

# Exploration of cell lysis in a bioreactor using *Escherichia coli* expressing single-chain variable-domain antibody fragments

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**Abstract** Cell lysis has long been a process problem in protein expression using bacterial hosts. To explore the pathways involved in cell lysis in a bioreactor, *Escherichia coli* strain W3110 was used as the host to express single-chain variable-domain antibody fragments (scFv). Under the same fermentation conditions, *E. coli* strain W3110 expressing a humanized scFv entered the viable but nonculturable (VBNC) state 6 h before the wild-type strain, and the accumulation of the scFv within the cells accelerated cell lysis. At the transcriptional level, the scFv not only altered the expression of *rpoH*, *dnaK*, *dnaJ*, *groES*, and *groEL* in the heat stress response, but also *rpoS*, *gadE*, and *gadX* in the acid stress response pathway and *sodA* and *katE* in the oxygen stress response pathway. During cell lysis in a fermenter, the expression of the membrane protein-encoding genes *ompA*, *ompC*, *ompW*, and *ompX* significantly decreased, and the high-level expression of *rpoE* was not sustained. These findings provide new insights into cell lysis as well as a theoretical foundation for improving fermentation conditions and engineering bacteria to minimize cell lysis during fermentation.

**Keywords** Bioreactor · Engineering cell lysis ·  $\sigma^E$  pathway · Stress response · Viable but nonculturable

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## Introduction

Industrial-scale production of single-chain variable-domain antibody fragment (scFv) expression in *Escherichia coli* was made possible by genetic engineering. To achieve large-scale microbial biomass production in fed-batch cultures, high cell density cultivation and efficient exogenous protein expression are required (Zarschler et al. 2013). However, cell lysis frequently occurs during the fermentation process because of diverse reactor microenvironments and the expression of heterologous transcripts and proteins. At present, study of the cell autolysis mechanism has mainly focused on the shaking flask level; however, in order to gain more target protein, the culture level of engineering strain expressing foreign protein often needs to enlarge its culture bioreactor level, so it is of great significance to study the effect of the specific expression of foreign proteins at the bioreactor level on cell activity and cell lysis (Sandén et al. 2003). The unpredictable cell lysis phenomenon in the large-scale culture process of *E. coli* often causes significant difficulty in the downstream purification process and leads to degradation of the target protein, eventually resulting in a decline in the output and quality of the target protein (Andersson et al. 1996).

Overexpressing foreign proteins impose different levels of toxicity to cells, resulting in physical defects and lower cellular activity. Simultaneously, the additional metabolic burden can result in a reduction in the metabolic energy required for effective protein and nucleic acid biosynthesis (Chou 2007). To explore the negative impact of cell lysis on host cells in a biological reactor, it is needed to understand the mechanisms underlying the stress-induced cell lysis caused by the expression of recombinant, exogenous proteins in an *E. coli* host. *Escherichia coli* has formed a sophisticated stress response mechanism during its evolution, and it is regulated by complex protein–protein interactions and protein phosphorylation

events that are governed by signal transduction pathways. These responses allow the bacteria to adapt to a variety of external and/or internal stimuli, including temperature, pH, osmolarity, nutrient concentrations, the production of secondary metabolites, and the accumulation of misfolded proteins in the periplasmic space (Sikdar et al. 2013). Furthermore, a recent study found that cell death and lysis in *E. coli* are regulated by programmed cell death, which is similar to apoptosis in eukaryotic cells. Based on a shaking flask culture, Murata et al. (2012) identified a  $\sigma^E$ -dependent cell lysis process, which is considered to be a survival strategy when cells suffer from adverse environmental stresses. When cells suffer from stresses during the early stationary phase of growth, most of them enter the viable but nonculturable (VBNC) state, and cell lysis is induced to remove these damaged cells, thereby maximizing the use of nutrients to maintain cell viability for prolonged periods.

However, current progress on understanding stress and cell lysis mechanisms is limited to the shake flask level, so a comprehensive understanding of the impact of exogenous protein expression on cell lysis in bioreactors is needed. To fill this knowledge gap, we have investigated the correlation between exogenous protein expression and cell lysis in a bioreactor, as well as the role of  $\sigma^E$ -directed stress response pathways on cell lysis, using an scFv-expressing *E. coli* W3110 system.

## Methods

### Strains, plasmids, and culture conditions

The strains used in this study are the *E. coli* W3110 wild-type strain and a W3110 strain carrying the vector pBZ001 (W3110/pBZ001). This vector contains the scFv gene (939 bp), the expression of which is driven by the T7A3 promoter, and an operator system consisting of two perfect palindromic sequences. The signal sequence used for the periplasmic production of scFv was derived from OmpA, and this sequence is cleaved when the recombinant protein is translocated across the cytoplasmic membrane, thereby allowing the mature protein to fold into a functional scFv. The strains were cultured overnight at 37 °C on Luria–Bertani (LB) plates. A single colony was picked and inoculated into 100 mL of Terrific Broth/Super Broth (TB/SB) (Table 1) seed medium in a 1-L Erlenmeyer flask and cultured at 37 °C with shaking at 230 rpm on an oscillatory shaker. When the optical density at 600 nm ( $OD_{600}$ ) reached 4, the seed culture was inoculated into 2 L of TB/SB fermentation medium in an ez-Control 5-L fermenter (Applikon, Delft, The Netherlands). The fermenter temperature was maintained at 30 °C  $\pm$  0.1 °C, and the pH was maintained at 7.0  $\pm$  0.05 by the addition of 17 % phosphoric acid or 25 % aqueous ammonia. To ensure that the level of dissolved oxygen (DO)

achieved 30 % oxygen saturation, the air flow rate was maintained at 2 volumes of air per volume of fermentation broth per minute (vvm); when necessary, 1 vvm of pure oxygen was provided. Before DO spiking, feeding was initiated using a precalibrated peristaltic pump at different flow rates (3.5 or 6 mL h<sup>-1</sup>l<sup>-1</sup>). Tetracycline was sterilized by filtration through a 0.2- $\mu$ m cellulose acetate membrane filter and added to a final concentration of 15  $\mu$ g mL<sup>-1</sup>. To induce the expression of exogenous scFv in *E. coli* W3110/pBZ001, a final isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) concentration of 100  $\mu$ M was achieved by injecting IPTG into the fermenter 1 h after feeding was initiated, and the fermentation process was continued for approximately 38 h after induction.

