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Minimizing inclusion body formation during recombinant protein production in *Escherichia coli* at bench and pilot plant scale

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Abstract

Many recombinant proteins partially aggregate into inclusion bodies during production in *Escherichia coli* in batch culture on defined medium. Production on complex medium, however, effectively prevented inclusion body formation of a β-galactosidase-HIVgp41 fusion protein for detection of anti-HIV antibodies which is produced at 42 °C under control of a temperature-inducible expression system. Cells pre-conditioned by cultivation on complex medium before induction showed faster growth, higher product concentration and reduced inclusion body formation even when producing on defined medium. In contrast, for human basic fibroblast growth factor (hFGF-2) produced under control of the phage T7-promoter, medium composition could not reduce inclusion body formation even at 30 °C. Here, slow production in high-cell density fed-batch mode using a defined medium with limited glucose feeding enabled the accumulation of 50 mg product per gram cell dry mass exclusively in the soluble cell fraction, resulting in a volumetric concentration of more than 4 g per litre hFGF-2. With the β-galactosidase fusion protein produced in fed-batch, over 100 mg of product per gram cell dry mass accumulated in the soluble cell fraction. With a cell density of 100 g cell dry mass per litre, this resulted in a volumetric concentration of 10 g per litre of soluble β-galactosidase-HIVgp41 fusion protein. Thus, two approaches to balance heterologous protein production and host physiology are presented, which fit the needs of lab bench or pilot plant, respectively.

Keywords: Inclusion bodies; Recombinant protein production; *Escherichia coli*; High-cell density; Cultivation; β-galactosidase fusion protein; hFGF-2

1. Introduction

Determinants of successful recombinant protein production, such as rate or duration of production and quality or stability of the product, depend on the physiology of the producer cell. This can be manipulated by metabolic engineering of the host cell, by genetic engineering of the expression vector, and, last but not least, by process engineering.

The factors that determine production rate, protein synthesis capacity and gene doses, vary with growth rate in opposite directions. On the one hand, higher specific growth rates during production [1] or even before induction [2,3] can increase recombinant protein production, presumably via higher ribosome content [4], but on the other hand decrease the cellular plasmid content [5]. Thus, the growth rate at which the production rate reaches a maximum depends on the system under study [5–10]. Generally, a fast production terminates earlier than a slower production, sometimes giving higher final product yields with the slower systems. This was observed comparing strong versus moderate promoter systems [11], high versus low growth temperatures [12], and fast versus slow growth in batch and fed-batch systems [13]. Possible reasons include early attainment of a steady state of product concentration, or stress affecting the cells to an extent that prevents further production. Thus, taking sustainability into account, conditions that maximise the production rate do not necessarily optimise the overall process. Moreover, fast production of heterologous protein may exceed the capacity of the host cell to handle the protein properly. Many recombinant proteins tend to form inclusion bodies upon production in *E. coli*. Thus, process conditions must be found which balance heterologous protein production and host physiology to optimise the overall yield of active product.

Medium composition influences recombinant protein production, giving higher growth rates and lower plasmid content in complex medium [14]. Addition of amino acids can reduce product degradation [15] by proteases induced during amino acid limitation [16]. Likewise, addition of casamino acids or peptone can enhance the stability or the

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synthesis of the recombinant protein [17]. Disadvantages of complex compounds include reduced solubility of glucose in peptone-containing feeding solution [18], or can result in reduced plasmid stability in case of amino acid supplementation [19]. Moreover, only a defined medium may be acceptable to guaranty the reproducibility and safety required for the production of protein pharmaceuticals. Thus, means to improve recombinant protein production on the bench scale may not fit the needs of large-scale processes.

In this study, the production of two proteins, a big tetrameric β-galactosidase-fusion protein of about 480 kDa and the small monomeric human basic fibroblast growth factor (hFGF-2) of 18 kDa is optimized. The fusion protein shows the same overall folding as the homologous β-galactosidase of *E. coli* but displays an antigenic peptide from the HIV gp41 envelope protein on the surface [20]. Binding of anti-peptide-antibodies modulates the enzymatic activity of the fusion protein, giving a sensitive homogenous assay for detection of HIV infection in blood samples [20]. The all-β-sheet protein hFGF-2 structurally resembles interleukin-1β and shows very slow folding kinetics [21]. Several therapeutic applications are suggested for hFGF-2, including wound healing or therapeutic angiogenesis [22]. We examined the influence of medium (complex versus synthetic) and cultivation regime (batch versus fed-batch) on production rate, sustainability of production, and prevention of in vivo aggregation of these two diverse proteins.

