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Production of antibody fragment (Fab) throughout *Escherichia coli* fed-batch fermentation process: Changes in titre, location and form of product

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Abstract

Background: Recombinant proteins, including antibodies and antibody fragments, often contain disulfide bond bridges that are necessary for their folding, stability and function. Production of disulfide-bond-containing proteins in the periplasm of *Escherichia coli* has been very useful, due to unique characteristics of the periplasm, for obtaining fully active and correctly folded products and for alleviating downstream processing.
Results: In this study, fed-batch cultivation of *Escherichia coli (E. coli)* for production of Fab D1.3, which is an anti-hen egg white lysozyme (HEWL) antibody fragment was carried out at 37ºC, and the bacterial cells were induced by adding 0.1 mM IPTG to the culture medium. Fermentor was sampled over the course of fermentation; the bacterial cells were centrifugally separated from the culture broth and subjected to osmotic shock (with excluding HEWL) and sonication procedures. The resulting fractions were analysed for Fab using a combination of ELISA, SDS-PAGE and Western blotting and changes in product titre, location, and form was assessed throughout growth. It was shown that osmotic shock released the Fab from the periplasm very efficiently and its efficacy was 20-45% more than sonication. This study demonstrates that, at high cell density cultivation in fermentor, target product can appear inside and outside the cells, depending on the time of induction. The maximum amount of Fab (47 mg/l) in the periplasm was reached at 14 hrs cultivation (4 hrs post induction), being suitable time for cell harvest, selective periplasmic extraction and downstream capture. The Fab increasingly leaked into the culture medium, and reached its maximum culture medium titre of ~78 mg/l after 6 hrs post induction. After 16 hrs cultivation (6 hrs post induction) the amount of Fab remained constant in different locations within and outside the cells. Western blot analysis of cell fractions showed that certain amount of the Fab was also produced in the cells as insoluble form.
Conclusions: In this work we showed that the production of Fab in the periplasm during high cell density cultivation of *E. coli* in fermentor can be challenging as the product may appear in various locations within and outside the cells. To exploit the advantages of the periplasmic expression systems for purification in downstream processing, bacterial cells should be harvested when they maintain.

Keywords: antibody fragment, cell disruption, *Escherichia coli*, fed-batch fermentation, periplasmic expression, recombinant protein.

INTRODUCTION

At present, recombinant proteins, including therapeutics derived from antigen-specific fragments of antibodies produced by recombinant processes, play an important role in pharmaceutical industry.

Antibody fragments (Fabs), lacking the glycosylated Fc constant regions, have the advantage over whole antibodies in applications requiring rapid tissue penetration and rapid clearance from the blood or kidney (Yokota et al. 1992). Antibody fragments are useful in diagnostic applications, tumour therapy and tumour imaging (Holliger and Hudgson, 2005; Wu and Senter, 2005) as a result of their small size. Development of bifunctional antibodies, through fusing the gene of antibody fragment to the gene of an effector protein (*e.g.* cytokine), have been a new approach for eliminating tumour cells without harming healthy cells (Neuberger et al. 1984; Boleti et al. 1995; Ortiz-Sánchez et al. 2008). In contrast to some of the heavily engineered antibody fragments, the Fab has native sequence; subsequently, it is less likely for the Fab to be immunogenic when used as therapy.

Due to these features, the production of antibody fragments is industrially highly demanded and the antibody fragment pipeline is expanding, with three therapeutic Fabs approved by US Food and Drug Administration (two of which, namely Ranibizumab and Certolizumab pegol, are manufactured in the periplasm of *E. coli*), and many in the active clinical pipeline and preclinical research (Nelson and Reichert, 2009; Nelson, 2010; Reichert, 2012).

E. coli was the first host used to produce recombinant DNA (rDNA) pharmaceuticals, enabling the approval of recombinant DNA (rDNA) human insulin (Humulin®, licensed by GENENTECH to ELI LILLY) in 1982 and marketing of Monsanto's bovine growth hormone (bGH) in 1994 (Swartz, 2001; Meyer and Schmidhalter, 2012).