### Determination of VBNC cell number

#### Sample treatment

Culture broth samples (1 mL) were collected aseptically at different fermentation stages based on the  $OD_{600}$  and culture time, and they were serially diluted 10-fold to an appropriate concentration using 0.01 M phosphate-buffered saline (PBS) for counting. One hundred microliters of the diluent was used for colony-forming unit (CFU) determinations by plating it onto LB agar plates and counting the number of colonies after 24 h of incubation at 37 °C (viable and culturable cell number). Meanwhile, an appropriate dilution (100  $\mu$ L) was centrifuged at 9000  $\times$  g at 4 °C for 3 min (FRESCOL-7, Thermo Fisher Scientific). The supernatant was discarded, and the pellet was washed twice with 0.01 M PBS.

#### CTC staining

5-Cyano-2,3-ditolyl tetrazolium chloride (CTC) fluorescent staining counting was conducted as described previously (Abe et al. 2007), with slight modifications. Viable bacteria cells transport electrons via respiration, thereby reducing CTC to CTC formazan (CTF), which results in the formation of a red fluorescent precipitate in the cell membrane. The washed pellets were resuspended in approximately 100  $\mu$ L of a 3 mM CTC solution (dissolved in 0.01 M PBS) and incubated for 30 min at 25 °C with shaking at 200 rpm in the dark.

#### Fluorescence microscopy count

As Hobbie et al. (1977) described, stained samples were subsequently harvested by filtration through a black polycarbonate membrane filter (0.02  $\mu$ m pore size, 13 mm diameter; Sartorius AG, Göttingen, Germany). The cell number was determined using a BX53F fluorescence microscope (Olympus, Tokyo, Japan) with a Fluotar objective (U Plan Semi-Apochromat objective 100 $\times$ /1.3, oil) and a wide-field eyepiece (10 $\times$ , focusable). An Olympus filter set (BP425-

**Table 1** The medium used in the study

Medium (w/v)	Constituents (g/L)	Source
LB plating medium	Tryptone 10, yeast extract 5, NaCl 10, agar 20, pH 7.0	Lessard (2013)
TB/SB seed medium	Tryptone 12, yeast extract 24, KH <sub>2</sub> PO <sub>4</sub> 2.31, K <sub>2</sub> HPO <sub>4</sub> 9.58, glycerin 10, pH 7.0	Lessard (2013)
TB/SB fermentation medium	Tryptone 24, yeast extract 48, KH <sub>2</sub> PO <sub>4</sub> 2.31, K <sub>2</sub> HPO <sub>4</sub> 9.58, glycerin 25, pH 7.0	This study
Booster feeding medium	Glycerin 714, yeast extract 50, (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> 25	This study

445 exc./FF593/BA628-640 em.) was used to detect CTF. At least 30 to 50 cells in ten randomly chosen areas on a filter were counted, and the average value and standard deviation were calculated. The cell number was calculated as follows:

$$E = \frac{X \times S_1}{S_2} \times \frac{1}{V}$$

where  $E$  is the cell number in the sample,  $X$  is the average cell number in the microscopic fields,  $S_1$  and  $S_2$  are the areas of the membrane and microscopic field, respectively, and  $V$  is the sample volume.

### RNA extraction, cDNA synthesis, and RT-PCR

Bacteria ( $5 \times 10^6$ – $10^7$  cells) in fermented broth were immediately centrifuged at  $8000 \times g$  (4 °C, 2 min), the supernatant was discarded, and the pellet was stored in liquid nitrogen for RNA extraction. Total RNA was isolated from *E. coli* W3110 cells using the RNAiso Plus Kit (TaKaRa, Shiga, Japan), according to the manufacturer's instructions. The amount and purity of the RNA were reflected by the absorbance at 260 and 280 nm ( $A_{260}$  and  $A_{280}$ , respectively), and the RNA purity was assessed by 2 % agarose gel electrophoresis. The primers used in our study were designed with Primer 5.0 software, and the sequences of the primers are given in Table 2. After removing the genomic DNA, total RNA was reverse-transcribed into cDNA, as described in the instructions of the PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa). Real-time polymerase chain reaction (RT-PCR) was conducted using an iCycler CFX96 quantitative PCR system (Bio-Rad, Hercules, CA, USA), SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) fluorescence quantitative reagents (TaKaRa), and individual eight-tube PCR strips (Bio-Rad). Each reaction mixture (10 µL) consisted of 5 µL SYBR® Premix Ex Taq™ II (2×), 0.4 µL of forward primer (10 µM), 0.4 µL of reverse primer (10 µM), and appropriate volumes of cDNA template and sterile double-distilled H<sub>2</sub>O. The reaction mixture was incubated at 95 °C for 30 s for predenaturation, followed by 40 cycles of amplification, which consisted of a denaturation step (95 °C for 5 s), an annealing step (60 °C for 30 s), and a denaturation step (95 °C for 30 s). Gene expression was calculated relative to

