Appl Microbiol Biotechnol (2006) 72: 968–974
DOI 10.1007/s00253-006-0357-y

Hal Alper · Kohei Miyaoku · Gregory Stephanopoulos

Characterization of lycopene-overproducing *E. coli* strains in high cell density fermentations

Received: 11 November 2005 / Revised: 20 January 2006 / Accepted: 27 January 2006 / Published online: 25 February 2006

Abstract Previous work identified two recombinant strains of *Escherichia coli* capable of significant lycopene overproduction. These strains were constructed by superimposing the deletion of three genes, selected through combinatorial and systematic searches of the metabolic landscape, onto a previously engineered strain overexpressing critical genes in the lycopene biosynthesis pathway. In this paper, we characterize the performance of these two strains in comparison to the parental, pre-engineered strain. Specifically, high cell density fermentations were performed after identifying optimized putative operating parameters. High oxygen levels and increased pH values were found to be critical for increasing both specific and volumetric product titers. Carbon balances suggest linkages between glutamate, NADPH, formate, and alanine levels with lycopene overproduction. Furthermore, lycopene production reached nearly 220 mg/l from approximately 27 g dry cell weight/l in these reactors, which is the highest value reported to date for *E. coli*.

Introduction

Engineering strains for the overproduction of isoprenoid-based molecules is of significant interest due to the diversified base of products accessible through this molecular gateway. Branching from the five-carbon unit of isopentenyl-pyrophosphate, it is possible to create carotenoids, quinones, and even precursors for desirable pharmaceuticals such as Taxol and Artemesinin (Huang et al. 2001; Martin et al. 2003). The carotenoids are of interest due to its antioxidant, UV protecting, and, natural food colorant properties (Lee and Schmidt-Dannert 2002). Lycopene is a major precursor to downstream, modified carotenoids (Sandmann 2002). Lycopene production in *Escherichia coli* requires the heterologous expression of the *crtEBI* genes to encode the polymerization of isopentenyl to the 40-carbon molecule of lycopene. However, like most secondary metabolites, these molecules are energetically expensive and produced in low yields before the stationary phase. The vast majority of product accumulation typically occurs in the stationary phase.

Recently, we have made advances in the creation of host strains capable of overproducing the red pigment carotenoid lycopene. By starting with a stoichiometric model of *E. coli* metabolism and a pre-engineered parental strain, we selected a number of single and multiple gene knockout targets which led to the creation of a triple knockout strain Δ*gdhA* Δ*aceE* Δ*fdhF* which exhibited a 40% increase in lycopene production in 15 h (Alper et al. 2005b). This stoichiometric or systematic analysis was complemented by a separate combinatorial gene knockout search mediated by random transposon mutagenesis yielding another set of gene knockout targets (Alper et al. 2005a). Upon creating two distinct classes of gene targets (systematic and combinatorial), we sought to uncover the topology of the metabolic landscape spanned by these two classes by creating and assaying 64 mutant strains whose genotypes span all combinations of these systematic and combinatorial gene knockout targets. The visualization of the metabolic landscape uncovered the presence of two globally optimum strains with respect to lycopene accumulation: one, a purely stoichiometric-based strain (Δ*gdhA* Δ*aceE* Δ*fdhF*), and the other, the result of combining the systematic and combinatorial targets (Δ*gdhA* Δ*aceE* Δ$_{p}$*yjiD*) (Alper et al. 2005a).

Initial shake-flask fermentations in glucose minimal media suggested that these two global maxima strains behaved similarly, with each producing 11,000 ppm (μg

H. Alper · K. Miyaoku · G. Stephanopoulos (✉)
Department of Chemical Engineering,
Massachusetts Institute of Technology,
Room 56-469, Cambridge
MA 02139, USA
e-mail: gregstep@mit.edu
Tel.: +1-617-2534583
Fax: +1-617-2533122

K. Miyaoku
Mitsubishi Chemical Group,
Science and Technology Research Center,
1000 Kamoshida-cho Aoba-ku,
Yokohama 227-8502, Japan

lycopene/g dry cell weight) over the course of a 48-h fermentation. However, a more optimized staged glucose feed experiment suggested that the Δ*gdhA* Δ*aceE* Δ*pyjiD* strain had a higher rate of production reaching 18,000 ppm in just 24 h (compared with 40 h for the Δ*gdhA* Δ*aceE* Δ*fdhF* strain) (Alper et al. 2005a). In this study, we sought to more rigorously characterize the production rates, profiles, and carbon balance of these engineered strains using high cell density fermentations with controlled glucose feeding.

