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Metabolic approaches for the optimisation of recombinant fermentation processes

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Abstract The aim of this work was the establishment of a novel method to determine the metabolic load on host-cell metabolism resulting from recombinant protein production in *Escherichia coli*. This tool can be used to develop strategies to optimise recombinant fermentation processes through adjustment of recombinant-protein expression to the biosynthetic capacity of the host-cell. The signal molecule of the stringent-response network, guanosine tetraphosphate (ppGpp), and its precursor nucleotides were selected for the estimation of the metabolic load relating to recombinant-protein production. An improved analytical method for the quantification of nucleotides by ion-pair, high-performance liquid chromatography was established. The host-cell response upon overexpression of recombinant protein in fed-batch fermentations was investigated using the production of human superoxide dismutase (rhSOD) as a model system. *E. coli* strains with different recombinant systems (the T7 and pKK promoter system) exerting different loads on host-cell metabolism were analysed with regard to intracellular nucleotide concentration, rate of product formation and plasmid copy number.

Introduction

The main goal of this work was the development of an efficient tool for monitoring the metabolic load relating to recombinant-protein production. The determination of significant nucleotides, especially the signal molecule ppGpp, was studied with a view towards optimising recombinant fermentation processes by matching the protein expression rate to the host-cell metabolic capacity.

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Advancements in genetic engineering and fermentation methodology offer the potential to produce large quantities of biologically active molecules used in therapy and food technology. The production of recombinant protein has been established in various host organisms. Bacteria, especially *Escherichia coli*, remain important to production, both in the laboratory and on an industrial scale (Hjorth et al. 1992; Glick 1995). However, the number of recombinant products on the market is small, because many industrial processes are still far from optimal. The limited efficiency of bioprocesses appears to be due to an incomplete understanding of the relationship between recombinant-protein expression and host-cell metabolism.

In the past, optimisation of recombinant processes was mainly attained by genetics-based solutions (Imanak 1986; Das 1990), and in some cases by modification of the fermentation strategies (Yee and Blanch 1992); insufficient attention was given to the biosynthetic capacity of the host-cell. Of the gene constructs currently available, many are too strong and exert such a significant burden on host-cell metabolism that vital cellular functions are impaired; others are rather weak and do not lead to full exploitation of host-cell metabolism. Even though it provides the protein-synthetic machinery, the specific building blocks and the energy, the host-cell's importance to recombinant protein biosynthesis has been widely underestimated (Bentley et al. 1990; Jensen and Carlsen 1990; George et al. 1992; Glick 1995). The optimum yield of recombinant protein is attained by tuning the expression rate in relation to the synthetic capacity of the host-cell. Therefore the determination of the metabolic load imposed by recombinant protein synthesis, in brief the development of a ''metabolic load sensor'', is the key issue in further process optimisation.

The usual standard process variables do not provide sufficient physiologically relevant information. Therefore key variables reflecting the metabolic state have to be identified. In addition to its metabolic capabilities, the bacterial cell contains highly sophisticated signal-processing machinery. In natural habitats, cells are

frequently exposed to environmental changes and have to adapt to different levels of metabolism. To cope with these complex tasks (involving the co-ordination of hundreds of widely distributed genes) cells have evolved a great variety of regulatory and signalling networks, as described in the operon–modulon–stimulon model (Neidhardt et al. 1990; Lengeler and Postma 1999).

One of these, the stringent response network, protects the cell from starvation by forming the signal and effector molecule guanosine tetraphosphate (ppGpp) (Cashel and Rudd 1987; Hecker and Riesenberg 1988; Neidhardt et al. 1990). As mentioned, overexpression of recombinant protein also leads to the depletion of building blocks and energy, thereby inducing this particular nutrient-starvation signal. Therefore monitoring of ppGpp should provide excellent information about the metabolic load relating to recombinant protein production. Further information can be obtained by quantifying nucleotides such as GTP (the precursor of ppGpp), ATP and ADP because of their role in energy metabolism. The analysis of these nucleotides is critical because nucleotide pools are highly dynamic, with turnover rates that respond immediately to any environmental change or disruption. This is particularly true for samples harvested from high-cell-density suspensions. Therefore a specific sampling device and a reliable sampling procedure for stabilising and extracting the nucleotides had to be developed. The multitude of nucleotides present in the cell extract approaches the limits of the separation performance of ion-pair, reversed-phase HPLC, making extensive optimisation necessary.

Materials and methods

Bacterial strains and plasmids

The *E. coli* K-12 strain MG1655 (Xiao et al. 1991) was used for the establishment of the nucleotide analysis. The *E. coli* strains JM105(pKKhSOD), HMS174(DE3)(pET3ahSOD) and HMS174 (DE3)(pET11ahSOD) were used for the expression of the model protein, human superoxide dismutase (rhSOD) (E.C. 1.15.1.1). rhSOD is a 32-kDa dimeric, highly soluble, non-inclusion-body-forming protein that is released into the cytoplasm; it is non-toxic to the host-cell. The strains were provided from the strain collection of the Institute of Applied Microbiology (Kramer et al. 1996).

