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ORIGINAL ARTICLE

Over-production of β-carotene from metabolically engineered *Escherichia coli*

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Abstract To produce recombinant β-carotene in vitro, synthetic operons encoding genes governing its biosynthesis were engineered into *Escherichia coli*. Constructs harboring these operons were introduced into either a high-copy or low-copy cloning vector. β-Carotene production from these recombinant *E. coli* cells was either constitutive or inducible depending upon plasmid copy number. The most efficient β-carotene production was with the low-copy based vector. The process was increased incrementally from a 5 l to a 50 l fermentor and finally into a 300 l fermentor. The maximal β-carotene yields achieved using the 50 l and 300 l fermentor were 390 mg l^{-1} and 240 mg l^{-1}, respectively, with overall productivities of 7.8 mg l^{-1} h^{-1} and 4.8 mg l^{-1} h^{-1}.

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Introduction

β-Carotene (C_{40}) functions as a provitamin A, rendering it a particularly important carotenoid for industrial purposes. Biosynthesis of β-carotene in non-carotenogenic bacteria, including *Escherichia coli*, is possible via introducing the bacterial *crt* gene cluster from *Erwinia* species or other carotenogenic bacteria. The *crt* genes originating from *Erw. uredovora* and *Erw. herbicola* were successfully used to trigger de novo biosynthesis of lycopene (*crtE, crtI,* and *crtB*), β-carotene (*crtE, crtI, crtB*, and *crtY*) and zeaxanthin (*crtE, crtI, crtB, crtY*, and *crtZ*) in *E. coli* and other non-carotenogenic organisms (Misawa et al. 1990; Miura et al. 1998). Addition of isopentenyl pyrophosphate (IPP), a precursor of β-carotene, to culture medium sometimes enhances carotenoid production (Harker and Bramley 1999; Matthews and Wurtzel 2000; Kim and Keasling 2001). Amplification of IPP isomerase (*idi*), which converts IPP into dimethylallyl pyrophosphate (DMAPP) in the carotenoid biosynthesis pathway, may also enhance carotenoid production in such cultures (Kajiwara et al. 1997; Wang et al. 1999).

In contrast to high-level recombinant protein production methods, metabolic engineering does not require a strong promoter/terminator expression system or a high-copy plasmid. In fact, using a strong expression system might result in metabolite imbalances due to enzyme over-expression, potentially disrupting normal host cell metabolism. The presence of a high-copy plasmid could also induce a significant shift in normal host cell metabolism, thereby increasing the risk of plasmid instability. Increased demand for nucleotides concomitant with plasmid DNA replication (Birnbaum and Bailey 1991) and with translation of plasmid-encoded genes (Bentley et al. 1990; Glick 1995; Vind et al. 1993) could account for such metabolic burden issues. Therefore to decrease this metabolic burden, the choice of an appropriate promoter/terminator expression system is critical. Ideally, such a system would be amenable to tight regulation as well as maintaining proper copy number, so as not to interrupt normal host cell metabolism.

Taking into account these considerations, we constructed inducible operons optimized for high-level production of β-carotene as a model carotenoid in *E. coli*. The process was then scaled-up gradually to accommodate β-carotene production in 5 l, 50 l and 300 l fermentors.

Materials and methods

Bacterial strains, genes and culture conditions

E. coli DH5α was used for basic gene manipulations and production of β-carotene. The *crt* genes (*crtE, crtI, crtB*, and *crtY*) governing β-carotene biosynthesis were cloned from *Erwinia uredovora* (ATCC(R) 19321). IPP isomerase (*idi*) was cloned from the *E. coli* K-12 strain W3110. Recombinant *E. coli* strains were then grown on 2× YT plates supplemented with ampicillin (100 μg ml^{-1}) at 25°C and then inoculated into a 500 ml baffled flask containing 50 ml of 2× YT broth media (100 μg ampicillin ml^{-1}). Primary cultivations were incubated for 27 h in a rotary shaking incubator at 37°C and 180 rpm. Production of β-carotene in transformants harboring the low-copy vector was induced with 1 mM arabinose, whereas *E. coli*

