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Characterization of the AlkS/P_{alkB}-Expression System as an Efficient Tool for the Production of Recombinant Proteins in *Escherichia coli* Fed-Batch Fermentations

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ABSTRACT: The availability of suitable, well-characterized, and robust expression systems remains an essential requirement for successful metabolic engineering and recombinant protein production. We investigated the suitability of the *Pseudomonas putida* GPo1-derived AlkS/P_{alkB} expression system in strictly aqueous cultures. By applying the apolar inducer dicyclopropylketone (DCPK) to express green fluorescent protein (GFP) from this system in *Escherichia coli* and analyzing the resulting cultures on single-cell level by flow cytometry, we found that this expression system gives rise to a homogeneous population of cells, even though the overall system is expected to have a positive feed-back element in the expression of the regulatory gene *alkS*. Over-expressing *E. coli*'s serine hydroxymethyltransferase gene *glyA*, we showed that the system was already fully turned on at inducer concentrations as low as 0.005% (v/v). This allows efficient mass production of recombinant enzymes even though DCPK concentrations decreased from 0.05% to 0.01% over the course of a fully aerated cultivation in aqueous medium. Therefore, we elaborated the optimum induction procedure for production of the biocatalytically promising serine hydroxymethyltransferase and found volumetric and specific productivity to increase with specific growth rate in glucose-limited fed-batch cultures. Acetate excretion as a result of recombinant protein production could be avoided in an optimized fermentation protocol by switching earlier to a linear feed. This protocol resulted in a production of a final cell dry weight (CDW) concentration of 52 g/L, producing recombinant GlyA with a maximum specific activity of 6.3 U/mg total protein.

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KEYWORDS: expression system; *alk* system; biocatalysis; positive feed-back; growth rate-dependent protein production

Introduction

Suitable recombinant expression systems are a fundamental prerequisite for the overproduction of recombinant proteins, for example, for therapeutic or biocatalytic purposes (Choi et al., 2006), or for rational metabolic engineering (Keasling, 1999). The production of recombinant proteins has become standard practice within the last decades. To improve the yield, a wide variety of host and expression systems and fermentation regimes have been developed and successfully implemented (Jana and Deb, 2005). Nevertheless, *Escherichia coli* remains an attractive recombinant host with cell dry weight (CDW) concentrations as high as 148 g/L CDW (Korz et al., 1995) and 145 g/L CDW (Horn et al., 1996) and a variety of expression systems available. However, disadvantages remain with frequently applied model *E. coli* expression systems, such as expensive and toxic inducers (e.g., isopropyl- β -thiogalactoside (IPTG) in the LacI/P_{lacZ}-system (Donovan et al., 1996)), catabolite repression which makes the use of glucose as carbon source in mineral media difficult (e.g., the LacI/P_{lacZ} (Stulke and Hillen, 1999) or the PrpR/P_{prpB} expression system (Lee et al., 2005)), the requirement for dedicated strains (as for the T7 system (Studier et al., 1990) or the GalR/P_{galP-2} system (Menzella and Gramajo, 2004)), or—on process level—difficult induction procedures (such as heat induction for C₁₈₅₇/ λ P_{LPR} system (Chao et al., 2002)). Moreover, some well established expression systems—LacI/P_{lacZ} and AraC/P_{araB}—can display bistable rather than graded expression behavior: upon induction they can produce two subpopulations of cells, one fully induced and the other not at all (Khlebnikov and Keasling, 2002; Ozbudak et al., 2004; Siegle and Hu, 1997). Although the expression response on

population level appears to be increasing with the inducer concentration, this reflects on single-cell level only the increase of one population over the other (Khlebnikov and Keasling, 2002; Khlebnikov et al., 2002; Ozbudak et al., 2004). This property has been attributed to an autocatalytic behavior of these systems, as the inducers—lactose and thio-methylgalactoside for the *lac* or arabinose for the *ara* system—also induce the expression of a transporter gene which in turn facilitates the entry of the inducer into the cell (Carrier and Keasling, 1999; Ozbudak et al., 2004). To allow truly graded responses, these systems require inducers not depending on transporter gene expression for membrane crossing, such as IPTG (Khlebnikov and Keasling, 2002), or decoupling of the regulation of the expression of the transporter gene from the actual expression system (Khlebnikov et al., 2000, 2001).

