The effect of different inducer concentrations (1 %, 0.2 %, 0.05 %, 0.02 % or 0.002 % arabinose, added at an OD_{600} of 1) was tested. Bacteria transformed with the empty vector (pLT72) or the vector coding for rhTNFα (pLT72-TNFα) under non-inducing conditions were used as controls (Fig. 3).

Growth of cultures induced with 1 % to 0.02 % arabinose was arrested following arabinose addition and more than 80% of the bacteria were plasmid deficient after 24 h. The final biomass concentration after 24 h was inversely proportional to the inducer concentration used, but higher for induced cultures transformed with the recombinant plasmid than the non-induced cultures or cultures containing empty vector (Fig. 3a). In contrast, growth of cultures induced with 0.002 % arabinose was only slightly inhibited (Fig. 3a), and more than 80 % of these bacteria had retained the plasmid after 24 h (Fig. 3b).

The concentration of rhTNFα in cells after 24 hours growth was similar in all cultures (Fig. 3c), independent of the arabinose concentration used, showing that the lowest concentration of arabinose, 0.002 %, was sufficient to fully induce the T7 expression system. In addition, the majority of the rhTNFα was found to be accumulated in the soluble protein fraction independently of the concentration of arabinose used to induce the cultures (data not shown).

The effect of changing the point of induction in shake-flasks was evaluated by inducing RP by adding 0.2% arabinose at an OD_{600} of 0.5, 2 or 3 (Supplemental Fig. S2). Unlike changing the inducer concentration, there were no large differences between cultures induced at different cell densities. Cultures induced at an OD_{600} of 0.5 grew more slowly after induction but reached higher cell densities than those cultures induced at an OD_{600} of 2 or 3 (Supplemental Fig. S2a). There were no significant differences in CFU or in rhTNFα productivity for cultures induced at an OD_{600} of 0.5, 2 or 3 and

plasmid loss was observed 24 hours post-inoculation for all the induced cultures as a result of the high concentration of arabinose used (Supplementary Fig. 2b and data not shown).

Effect of temperature on rhTNFα productivity

As well as lowering inducer concentration, stress minimisation can be achieved by decreasing the temperature of growth, thus slowing growth and protein production rates. This has been previously been shown to favour accumulation of recombinant proteins in a soluble form [6,14]. Cultures were grown as before in TB supplemented with casamino acids, but at temperatures of 37 °C, 30 °C, or 25 °C. Expression of rhTNFα was induced by the addition of arabinose to a final concentration of 0.2 % at OD_{600} = 1, cell samples were harvested 4 hours after induction, separated into soluble and insoluble fractions and analysed by SDS-PAGE (Fig. 4a). The proportion of rhTNFα in the soluble fraction increased as the growth temperature decreased.

To investigate the interplay between inducer concentration and temperature of growth, further cultures were grown as before at 25 °C and at OD_{600} = 0.5 induced with concentrations of arabinose between 0.2 % and 0.001 %. Plasmids were retained for 24 h by uninduced cultures or cultures transformed with the empty vector (Fig. 4b). Although >90 % of bacteria induced with 0.2 % arabinose had lost the plasmid within 24 h, plasmids had been retained by most of the bacteria at much higher induced concentrations at 25 °C than at 30 °C (Compare Figs. 3b and 4b). Production of rhTNFα production was fully induced with 0.005 % and 0.002 % arabinose, but was suboptimally induced at 0.001 % arabinose (Fig. 4c).

Intensification of rhTNFα production in fed-batch fermentations

The optimal conditions for the production of rhTNFα defined during shake-flask studies were transferred to 5 L fed-batch fermentations using medium A, a semi-defined culture medium formulation obtained from Cobra Biologics, supplemented with 2 % casamino acids. Production of rhTNFα was induced with arabinose to a final concentration of 0.005 % at an OD_{600} of 0.5 after 3 hours of growth (Fig. 5). The culture grew after inoculation reaching a final OD_{600} of 90.8 after 48 hours (Fig. 5a). Although μ initially exceeded 0.4 during the initial batch phase of growth, it

decreased below 0.4 after 6 hours. The CFU dropped after 7 hours growth, and plasmid retention dropped below 90 % after 11 hours (Fig. 5b). After 48 hours, only 2 % of the bacteria had retained the plasmid. Although rhTNFα had accumulated to 22 % of cellular protein after 26 h, it did not significantly increase after that point (Fig. 5c). The increase in biomass between 26 h and 48 h did not correlate with an increase in rhTNFα productivity, as expected since the plasmid retention of the culture was low and decreasing. Production of the 31 kDa APH protein had also accumulated by 9 h post-induction. Analysis of soluble and insoluble cellular fractions revealed that the majority of rhTNFα was present in the soluble fraction (Fig. 5d).

Taken together, these data revealed that the fermentation conditions as defined in shake-flask studies were a good starting point for fermentation development, as rhTNFα was generated in the soluble fraction in significant quantities and induction of rhTNFα production did not cause growth arrest or immediate decreases in CFU or plasmid retention. However, harvesting the culture after 30 h when the percentage of cellular protein that was rhTNFα was the greatest (25 %) would not have resulted in high overall rhTNFα yield due to the low biomass (OD_{600} < 40). This indicates that allocation of resources to growth and RPP was unbalanced. In addition, production of APH from pLT72-TNFα could have increased the metabolic burden on the cells.

Improvement of vector design for minimisation of APH synthesis

Although high yields of rhTNFα were obtained in bacteria transformed with plasmid pLT72-TNFα, plasmid deficient bacteria were detected 24 h post-induction under all growth conditions tested. Attempts were therefore made to decrease the stress on the host further by decreasing expression of AHP, which was suspected to be the abundant 31 kDa protein detected by SDS PAGE. Three modified plasmids were constructed (Fig. 6a), and plasmid retention and rhTNFα accumulation during growth in small scale batch cultures were compared with those of the original plasmid. For these experiments, bacteria were grown at 30 °C in Terrific Broth and RP was induced with 0.02 % arabinose. These conditions were known from previous experiments to show high plasmid loss, so any improvement in plasmid retention would indicate reduced stress [6].

In contrast to the 20 % plasmid retention by pLT72-TNFα transformant, 40 % of the bacteria transformed with plasmid pLT72-T7t-TNFα in which the T7 terminator was cloned downstream of the TNFα gene had retained the plasmid 24 h post-induction (Fig. 6b). Similar results were obtained with plasmid pLT72-T7tKanT2t-TNFα with both the T7 and the T2 terminators after the TNFα gene and the kanamycin gene in reverse orientation. However, plasmid retention was further improved (~80 %) in cultures carrying the vector with the T7 terminator sequence and the kanamycin resistance gene in the opposite orientation to the rhTNFα gene (pLT72-T7tKan-TNFα). SDS-PAGE analysis of whole cell proteins at different time points during growth revealed no significant differences between the four vectors in terms of production of rhTNFα (Fig. 6c). The abundance of the 31 kDa protein was slightly lower with the vector containing the T7 terminator sequence (pLT72-T7t-TNFα); and decreased further for vectors with the sequence coding for kanamycin resistance gene in reverse orientation (pLT72-T7tKan-TNFα and pLT72-T7tKanT2-TNFα). These data confirm the identity of the 31 kDa protein as APH. Plasmid retention data identified pLT72-T7tKan-TNFα as the optimal construct due to its low rate of plasmid loss, indicative of lowered stress.

Improvement of fed-batch fermentation using the improved expression vector

The fed-batch fermentation was repeated with pLT72-T7tKan-TNFα, containing the T7 terminator sequence and the gene coding for the kanamycin resistance gene in reverse orientation (Supplemental Fig. S3). As before, the culture grew continuously, reaching a final OD_{600} of 68 after 48 h (Supplemental Fig. S3a), although the OD_{600} only increased slightly after 33 h. The specific growth rate during the batch phase was comparable to the previous fermentation. CFU values decreased slightly less following induction (Supplemental Fig. S3b) compared to the fermentation using pLT72-TNFα (Fig. 5a), whereas plasmid retention remained at close to 100 % throughout.

The quantity of rhTNFα was comparable to the fermentation using pLT72-TNFα, with 25 % of cellular protein being rhTNFα after 27 h (Supplemental Fig. S3c). After 48 h, the majority (>80 %) of rhTNFα was in the soluble fraction. The use of the improved vector design helped to minimise plasmid loss during fermentation, avoiding the overgrowth of plasmid-free cells, and therefore, non-productive

cells at the last stages of the fermentation process. However, this did not significantly increase the quantity of rhTNFα generated.

Removal of casamino acids from fed-batch growth medium

Good Manufacturing Practices (cGMP) for pharmaceutical products recommend that animal-derived products should be excluded from bacterial culture medium to eliminate risks from zoonotic viruses and transmissible spongiform encephalopathies. To develop a GMP compliant fermentation process, casamino acids (a complex medium component derived from casein) were removed from medium A and replaced with 14 $g \cdot L^{-1}$ of ammonium sulphate and 0.3 $g \cdot L^{-1}$ of calcium chloride. Bacteria transformed with plasmid pLT72-T7tKan-TNFα were induced at an OD_{600} of 0.5 with 0.005 % arabinose and sampled for up to 32 h post-induction. The biomass concentration increased steadily reaching a final OD_{600} of 72 (Supplemental Fig. S4a). However, no great increase of the cell biomass was observed after 32 h, and in contrast to previous fermentations the growth rate decreased immediately following rhTNFα induction, (Fig. 5 & Supplemental Fig. S3). There was no decrease in CFU following induction of RPP (Supplemental Fig. S4b), but in contrast to 100 % retention of the plasmid in the presence of casamino acids, plasmid-free bacteria started to overgrow the culture after 9 h growth (Supplemental Fig. S3). The concentration of rhTNFα increased after induction, reaching a maximum of 26 % of total cell protein after 24 h growth (Supplemental Fig. S4c). The concentration of rhTNFα decreased after 24 h, presumably due to outgrowth of plasmid-free, non-productive cells. The concentration of rhTNFα in the soluble fraction was lower than in previous fermentations, with only 50 % of the rhTNFα in the soluble fraction after 48 h. Thus removal of casamino acids was detrimental to the fermentation process. Ben-Bassat *et al.* [15] and Hoffmann *et al*. [11] reported that casamino acids increased recombinant protein yields and/or stability, presumably due to enhanced supply of amino acids allowing more rapid protein synthesis without the need for generation of amino acids *de novo*. Casamino acids contains more free amino acids and more peptide fragments of smaller molecular mass (<250 Da) than the yeast extract also present in the medium [16].