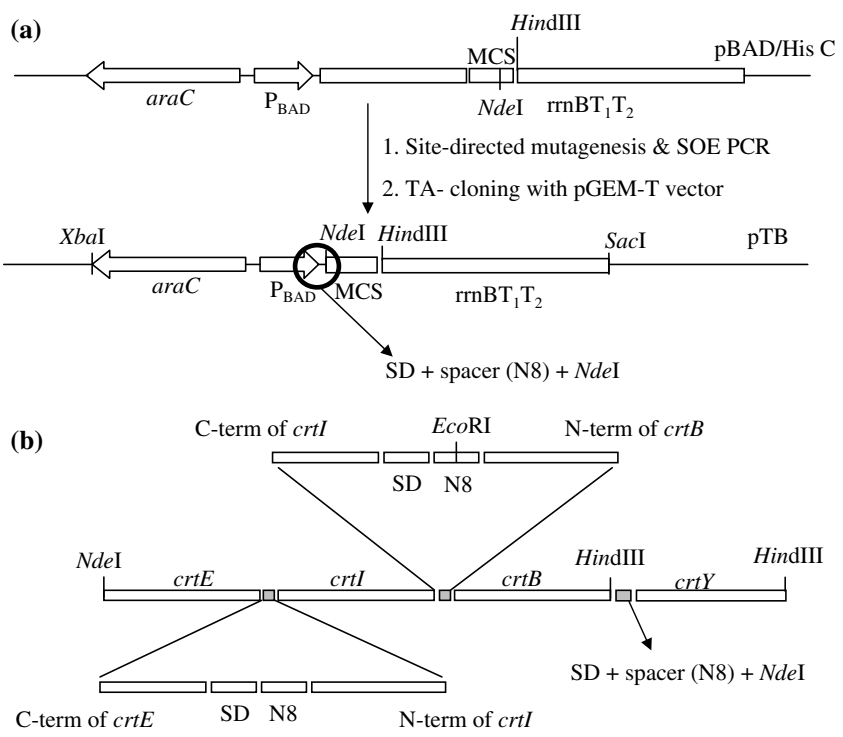

Fig. 1 Descriptions of the *araBAD* expression cassette and the synthetic *crt* operon. (**a**) Schematic diagram of the *araBAD* expression cassette; (**b**) Map of the synthetic operon composed of *crtE, crtI, crtB and crtY* from *Erw. uredovora*

harboring the high-copy vector were grown in the absence of arabinose.

Construction of synthetic *crt* operons and vectors

The *araBAD* promoter/terminator expression system used in this study was derived from pBAD/His C (Invitrogen) with some modifications (Fig. 1a). For the construction of pTB, an expression vector harboring the *araBAD* promoter/terminator cassette, we performed site-directed mutagenesis and splicing via overlapping extension (SOE) PCR, followed by cloning of the resultant PCR products into pGEM-T vector (Promega). Figure 1b details the construction method yielding synthetic *crt* operons. Briefly, amplified products encoding crtE(SD) and crtI(SD) were used as templates to acquire crtEI using SOE PCR. After digestion of crtEI with *Nde*I & *Hin*dIII, the fragment was incorporated into pTB that had been digested with the same enzymes (designated pTB-EI). Plasmid pTB-EIB was then constructed by ligating the amplified crtB product, into the *Eco*RI/*Hin*dIII site of pTB-EI. Finally, the β-carotene-producing vector pTB-EIBY was constructed by inserting the crtY PCR product into the dephosphorylated *Hin*dIII site of pTB-EIB. To construct the low-copy vector pTB-EIBYrop, we inserted the *rop* gene from pBR322, into the dephosphorylated *Spe*I site of pTB-EIBY. A vector containing *idi*, designated pAT-rc::idi, was constructed by inserting a *trc* cassette harboring the *idi* gene from *E. coli* W3110 into