Here, we characterize another expression system, the AlkS/*P_{alkB}* system originally derived from *Pseudomonas putida* GPo1 (van Beilen et al., 1994, 2001), for biotechnological purposes in strictly aqueous media. AlkS senses the presence of alkanes and induces the expression of genes from its cognate promoter *P_{alkB}* (Kok et al., 1989), but also from its own promoter *P_{alkS2}* (Canosa et al., 2000). Natural inducers of the system are *n*-alkanes (*C*₆–*C*₁₂) (Grund et al., 1975; van Beilen et al., 1994), but the system can also be induced by the ketone dicyclopropylketone (DCPK) (Grund et al., 1975; Sticher et al., 1997), which is well water soluble and less volatile. These inducers do not require transport proteins to enter the cell. The AlkS/*P_{alkB}* system has been successfully applied for whole-cell biocatalysis in two-liquid phase cultures, where an additional organic phase could serve as a reservoir for the inducer *n*-octane at rather high concentrations, which would otherwise be quickly stripped from strictly aqueous cultivations (Bühler et al., 2003a,b; Panke et al., 1999, 2000). The various parts of the system have been assembled into a series of convenient and self-sufficient expression vectors (Panke et al., 1999; Smits et al., 2001), which can be freely exchanged between *E. coli* strains without additional strain engineering efforts. The strength of the promoter is comparable to the strong *tac* system, and it is tightly shut in the uninduced situation (Panke et al., 1999, 2000). Carbon catabolite repression—although present in the wild-type—does not occur in *E. coli* (Staijen et al., 1999; Yuste et al., 1998). First efforts to extend the expression system into protein production in *E. coli* in aqueous systems involved the production of oxygenases: One part, StyA, of a two-component styrene monooxygenase, StyAB, was produced, but to relatively low cell densities (Otto et al., 2004). Other proteins were a two-component xylene oxygenase, the membrane protein part of which had a major impact on the host physiology (Looser et al., 2005), and a chlorobenzene dioxygenase (Yildirim et al., 2004, 2005) which was used for concomitant biotransformations and the toxic effects of the product interfered with the cultivation.

In order to explore the more general suitability of the AlkS/*P_{alkB}* expression system for metabolic engineering or

protein production purposes in aqueous systems, we characterized the response of the expression system to different inducer concentrations and whether the culture is homogeneously induced, and subsequently elaborated an optimized induction protocol for the overproduction of enzymes for biocatalysis. To prevent additional interference of the synthesized protein with cell physiology, we overproduced an intracellular *E. coli* protein with promising potential as a biocatalyst: GlyA, a serine hydroxymethyltransferase, which is a functional member of the group of glycine-dependent aldolases (Silvestri et al., 2003).

Materials and Methods

Chemicals

All chemicals were either from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland) or Roth (Reinach, Switzerland) unless mentioned otherwise. Restriction enzymes were from New England Biolabs (Beverly, MA).

Strains and Plasmids

Relevant strains and plasmids used in this study are listed in Table I. Standard cloning procedures were performed as described (Sambrook and Russell, 2001). Helper plasmid pSPZ4 was constructed by digestion of pSPZ3 with *PacI* and *BamHI* and ligation to a polylinker 5' TAACCTAGGGGC GCGCC 3' and 5' GATCGGCGCGCCCCTAGGTATAAT 3' maintaining the *PacI* site inside *alkS* next to an *AvrII* and an *AscI* site. To eliminate an additional *NdeI* site on pSPZ4, site-directed mutagenesis was performed (CATATG changed to CATAAG) using the Quik Change XL Site-Directed Mutagenesis Kit from Stratagene (Amsterdam, The Netherlands), yielding pSPZ4ΔN, which facilitates future cloning procedures. The *glyA* gene was amplified by PCR from *E. coli* W3110 genomic DNA, adding on the 5' end an *EcoRI* and *NdeI* site and on the 3' end a *BamHI* site. The PCR product was cloned into pSPZ1(+) as an *EcoRI/BamHI* fragment. The *glyA* gene was removed from the resulting plasmid pESM1 as a *BamHI/NdeI* fragment and inserted into equally digested plasmid pSPZ2Not, resulting in pESM2. From there, a *PacI/AscI* fragment covering the 5' part of *alkS** (the star indicates an *alkS* gene in which an internal *NdeI* site has been silently mutated (Panke et al., 1999)) and the *glyA* gene was excised and inserted into pSPZ4ΔN, where it reconstituted the *alkS** gene. This resulted into the functional expression plasmid pESM3.

Plasmid pGEM-7Zf(+) was digested with *NsiI/EcoRI* and ligated to a polylinker consisting of the oligonucleotides: 5' TCATATGGTTCGACGGATCCAAGCTTCACGTGCTCGAGGGCGCGCCG 3' and 5' AATTCGGCGCGCCCTCGAGCACGTGAAGCTTGGATCCGTCGACCATATGATGCA 3'. This linker was excised as *NdeI/AscI* fragment and ligated into equally digested pESM3 to yield pESM5, a vector

