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ORIGINAL PAPER

# Effects of temperature shifts and oscillations on recombinant protein production expressed in *Escherichia coli*

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**Abstract** *Escherichia coli* is widely used host for the intracellular expression of many proteins. However, in some cases also secretion of protein from periplasm was observed. Improvement of both intracellular and extracellular production of recombinant protein in *E. coli* is an attractive goal in order to reduce production cost and increase process efficiency and economics. Since heat shock proteins in *E. coli* were reported to be helpful for protein refolding and hindering aggregation, in this work different types of single and periodic heat shocks were tested on lab scale to enhance intracellular and extracellular protein production. A single heat shock prior to induction and different oscillatory temperature variations during the induction phase were executed. The results showed that these variations influence protein production negatively. In other words, 45 and 50 % reduction in extracellular protein production were observed for the single heat shock and oscillated temperature between 35 and 40 °C, respectively. However, the oscillatory temperature approach introduced in this study is recommended as a tool to quantitatively analyze the effects of inhomogeneous temperature on cell physiology and productivity in large-scale bioreactors.

**Keywords** Heat shock · Temperature oscillation · Intracellular protein · Protein release

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## Introduction

The bacterium *Escherichia coli* is one of the most extensively used microorganisms for genetic modification and production of recombinant products. In comparison to other hosts it provides many advantages like fast growth on inexpensive media compared to media used for mammalian cells, rapid biomass production and simple process scale up [1]. *E. coli* usually does not excrete proteins into the extracellular medium mainly due to the presence of the outer membrane that is not permeable to proteins. Thus, the first step in recovery of the intracellular protein is cell disruption [2]. Advances in upstream processing have been successful in achieving high product titers, and downstream costs now typically dominate the overall manufacturing costs. Improvement of upstream productivity, however, may improve the overall process economics in case product can be released to the medium and therefore downstream unit operations can be skipped [3]. Several attempts were made for targeting recombinant protein release into the medium. One of the widely used approaches is the transport of proteins to the periplasm [4]. This approach necessitates synthesis of soluble protein inside of the cytoplasm and translocation of the soluble form into the periplasm. The translocated products in the periplasm then can be released selectively by crossing outer cell wall while keeping the cytoplasm intact [5].

Improper folding of overexpressed proteins leads to formation of aggregates and inclusion bodies which greatly reduces the protein yields in *E. coli*. A strategy to enhance expression of protein in soluble form is to increase the concentration of intracellular heat shock proteins [1, 6–9]. These proteins form a mechanism which avoids misfolding and aggregation of proteins and are synthesized in response to heat stress [1, 6, 10]. It was reported that the solubility of intracellular target protein, KsgA-DeltaN, was increased when an *E. coli* culture, cultivated in shake flask, was

exposed to a step change in temperature (37–42 °C) before induction. However, the effectiveness of the method was found to be protein dependent [11]. The shift in temperature (from 30 to 42 °C) at the beginning of induction phase of pPreS2-*S′*-β-galactosidase production resulted in 2- to 3-fold higher recovery of the active enzyme. The experiments were carried out in shake flasks [12]. Periodic temperature variations, a simple sinusoidal wave model, were employed to trigger the volumetric production of plasmid-based measles vaccine (pcDNA3F) [13]. Reduced growth temperature (30 °C instead of 37 °C) in cultivation of *E. coli* as well as *Pichia pastoris* (20 °C instead of 30 °C) enhanced specific production of antibody Fab fragments. However, this was at the cost of biomass production [13–15].

Although a couple of studies have been done so far in which temperature oscillation and shifts were reported as a means of improving intracellular solubility of target protein, they were not even executed and tested in lab-scale bioreactors in a quantitative manner. This demonstrates that this approach is far from being implemented in real commercial production plants.