Fermentation with an alternative medium composition

The semi-defined medium C is fully GMP compliant and has been used successfully both by pharmaceutical companies and to produce model proteins in *E. coli* fed-batch fermentations [7,9,17]. This medium was therefore used in 5 L fed batch fermentations to generate rhTNFα from bacteria transformed with plasmid pLT72-T7tKan-TNFα. As before, the addition of the feed was started 10 hours after inoculation and the specific growth rate (μ) during the fed batch phase was maintained at 0.1 h^{-1} by the use of an exponential feeding profile. The culture was induced at an OD_{600} of 0.5 by the addition of arabinose to a final concentration of 0.005 %. The pH was maintained at 7.0 by the addition of 20 % NH_4OH or 5 M HCl.

Exponential growth in medium C was faster than in media A or B, peaking at μ = 0.815 h^{-1} (Fig. 7a). Unlike previous fermentations, the growth rate increased following induction of RPP, then decreased. CFU measurements consistently increased throughout the fermentation (Fig. 7b) and plasmid retention remained at around 100 % throughout. The concentration of rhTNFα increased gradually after induction, reaching a maximum of 30 % of the total cell protein between 24 and 30 h of growth (Fig. 7c). At the end of the fermentation, rhTNFα constituted 25 % of the total cell protein, being primarily accumulated in the soluble fraction, with less than 30 % of rhTNFα in the insoluble fraction. Overall, this fermentation process successfully generated a greater yield of rhTNFα than previous fermentations, the majority being accumulated in a soluble form, minimising plasmid loss and using a culture medium compliant with cGMP guidelines.

Bioassay of rhTNFα activity

The final goal of any fermentation process is the production of a soluble and active product, and protein solubility is often a good indication of activity. However, this statement is not always true, and recombinant proteins might accumulate in a soluble form but with an incorrect conformation, which limits or abolishes biological activity. Therefore, the activity of rhTNFα was measured using a bioassay [18]. rhTNFα was purified from the cell paste generated in fermentation 1 (Fig. 5; purified rhTNFα shown in Supplemental Fig. S1). The C3H mouse fibrosarcoma cell line L929, which is sensitive to hTNFα, was used to evaluate the activity of the purified rhTNFα. The basis of the

bioassay is that unlike dead cells killed by hTNFα, live L929 cells are stained by the dye crystal violet (CV).

The activity of the purified rhTNFα was calculated as the percentage of cytotoxicity by comparing the amount of dye taken up by cells incubated with different quantities of rhTNFα. rhTNFα samples purified from fermentation 1 had a 50 % cytotoxicity (LD_{50}) value at a concentration of 0.349 ± 0.035 $ng \cdot mL^{-1}$. This in within the concentration range of 0.05 to 20 $ng \cdot mL^{-1}$ in which for most *in vitro* applications hTNFα exerts its biological activity. Comparison with LD_{50} values for rhTNFα standards (Life Technologies) revealed that the specific activity of the purified rhTNFα was 2.8×10^6 $U \cdot mg^{-1}$. Overall, the result of the cytotoxicity assay showed that the optimization of the fermentation conditions have led to the successful production of soluble and active product and the rhTNFα produced during by fed-batch fermentation was active and stable.

In summary, stress minimisation has been demonstrated to be an effective tool for the optimisation of the production of the human biopharmaceutical rhTNFα. Data generated in shake-flask experiments allowed design of intensified bioreactor cultures in which RPP and growth could be balanced, leading to high quantities of both rhTNFα and biomass. Balanced growth, allowing RP to accumulate along with biomass and thus maintaining cell health and viability, is important not only for biomass and RP accumulation during the fermentation, but also for harvest. Unhealthy or stressed bacteria are more difficult to harvest by centrifugation and subsequent downstream processing steps [5].This strategy also enables more flexible scheduling of fermentations.

AUTHOR STATEMENTS

Funding information: This study was funded by Innovate UK, the UK Biotechnology & Biological Sciences Research Council and the UK Engineering & Physical Sciences Research Council under the KTP scheme, grant number KTP 9044. The funding bodies played no role in the design of the study and collection, analysis, and interpretation of data or in writing the manuscript.

Acknowledgements: We thank Bruce Humphrey (Cobra Biologics) for plasmids and Nicola Barison (Cobra Biologics) for assistance with protein purification.

Conflicts of interest: The authors declare that they have no competing financial interests.

REFERENCES

1. **Spadiut O, Capone S, Krainer F, Glieder A, Herwig C.** Microbials for the production of monoclonal antibodies and antibody fragments. *Trends Biotechnol*. 2014; 32: 54-60.

2. **Overton TW**. Recombinant protein production in bacterial hosts. *Drug Discov Today*. 2014; 19: 590-601.

3. **Singh SM, Panda AK**. Solubilization and refolding of bacterial inclusion body proteins. *J Biosci Bioeng*. 2005; 99: 303–310.

4. **Sørensen HP, Mortensen KK**. Soluble expression of recombinant proteins in the cytoplasm of *Escherichia coli. Microb Cell Fact*. 2005;4:1

5. **Hsu CC, Thomas ORT, Overton TW**. Periplasmic expression in and release of Fab fragments from *Escherichia coli* using stress minimization. *J Chem Tech Biotechnol*. 2016; 91:815-822

6, **Sevastsyanovich Y, Alfasi S, Overton T, Hall R, Jones J, Hewitt C, Cole J**. Exploitation of GFP fusion proteins and stress avoidance as a generic strategy for the production of high-quality recombinant proteins. *FEMS Microbiol Lett*. 2009; 299: 86-94.

7. **Wyre C, Overton TW**. Use of a stress-minimisation paradigm in high cell density fed-batch *Escherichia coli* fermentations to optimise recombinant protein production. *J Ind Microbiol Biotechnol*. 2014; 41: 1391-1404.

411 8. **Horiuchi T, Mitoma H, Harashima S, Tsukamoto H, Shimoda T**. Transmembrane TNF-α:
412 structure, function and interaction with anti-TNF agents. *Rheumatology (Oxford).* 2010; 49:1215-
413 1228.

414 9. **Hodgson IJ, Lennon CDJ, Kara VL.** Expression system. European patent EP2695943 (A1)

415 10. **Oshima T, Tanaka S, Matsukura S.** Expression vector for human TNF. US patent
416 US4871663

417 11. **Hoffmann F, van den Heuvel J, Zidek N, Rinas U.** Minimizing inclusion body formation during
418 recombinant protein production in *Escherichia coli* at bench and pilot plant scale. *Enz Microbial*
419 *Tech*. 2004; 34:235-241.

420 12. **Studier FW.** Use of bacteriophage T7 lysozyme to improve an inducible T7 expression system.
421 *J Mol Biol.* 1991; 219: 37-44.

422 13. **Sevastsyanovich YR, Alfasi SN, Cole JA.** Sense and nonsense from a systems biology
423 approach to microbial recombinant protein production. *Biotechnol Appl Biochem*. 2010; 55: 9-28.

424 14. **Alfasi SN.** Physiological aspects underpinning recombinant protein production in *Escherichia*
425 *coli.* PhD Thesis. University of Birmingham; 2011.

426 15. **Ben-Bassat A, Dorin G, Bauer K, Lin L.** Method of improving the yield of heterologous
427 protein produced by cultivating recombinant bacteria. US patent US4656132 A

428 16. **Beckton, Dickinson and Company.** BD Bionutrients Technical Manual, 4th Edition.
429 http://www.bd.com/ds/technicalCenter/misc/lcn01558-bionutrients-manual.pdf Accessed on 7 June
430 2017.

431 17. **Want A, Thomas OR, Kara B, Liddell J, Hewitt CJ.** Studies related to antibody fragment
432 (Fab) production in *Escherichia coli* W3110 fed-batch fermentation processes using multiparameter
433 flow cytometry. *Cytometry A*. 2009; 75: 148-154.

18. **McGee ZA, Clemens CM**. Effect of bacterial products on tumor necrosis factor production: quantitation in biological fluids or tissues. *Methods Enzymol*. 1994; 236:23-31.

19. **Passarinha L, Bonifacio M, Queiroz J.** Application of a fed-batch bioprocess for the heterologous production of hSCOMT in *Escherichia coli*. *J Microbiol Biotechnol*. 2009; 19: 972-981.

20. **Babaeipour V, Shojaosadati S, Robatjazi S, Khalilzadeh R, Maghsoudi N.** Over-production of human interferon-γ by HCDC of recombinant *Escherichia coli*. *Process Biochem*. 2007; 42:112-117.

FIGURE LEGENDS

Figure 1. Selection of culture medium for the optimisation of the production of rhTNFα. *E. coli* BL21-T7 carrying the empty vector (pLT72) or the vector encoding rhTNFα (pLT72-TNFα) were grown at 30 °C in LB (a,c,e) or TB (b,d,f); half of cultures were induced with 0.2 % arabinose at an OD_{600} ≈ 1. Samples were taken at regular intervals and (a,b) OD_{600}, (c,d) CFU and (e,f) plasmid retention determined. Data shown are single values for CFU and plasmid retention and mean values from replica flasks for OD_{600}, error bars are ±1 SD.