Nearly 30% of currently approved recombinant therapeutic proteins are produced in *E. coli* (Huang et al. 2012) as the host of choice due to its rapid growth on simple inexpensive media, high-yield production and well-known physiology and genetics (Swartz, 2001; Meyer and Schmidhalter, 2012). Proteins of therapeutic value, including antibodies and antibody fragments, often contain disulfide bond bridges that are necessary for their folding, stability and/or function. Correct formation of disulfide bridges is a main problem when expressing these proteins in bacterial cells, in particular, when a protein of interest contains non-consecutive disulfide bonds in its final folded structure necessitating the action of protein disulfide isomerases (Berkmen et al. 2007). Over-expression of these proteins in the cytoplasm of *E. coli* leads to the accumulation of inclusion bodies (IBs), due to lack of oxidizing environment in the cytoplasm needed for disulfide bond formation and appropriate protein folding, which contain the target protein in an misfolded and malfunctioned form (Carrió and Villaverde, 2002; Heeboll-Nielsen et al. 2003; Manzur et al. 2006; Ventura and Villaverde, 2006; Hariprasad, et al. 2009; Kim et al. 2011).

The problem of the vulnerability to incorrect disulfide bond formation can be solved by directing the recombinant proteins into the periplasm of *E. coli*, and it has been demonstrated that production of large quantities of correctly folded soluble recombinant proteins is feasible by targeting them to the periplasm of this bacterium (Carvalho et al. 1998; Yoon et al. 2010; Arredondo and Georgiou, 2011; Jin et al. 2011). Promoters and secretion signals from a few outer membrane proteins have been used to direct recombinant proteins into the periplasm (Moir and Mao, 1990; Carter et al. 1992; Joly et al. 1998; Chen et al. 2004; Laird et al. 2005; Yoon et al. 2010). The oxidizing environment of the periplasm in cooperation with the periplasmic disulfide oxidoreductases/isomerases (Berkmen et al. 2007) and periplasmic chaperones (Betton, 2007) assists the formation of correctly folded recombinant proteins. Also, the periplasm can be manipulated by being exposed to extracellular chemicals assisting protein folding (Wunderlich and Glockshuber, 1993; Barth et al. 2000; Sandee et al. 2005). In addition, secreting a protein into the periplasmic space can alleviate downstream processing (DSP), owing to reduced proteolytic degradation and low total cell protein in the periplasm (Park and Lee, 1998). This secretory process also allows elimination of the amino-terminal signal sequence leading to the appearance of mature proteins (Yoon et al. 2010). Thus, secretion of recombinant proteins into the periplasm of *E. coli* has been useful for production of properly folded pharmaceutical proteins.

This study describes that manufacturing of periplasmic proteins can be tricky and a feature of periplasmic secretion systems in *E. coli* is that the product may appear in various locations within and outside the cells. Therefore, monitoring of the titre, location and form of a target protein is required throughout a process of high cell density cultivation of *E. coli*. It is very crucial to discern the point at which the majority of a target protein is placed in the periplasmic space of bacterial cells during fermentation process in order to harvest the cells and exploit the advantages of the periplasmic expression systems for DSP. In this work, experiments were carried out to show how anti-lysozyme Fab D1.3 titre, location, and form varied over the course of fed-batch fermentation. Also experiments

were done to compare the efficiency of osmotic shock and mechanical cell disruption methods for the recovery of the antibody fragment from *E. coli.*

MATERIALS AND METHODS

Materials

Escherichia coli W3110 (ATCC 27325) transformed with a plasmid containing the gene for the anti-lysozyme Fab D1.3 under the control of the lac-based expression system (Hodgson et al. 2007) was provided by Merck Sharp and Dohme Ltd. (Billingham, UK) and maintained at -80ºC in LB medium supplemented with 20% v/v glycerol.

Nutrient agar and phosphate buffered saline (PBS) were purchased from Oxoid (Basingstoke, Hampshire, UK). Yeast extract and trypton were obtained from Becton, Dickinson and company (BD) (Sparks, MD, USA). Sodium dodecyl sulphate (SDS) and Laemmli electrophoresis and running buffers were attained from Bio-Rad Laboratories (Hercules, CA, USA). Ammonium hydroxide; phosphoric acid; magnesium sulphate; calcium chloride dehydrate; manganese (II) sulphate fourhydrate; and HPLC grade methanol were purchased from Fisher Scientific (Loughborough, Leicestershire, UK). TMB (3,3′,5,5′-tetramethylbenzidine) microwell peroxidase substrate (2-C) and TMB membrane peroxidase substrate system (3-C) were obtained from KPL (Gaithersburg, MD, USA). Hybond-P PVDF membrane was obtained from Amersham Biosciences (Uppsala, Sweden). Bicinchoninic acid (BCA) protein assay kit and Isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Thermo Scientific (Rockford, IL, USA). Tetramethylethylenediamine (TEMED) was supplied by Melford Laboratories (Suffolk, UK). Ammonium sulphate; glycerol; potassium phosphate monobasic and dibasic; citric acid; ferrous sulphate heptahydrate; zinc sulphate; sodium molibdate dehydrate; copper (II) sulphate; boric acid; tetracycline; sodium chloride; sodium hydroxide; Tween 20; ethylenediaminetetraacetic acid (EDTA); sucrose; Trizma-base; sodium phosphate monobasic; sodium phosphate dibasic; sodium carbonate; sodium hydrogen carbonate; glycine for electrophoresis; chicken egg white lysozyme (HEWL); albumin from bovine serum (BSA); and anti-IgG (Fab specific) - peroxidise antibody produced in goat, as well as all other chemicals used in this study were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