Hence, the aim of this work was to quantitatively analyze the effect of heat shock and periodic temperature variations on intracellular soluble target protein production in lab-scale bioreactors. The target protein in this work was recombinant alkaline phosphatase which is synthesized by a rhamnose-inducible expression system using *E. coli* as the host. Since more intracellular soluble proteins could improve protein release, the second goal was to test if the above-mentioned methodology enhances protein release. In addition, this study aimed at proposing a strategy to quantify the effects of temperature variations, which are probable to occur in large-scale bioreactors due to the inhomogeneities, on product location.

## Materials and methods

### Biological model system

A proprietary *E. coli* strain, with a plasmid coding recombinant alkaline phosphatase was used as a model system. It carried a plasmid with a rhamnose-inducible expression system coding for model reporter protein alkaline phosphatase, which is not able to metabolize rhamnose. The media composition for batch and preculture as well as feed medium composition were described somewhere else [16].

### Experimental setup and approach

Experiments were done using a 3 l highly instrumented autoclavable laboratory bioreactor (Infors, Switzerland) connected to the Process Information Management System (Lucullus, Biospectra AG, Switzerland) for online process monitoring and control. The bioreactor was equipped with temperature, dissolved oxygen and pH control system which facilitated controlling of process parameters accurately. The cultivation parameters were set to 37 °C and 1,500 rpm of stirrer speed with 2 vvm air flow. pH was adjusted by means of PID controller which added $NH_4OH$ to keep the pH at 7. In the case of lower dissolved oxygen concentration than 30 % of the saturated value, pure oxygen was added into the inlet air controlled by mass flow controller. The off gas passed through the gas analyzer (Servomex, M. Müller AG, Switzerland) to quantify $CO_2$ and $O_2$ content, using infrared and paramagnetic principles, respectively.

The 16.5 ml of the inoculum was transferred to the bioreactor to inoculate 1 l batch media. After the batch phase, which was finished when the downward trend in $CO_2$ and upward trend in $O_2$ were observed, a peristaltic pump was started to exponentially feed glycerol into the culture so that specific growth rate was kept constant. After the fed batch phase of 9 h, strain was induced by rhamnose solution and the glycerol feed was switched to the designed feeding profile.

Three pairs of experiments were designed to achieve the goals of this research. Pair 1 (P1) involves two runs (P1–R1 and P1–R2) with the difference only in temperature profile in fed batch phase (Fig. 1). In the stressed run (P1–R2) a temperature shift from 35 to 40 °C was carried out for 3 h as a heat shock prior to induction (Fig. 1a), based on similar approach, by which more soluble target protein (KsgA-DeltaN) was observed, was applied by Chen et al. [11]. Both P1–R1 and P1–R2 runs had the same post-induction feed profile in which the flow rates were adjusted initially according to the same substrate uptake rate (Fig. 1b). In Pair 2 (P2), experiments were designed to be different in the post-induction temperature profile (Fig. 2). In run P2–R2, the temperature oscillated triangularly between two fixed temperatures as shown in Fig. 2a. The oscillation period of 15 min were chosen because of the relevant mixing times in large-scale bioreactors [17] on one hand and considering the heating and cooling capacity of the fermenter, on the other hand. Both P2–R1 and P2–R2 runs had the same post-induction feed profiles (Fig. 2b). Pair 3 (P3) was similar to Pair 2 with the difference in the range the temperature was oscillated as well as in the period of oscillation. The lower and upper bound of the temperature was designed as 35 and 42 °C, respectively, and oscillation period of 30 min was considered (Fig. 3a). In order to hinder proteolysis and to improve intracellular soluble target protein content, the feed rate was triangularly oscillated between two fixed flow rates in both runs (Fig. 3b). This oscillated feed strategy was introduced

before [18] and said to positively affect intracellular soluble protein synthesis by reducing synthesis of proteolytic enzymes.

Oscillatory feeding profile was carried out by means of a feed-forward flow controller. That is, the pump set point corresponding to the designed feed flow rate was sent to the peristaltic pump (LAMBDA Laboratory Instruments, Switzerland). The pump was calibrated accurately before the experiment.