2. Materials and methods

2.1. Strains and plasmids

Escherichia coli BL26 (Novagen, Madison, WI, USA), a *lacZ*-deleted derivative of BL21, was used as host for the plasmid pNF795gpC, coding for β-galactosidase carrying an antigenic peptide from HIV gp41 that is inserted at amino acid position 795 of β-galactosidase [20]. Expression of the fusion gene was under the control of the p_R promoter of bacteriophage lambda, repressed by the temperature-sensitive repressor cI857 and induced by temperature shift from 30 to 42 °C.

The IPTG inducible T7 polymerase expression system pET29c(+)hFGF-2 was constructed by cloning the *Nde*1-*Bam*H1 hFGF-2 fragment of pJHLbFGF [23] into pET29c(+), which was also digested with *Nde*1 and *Bam*H1. The strain BL21(DE3) and plasmid pET29c(+) for expression were obtained from Novagen (Madison, WI, USA).

2.2. Shake flask experiments

Shake flask experiments with BL26 pNF795gpC were done in 10 ml complex LB medium (10 g l^{-1} bactotryptone, 5 g l^{-1} yeast extract, 7 g l^{-1} NaCl) supplemented with 50 μg ml^{-1} ampicillin, or in a defined glucose mineral salt medium [24] containing 10 g l^{-1} glucose and supplemented with 50 μg ml^{-1} ampicillin. Cultures were inoculated with 1% (v/v) of overnight preculture in the respective medium, grown to OD_{600} of 0.4–0.6 and induced by transfer from 30 to 42 °C. For change of medium, cells were collected by centrifugation (15 min at 4000 × *g*), and the pellets were resuspended in fresh medium. For supplementation experiments, ten times concentrated solutions, i.e. 10× LB or 100 g l^{-1} glucose, were added to 10% (v/v) of the culture volume.

2.3. High-cell density cultivation and on-line analysis

The preparation of the defined medium using glucose as carbon source and the high-cell density fed-batch cultivation strategy has been described before [24]. After termination of the batch growth phase, an exponentially increasing feeding rate of the concentrated glucose/salt solution aimed at a specific growth rate of $\mu_{set} = 0.12\,h^{-1}$ at 30 °C and $\mu_{set} = 0.08\,h^{-1}$ after temperature shift to 42 °C for induction of BL26 pNF795gpC. For production of hFGF-2 with BL21(DE3) pET29c(+)hFGF-2, the temperature was 30 °C, the set growth rate was kept constant at $\mu_{set} = 0.12\,h^{-1}$, and induction was achieved by addition of IPTG to a final concentration of 0.5 mmol l^{-1}. Induction of recombinant protein synthesis was started in both cultures when the OD_{600} reached 100. Details of the cultivations are specified in the figure captions. Biomass, specific growth rate, and yields of biomass and carbon dioxide were estimated from *on-line* measured ammonia and glucose consumption rates and off-gas analysis data as described [25].

2.4. Off-line analysis

Cell growth was also monitored by turbidity at a wavelength of 600 nm of samples appropriately diluted with medium; cell dry mass was determined from washed cell pellets collected in preweight Eppendorf tubes after drying at 40 °C under vacuum. The specific growth rate was also calculated *off-line* from the change in time of the natural logarithm of the cell dry mass.

For protein determination, cell pellets were collected by centrifugation, stored at −70 °C, resuspended in 50 mmol l^{-1} sodium phosphate buffer (pH 7) to OD_{600} = 4.5 and disrupted by sonication on ice. Soluble and insoluble cell fractions were separated by centrifugation (45 min at 38,000 × *g* and 4 °C), analyzed by SDS–PAGE, and specific protein concentrations quantified by densitometry assuming a constant content of cellular proteins of 550 milligram protein per gram cell dry mass [26]. Relative productivity q_P (mg g^{-1} h^{-1}) was calculated as the change of the specific concentration *c* (mg g^{-1}) of fusion protein multiplied with the optical density OD at the respective sampling times *t* (h), and normalized with the optical density at the time of

induction OD_{ind} Eq. (1).

$$q_P = \frac{1}{OD_{ind}} \frac{c_{t2} \cdot OD_{t2} - c_{t1} \cdot OD_{t1}}{t_2 - t_1} \quad (1)$$

3. Results and discussion

3.1. Influence of medium composition on the production of a β-galactosidase fusion protein in shake flask experiments

A fusion protein of β-galactosidase carrying an antigenic peptide from gp41 of HIV was produced in *E. coli* BL26 pNF795gpC from a temperature inducible expression system on complex medium containing yeast extract and protein hydrolysate, or on defined mineral salt medium with glucose as sole carbon source (Fig. 1). The specific product concentration obtained on defined medium was considerably higher than on complex medium (Fig. 1B), whereas the cell growth was slower (Fig. 1A). While the relative productivity was initially similar on both media, on defined medium growth and production was sustained for a longer period with a considerably higher final productivity (Fig. 1C). However, up to 40% of the fusion protein produced on defined medium accumulated in the insoluble cell fraction, compared to less than 5% final accumulation as inclusion body on complex medium (Fig. 1D).

