J Ind Microbiol Biotechnol (2010) 37:793–803
DOI 10.1007/s10295-010-0724-7

ORIGINAL PAPER

Transient metabolic modeling of *Escherichia coli* MG1655 and MG1655 Δ*ackA-pta*, Δ*poxB* Δp*ppc ppc*-p37 for recombinant β-galactosidase production

Marjan De Mey · Gaspard J. Lequeux · Joeri J. Beauprez · Jo Maertens · Hendrik J. Waegeman · Inge N. Van Bogaert · Maria R. Foulquié-Moreno · Daniel Charlier · Wim K. Soetaert · Peter A. Vanrolleghem · Erick J. Vandamme

Received: 20 August 2009 / Accepted: 10 April 2010 / Published online: 4 May 2010

Abstract *Escherichia coli* is one of the most widely used hosts for the production of recombinant proteins, among other reasons because its genetics are far better characterized than those of any other microorganism. To improve the understanding of recombinant protein synthesis in *E. coli*, the production of a model recombinant protein, β-galactosidase, was studied in response to the constitutive overexpression of the anaplerotic reaction afforded by PEP carboxylase. To this end, an IPTG wash-in experiment was performed starting from a well-defined steady-state condition for both the wild-type *E. coli* and a mutant with a defective acetate pathway and a constitutively overexpressed *ppc*. In order to compare the dynamics of the fluxes over time during the wash-in experiment, a method referred to as transient metabolic flux analysis, which is based on steady-state metabolic flux analysis, was used. This allowed us to track the intracellular changes/fluxes in both strains. It was observed that the flux towards fermentation products was 3.6 times lower in the *ppc* overexpression mutant compared to the wild-type *E. coli*. In the former on the other hand, the PPC flux is in general higher. In addition, the flux towards β-galactosidase was higher (12.4 times), resulting in five times more protein activity. These results indicate that by constitutively overexpressing the anaplerotic *ppc* gene in *E. coli*, the TCA cycle intermediates are increasingly replenished. The additional supply of these protein precursors has a positive result on recombinant protein production.

Keywords *Escherichia coli* · Recombinant protein · Transient MFA

Electronic supplementary material The online version of this article (doi:10.1007/s10295-010-0724-7) contains supplementary material, which is available to authorized users.

M. De Mey (✉) · J. J. Beauprez · H. J. Waegeman · I. N. Van Bogaert · W. K. Soetaert · E. J. Vandamme
Laboratory of Industrial Microbiology and Biocatalysis, Department of Biochemical and Microbial Technology, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000 Ghent, Belgium
e-mail: Marjan.DeMey@UGent.be
URL: http://www.limab.UGent.be

G. J. Lequeux · J. Maertens · P. A. Vanrolleghem
BIOMATH, Department of Applied Mathematics, Biometrics and Process Control, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000 Ghent, Belgium

M. R. Foulquié-Moreno · D. Charlier
Laboratory for Genetics and Microbiology, Department of Applied Biological Sciences, Faculty of Sciences, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Elsene, Belgium

Present Address:
M. R. Foulquié-Moreno
Laboratory of Molecular Cell Biology, Department of Molecular Microbiology (VIB), Katholieke Universiteit Leuven, Kasteelpark Arenberg 31, bus 2438, 3001 Heverlee, Belgium

Introduction

The growing environmental concerns and the awareness that the world's oil supplies are limited are factors prompting the chemical and biotechnological industries to explore nature's richness in search of methods to replace petroleum-based synthetics for the development of a bio-based economy [1]. An entire branch of biotechnology, known as industrial biotechnology, is devoted to this. It uses living cells and enzymes to synthesize a wide range of

products such as bulk and fine chemicals, food ingredients, pharmaceutical ingredients, bio-fuels, bio-plastics, etc., from renewable resources, using a wide range of production hosts varying from bacteria (e.g., *Escherichia coli*, *Corynebacterium glutamicum*), yeasts (e.g., *Saccharomyces cerevisiae*, *Pichia pastoris*) to fungi (e.g., *Penicillium chrysogenus*).

One type of these high-added value products is recombinant proteins. Since the introduction of recombinant DNA technology, the production of these recombinant proteins has become increasingly important. However, the development of a production process for recombinant proteins is not always straightforward. Common hurdles to take are the choice of the production host (e.g., prokaryote/eukaryote, strain), the choice of expression (e.g., constitutive/inducible, length of protein), and the choice of the protein purification procedure. An approach to handle these difficulties is given in Gräslund et al. [2].

The organism *Escherichia coli* is predominantly used for recombinant protein production because it is biochemically and molecularly well characterized, and it grows fast on inexpensive substrates [3, 4]. However, besides the problems related to the post-translational modification of eukaryotic proteins and the formation of inclusion bodies, the production of acetate as a byproduct during fermentation is a common difficulty when using *E. coli*.

Hence, many approaches to optimize the protein production process focus on the reduction of acetate because it severely hampers growth [5], inhibits protein formation [6] and implies a waste of carbon [7]. It is believed that this formation of acetate in *E. coli* cultures is caused by two mechanisms: (1) the activation of the fermentation pathways by a (local) lack of dissolved oxygen (mixed acid fermentation); (2) the imbalance between the rapid glucose uptake and its conversion into biomass and products (crabtree effect) [8].

The state-of-the-art strategies to reduce acetate formation anticipate these two mechanisms by modifying the bioprocess conditions or the genotype of the production host. The former strategies comprise the limitation of the glucose uptake rate by applying specific glucose feeding patterns, the application of alternative substrates, the addition of supplements to the medium, the control of a range of fermentation parameters and the application of systems to remove acetate from the fermentation broth [7–15]. The latter strategies are based on the alteration of the central metabolism of *E. coli*. These strategies mainly target the glucose uptake mechanism, the acetate pathway and the pyruvate branch point (starting point to direct the carbon flow to acetate) [7, 12, 13, 16–26].

Another strategy to increase recombinant protein production is to preserve sufficient precursors of the amino acids, the building blocks of proteins, in the central metabolism. Two main amino acid precursors are oxaloacetate and α-ketoglutarate, and they serve as precursor for 11 amino acids involved in protein synthesis. Moreover, these intermediates play an important role in several reactions resulting in key components of the cellular metabolism such as glycolysis, gluconeogenesis and the citric acid cycle. March et al. [7] proposed an approach to increase protein production and reduce acetate formation by providing the cell with improved metabolic means to replenish these TCA cycle intermediates. They expressed the heterologous anaplerotic enzyme pyruvate carboxylase of *Rhizobium etli* in *E. coli*, which had a positive influence on recombinant protein production [7].