Table 1 List of PCR products, primers and vectors used in this study

Primer name	Sequence (5′ → 3′)	Template PCR Product
F-araC/*Xba*I	caatctagataattatgacaacttgacg	pBAD/His A
R-BAD/*Nde*I	gatctcgagatcgatcatatgtaattcctcctg	araC-BAD
F-MCS	caggaggaattacatatg atcgat ctcgagatctgcagc	pBAD/His A
R-Ter	gagagctccagataaaacgaaag	Ter
F-araC/*Xba*I	caatctagataattatgacaacttgacg	araC-BAD, Ter
R-Ter	gagagctccagataaaacgaaag	araC-BAD/Ter
F-crtE/*Nde*I	agcatatgacggtctgcgcaaa	*Erw. uredovora*
R-crtE(SD)	attgtaatcctccttattaactgacggcagcg	crtE(SD)
F-crtI(SD)	taaggaggattacaatatgaaaccaactacggta	*Erw. uredovora*
R-crtI(SD)/*EH*	gaagctt gaattcctccttatcatatcagatcctccagtact	crtI(SD)
F-crtE/*Nde*I	agcatatgacggtctgcgcaaagaagctt	crtE(SD), crtI(SD)
R-crtI(SD)/*EH*	gaattcctccttatcatatcagatcctccagtact	crtEI
F-crtB(N8)/*E*	cagaattcaatatgaataatccgtcgttactc	*Erw. uredovora*
R-crtB/*HE*	aggaattc aagcttctagagcgggcgctgccagag	crtB
F-crtY/*H*	caaagcttaaggaggattacaatatgcaaccgcattatgatctgattctc	*Erw. uredovora*
R-crtY/*H*	caaagctttaacgatgagtcgtcat	crtY
F-rop/*Spe*I	caactagtacacggaggcatcagtgac	pBR322
R-rop/*Spe*I	ggactagtatgtgtcagaggttttcacc	rop
Vector name	Descriptions	References
pBAD/His C	Commercial cloning vector, araBAD cassette	Invitrogen
pGEM-T vector	Commercial cloning vector	Promega
pBR322	Commercial cloning vector, low-copy, ColE1	NEB[1]
pACYC 177	Commercial cloning vector, low-copy, p15A	NEB[1]
pTB	pGEM-T vector harboring araBAD cassette	This study
pTB-EI	pTB harboring *crtE* and *crtI* in MCS region	This study
pTB-EIB	pTB-EI harboring *crtB* in MCS region	This study
pTB-EIBY	pTB-EIB harboring *crtY* in MCS region, high-copy	This study
pTB-EIBYrop	pTB-EIBY harboring *rop* gene, low-copy	This study
pTrc::idi	pTrc(BP) harboring idi in MCS region, w/o $lacI^q$	Laboratory stock
pATrc::idi	pACYC177 harboring trc::idi, low-copy	This study

Underline is a recognition site of restriction enzyme

[1]New England Biolabs

the *Bam*HI/*Pst*I site of pACYC177 (New England Biolabs). The names of the PCR products, primers, and vectors are listed in Table 1.

Extraction and quantification of β-carotene

β-Carotene was extracted from *E. coli* with acetone at 55°C for 15 min, as previously described (Kim and Keasling 2001). Cell extracts were analyzed and quantified by HPLC on a C_{18} 5 μm column with ethanol/methanol (70:30, v/v) at 1 ml min^{-1}.