Figure 2. Accumulation of rhTNFα in cultures grown in LB or TB culture media. *E. coli* BL21-T7 carrying the empty vector (pLT72) or the vector encoding rhTNFα (pLT72-TNFα) were grown in (a) LB or (b) TB at 30 °C; half of cultures were induced with 0.2 % arabinose at an OD_{600} ≈ 1 and samples were taken at regular intervals. Whole cell lysates were separated by SDS-PAGE and protein stained with colloidal blue. M, marker; BI, before induction. The quantity of rhTNFα is expressed as a percentage of whole cell protein at the bottom of the gel. Samples collected at 24 hours were also fractionated to obtain soluble (24S) and insoluble (24I) cell fractions facilitated by the addition of lysozyme (shown on right). The ~31kDa protein presumed to be APH is shown on the right.

Figure 3. Effect of the inducer concentration on the production of rhTNFα. (a) Growth of *E. coli* BL21-T7 carrying the empty vector (pLT72, non-induced) or pLT72-TNFα, incubated at 30 °C and induced with between 1 % and 0.002 % arabinose at OD_{600} ≈ 1. (b) Plasmid retention after 24 hours

of growth as in (a). (c) SDS-PAGE showing accumulation of rhTNFα from whole cell lysates obtained 24 hours post-inoculation. Data shown are mean values from two replica flasks for OD_{600} and plasmid retention, error bars are ±1 standard deviation.

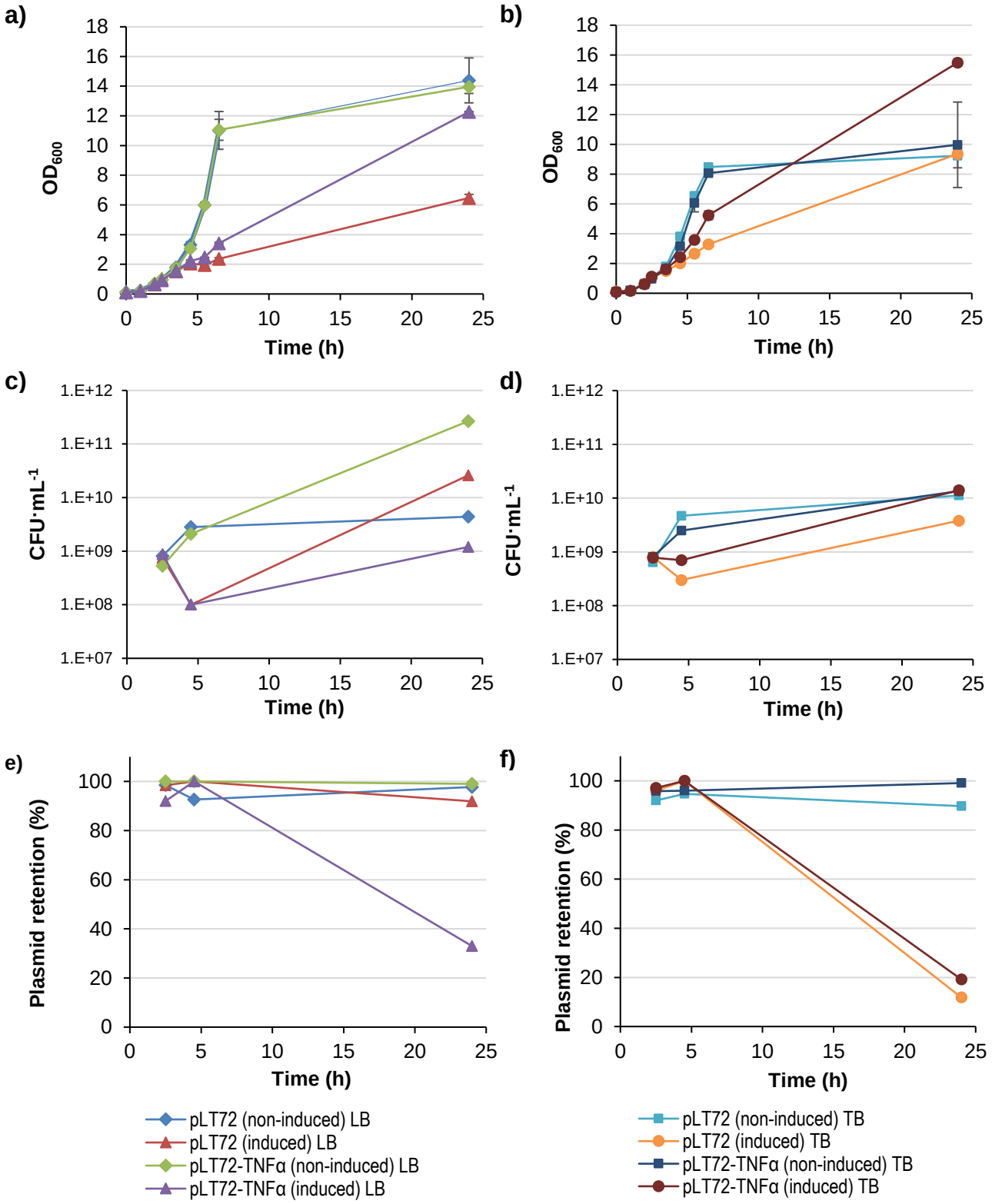
Figure 4. Effect of the temperature and inducer concentration on the production of rhTNFα. (a) SDS-PAGE gel showing the accumulation of the rhTNFα in the soluble (Sol) and insoluble (Ins) fractions 4 hours after induction. Cultures were induced with 0.2 % arabinose and grown at 37 °C, 30 °C or 25 °C; samples were fractionated and rhTNFα quantified by densitometry to give the percentage of rhTNFα in the soluble and insoluble fractions. (b) Plasmid retention of cultures carrying either the empty vector (pLT72) or pLT72-TNFα were grown at 25 °C under non-induced conditions or induced at $OD_{600} \approx 0.5$ with 0.2 % to 0.001 % arabinose. Data shown are mean values from two replica flasks, error bars are ±1 standard deviation. (c) SDS-PAGE gel showing the accumulation of rhTNFα from whole cell lysates after 24 hours of growth as in (b).

Figure 5. Production of rhTNFα by fed-batch fermentation. *E. coli* BL21-T7 pLT72-TNFα was grown at 25 °C in medium A and induced with 0.005 % arabinose at $OD_{600} \approx 0.5$ (t = 3h; solid arrow). Feed was started at t = 10h (dashed arrow). (a) Growth as determined using OD_{600} and specific growth rate. (b) CFU and plasmid retention. (c) SDS-PAGE analysis of whole cell lysates. The 3h sample is immediately before induction. rhTNFα and APH are indicated, along with densitometric analysis of the percentage of cellular protein that is rhTNFα. The "–ve" sample is *E. coli* BL21-T7 pLT72 (empty vector) after 24 hours of growth (non-induced). (d) Samples were separated into soluble and insoluble fractions. rhTNFα and lysozyme are indicated.

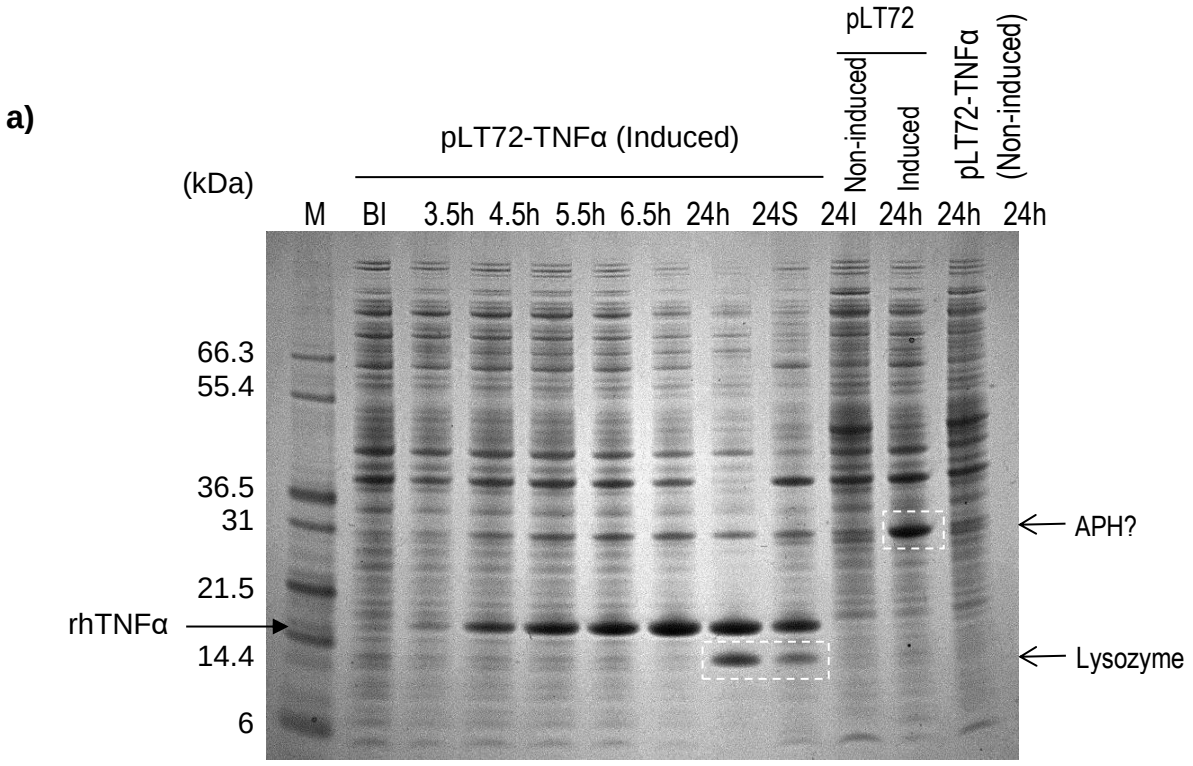
Figure 6. Optimisation of vector design to confirm the identity of the 31 kDa protein as APH and to minimise its production. (a) Schematic of the new vector designs. Arrows indicate genes, stem-loops terminators (T7t and T2t). (b) Plasmid retention after 24 hours growth of cultures carrying the empty vector (pLT72) or the 4 vectors as in (a) grown at 30 °C under non-inducer or induced (0.02 % arabinose at $OD_{600} \approx 0.5$) conditions. Data shown are mean values from two replica flasks, error bars are ±1 standard deviation. (c) SDS-PAGE gel showing the accumulation of rhTNFα and APH in whole cell lysates after 4 h, 8 h, 10 h, 12 h and 24 h growth as in (b).