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Characteristics	Source or reference
<i>E. coli</i> strains		
W3110	F ⁻ , λ ⁻ , IN(<i>rrnd-rrnE</i>)1	Gentic Stock Center, Yale University, USA
DH5α	<i>supE44 ΔlacU169 (Φ80 lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Gentic Stock Center, Yale University, USA
XL10-Gold	<i>Tet^rΔ (mcrA)183 Δ(mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F' proAB lacI^qZ ΔM15 Tn10 (Tet^r) Amy Cam^r]</i>	Stratagene
JM101	<i>SupE thi Δ(lac-proAB) F' [traD36 proAB⁺lacI^qlacZ ΔM15]</i>	New England Biolabs
Plasmids		
pGEM-7Zf(+)	<i>lacZα</i> , phage f1ori, Ap ^r	Promega (Madison, WI)
pGFPuv	pUC, <i>lacZ</i> , <i>gfpuv</i> , Ap ^r	Clontech (Mountain View, CA)
pSPZ1(+)	pGEM-7Zf(+) with a new polylinker, Ap ^r	Panke et al. (1999)
pSPZ2Not	pUC18 derivative with <i>alkBp</i> and T4 transcriptional terminator, Ap ^r	Panke et al. (1999)
pSPZ4	pBR322 derivative with incomplete <i>alkS</i> [*] and a polylinker, Km ^r	This study
pSPZ4ΔN	Same as pSPZ4, but <i>NdeI</i> site deleted, Km ^r	This study
pESM1	pSPZ1(+) containing <i>glyA</i> , Ap ^r	This study
pESM2	pSPZ2Not containing <i>glyA</i> , Ap ^r	This study
pESM3	AlkS expression vector, containing <i>glyA</i> , Km ^r	This study
pESM4	pGEM-7Zf(+) with a new polylinker, Ap ^r	This study
pESM5	AlkS expression vector with a new polylinker, Km ^r	This study
pGFPuvΔN	Same as pGFPuv, but GFP- internal <i>NdeI</i> site deleted, Ap ^r	This study
pESM7	AlkS expression vector, containing <i>gfpuv</i> ΔN	This study

containing the AlkS/*P_{alkB}* expression system and a multiple cloning site.

A mutation was introduced into the *gfpuv* gene in order to eliminate its internal *NdeI* restriction site without altering the amino acid sequence using the QuikChange XL Site-Directed Mutagenesis Kit. The mutated *gfpuv* gene was amplified by PCR, flanking the gene with *NdeI* and *AscI* restriction sites, which enable the cloning into *NdeI/AscI* digested pESM3, resulting in vector pESM7.

Induction Experiments

The induction experiments with *E. coli* XL10 (pESM7), addressing homogeneous expression from the AlkS/*P_{alkB}* expression system, were performed in LB medium, supplemented with 50 μg/mL kanamycin at 30°C. Cells were taken from a common preculture, inoculated into 50 mL cultures, and grown to an OD₆₀₀ of 0.3 prior to induction with DCPK to concentrations specified in the text. Cells were collected after 4 h by centrifugation, washed with phosphate buffered saline (PBS), centrifuged again, and resuspended in PBS. Prior to the analysis, the cells were diluted to an OD₆₀₀ between 0.05 and 0.1 in PBS and filtered through a 50 μm pore size sieve (Partec GmbH, Münster, Germany). Flow cytometry was performed on a Becton Dickinson FACS Aria (BD Biosciences, San Jose, CA) flow cytometer with an emission wavelength of 488 nm and a 530/30 nm wavelength band pass filter. Each sample was processed at a rate between 12 and 15,000 events/s and 200,000 events were analyzed.

Induction experiments with *E. coli* JM101 (pESM3) expressing *glyA*, addressing the potential of the expression system for protein production, were performed in M9^{*}

medium (Panke et al., 1999) containing 5 g/L glucose, 1 mL/L of trace element solution US (Panke et al., 1999) and supplemented with 5 mg/L thiamine. Cells were grown in a common preculture at 30°C and then diluted to an OD₆₀₀ of 0.1 into 50 mL cultures, where they were induced at an OD₆₀₀ of 0.3 by the addition of DCPK to concentrations specified in the text. Cells were harvested 6 h after induction by centrifugation and stored at -20°C until determination of enzymatic activity.

All concentrations reported for DCPK refer to a volume to volume ratio.

Fed-Batch Fermentations

The defined mineral medium used for precultures of the fermentation and the fed-batch fermentations themselves contained (g/L): glucose, 10 (5 for the preculture medium); (NH₄)₂HPO₄, 3.0; MgSO₄·7H₂O, 0.7; KH₂PO₄, 10; Na₂HPO₄, 1.5; CaCl₂·2H₂O, 0.03; citric acid, 1.0; thiamine, 0.02; kanamycin, 0.05; and trace element solution TES, 7 mL. The pH was adjusted to 7.0 by the addition of 10 N NaOH. TES contained (g/L): CaCl₂·2H₂O, 2.28; FeSO₄·7H₂O, 10; ZnSO₄·H₂O, 1.4; MnSO₄·H₂O, 0.38; CuSO₄·5H₂O, 1; MoO₄Na₂·2H₂O, 0.5; Na₂B₄O₇·10H₂O, 0.23; 35% HCl 10 mL. The feeding solution contained (g/L): glucose, 500; MgSO₄·7H₂O, 20; thiamine, 0.2; and TES 6 mL. Glucose, MgSO₄, and CaCl₂ were autoclaved separately, while the trace element solution, thiamine, and kanamycin were added by sterile filtration. For fermentations, the strains were taken from a glycerol stock stored at -80°C and plated on LB_{Km} agar. Some colonies were then transferred to an M9_{Km} agar plate with glucose and incubated overnight at 37°C to ensure maintenance of *E. coli* JM101's F-plasmid