Fed-batch fermentation process

Bacterial cells from the cell bank, stored at -80ºC, were propagated overnight on nutrient agar supplemented with 15 mg/l tetracycline. Starting cultures were prepared by inoculating 100 ml aliquots of Luria Bertani (LB) broth (comprising 1% NaCl, 0.5% yeast extract and 1% tryptone) containing 15 mg/l tetracycline with a fresh single colony of *E. coli* and shaking at 37ºC and 200 rpm for 13 hrs.

Fermentation was conducted using a 10 l Electrolab fermentor (Tewkesbury, UK). A complex medium (3.3 l initial volume) was employed. The composition of the complex medium is as follows: 14 g/l $(NH_4)_2SO_4$; 35 g/l glycerol; 20 g/l yeast extract; 2.0 g/l KH_2PO_4; 16.5 g/l K_2HPO_4; 7.5 g/l citric acid; and 1.5 ml/l H_3PO_4. The pH of the medium was adjusted to 7.0 using NH_4OH, and the medium was sterilised in autoclave at 121ºC for 0.5 hrs. After cooling to 37ºC, post sterilisation and trace metal additions (Table 1 and Table 2) were aseptically added to the vessel. The fermentor was inoculated with 200 ml (~4% inoc./medium ratio) from a 13-hrs shake flask inoculum, and operated in batch mode. The starting conditions were: temperature 37ºC; agitator speed 250 rpm; air-flow rate 1.0 vvm; pH 7.0. Agitation rate was increased during the fermentation (up to maximum value of 1500 rpm), in order to sustain the dissolved oxygen tension on a set point of 30%. The pH was maintained at 7.0 throughout the fermentation by the automatic addition of NH_4OH. Once glycerol was depleted, being indicated by an increase in the dissolved oxygen measured in the fermentor (7.5 hrs cultivation), the fed-batch fermentation mode was initiated by using glycerol/magnesium sulphate feed (containing 714 g/l glycerol and 7.4 g/l $MgSO_4 \times 7H_2O$) at a constant rate of 11 g glycerol/l/h.

Fab D1.3 expression was induced by the addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM after 10 hrs cultivation (OD_{600nm}~50). The fermentor was sampled hourly; cell fractionation was made by sonication and osmotic shock, as described below, and various fractions were stored at -20ºC for further SDS-poly-acrylamide gel electrophoresis (SDS-PAGE), enzyme-linked immunosorbent assay (ELISA) and Western blot analyses.

Table 1. Components which aseptically added after sterilisation and cooling of the medium to 37ºC prior to inoculation of the fermentor.

Component	Concentration required
1 M $MgSO_4$ x $7H_2O$ solution	10 ml/l
1 M $CaCl_2$ x $2H_2O$ solution	2 ml/l
Antifoam AF204	0.2 ml/l
15 mg/ml Tetracycline	1 ml/l

Table 2. Trace metal composition, 34 ml solution was sterilised through a 0.22 µm sterile filter into a suitable sterile container and added into 1 L medium in the fermentor.

Component	Concentration required
$FeSO_4$ x $7H_2O$	3.36 g/l
c$ZnSO_4$ x $7H_2O$	0.84 g/l
$MnSO_4$ x H_2O	0.51 g/l
Na_2MoO_4 x $2H_2O$	0.25 g/l
$CuSO_4$ x $5H_2O$	0.12 g/l
H_3BO_3	0.36 g/l
Conc. H_3PO_4	48 ml/l

Biomass estimation and viability counts

Optical density measurements were performed at a wavelength of 600 nm in a KONTRON UNIKON 922 UV-VIS spectrophotometer (KONTRON Northstar Scientific, Bardsey, UK), using PBS for dilution of culture samples (readout range of 0.2 to 0.8 OD_{600nm} units).