Figure 7. Production of rhTNFα using an alternative fed-batch medium composition. *E. coli* BL21-T7 pLT72-T7tKan-TNFα was grown at 25 °C in medium C and induced with 0.005 % arabinose at an OD_{600} ≈ 0.5 (t = 5h; solid arrow). Feeding was started at t = 10h (dashed arrow). (a) Growth as determined using OD_{600} and specific growth rate. (b) CFU and plasmid retention. (c) SDS-PAGE analysis of whole cell lysates. The 5h sample is immediately before induction. rhTNFα is indicated, along with densitometric analysis of the percentage of cellular protein that is rhTNFα. The "–ve" sample is *E. coli* BL21-T7 pLT72 (empty vector, non-induced) after 24 hours of growth. Samples obtained at t = 48h were also fractionated into soluble (Sol) and insoluble (Ins) fractions to determine the quantities of soluble and insoluble rhTNFα.

FIGURE 1


a)
b)
c)
d)
e)
f)
OD600
Time (h)
CFU·mL-1
Plasmid retention (%)
pLT72 (non-induced) LB
pLT72 (induced) LB
pLT72-TNFα (non-induced) LB
pLT72-TNFα (induced) LB
pLT72 (non-induced) TB
pLT72 (induced) TB
pLT72-TNFα (non-induced) TB
pLT72-TNFα (induced) TB

502 **FIGURE 2**



503

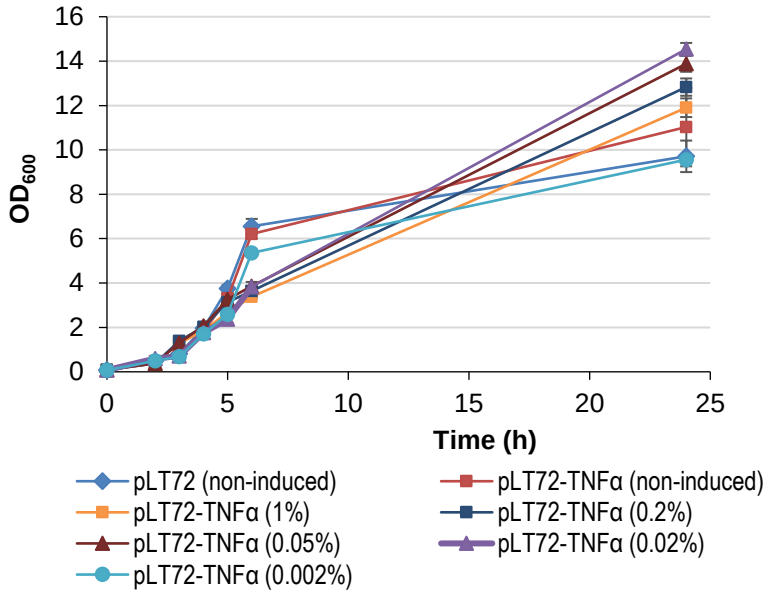
504

505

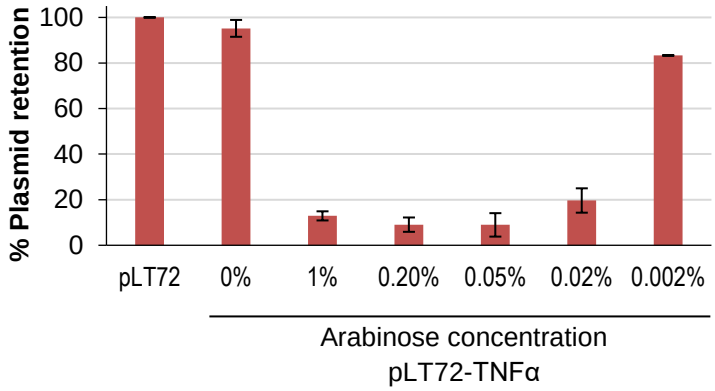
506

FIGURE 3

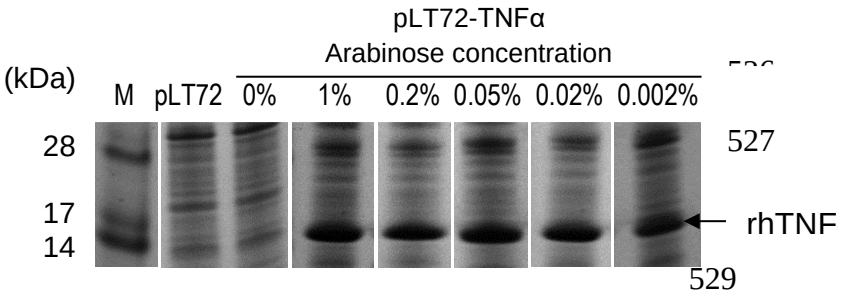
a)


16
14
12
10
8
6
4
2
0
OD600
0
5
10
15
20
25
Time (h)
pLT72 (non-induced)
pLT72-TNFα (non-induced)
pLT72-TNFα (1%)
pLT72-TNFα (0.2%)
pLT72-TNFα (0.05%)
pLT72-TNFα (0.02%)
pLT72-TNFα (0.002%)

b)


% Plasmid retention
100
80
60
40
20
0
pLT72
0%
1%
0.20%
0.05%
0.02%
0.002%
Arabinose concentration
pLT72-TNFα

c)

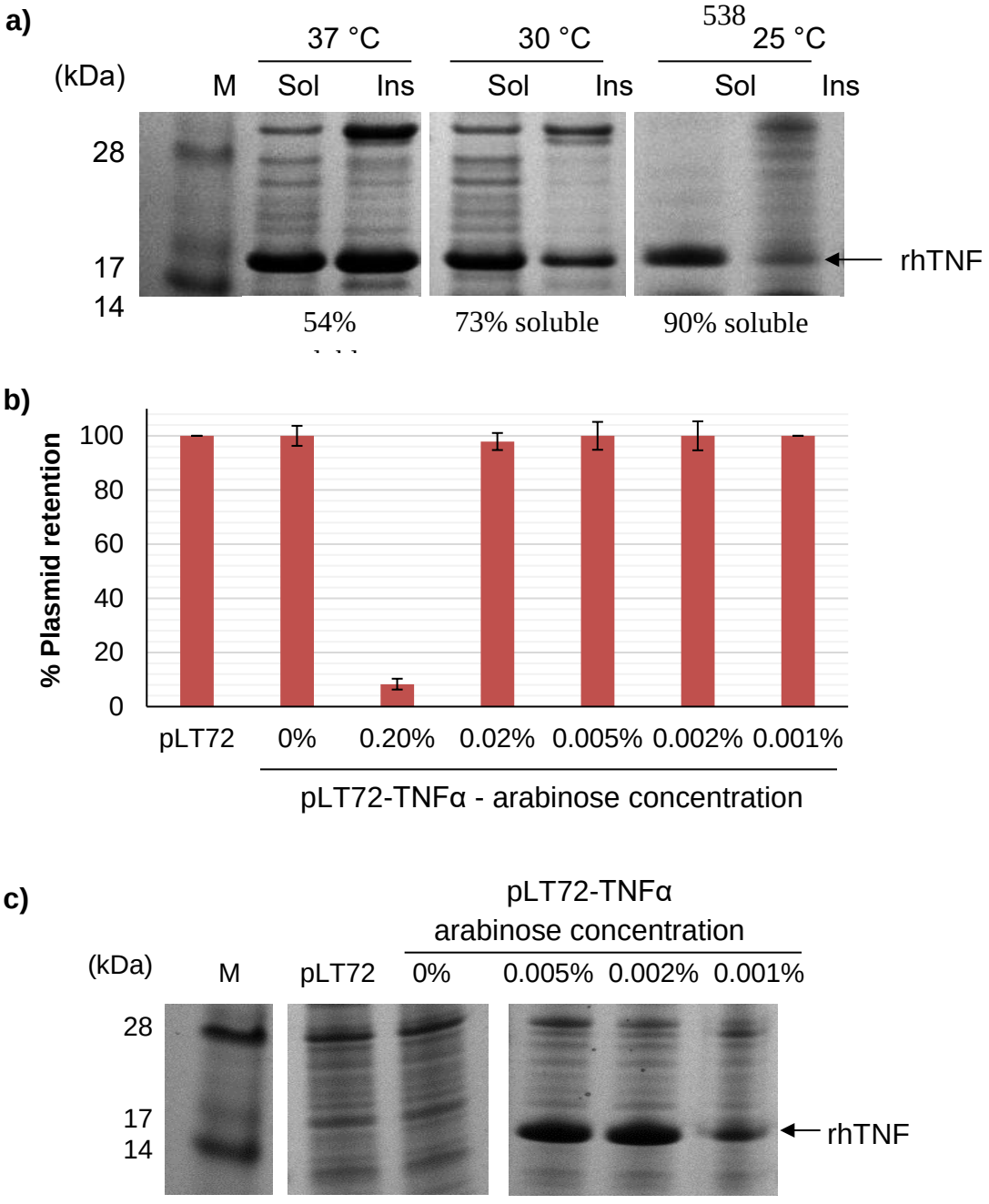

pLT72-TNFα
Arabinose concentration
(kDa)
M
pLT72
0%
1%
0.2%
0.05%
0.02%
0.002%
28
17
14
rhTNF