An alternative approach to provide sufficient TCA-cycle intermediates is to overexpress PEP carboxylase, the principal anaplerotic pathway in *E. coli*, during growth on glucose, which converts phosphoenolpyruvate (PEP) into oxaloacetate. Mutants overexpressing PEP carboxylase (coded by *ppc*) showed a significant reduction in acetate excretion, and consequently these mutants are promising to improve recombinant protein production in aerobic *E. coli* cultures [13, 27].

Next to these biochemical and metabolic points of view, model-based approaches such as metabolic flux analysis (MFA) have also helped to analyze the phenotypic behavior. Metabolic flux analysis permits calculation of the intracellular fluxes by measuring the exchange fluxes with the environment, assuming that the reactor is in a (pseudo) steady state. Recently, it has been attempted to apply this method to analyze cell cultures under transient conditions, which are typically used for recombinant protein production.

Varma and Palsson [28] applied flux balance analysis (FBA) in a non-stationary case (fed-batch) by iteratively solving the model for maximal biomass production. At each time point, the available substrate is calculated from the results of the FBA model in the previous time step. In this way, the time profiles of cell density, glucose and by-products could be quantitatively predicted. The concept of dynamic FBA was further developed by Mahadevan et al. [29]. They formalized the methodology of Varma and Palsson [28] and named it static optimization-based DFBA. They introduced a new method, called dynamic optimization-based DFBA, in which the optimization is performed over the entire time period of interest in order to obtain time profiles of the cellular fluxes. The method of dynamic optimization-based DFBA was combined with the concept of minimization of metabolic adjustment (MOMA) to model myocardial energy metabolism [30]. This alternative goal function proposed by Segrè et al. [31] aims at better predicting the behavior of mutated strains by assuming that a mutant strain will not radically alter its behavior after a genetic modification, but will behave as similar as possible to the wild type. The results show that metabolic states of the

metabolic networks are not always optimal, with respect to growth maximization, during transient perturbations.

Hence, to improve the understanding of recombinant protein synthesis in *E. coli*, we analyzed the production of the model recombinant protein β-galactosidase in response to the constitutive overexpression of PEP carboxylase. Therefore, a *ppc* overexpressing mutant of *E. coli* Δ*ackA-pta*, Δ*poxB* was created in which the natural *ppc* promoter was replaced with a strong constitutive artificial promoter [32].

In this work, transient MFA is applied to an overdetermined system under transient conditions, assuming an intracellular pseudo steady state. At each time point, the exchange fluxes are calculated and used as inputs for the metabolic flux model. In this way, the intracellular fluxes during the transient can be revealed.

Materials and methods

Bacterial strain and plasmids

Escherichia coli MG1655 [λ^-, F^-, *rph*-1, (*fnr*$^-$ 267)del] was obtained from the Netherlands Culture Collection of Bacteria (NCCB). *Escherichia coli* MG1655 Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37 [λ^-, F^-, *rph*-1, (*fnr*$^-$ 267)del, Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37] was constructed in the Laboratory of Genetics and Microbiology (MICR) using the method of Datsenko and Wanner [33]. Plasmid pTrcHisTopoLacZ was obtained from Invitrogen (Merelbeke, Belgium). This expression plasmid contains the model recombinant protein β-galactosidase coded by the gene *LacZ*.

Culture conditions, sampling and sample analysis

Carbon-limited chemostat experiments of *E. coli* were performed in 2-l Biostat B culture vessels (Sartorius-BBH Systems, Melsungen, Germany) with 1.5 l working volume as described before [27]. Sampling for CDW, extracellular measurements and sample analysis was done as described elsewhere [27].

Wash-in experiment

After the cells attained steady state, the medium was switched to the same medium that additionally contained 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to induce the p*trc* promoter on the pTrcHisTopoLacZ plasmid.

During the subsequent 7 h, samples for OD_{600}, extracellular measurements and β-galactosidase activity were taken every 15 min using a rapid sampling loop and a cold stainless bead sampling method, immediately followed by centrifugation.

Data analysis

Glucose and organic acids were determined by High Performance Liquid Chromatography (HPLC) on a Varian Prostar HPLC system (Varian, Sint-Katelijne-Waver, Belgium), and phosphate determination was performed as described before [27].

Quantification of the total nitrogen content in the culture medium was performed using the LCK238 kit from HACH Lange GmbH (Mechelen, Belgium) as described by the supplier.

β-galactosidase assay

Cell lysis using the EasyLyse™-kit (Epicentre® Biotechnologies, BIOzymTC, Landgraaf, The Netherlands) was performed as recommended by the supplier in the EasyLyse Bacterial Protein Extraction Solution manual. β-galactosidase activity was assayed as described by Miller [34] with some modifications. Fifty microliters of sample was added to 200 µl 16 mM ortho-nitrophenyl-galactopyranoside (ONPG) (in phosphate buffer: 100 mM phosphate, 1 mM $MgCl_2$, pH 7.4). The absorbance at 415 nm was measured in a microplate reader (680 XR microplate reader, Bio-Rad, Eke, Belgium) for 10 min. A calibration curve was made for each batch of measurements using a stock solution of 1 U/µl commercial β-galactosidase of *Escherichia coli* (Fluka, Bornem, Belgium). This stock solution was used to make a standard series from 0 to 0.003 U/µl.

Transformation

The expression plasmid pTrcTopoHisLacZ (Invitrogen, Merelbeke, Belgium) was transformed in competent cells of MG1655 and MG1655 Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37 using the simplified procedure of Hanahan [35].

Transient metabolic flux analysis

Transient metabolic flux analysis was used to analyze the cellular behavior during the applied transient. This is allowed assuming that the cells themselves are already in pseudo steady state.

To this end, at each sampling time instant transient MFA was performed. In order to calculate the intracellular flux distribution using MFA, the exchange rates are required. However, because during the transient the reactor itself is not yet in a pseudo steady state, an additional term reflecting the derivative of the concentration data to the time has to be added to determine the exchange rates. The exchange reaction rate can then be calculated as:

$$r_p = \frac{dC}{dt} + D(C - C_{in}) \quad (1)$$

where C is the concentration of a component (g l^{-1}), D the dilution rate (h^{-1}), C_{in} the concentration of the component in the influent and r_p the exchange rate of the considered compound (g l^{-1} h^{-1}). C and $\frac{dC}{dt}$ have to be estimated from the measured data.