E. coli JM105(pKKhSOD)

The expression plasmid is based on the plasmid vector pKK223-3 (Pharmacia). The origin of replication and the ampicillin-resistance gene were derived from plasmid pBR322. The expression vector contains the *tac* promoter. The coding region of rhSOD is equivalent to the nucleotide sequence of a human cDNA. For this expression vector, an *E. coli* JM105 host was used (Yanisch-Perron et al. 1985).

T7 expression system

The T7 expression system uses the RNA polymerase of bacteriophage T7 to express cloned DNA in *E. coli* HMS174(DE3) (Studier and Moffat 1986; Studier et al. 1990). DE3 is a λ derivative that carries the gene for T7 RNA polymerase under the control of the *lacUV5* promoter. The T7 promoter in the pET3 vector is derived from the ϕ10 promoter. For tight control of the expression in the non-induced state, a pET vector with a combined T7/*lac* promoter containing a 25-bp *lac* operator sequence is available (pET11), providing tight control of the target gene. The gene for rhSOD was cloned into the vectors pET3a and pET11a (Lüttich 1991).

Media and growth conditions

The nutrient medium for batch cultivation was as follows (g l^{-1}): glucose monohydrate, 16.5; KH_2PO_4, 3.0; $K_2HPO_4 \cdot 3H_2O$, 6.0; yeast extract, 0.05; tryptophan, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.05; trisodium citrate, 1.25; $(NH_4)_2SO_4$, 2.25; NH_4Cl, 1.85; trace-element solution, 250 µl (prepared in 5 N HCl (g l^{-1}): $FeSO_4 \cdot 7H_2O$, 40.0; $MnSO_4 \cdot H_2O$, 10.0; $AlCl_3 \cdot 6H_2O$, 10.0; $CoCl_2$, 4.0; $ZnSO_4 \cdot 7H_2O$, 2.0; $Na_2MoO_2 \cdot 2H_2O$, 2.0; $CuCl_2 \cdot 2H_2O$, 1.0; H_3BO_3, 0.50); $CuCl_2 \cdot 2H_2O$, 0.02; $ZnSO_4 \cdot 7H_2O$, 0.017). The nutrient medium for fed-batch cultivation was as follows (g l^{-1}): glucose monohydrate, 113; KH_2PO_4, 3.0; $K_2HPO_4 \cdot 3H_2O$, 6.0; $MgSO_4 \cdot 7H_2O$, 3.34; $CaCl_2 \cdot 2H_2O$, 0.34; trisodium citrate, 8.6; $(NH_4)_2SO_4$, 15.47; NH_4Cl, 12.72; trace-element solution, 1720 µl; $CuCl_2 \cdot 2H_2O$, 0.138; $ZnSO_4 \cdot 7H_2O$, 0.11. The cells were grown in a 20-l, computer-controlled bioreactor (MBR Bioreactor AG, Wetzikon, Switzerland) equipped with standard measurement and control units (pH, pO_2, temperature, rpm) at 37 °C using fed-batch cultivation with an exponential substrate feed providing a growth rate of 0.1 h^{-1} for 3.5 generations. The starting volume was 4 l. The inoculum, a deep-frozen (−80 °C) working cell bank (1 ml, $OD_{600} = 1$) was transferred aseptically to the fermenter. Exponential feed was started when the cells entered the stationary phase. Dissolved oxygen was maintained at 50% saturation, the pH was controlled at a set point of pH 7 by the addition of potassium hydroxide (10% w/v), and foam was controlled by the addition of 5% anti-foam (Glanapon 2000; Bussetti, Vienna). Recombinant protein expression was induced 2.5 generations after the start of feeding by the addition of 0.4 g l^{-1} isopropyl-β-D-1-thiogalactopyranoside (IPTG).

Analytical methods

Optical density (OD) was measured at 600 nm. Bacterial dry matter (BDM) was determined by centrifugation of 10 ml cell suspension, resuspension in distilled water followed by centrifugation, and resuspension for transfer to a pre-weighed beaker, which was then dried at 105 °C for 24 h and re-weighed. For the determination of the content of recombinant protein (rhSOD), aliquots of approximately 1.0 mg BDM of the samples were centrifuged in Eppendorf tubes (3000 *g*, 10 min); the supernatants were removed and the insides of the tubes were carefully blotted dry and frozen at −20 °C. The rhSOD content was measured using the enzyme-linked immunosorbent assay (ELISA) method described previously (Bayer et al. 1990). Plasmid copy number (PCN) was calculated from plasmid and chromosomal DNA. Plasmid DNA was isolated from the bacterial cells with a commercial mini-prep kit (PLAS mix, TALENT srl, Trieste, Italy) and quantified by capillary electrophoresis (Breuer et al. 1998). Chromosomal DNA was determined using a fluorescence assay with dye H33258 (Hoechst) after cell disintegration with lysozyme and sodium dodecyl sulphate (SDS) (Rymaszewski et al. 1990). The relationship between plasmid-free segregants and plasmid-containing cells was estimated by plating on NB plates and NB plates containing ampicillin (100 mg g^{-1}). Colony-forming unit (cfu) counts were done after 48 h incubation.