## Off-line analytics

Cell mass concentration was quantified gravimetrically. Two milliliters of culture broth were added to pre-weighted glass tubes, followed by immediate centrifugation at 5,000 rpm for 10 min at 4 °C using a Sigma 3K30 centrifuge with the rotor 11156. The supernatant was removed from the top and used for different extracellular protein measurement. The pellets were re-suspended twice in 2 ml of distilled water using a vortex to wash them and they were centrifuged again under the same conditions mentioned before. The last supernatants were removed carefully and the tubes were dried for 72 h at 105 °C. Tubes were kept in a desiccator before being weighed. During the whole process, the samples were stored on ice to avoid and stop further reactions.

Total extracellular protein concentration was determined at 562 nm by Bicinchoninic Acid Kit for Protein Determination (Sigma, BCA1-1KT). Each fermentation was done once. All analytical procedures were performed in duplicate and the averages are shown in the figures.

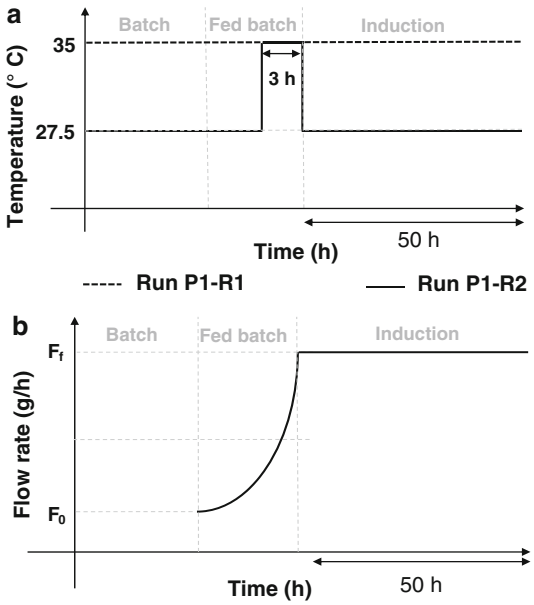


**Fig. 1** **a** Temperature setpoint of Pair 1; run P1–R1: the run with the constant temperature throughout the experiment (non-stressed run). run P1–R2: the run with heat shock in fed batch phase (stressed run). **b** Feed profile in Pair 1. In both stressed and non-stressed runs the same feed profiles were conducted

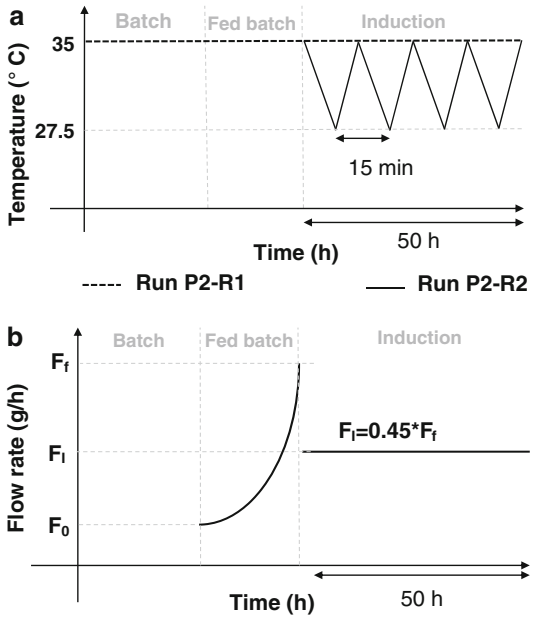


**Fig. 2** **a** Temperature setpoint in Pair 2; P2–R1: the run with constant temperature throughout the experiment (non-stressed run). P2–R2: the run with oscillatory temperature profile in induction phase (stressed run). **b** Feed profile of Pair 2; in both stressed and non-stressed runs the same feeding profiles were conducted