As the derivative of the concentration data to the time is very sensitive to random variations on the measurements, polynomials were fitted through it. A polynomial can be represented as:

$$\hat{C} = \sum_{i=0}^{d} a_i t^i \quad (2)$$

where $\hat{C}$ is the estimated concentration, t is the time, a_i is a parameter, and d is the degree of the polynomial. Besides polynomials, also a sigmoid curve was used to fit the data:

$$\hat{C} = a_1 + a_2 e^{\frac{a_3 - t}{a_4}}. \quad (3)$$

Again $\hat{C}$ is the estimated concentration, a_i is a parameter, and t is the time. The time series of metabolite concentration data (e.g., biomass, succinate, glucose, etc.) was separately fitted to polynomials, of which the degree varied between 0 and 30, and to the sigmoid curve. Hence, a total of 32 curves were fitted for each metabolite measured. Fitting was performed using standard least square fitting algorithms as found in the SciPy toolbox of Python [36].

The curve (polynomial or sigmoid curve) with the smallest sum of absolute errors between the measured data and the predicted data was retained (for very noisy data, the selection of the curve was manually done). From this selected curve, smoothened metabolite concentrations and the change in metabolite concentration over time were calculated, on the basis of which the consumption and production rates (Eq. 1) can be determined.

The metabolic model used to analyze the transient is described in De Mey et al. [27]. A β-galactosidase synthesis reaction had to be added to the model. The final model contains 138 reactions and 152 metabolites, of which 12 were considered exchangeable with the environment: ammonia (NH_3), phosphoric acid (PiOH), biomass (Biom), glucose (GLC), lactate (Lac), oxaloacetate (OAA), succinic acid (Suc), oxygen (O_2), carbon dioxide (CO_2), water (H_2O), sulphuric acid (H_2SO_4) and β-galactosidase (BGalAse).

Results and discussion

To investigate whether blocking the acetate pathway and constitutively overexpressing the anaplerotic pathway afforded by PEP carboxylase have a positive effect on recombinant protein production in *E. coli*, a *ppc* overexpression mutant of *E. coli* Δ*ackA-pta*, Δ*poxB* was created in which the natural *ppc* promoter was replaced with a strong constitutive artificial promoter [32]. By using qPCR and an enzymatic assay, it was proven that the mutant showed 4.49 (4.12–4.89) more *ppc* expression and 4.163 ± 0.013 more phosphoenol pyruvate carboxylase activity, respectively, compared to the wild type. The capacity to produce recombinant proteins of this mutant was compared to the wild-type strain. Therefore, the commercially available expression plasmid pTrcHisTopoLacZ was transformed in competent cells of MG1655 and of MG1655 Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37. Insertion of the plasmid was confirmed by antibiotic resistance and control restriction digest on the purified plasmid with restriction enzymes NcoI (New England Biolabs, The Netherlands) and AlwnI (New England Biolabs, The Netherlands) in NEB Buffer 4 (New England Biolabs, The Netherlands) at 37°C. The resulting DNA fragment pattern was verified on a 1.2% agarose gel in TAE buffer (50× stock solution, Invitrogen, Merelbeke, Belgium) (data not shown).

This study thus clearly illustrates the usefulness of fine tuning gene expression [32]. Instead of introducing a gene encoding for a non-native protein on a plasmid, a more potent promoter for *ppc* was introduced in the genome. This is in contrast to March et al. [7], who used a plasmid encoding for the non-native anaplerotic enzyme pyruvate carboxylase of *Rhizobium etli*. In this way, typical problems related to the introduction of non-native genes can be avoided, and the metabolic potential of the metabolism is being fully exploited.

The pursued approach has several advantages over the one followed by March et al. [7]. The development of promoter libraries, consisting of constitutive promoters of varying strength, has allowed the fine tuning of gene expression. Whereas the approach discussed in this study enables the direct use of each expression plasmid for the production of a heterologous protein, the approach of March et al. [7] consists of two plasmids with different *ori*, one bearing the pyruvate carboxylase of *Rhizobium etli* gene and one bearing the heterologous protein gene, in order to maintain both plasmids in the cell and hence expression of *pyc* and the heterologous protein. In addition, two different antibiotics have to be used to keep selective pressure on both plasmids. Moreover, the anaplerotic enzymes used to enhance the flux towards oxaloacetate catalyze different reactions, and as a consequence they have different metabolic implications. Both enzymes yield a bypass to oxaloacetate and inorganic phosphate. Whereas the non-native pyruvate carboxylase of *R. etli* requires pyruvate, bicarbonate and additional energy (ATP), the native phosphoenolpyruvate carboxylase of *E. coli* has PEP and CO_2 as substrate.

Monitoring β-galactosidase production

For both strains an IPTG wash-in experiment was performed to evaluate the potential advantageous effect of the overexpression of phosphoenolpyruvate carboxylase and the elimination of the acetate pathway on the production of the recombinant protein β-galactosidase. Therefore, a chemostat experiment at a dilution rate of 0.1 h^{-1} was performed. Once the steady state was established in the reactor, the medium was supplemented with 1 mM IPTG, which induces the p*trc* promoter of the pTrcHisTopoLacZ plasmid, and the behavior of the reactor was monitored during the subsequent 7 h by sampling every 15 min. In what follows, all time references are in relation to the moment of the medium switch, which coincides with the continuous addition of IPTG to the reactor and is indicated by 0 h. In Fig. 1, the evolution of the biomass, the % CO_2, the (intracellular) β-galactosidase activity and the residual glucose concentration for the experiments with MG1655 and MG1655 Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37 during the 7 h monitoring period are depicted. The β-galactosidase activity was also measured in the supernatant of the broth, but no extracellular activity could be detected.

During the monitored time interval, the evolution of the biomass concentration (Fig. 1a) is similar for both strains. First, there is a slight increase in biomass concentration followed by a stagnation. Subsequently, there is a second increase followed by a decrease around 4 h after the start of induction. Figure 1b shows for both strains a distinct increase in % CO_2 during the first 30 min followed by a gradual increase. After 3.5 h, a difference between the two strains can be noticed for % CO_2. While for MG1655 the % CO_2 keeps increasing until 5 h followed by an exponential drop to 0.65, the % CO_2 of MG1655 Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37 drops immediately, but not as low as for MG1655, and then fluctuates around 0.9%.