Over-production of β-carotene using 5 l, 50 l and 300 l fermentors

Fed-batch cultures were established in 5 l, 50 l and 300 l fermentors (Kobiotech, Korea) con-

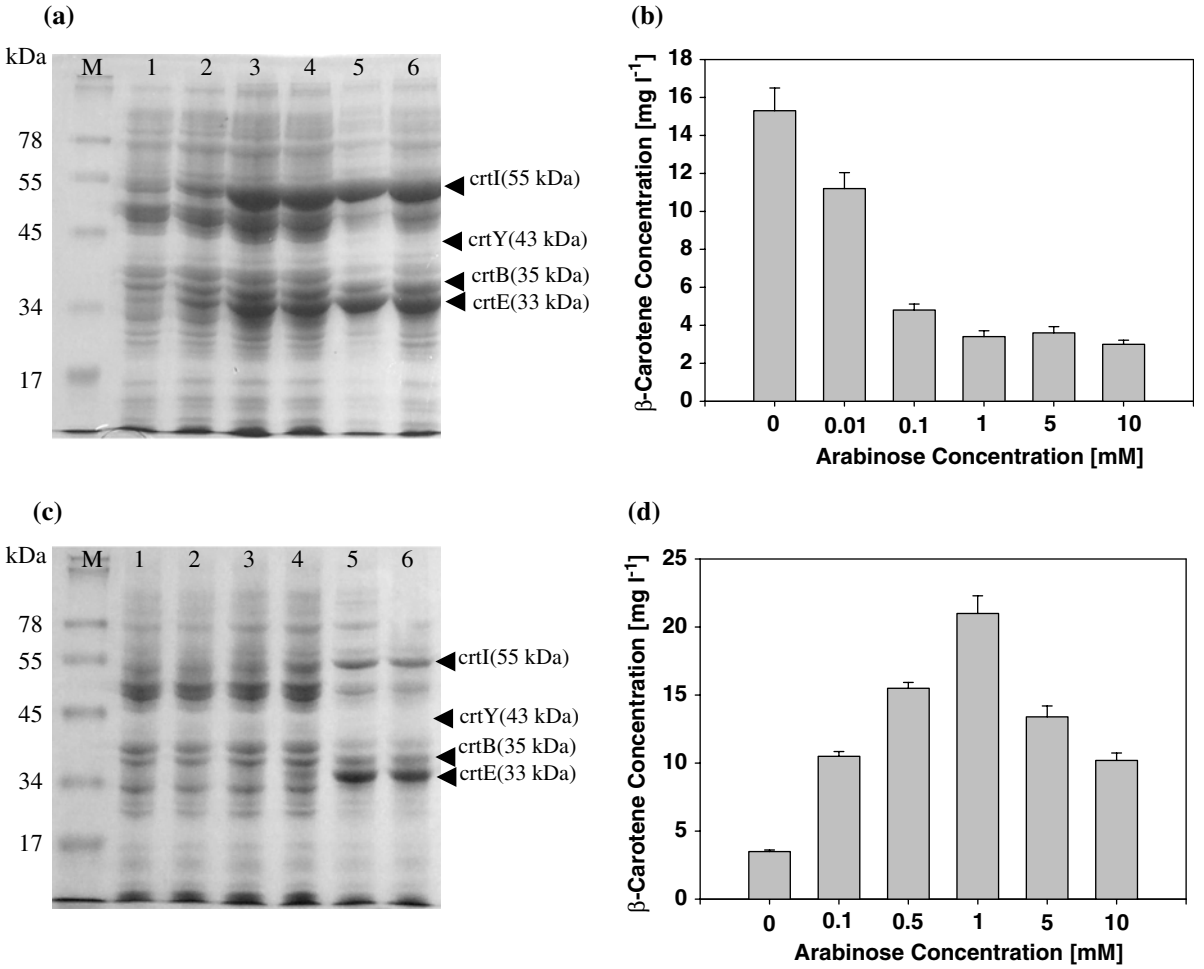

Fig. 2 Gene expression patterns and yields of β-carotene associated with DH5α harboring either high-copy (pTB-EIBY) or low-copy (pTB-EIBYrop) plasmid. The protein bands corresponding to *crtB* and *crtY* on both SDS-PAGE gels were not clearly detectable when compared with the negative control. (**a**, **b**) *E. coli* DH5α harboring pTB-EIBY; (**c**, **d**) *E. coli* DH5α harboring pTB-EIBYrop; (**a**, **c**) Results of SDS-PAGE analysis of cultures induced by different arabinose concentrations; (**b**, **d**) β-Carotene yields (mg l^{-1}) extracted from cultures induced by a range of arabinose concentrations. In (**a**), M, See Blue® plus2 Pre-stained Standard (Invitrogen); lane 1, no arabinose (control); lane 2, 0.01 mM arabinose; lane 3, 0.1 mM arabinose; lane 4, 1 mM arabinose; lane 5, 5 mM arabinose; lane 6, 10 mM arabinose. In (**c**), M, See Blue® plus2 Pre-stained Standard (Invitrogen); lane 1, no arabinose (control); lane 2, 0.1 mM arabinose; lane 3, 0.5 mM arabinose; lane 4, 1 mM arabinose; lane 5, 5 mM arabinose; lane 6, 10 mM arabinose

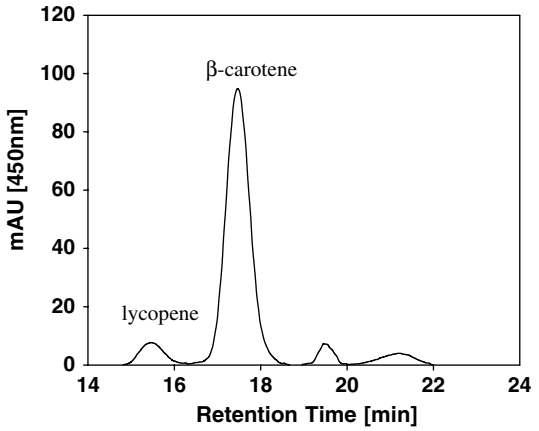