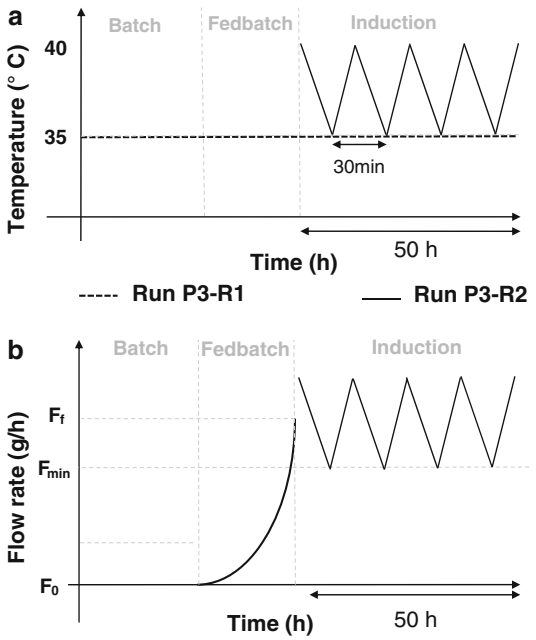


**Fig. 3** **a** Temperature setpoint of Pair 3; P3–R1: the run with the constant temperature throughout the experiment (non-stressed run). P3–R2: the run with the oscillatory temperature profile (stressed run). **b** Feed profile of Pair 3. In both stressed and non-stressed runs the same feed profiles were conducted

### Basis for comparison

All online and off-line measurements were pretreated according to the strategy proposed by Herwig et al. [16] to assure that the data used for further comparison and studies provide an accurate representation of what is happening in the process. Using pretreated data, elemental balances (carbon balance and degree of reduction balance) closed within the uncertainty of 10 %. The pretreatment procedure involves reconciliation of the measurements within their expected error bound so that they fulfill the carbon and degree of reduction balance. On this basis, the presented results can be concluded as being significant.

The runs were compared on the basis of cumulative specific production of extracellular protein (g total extracellular soluble protein/g biomass) and intracellular protein content (total intracellular soluble protein/g biomass). The former represents how much protein was released per biomass and the latter shows how much intracellular protein was produced per biomass. The assumption behind these objective functions is that the measured total extracellular soluble protein concentration is mainly the concentration of target protein. This assumption was proven by performing SDS gels for all samples. The gels showed that more than 90 % of the total extracellular soluble protein consists of target protein (patent application pending). Therefore, total extracellular protein was taken as total target protein and then was corrected by biomass in order to make different runs comparable. Similarly, SDS gels showed that intracellular soluble target protein has the same fraction in all samples taken after induction of the culture; therefore, the measured total intracellular soluble protein concentration is always proportional to total intracellular soluble target protein with the same factor. All results were plotted versus time which was counted from the induction moment. This means that the induction moment was set as time zero. By doing so, it was possible to compare the experiments only in induction phase. Moreover, all cultures had the same background and physiological state before induction. The same procedure, medium and strain sources were used to carry batch and fed batch phases out. The cultures were kept well at the same designed operating conditions. In all runs, substrate was fed exponentially with an identical exponent, that is, the cultures were grown with a same specific growth rate. This guaranties achieving the same physiological state before induction.

## Results and discussion

### Execution of temperature profile

To demonstrate that the temperature and feeding regime were executed as designed, the set points and actual executed temperatures for both P2–R2 and P3–R2 runs are shown in Fig. 4. The executed temperature profiles showed a close proximity to the set points. The presented profiles belong to a short period of time; however, identical profiles were observed throughout the induction phase.

### Pair 1: single heat shock from 35 to 40 °C

Figure 5a shows significant difference in the cumulative specific protein production (CSPP) between run P1–R1 and run P1–R2. This difference became larger until the middle of induction phase (at about 20 h) and thereafter remained almost unchanged. In the non-stressed run (the run without heat shock: P1–R1), CSPP increased sharply during first 15 h from 0 to about 2.5 g/g and then continued to increase slowly from 2.5 to about 3.5 g/g in 35 h. However, in the stressed run (the run with heat shock: P1–R2), CSPP increased at an almost constant rate from 0 g/g in the beginning to about 2.5 g/g at 50 h. The intracellular protein contents of these two runs are illustrated in Fig. 5b. Initial intracellular protein content of the stressed run was