**Table 2** The sequences of the primers used in real-time polymerase chain reaction (RT-PCR)

Primers	Sequences (5' to 3')	Source
16s-rRNA-F	ACGACCAGGGCTACACAC	This study
16s-rRNA-R	ACTACGACGCACTTTATGAGG	This study
RpoH-F	CCCGTGAAGTGGGCGTAACC	This study
RpoH-R	TGGCTGTCGGAATCGTCGTC	This study
DnaK-F	ATGAACATCAAAGTGACTCGTG	This study
DnaK-R	TTCTGAACCATTGGCATAACG	This study
GroEL-F	GTACCATCTCCGCTAACTCC	This study
GroEL-R	CGAACTGCATACCTTCAACC	This study
GroES-F	GCGTCACCCATAACAGATAC	This study
GroES-R	AAATAACGATGTCGCCAAC	This study
DnaJ-R	CCATAGCGAAGTTGATCGGGAC	This study
RpoS-F	AGCCGTATGCTTCGTCTTAAC	This study
RpoS-R	TATCGTCATCTTGCCTGGTATC	This study
H-NS-F	GTATTGACCCGAACGAACTG	This study
H-NS-R	GATTTACCTTGCTCATCCATTG	This study
GadE-F	GAACAACGATTCCGACAAGG	This study
GadE-R	TTTCGTGATTATCTTCAACTGC	This study
GadX-F	ACGCCCACAGAGTATCAGGAG	This study
GadX-R:	TTCCGCAGAACGGTCAGTG	This study
KatE-F	TGTGGGAAGCCATTGAAGCAG	This study
KatE-R	AGCACCATTTTCCGACACG	This study
SodA-F	GTCTGAAAAAAGGCACCACCC	This study
SodA-R	TCACCCATCAGCGGAGAATC	This study
RpoE-F	GGTCCAGAAGGGAGATCAGAAAGC	This study
RpoE-R	AACATCACCCGACGGCACATAG	This study
OmpA-F	TGAGCCTGGGTGTTTCTTAC	This study
OmpA-R	CAGAGCAGCCTGACCTTCC	This study
OmpC-F	CGGCAACCCATCTGGTGAAG	This study
OmpC-R	GCAGCGGTGTTCTGAGCATC	This study
OmpW-F	AGGTGGGGGTTGATTATCTG	This study
OmpW-R	TACGCTATCGTGTGCTGTG	This study
OmpX-F	CACTGAATACCCGACCTACAAAC	This study
OmpX-R	CTCGTAAGAGAAGTCCAGAGCAAC	This study

16sRNA as the reference gene; *rpoH*, *dnaK*, *dnaJ*, *groEL*, and *groES* are the genes of the heat stress response pathway; *rpoS*, *H-NS*, *gadE*, and *gadX* are the genes of the acid stress response pathway; *katE* and *sodA* are the genes of the oxygen stress response pathway; *rpoE*, *ompA*, *ompC*, *ompW*, and *ompX* are the genes of the  $\sigma^E$ -dependent cell lysis pathway

that of 16S rRNA expression using the  $2^{-\Delta\Delta CT}$  method. Three independent RT-PCR replicates were performed. Relative gene expression was shown as the ratio of gene expression in *E. coli* W3110/pBZ001 and wild-type *E. coli* W3110 at the same culture time.

### The degree of cell lysis

We defined the degree of cell lysis as the ratio between the reduction of maximum biomass at time  $t$  and the maximum biomass, which occurred when *E. coli* W3110/pBZ001 and wild-type *E. coli* W3110 entered stationary phase after 15 h of incubation:

$$\text{Cell lysis degree} = \frac{\text{OD}_{600}(15 \text{ h}) - \text{OD}_{600}(t)}{\text{OD}_{600}(15 \text{ h})}$$

### Secreted and intracellular scFv levels as determined by an enzyme-linked immunosorbent assay

Ninety-six-well microtiter plates were coated with 100  $\mu\text{L}$  of human serum albumin (HAS) ( $25 \mu\text{g mL}^{-1}$ ) in 50 mM carbonate buffer at pH 9.6 and incubated at 4 °C overnight. The plates were emptied and washed five times with PBS-Tween-20 (PBS-T) washing solution (0.15 M sodium phosphate buffer, pH 7.4, supplemented with 0.05 % Tween-20). One hundred and fifty microliters of 20 % (w/v) bovine serum albumen solution in PBS-T buffer was added and incubated for 1 h at 37 °C to block the unoccupied sites on the surfaces of the polystyrene wells. The plates were emptied and washed as described above. Antibody samples (the fermentation supernatant) and controls (scFv standard after protein A purification) were diluted in phosphate buffer, pH 7.4, and 50  $\mu\text{L}$  of the antibody and control diluents were preincubated for 1 h at 37 °C. The plates were washed as before. Goat anti-mouse horseradish peroxidase-conjugated secondary antibody, diluted 1:1000, was added (50  $\mu\text{L}$  per well) to the plates. The plates were incubated for 1 h at 37 °C, emptied, and washed. One hundred microliters of substrate and chromogenic reagent were mixed for 15 min at 37 °C in the dark, and 100  $\mu\text{L}$  of 2 M  $\text{H}_2\text{SO}_4$  was added to stop the reaction. The absorbance at 450 nm was read by a microplate reader.

