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Reduction of N-terminal methionylation while increasing titer by lowering metabolic and protein production rates in *E. coli* auto-induced fed-batch fermentation

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Abstract A standard fed-batch fermentation process using 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) induction at 37 °C in complex batch and feed media had been developed for manufacturing of a therapeutic protein (TP) expressed in inclusion bodies (IBs) by *E. coli* BL21 (DE3) driven by T7 promoter. Six unauthentic TP N-terminal variants were identified, of which methionylated TP (Met-TP) ratio was predominant. We hypothesized that lowering metabolic and protein production rates would reduce the Met-TP ratio while improving TP titer. The standard process was surprisingly auto-induced without added IPTG due to galactose in the complex media. Without changing either the clone or the batch medium, a new process was developed using lower feed rates and auto-induction at 29 °C after glucose depletion while increasing induction duration. In comparison to the standard process, the new process reduced the unauthentic Met-TP ratio from 23.6 to 9.6 %, increased the TP titer by 85 %, and the specific production yield from 210 to 330 mg TP per gram of dry cell weight. Furthermore, the TP recovery yield in the purified IBs was improved by ~20 %. Adding together, ~105 % more TP recovered in the purified IBs from per liter of fermentation broth for the new process than the standard process. The basic principles of lowering metabolic and production rates should be applicable to other recombinant protein production in IBs by fed-batch fermentations.

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Introduction

Recombinant therapeutic proteins are mainly produced by cell culture, yeast, and *E. coli* [5, 8, 29, 30]. Because of its rapid growth, well-known genetics and physiology, and low cost for manufacturing, the *E. coli* expression system has a great advantage over cell culture and yeast for production of therapeutic proteins of small size which do not need complicated glycosylation [5, 11–13, 18, 20, 22, 28]. For example, nearly 30 % of the therapeutic proteins approved by the FDA were produced in *E. coli* [12, 30]. In general, recombinant proteins produced by *E. coli* can be expressed in an insoluble form as inclusion body (IB) in the cytoplasm, in a soluble form with periplasmic expression, and/or extracellular secretion [18]. The secretion of protein into the extracellular medium is rare, and periplasmic expression often results in a low level of recombinant protein expression [20]. Significantly higher protein titers have been achieved when the recombinant proteins are expressed in IBs than those expressed in the soluble form. The clones with recombinant proteins expressed in IBs are easier to make than those expressed in the soluble form. Therefore, IB formation is widely used for industrial production of therapeutic proteins as long as IBs can be solubilized and refolded at a reasonable cost [12, 20].

It is common to find N-terminal modifications of recombinant proteins produced in IBs by *E. coli*. These modifications potentially affect stability, function, and degradation of the therapeutic protein [17, 34, 35]. For example, it is found that the authentic human interleukin-1

Table 1 Concentrations and feed rates of the standard and new feeds

	Concentration (g/l)			Feed rate (g/l/h)[a]		
	Glycerol	Soy peptone	Yeast extract	Glycerol	Soy peptone	Yeast extract
Standard feed	40	100	100	3.4	8.5	8.5
New feed	150	150	150	2.5	2.5	2.5

[a] The feeding rates were calculated based on the initial batch medium volume of 6 l

β (IL-1β) is 400–600 % more active than the unauthentic methionylated IL-1β [34]. Protein synthesis, starting at the N-terminus, usually begins with N-formyl methionine [31]. As the first few amino acids of the protein translocate through the exit, the N-terminal formyl group is removed by peptide deformylase, followed by the removal of the N-terminal methionine by methionine amino-peptidase. These enzymes may not fully remove those residues, resulting in methionylation of the target protein when the protein production rate is too high [4], especially with a strong promoter like T7 [25, 31]. Other N-terminal modifications such as gluconoylation [1], phosphogluconoylation [9], and acetylation [32] are also reported in recombinant protein production by *E. coli*. Genetic approaches by changing *E. coli* clones have been reported for effective control of unauthentic variants [1, 17, 23]. From an industrial point of view, optimizing operation conditions is more practical than modifying a production clone for an existing therapeutic protein manufacturing process due to regulatory constraints. To our knowledge, the impact of fed-batch fermentation conditions on N-terminal variant profiles has not been studied in detail.