535 **FIGURE 4**

536

537

538



539 FIGURE 5

540

541

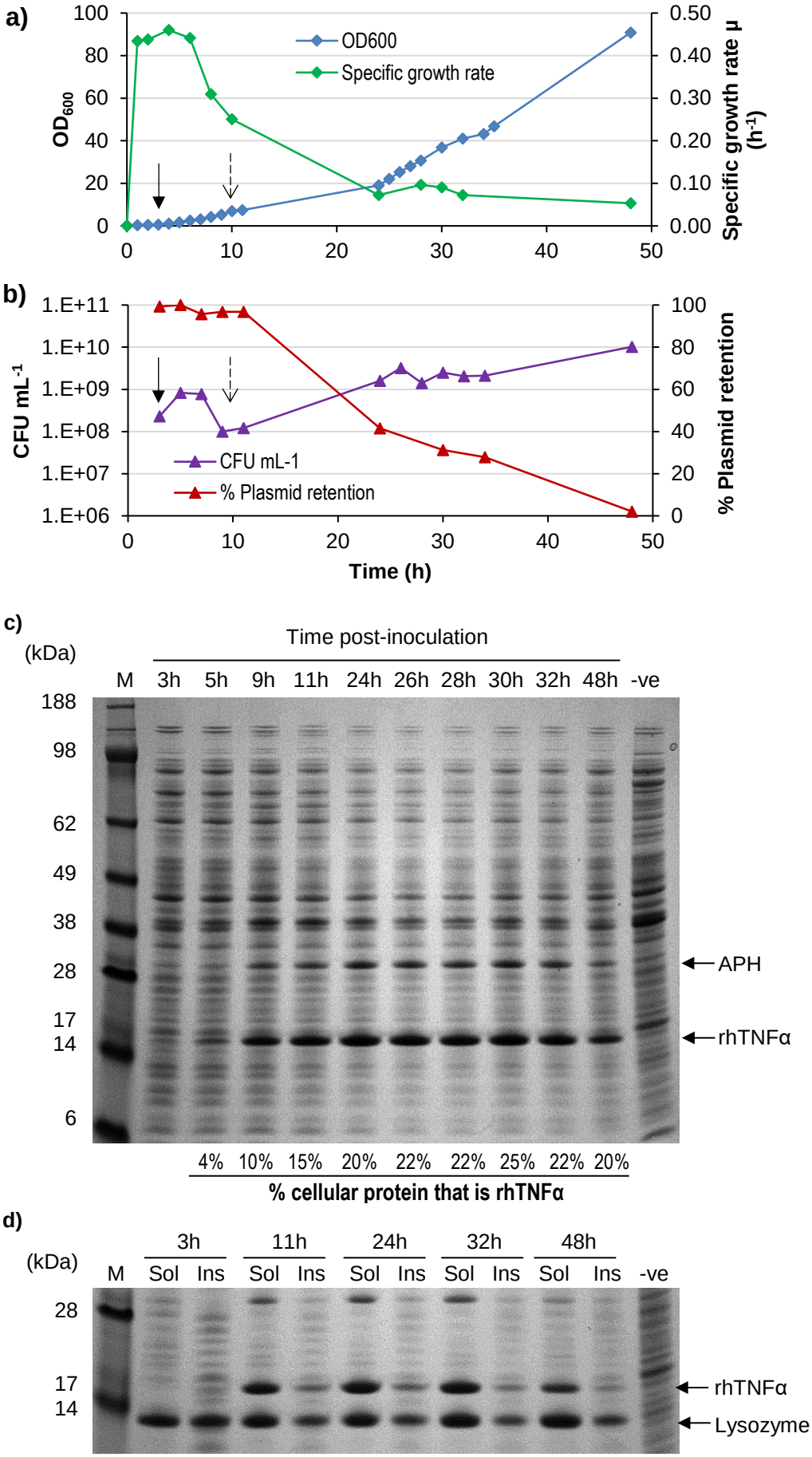
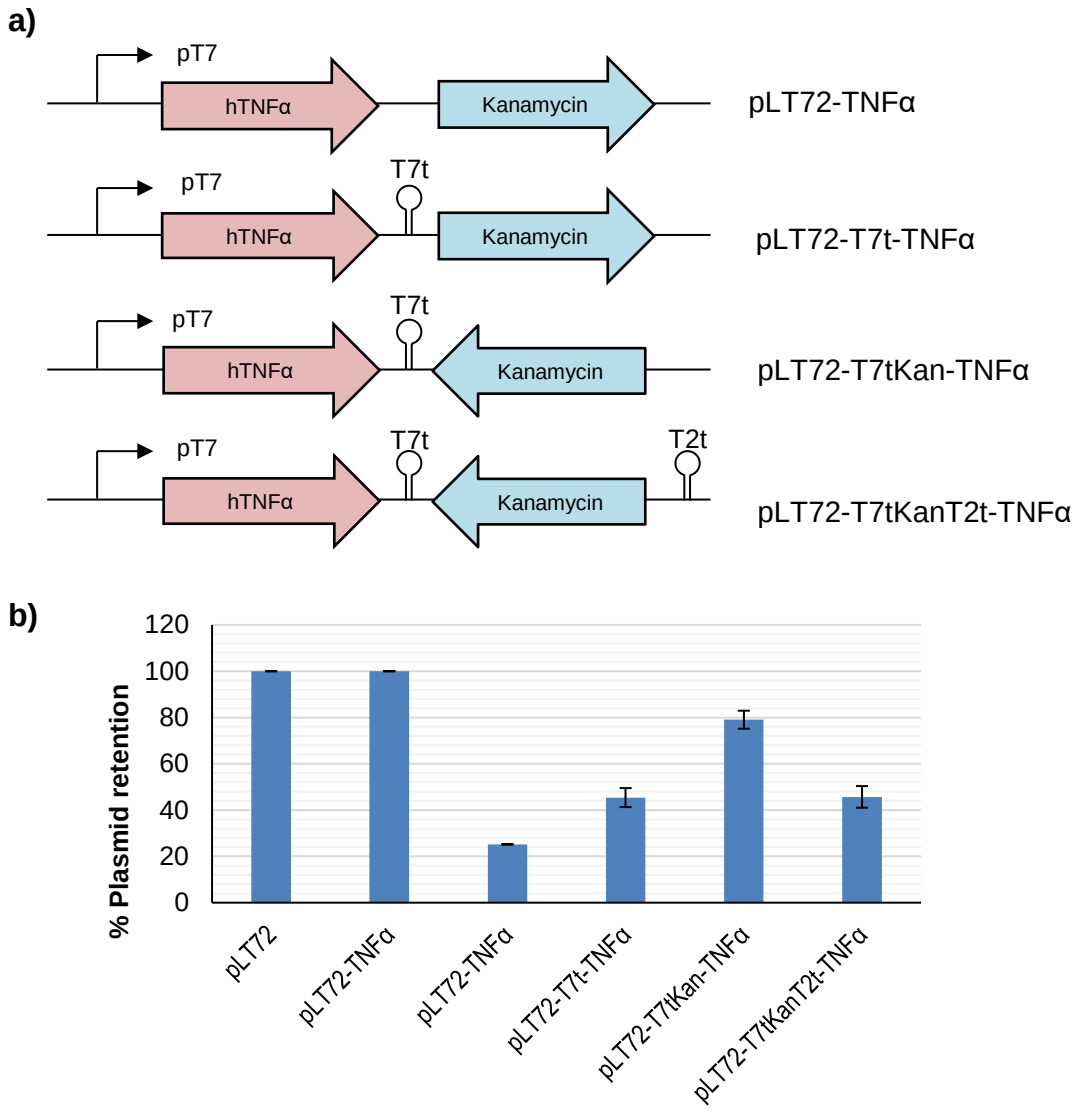