Addition of a concentrated glucose solution to the complex medium upon induction did not increase the specific product concentration (Fig. 1B), indicating that the low product concentration in complex medium is not due to depletion of carbon sources. This was also concluded by MacDonald and Neway [27], who replaced spent medium in perfusion cultures of recombinant *E. coli*. Addition of a concentrated solution of the complex medium components to the defined medium accelerated culture growth to the rate observed in complex medium (Fig. 1A), but strongly reduced the specific concentration of the fusion protein (Fig. 1B). While this treatment hardly changed the relative productivity of total fusion protein (Fig. 1C), the final volumetric yield of *soluble* fusion protein increased by 30% (data not shown).

The components of the complex medium were individually added to defined medium upon induction, and bactotryptone was as efficient as the complete medium in promoting growth and reducing aggregation. Yeast extract was a little less effective, whereas NaCl or several inorganic nitrogen sources had no effect (data not shown).

To check the effect of pre-induction conditions, the medium was changed by centrifugation of the culture and resuspension of the cells in fresh medium before transfer to the induction temperature. Cells producing on complex medium grew two to three times faster than cells producing on defined medium, and had 40% lower product level (Table 1) similar to the unperturbed culture described above. The pre-induction growth medium had a strong effect on the product quality, and the effect was identical with both production media: “Skimped” cells, i.e. cells cultivated on the defined medium before induction, are compared to “coddled” cells that were cultivated on complex medium before induction, comparing those cultures that were afterwards resuspended in the same medium for production. After 1.5 h of production, the fusion protein concentrations and the growth rates of the “skimped” cells were 20–25%

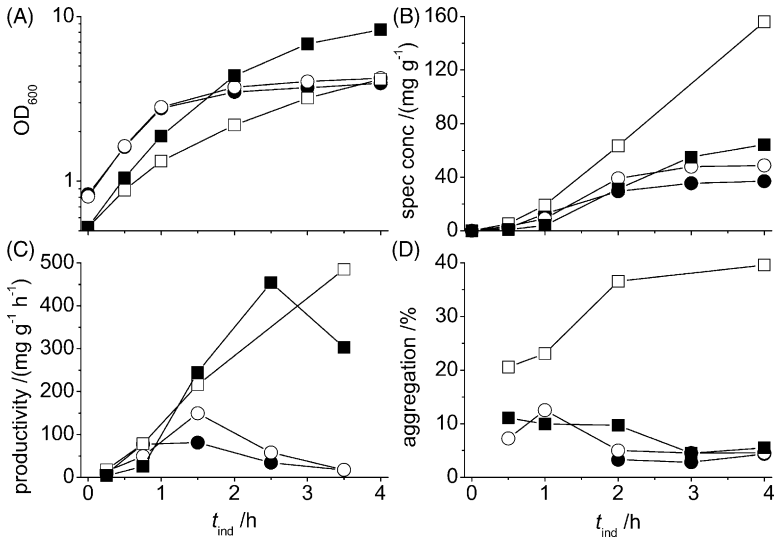

Fig. 1. Profiles of growth, production of the β-galactosidase-HIVgp41 peptide fusion protein, and aggregation on medium of different compositions. (A) Growth after induction was followed by the optical density at 600 nm, (B) specific concentration of the β-galactosidase-HIVgp41 peptide fusion as determined from densitometry of Coomassie stained SDS–PAGE gels, (C) relative productivity, and (D) percentage of aggregated fusion protein, determined from SDS–PAGE analysis of disrupted cells separated by centrifugation. Medium: (□) defined medium, (■) defined medium plus 10% (v/v) 10× concentrated solution of complex LB medium upon induction, (○) complex LB medium, (●) complex LB medium plus 10% (v/v) glucose solution (100 g l^{-1}) upon induction.

Table 1
Effect of medium before and after induction on growth, production and aggregation of the β-galactosidase-HIVgp41 peptide fusion protein

Medium		Specific growth rate ($\mu\,h^{-1}$)		Fusion protein		
Before induction	After induction	Before induction 30 °C	After induction 42 °C	Total (mg g^{-1}[a])	Insoluble	
					mg g^{-1}[a]	%[b]
Complex	Complex	0.72 ± 0.02[c]	1.51 ± 0.01	19 ± 6	nd[d]	0
	Glucose salt		0.64 ± 0.05	31 ± 9	2.8 ± 1	≈10
Glucose salt	Complex	0.38 ± 0.01	1.17 ± 0.04	14 ± 3	nd[d]	0
	Glucose salt		0.50 ± 0.03	23 ± 5	4.6 ± 2	≈20

The specific growth rate was determined from the optical density at 600 nm, specific concentration of fusion protein from densitometry of SDS–PAGE of samples taken 1.5 h post-induction.
[a] β-Galactosidase-HIVgp41 peptide fusion protein as milligram per gram cell dry mass.
[b] Insoluble β-galactosidase-HIVgp41 peptide fusion protein as percentage of total fusion protein.
[c] 95% confidence interval from three parallel experiments.
[d] Below detection limit.

lower than those of "coddled" cells (Table 1), using both defined or complex media for resuspension in the production phase. Moreover, despite lower total concentration of fusion protein, "skimped" cells showed higher tendency to form inclusion bodies when producing the fusion protein on defined medium (Table 1).