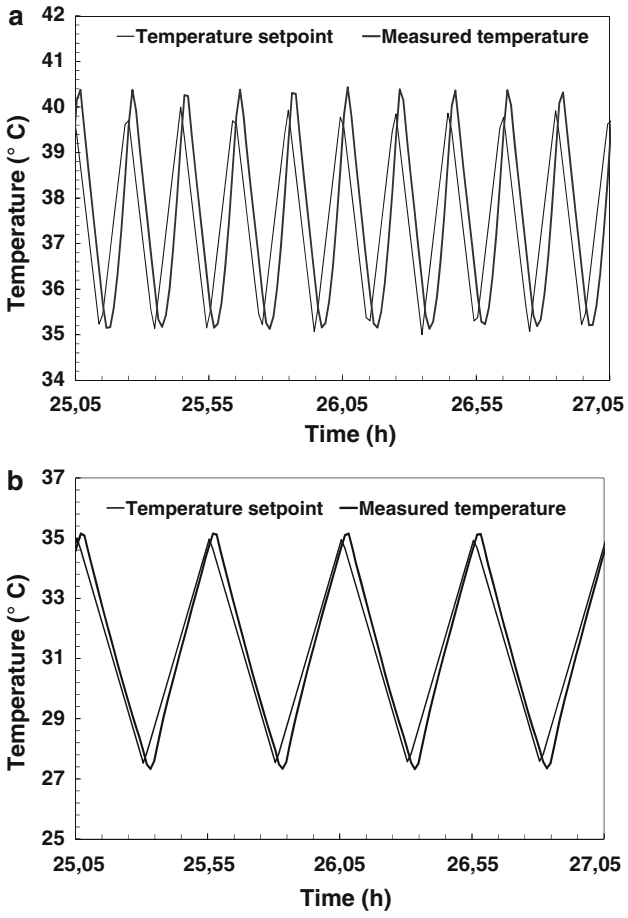


**Fig. 4** **a** Real executed oscillatory temperature profile and temperature set point of run P2–R2. **b** Real executed oscillatory temperature profile and temperature set point of run P3–R2

significantly higher than that of non-stressed run (0.51 g/g in P1–R2 in comparison with 0.39 in P1–R1). The difference between two runs became smaller over the induction phase, so that almost no difference was observed at 25 h.

One of the main consequences reported for the heat shock is the synthesis of heat shock proteins (HSPs) [9, 10]. Major HSPs are molecular chaperons, including Dnak, DnaJ and GrpE, GroEl and GroEs and proteases. Proteases are able to degrade the expressed foreign protein inside recombinant cell. However, HSPs play an important role in preventing aggregation and refolding proteins [1]. In run P1–R2 it seems that proteases which were induced by heat shock degraded the foreign protein. Protein release includes expression of soluble proteins in cytoplasm followed by translocation into the periplasm and then release into the extracellular medium [5]. Because of the need for soluble protein in translocation, there may be a connection between proteases induced by heat shock and subsequent proteolysis which reduces the protein available for translocation and release. This may be the reason for less protein release in runs P1–R2 compared to run P1–R1. In other words, the heat shock prior to induction executed in this work was not able to enhance protein release.