Materials and methods

Bacterial strains and media

E. coli K12 PT5-*dxs*, PT5-*idi*, PT5-*ispFD*, provided by the DuPont company (Yuan et al. 2006), was used as the lycopene expression strain when harboring the pAC-LYC plasmid containing the *crtEBI* operon (Cunningham et al. 1994). Over-expression of *dxs*, *idi*, and *ispFD* were chromosomally incorporated without an antibiotic marker through promoter delivery (Yuan et al. 2006). Reactor seed cultures were grown overnight in 50-ml cultures at 37°C with 225 rpm orbital shaking in either 2× M9-minimal medium (Alper et al. 2005a) or R-medium (Riesenberg et al. 1991) containing 5 g/l D-glucose and 68 μg/ml chloramphenicol. All experiments were performed in replicate to validate data and assess statistical variability. Cell density was monitored spectrophotometrically at 600 nm. M9 minimal salts were purchased from US Biological and all remaining chemicals were from Sigma-Aldrich.

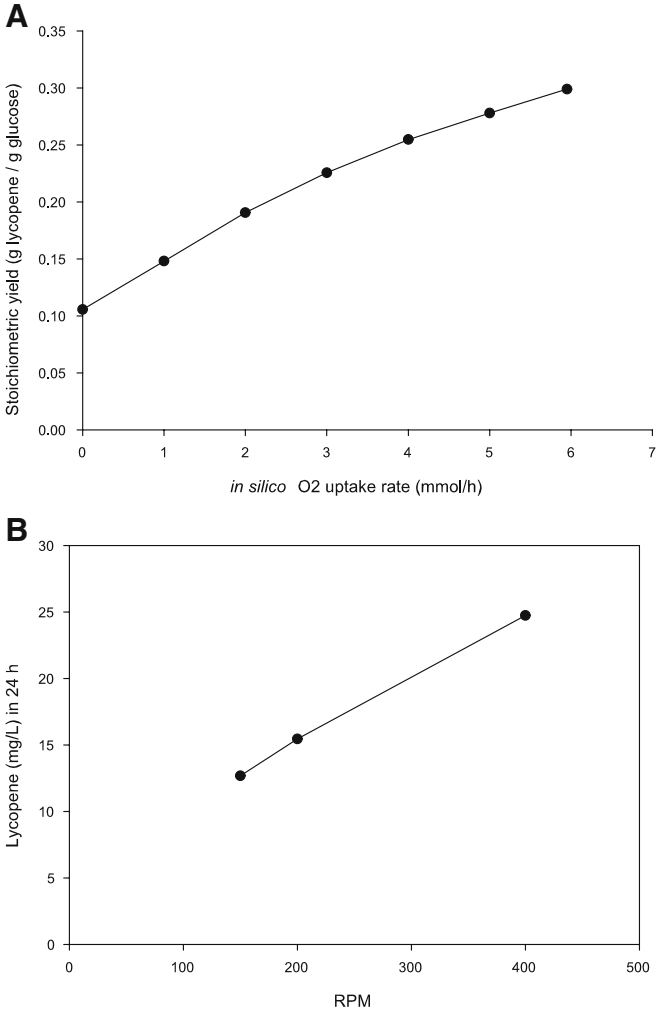

Fig. 1 *In silico* and fermentation-based investigation on the impact of oxygen on lycopene production. **a***In silico* analysis using global stoichiometric models indicates that the maximum stoichiometric yield (g lycopene/g glucose) increases as a function of the oxygen uptake rate. **b** Trial batch fermentations for the Δ*gdhA* Δ*aceE* Δ*pyjiD* strain in 2× M9 media with a staged glucose feed show an increasing trend of volumetric lycopene production as a function of agitation. These results qualitatively match the *in silico* predictions