Dry cell weight (DCW) was measured as follows: 1 ml fermentation samples were centrifuged in pre-dried and pre-weighed 1.5 ml Eppendorf tubes using a Eppendorf microcentrifuge (Eppendorf Cenrifuge 5415D, Eppendorf, Hamburg, Germany) operated at 15800 g for 5 min. The cell pellets were resuspended in 1 ml distilled water and then re-centrifuged. The supernatants were carefully discarded, the pellets were dried overnight in a hotbox oven (Gallenkamp, Riley Industries Ltd, Aldridge, UK) at 100ºC, and the tubes were weighed once again. The biomass content in each tube was then calculated by difference.

Serial dilutions of fermentation samples were prepared aseptically in PBS for viability counts. One hundred microlitres aliquots of serial dilutions were plated onto LB agar plates supplemented with 15 mg/l tetracycline, and following overnight incubation at 37ºC, the number of colony forming units (CFU) was measured by counting colonies on plates containing less than 300 colonies according to following formula.

CFU/ml = No. of colonies x dilution factor x 10

Cell fractionation

Osmotic shock. Cell pellets obtained from 1 ml fermentation samples were resuspended in 1 ml of an osmotic shock solution (designated OS_1) comprising 20 mM Tris-HCl buffer, pH 8.0, supplemented with 2.5 mM EDTA and 20% sucrose. After a static incubation on ice for 600 sec, the cells were harvested by centrifugation (15800 g, 120 sec) and the supernatants were immediately frozen at -20ºC. The resulting cell pellets (spheroplasts) were then resuspended in 1 ml of a second osmotic shock solution (designated OS_2) lacking sucrose (*i.e.* 20 mM Tris-HCl buffer pH 8.0, containing 2.5 mM EDTA) and incubated on ice for 600 sec. Following centrifugation (15800 g, 120 sec), the supernatants were then removed and stored at -20ºC until required. The pellet was washed with a high salt buffer as described later.

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Sonication. Cell pellets obtained from 5 ml fermentation samples were resuspended in 5 ml of 100 mM Tris-hydrochloride buffer, pH 8.0. The resulting cell suspensions were then disrupted at 4ºC by ultrasonication (ultrasonic liquid processor XL, Heat System Incorporated, Farmingdale, USA) at 55% amplitude for 300 sec (15 cycles, each cycle lasted 10 sec with intervals of 10 sec between the cycles) to release intracellular protein. Successful cell disruption was confirmed with the aid of a phase contrast light microscopy. The bacterial cell debris was separated from the supernatants by centrifugation at 15800 g for 300 sec. The supernatant was frozen at -20ºC for further analyses, and the pellet was used for high salt washing step as described below.

Washing cell pellets with high salt buffer. The cell debris obtained from sonication cell disruption was resuspended in 5 ml of 20 mM sodium phosphate buffer supplemented with 0.5 M NaCl, pH 8.0. The suspensions were agitated for 1 hr and centrifuged at 15800 g for 300 sec. The supernatant samples were stored at -20ºC for further analysis.

Analytical techniques

SDS-poly-acrylamide gel electrophoresis (SDS-PAGE) and Western blotting. SDS-PAGE was performed on 15% gels at 150 V/20mA for 2.5 hrs using Laemmli electrophoresis and running buffers. Prior to electrophoresis, all samples were diluted 1:1 with the sample buffer, boiled for 120 sec, and then centrifuged briefly at high speed in a microcentrifuge, before loading into the wells of the gel.

For Western blotting, the stacking gel was removed and the resolving gel was soaked in the protein transfer buffer (3.03 g/l Trizma-base, 14.4 g/l glycine and 200 ml/l methanol) for 0.3 hrs. Hybond-P PVDF membrane and the filter paper were cut to the dimension of the gel, soaked in 100% (v/v) methanol for 10 sec, washed in distilled water for 300 sec, and equilibrated in the protein transfer buffer for 0.3 hrs. The electroblotting cassette was assembled and placed between the electrodes in the blotting unit according to the manufacturer's instructions (Mini Trans-Blot electrophoretic Transfer Cell, Bio-Rad, CA, USA). The band-transfer was carried out initially for 1 hr at 100 V, with cold transfer buffer at 4ºC, and was then perpetuated at 4ºC, at 30 V, overnight. Following transfer, the membrane was removed from the blotting cassette, the orientation of the gel on the membrane was marked, and the membrane was rinsed briefly in phosphate buffered saline (PBS; 11.5 g/l di-sodium hydrogen orthophosphate, 2.96 g/l sodium dihydrogen orthophosphate, and 5.84 g/l sodium chloride; pH 7.5). For immunodetection, non-specific binding sites on the membrane were blocked using 3% (w/v) BSA made in PBS, for 1 hr, and then briefly rinsed twice with PBS containing 0.1% (v/v) Tween 20. The blot was washed with an excess volume of PBS/0.15 (v/v) Tween 20 for 300 sec and incubated with anti-IgG (Fab specific) - peroxidise antibody (which was diluted down in PBS 10000-fold) for 1 hr. The blot was washed three times with PBS/Tween 20, and the enzyme substrate (TMB membrane peroxidise substrate system (3-C)) solution was added to the blot and incubated at room temperature until the desired band intensity was achieved.