## Results and discussion

### scFv expression caused cell lysis in *E. coli* W3110/pBZ001

The prebatch cultivation results showed that DO levels always spiked after 11.5 h  $\pm$  1 h of inoculation, which indicates that the glycerol in the culture was exhausted (data not shown). Therefore, feeding was started at 9 h. One hour after feeding

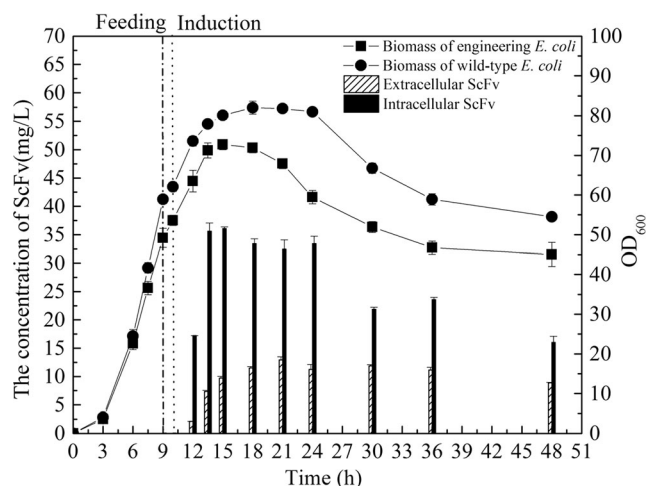
was initiated, scFv expression was induced by 1 mM IPTG when the  $\text{OD}_{600}$  was greater than 40. Although *E. coli* W3110/pBZ001 grew slightly slower than the wild-type W3110 strain, they roughly entered stationary phase after 15 h of induction. Obvious cell lysis of *E. coli* W3110/pBZ001 started at 18 h, whereas lysis of the wild-type strain began at 12 h. At 24 h, the degree of cell lysis of *E. coli* W3110/pBZ001 reached 18.3 %, which was significantly higher than that of the wild-type strain (Fig. 1).

The expression of scFv in the pBZ001 vector was regulated by the T7A3 promoter. This expression system was characterized by weak basal activity, which was the reason why scFv was barely detected in cell supernatants and the fermentation broth prior to induction. Within 3.5 h after induction, scFv production was rapidly induced at the rate of  $0.906 \text{ mg g}^{-1} \text{ DCW h}^{-1}$ . Most of the products are intracellular accumulated and less than one-fifth of the total amount of scFv was secreted into the extracellular space; no scFv expression was detected in the wild-type strain (Fig. 1).

As shown in Fig. 1, introducing the plasmid containing the exogenous scFv gene slightly inhibited cell growth. Moreover, it was obvious that the high-level expression of scFv after induction led to early cell lysis. These problems in an *E. coli* host can be a major bottleneck that hampers heterologous protein production in bioreactors (Schlegel et al. 2013). However, the cell lysis phenomenon is not obvious in a shake flask. The stability culture stage can be maintained for a long time (>72 h) and the cell density decreases little in the decline phase (data not shown).

### Cell lysis mainly occurs in VBNC cells in a bioreactor

The VBNC state of bacteria was proposed by Xu et al. (1982), and it represents cells that have lost the ability to form colonies

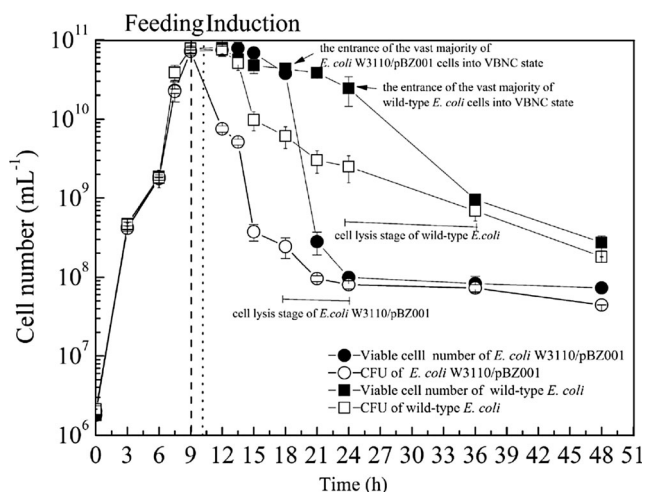


**Fig. 1** Comparison of cell lysis and single-chain variable-domain antibody fragment (scFv) expression between *Escherichia coli* W3110/pBZ001 and the wild-type strain

on a medium when they suffer from stresses; however, their cell structure (e.g., cell membrane integrity), metabolic activity (such as respiration, biochemical substance absorption, etc.), DNA synthesis, and the expression of specific mRNA genes that are characteristic of living cells remain intact. Once a suitable environment is restored, VBNC cells can be transformed into the cultivable state.

As shown in Fig. 2, after scFv overexpression in W3110/pBZ001, the number of CFU immediately decreased exponentially, while a slight reduction in living cells occurred from 10 to 18 h, indicating the entrance of the vast majority of cells into the VBNC state. A significant reduction in living cells and a slight CFU decrease, which accompanied the cell lysis, was detected after 18 h, indicating that cell lysis mainly occurs in cells entering the VBNC state. A similar conclusion was drawn for the wild-type strain. Most VBNC cells accumulated in the wild-type strain after 15 h and substantially lysed at 24 h. This is consistent with the assumption proposed by Noor et al. (2009) that VBNC cells are formed during the stationary phase and, in turn, are lysed by a  $\sigma^E$ -dependent process.