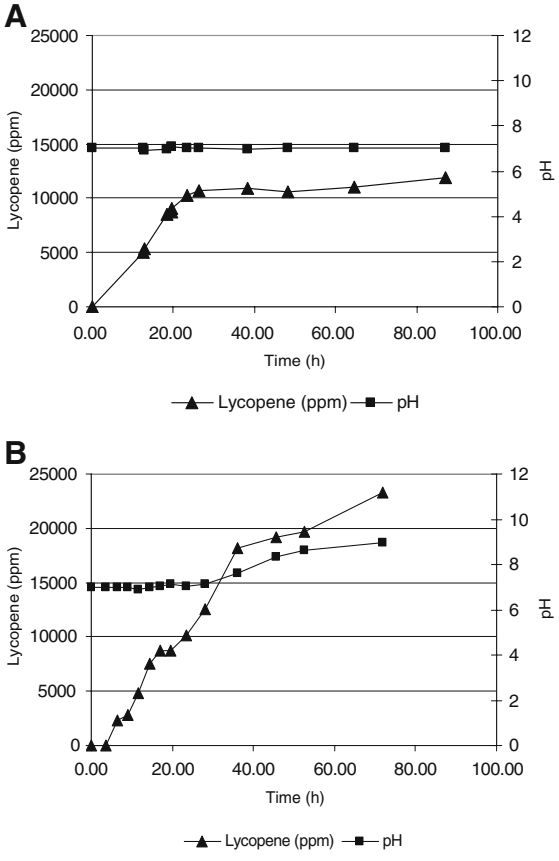

Fig. 2 Impact of pH control on lycopene production. pH control strategies were varied for Δ*gdhA* Δ*aceE* Δ*pyjiD* cultures in a 2× M9 media batch fermentation using a staged glucose feed. **a** For the case of a constant pH of 7.0 controlled with both acid and base, the specific lycopene production (in parts per million) plateaus after around 24 h. However, cell density continues to increase after this point and, therefore, lycopene production increases at the same rate at which it is being diluted by growth. **b** When the reactor is only controlled with base and allowed to be at or above pH 7.0, the lycopene production shows significant increases past the 24-h timepoint, which correlates to drastic increases in the pH level; however, it is not accompanied by a significant increase in cell density. A comparison of the profiles suggests that partial pH control is a more favorable condition for increasing specific yields

Fermentation conditions

All fed-batch fermentations were conducted in 1.5-l Applikon vessels containing an initial volume of 500 ml (for M9-based cultures) or 600 ml (for R-media-based cultures). A starting inoculum of 4% by volume (for M9-cultures) and 1% by volume (for R-media) was used. pH was measured and controlled online using NH_4OH and HCl, as appropriate for the desired control strategy. Temperature was regulated and controlled at 37°C through water bath circulation. Glucose concentration was monitored and controlled online at a setpoint of 0.45 g/l using a YSI 2700 biochemistry analyzer connected to a pump with a feedstock of 200 g/l glucose with 0.1% antifoam (the set sampling interval was 20 min). Agitation speed was increased every 2 h for the high cell density fermentations to obtain a linear increase from 400 to 1,100 rpm. Samples were taken every 2 h for organic acid, amino acid, lycopene, and biomass measurements. Online off-gas analysis was performed using an MGA1600 mass spectrometer. Inlet air was supplied for aeration at a pressure of 15 psig.

Lycopene assays

Intracellular lycopene was extracted from 1 ml of bacterial culture for lower cell densities (below 30) and from 100 μL of bacterial culture for cell densities above 30. The cell pellet was washed and then extracted in 1 ml of acetone at 55°C for 15 min with intermittent vortexing. The lycopene content in the supernatant was quantified through absorbance at 475 nm (Alper et al. 2005a), and concentrations were calculated through a standard curve and appropriate dilution factor. The entire extraction process was performed in reduced light conditions to prevent photobleaching and degradation. Cell mass was calculated by correlating dry cell with OD600 for use in parts per million (mg lycopene/g dry cell weight) calculations.