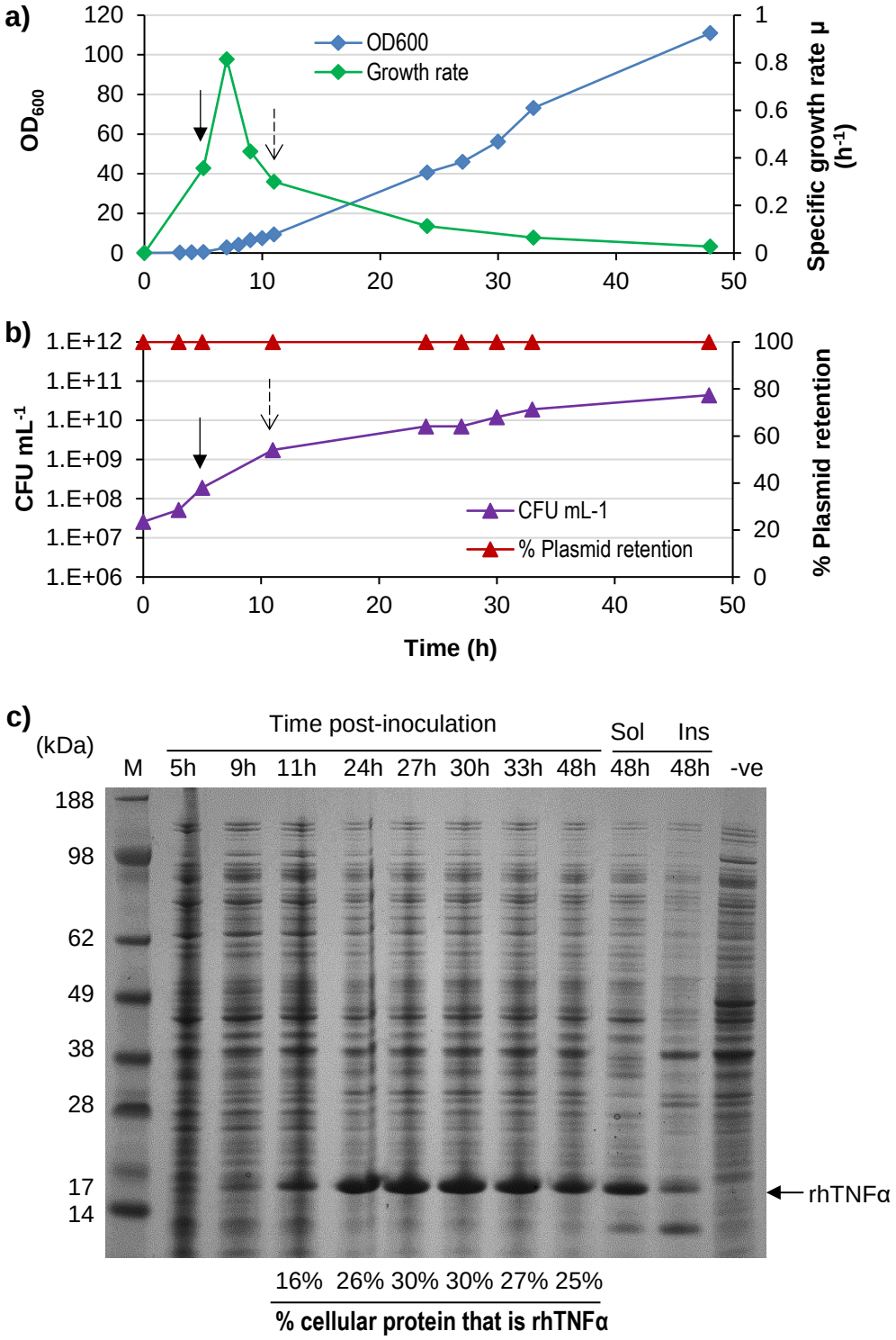
FIGURE 6


a)
pT7
hTNFα
Kanamycin
pLT72-TNFα
T7t
pLT72-T7t-TNFα
pLT72-T7tKan-TNFα
T2t
pLT72-T7tKanT2t-TNFα
b)
% Plasmid retention
120
100
80
60
40
20
0
pLT72
pLT72-TNFα
pLT72-T7t-TNFα
pLT72-T7tKan-TNFα
pLT72-T7tKanT2t-TNFα
c)
(kDa)
Non-induced
Induced (0.02%)
M
4h
8h
10h
12h
24h
38
APH
28
rhTNFα
17
14
pLT72-T7tKan-TNFα
pLT72-T7tT7tKanT2t-TNFα
pLT72 (Non-ind.)
pLT72 (Induced)

549 FIGURE 7

550

551



Supplemental information for:

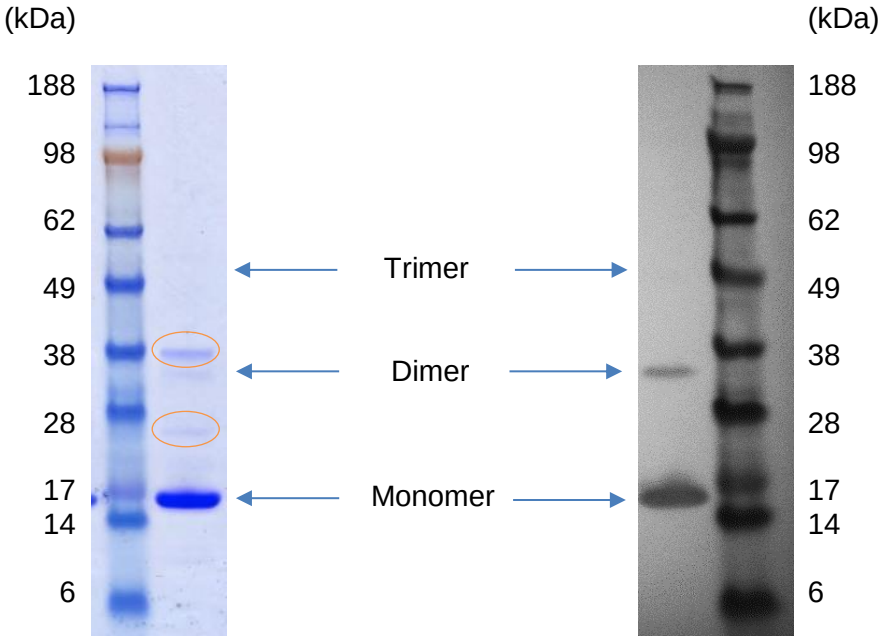
Optimising host cell physiology and stress avoidance for the production of recombinant human tumour necrosis factor α in *Escherichia coli*

T Selas Castiñeiras[1,2,3], SG Williams[1], A Hitchcock[1], JA Cole[3,4], DC Smith[1], TW Overton[2,3*].

[1]Cobra Biologics, Stephenson Building, The Science Park, Keele ST5 5SP, UK.

[2]School of Chemical Engineering, [3]Institute of Microbiology & Infection, and [4]School of Biosciences, The University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

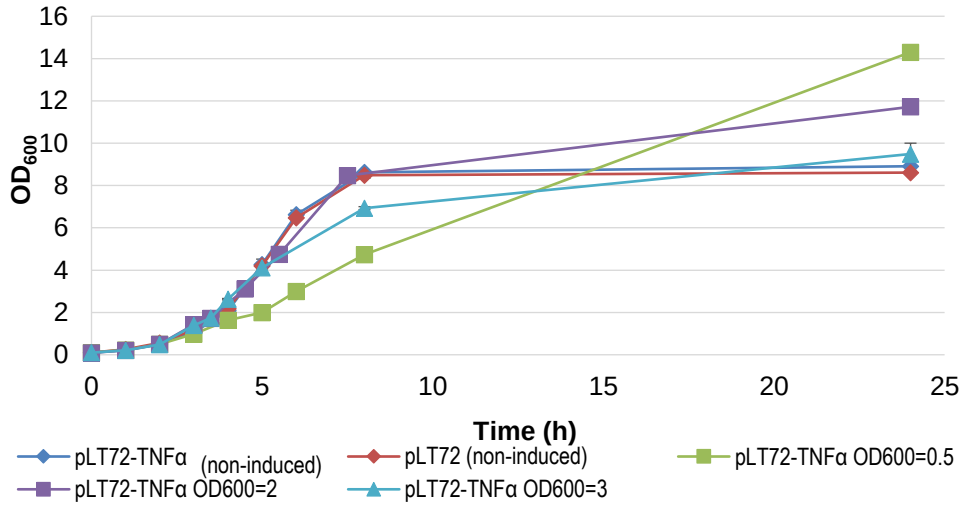
* To whom correspondence should be sent: t.w.overton@bham.ac.uk



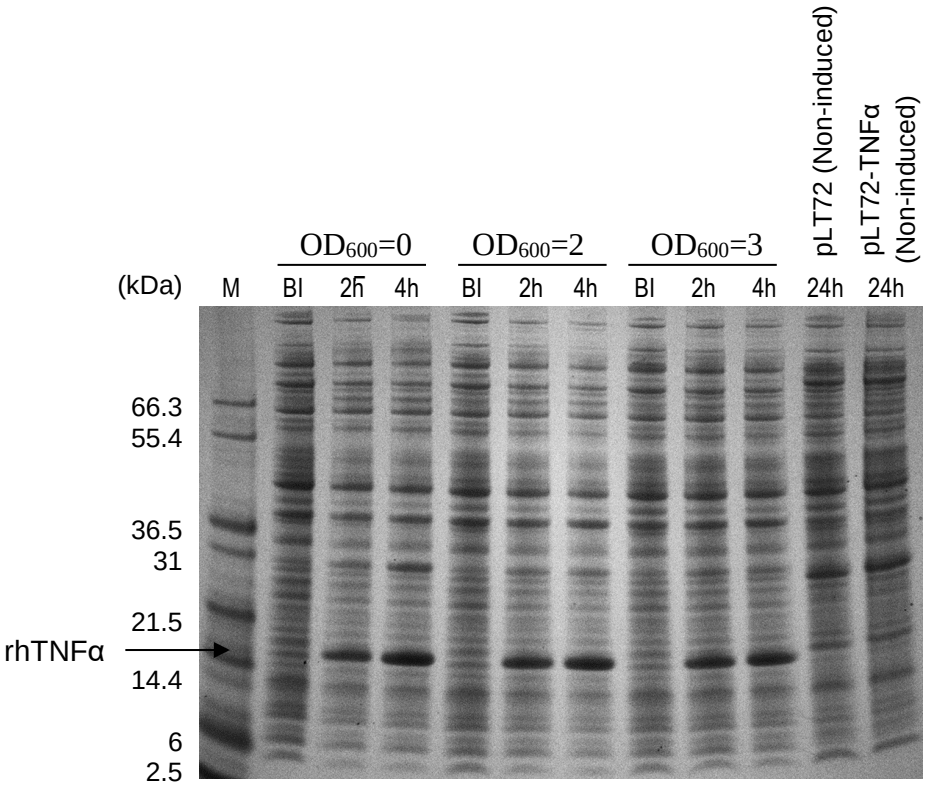
Supplemental Figure S1. Final material obtained after the purification process of rhTNFα. The rhTNFα accumulated mainly in the monomeric form. The presence of the rhTNFα dimer and trimer could also be detected by SDS-PAGE (left) and western blot (right). Bands corresponding to other contaminant proteins could also be observed (orange circles). The final product had a purity greater than 95 % as determined by densitometry. The western blot was developed using an anti-TNFα antibody which confirmed the identity of the monomer, dimer and trimer forms of the rhTNFα produced by fed-batch fermentation.