In both cases the cells gradually produce more β-galactosidase during the first 2 h, which is reflected in the increased enzyme activity, followed by a stagnation of the β-galactosidase production (Fig. 1c). This increase in β-galactosidase production is due to the increasing concentration of IPTG in the reactor (wash-in experiment). After about 2 h, the concentration of IPTG in the reactor

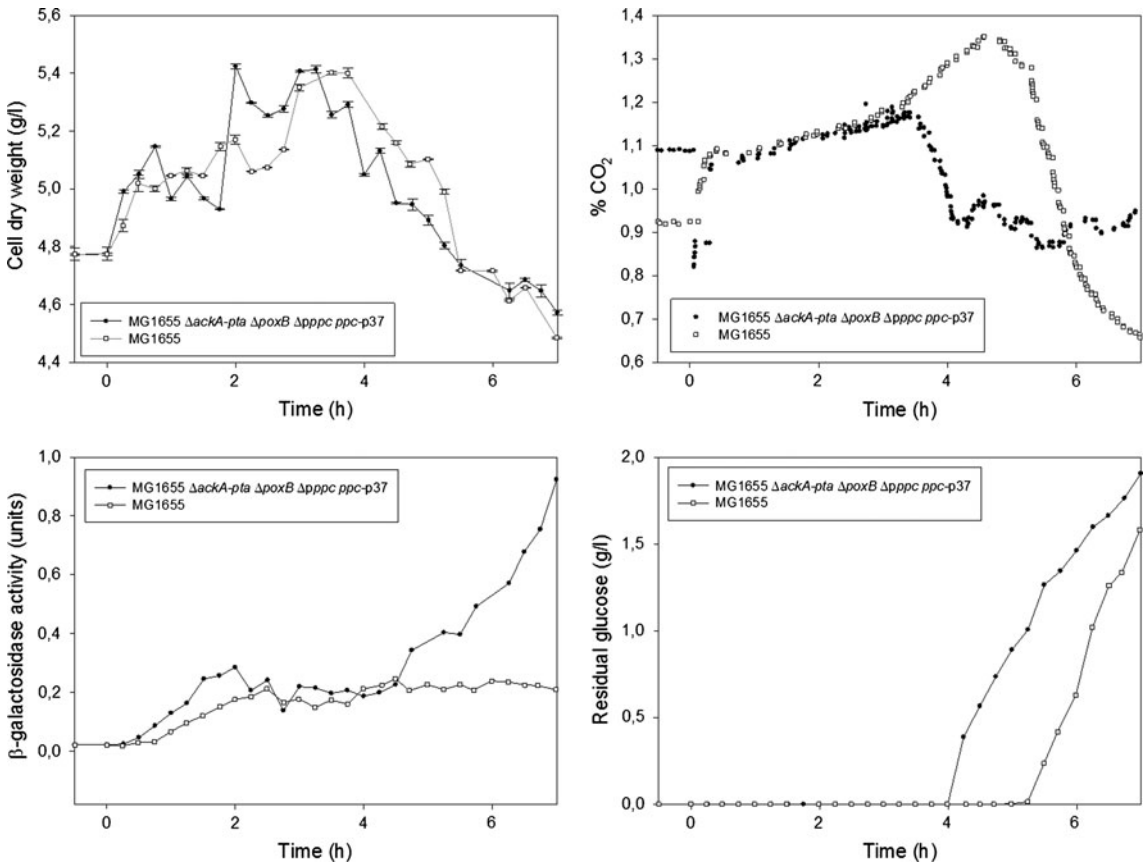

Fig. 1 Evolution of biomass (cell dry weight, *top left*), % CO_2 (*top right*), β-galactosidase activity (*bottom left*) and residual glucose (*bottom right*) at the initial steady state (time $t = 0$ h) and during the induction of pTrcHisTopoLacZ for the MG1655 culture (*open symbols*) and MG1655 Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37 culture (*closed symbols*), respectively

reaches 0.18 mM. This concentration probably suffices to saturate induction and consequently a further increase in the IPTG concentration has no further positive effect on the production of the recombinant protein (stagnation of recombinant protein production both for the wild-type and the mutant strain). This is in agreement with literature data. For the induction of non-toxic genes, it is observed that an IPTG concentration of about 0.1–0.2 mM is sufficient to saturate induction, and higher IPTG concentrations do not result in a higher expression. This increase in β-galactosidase during the first 2 h is faster and more pronounced in MG1655 Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37, which indicates that this strain is a better recombinant protein producer. Whereas the β-galactosidase production in MG1655 keeps constant after 2 h, the β-galactosidase production in MG1655 Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37 keeps increasing. At the end, after 7 h of induction the β-galactosidase production in MG1655 Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37 is approximately five times higher compared to the β-galactosidase production in MG1655.

In addition, differences in the glucose consumption (Fig. 1d) can be observed between the two different strains. After 4 h, the glucose consumption of MG1655 Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37 slows down, whereas the glucose consumption of MG1655 only decreases after 5 h.

A plausible explanation for the observed differences in recombinant protein production between the two strains is the following: at the start of the wash-in experiment, the concentration of IPTG gradually increases in the broth and consequently protein production increases. The increase in protein production is higher in MG1655 Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37, suggesting that this strain is a better recombinant protein producer compared to MG1655, which is probably due to the larger pool of precursors in the Krebs cycle as a result of the constitutive *ppc* (over)expression. After 2 h, a suboptimal production capacity of the cells is reached; the production capacity thereafter declines a bit followed by a stagnation. During the transient, MG1655 Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37 grows slower than MG1655, which is likely due to the *ppc* overexpression because more PEP will be converted to oxaloacetate [PEP carboxylase competes with the phosphotransferase system (PTS) for the available PEP]. In addition, it is likely that this strain is less affected by stress as a result of the withdrawal of amino acids and their precursors required for the IPTG induced recombinant protein production, because of the superior means to fuel the pool of oxaloacetate and α-ketoglutarate, two main amino acid precursors. Hence, this strain probably adapts better to the stress situation, resulting in a steady increase in β-galactosidase production, whereas the β-galactosidase production of MG1655 stagnates. This evolution and the differences between the wild-type and the mutant are confirmed by the Western blot of the intracellular crude cell extract (data not shown).

Transient metabolic flux analysis

To elucidate the altered flux distribution in the mutant strain in comparison with the wild-type metabolic modeling, e.g., metabolic flux analysis is useful. However, metabolic flux analysis, which yields a snapshot of the metabolic state in a particular condition, is typically used under steady-state conditions (chemostat experiments), i.e., in conditions where all variables remain constant over time.

In order to compare the flux distribution changes during the transient, a method was used, which is referred to as transient metabolic flux analysis, which is based on steady-state metabolic flux analysis.

The application is permitted, because an intracellular pseudo steady state can also be assumed under certain dynamic conditions, because of the relatively small time constants of cellular processes, e.g., mass action and the metabolic adaptation to novel conditions, in comparison with processes affecting the observed environmental conditions. Perturbation experiments performed in *E. coli* [37–40] showed that the intracellular metabolite pools reach a new pseudo steady state already after 20 s. Hence, an intracellular pseudo steady state can be assumed during the transient; thus, though the environmental conditions vary, MFA can be applied.