that carries the proline synthesis genes. A single colony from such a plate was used to inoculate 4 mL LB_{K_m} which was then incubated for 12 h at 37°C. The LB_{K_m} culture (2.5 mL) was used to inoculate 100 mL of the preculture medium. This seed culture was incubated for 11 h at 30°C and used then for the inoculation of the reactor. All fermentations were carried out in a 5 L Biostat A bioreactor (Sartorius BBI Systems GmbH, Melsungen, Germany) with a starting volume of 3 L. When the glucose in the medium was depleted, the feeding solution was added exponentially following the equation (adapted from (Lee, 1996)):

$$\text{Feed}(t) = \left(\frac{\mu_{\text{set}}}{Y_{X/S}} + m \right) C_{X0} \frac{V_0}{C_S} e^{\mu_{\text{set}} t} \quad (1)$$

where *Feed* (in L/h) is the feeding rate, μ_{set} (h⁻¹) is the desired specific growth rate, $Y_{X/S}$ (g/g) is the cell yield on carbon substrate (assumed to be 0.5 for glucose), m (0.04 g/g·h) is the specific maintenance coefficient (Reiling et al., 1985), C_{X0} (g/L) is the CDW concentration at the beginning of the feed, V_0 (L) is the liquid volume in the reactor at the start of the feed, C_S (g/L) is the concentration of glucose in the feeding solution, and t (h) is the time that has elapsed since the start of the feed. To maintain a dissolved oxygen value of above 20% of saturation, first the stirrer speed and then the aeration rate were increased to maximum values of 1,200 RPM and 2 volume per volume per minute (vvm), respectively. After that, the feed regime was changed from exponential to linear when the dissolved oxygen concentration reached below 20% or an accumulation of acetate in the fermentation broth was detected as specified in the text. Temperature, pH, and oxygen saturation were measured online. The pH was maintained at 7.0 by the automatic addition of 25% (wt/vol) NH₄OH or 1 M H₂SO₄. Foaming was suppressed by the initial addition of 5 mL/L 20% polypropylenglycol (PPG 2000). The fermentation parameters were controlled by the Micro DCU 300 control unit and the MFCS/win 2.0 software package of Sartorius BBI Systems.

Off-Line Analytical Procedures

Growth was monitored by measuring the optical density at 600 nm. CDW was determined by centrifuging 1.5 mL of cell suspension in preweighed 2 mL Eppendorf tubes, washing, and re-centrifuging, drying at 95°C for at least 24 h and then transferring into a desiccator and cooling to room temperature. Glucose, acetate, and ammonia concentrations were determined by enzymatic test-kits (R-Biopharm, Darmstadt, Germany). DCPK concentration in the medium was determined by HPLC on a ProntoSil Eurobond C18 124 × 4.0 mm column (Bischoff Chromatography, Leonberg, Germany) using a gradient from 15% to 40% (v/v) acetonitrile in water over 12 min with detection at 210 nm on a Merck Hitachi LaChrom HPLC system.

For determination of GlyA activity, samples were resuspended in buffer G (20 mM Tris (pH 8.0) containing 10 mM dithiothreitol (DTT)). Cell walls were disrupted by grinding with glass beads in a mixer-mill (Retsch, Haan, Germany) for 90 s at a frequency of 30 Hz. Cell debris was removed by centrifugation at 10,000g for 10 min. Total protein concentration was measured using the Bradford assay from BioRad (Hercules, CA) against BSA as standard and analyzed by SDS–PAGE. The activity of the serine hydroxymethyltransferase was quantified using 3-phenylserine as substrate and the assay was performed as described (Ulevitch and Kallen, 1977).

For determination of inclusion bodies and estimating the amount of recombinant protein produced, frozen cell pellets were resuspended in 50 mM sodium phosphate buffer (pH 7.0) to OD₆₀₀ = 20–30 and disrupted in a French press. Soluble and insoluble fractions were separated by centrifugation for 30 min at 40,000g and 4°C and further analyzed by SDS–PAGE. Purification of GlyA was performed on an Äkta prime system (GE Healthcare, Uppsala, Sweden). One hundred microliters of the soluble fraction were loaded on an anion exchange column (1 mL HiTrap Q FF, GE Healthcare) equilibrated with buffer G. Elution was carried out with an increasing KCl gradient in buffer G. GlyA was eluted at a KCl concentration of 250 mM.