SUPPLEMENTAL FIGURE S2.

a)

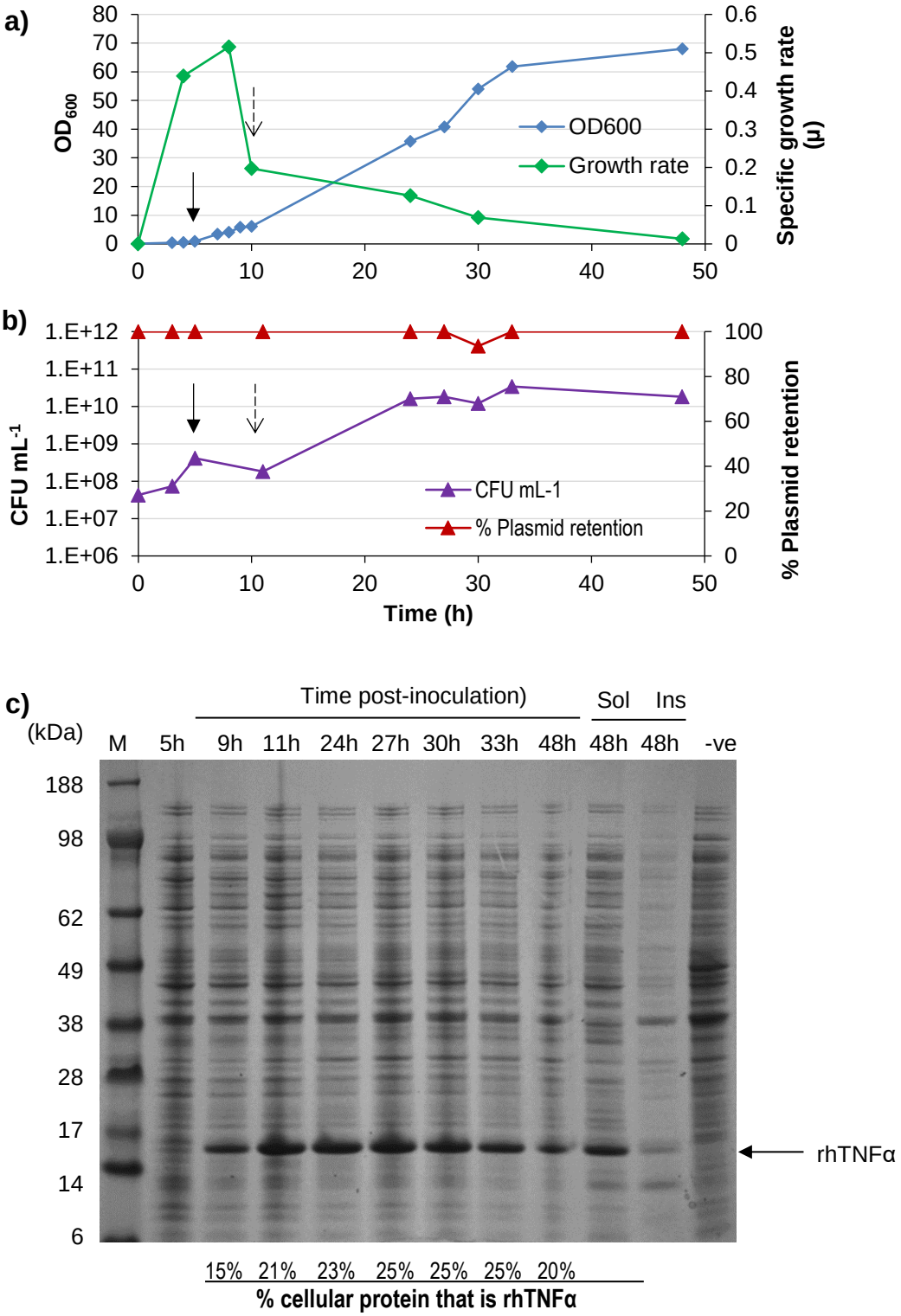


b)



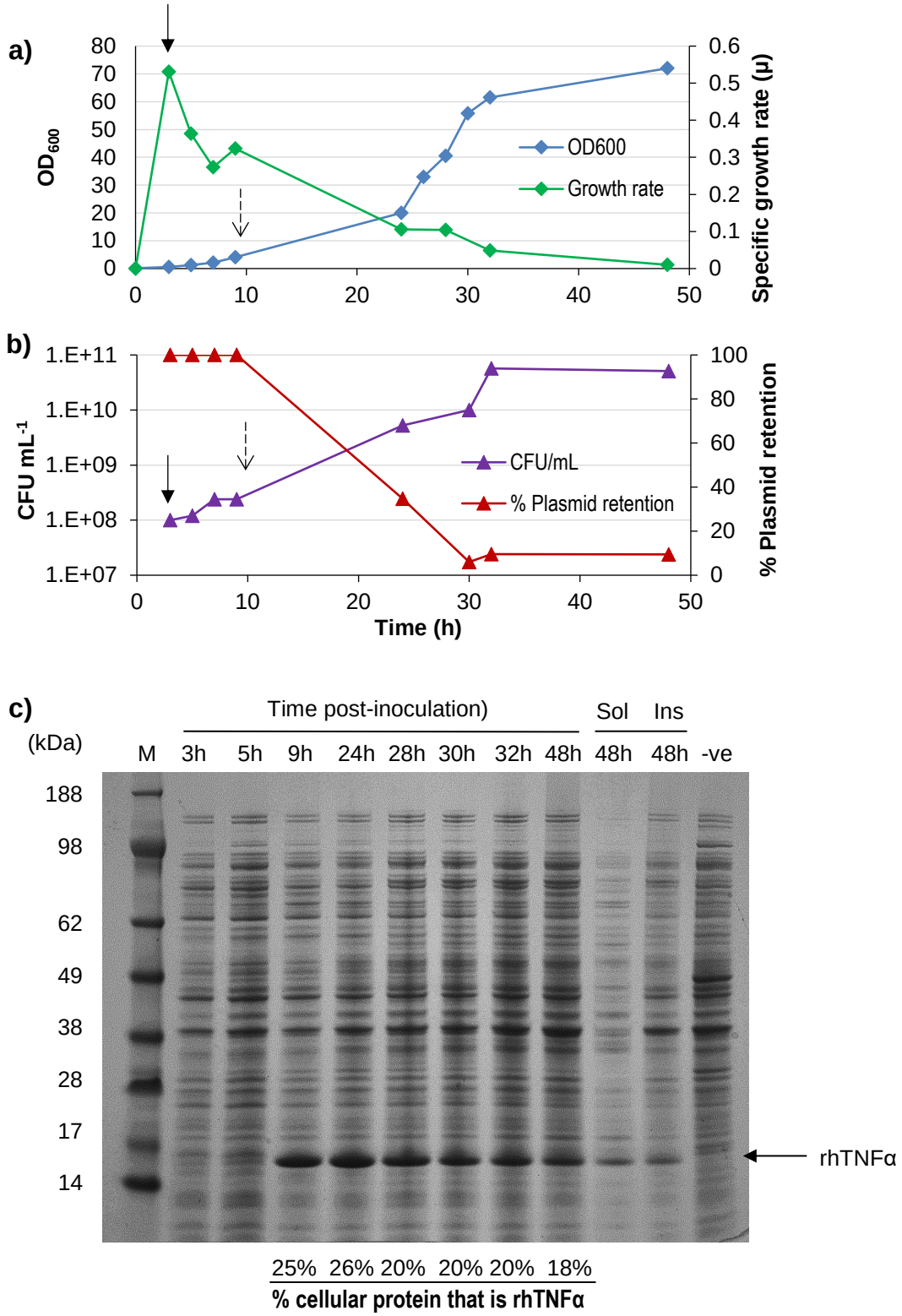
Supplemental Figure S2. The effect of the induction point on the production of rhTNFα. (a) Growth of *E. coli* BL21-T7 carrying the empty vector (pLT72) or the vector coding for rhTNFα (pLT72-TNFα) incubated at 30 °C; cultures were induced with 0.2 % arabinose at OD_{600} ≈ 0.5, 2 or 3. (b) SDS-PAGE showing accumulation of rhTNFα from whole cell lysates before induction (BI), 2 hours and 4 hours after induction. Data shown are mean values from two replica flasks for OD_{600}, error bars are ±1 standard deviation.

SUPPLEMENTAL FIGURE S3



Supplemental Figure S3. Production of rhTNFα by fed-batch fermentation using optimised expression vector pLT72-T7tKan-TNFα. *E. coli* BL21-T7 pLT72-T7tKan-TNFα was grown at 25 °C in medium A and induced with 0.005 % arabinose at an OD_{600} ≈ 0.5 (t = 5h; solid arrow). Feeding was started at t = 10h (dashed arrow). (a) Growth as determined using OD_{600} and specific growth rate. (b) CFU and plasmid retention. (c) SDS-PAGE analysis of whole cell lysates. The 5h sample is immediately before induction. rhTNFα is indicated, along with densitometric analysis of the percentage of cellular protein that is rhTNFα. The "–ve" sample is *E. coli* BL21-T7 pLT72 (empty vector, non-induced) after 24 hours of growth. Samples obtained at t = 48h were also fractionated into soluble (Sol) and insoluble (Ins) fractions to determine the quantities of soluble and insoluble rhTNFα.