Sandwich ELISA method for Fab D1.3. For this purpose, each well of a 96-well microtiter plate was coated overnight at 4ºC with 100 µl of 0.1% HEWL, made in coating buffer (1.59 g/l Na_2CO_3 and 2.93 g/l $NaHCO_3$). Blocking was made with 200 µl PBS-BSA (1 PBS tablet/1 g BSA in 100 ml distilled water) per well, with shaking (500 rpm) in a microplate incubator shaker (Infors AG type AK120, Infors HT, Bottmingen Switzerland) for 1 hr, at 37ºC. The wells were then washed three times with 300 µl wash buffer (1 PBS tablet and 100 µl Tween 20 in 100 ml distilled water) per well and tap dried. Both standards and samples (100 µl), having been diluted in PBS on a separate plate, were transferred to the washed ELISA plate, incubated at 37ºC, 500 rpm for 1 hr, washed again with the wash buffer three times and tap dried. The plate was then loaded with 100 µl anti-IgG (Fab specific) - peroxidase antibody produced in goat, which was diluted 10000-fold in the block buffer, incubated at 37ºC, 500 rpm for 1 hr, and then washed three times with the wash buffer and tap dried, before 100 µl of peroxidase substrate was added to each well. The reaction was stopped after 600 sec by adding 100 µl of 1 M H_3PO_4 to each well, and the plate was read at the wavelength of 450 nm using a microplate reader (Promega Glomax-Multi detection system, Turner BioSystems Inc., Sunnyvale, CA, USA).

RESULTS AND DISCUSSION

During fed-batch fermentation, various parameters including OD_{600nm}, CFU, DCW, were measured, and these are presented in Figure 1. It is evident from this figure that optical density reached the plateau

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after 13 hrs cultivation (3 hrs post induction with 0.1 mM IPTG), and started to reduce after 16 hrs cultivation (6 hrs post induction); however, there was a drop in CFU/ml after 2 hrs induction. Ideally, when cells are healthy, it is expected that both turbidity and CFU/ml follow each other. In this work, there was approximately 10-fold drop in CFU/ml for slight increase in OD_{600nm} between 2 and 5 hrs induction. The existence of 'viable but nonculturable' bacterial cells can be the reason for the CFU/ml drop after 2 hrs induction with 0.1 mM IPTG. The term 'viable but nonculturable' has been used for those bacterial cells which have detectable metabolic function (*i.e.* cells are alive and contributed to the production), but do not undergo cell division on routinely employed bacteriological media (Roszak and Colwell, 1987). Sundström et al. (2004) studied a fermentation process for the production of the recombinant fusion protein, promegapoietin (PMP), in *E. coli* and reported that after induction the number of CFU/ml dropped to approximately 10 per cent of its maximum value while the biomass concentration continued to increase. By doing flow cytometric analysis and measuring intracellular concentration of PMP, they demonstrated that the cells were alive and contributed to the production, and the drop in the number of CFU/ml indicated a loss of cell division ability rather than cell death. DCW figure was constantly increased during 16 hrs fed-batch cultivation, and afterwards there was an insignificant rise in this figure to the end of fermentation.

It has been reported that specific growth rate (μ) affects the amount of soluble excreted target product and that secretion of target product to the periplasmic space and culture media is increased at μ values lower than 0.25 h^{-1} before induction, in fed batch fermentation process with an exponential feeding strategy (Puertas et al. 2010). In the present work, the specific growth rate at the moment of induction was 0.19 h^{-1}. However, few hours after induction the cell growth was compromised, as it is evident from the CFU figure, and consequently the specific growth rate was dropped markedly.

Two fractionation methods, including osmotic shock and sonication, were utilised to disrupt the bacterial cells present in the samples taken through the course of fed-batch fermentation. Subsequent cell disruptions, the titre of soluble Fab D1.3 in each fraction was quantified using ELISA. Figure 2 shows the titre of Fab in the culture broth medium and in the osmotic shock solutions 1 and 2 (OS_1 and OS_2) at the point of induction and later.