Organic and amino acid measurements

A Bio-Rad Aminex HPX-87H reverse phase column with a Hewlett Packard 1050 high-performance liquid chromatography (HPLC) system was used to measure acetate and formate. The column was run in an isocratic mode at a flow rate of 0.6 ml/min with a mobile phase consisting of 0.005 N H_2SO_4 at 45°C. Detection was done by UV absorbance at 210 nm. Amino acids were analyzed as *ortho*-phthaldialdehyde derivatives using an AminoQuant (Agilent Technologies, Palo Alto, CA, USA) 2.1-mm bore reversed phase column with a Hewlett Packard 1050 HPLC system which allowed full automation of derivatization, chromatography, data acquisition, and data evaluation. Detection was done by UV absorbance at 338 nm, and the column was run at 40°C in a gradient mode at a flow rate of 0.45 ml/min consisting of two buffers: buffer A contained 20 mM Na-acetate, 50 mM tetrahydrofuran (HPLC-grade, Fluka Chemical, Ronkonkoma, NY, USA), and 2 mM triethylamine (HPLC-grade, Fulka Chemical) at pH 7.2. Buffer B contained 20 mM Na-acetate, pH 7.2, methanol

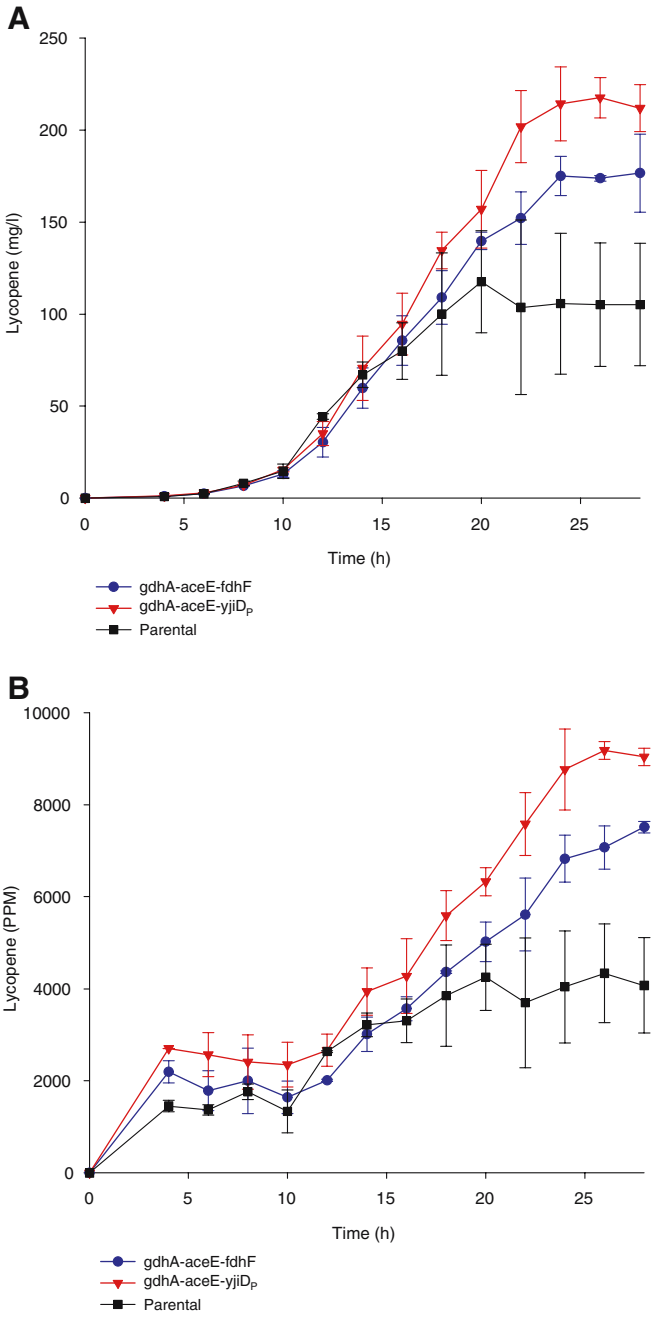

Fig. 3 Lycopene production in the reactors. **a** Lycopene (presented in milligrams/liter) accumulates as a growth-associated product and growth-independent product. In the parental strain, lycopene levels remain nearly constant after 15–18 h. However, the two engineered strains both exhibit significant increases in lycopene levels well after cell growth stops. Furthermore, the production profiles of the two engineered strains are distinct. **b** The specific lycopene content in parts per million (μg/g dcw h). This *graph* illustrates the significant difference in the lycopene content per cell in the engineered strains compared with the parental strain. As with the volumetric productivity, accumulation continues for the engineered strains after the 15- to 18-h timepoints. Average values for the replicate experiments are presented in both graphs

(HPLC-grade, Mallincroft), and acetonitrile (HPLC-grade, Mallincroft) at a volumetric ratio of 20:20:40.