SUPPLEMENTAL FIGURE S4



Supplemental Figure S4. Production of rhTNFα by fed-batch fermentation without the addition of casamino acids. *E. coli* BL21-T7 pLT72-T7tKan-TNFα was grown in medium B at 25 °C and induced with 0.005 % arabinose at an OD_{600} ≈ 0.5 (t = 3h; solid arrow). Feeding was started at t = 10h (dashed arrow). (a) Growth as determined using OD_{600} and specific growth rate. (b) CFU and plasmid retention. (c) SDS-PAGE analysis of whole cell lysates. The 3h sample is immediately before induction. rhTNFα is indicated, along with densitometric analysis of the percentage of cellular protein that is rhTNFα. The "–ve" sample is *E. coli* BL21-T7 pLT72 (empty vector, non-induced) after 24 hours of growth. Samples obtained at t = 48h were also fractionated into soluble (Sol) and insoluble (Ins) fractions to determine the quantities of soluble and insoluble rhTNFα.

Supplemental methods

Fed-batch fermentation

Starter cultures were grown in 10 mL of LB broth with 50 μg·mL^{-1} kanamycin at 25 °C and 200 rpm until OD_{600} = 2. Starter cultures were used to inoculate a 1 L baffled shake-flask containing 200 mL of LB broth with 50 mg·L^{-1} kanamycin, and were grown at 25 °C and 200 rpm to an OD_{600} between 4 and 6.

A 7 L total volume (5 L working volume) bench-top fermenter (Applikon ADI 1010 Bio controller) equipped with 3 Rushton impellers and 4 baffles was used for fermentation experiments. The aeration rate was constant at 1 volume air per volume medium per minute (vvm) and the dissolved oxygen tension (DOT) was maintained above 20 % being controlled by the stirrer speed (200 - 1250 rpm). All fermentations began with an initial volume of 3 L batch salts, sterilised in the vessel by autoclaving for 20 minutes at 121 °C. Once cooled, post-autoclave additions and trace element solutions were added. Two litres of feed solution was prepared and sterilised by filtration (0.22 μm filter). For medium A (Cobra biologics), the batch salts contained: 13.3 g·L^{-1} K_2HPO_4, 4 g·L^{-1} $(NH_4)_2SO_4$, 1.7 g·L^{-1} citric acid, 10 g·L^{-1} Bacto™ yeast extract and 0.16 mL·L^{-1} PPG 2000. The post-autoclave additions were: 1 mL·L^{-1} trace elements solution A (comprising 5 g·L^{-1} citric acid, 2 g·L^{-1} $CoCl_2·6H_2O$, 1.2 g·L^{-1} $CuCl_2·2H_2O$, 2.5 g·L^{-1} H_3BO_3, 2 g·L^{-1} $Na_2MoO_4·2H_2O$, 1.2 g·L^{-1} $MnCl_2·4H_2O$), 10 mL·L^{-1} trace elements solution B (comprising 6 g·L^{-1} $FeSO_4·7H_2O$, 0.84 g·L^{-1} EDTA·$2H_2O$ and 0.8 g·L^{-1} $ZnCl_2$), 10 g·L^{-1} glycerol, 1.2 g·L^{-1} $MgSO_4·7H_2O$, 2% (w/v) casamino acids and 1 mL·L^{-1} 50 mg · mL^{-1} kanamycin stock. The feed solution contained 600 g·L^{-1} glycerol, 5 g·L^{-1} $MgSO_4·7H_2O$, 50 g·L^{-1} yeast extract, 10 g·L^{-1} KH_2PO_4, 2.1 g·L^{-1} K_2HPO_4, 2% (w/v) casamino acids, 1 mL·L^{-1} 50 mg·mL^{-1} kanamycin stock and 0.5 mL·L^{-1} 20 % arabinose stock. For medium B, casamino acids were omitted and replaced with 14 g·L^{-1} $(NH_4)_2SO_4$ and 0.3 g·L^{-1} of $CaCl_2·2H_2O$.

For medium C [17], the batch salts contained 14 g·L^{-1} $(NH_4)_2SO_4$, 35 g·L^{-1} glycerol, 20 g·L^{-1} Bacto^{TM} yeast extract, 2 g·L^{-1} KH_2PO_4, 16.5 g·L^{-1} K_2HPO_4, 7.5 g·L^{-1} citric acid, 1.5 mL · L-^{1} concentrated H_3PO_4 and 0.66 mL·L^{-1} PPG 2000. The post-autoclave additions were 34 mL·L^{-1} trace elements solution (comprising 3.36 g·L^{-1} $FeSO_4·7H_2O$, 0.84 g·L^{-1} $ZnSO_4·7H_2O$, 0.15 g·L^{-1} $MnSO_4·H_2O$, 0.25 g·L^{-1} $Na_2MoO_4·2H_2O$, 0.12 g·L^{-1} $CuSO_4·5H_2O$, 0.36 g·L^{-1} H_3BO_3 and 48 mL·L^{-1} concentrated H_3PO_4), 10 mL·L^{-1} 1 M $MgSO_4·7H_2O$, 2 mL·L^{-1} 1 M $CaCl_2·2H_2O$ and 1 mL·L^{-1} 50 mg·mL^{-1} kanamycin stock. The feed contained 714 g·L^{-1} glycerol, 30 mL·L^{-1} 1 M $MgSO_4·7H_2O$, 1 mL·L^{-1} 50 mg·mL^{-1} kanamycin stock and 0.5 mL·L^{-1} 20 % arabinose stock.

The pH was maintained at 6.8 by the addition of 5 M NaOH and 5 M HCl for fermentations using media A and B, and at 7.0 by the addition of 5 M HCl or 20 % NH_4OH for medium C. Polypropylene glycol (PPG) antifoam was added when required. Fed-batch fermentations were monitored using BioXpert® software (Applikon). The inoculum was added to an initial OD_{600} of 0.1. The fermentation was carried out at a temperature of 25 °C and the culture was induced with 0.005 % arabinose at an OD_{600} of 0.5. The feed solution was started 10 h after inoculation at an exponential feed rate to achieve a specific growth rate of 0.1 h^{-1}, calculated using equation 1:

$$F = \left(\frac{1}{S}\right) \times \left(\frac{\mu}{Y_{XS}} + m\right) \times X_0 \times e^{\mu t} \qquad (1)$$

F is the feed rate in L·h^{-1}, S is the substrate concentration in the feed in g·L^{-1}, μ is the required specific growth rate in h^{-1}, Y_{XS} is the yield coefficient in g biomass per g carbon source, m is the maintenance coefficient, X_0 is the biomass in g and t is time. Values for Y_{XS} and m were obtained from the literature, 0.22 [19] and 0.025 [20], respectively.

SDS-PAGE

4-12 % Bis-Tris NuPAGE SDS-PAGE gels (Life Technologies) were generally used to evaluate the production of recombinant proteins. Seven microliters of protein sample were mixed with 2 µL of 4x NuPAGE LDS sample buffer (Life Technologies) and 1 µL of 10x NuPAGE sample reducing agent (Life Technologies), and heated for 10 min at 70 °C. 1x electrophoresis running buffer was prepared by diluting 20x NuPAGE MES SDS running buffer (Life Technologies) in deionised water. For reducing protein electrophoresis, 0.5 mL of NuPAGE antioxidant (Life Technologies) was added to 200 mL of running buffer and used to fill the inner chamber of the electrophoresis tank. SDS-PAGE gels were run for at least 45 minutes at 200 V according to the manufacturers' protocol. Molecular size markers were used: Mark12™ Unstained Protein Standard (Life Technologies) or SeeBlue® Plus2 Pre-Stained Protein Standard (Life Technologies). SDS-PAGE gels were stained using Colloidal Blue Staining (Life Technologies). SDS-PAGE gels were submerged in the fixing solution, (40 % (v/v) methanol, 10 % (v/v) glacial acetic acid) for 10 minutes, the staining solution A (20 % (v/v) methanol and 20 % (v/v) staining solution A) for 10 minutes, then staining solution B was added to a final concentration of 5 % (v/v). SDS-PAGE gels were stained for a minimum of 3 hours and de-stained with deionised water for at least 12 hours.

Western blotting

SDS-PAGE gels were run as above and transferred to a 0.2 µm nitrocellulose membrane (Life Technologies) using the Xcell II blot module at 30 V for 1 h (Life Technologies). Transfer buffer was prepared by the addition of 20x NuPAGE transfer buffer (Life Technologies), 10% of methanol (v/v) and 1 $mL \cdot L^{-1}$ NuPAGE antioxidant. Membranes were blocked in 5 % (w/v) skimmed milk powder (Sigma-Aldrich) in PBS for at least 1 hour. For the detection of rhTNFα, the blot was incubated with an anti-TNFα antibody (ab9635, Abcam) using 1:2500 dilution in 5% (w/v) skimmed milk (Sigma-Aldrich) in PBS for 1 h, washed with 0.05% Tween-

20 in PBS and successively incubated with an anti-rabbit IgG antibody conjugated with horseradish peroxidase (HRP; Sigma-Aldrich) using 1:3000 dilution for an hour. Western blots were developed using 3,3′,5,5′-tetramethylbenzidine (TMB) substrate for HRP (Sigma-Aldrich).

Quantification of rhTNFα from SDS-PAGE

AlphaEase® software (Alpha Innotech) was used to calculate the quantity of rhTNFα as a percentage of total cell protein (TCP). Gels were photographed using an AlphaImager (Alpha Innotech) and images subjected to background subtraction using the default settings for peak-to-peak background subtraction. The percentage of soluble and insoluble recombinant protein was calculated by the software package. All samples were normalised by OD_{600} before loading on the SDS-PAGE gel, so each lane contained equivalent biomass.

rhTNFα reference material obtained from Life Technologies was used to quantify the concentration of rhTNFα obtained at the end of each fermentation by densitometry from samples. A standard curve was generated by loading different concentrations of rhTNFα reference material on an SDS-PAGE gel. The concentration of rhTNFα from fermentation samples was quantified using a standard curve with the AlphaEase® software. The rhTNFα yields were calculated to obtain the final yield based on the final OD_{600} of the culture.