The existence of Fab D1.3 in OS_1 corroborates the periplasmic production of the protein, while the Fab titration in OS_2 shows the amount remained in the cytoplasm of the bacterial cells.

As illustrated in this figure, a low amount (approximately 4 mg/l) of the Fab was observed in the periplasm and culture broth together just before induction with 0.1 mM IPTG (*i.e.* 10 hrs cultivation), indicating that the promoter was induced to an insignificant extent in the absence of the real inducer, *i.e.* IPTG. The amount of Fab D1.3 in the periplasm increased after induction and reached a maximum titre of 47 mg/l after 4 hrs post induction. The Fab secretion and/or leakage into the culture broth was started after 2 hrs post induction (the point after which the bacterial cells appeared to be 'viable but nonculturable' as discussed earlier), sharply reached a maximum of about 79 mg/l after 6 hrs post induction, and stopped afterwards. Besides after 6 hrs post induction, the Fab titration in either periplasm or cytoplasm remained almost constant, indicating that soon after the bacterial cells become unhealthy, based on OD_{600nm} measurements shown in Figure 1, both cellular production and discharge of the recombinant protein are stopped in the cells.

To detect how much Fab is attached to the bacterial cell pellets after osmotic shock treatment, the post-osmotic shock pellet samples were washed with a high salt buffer (sodium phosphate buffer containing 0.5 M NaCl, pH 8.0) to dissociate any electrostatically-bond Fab from the pellet, and centrifuged. ELISA analysis of the resulted soluble fractions showed that about 1.2 mg/l Fab D1.3 was electrostatically attached to the pellet and released by this washing procedure (Figure 3).

To perceive the efficiency of the osmotic shock treatment and in order to show if any Fab D1.3 was left in the bacterial cell pellets disrupted by osmotic shock and washed with the high salt buffer, these pellets were subsequently disrupted by sonication. As shown in Figure 4, there was inconsiderable amount (5.9%) of soluble Fab remained in these pellets. This indicates that soluble Fab can be very effectively released from the cells by this osmotic shock procedure.

Also, cell disruption by sonication was directly performed on the samples taken from the fermentor throughout the fed-batch fermentation process. This mechanical cell disruption experiment was conducted to measure the amount of soluble Fab in the cytoplasm and periplasm jointly released by

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this technique, to compare the efficiency of two different cell disruption methods for the soluble Fab release from bacterial cells, and to confirm any insoluble Fab in the cells. ELISA results for soluble samples obtained from sonication disruption have been illustrated in Figure 5. By comparing ELISA results of osmotic shock and sonication disruptions, it is evident that the amount of Fab recovered from the bacterial cells by sonication was approximately 20 to 45% less than Fab recovered by osmotic shock (*i.e.* OS_1 plus OS_2) treatment. This denoted that sonication had deleterious impact on the Fab, afflicted its ability to attach to HEWL being coated onto ELISA plate. Sonication produces gas bubbles collapsing in a process known as cavitation, in which a bubble in a liquid rapidly collapses and consequently produces a shock wave, generating extremely high local temperature, high sheer pressure, and free radicals H° and OH° from sonolysis of water which can jointly cause protein destabilising (Hawkins and Davies, 2001; Stathopulos et al. 2004; Kohno et al. 2011). Therefore, lacking deleterious effects of sonication, osmotic shock seems to be a more effectual method for Fab recovery from these bacterial cells.

Sonication cell disruption samples were also analysed by Western blotting (Figure 6). Pellets, obtained from centrifugation of samples after sonication were analysed by Western blotting. Western blot analysis of these pellet samples demonstrated that a certain amount of the Fab was produced as insoluble form in these bacterial cells during fed-batch fermentation process.