To this end, seven metabolites were measured: Biom, GLC, Lac, Suc, O_2, CO_2 and BGalAse. There are 144 independent equations in the model and $138 + 12 = 150$ unknowns. Thus, at least six measurements should be performed to solve the model. In this case there were seven so that some measurements could be balanced [41].

Derivatives are very sensitive to random variations and noise in the data. Therefore, rather than using raw data for Eq. 1, polynomials were fitted to the data. It was opted to fit one polynomial, since this seemed sufficient to capture the dynamics in the measurements. Figure 2 shows the polynomials fitted to the data.

Using the polynomials fitted to the measured data, the measured metabolite concentration in the medium and in the broth, the flux distribution can be calculated at each time point similar to steady-state MFA. The most salient results are depicted in Fig. 3. This figure displays the dynamics in the relative fluxes of the central metabolism, the fermentation pathways and the recombinant protein synthesis pathway during the wash-in experiment.

As can be seen in Fig. 3a, the initial steady-state flux (time = 0 h) through the phosphotransferase system (PTS) was smaller in *E. coli* MG1655 Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37 as expected, which is probably due to the *ppc*

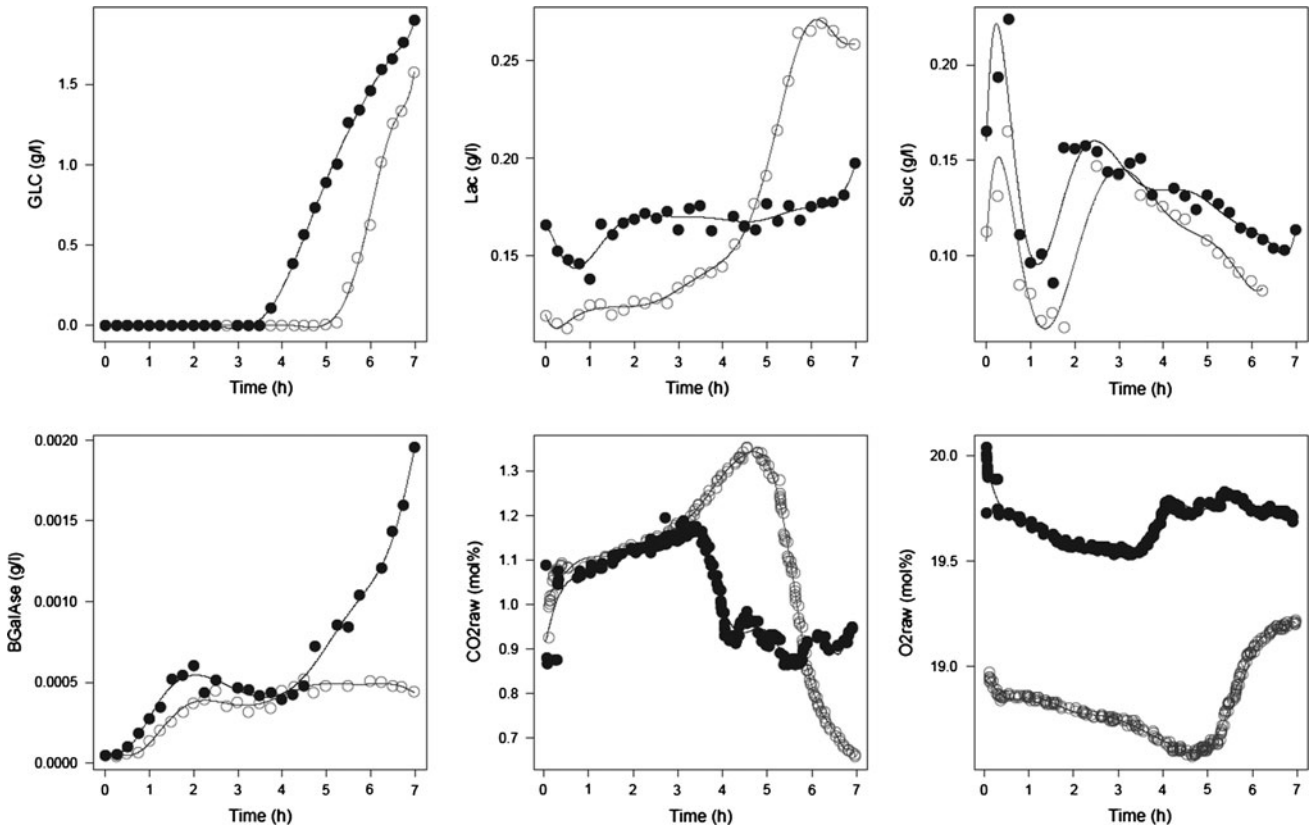

Fig. 2 The data collected during the 7-h monitoring period for glucose (GLC), lactose (Lac), succinate (Suc), β-galactosidase (BGalase), % CO2 and % O2 for the *E. coli* MG1655 culture and the *E. coli* MG1655 Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37 culture represented by an *open circle* and *closed circle*, respectively. The *curves* represent the polynomial fit to the raw metabolite data. Time $t = 0$ h refers to the start of the wash-in experiment and the concurrent induction of β-galactosidase expression by IPTG

overexpression and the concurrently stronger competition with PTS for the available PEP. This is in good agreement with the literature where *ppc* overexpression mutants are reported to have a reduced specific glucose consumption rate [27, 42]. However, from 1 h after induction, the PTS flux was higher in *E. coli* MG1655 Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37, except for 2 h between 4 and 6 h.

The flux towards fermentation products (lactate) was at steady state (time = 0 h) smaller in *E. coli* MG1655 Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37 (see Fig. 3d). This was also observed previously for *ppc* overexpressing *E. coli* strains grown on glucose during anaerobic [42] and aerobic batches [27]. During the monitoring period, the flux towards fermentation products followed roughly the same dynamics in both strains until 4 h. From that point on, the flux towards fermentation products was maintained in *E. coli* MG1655 Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37, whereas it first drastically increased in the wild-type *E. coli* and then seemed to decrease again. After circa 7 h protein production, the relative flux towards fermentation products (lactate) is 3.6 times higher in the wild-type *E. coli* than in MG1655 Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37.