Optimization of feeding and induction conditions is critical to increase titer of many recombinant proteins in *E. coli* [6, 16, 20]. It is known that a high protein production rate positively affects aggregation in vivo. High temperature, high cell density, and high concentrations of inducers promote IB formation [7]. Therefore, a high protein production rate induced at a high temperature and with a high concentration of inducer is a general strategy for recombinant protein production in IBs by *E. coli* [15, 21]. However, this strategy causes metabolic burden and thus may reduce protein production yield [3, 26]. Although lowering the metabolic rate, as lowering temperature below 30 °C, is quite often used to increase soluble protein production yield and titer [6, 12], to our knowledge, this strategy to lower the metabolic rate has not been reported for titer and quality improvement of recombinant proteins in IBs by *E. coli*.

In this study, we scaled down a standard manufacturing process of a proprietary therapeutic protein (TP), produced in IBs by *E. coli* BL21 driven by a strong T7 promoter in complex media and induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37 °C, to 10-l fermentors for process improvement. The standard process driven by a strong promoter and induced by a high IPTG concentration at high temperature would result in high metabolic and protein production rates, which would cause high methionylation and would cause metabolic burden and thus reduce protein yield. Therefore, we hypothesized that lowering metabolic and protein production rates would reduce the methionylated-TP (Met-TP) ratio and improve the TP titer. To lower metabolic and protein production rates, we reduced feed rates for each of the feed components, the induction temperature, and utilized auto-induction without IPTG. IB recovery was also performed to evaluate the impact of those changes on the purified IB quality and yield.

Materials and methods

Bacterial strain, media, and cultivation conditions

E. coli BL21 (DE3) (Novagen) with T7 promoter and kanamycin resistance was used for TP production. The DNA sequence encoding TP (Bristol-Myers Squibb proprietary) with ~100 amino acids was cloned into the expression plasmid pET 9a (Novagen), which was used to transform the host *E. coli* BL21 (DE3) for the protein expression in IBs.

All fermentation experiments were performed in stainless-steel 10-l fermentors (B Braun C10-2). The batch medium contained 20 g/l glucose, 10 g/l glycerol, 30 g/l soy peptone (Nu-Tek), 15 g/l yeast extract (Bio Springer), minimal salts, and antifoam. The basal batch medium containing soy peptone, yeast extract, antifoam, and some minimal salts were in situ steam-sterilized in the 10-l fermentors at 121 °C for 30 min. Glucose, glycerol, and the rest of the minimal salts were separately autoclaved at 121 °C for 30 min and then added into the fermentors as supplements. The feed media (Table 1) were autoclaved at 121 °C for 30 min. Finally, concentrated kanamycin sulfate solution at 100 g/l after 0.2 μm filter-sterilization was added into the batch and feed media at a final concentration of 0.1 g/l before inoculation.

For the standard process, 6 ml of the seed culture grown in the batch medium with an OD_{600} value of 4–7 was

inoculated into the 10-l fermentor containing 6 l of the batch medium. The fermentor culture was cultivated at 37 °C with a backpressure of 0.3 bar throughout the entire fermentation. Dissolved oxygen (DO) was controlled by a cascade mode first with agitation from 200 to 1,350 revolutions per minute (rpm) and then with aeration from 6 to 12 standard liters per minute (slpm). The pH was controlled at pH 6.9 by NaOH and H_3PO_4. The standard feed at 510 ml/h was initiated at glucose depletion in the batch medium. The culture was induced by 1 mM IPTG 1 h after feed initiation and harvested at elapsed fermentation time (EFT) 16 h.

For the new process, the same seed, batch medium, DO control, and pH control as those used in the standard process were applied. Major changes were made in the feeding and induction conditions. The new feed (Table 1) at 100 ml/h was started with a temperature shift from 37 to 29 °C at the glucose depletion. The culture was auto-induced without IPTG and harvested at EFT 28 h.