Fig. 3 HPLC analysis characterizing cell extracts from *E. coli* DH5α co-transformants harboring pTB-EIBYrop and pATrc::idi

taining 2× YT medium with 2% (w/v) glycerol at 25°C and 37°C. Seed culture was prepared in a 1-l flask containing 200 ml of 2× YT medium plus 2% (w/v) glycerol. The pH was maintained at 7.0 by adding 28% (v/v) NH_4OH. The dissolved O_2 concentration was maintained at near 30% air saturation using sufficient aeration (1 vvm) and by manually controlling the stirrer speed up to 800 rpm. Nutrient feeding solution was added into the fermentor using a pH-stat feeding strategy with a defined nutrient feeding solution (70% (w/v) glycerol plus 2% (w/v) $MgSO_4 \cdot 7H_2O$). When the pH rose to a value greater than its set point (7.0) by 0.1 due to glycerol depletion, an appropriate volume of feeding solution was then added to increase the glycerol culture broth concentration to 1 g l^{-1}. During the fed-batch fermentation, we induced *crt* genes by adding 1.5 mM arabinose when the cell dry weight reached approximately 2.56 g l^{-1}. Residual glycerol levels were measured using a free glycerol reagent (Sigma, F6428).

Results and discussion

β-Carotene production patterns vary between *E. coli* harboring high-copy versus low-copy vector