Determination of nucleotides

Sampling procedure and extraction

Samples for nucleotide analysis were withdrawn from the fermenter using a specifically designed sampling device. A three-way, low-

dead-volume valve is mounted into the fermenter to provide immediate and vigorous contact between the sample and the stabilising reagents present in a 10-ml disposable sampling syringe. The device is designed for aseptic operation, allowing steam sterilisation following sample removal.

The sample-preparation procedure was as follows: a disposable 10-ml syringe was filled with approx. 1.0 ml 35% perchloric acid (PCA) containing 80 mM EDTA pre-weighed and chilled to 0 °C in an ice-water bath. Then a sample (approx. 4.0 ml) was collected and, after mixing, the syringe was re-weighed to allow sample volume calculation based on a density of 1.0 g ml^{-1}. The mixture was transferred to an ice-cold test tube and mixed by vortexing at 5.0-min intervals for 15 min. Afterwards the samples were neutralised by the addition of 3.0 ml 2 M potassium phosphate buffer (pH 8.2). Following centrifugation at 3000 *g* for 10 min at 4 °C, to remove cell debris, precipitated protein and potassium perchlorate, the supernatant was transferred to an ice-cold test tube and frozen at −80 °C.

To ensure the efficiency of the sample-preparation procedure the following reagents, chilled to 0 °C, were tested: TCA (20% trichloroacetic acid, 2 mM EDTA), PCA (35% perchloric acid, 80 mM EDTA), FA (2 M formic acid, 20 mM EDTA), ethanol (96% at −20 °C) and form + KOH (1.9% formaldehyde for stabilisation and then 0.2 M KOH). The ATP bioluminescence assay (Luminometer model 1250, LKB Wallac, Sweden; ATP assay kit Boehringer, CLS 567 736) was used as a reference for process validation.

HPLC analysis of nucleotides

ppGpp and other stress-relevant nucleotides were separated and quantified by ion-pair reversed-phase high-performance liquid chromatography (HPLC). HPLC was performed using a Pharmacia LKB system consisting of two pumps (2150), a high-pressure mixing valve, an LC controller (2152), an HP 1100 autosampler, an absorbance detector (2238) and a diode array (HP DAD 1100). Ion-pair reversed-phase chromatography was carried out with a Supelcosil LC-18T column (150-mm × 4.6-mm i.d., 3 μm particle size) connected with a Supelguard LC-18T guard column (5 μm particle size) (Supelco). The temperature was set to 40 °C. A flow rate of 1.5 ml min^{-1} and absorbance detection at 254 nm or a diode array were employed. The mobile phases consisted of two eluents: buffer A (100 mM KH_2PO_4/K_2HPO_4 pH 6, containing 5 mM tetrabutyl ammonium dihydrogen phosphate and 50 mM EDTA, final pH 5.3) and buffer B (buffer A (82%) with acetonitrile (18%), final pH 5.9). All buffer solutions were filtered through a 0.2-μm filter (Millipak 20; Millipore). Both buffers were stored under helium pressure during the period of use. The chromatographic conditions were as follows: the gradient was 5–40% buffer B for 36 min, 40–100% buffer B for 15 min, 100% buffer B for 5 min, 100–5% buffer B for 4 min, and then equilibration at 5% buffer B for 3 min to restore the initial conditions. In case of insufficient separation performance, the column was rinsed using several cycles of a water/methanol gradient.

Prior to HPLC injection the sample was filtered through a 0.2-μm syringe filter (Millex GV; Millipore). The samples are stable for at least 24 h at room temperature. The injected amount of sample was equivalent to approximately 0.3 mg BDM. Peaks were identified by comparison of retention times of spiked samples by UV absorbance at 254 nm, and in one particular case by on-line scanning with UV spectra (DAD). Quantification was carried out by integration of peak area (software package Turbochrom 3, PE Nelson Systems, Cupertino, CA, USA). Each sample was analysed at least twice. All standard curves were achieved with nucleotide standards in the range 100–2500 pmol in which the peak area shows good correlation with the amount present. For the preparation of approx. 0.5 mM stock solutions of standard nucleotides the individual nucleotides were dissolved in 100 mM KH_2PO_4/K_2HPO_4 (pH 7) buffer and stored at −80 °C. The precise content was determined using specific UV absorption.

All chemicals were obtained from Merck, Fluka or Sigma. The procedure for the synthesis and purification of ppGpp was as described previously (Mueller 1995).