IB recovery

Cells from fermentation broth were collected by centrifugation at 12,400 × *g* and 10 °C for 10 min. The cells were resuspended in the homogenization buffer containing 50 mM Tris, 0.5 M NaCl, and 5 mM EDTA·2Na·2H_2O at pH 8.0. The cell suspension was lysed in two passes through a microfluidizer (Microfluidics 110Y) at 1,000 bar. The lysate was centrifuged at 12,400 × *g* and 10 °C for 10 min. The IB pellets were washed with PBS buffer containing 0.1 % v/v Tween 20 by mixing and centrifugation twice. The IB pellets were then washed with DI water by mixing and centrifugation twice. Finally, the purified IB pellets were resuspended in DI water.

HPLC assays for TP titer and N-terminal variants

An arbitrary titer used for evaluation of recombinant protein expression was defined as a volumetric titer (an arbitrary unit mass per liter), which was proportionally converted from a real volumetric titer (gram per liter).

HPLC samples were prepared as the same described in our previous study [33]. In brief, whole fermentation broth sample was lysed with a lysis buffer (Novagen). IBs in pellets were washed with Tris buffer and DI water, respectively. The washed IB pellets were dissolved in the solubilization buffer for TP titer and N-terminal variant-profiling assays.

TP titer was measured using an Agilent 1100 reverse-phase HPLC (RP HPLC) as described in our previous study [33]. The TP and all its variants were not separated by this HPLC method and were eluted as one peak at ~14.4 min. The TP volumetric titer was calculated by comparison of the peak area with a purified TP standard and expressed as an arbitrary unit mass per liter.

The N-terminal variant profile for the final purified TP product was analyzed by an RP HPLC. The separation was performed in C18 3 μm HPLC column by linear gradient of mobile phase B (0.05 % TFA in 80 % CAN) from 40 to 45 % for 35 min. The N-terminal variants were detected at 214 nm by UV detector and the mass were confirmed by intact mass spectrometer.

The TP protein-related N-terminal variants for in-process samples were measured using ultrahigh pressure liquid chromatography (UPLC) system (Waters) equipped with Acquity C18 1.7 μm Column, 2.1 × 100 mm (Waters). Analyses were performed with a column temperature of 60 °C and the protein was monitored at 214 nm with Tunable UV detector (Waters). Mobile phase A was prepared with 0.02 % TFA in water and mobile phase B was prepared with 0.02 % TFA in 80 % acetonitrile. Protein was eluted with a flow rate of 0.2 ml/min using a linear solvent gradient with 39–43 % B from 0 to 16 min, 43–70 % B from 16 to 18 min, 70–100 % B from 18 to 20 min, 100 % B from 20 to 22 min, 100–39 % B from 22 to 22.1 min, 39 % B from 22.1 to 25 min. The eluted peaks were assigned according to the N-terminal modification, which was verified by mass spectrometry. Five TP variants were quantified, namely TP, methionylated TP (Met-TP), formylmethionylated TP (fMet-TP), gluconoylated TP (Glu-TP), and phosphogluconoylated TP (PhoGlu-TP). The peak area was integrated to compare the ratios of N-terminal variants. The ratio of each variant expressed as percentage was calculated from the peak area of the variant over the sum of the areas of all variants.

CO_2 assay and calculation of CO_2 evolution rates

CO_2% (v/v) in the out gas was measured by process mass spectrometer (Questor GP, ABB Extrel). The CO_2 evolution rate (CER) was calculated as

$$\mathrm{CER} = \frac{\mathrm{P}}{\mathrm{RTV}}\dot{Q}(\mathrm{CO_2out} - \mathrm{CO_2air})$$

[24].

Other assay methods

Glucose in the supernatant of fermentation broth was analyzed offline with the YSI biochemical analyzer (YSI 2700). Glycerol was analyzed offline using HPLC equipped with a Bio-Rad Aminex HPX-87H column controlled at 50 °C. The mobile phase is 4 mM H_2SO_4 with a flow rate of 0.6 ml/min. Glycerol is detected by a refractive index detector.