Lin (2000) expressed yeast  $\alpha$ -glucosidase in *E. coli* as a model system to obtain a more comprehensive understanding of the cell physiological response to the overexpression of heterologous genes during glucose-limited, fed-batch cultures. They found that the synthesis of recombinant proteins inhibited the synthesis of cellular housekeeping proteins, as there was a competition for RNA polymerase, ribosomes, and translation factors. As a result, there was a decrease in different cellular metabolic reactions, such as replication, transcription, translation, glucose uptake, and respiration, and this competitive effect led to the inhibition of cell growth. Thus, the host cells became VBNC cells, which maintain metabolic



**Fig. 2** The relationship between the occurrence of cell lysis and the reduction of the viable but nonculturable (VBNC) number in *E. coli* W3110/pBZ001 and the wild-type strain. The VBNC number is equal to the difference between the viable cell number and the colony-forming unit (CFU) number

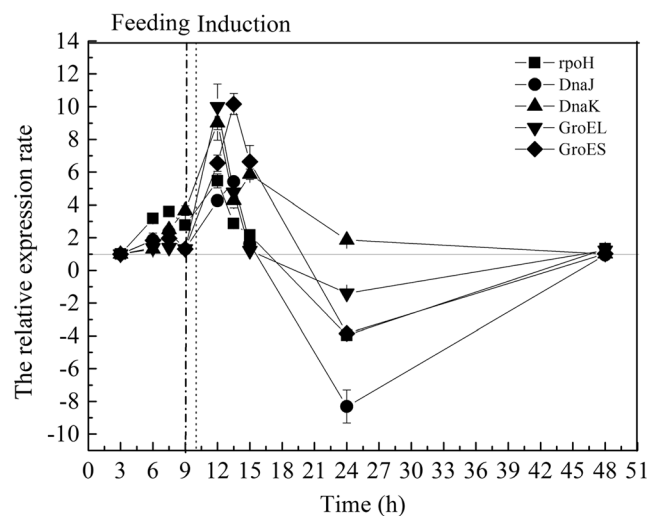
activity, but irrevocably lose their ability to grow (Lin 2000). Therefore, we assume that the lysis of *E. coli* W3110/pBZ001 cells observed in our study occurred mainly in VBNC cells.

## The stress response and $\sigma^E$ -dependent cell lysis in W3110/pBZ001

### The heat shock response

Figure 3 compares the expression of heat shock response genes between W3110/pBZ001 and wild-type strain in an identical culture system. From 3 to 15 h after induction, the expression of *rpoH* was notably higher in W3110/pBZ001 than in the wild-type strain, reaching a maximum 12 h after induction and undergoing a dramatic decline after 15 h when cell lysis began. The expression of *dnaK*, *dnaJ*, *groES*, and *groEL*, which are controlled by *rpoH*, showed the same tendency. The expression of all genes in the two strains tended to converge to the same level at the end of the fermentation (48 h), indicating that their metabolic activity stagnated after cell lysis.

The heat shock response in *E. coli* is self-regulated under adverse conditions to enable survival in various microenvironments (Arsène et al. 2000). The *rpoH* gene, encoding the  $\sigma^{32}$  regulator, is responsible for degrading misfolded proteins, preventing protein misfolding, or refolding misfolded proteins by regulating the DnaK/J–GrpE and GroEL–GroES chaperone pairs (Hoffmann and Rinas 2004; Kumar and Sourjik 2012). By binding to hydrophobic regions of unfolded polypeptides, the role of heat shock proteins, such as DnaK/DnaJ in prokaryotes, is to maintain protein solubility and prevent aggregation, while GrpE functions as a trigger to separate DnaK/DnaJ/polypeptide complexes via nucleotide exchange



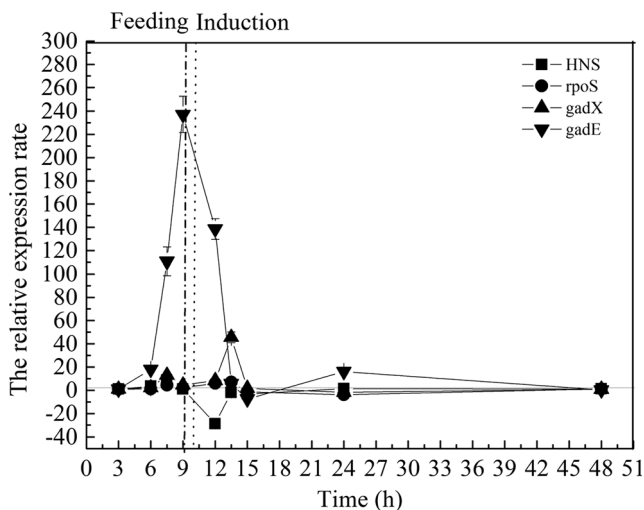
**Fig. 3** The relative expression of heat stress response genes in *E. coli*. The relative expression is the ratio of gene expression in *E. coli* W3110/pBZ001 and wild-type *E. coli* at the same time point

(Hartl et al. 2011; Noor 2015). After IPTG induction in a fermentation culture, the expression of *rpoH*, *dnaK*, *dnaJ*, *grpE*, *groEL*, and *groES* in *E. coli* W3110/pBZ001 significantly increased compared with that in the wild-type strain at each time point, indicating that the heat shock response of W3110/pBZ001 cells was intensely triggered by scFv overexpression.

#### The acid stress response

Figure 4 compares the dynamics of the expression of *H-NS*, *rpoS*, *gadE*, and *gadX* in the acid stress response pathway. The mRNA level of *H-NS*, the senior negative regulator in the acid resistance (AR) regulating system (Krin et al. 2010), substantially decreased 2 h after the induction of scFv expression in *E. coli* W3110/pBZ001, while that of *rpoS* and *gadX*, the general regulators of the stress response (Talukder et al. 1996) and the retrograde transport gene in the AR system, increased by approximately 6- and 8-fold, respectively, compared with the wild-type strain. It is noteworthy that the transcriptional level of *gadE*, encoding GadE, the central activation element of the glutamate-dependent AR system, increased by more than 200-fold dramatically before the induction and remained at a high level 2 h later and then declined 5 h eventually.