At the initial steady state, the flux through the anaplerotic pathway PEP carboxylase is as expected to be two times higher in the mutant compared to the wild-type *E. coli*, which is probably due to constitutive overexpression of *ppc* (see Fig. 3f). These results are in good agreement with the literature where a *ppc* overexpression mutant was described in which the flux through PEP carboxylase was twice as high [42] and was 40% of the specific glucose consumption rate. The differences with these literature data are probably due to the constitutive expression used in this study with a strong artificial promoter [32] compared to the inducible expression applied in [42]. During the wash-in experiment, the flux through PEP carboxylase is in general higher in the *E. coli* MG1655 Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37 compared to the wild type, and after 7 h of induction, it is 10.3 times higher. On the other hand, the flux through pyruvate kinase is higher in the wild-type strain at the initial steady state, which is likely due to the increased availability of phosphoenolpyruvate (see Fig. 3c). This is also reflected in larger lactate production in the wild-type strain compared to the *E. coli* MG1655 Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37 mutant (see

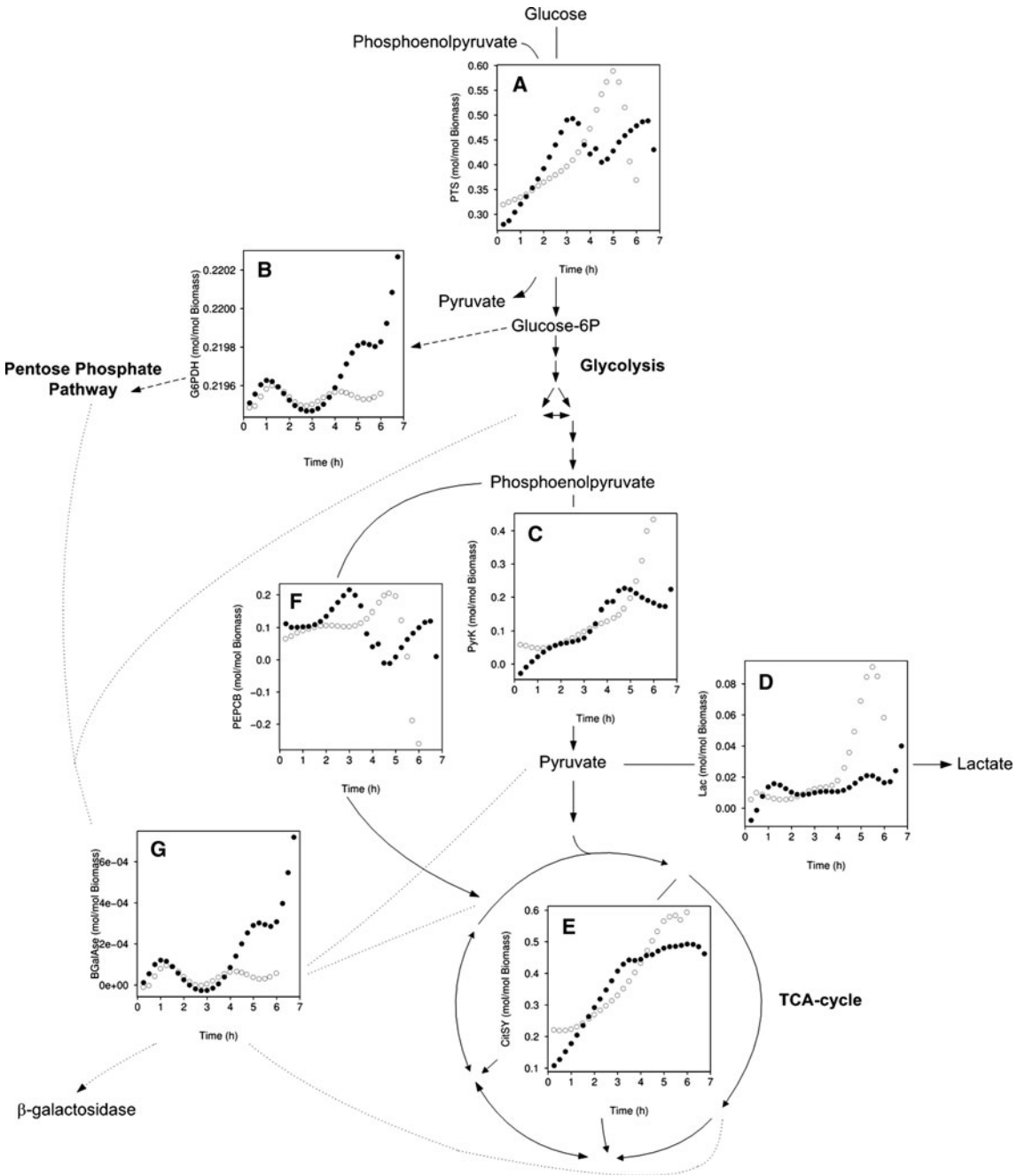

Fig. 3 The evolution of the relative fluxes, calculated with transient MFA, for the reactions phosphotransferase system (PTS) (subfigure **a**), glucose-6P dehydrogenase (G6PDH) (subfigure **b**), phosphoenol pyruvate carboxylase (PEPCB) (subfigure **f**), pyruvate kinase (PYRK) (subfigure **c**), lactate production (Lac) (subfigure **d**), citrate synthase (CitSY) (subfigure **e**) and *β*-galactosidase synthesis (BGalAse) (subfigure **g**) at the initial steady state (time $t = 0$ h) and during the 7 h monitoring period for the *E. coli* MG1655 culture (*open circle*) and the *E. coli* MG1655 Δ*ackA-pta*, Δ*poxB*, Δ*pppc ppc*-p37 culture (*closed circle*). Time $t = 0$ h refers to the start of the wash-in experiment and the concurrent induction of *β*-galactosidase expression by IPTG. The central metabolism is represented by *solid lines*, the pentose phosphate pathway is represented by *dashed lines*, and the reactions towards *β*-galactosidase are represented by *dotted lines*

Fig. 3d). In addition, the flux through the TCA-cycle is higher in the wild-type strain compared to the *E. coli* MG1655 Δ*ackA-pta*, Δ*poxB*, Δ*pppc ppc*-p37 mutant (see Fig. 3e).

A limited production of *β*-galactosidase due to leak expression can be observed before the start of the IPTG addition (see Fig. 3g). At steady state ($t = 0$ h), the flux towards recombinant protein production is 7 times higher

in *E. coli* MG1655 Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37 compared to the wild type, resulting in a slightly higher recombinant protein production. During the monitoring period, the flux towards β-galactosidase followed the same dynamics for both strains during the first 4 h of induction. From that point on, the flux towards β-galactosidase was maintained in the wild type, whereas it steeply increased in the *E. coli* MG1655 Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37. After 7 h of induction of protein expression, the flux towards β-galactosidase is 12.4 times bigger in MG1655 Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37. This indicates that the overexpression of an anaplerotic pathway afforded by PEP carboxylase has a positive effect on recombinant protein production.