Results

Determination of nucleotides

In this paper we report the development of an improved method consisting of sample preparation and an HPLC analytical procedure for the determination of ppGpp, GTP, ADP and ATP (with the objective of evaluating the metabolic load due to recombinant protein biosynthesis). To evaluate the metabolic state of the cells in a fermentation sample, it is necessary to achieve immediate and full inactivation of the metabolic processes. Furthermore, the simultaneous determination of the relevant nucleotides is necessary in order to obtain more complete set of information on the metabolic state. Previously published methods (Lundin and Thore 1975; Little and Bremer 1982; Payne and Ames 1982; Meyer and Wagner 1985; Stocchi et al. 1985; Federn and Ristow 1986; Perret 1986; Ryll and Wagner 1991) proved to be unsatisfactory with respect to both the speed and efficiency of inactivation and the simultaneous determination of the relevant analytes. We describe an accurate method that can be used for routine off-line fermentation control.

Sampling procedure and extraction of nucleotides

In view of the high turnover of nucleotides and, in particular, the potential formation of ppGpp because of starvation during sampling a specific sampling procedure had to be established. It was necessary to stabilise the nucleotide pool and prevent leakage prior to and during cell harvesting. This was achieved by the application of a special low-dead-volume bioreactor sampling device.

Different sample-preparation reagents (TCA, PCA, HCOOH, EtOH, H_2SO_4, H_3PO_4 and KOH mixed with formaldehyde) commonly used to stop cell metabolism were tested with respect to the yield of nucleotides, the stability of the treated sample, compatibility with the subsequent ion-pair reversed-phase HPLC analysis, and the reproducibility of results. ATP, which is usually rapidly consumed during the sampling process, because of the absence of aeration, was used as the key analyte. To assess the effectiveness of the individual reagents, the ATP content of standardised *E. coli* MG1655 cells (grown in chemostat culture at a dilution rate of 0.2 h^{-1} at cell densities of 2.5–3 g l^{-1}) was determined by bioluminescence assay.

The results obtained, shown in Fig. 1, indicate that the application of TCA, PCA, HCOOH and H_2SO_4 provides a high ATP yield within a 9% range, i.e. from 8.0–8.7 μmol g^{-1} BDM, while ethanol, H_3PO_4 and al-

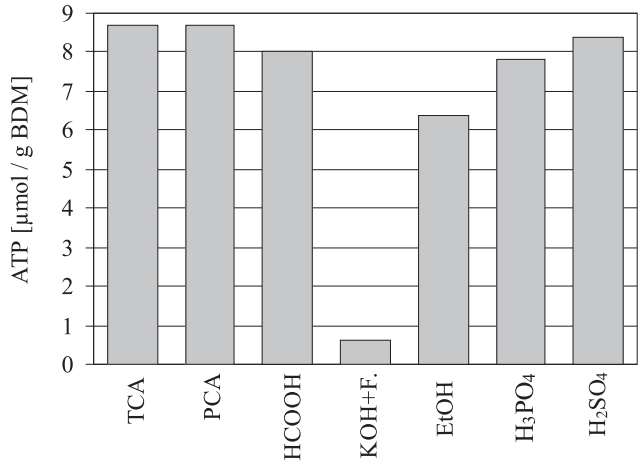

Fig. 1 Validation of effectiveness of different extraction reagents for nucleotides

kaline formate reagents yielded significantly lower ATP values. Afterwards, the compatibility of TCA, PCA and HCOOH extraction reagents with the ion-pair reversed-phase HPLC separation method was examined. H_2SO_4 extraction was eliminated because of its corrosive properties, although the yield efficiency was equal to that of the other reagents. The TCA, PCA and HCOOH cell extracts were neutralised by the addition of potassium phosphate buffer and then centrifuged. In the case of TCA extraction, ATP could not be detected by ion-pair reversed-phase HPLC, probably because of the high ionic strength of the TCA interfering with the ion-pair mechanism. This is not the case with PCA because potassium perchlorate is precipitated after neutralisation and removed by centrifugation. Therefore the PCA extraction method was chosen because of its superior yield of ATP compared to HCOOH and because of its compatibility with the following ion-pair reversed-phase HPLC method.

Subsequently, the PCA extraction method was improved. The yield of nucleotides could be increased by periodical mixing of the cell extract at 5.0-min intervals within a 15-min period. By means of the bioluminescence assay it was proved that there was no loss of ATP after the neutralisation of the PCA extract with potassium buffer (data not shown). (PCA extracts of bacteria have been stored for several months at −80 °C without any loss of separation properties). The respective nucleotides remained completely stable for at least 24 h at room temperature (data not shown), something that is critical to the application of an autosampler.