Results

Adaptation of the Expression System

We wanted to investigate the AlkS/*P_{alkB}* expression system for its capacity to produce a homogeneously induced culture of recombinant *E. coli* cells and its suitability for metabolic engineering and recombinant protein production in strictly aqueous systems. Therefore, we adapted the pSPZ-series of expression vectors (Panke et al., 1999) for direct insertion of recombinant genes as *NdeI*/*AscI* fragments and inserted a variant of the *gfp* gene (Cramer et al., 1996) and *E. coli*'s *glyA* gene encoding a functional threonine aldolase (Kimura et al., 1997) (Table I).

Homogeneous Expression of GFP

To examine the homogeneity of the expression response of recombinant *E. coli* cells to induction by DCPK, the synthesis of the GFPuv protein under control of the AlkS/*P_{alkB}* expression system was analyzed by flow cytometry (Fig. 1). Cells were induced by different concentrations of DCPK and analyzed for their GFPuv level after 4 h (which is sufficient for full induction under conditions of unlimited growth (Panke et al., 1999)). The level of GFPuv increased from the situation without induction via 0.001% DCPK to 0.05% DCPK and did not further increase with the inducer concentration. At those DCPK concentrations that are relevant for typical production processes (≥0.01%, see below), only one population of cells could be observed in all

Figure 1. Histogram showing the number of cells and their fluorescence intensity against the concentration of the inducer DCPK. Cells were analyzed by flow cytometry 4 h after addition of DCPK.

three induced situations. Furthermore, the strength of the induction was a function of the inducer concentration. Clearly, the response had a reduced variance when the inducer concentration became higher.

Expression of Serine Hydroxymethyltransferase

Functional expression of GlyA in *E. coli* JM101 (pESM3) was verified in shake flasks with mineral medium and glucose as the sole carbon source and the inducer concentration varying from 0.001% to 0.1%. Six hours after induction with 0.05% DCPK, far more than 20% of the total cell protein was GlyA as estimated from SDS-PAGE (see Fig. 2), resulting in specific activities in the order of 2 U/mg of total protein. However, 5%–10% of the recombinant protein were found in the insoluble fraction (data not shown). Increasing the DCPK concentration further did not result in an increase of specific activity, but in a decreased CDW concentration. On the other side, the addition of as little as 0.001% DCPK resulted in the overexpression of *glyA*, although to a lower level. Induction with 0.005% gave a similar expression level as with 0.05% (Fig. 2), confirming the results obtained with GFPuv. From these results, it appeared that the AlkS/P_{alkB} expression system was fully turned on above DCPK concentrations of 0.005%.

DCPK Concentration Decrease During Fermentation

Considering the results from above, we investigated the behavior of the inducer concentration in fully aerated cultures of *E. coli*. After the addition of 0.05% DCPK to a

Figure 2. Specific activity of GlyA as a function of the inducer concentration and corresponding SDS-PAGE. Cells were harvested 6 h after induction. (M: marker; 0–0.1: concentration of added DCPK in %).

fed-batch culture of plasmid-free *E. coli* JM101, a concentration drop-over time was indeed observed (Fig. 3). At an airflow of 1.5 vvm, the concentration of DCPK decreased continuously to 0.01% over 11 h. At this concentration, the AlkS/*P_{alkB}* expression system is still fully induced according to the previous shake flask experiments (Fig. 2). As can be seen from Figure 3, this result was confirmed by a number of subsequent cultivations of *E. coli* JM101 (pESM3), expressing *glyA*. The patterns of DCPK concentrations were similar for all these fermentations, indicating that the decrease in DCPK concentration was not due to any plasmid-encoded function. This was further supported when an experiment was performed under the same conditions as for the fermentations but without inoculation: the DCPK concentration decreased in a very similar fashion (Fig. 3).

Fed-Batch Cultivations With Induction at Different Growth Rates

An often applied method of operation for the production of recombinant proteins is the induction of the expression system towards the end of the fermentation, with optimized variations adapted to the particular needs of the specific process and product (Sanden et al., 2003; Zabriskie and Arcuri, 1986). This way, a high-cell density can be achieved before induction and the resulting additional metabolic burden, which, for example, reduces the risk of plasmid loss (Kyslik et al., 1993). However, at such a late stage of fermentation, the specific growth rate is already rather low, often even below 0.05 h^{-1} , due to the difficulties in supplying enough oxygen. Most of the energy consumption of the cells then goes into maintenance (Sanden et al., 2003) and the additional request of protein production results in further stress. To elucidate the effect of the growth rate on

Figure 3. Decrease of the DCPK concentration in the fermentation broth. Samples from three different fermentations of *E. coli* JM101: (●) plasmid-free, DCPK addition at growth rate $\mu = 0.15 \text{ h}^{-1}$; dashed line: corresponding airflow into the reactor in vvm; (▲) containing plasmid pESM3, induction at growth rate $\mu = 0.15 \text{ h}^{-1}$; (◆) containing plasmid pESM3, induction at growth rate $\mu = 0.2 \text{ h}^{-1}$; (○) from fermentation without inoculation (lines drawn only to show trend).

recombinant protein expression employing the AlkS/*P_{alkB}* expression system, cells were induced during fed-batch fermentations at different specific growth rates of 0.05, 0.15, and 0.2 h^{-1} .