*H-NS* is believed to promote the degradation of *rpoS* mRNA and suppress the expressions of certain regulators, such as *gadX*, *adiY*, and *cadC* in amino acid-dependent AR systems (Krin et al. 2010; Talukder et al. 1996). During the phase of rapid scFv synthesis (10–13.5 h of induction), the expression of *H-NS* decreased and removed the inhibition of



**Fig. 4** The relative expression of acid stress response genes in *E. coli*. The relative expression is the ratio of gene expression in *E. coli* W3110/pBZ001 and wild-type *E. coli* at the same time point

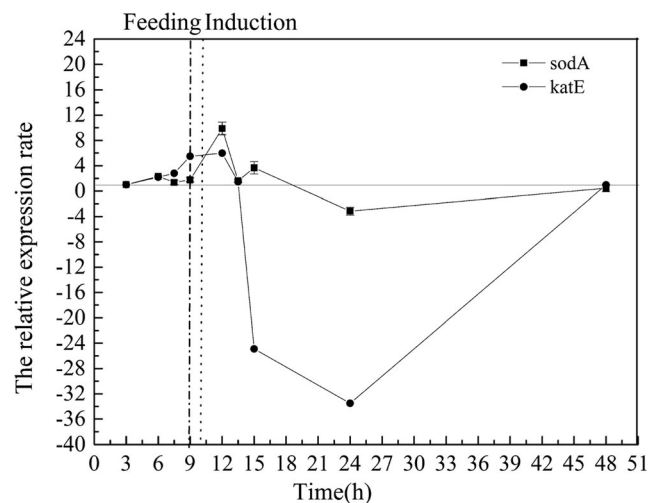
*rpoS* expression, directly or indirectly, and promoted the expression of the *gadX* and *gadE* genes in the glutamate-dependent AR system to cope with changes in the intracellular pH caused by scFv overexpression.

#### The oxygen stress response

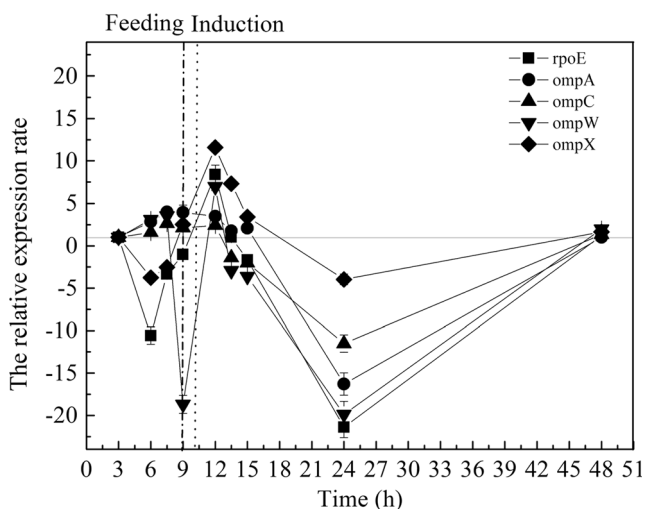
Figure 5 compares the dynamics of two major oxygen stress response genes between the W3110/pBZ001 and wild-type strains. *sodA* and *katE*, encoding superoxide dismutase and catalase HP11, respectively, can cope with protein carbonylation damage caused by sudden oxygen stresses (Dukan and Nyström 1999). Two hours after the induction of scFv expression in the W3110/pBZ001 strain, the mRNA levels of *sodA* and *katE* increased by approximately 10- and 6-fold, respectively. However, the expression of *sodA* in the W3110/pBZ001 strain declined 3 h later to an extremely low level compared with that of the wild-type strain, and reached its minimum level at 24 h, while the expression of *katE* in the W3110/pBZ001 strain also decreased by approximately 3-fold at 24 h (Fig. 5).

#### The $\sigma^E$ -dependent cell lysis and repair pathway

Figure 6 shows the expression patterns of the *rpoE*, *ompA*, *ompC*, *ompW*, and *ompX* genes during  $\sigma^E$ -dependent cell lysis, which was discovered by Murata et al. (2012), in the W3110/pBZ001 and wild-type strains. Prior to IPTG induction, the mRNA level of *rpoE*, which encodes the  $\sigma^E$  transcription factor, was lower in the W3110/pBZ001 strain than in the wild-type strain, and it increased by approximately 8-



**Fig. 5** The relative expression of oxygen stress response genes in *E. coli*. The relative expression is the ratio of gene expression in *E. coli* W3110/pBZ001 and wild-type *E. coli* at the same time point



**Fig. 6** The relative expression of cell lysis genes governed by  $\sigma^E$  in *E. coli*. The relative expression is the ratio of gene expression in *E. coli* W3110/pBZ001 and wild-type *E. coli* at the same time point

fold 2 h after induction. The enhanced mRNA *rpoE* levels eventually decreased to a significantly low level 14 h after induction. The mRNAs of *ompA* and *ompC*, which encode the two main outer membrane proteins in *E. coli*, remained at high levels in the W3110/pBZ001 strain up to 12 h of induction, and then decreased to extremely low levels. Prior to induction, the mRNA levels of *ompW* and *ompX* in the W3110/pBZ001 strain were approximately 18-fold lower after 9 h of incubation and 2.5-fold lower after 7.5 h of incubation, respectively. However, the expression of *ompW* and *ompX* significantly increased after induction, but decreased after 5 h, compared with that in the wild-type strain.