Results

Determination of optimal fermentation parameters

Previous work (Alper et al. 2005a) indicated that dry cell weight (dcw) specific lycopene productivity (as measured in micrograms of lycopene per cell mass) could be increased by altering salt concentrations and optimizing glucose feeding profiles. A 2× M9 medium was found to be optimal for our purposes (Alper et al. 2005a). Before optimizing the glucose feed, we sought out to investigate how additional key fermentation control parameters such as agitation speed and pH influence the production profiles of lycopene. All reactors were set at 37°C, which is the optimal temperature for cell growth.

Agitation speed was investigated as a putative bioreactor parameter responsible for controlling dissolved oxygen content and maximum cell density. Stoichiometric analysis (Alper et al. 2005b) suggested that volumetric lycopene yield in *E. coli* from glucose increases with oxygen uptake rate due to the large energy requirement of lycopene production (eight CTPs and eight ATPs per mole) (Fig. 1a). We carried out a series of cultures at various agitation speeds, and Fig. 1b presents the volumetric production level of lycopene after 24 h as a function of the sampled agitation speeds. This timepoint was chosen as it represents the time at which glucose had been fully utilized from the last pulsed feed. The increasing trend and higher volumetric productivities at increased agitation speeds matched qualitatively with the findings of stoichiometric analysis. These results indicated that the lycopene fermentations should be run under aerobic conditions during times of balanced growth and glucose utilization.

The control of pH in a bioreactor can influence cell growth and, consequently, secondary metabolite production. Furthermore, pH changes in a reactor could introduce adverse effects on the growth rate of the culture. Varying strategies of double-side and partial (single-side) pH control were tested with respect to lycopene production. As an illustration of the pH effect, a fully controlled pH 7.0 strategy was compared with a partial (only base) control strategy. Figure 2a depicts the time profile of lycopene production under a constant pH 7.0 controlled through the addition of NH_4OH and HCl. While specific lycopene productivity remains constant for times after 24 h, the cell density is still increasing. As a result, lycopene is being produced at the same rate at which it is being diluted by growth. Conversely, the profile of specific lycopene productivity under partial pH control continues to increase after 24 h and is accompanied by a significant pH increase in the stationary/production phase (Fig. 2b). This phenomenon is a good example of a supporting evidence that reduced growth rate can result in increased specific productivity levels. The observed pH increase correlated with an increased specific lycopene productivity. Under this control strategy, the total cell density did not increase appreciably after 24 h. Therefore, the results suggest that a partial pH control with only base addition allowed and with a starting pH of 7.0 had the better production profile of lycopene.

High cell density fermentations

As lycopene is stored as an intracellular product in the membrane (Fraser and Sandmann 1992), special considerations must be made for identifying optimal parameters. Optimal parameters must be able to promote lycopene production during both growth phase and stationary phase. During growth phase, a primary concern is the volumetric production (milligrams of lycopene per liter in the reactor)

Table 1 Growth and lycopene phenotypes of strains in fed-batch reactor

		Parental	Δ*gdhA* Δ*aceE* Δ*fdhF*	Δ*gdhA* Δ*aceE* Δ$_{\text{P}}$*yjiD*
Growth phenotype	μ (h^{-1})	0.504 (.003)	0.408 (.016)	0.446 (.019)
	Maximum OD_{600}	92.1 (4.4)	89.5 (3.8)	84.6 (1.7)
	Biomass (g dcw/l)	29.0 (1.4)	28.2 (1.2)	27.0 (0.5)
Lycopene phenotype	Specific production (μg/g dcw h)	242.9 (70.1)	388.0 (30.6)	496.3 (15.6)
	Bioreactor productivity (mg/bioreactor h)	12.3 (2.8)	13.7 (0.5)	17.9 (0.6)
	Total produced (mg) (24 h)	139.1 (39.4)	186.2 (20.4)	228.5 (10.6)
	Maximum (mg/l)	132.7 (38.3)	176.5 (12.8)	221.6 (7.3)
	Overall yield of reactor (mg/g glucose) (24 h)	1.58 (0.43)	2.15 (0.26)	2.61 (0.11)
Side-products (24 h)	Acetate (g/l)	14.84 (7.56)	13.26 (0.38)	10.00 (1.97)
	Alanine (mM)	0.59 (0.44)	0.38 (0.31)	0.28 (0.29)
	Formate (g/l)	0.60 (0.15)	1.16 (0.58)	0.44 (0.22)
	Glutamate (mM)	10.08 (1.19)	5.80 (2.99)	2.47 (0.86)