Volumetric Productivity as a Function of Specific Growth Rate

In Figure 4, time profiles of the CDW concentration and of the specific activity of the recombinant GlyA are shown for fermentations with different predefined specific growth rates

Figure 4. Fed-batch cultivations of *E. coli* JM101 (pESM3) showing the time course of the cell dry weight (CDW) concentration and the specific activity of GlyA. Induction was performed at different specific growth rates. **A:** growth rate 0.05 h^{-1} ; **(B)** growth rate 0.15 h^{-1} ; **(C)** growth rate 0.2 h^{-1} . Arrows: (1) start of exponential feed; (2) induction with 0.05% DCPK; (3) setting feed linear; (4) second addition of 0.05% DCPK.

μ_{set} . Within the first few hours after induction, the cells produced the recombinant enzyme more rapidly at a higher growth rate. The maximum specific activity at the slow specific growth rate of 0.05 h^{-1} was 5.5 U/mg total protein after 19.6 h of induction at a CDW of 26.9 g/L . This fermentation at a low specific growth rate received a second addition of DCPK 9.4 h after the first one to increase the inducer concentration again to 0.05% (Fig. 4A). The maximum specific activity value increased slightly when μ_{set} was increased to 0.15 h^{-1} , to 5.9 U/mg total protein at a CDW of 29.2 g/L after 11.4 h (Fig. 4B), and reached its maximum value at the highest growth rate examined, $\mu = 0.2 \text{ h}^{-1}$, when 6.4 U/mg total protein was reached 7.4 h after induction at a CDW of 30.1 g/L (Fig. 4C). From this data, and by comparing the specific production rates (Fig. 7), induction at a high growth rate should be most favorable.

Acetate and Glucose Concentrations During Fermentations

Acetate and glucose concentrations during fed-batch fermentations are shown in Figure 5. The increase in concentration of acetate during the initial batch phase was expected as a result of overflow metabolism. However, we observed a second increase of acetate concentration up to 1.3 g/L during cultivation after the cells were induced at specific growth rates $\mu = 0.2$ or 0.15 h^{-1} . We therefore performed control fermentations with either *E. coli* JM101 or *E. coli* JM101 (pESM5). No accumulation of acetate could be observed after the addition of 0.05% DCPK to the plasmid-free *E. coli* JM101 fermentation ($\mu_{\text{set}} = 0.15 \text{ h}^{-1}$), excluding any toxic or stress inducing effects of DCPK on *E. coli* as the cause of acetate formation. Plasmid pESM5 contains the functional $\text{AlkS}/P_{\text{alkB}}$ expression system, but no recombinant gene is under the control of the P_{alkB} promoter. The addition of 0.05% DCPK to a culture of *E. coli* JM101 harboring pESM5 ($\mu_{\text{set}} = 0.17 \text{ h}^{-1}$) did not provoke excretion of acetate either. In the fermentation with induction at low specific growth rate $\mu = 0.05 \text{ h}^{-1}$, initially no acetate formation could be observed, whereas after a second pulse of DCPK at a later stage of cultivation, also in this case the cells began to form acetate. Following the accumulation of acetate in the induced cultivations at specific growth rates of either 0.15 or 0.2 h^{-1} , also the accumulation of glucose in the medium could be observed (Fig. 5B). Changing the feed regime from exponential to linear caused a concentration drop of glucose as well as acetate in the fermentation broth. The glucose accumulation was not observed in the control experiments, neither with plasmid-free nor pESM5-containing cells.

Recombinant Protein Production With an Optimized Protocol

Based on the previous results, we performed an optimized fermentation (Fig. 6), considering the constraints of sufficient oxygen supply and avoiding the formation of

Figure 5. Time profile of the acetate and glucose concentrations of fed-batch fermentations of *E. coli* JM101 (●) plasmid-free, DCPK addition at growth rate $\mu = 0.15 \text{ h}^{-1}$; (▲) containing plasmid pESM3, induction at growth rate $\mu = 0.15 \text{ h}^{-1}$; (◆) containing plasmid pESM3, induction at growth rate $\mu = 0.2 \text{ h}^{-1}$. Arrows: (1) start of exponential feed; (2) addition of 0.05% DCPK; (3) setting feed linear, (A) at $\mu = 0.2 \text{ h}^{-1}$, (B) at $\mu = 0.15 \text{ h}^{-1}$, (C) at $\mu = 0.15 \text{ h}^{-1}$, plasmid-free.