Dry cell weight (DCW) was measured by adding 1 ml of fermentation broth into a 1.5-ml Eppendorf tube. After centrifugation at 3,500 × *g* for 3 min, the pellet was resuspended in 1 ml of DI water. After centrifugation at 3,500 rpm for 3 min, the pellet was dried in an oven under vacuum at 80 °C overnight. The net weight of the dried pellet was considered as DCW (g) per ml of broth. The specific protein production yield, expressed as mg TP produced per gram of DCW, was calculated by HPLC titer (mg/ml)/DCW (g/ml).

IB assays were performed as follows. Approximately 1 ml sample of the purified IB suspension was centrifuged at 16,100 × *g* and 10 °C for 10 min. The pellet was dissolved with the solubilization buffer by shaking on a Thermomixer R at 1,400 rpm for 30 min or until pellets were dispersed. After centrifugation at 16,100 × *g* and 10 °C for 10 min, the supernatant was diluted as required with the resolubilization buffer for TP titer, and total protein concentration. The insoluble residues were accurately weighed to 0.1 mg and % insoluble residues were calculated as weight of insoluble residues/weight of pellet × 100 %.

Total protein concentration was calculated from the absorbance at 280 nm of the supernatant diluted as required, using the formula: mg Protein/ml = A_{280} × 0.77 × dilution factor. The purity of IBs was expressed as HPLC titer (mg/ml)/total protein (mg/ml).

The TP recovery yield in purified IBs was equal to the amount of TP (g, quantified by HPLC titer assay) in the IBs purified from 1-l broth divided by the final fermentation HPLC titer (g/l).

Results and discussion

Standard process

Initially, a standard process was adopted according to a general strategy for production of recombinant protein expressed in IBs by *E. coli*: using high temperature (37 °C) and high IPTG concentration (1 mM) to push for a high protein-production rate.

TP N-terminal variant profile analysis

The TP N-terminal variant profile was analyzed with RP-HPLC for the purified TP product by the standard process (Fig. 1a). We found that authentic TP was predominant with six unauthentic variants, which were confirmed by intact mass spectrometer. Met-TP was the major unauthentic TP variant. Since methylated TP and acetylated TP variants were present in trace amounts (Fig. 1a), these two variants were not quantified in this study. In addition, the TP and its N-terminal variant profiles were monitored through fermentation, recovery, and purification processes. To shorten analytical time, the TP N-terminal variant profiles for the fermentation samples were analyzed by UPLC (Fig. 1b). At EFT 10 h or early induction, the authentic TP ratio was greater than 90 %, while all other TP variants were very low. The TP ratio decreased with time and reached ~60 % at EFT 18 h, while all unauthentic TP variant ratios increased (Fig. 1b). The increase of Met-TP and fMet-TP may be due to the high protein production rate by the standard process and thus peptide deformylase and methionine amino-peptidase would not remove those residues [4]. During the recovery and purification processing, the TP and its variant profiles did not change. Therefore, it is important to control TP variant profiles during the fed-batch fermentation.

Fermentation profiles

The standard fed-batch process was controlled at 37 °C throughout the entire fermentation (Fig. 2a). After inoculation, DO gradually decreased to the setpoint of 30 %, when the agitation increased gradually to max and followed by increasing air flow rates by a cascade control mode. The glucose in the batch medium was depleted at EFT ~7 h, indicated by spiking of DO above 33 % and pH above 6.95, which was mainly due to the shift of carbon source from glucose to glycerol. The glucose concentration of <0.2 g/l was confirmed by the YSI assay at the glucose depletion. The standard feed was then initiated at 510 ml/h throughout the rest of the fermentation. Both the agitation and the air flow rate reached a maximum until the glycerol depletion, indicated by a DO spike above 40 % followed by a decrease of air flow and agitation rates at ~8.5 h (Fig. 2a). The glycerol concentration of <0.2 g/l was confirmed by the HPLC assay at the glycerol depletion. After 1 h post-feeding, 1 mM of IPTG was added for induction of TP expression for 6–8 h. CO_2% (v/v) in the exhaust gas, an indicator of metabolic rate, reached a maximum at EFT 6–8 h, and decreased to 7–8 % after glycerol depletion (Fig. 2b). The decrease of CO_2% was mainly due to less C source available after glycerol depletion. The TP fermentation titer increased with time and leveled off at an arbitrary titer of ~6,500 after ~6 h of induction, while the cell biomass, as expressed in OD_{600}, showed a similar trend (Fig. 2b).