HPLC analysis of nucleotides

For the quantification of nucleotides by HPLC, several methods (such as ion-exchange, reversed-phase and ion-pair reversed-phase mechanisms) have been described. Here we report an improved ion-pair reversed-phase HPLC method that allows the simultaneous separation of ppGpp together with other nucleotides relevant to the stress response (GTP, ADP, ATP) within 60 min.

Mixtures of authentic nucleotides (ATP, ADP, AMP, ppGpp, GTP, GDP) were used in preliminary experiments to establish the optimal conditions required for good resolution of the specific nucleotides. Good separation was achieved by using a gradient of increasing acetonitrile concentration and pH (data not shown). Further runs with cell extracts and authentic nucleotides produced an unacceptable loss in separation performance, probably caused by matrix effects and the higher number of analytes. Retention times and peak resolution, especially of ATP and ppGpp, shifted significantly from run to run, and peak overlay was observed. Individual runs even showed changes in the peak pattern. Modification of the gradient and elution buffers could not solve these problems (data not shown). However, reproducibility was significantly improved by the addition of 50 mM EDTA to the mobile phase for chelation of interfering heavy-metal ions. Finally, the effect of temperature (20, 30, 35, 40, 45, 50 °C) on peak resolution was investigated. Depending on the nature of the individual nucleotide, an increase in temperature led to a reduction in retention time. Optimum separation of the relevant nucleotides was attained at 40 °C. Reproducible retention times and baseline resolution of nucleotide standards (Fig. 2) and complex bacterial cell extracts (Fig. 3) were attained. The order of elution of compounds in the cell extract was the same as that in the standard nucleotide mix. However, individual compounds of complex cell extracts showed shorter retention times because of matrix effects. Spiking of bacterial cell extracts with authentic nucleotides and on-line scanning of UV spectra (diode array) were used to confirm peak identification and to search for potential interactions between cellular extract components. The linear response of the spiked samples excluded interference.

The accuracy of the improved nucleotide analysis was confirmed using the ATP bioluminescence assay as a

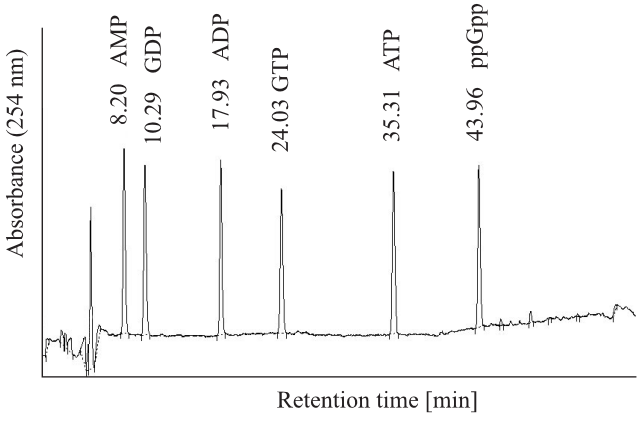

Fig. 2 Separation of authentic nucleotides (approximately 1 nmol each) by ion-pair reversed-phase high-performance liquid chromatography

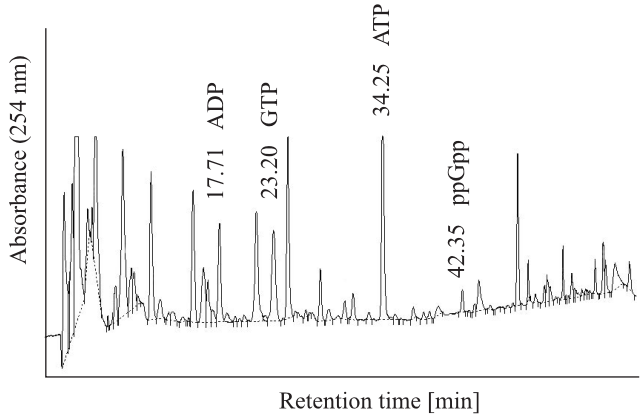

Fig. 3 Chromatogram of an *Escherichia coli* cell extract after perchloric acid extraction

reference. ppGpp, ATP, ADP and GTP were quantified over a concentration range of 0.1–250 μM in the injected sample, which is equivalent to a concentration of up to 1.5 μmol ppGpp per g BDM and 10 μmol ATP per g BDM. Evaluation of run-to-run precision was carried out with a homogenous bacterial cell extract from five consecutive runs. Data and a statistical assessment are given in Table 1. The reproducibility of the complete analytical procedure, including sampling, PCA extraction and HPLC, was tested by three independent operators (A, B, C) in three repetitions, using ATP as the reference analyte (Table 2).

Analysis of nucleotides for monitoring recombinant fermentation processes

The applicability of the improved ppGpp analysis was assessed in a series of fermentations using three different *E. coli* expression systems that exert different loads on host-cell metabolism. For the evaluation of basic relationships between the concentrations of specific nucleotides, the recombinant protein expression rate (qP) and PCN, the production of the model protein recombinant human superoxide dismutase (rhSOD) was investigated in otherwise identical fed-batch fermentations ($\mu = 0.1\ h^{-1}$) (Figs. 4, 5).