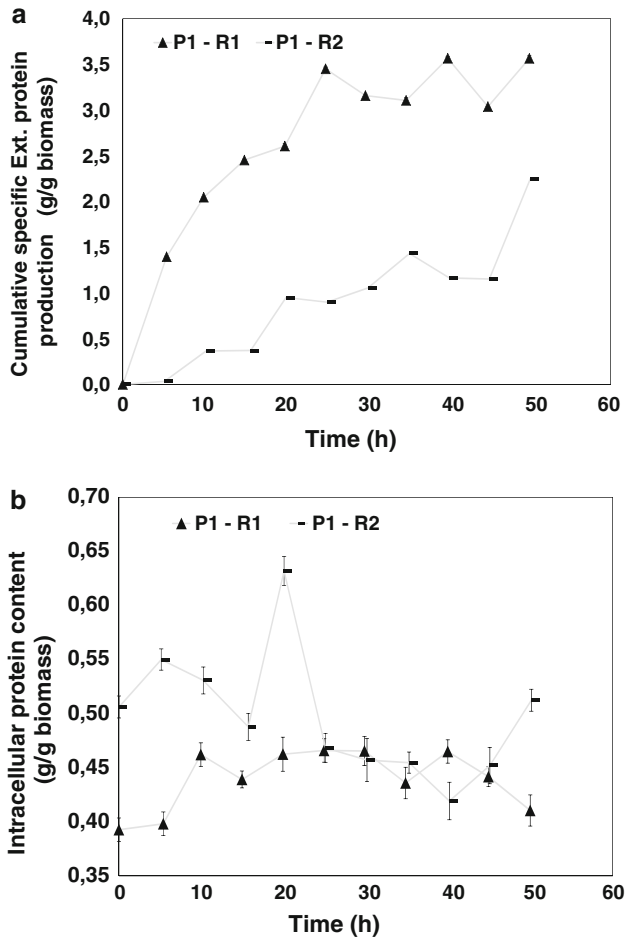


**Fig. 5** **a** Cumulative specific production of extracellular protein of Pair 1: total extracellular soluble protein produced per biomass. **b** Intracellular protein content of Pair 1: total intracellular soluble protein per biomass. P2–R1: the run with constant temperature throughout the experiment (non-stressed run). P2–R2: the run with oscillatory temperature profile in induction phase (stressed run). In both stressed and non-stressed runs the same feed profiles, that is constant flow rate, were conducted

It was reported that a temperature upshift from 30 to 40 °C consists of rapid induction of HSPs of *E. coli* followed by adaptation phase where HSPs were synthesized less to reach a steady-state level [1]. Higher total intracellular soluble protein content measured after the heat shock (Fig. 5b, beginning of induction phase: 0 h) seemed to be the result of induction of HSPs by heat shock. At 25 h onwards, total intracellular soluble protein content reached an almost constant level. This could be an evidence for the fact that the synthesis of HSPs were followed by an adaptation phase in which they were less synthesized. In order to induce continuous synthesis of HSPs and consequently benefiting from their presence in cytoplasm for higher refolding and soluble foreign protein, an experiment (P2–R2) was designed to conduct periodic heat shock (temperature oscillation).

Pair 2: triangular post-induction temperature oscillation between 27.5 and 35 °C

In contrast to Pair 1, CSPP did not differ significantly in both P2–R1 and P2–R2 experiments. The onset of deviation was at about 25 h and the difference was increased thereafter (Fig. 6a). Intracellular protein content in non-stressed run (P2–R1, Fig. 6b) was less than those of stressed run (P2–R2). Although both experiments had nearly the same initial intracellular protein content, the difference of about 0.1 g/g was observed throughout the induction phase. Therefore, due to the temperature oscillation, run P2–R2 had higher protein content.

Despite higher intracellular protein content in run P2–R2 (Fig. 6b), the CSPP was almost the same as run P2–R1 (Fig. 6a). Higher CSPP which was observed after 25 h proved to be the consequence of cell lysis that occurred in this period (patent application pending). Although periodic heat shock between 27.5 and 35 °C resulted in higher intracellular protein content (Fig. 6b; run P2–R2), this seems to be not strong enough to trigger more extracellular protein production (Fig. 6a; run P2–R2).