TP production can be auto-induced by the standard process without IPTG

In order to improve the standard process for TP production, we started the fermentation optimization with lower IPTG concentrations, since high IPTG concentrations would

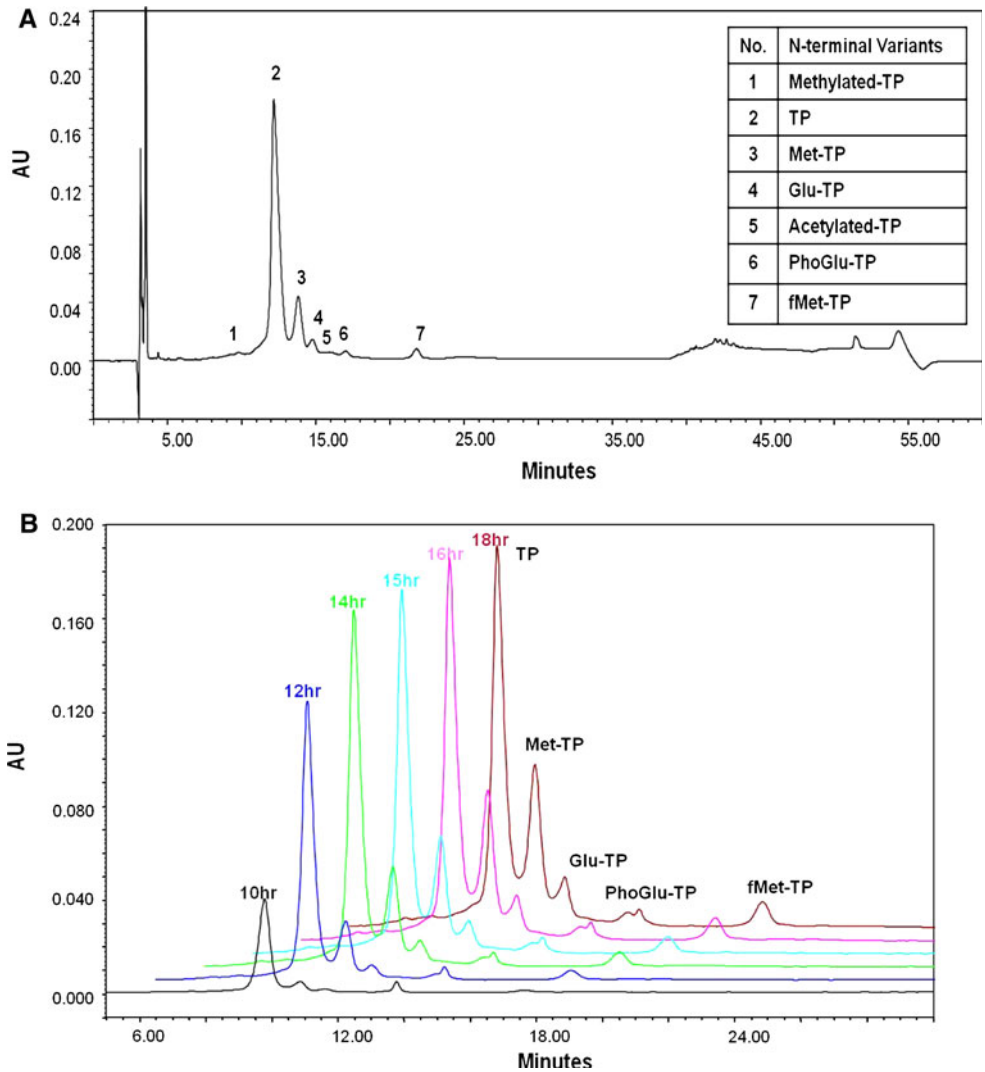

Fig. 1 TP N-terminal variant profile analysis. **a** RP HPLC chromatogram of TP and its N-terminal variant profile for the purified TP product after downstream processing. **b** An overlay of UPLC chromatograms of in-process fermentation samples ranging EFT 10 to 18 h for the standard process with *E. coli* BL21