The  $\sigma^E$  transcription factor was originally defined in a study of the heat shock factor  $\sigma^H$  and  $\sigma^E$  regulates genes, which are required to repair abnormally folded periplasmic and membrane proteins that occur in response to thermal stress or other external stimuli (Ades 2008). Murata et al. (2012) presented a complete  $\sigma^E$ -dependent cell lysis pathway. Because of the intolerable accumulations of unfolded proteins in the periplasmic space or the cell membrane induced by sudden stresses, the  $\sigma^E$  protein in the cytoplasm is activated to upregulate the transcription of the small RNA *MicA* and *rybB*, which reduce the expression of genes encoding outer membrane proteins such as *ompA*, *ompC*, and *ompW*, resulting in defects in the outer membrane and ultimately leading to cell lysis. This  $\sigma^E$ -dependent cell lysis proceeds in a cascade following *rpoE* overexpression during the logarithmic and stationary phases in shake flask cultures. However, the sustained high level of expression of *rpoE* does not occur during dramatic cell lysis in bioreactor cultures, indicating that there are alternative ways of governing cell lysis in *E. coli*. *OmpA* is an important outer membrane structural protein that maintains

cell morphology and transfers hydrophilic compounds across the cell membrane (Danoff and Fleming 2011), and *OmpC* is the main cation-selective porin that enables the entry of small molecules into the bacteria (Baslé et al. 2006; Joseph Sahaya Rajan et al. 2015). In Gram-negative bacteria, *OmpW* encodes a small  $\beta$ -sheet membrane protein that allows the bacteria to adapt to environmental changes (Brambilla et al. 2014), while the mechanism that controls *OmpX* expression is not clear. The levels of the mRNAs that encode these four proteins increased with the enhanced expression of *rpoE* during the early stage of induction (about 10 to 15 h) in the fermentation culture of W3110/pBZ001. When cells are exposed to extracytoplasmic stresses caused by the rapid accumulation of exogenous proteins,  $\sigma^E$  induces its own expression in response to *RseB* or *RseA* degradation by the *DegS* and *YaeL* proteinases, which leads to the repair of the outer membrane (Missiakas et al. 1997). Five hours after induction, the expression of genes encoding outer membrane proteins in *E. coli* W3110/pBZ001 was significantly lower than that in the wild-type strain. This phenomenon may be caused by the decreased synthetic capacity of the outer membrane proteins and the negative regulatory effect of cell lysis on the expression of these genes (Nitta et al. 2000), which, in turn, led to earlier cell lysis.

Numerous advances have been achieved in the engineering of  $\sigma$  transcriptional regulators, which have provided us with an effective means by which to tune the regulatory networks of bacteria, improve bacterial stress resistance, and increase the yields of desired products (Tripathi et al. 2014). Recently, Dragosits et al. (2012) constructed a novel, self-regulatory protein production system, which controls recombinant protein production through a stress-induced, negative feedback mechanism. To screen for molecular targets that increase cell resistance to stresses and to control cell lysis, further exploration of the mechanisms underlying the  $\sigma$ -dependent stress response and cell lysis is needed. Such knowledge could help to maximize the yields of heterologous proteins by balancing exogenous protein expression and cell growth.

## Conclusion

The present study investigated how the induction of single-chain variable-domain antibody fragment (scFv) expression in *Escherichia coli* W3110/pBZ001 influenced the viability of host cells and their cell lysis pattern in a 5-L bioreactor. Furthermore, the transcriptional levels of genes related to the general stress response and cell lysis were compared between the W3110/pBZ001 and wild-type strains throughout identical fermentation processes. The scFv protein was rapidly synthesized from 10 to 13.5 h of induction, and most of the protein

accumulated in the intracellular space until 24 h (Fig. 1). The viable but nonculturable (VBNC) cell number in W3110/pBZ001 began to increase rapidly 10 h after induction, and reached its maximum of approximately  $7.34 \times 10^{10}$  colony-forming units (CFU)  $\text{mL}^{-1}$  at 13.5 h after induction, while the rapid increase in the VBNC cell number in the wild-type strain was postponed for 6 h (Fig. 2). Because of scFv overexpression, multiple stress response pathways in *E. coli* W3110/pBZ001 were activated to accommodate the microenvironmental changes, as evidenced by the results shown in Figs. 3, 4, 5, and 6. VBNC cells lysed after 18 h of scFv induction in the W3110/pBZ001 strain, which was followed by a loss of cell viability, as evidenced by the substantial decrease in the expression of stress response genes (from 15 to 24 h of induction).  $\sigma^E$  participated in cell membrane repair, but no overexpression was detected during cell lysis (from 18 to 24 h of induction in the W3110/pBZ001 strain).

According to the findings of this study, we assume that recombinant *E. coli* cell lysis occurs mainly in VBNC cells. Comprehensive investigations of VBNC cell formation and changes in bioreactor conditions are essential for the industrial-scale production of scFv (Chou 2007). Further study is required in order to assess the effects of external stresses, such as process design parameters, on recombinant cells, as well as to understand the variations in the cellular profiles of VBNC cells (Lin 2000), which may contribute to the definition of the physiological states of the cells, i.e., survival or cell lysis.