Table 1 Run-to-run precision of the high-performance liquid chromatography (HPLC) method using an *Escherichia coli* cell extract. Values are expressed as mean values and standard deviations ($n = 5$). t_R retention time, *VC* variation coefficient

Nucleotide	$t_R \pm$ SD (min)	Concentration $\pm$SD (μmol g^{-1})	VC (%)
ADP	20.74 ± 0.14	0.82 ± 0.043	5.24
GTP	26.03 ± 0.12	3.32 ± 0.037	1.11
ATP	37.86 ± 0.08	5.76 ± 0.020	0.33
ppGpp	45.13 ± 0.08	1.01 ± 0.016	1.62

Table 2 Reproducibility of the complete analytical procedure (sampling, perchloric acid extraction, HPLC) shown by ATP determination

Operator	ATP (μmol g^{-1})	Mean ± SD	VC (%)
A-1	7.39		
A-2	7.29		
A-3	7.31	7.33 ± 0.04	0.6
B-1	7.16		
B-2	7.09		
B-3	7.30	7.19 ± 0.09	1.25
C-1	7.05		
C-2	7.13		
C-3	7.11	7.09 ± 0.03	0.49
Total		7.20 ± 0.12	1.61

Fermentation of E. coli HMS174(DE3)(pET3ahSOD)

E. coli HMS174(DE3)(pET3ahSOD) uses the very strong T7 expression system and shows a considerable basal expression level under non-induced conditions and lethal overexpression after induction. The ppGpp concentration during the non-induced fed-batch phase reflects the metabolic load and correlates with the level of expression rate qP. Under those conditions, growth at $\mu = 0.1\ h^{-1}$ and recombinant protein formation could be maintained in parallel and a considerable yield of about 100 mg rhSOD g^{-1} BDM (14% of total protein) was attained.

Immediately after induction (IPTG) dramatic increases in the product-formation rate, PCN and ppGpp level are observed. The specific

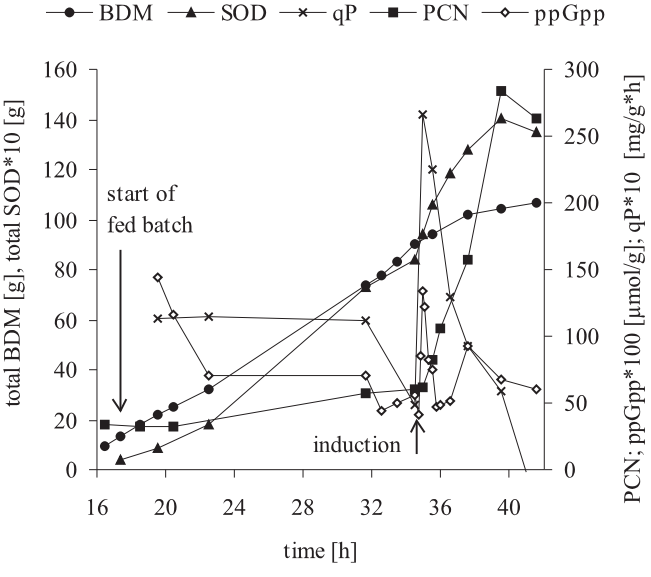

Fig. 4 Fed-batch fermentation for human superoxide dismutase production in *E. coli* HMS174(DE3)(pET3ahSOD)