We successfully constructed a synthetic *crt* operon containing each open reading frame following a SD sequence (AGGAGG) and a spacer (N8). To optimize induction conditions, we exposed *E. coli* cultures harboring pTB-EIBY and pTB-EIBYrop (respectively) to a range of arabinose concentrations (Fig. 2). In the case of pTB-EIBY, a high-copy vector, gene expression sharply increased upon induction with 0.1 mM arabinose (Fig. 2a), yet β-carotene production decreased in response to arabinose dose (Fig. 2b). Prior studies demonstrated that carotenoid production in *E. coli* without induction is possible when leaky expression of *crt* genes is driven by lac P/O on a high-copy plasmid (Umeno et al. 2002). However, cells containing the low-copy vector pTB-EIBYrop clearly responded to arabinose dose, where maximal yields of β-carotene resulted upon induction with 1 mM arabinose (Fig. 2d). Still, we experienced difficulty in detecting protein bands induced in cultures treated with 1 mM arabinose (Fig. 2c). Furthermore, over-expression of *crt* genes correlated with repressed β-carotene production. While maximal expression of β-carotene biosynthesis genes was inducible in both populations, our findings demonstrate that intermediate expression levels of *crt* genes are necessary to decrease metabolic burden and/or achieve specific intracellular conditions. Novick and Weiner (1957) referred to this phenomenon as ''all-or-none'' or autocatalytic gene expression. Siegele and Hu (1997) reported similar autocatalytic behavior for the *ara* operon. Our data indicate that β-carotene production resulted from leaky expression of *crt* genes in the case of cells containing the high-copy vector, or by inducible expression in cells with the low-copy vector. Therefore to accomplish optimal β-carotene production levels, the low-copy based vector proved most efficient in our hands. These findings are similar to those published previously by another group (Jones et al. 2000).

HPLC analysis of cell extracts from DH5α harboring pTB-EIBYrop and pATrc::idi

Using HPLC analysis, we determined the β-carotene profile in extracts of cells co-transformed with pTB-EIBYrop and pATrc::idi. As described in Fig. 3, the profile of cell extracts at