cause high metabolic burden. Surprisingly, there was no difference in TP production and N-terminal variant profiles when testing different IPTG concentrations at 1, 0.1, 0.01, and 0 mM. Therefore, TP production can be auto-induced by the standard process in the complex media. Currently, complex media are popularly used in industry because they offer flexibility and enable both high cell density and protein yield in most of production processes [12]. It is not uncommon that auto-induction of recombinant protein expression has been observed in *E. coli* BL21 using complex media [10, 14, 27, 33]. Furthermore, we found that 1 kg of the soy peptone contains 7.07 g of galactose and 0.4 mM galactose can cause a high level of protein expression, since *E. coli* BL21 cannot degrade galactose in a previous study [33]. After calculation, it was found that 1.2–2.3 mM galactose was present in the standard process in this study. This is why TP production can be auto-induced by the standard process without IPTG.

There is a concern about IPTG used in industrial-scale production of therapeutic proteins, because of its cost and potential impact on final product quality [11, 13]. The advantages of auto-induction over IPTG induction are that there is no need to monitor cell growth for controlling IPTG addition timing and to worry about the potential negative impact of IPTG on final product quality [27, 33]. Therefore, we believe that the auto-induction process is better than the standard process for TP manufacturing.

Galactose is a weaker inducer and may result in a lower metabolic burden than IPTG [2, 19]. Therefore, galactose-mediated auto-induction for the standard process would have a lower metabolic rate than 1 mM IPTG induction. The reason we did not see a difference in titer between IPTG and auto-induction could be due to the fact that the high feed rate and high temperature masked the effect of different inducers on TP production for the standard process.