In contrast to March et al. [7], who used a plasmid encoding for the non-native anaplerotic enzyme pyruvate carboxylase of *Rhizobium etli*, we fully exploited the metabolic potential of *E. coli*. The stoichiometry of these reactions is different. Whereas the pyruvate carboxylase of *Rhizobium etli* converts pyruvate, bicarbonate and ATP to oxaloacetate and inorganic phosphate, the phosphoenolpyruvate carboxylase of *E. coli* converts PEP and CO_2 to oxaloacetate and anorganic phosphate. Thus, the latter directly influences both the glucose uptake rate, due to the competition of Ppc and PTS for the available PEP, and more efficiently avoids the production of fermentation products, e.g., lactate, because PEP is converted to oxaloacetate rather than pyruvate, which is a direct precursor of both lactate and acetate.

A detailed insight into the flow of carbon throughout the metabolic network was obtained using transient metabolic flux analysis. The results clearly demonstrate the correlation between the Ppc flux and recombinant protein production. This is in contrast to March et al. [7], who only obtained an outside view about the cell's functioning under the perturbed genotype. The obtained results confirm the premise that by improving the metabolic capacity to replenish the TCA intermediates, recombinant protein production can be improved. An alternative to further improve heterologous protein production is overexpression of the *pck* gene, which encodes for phosphoenolpyruvate carboxykinase. This enzyme catalyzes the conversion of phosphoenolpyruvate, CO_2 and ADP to oxaloacetate and ATP, which is energetically favorable for the cell compared to phosphoenolpyruvate carboxylase or pyruvate carboxylase. In *E. coli*, however, this reaction occurs predominantly in the reversed direction. In *Anaerobiospirillum succiniciproducens* and *Mannheimia succiniciproducens*, in contrast, this reaction occurs predominantly is the desired direction. A possible strategy could be to delete the endogenous *pck* gene and to knock-in the heterologous *pck* gene of *A. succiniciproducens* or *M. succiniciproducens* in front of an artificial constitutive promoter.

Conclusion

To improve our understanding of recombinant protein synthesis in *E. coli*, we analyzed the production of the model recombinant protein, β-galactosidase, in response to the constitutive overexpression of an anaplerotic reaction (PEP carboxylase). To compare the production of recombinant β-galactosidase of *E. coli* MG1655 with and without the increased constitutive phosphoenolpyruvate carboxylase activity and cutting off the acetate pathway, a chemostat experiment at a dilution rate of 0.1 h^{-1} was performed for both strains prior to protein induction. During the subsequent 7 h, the p*trc* promoter of the pTrcHisTopoLacZ plasmid was induced with 1 mM IPTG. In order to compare the dynamics of the fluxes over time during the induction of recombinant protein production, transient metabolic flux analysis was applied. This revealed that the relative flux towards fermentation products was higher in the wild-type *E. coli* than in MG1655 Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37. In MG1655 Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37, on the other hand, the flux towards β-galactosidase was significantly higher in MG1655 Δ*ackA-pta*, Δ*poxB*, Δp*ppc ppc*-p37, resulting in five times more protein activity.

Acknowledgments The authors wish to thank Ellen Van Horen for the analysis. The authors wish to thank the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen) for financial support in the framework of a Ph.D. grant (B/04316/01) to M. De Mey and for support via the MEMORE project (040125). The authors also wish to thank the Fund for Scientific Research-Flanders (FWO-Vlaanderen) for support (FWO-project G.0184.04). J. Maertens was a research assistant for the Fund for Scientific Research-Flanders (FWO-Vlaanderen) (2003–2007).

References

1. Frazzetto G (2003) White biotechnology. EMBO Reports 4(9):835–837
2. Gräslund S, Nordlund P, Weigelt J, Hallberg BM, Bray J, Gileadi O, Knapp S, Oppermann U, Arrowsmith C, Hui R, Ming J, dhe-Paganon S, Park H-w, Alexei S, Yee A, Edwards A, Vincentelli R, Cambillau C, Kim R, Kim S-H, Rao Z, Shi Y, Terwilliger TC, Kim C-Y, Hung L-W, Waldo GS, Peleg Y, Albeck S, Unger T, Dym O, Prilusky J, Sussman JL, Stevens RC, Lesley SA, Wilson IA, Joachimiak A, Collart F, Dementieva I, Donnelly MI, Eschenfeldt WH, Kim Y, Stols L, Wu R, Zhou M, Burley SK, Emtage JS, Sauder JM, Thompson D, Bain K, Luz J, Gheyi T, Zhang F, Atwell S, Almo SC, Bonanno JB, Fiser A, Swaminathan S, Studier FW, Chance MR, Sali A, Acton TB, Xiao R, Zhao L, Ma LC, Hunt JF, Tong L, Cunningham K, Inouye M, Anderson S, Janjua H, Shastry R, Ho CK, Wang D, Wang H, Jiang M, Montelione GT, Stuart DI, Owens RJ, Daenke S, Schütz A, Heinemann U, Yokoyama S, Büssow K, Gunsalus KC (2008) Protein production and purification. Nat Meth 5(2):135–146
3. Choi JH, Lee SY (2004) Secretory and extracellular production of recombinant proteins using *Escherichia coli*. Appl Microbiol Biotechnol 64:625–635