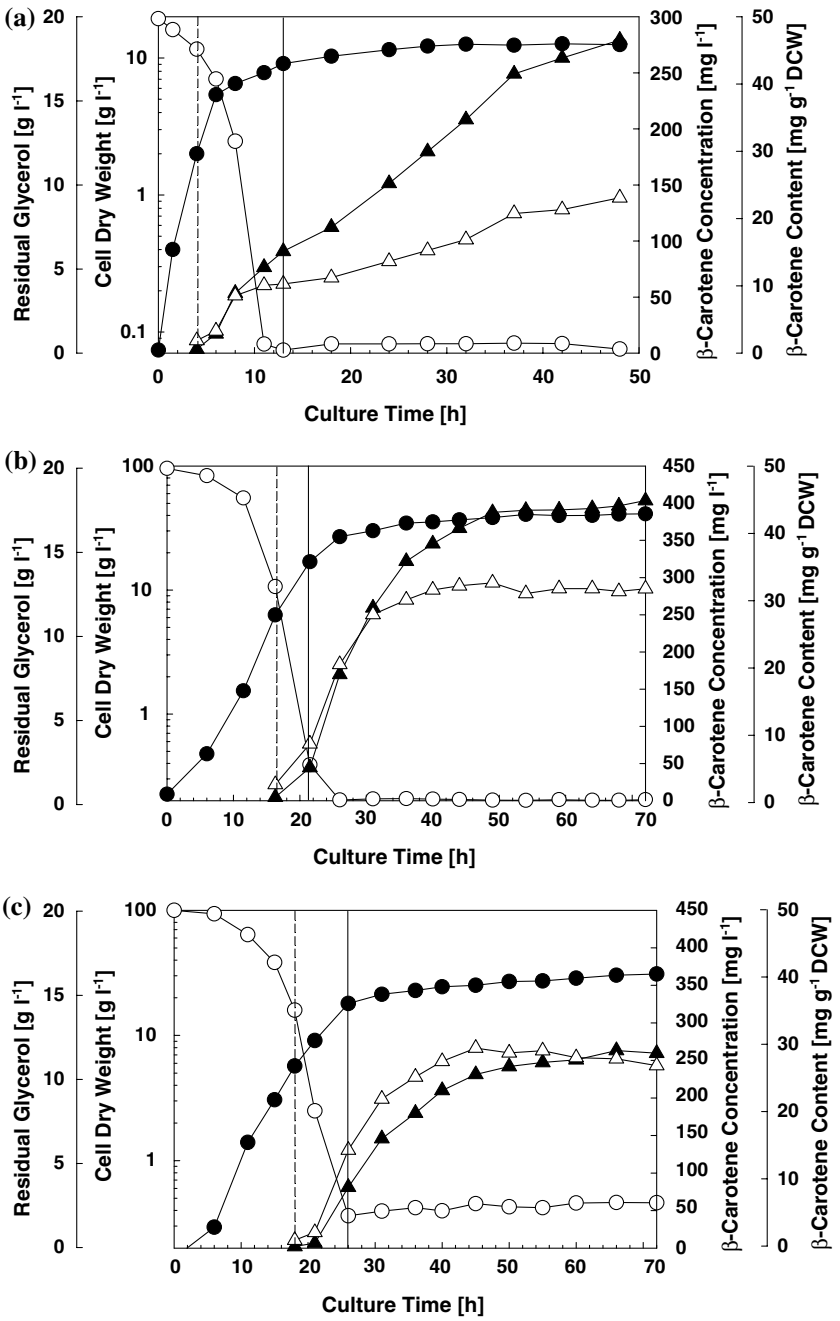

Fig. 4 Results of the scale-up process for the production of β-carotene in 5, 50, and 300 l fermentors. (**a**) Result of culture at 37°C in a 5 l fermentor; (**b**) Result of culture at 25°C in a 50 l fermentor; (**c**) Result of culture at 25°C in a 300 l fermentor. Dashed line and solid line indicate induction time and feeding start time, respectively

λ_{max} = 450 nm indicated a major and a few minor peaks. In comparison to standard carotenoids, the major peak at 17.5 min was identified as β-carotene and the peak at 15.4 min was confirmed to be lycopene. These data suggest that the synthetic operon in these cells is sufficient to convert most available lycopene into β-carotene.

Scale-up process for the production of β-carotene

We used co-transformed *E. coli* harboring pTB-EIBYrop and pATrc::idi for high-level β-carotene production. During the fermentation process, glycerol served as the primary carbon source

instead of glucose. Glucose was not chosen due to decreased cAMP levels resulting from catabolite repression of the P_{BAD} promoter (Guzman et al. 1995). As illustrated in Fig. 4, we carried out the fermentation process at two different temperature, 25°C and 37°C. Our findings indicate that 5-l jar fermentation at the lower temperature resulted in 1.5-fold β-carotene yields (data not shown). These data agree with previously reported results demonstrating high carotenoid yields obtained during a growth period of 48 h at 28°C (Sandman et al. 1999). In our experiments, β-carotene accumulated slowly after feeding in cultures incubated at 37°C (Fig. 4a), whereas accumulation proceeded more rapidly in cultures fed and maintained at the lower temperature (Fig. 4b, c). At 50 h incubation, the β-carotene yield (mg l^{-1}) from these scaled-up cultures (5, 50, and 300 l) was 270, 390 and 240 mg $l^{-1}$and the productivity (mg l^{-1} h^{-1}) levels were 5.4, 7.8 and 4.8 mg l^{-1} h^{-1}, respectively. Considering that β-carotene productivity from cultures grown in the 5 l fermentor at low temperature was 7 mg l^{-1} h^{-1} (data not shown), the 50 l fermentor process performed relatively well. In the case of the pilot-scale fermentor, the yield and the productivity of β-carotene decreased slightly compared with the 5 l and 50 l cultures. While we could not pinpoint the underlying cause of this repressed production, the sole difference that we noted was residual glycerol at 2–3 g l^{-1} in the 300 l culture. Therefore, modulating the feeding speed may improve the yield and productivity in pilot-scale fermentation processes.

For these studies, we engineered *E. coli* with a synthetic operon constructed to produce β-carotene, then confirmed its efficacy by HPLC analysis. In addition, we utilized these engineered cells to produce β-carotene on an industrial scale via an iterative process transitioning cultures from a 5 l to a 50 l, and finally to a 300 l fermentation format. Finally, we expect that the synthetic operon constructed for this study will be easily modified to produce other carotenoids via similar metabolic pathways, such as lycopene, zeaxanthin, and astaxanthin.

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