The average growth rates and lycopene production rates are presented from duplicate high cell density fermentations. Numbers in parenthesis represent the standard deviation of these values from two separate trials for each strain. The two optimized strains exhibit increased production rates and decreased growth rates compared with the parental strain. The engineered strains were also more stable and reproducible as exhibited by the smaller standard deviations in all categories

and optimization of product yield (grams of lycopene per gram of glucose). During stationary phase, the specific productivity (microgram lycopene per gram dry cell weight) will increase as cell growth has ceased. The identification of bioreactor conditions which encourage both modes of lycopene production is important in creating an optimized process. The trade-off relationship between cell growth rate and specific lycopene productivity is an apparent limitation in obtaining high lycopene volumetric productivity, which is an industrially important process metric. To release this limitation, fed-batch fermentations were conducted that allowed high cell density to be obtained during the growth phase followed by production during the stationary phase.

Fermentations of the parental control strain along with the two engineered triple knockout strains were conducted using a controlled glucose feed with partial pH control (only base addition) and high aeration rates (by continually increasing the revolutions per minute). This feeding and control strategy was optimized based on the trial fermentations described above. The production profiles of lycopene in milligram per liter and parts per million are presented in Fig. 3a,b, respectively. All strains exhibit a nearly similar growth-associated volumetric lycopene production rate. However, significant differences between the engineered strains and the parental strain are seen after around 15–18 h from the start of the reaction. Lycopene accumulations (both specific and volumetric) remain constant after 15–18 h for the parental strain. However, significant increases in the volumetric productivity and specific productivity are seen in both engineered strains. Furthermore, it is clear that the production profiles are indeed distinct for these two strains, suggesting evidence that the different genotypes are leading to different production phenotypes. The fermentation metrics for the growth characteristics and lycopene production rates are summarized in Table 1. The two optimized strains exhibit increased productivities and decreased growth rates