It was assumed that the lower and upper temperature bounds in Pair 2 were too low that insufficient HSPs were induced for triggering more soluble target protein and more protein release. Hence, the authors designed an experiment in which higher levels of lower and upper bounds of temperature variation were chosen. Moreover, previous work of the authors, which was reported elsewhere [18],

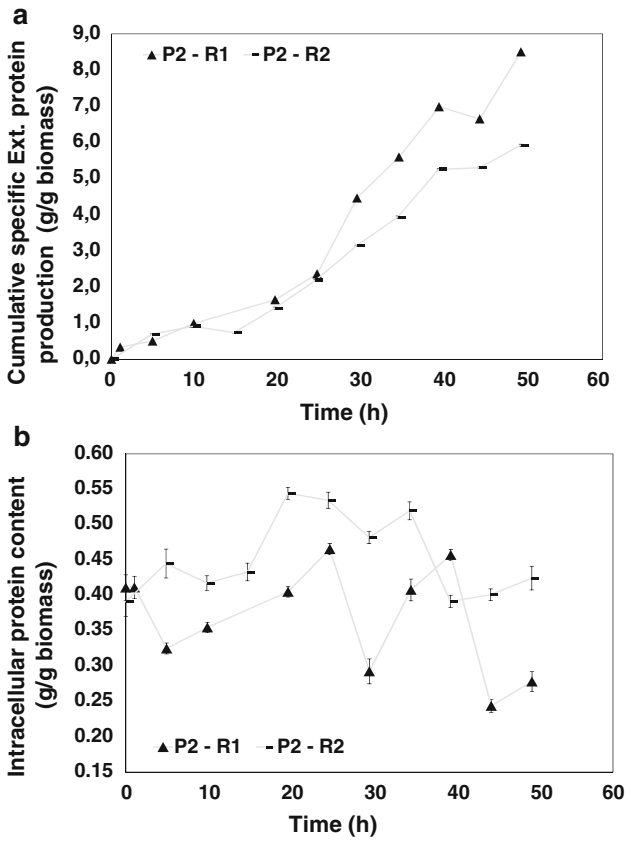


**Fig. 6** **a** Cumulative specific production of extracellular protein of Pair 2: total extracellular soluble protein produced per biomass. **b** Intracellular protein content of Pair 2: total intracellular soluble protein per biomass. P2–R1: the run with constant temperature throughout the experiment (non-stressed run). P2–R2: the run with oscillatory temperature profile in induction phase (stressed run). In both stressed and non-stressed runs the same feed profiles, that is constant flow rate, were conducted

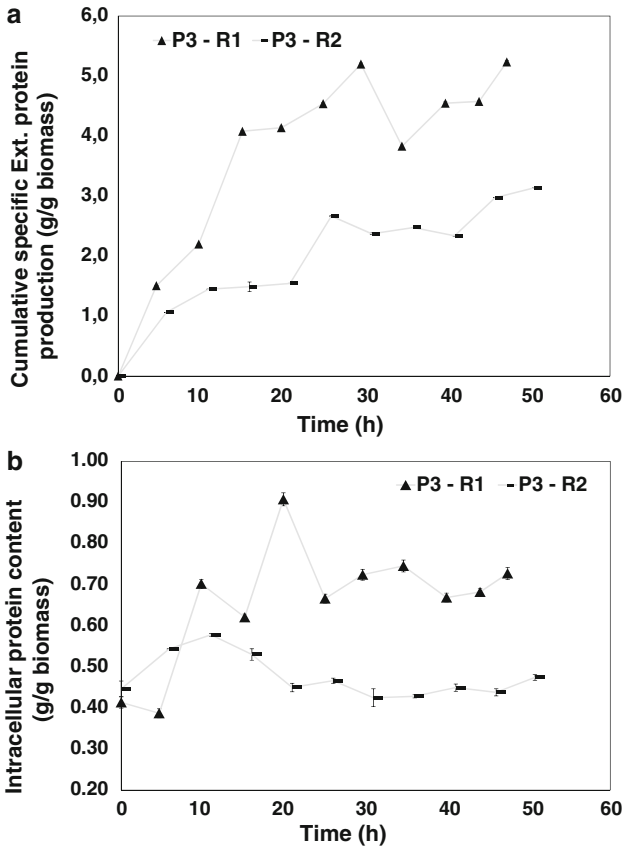


**Fig. 7** **a** Cumulative specific production of extracellular protein of Pair 1: total extracellular soluble protein produced per biomass. **b** Intracellular protein content in Pair 3: total intracellular soluble protein per biomass. P3–R1: the run with the constant temperature throughout the experiment (non-stressed run). P3–R2: the run with the oscillatory temperature profile (stressed run). In both stressed and non-stressed runs the same feed profiles, that is oscillatory feeding, were conducted

showed that oscillatory feeding rate can hinder expression of proteolytic enzymes. Hence Pair 3 was designed and executed in such a way that both oscillatory feeding and oscillatory temperature profiles were carried out simultaneously.