acetate after induction. In the fed-batch phase, the cells were grown at a growth rate of 0.2 h^{-1} up to a cell density of 20 g/L before induction with 0.05% DCPK. The feed was maintained exponential for one more hour and then, as oxygen transport capacity of the reactor reached its maximum, set to linear. After 9.5 h of linear feed, a final CDW of 51.7 g/L was reached, resulting in a final specific activity of 5.2 U/mg total protein, whereas the maximum specific activity of 6.3 U/mg total protein was observed 6.5 h after induction at a CDW of 41.6 g/L . The specific production rate, however, dropped very fast as can be seen in Figure 7. The amount of produced recombinant protein has been estimated to be 120 mg/g CDW . The specific activity of GlyA decreased at elongated fermentation times,

Figure 6. Fed-batch cultivation of *E. coli* JM101 (pESM3) with induction at optimized conditions. **A:** Time course of CDW and specific GlyA activity. **B:** Time profile of the corresponding glucose, acetate, and ammonia concentrations in the fermentation broth. Arrows: (1) start exp. feed ($\mu_{\text{set}} = 0.2 \text{ h}^{-1}$); (2) induction with 0.05% DCPK; (3) setting feed linear; (4) reduced linear feed.

most probably because of some proteolytic activity of the stressed cells as the growth rate steadily decreased because of the linear feed. Only a minor part of the recombinant protein was found in the insoluble fraction due to aggregation (data not shown).

Discussion

The AlkS/*P_{alkB}* expression system has the potential to overcome several drawbacks associated with current recombinant expression systems, such as carbon catabolite repression, strain dedications, or complex or difficult to control induction procedures. However, it has been designed for preparative applications in two-liquid phase cultivations (Panke et al., 1999) where a second organic phase could serve as a reservoir for the required apolar inducer (Bühler et al., 2003a,b; Panke et al., 1999, 2000). In this work, we investigated its suitability for metabolic engineering or protein production purposes in strictly

Figure 7. Specific GlyA production rates in fermentations of *E. coli* JM101 (pESM3) with induction at different specific growth rates. (■) at growth rate 0.2 h^{-1} ; (▲) at growth rate 0.15 h^{-1} ; (◆) at growth rate 0.05 h^{-1} ; (●) optimized fermentation.

aqueous systems. First we addressed the question whether the system would produce a monostable or a bistable (“all or none” (Carrier and Keasling, 1999)) expression response, as this would interfere with the usefulness of the system for metabolic engineering purposes (Lee and Keasling, 2005). It has been shown that a positive feed-back characteristic of the expression system is a necessary—though not sufficient—requirement for bistable expression behavior (Becskei et al., 2001; Ozbudak et al., 2004). In the AlkS/*P_{alkB}* system, such a characteristic is potentially introduced by induction of *alkS* expression by the activated gene product itself, which has been confirmed in a *Pseudomonas* background (Canosa et al., 2000). Consequently, bistable behavior cannot a priori be excluded.

However, when single cells producing GFPuv under AlkS/*P_{alkB}* control were analyzed for fluorescence, we found only one population of cells over the range from 0.001% to 0.1% of inducer, suggesting that the specific parameters of the AlkS/*P_{alkB}* expression system in *E. coli*—such as the extent of the autocatalytic behavior of the *alkS* expression and potential AlkS cooperative behavior at *P_{alkB}*, maximal activity of activated AlkS, and ratio of activities of induced over non-induced state—do not result in bistable behavior (Becskei et al., 2001; Carrier and Keasling, 1999; Ozbudak et al., 2004). As the translational apparatus is thought to be saturated at full induction, pretranslational sources of fluctuation hardly matter anymore, in particular differences in plasmid copy number per cell of pBR-type plasmids (Summers and Sherratt, 1984). This could explain the reduced variance at higher inducer concentrations (Fig. 1).

The fluorescence analysis also indicated that the AlkS/*P_{alkB}* expression system operates already at full expression at relatively low inducer concentrations of 0.05%. Taking

additionally into account the *glyA* expression data (Fig. 2), this value is as low as 0.005%. Consequently, it will be very difficult to operate this expression system in *E. coli* in another fashion than fully expressed. Considering the concentration drop of the inducer over one cultivation from 0.05% to 0.01% (Fig. 3), it will be rather cumbersome to implement control systems that allow maintaining the inducer concentration at a level required for submaximal expression. Consequently, in order to exploit the AlkS/*P_{alkB}* system for metabolic engineering purposes, future work will need to expand the inducer concentration window to elicit intermediate expression responses, most probably by redesigning *alkS* expression.

On the other hand, the fact that the system is already fully turned on above DCPK concentrations of 0.005% and no DCPK-toxicity effects were observed up to concentrations of 0.05%, there is a considerable range over which the concentration of the relatively volatile DCPK can drop in aerated cultivations before the expression level is affected. This indicates that the AlkS/*P_{alkB}* expression system might be useful for the mass production of proteins where full induction of the system is normally the experimental method of choice anyway.

When comparing the maximum specific GlyA activities obtained from shake flask or from fed-batch cultivations, the GlyA activities from the shake flask experiments were around threefold lower. But by looking at the specific production rates, calculated to be 280 U/g·h in the shake flask, the results indicate a possible advantage of induction at a high specific growth rate of 0.32 h⁻¹. However, also the formation of inclusion bodies was observed in shake flasks, contributing to—but not fully explaining—the lower specific activity.