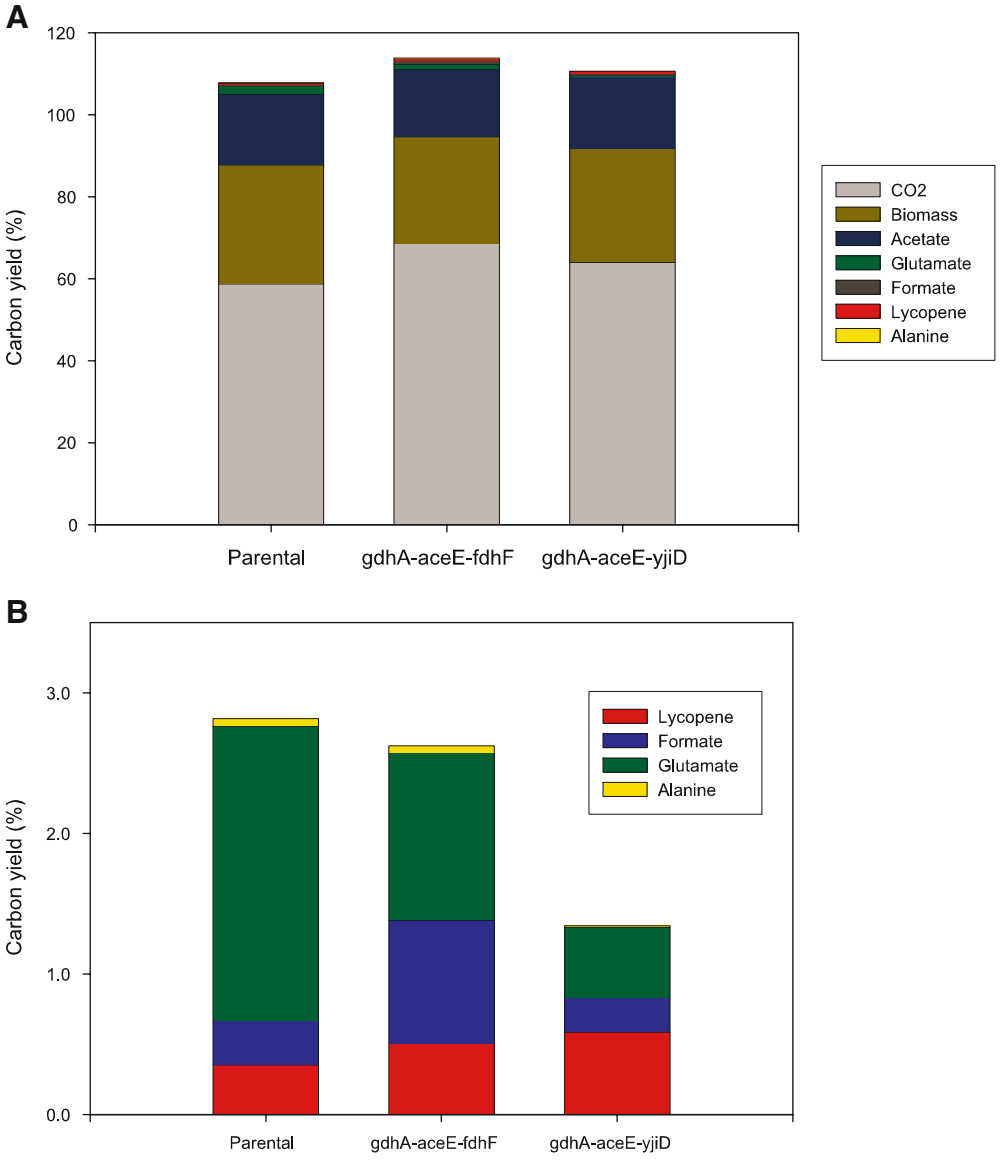

Fig. 4 Carbon yield balances for fermentors. Organic and amino acids, biomass, and lycopene were measured off-line, while carbon dioxide was measured online. **a** The average final carbon yields are presented for each of the strains. Most of the glucose fed to the reactor went to carbon dioxide and biomass. **b** A more detailed analysis of some contributors to the carbon balance indicates a significant glutamate level in the parental strain. Furthermore, all three side-components (glutamate, formate, and alanine) were reduced in the Δ*gdhA* Δ*aceE* Δ_{P}*yjiD* triple mutant

compared with the parental strain. The engineered strains also yielded more robust and reproducible fermentations as is exhibited by the smaller standard deviations in all categories. The fermentor pH was controlled according to the one-sided (partial) control strategy using only base (NH_4OH) addition. Similar to the trial profile in Fig. 2a, the pH did increase during the production phase to reach values upwards of 8.0 at around the 24-h timepoint.

Carbon balances

Samples were taken from the bioreactors in 2-h intervals to measure organic and amino acid levels as well as the lycopene level and biomass. The total carbon balances (Fig. 4a) suggest similar distributions of carbon among the products of all the strains with a slight increase in carbon dioxide contribution for the engineered strains. However, a more detailed analysis of marginal contributors to the carbon balance (Fig. 4b) reveals significant differences in the carbon fluxes. A significant amount of glutamate was detected in the media for the parental strain over the two engineered strains. Furthermore, lycopene production was inversely related to the level of glutamate secreted by the various strains. Stoichiometric analysis also previously indicated that a reduction in the glutamate flux (especially through *gdhA*) is related to an increase in lycopene production, presumably through the resulting increase in NADPH availability (Alper et al. 2005a). Furthermore, formate levels were substantially higher in the Δ*gdhA* Δ*aceE* Δ*fdhF* mutant, which is designed to limit the loss of carbon (particularly pyruvate) through formate and its subsequent byproducts. It is interesting to note that the other engineered strain (Δ*gdhA*, Δ*aceE*, Δ_P*yjiD*) showed a substantial decrease in both formate and glutamate levels, perhaps providing some clues to the function of the hypothetical protein encoded by *yjiD*. Moreover, alanine was also detected in the media and, despite its small contribution, was found to contribute fivefold less in the Δ*gdhA*, Δ*aceE*, Δ_P*yjiD* strain than the other two strains, which were nearly similar.