3.2. Production of the β-galactosidase fusion protein in high-cell density cultivation

For pilot-scale production of the β-galactosidase fusion carrying the antigenic peptide from gp41 of HIV, a fed-batch protocol established for the production of recombinant proteins was used. After a batch phase on defined medium, a glucose solution was fed with an exponentially increasing rate aiming at a specific growth rate of $\mu_{set} = 0.12\,h^{-1}$, which corresponds to about 25% of the maximum growth rate at 30 °C. When reaching an OD_{600} of 100, the feeding was restarted with $\mu_{set} = 0.08\,h^{-1}$ and the temperature was raised to 42 °C to induce the fusion protein synthesis.

After induction, the specific product concentration increased permanently, finally reaching 120 mg of total fusion protein per gram cell dry mass, corresponding to 20% of the total cell protein (Fig. 2A). These high amounts of fusion protein were mainly maintained in the soluble cell fraction, as less than 15% of the fusion protein produced was aggregated at the end of the cultivation (Fig. 2A). In this high-cell density cultivation, the maximum specific relative productivity, calculated as described in Section 2 for comparison with productivities obtained in shake flask cultures, reached about 70 mg per hour and gram cell dry mass four hours after induction and declined afterwards (Fig. 2A). This was about six times lower than in the shake flask experiments (Fig. 1C) due to the lower growth rate and hence lower protein production rate in the glucose limited fed-batch cultivation.

Growth was monitored *off-line* by cell dry mass and *on-line* by balancing the alkali consumed for pH maintenance (Fig. 2B). The specific growth rate was close to the set value of $\mu_{set} = 0.12\,h^{-1}$ during the fed-batch phase before induction. After induction, the specific growth rate

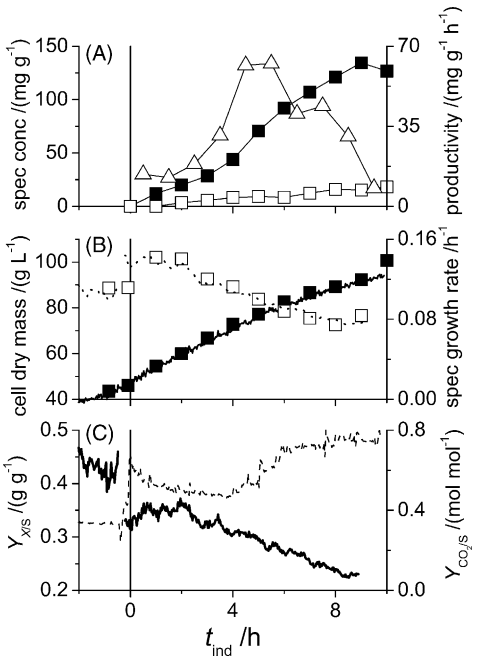

Fig. 2. Production of the β-galactosidase-HIVgp41 peptide fusion protein in high-cell density cultivation. After batch (initial glucose concentration 30 g l^{-1}, 13.25 h) and first fed-batch phase ($\mu_{set} = 0.12\,h^{-1}$ for 10.75 h) at 30 °C, the feeding rate was adjusted ($\mu_{set} = 0.08\,h^{-1}$) starting 25 min before induction. When the new feeding rate was reached 15 min later, the temperature shift was initiated after 3 min. The induction temperature of 42 °C was reached after 7 min. The time is given relative to this time point of induction t_{ind}. (A) Product accumulation: The specific fusion protein concentration was estimated densitometrically from Coomassie stained SDS–PAGE in the total cell extract (■) and in the insoluble cell fraction (□), separated by centrifugation of disrupted cells. The relative productivity (△) was determined as in Fig. 1C from Eq. (1). (B) Growth profile: cell density (■, —) and specific growth rates (□, ...) were determined *off-line* (■, □) from cell dry mass and culture volume, and estimated *on-line* (—,...) from alkali consumption and bioreactor weight as described in [25]. (C) Yields of biomass (—) and carbon dioxide on glucose (..., in mol CO_2 per mol carbon in glucose) calculated from the rates of alkali consumption, carbon dioxide evolution and glucose feeding as described in [25].

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