The problem of producing inactive forms of GlyA was no longer present—or at least significantly reduced—when we investigated mass-production of the enzyme in fed-batch cultivations with respect to suitable patterns of induction (Fig. 4). It is known that the activity and the concentration of components of the cellular protein synthesis machinery show a direct relation to the actual growth rate (Neubauer and Winter, 2001), for instance, the number of ribosomes and the fraction of functioning ribosomes is increasing with increasing growth rate (Yun et al., 1991, 1996). This suggests that, as a general rule, recombinant protein production is most efficient at higher growth rates. However, simulations suggested that the optimum growth rate for maximum expression of a cloned gene in recombinant microorganisms is also depending on the stability of the recombinant product (Lee and Bailey, 1984). Furthermore, other factors than the expression system such as the stress situation and the proteolytic activity affect the efficiency of recombinant protein production (Neubauer and Winter, 2001). The literature provides studies that found no correlation between growth rate and protein production (Harder et al., 1994; Shin et al., 1997), whereas others found a distinct optimum for the specific growth rate (Flickinger and Rouse, 1993; Hellmuth et al., 1994; Riesenberg et al., 1990) or a

maximum production rate at a high growth rate (Cheng et al., 2003; Sanden and Larsson, 2001; Sanden et al., 2003).

As the fundamental reasons for these different observations remain unclear, the influence of the specific growth rate needs to be determined. We limited our investigations to a maximum specific growth rate of 0.2 h⁻¹ as formation of acetate is a well-known phenomenon for *E. coli* cultures at growth rates between 0.17 h⁻¹ (Korz et al., 1995) and 0.23 h⁻¹ (Rothen et al., 1998), which should be avoided because of its inhibitory effect on growth and on product formation (Jensen and Carlsen, 1990). Furthermore, our reactor system showed a *K_La* of roughly 250 h⁻¹ at high-cell densities (data not shown), which indicates that it is difficult to maintain a growth rate larger than 0.2 h⁻¹ for longer than 4 h or one generation time, which we considered the minimum time period to follow the development of the specific GlyA activity.

Our experiments confirmed that induction at a higher growth rate indeed is advantageous for the production of GlyA as a higher specific activity could be achieved in an even shorter time frame than in cultivations with induction at a low growth rate (Fig. 4). However, the production rate at the highest growth rate showed a sharp drop within the first 4 h after induction, whereas at low growth rates, a smoother decline was observed (Fig. 7). We also observed a strong increase in acetate formation after the cells were induced. The data suggest that this depended on recombinant protein production alone as no acetate was detected in the control fermentations: neither the addition of DCPK to a cultivation of *E. coli* JM101 (Fig. 5A) nor to a fermentation of *E. coli* JM101 (*pESM5*) (vector without a gene expressed from the *P_{alkB}* promoter; data not shown) resulted in additional acetate formation. This excluded the metabolic burden of the plasmid replication alone (Bentley et al., 1990) and any stress induced by DCPK directly as the main cause for acetate formation.

Acetate formation after induction also depended on the growth rate: at a growth rate of 0.2 h⁻¹, the cells started earlier with acetate excretion than at a growth rate of 0.15 h⁻¹. A simple explanation might be that the stress induced by the expression of recombinant proteins led to a reduction in the specific growth rate and the glucose consumption rate (Oh and Liao, 2000), so that the glucose feed rate no longer matched the actual growth rate of the cells, however, we cannot completely exclude accompanying cell lysis. This would have led to an increase of the glucose concentration in the fermentation broth, a typical prerequisite for acetate formation. In the optimized fermentation, acetate formation was avoided by changing the feed regime to linear 1 h after induction. This way, we exploited at least in the beginning, the high capacity of the cell for recombinant protein production, stemming from the high preinduction specific growth rate, and at the same time avoiding the discrepancy between feed rate and the glucose uptake rate of the cells (Fig. 6).

In this contribution, we addressed expression behavior of the AlkS/*P_{alkB}* system and the development of a suitable

production strategy in recombinant *E. coli* strains. This system unites an attractive number of suitable properties for recombinant expression systems, such as the independence from carbon catabolite repression, the availability of self-contained vectors, a truly inducer concentration-dependent behavior, and a cheap inducer. The volatility of the inducer and the small window of useful inducer concentrations will make the adjustment of expression states below full induction cumbersome and need to be addressed in the future. Finally, the only moderate final CDW yield achieved in the current fermenter system would require further optimization of the mixing regime or switching to oxygen-enriched air to increase oxygen transfer.

In summary, we could demonstrate that the AlkS/P_{alkB} system is an efficient system for the recombinant production of enzymes, for example, for the serine hydroxymethyltransferase GlyA, that has shown much potential in diastereoselective carbon–carbon bond formation (Kimura et al., 1997; Saeed and Young, 1992).

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