4. Makrides SC (1996) Strategies for achieving high-level expression of genes in *Escherichia coli*. Microbiol Rev 60(3):512–538
5. Luli GW, Strohl WR (1990) Comparison of growth, acetate production, and acetate inhibition of *Escherichia coli* strains in batch and fed-batch fermentations. Appl Environ Microbiol 56(4):1004–1011
6. Jensen EB, Carlsen S (1990) Production of recombinant human growth hormone in *Escherichia coli*: expression of different precursors and physiological effects of glucose, acetate, and salts. Biotechnol Bioeng 36(1):1–11
7. March JC, Eiteman MA, Altman E (2002) Expression of an anaplerotic enzyme, pyruvate carboxylase, improves recombinant protein production in *Escherichia coli*. Appl Environ Microbiol 68(11):5620–5624
8. Akesson M, Karlsson EN, Hagander P, Axelsson JP, Tocaj A (1999) On-line detection of acetate formation in *Escherichia coli* cultures using dissolved oxygen responses to feed transients. Biotechnol Bioeng 64(5):590–598
9. Akesson M, Hagander P, Axelsson JP (2001) Probing control of fed-batch cultivations: analysis and tuning. Control Eng Pract 9(7):709–723
10. Akesson M, Hagander P, Axelsson JP (2001) Avoiding acetate accumulation in *Escherichia coli* cultures using feedback control of glucose feeding. Biotechnol Bioeng 73(3):223–230
11. Chen X, Cen P, Chen J (2005) Enhanced production of human epidermal growth factor by a recombinant *Escherichia coli* integrated with in situ exchange of acetic acid by macroporous ion-exchange resin. J Biosc Bioeng 100(5):579–581
12. Eiteman MA, Altman E (2006) Overcoming acetate in *Escherichia coli* recombinant protein fermentations. Trends Biotechnol 24(11):530–533
13. Farmer WR, Liao JC (1997) Reduction of aerobic acetate production by *Escherichia coli*. Appl Environ Microbiol 63(8):3205–3210
14. Fuchs C, Koster D, Wiebusch S, Mahr K, Eisbrenner G, Markl H (2002) Scale-up of dialysis fermentation for high cell density cultivation of *Escherichia coli*. J Biotechnol 93(3):243–251
15. Nakano K, Rischke M, Sato S, Maerkl H (1997) Influence of acetic acid on the growth of *Escherichia coli* K12 during high-cell-density cultivation in a dialysis reactor. Appl Microbiol Biotechnol 48(5):597–601
16. Aristidou AA, San K-Y, Bennett GN (1994) Modification of central metabolic pathway in *Escherichia coli* to reduce acetate accumulation by heterologous expression of the *Bacillus subtilis* acetolactate synthase gene. Biotechnol Bioeng 44(8):944–951
17. Aristidou AA, San K-Y, Bennett GN (1995) Metabolic engineering of *Escherichia coli* to enhance recombinant protein production through acetate reduction. Biotechnol Prog 11(4):475–478
18. Aristidou AA, San K-Y, Bennett GN (1999) Metabolic flux analysis of *Escherichia coli* expressing the *Bacillus subtilis* acetolactate synthase in batch and continuous cultures. Biotechnol Bioeng 63(6):737–749
19. Contiero J, Beatty CM, Kumari S, DeSanti CL, Strohl WR, Wolfe AJ (2000) Effects of mutations in acetate metabolism on high-cell-density growth of *Escherichia coli*. J Ind Microbiol Biotechnol 24(6):421–430
20. De Anda R, Lara AR, Hernandez V, Hernandez-Montalvo V, Gosset G, Bolivar F, Ramirez OT (2006) Replacement of the glucose phosphotransferase transport system by galactose permease reduces acetate accumulation and improves process performance of *Escherichia coli* for recombinant protein production without impairment of growth rate. Metab Eng 8(3):281–290. doi:10.1016/j.ymben.2006.01.002
21. De Mey M, De Maeseneire S, Soetaert W, Vandamme E (2007) Minimizing acetate formation in *E. coli* fermentations. J Ind Microbiol Biotechnol 34:689–700. doi:10.1007/s10295-007-0244-2 (ISSN0169-4146)
22. Han C, Zhang WC, You S, Huang LY (2004) Knockout of the *ptsG* gene in *Escherichia coli* and cultural characterization of the mutants. Sheng Wu Gong Cheng Xue Bao 20(1):16–20
23. Hannig G, Makrides SC (1998) Strategies for optimizing heterologous protein expression in *Escherichia coli*. Trends Biotechnol 16(2):54–60
24. Kleman GL, Strohl WR (1994) Acetate metabolism by *Escherichia coli* in high-cell-density fermentation. Appl Environ Microbiol 60(11):3952–3958
25. Koo TY, Park TH (1999) Increased production of recombinant protein by *Escherichia coli* deficient in acetic acid formation. J Microbiol Biotechnol 9(6):789–793
26. San K-Y, Bennett G-N, Aristidou A-A, Chou C-H (1994) Strategies in high-level expression of recombinant protein in *Escherichia coli*. Ann NY Acad Sci 721:257–267
27. De Mey M, Lequeux GJ, Beauprez JJ, Maertens J, Van Horen E, Soetaert WK, Vanrolleghem PA, Vandamme EJ (2007) Comparison of different strategies to reduce acetate formation in *Escherichia coli*. Biotechnol Prog 23(5):1053–1063
28. Varma A, Palsson BO (1994) Stoichiometric flux balance models quantitatively predict growth and metabolic by-product secretion in wild-type *Escherichia coli* W3110. Appl Environ Microbiol 60(10):3724–3731
29. Mahadevan R, Edwards JS, Doyle F Jr (2002) Dynamic flux balance analysis of diauxic growth in *Escherichia coli*. Biophys J 83(3):1331–1340
30. Luo R-Y, Liao S, Tao G-Y, Li Y-Y, Zeng S, Li Y-X, Luo Q (2006) Dynamic analysis of optimality in myocardial energy metabolism under normal and ischemic conditions. Mol Syst Biol 2:71
31. Segrè D, Vitkup D, Church GM (2002) Analysis of optimality in natural and perturbed metabolic networks. PNAS USA 99:15112–15117
32. De Mey M, Maertens J, Lequeux GJ, Soetaert WK, Vandamme EJ (2007) Construction and model-based analysis of a promoter library for *E. coli*: an indispensable tool for metabolic engineering. BMC Biotechnol 7:34
33. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K12 using PCR products. PNAS USA 97(12):6640–6645
34. Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor Press, Cold Spring Harbor
35. Hanahan D, Jessee J, Bloom FR (1991) Plasmid transformation of *Escherichia coli* and other bacteria. Meth Enzymol 204:63–113
36. Dalcin L, Paz R, Storti M, D'Elia J (2008) MPI for python: performance improvements and MPI-2 extensions. J Parallel Distributed Comput 68(5):655–662
37. Buchholz A, Hurlebaus J, Wandrey C, Takors R (2002) Metabolomics: quantification of intracellular metabolite dynamics. Biomolecular Eng 19:5–15
38. Chassagnole C, Noisommit-Rizzi N, Schmid JW, Mauch K, Reuss M (2002) Dynamic modeling of the central carbon metabolism of *Escherichia coli*. Biotechnol Bioeng 79(1):53–73
39. Hoque A, Ushiyama H, Tomita M, Shimizu K (2005) Dynamic responses of the intracellular metabolite concentrations of the wild-type and *pykA* mutant *Escherichia coli* against pulse addition of glucose or NH_3 under those limiting continuous cultures. Biochem Eng J 26:38–49
40. Schaub J, Mauch K, Reuss M (2008) Metabolic flux analysis in *Escherichia coli* by integrating isotopic dynamic and isotopic staionary ^{13}C labeling data. Biotechnol Bioeng 99(5):1170–1185

41. Lequeux G, Van der Heijden R, Van den Broeck S, Vanrolleghem PA (2004) Computational methods to determine conserved moieties and parallel pathways in metabolic network models. In: Proceedings of 9th IFAC conference on computer applications in biotechnology CAB9, Conference, Nancy, France

42. Gokarn RR, Eiteman MA, Altman E (2000) Metabolic analysis of *Escherichia coli* in the presence and absence of the carboxylating enzymes phosphoenolpyruvate carboxylase and pyruvate carboxylase. Appl Environ Microbiol 66(5):1844–1850