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## References

- Abe A, Ohashi E, Ren HF, Hayashi T, Endo H (2007) Isolation and characterization of a cold-induced nonculturable suppression mutant of *Vibrio vulnificus*. *Microbiol Res* 162:130–138
- Ades SE (2008) Regulation by destruction: design of the sigma(E) envelope stress response. *Curr Opin Microbiol* 11:535–540
- Andersson L, Strandberg L, Enfors SO (1996) Cell segregation and lysis have profound effects on the growth of *Escherichia coli* in high cell density fed batch cultures. *Biotechnol Prog* 12:190–195
- Arsène F, Tomoyasu T, Bukau B (2000) The heat shock response of *Escherichia coli*. *Int J Food Microbiol* 55:3–9
- Baslé A, Rummel G, Storici P, Rosenbusch JP, Schirmer T (2006) Crystal structure of osmoporin OmpC from *E. coli* at 2.0 Å. *J Mol Biol* 362:933–942
- Brambilla L, Morán-Barrio J, Viale AM (2014) Expression of the *Escherichia coli* ompW colicin S4 receptor gene is regulated by temperature and modulated by the H-NS and StpA nucleoid-associated proteins. *FEMS Microbiol Lett* 352:238–244
- Chou CP (2007) Engineering cell physiology to enhance recombinant protein production in *Escherichia coli*. *Appl Microbiol Biotechnol* 76:521–532
- Danoff EJ, Fleming KG (2011) The soluble, periplasmic domain of OmpA folds as an independent unit and displays chaperone activity by reducing the self-association propensity of the unfolded OmpA transmembrane  $\beta$ -barrel. *Biophys Chem* 159:194–204
- Dragosits M, Nicklas D, Tagkopoulos I (2012) A synthetic biology approach to self-regulatory recombinant protein production in *Escherichia coli*. *J Biol Eng* 6(1):1754–1760
- Dukan S, Nyström T (1999) Oxidative stress defense and deterioration of growth-arrested *Escherichia coli* cells. *J Biol Chem* 274:26027–26032
- Hartl FU, Bracher A, Hayer-Hartl M (2011) Molecular chaperones in protein folding and proteostasis. *Nature* 475:324–332
- Hobbie JE, Daley RJ, Jasper S (1977) Use of nucleopore filters for counting bacteria by fluorescence microscopy. *Appl Environ Microbiol* 33:1225–1228
- Hoffmann F, Rinas U (2004) Roles of heat-shock chaperones in the production of recombinant proteins in *Escherichia coli*. *Adv Biochem Eng Biotechnol* 89:143–161
- Joseph Sahaya Rajan J, Chinnappan Santiago T, Singaravel R, Ignacimuthu S (2015) Outer membrane protein C (OmpC) of *Escherichia coli* induces neurodegeneration in mice by acting as an amyloid. *Biotechnol Lett*. doi:10.1007/s10529-015-2025-8
- Krin E, Danchin A, Soutourina O (2010) Decrypting the H-NS-dependent regulatory cascade of acid stress resistance in *Escherichia coli*. *BMC Microbiol* 10:273–282
- Kumar M, Sourjik V (2012) Physical map and dynamics of the chaperone network in *Escherichia coli*. *Mol Microbiol* 84:736–747
- Lessard JC (2013) Growth media for *E. coli*. *Methods Enzymol* 533:181–189
- Lin H (2000) Cellular responses to the induction of recombinant genes in *Escherichia coli* fed-batch cultures. PhD dissertation, Universitäts und Landesbibliothek
- Missiakas D, Mayer MP, Lemaire M, Georgopoulos C, Raina S (1997) Modulation of the *Escherichia coli* sigma(E) (RpoE) heat-shock transcription-factor activity by the RseA, RseB and RseC proteins. *Mol Microbiol* 24:355–371
- Murata M, Noor R, Nagamitsu H, Tanaka S, Yamada M (2012) Novel pathway directed by sigma(E) to cause cell lysis in *Escherichia coli*. *Genes Cells* 17:234–247
- Nitta T, Nagamitsu H, Murata M, Izu H, Yamada M (2000) Function of the sigma(E) regulon in dead-cell lysis in stationary-phase *Escherichia coli*. *J Bacteriol* 182:5231–5237
- Noor R (2015) Mechanism to control the cell lysis and the cell survival strategy in stationary phase under heat stress. *Springerplus* 4:599
- Noor R, Murata M, Yamada M (2009) Oxidative stress as a trigger for growth phase-specific sigma(E)-dependent cell lysis in *Escherichia coli*. *J Mol Microbiol Biotechnol* 17:177–187
- Sandén AM, Prytz I, Tubulekas I, Förberg C, Le H, Hektor A, Neubauer P, Pragai Z, Harwood C, Ward A, Picon A, De Mattos JT, Postma P, Farewell A, Nyström T, Reeh S, Pedersen S, Larsson G (2003) Limiting factors in *Escherichia coli* fed-batch production of recombinant proteins. *Biotechnol Bioeng* 81:158–166
- Schlegel S, Rujas E, Ytterberg AJ, Zubarev RA, Luirink J, de Gier JW (2013) Optimizing heterologous protein production in the periplasm of *E. coli* by regulating gene expression levels. *Microb Cell Fact* 12:24–36



- Sikdar R, Simmons AR, Doerrler WT (2013) Multiple envelope stress response pathways are activated in an *Escherichia coli* strain with mutations in two members of the DedA membrane protein family. *J Bacteriol* 195:12–24
- Talukder AA, Yanai S, Nitta T, Kato A, Yamada M (1996) RpoS-dependent regulation of genes expressed at late stationary phase in *Escherichia coli*. *FEBS Lett* 386:177–180
- Tripathi L, Zhang Y, Lin Z (2014) Bacterial sigma factors as targets for engineered or synthetic transcriptional control. *Front Bioeng Biotechnol* 2:33
- Xu H-S, Roberts N, Singleton FL, Attwell RW, Grimes DJ, Colwell RR (1982) Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. *Microb Ecol* 8:313–323
- Zarschler K, Witecy S, Kapplusch F, Foerster C, Stephan H (2013) High-yield production of functional soluble single-domain antibodies in the cytoplasm of *Escherichia coli*. *Microb Cell Fact* 12:97–104