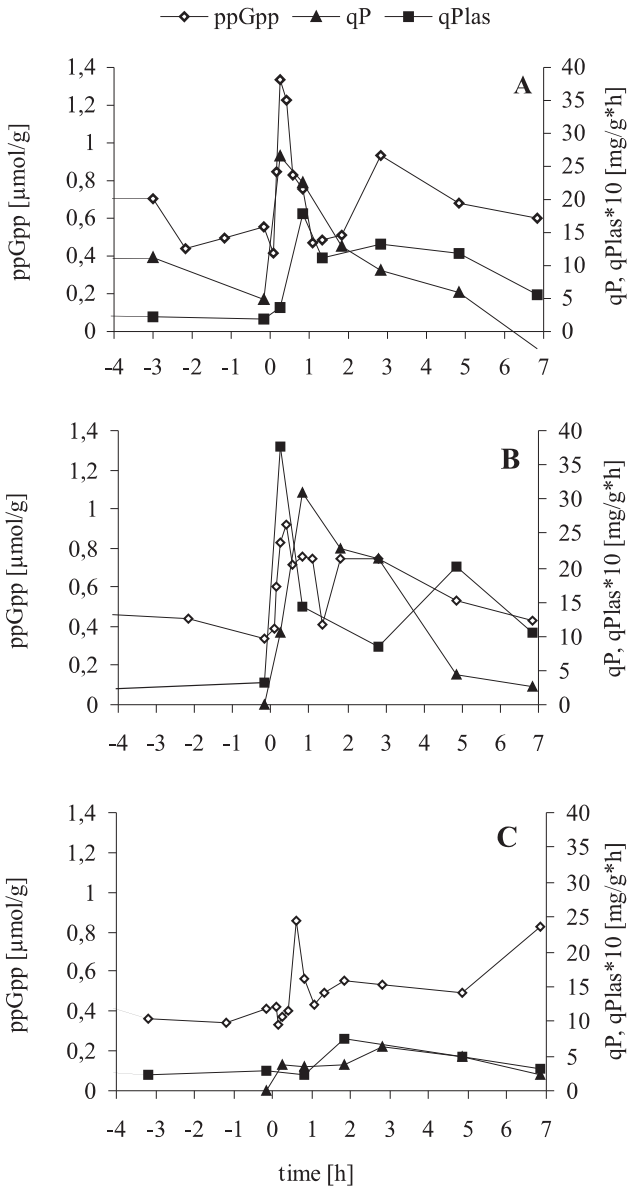

Fig. 5A–C Comparison of different expression systems after induction at time zero. **A** *E. coli* HMS174(DE3)(pET3ahSOD); **B** *E. coli* HMS174(DE3)(pET11ahSOD); **C** *E. coli* JM105(pKKhSOD)

rhSOD concentration increased up to 134 mg g^{-1}, i.e. to approx. 20% of total protein. An overload of host-cell metabolism (as it relates to recombinant protein production) was immediately detected in the form of an increase in the ppGpp concentration. However, the determination of ADP, ATP and GTP provides less significant information about the metabolic load (data not shown). During the fed-batch phase, the pool of ATP was quite low (about 3.5 µmol g^{-1}) and was largely constant. The induction led to a short decrease in the ATP content, which quickly recovered. The concentration of GTP (approx. 3.0 µmol g^{-1}) was not influenced immediately by induction. After a transient rise in ppGpp concentration, an increase in the GTP level (to approx. 4.2 µmol g^{-1}), resulting from the inhibition of protein biosynthesis by ppGpp, was observed.

Table 3 Segregation of plasmid-free cells in fed-batch fermentation of *E. coli* HMS174(DE3)(pET3ahSOD). *cfu* Colony-forming unit

	cfu ml^{-1}	
	Prior to induction	7 h after induction
± plasmids	2.1×10^{10}	5.9×10^{8}
+ plasmids	2.2×10^{10}	1.1×10^{8}

The growth inhibition of the producing cells favoured the overgrowth of plasmid-free cells, as the number of cfu 7 h after induction shows (Table 3). Furthermore, after induction a dramatic increase in the PCN, from 60 to 300 copies per cell, was observed. The specific plasmid-formation rate, *q*Plas (mg plasmid DNA g^{-1} h^{-1}), increased from 0.2 to 1.8 (Fig. 5A), whereby the peak maximum for *q*Plas appeared later than *q*P and ppGpp. It should be emphasised that, in the first instance, *q*Plas remained constant at about 1.2 mg g^{-1} h^{-1} for 5 h and could even be maintained longer than recombinant protein formation. From the data for plasmid and total DNA it can be calculated that plasmid DNA was formed at the expense of chromosomal DNA (data not shown).

Fermentation of E. coli HMS174(DE3)(pET11ahSOD)

E. coli HMS174(DE3)(pET11ahSOD) also uses the T7 expression system. In contrast to the previous gene construct, transcription of the target plasmid DNA is tightly controlled by an additional *lacUV5* promoter. This modification was made to enable the determination of the metabolic load after induction of previously non-producing cells. In the non-induced state the metabolic load exerted by the genetic modification is very low and caused mainly by plasmid replication and expression of the antibiotic-resistance genes (Fig. 5B). After induction, a significant overload of host-cell metabolism is observed. In comparison to *E. coli* HMS174(DE3)(pET3ahSOD), a higher expression rate *q*P (31 mg g^{-1} h^{-1}) and a longer expression period were observed after induction. This is probably because of the lower metabolic burden prior to induction. However, the total yield of recombinant protein (64 mg g^{-1}) was considerably lower than with *E. coli* HMS174(DE3)(pET3ahSOD), which produces recombinant protein both in the non-induced state and in the induced state.

As in the previous experiments, the metabolic burden following induction could be detected immediately as an increase in ppGpp (0.9 µmol g^{-1}). Although *q*P is higher, the amount of ppGpp is significantly lower (Fig. 5, A and B). This phenomenon might also be connected with the lower burden prior to induction.

Plasmid replication also behaved differently from fermentation of *E. coli* HMS174(DE3)(pET3ahSOD). It increased immediately after induction and attained a

rate that was twice as high. Growth was severely reduced after induction, because of the high *q*P and *q*Plas, and could be maintained only for one generation (data not shown).