Discussion

Lycopene-overproducing strains were previously engineered by the combination of systematic and combinatorial gene knockout targets. In particular, two strains emerged as potentially equal global maxima. By performing these fermentations, it was possible to discover significant differences in the lycopene production and the byproduct profiles of the two engineered strains. In addition, by investigating putative bioreactor parameters and byproduct profiles, it was possible to validate some trends suggested by previous stoichiometric modeling. In particular, the importance of oxygen, growth rate, and glutamate flux on lycopene production were seen through these various fermentations. Consequently, these stoichiometric models provided key insight into the development of fermentation strategy and strain development. Furthermore, these results suggest a key role for glutamate modification as an essential component of any carotenoid-overproducing strain.

These studies confirmed that the production profiles of the two engineered strains are indeed distinct, suggesting that the different genotypes are leading to a different production phenotype. A further analysis of the secreted metabolite levels was able to suggest some of the underlying factors related to lycopene production. Recently, we have analyzed these strains at the level of gene transcription and metabolomic profiles in an effort to gain a further understanding of the mode of action of these gene knockouts. These preliminary results suggest that the true phenotype of these strains must be assessed at multiple dimensions rather than strictly by the production level of lycopene. Furthermore, rational design using the collected metabolomic and genomic data and additional explorations of the metabolic landscape can further identify gene knockout or over-expression targets which may optimize these strains further and increase lycopene yields. Recent experiments (through the introduction of additional genes) with these strains in smaller-scale fermentations yielded similar titers for downstream carotenoids such as b-carotene (data not shown). These preliminary results provide the prospect of using these host strains for the production of other chiral or non-natural carotenoids which are inaccessible through chemical synthesis. Nevertheless, by the end of this study, the optimized high cell density fermentations of these engineered *E. coli* strains resulted in titers of around 220 mg/l. These titers are the highest reported in literature to date for the production of 40-carbon carotenoids in *E. coli*.

Acknowledgements We acknowledge financial support of this work by the DuPont-MIT Alliance. Hal Alper and Kohei Miyaoku contributed equally to this work.

References

Alper H, Miyaoku K, Stephanopoulos G (2005a) Construction of lycopene-overproducing *E. coli* strains by combining systematic and combinatorial gene knockout targets. Nat Biotechnol 23(5):612–616

Alper H, Yong-Su J, Moxley J, Stephanopouloz G (2005b) Identifying gene targets for the metabolic engineering of lycopene biosynthesis in *Escherichia coli*. Metab Eng 7 (3):155–164

Cunningham FX Jr, SZ, Chamovitz D, Hirschberg J, Gantt E (1994) Molecular structure and enzymatic function of lycopene cyclase from the cyanobacterium *Synechococcus* sp strain PCC7942. Plant Cell 6(8):1107–1121

Fraser P, Sandmann G (1992) In vitro assays of three carotenogenic membrane-bound enzymes from *Escherichia coli* transformed with different *crt* genes. Biochem Biophys Res Commun 185 (1):9–15

Huang Q et al (2001) Engineering *Escherichia coli* for the synthesis of taxadiene, a key intermediate in the biosynthesis of taxol. Bioorg Med Chem 9:2237–2242

Lee PC, Schmidt-Dannert C (2002) Metabolic engineering towards biotechnological production of carotenoids in microorganisms. Appl Microbiol Biotechnol 60(1):1–11

Martin VJJ et al (2003) Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. Nat Biotechnol 21(7):796–802

Riesenberg D et al (1991) High cell density cultivation of *Escherichia coli* at controlled specific growth rate. J Biotechnol 20(1):17–27

Sandmann G (2002) Combinatorial biosynthesis of carotenoids in a heterologous host: a powerful approach for the biosynthesis of novel structures. ChemBioChem 3(7):629–635

Yuan LZ et al (2006) Chromosomal promoter replacement of the isoprenoid pathway for enhancing carotenoid production in *E. coli*. Metab Eng 8(1):79–90