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Figures

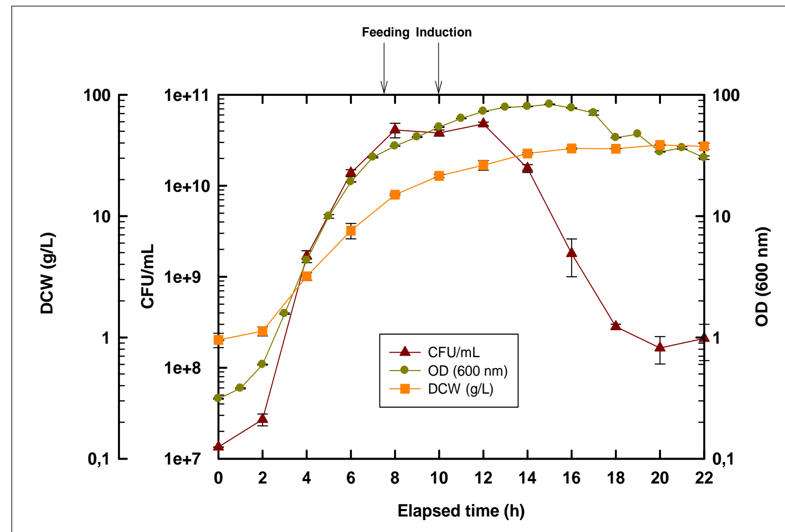

Fig. 1 Optical density (OD_{600nm}), CFU and DCW profiles for fed-batch fermentation of recombinant *E. coli* W3110 at 37ºC for production of Fab D1.3. Induction with 0.1 mM IPTG was performed after 10 hrs of cultivation (OD~50). Results for OD and CFU are averages of two measurements; results for DCW are averages of three measurements.

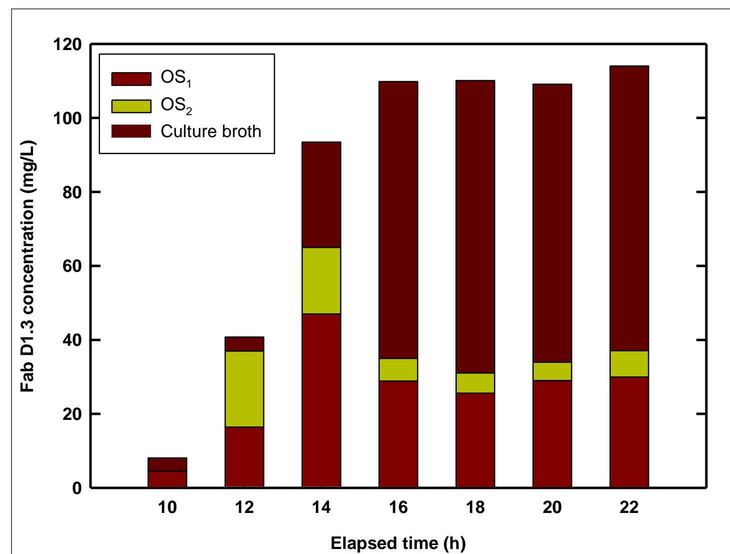

Fig. 2 ELISA of soluble samples arising from osmotic shock fractionation. Induction was done by 0.1 mM IPTG after 10 hrs cultivation (OD~50). Amount of Fab was quantified in osmotic solution 1, osmotic solution 2, and culture broth.

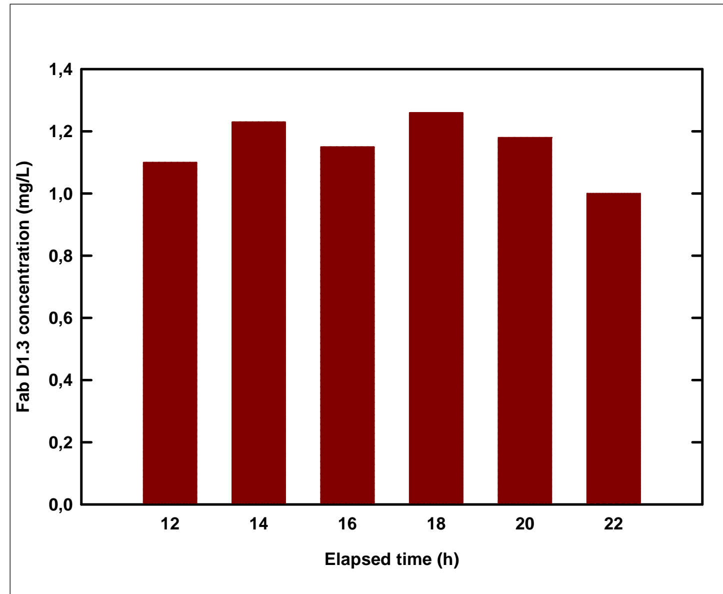

Fig. 3 ELISA analysis of soluble fractions obtained from washing post-osmotic shock bacterial cell debris with PBS supplemented with 0.5 M NaCl, pH 8.0.