Pair 3: post-induction temperature oscillation between 35 and 40 °C with simultaneous post-induction flow rate oscillation

Similar to the other two previous pairs of experiment, the difference in CSPP between both P3–R1 and P3–R2 runs was increased in the course of induction phase (Fig. 7a). Three times more CSPP was observed at about 20 h in non-stressed run [1.3 g/g in stressed run (P3–R2) and 4.1 g/g in non-stressed run (P3–R1)]. However, the twofold higher CSPP in non-stressed run was observed after 25 h. Intracellular protein content illustrated in Fig. 7b indicates that protein content of biomass was lower in stressed run (P3–

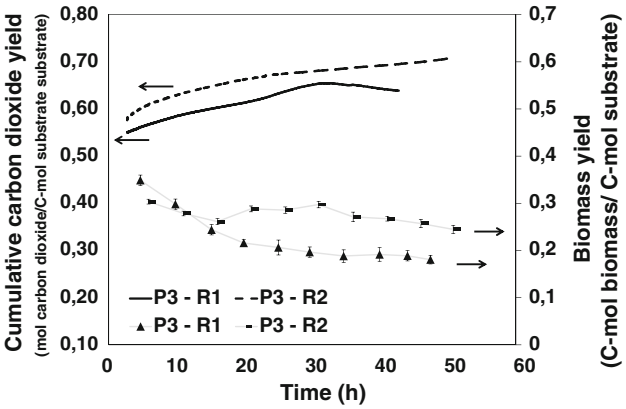


**Fig. 8** Biomass and carbon dioxide yield for run P3–R1 (the run with oscillatory post-induction feed profile but with constant post-induction temperature profile) and run P3–R2 (the run with oscillatory post-induction feeding profile and oscillatory post-induction temperature profile)

R2). In run P3–R2, after a slight increase in protein content, it diminished at about 10 h and then remained almost constant. Intracellular protein content of non-stressed run was about 0.25 g/g higher than that of stressed run at 25 h onwards (Fig. 7b). Hence, the temperature oscillation even in higher temperature ranges cannot help intracellular and extracellular target protein production. Lesser protein content in run P3–R2 can be explained by the higher biomass and carbon dioxide yield that caused to waste carbon source into biomass and carbon dioxide instead of protein (Fig. 8). Figure 8 shows that both biomass yield and carbon dioxide yield in stressed run (P3–R1) were greater than those of non-stressed run (P3–R2).

## Conclusions

The goal of this work was to benefit from HSPs to enhance intracellular target recombinant protein production and target recombinant protein release in *E. coli*. Such ideas were successfully tested by other researchers in shake flask scale for other target proteins than the one used in this work, as mentioned in the "Introduction". However, commercialization necessitates experiments in larger scales. Hence, in this work different types of single and periodic heat shocks were conducted on lab scale to improve intracellular and extracellular target protein production.

Heat shock executed in this study was a single upshift in temperature from 35 to 40 °C for 3 h before induction. This did not result in improved intracellular protein expression as well as enhanced protein release. The oscillated temperature between 27.5 and 35 °C, as a repeated heat shock, was able to increase intracellular soluble protein but not the extracellular one. However, this increase was not notable. Temperature oscillation between 35 and 40 °C (P3–R2) had a negative influence on protein release and reduced it by 40 %.

For the model system under study the periodic and single heat shocks were not beneficial in terms of protein release and intracellular protein production. However, oscillatory temperature profile can be recommended as a strategy to quantitatively analyze the behavior of biological system under inhomogeneous temperature conditions in large-scale bioreactors.

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