Fermentation of E. coli JM105(pKKhSOD)

The pKK- system is a weak producing system. From the behaviour of *q*P, *q*Plas and ppGpp (Fig. 5C) it can be concluded that the metabolic load due to recombinant protein formation is very low and that the host-cell metabolism is never overburdened. Growth and recombinant product formation can be maintained in parallel even in the fully induced state. Formation of ppGpp follows induction and becomes elevated for a short period of time.

Discussion

This work proves, using various *E. coli* recombinant systems, that the amount of the starvation signal molecule ppGpp is correlated to the host-cell metabolic load imposed by recombinant protein production. Knowledge of the metabolic state of the host-cell is important for systematic optimisation of recombinant fermentation processes. The yield of recombinant protein is determined by the mutual interplay between the strength of the genetic system, the gene dosage (PCN) and the host-cell metabolic capacity. To achieve a high yield of recombinant protein, it is common practice to use strong expression systems, which are (in reality) often too strong and lead to rapid loss of host-cell metabolic activity or even to cell death after induction. Since recombinant protein production shares the protein-synthesis machinery, the precursors and the energy of host-cell metabolism, the yield will also be low. Therefore monitoring of ppGpp should be used to determine the load limits of host-cell metabolism. Furthermore, the recombinant protein expression rate can be adapted for optimal exploitation of the host-cell's synthetic capacity. The reduction of the promoter strength of strong expression systems, e.g. by feeding limiting quantities of inducer or using alternative inducers with lower affinity for the specific repressor molecule, is an effective way of tuning the expression rate.

In this respect, the establishment of an improved method for the determination of stress-relevant nucleotides in complex bacterial cell extracts (including an effective PCA-extraction procedure and quantification by ion-pair reversed-phase HPLC) provides a powerful tool for monitoring metabolic load. PCA extraction allows immediate fixation of the actual physiological state, separation of relevant nucleotides by HPLC in a single run within 60 min; baseline separation enables reliable quantification and the method can be applied to rapid, routine analysis. PCA extraction was proved to be superior to the frequently described formaldehyde inactivation and KOH extraction (Little and Bremer 1982) and had no disadvantages with regard to the stability of highly phosphorylated nucleotides. Alkaline extraction, even after neutralisation, leads to increased degradation of nucleotides, NADH and NADPH at room temperature, so extracts have to be analysed immediately (Lundin and Thore 1975; Brugidou et al. 1991; Mueller et al. 1996). The proven stability of the analytes and the high reproducibility of the whole procedure (Tables 1, 2) enable a high throughput of samples by the application of partly automated analytical equipment such as autosamplers.

The results of this work clearly demonstrate that high rates of recombinant protein expression exceed the metabolic capabilities of host-cell metabolism and thereby trigger the stringent response by the formation of ppGpp. At lower levels of metabolic load, recombinant protein production can be maintained in parallel with growth. Under these conditions, the level of ppGpp is significantly correlated to the specific expression rate, *q*P, as shown in the fermentations of *E. coli* JM105(pKKhSOD) and *E. coli* HMS174(DE3)(pET3ahSOD) before induction (Fig. 5A, C); the levels of the other nucleotides (ADP, ATP and GTP) are less well correlated to the metabolic state than expected. However, the stringent-response network, which enables wild-type strains to survive environmentally induced starvation, does not provide sufficient protection to overcome the metabolic overload generated by high levels of recombinant protein expression. A major reason for this phenomenon could involve the complex role of uncharged tRNA, which triggers the formation of ppGpp (Neidhardt et al. 1990) and also enhances plasmid replication. Overamplification of ColE1-type plasmids is caused by the accumulation of particular uncharged tRNAs, which interfere in plasmid replication-control mechanisms through binding to the antisense RNA I and/or RNA II (Wróbel and Wegrzyn 1998). For process optimisation, it is important to consider that, after induction, the recombinant protein expression rate is raised, the pools of monomers are thus depleted, and levels of uncharged tRNAs are further increased. These, in turn, enhance plasmid amplification and transcription of mRNA, as T7 RNA polymerase can bind to its own promoters on the plasmid without further control, thus permanently inducing transcription. The results show that monitoring of the PCN is absolutely necessary for optimal tuning of recombinant protein expression and that the roles of the individual tRNAs require further investigation.

To sum up, it can be said that the amount of ppGpp reflects the load level of host-cell metabolism and thus provides important information for the strategic analysis of systematic process optimisation. Monitoring of ppGpp will be extremely useful with strong expression systems, such as *E. coli* HMS174(DE3)(pET3ahSOD) and *E. coli* HMS174(DE3)(pET11ahSOD), that have to be down-regulated to the load limits of host-cell metabolism by induction control. In future, the availability of specific tools, such as a metabolic load sensor, will

speed up the identification of 'bottlenecks' in industrial process development.

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