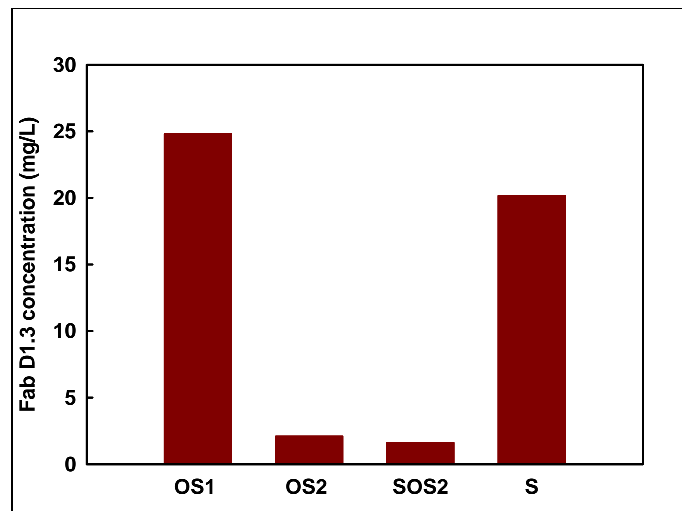

Fig. 4 ELISA analyses of soluble samples from osmotic shock and sonication treatments originating from 21 hrs culture. OS1: osmotic shock solution 1, OS2: osmotic solution 2, SOS2: sonication after OS2 and S: sonication.

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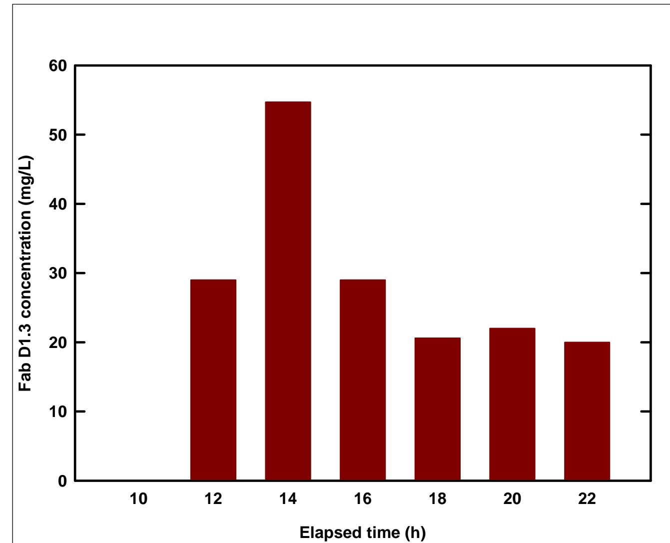

Fig. 5. ELISA of soluble samples arisen from sonication cell disruption. Induction with IPTG was after 10 hrs cultivation (OD~50).

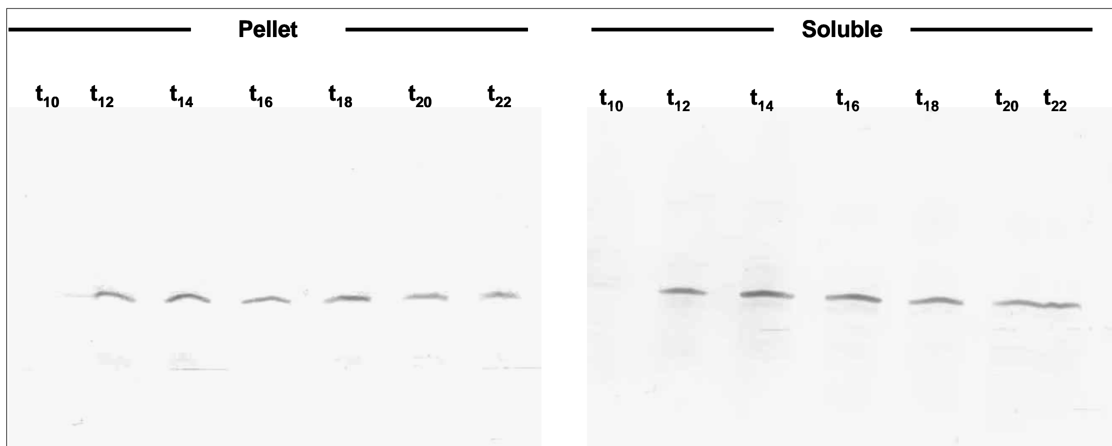

Fig. 6 Western blot analyses of soluble and insoluble samples arising from sonication cell disruption. Prior to Western blotting, the samples were subjected to SDS-PAGE. Induction with 0.1 mM IPTG was after 10 hrs cultivation (OD~50). Insoluble samples derived from sonication treatment were washed in a high salt buffer (sodium phosphate buffer containing 0.5 M NaCl, pH 8.0), centrifuged, and the obtained pellet samples were then analysed.