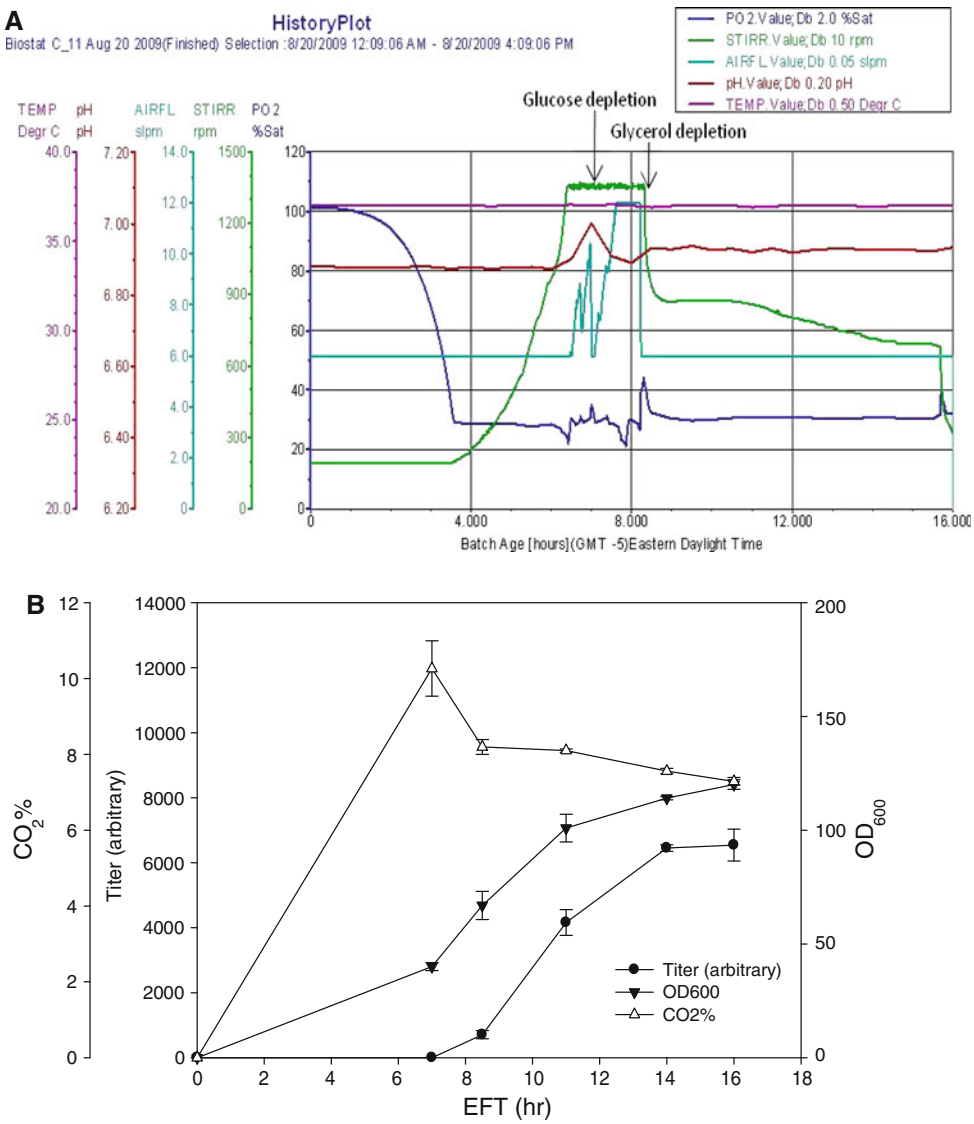

Fig. 2 The fermentation profiles for the standard process in 10-l fermentors. **a** Online monitoring data using B Braun MFCS/win software for DO% (PO_2), agitation rate (STIRR), air flow rate (AIRFL), pH, and temperature (TEMP), only one 10-l fermentor run was displayed as an example, while other two 10-l fermentor runs were similar. **b** Offline measurement data for OD_{600}, TP titer (arbitrary) and out gas CO_2% (v/v), values are reported as average ± standard deviation ($n = 3$)

New process

In order to test our hypothesis that lowering metabolic and protein production rates would improve both TP titer and reduce Met-TP ratio, we designed experiments using lower induction temperatures and feed rates. It was first found that there was no improvement of TP titer by either reducing temperature to 35, 32, and 30 °C separately or reducing feed rates alone (data not shown). Nevertheless, the high feed rate would be predominant and mask the temperature effect on TP production for the temperature induction experiment alone, while the high temperature would be predominant and mask the feed rate effect on TP production by changing feed rate only. Therefore, we tried to change induction temperature and reducing feed rates simultaneously, which worked well for TP production and N-terminal variant profiles in IB form by auto-induced fed-batch process. After empirical optimization of the induction temperature, feed rates for each feed component, and harvest timing, the final new process with a new feed rate at 100 ml/h, and a 6-l start batch volume was auto-induced at 29 °C after glucose depletion and harvested at EFT 28 h.

Fermentation profiles

Since there was no change in batch medium and process control before glucose depletion, the fermentation profiles for agitation, DO%, air flow rate, and pH for the new process (Fig. 3a) were similar to those for the standard process (Fig. 2a). At glucose depletion, the temperature was lowered from 37 to 29 °C for the new process and the new feed was initiated at 100 ml/h. Because of lower temperature and lower feed rates for all feed components (Table 1), the agitation and air flow rates for the new process (Fig. 3a) were lower than those for the standard

process (Fig. 2a) between glucose and glycerol depletion. It also took a longer time for glycerol to be depleted for the new process, although the new feed rates for glycerol, soy peptone, and yeast extract were lower (Table 1). After glycerol depletion, although air flow rate was maintained at min of 6 slpm for both the new and standard processes, the agitation rates for the new process were stable at ~480 rpm, which were lower than 650–900 rpm for the standard process.

N-terminal variant profile

The new process significantly improved TP N-terminal variant profiles (Table 2). The authentic TP ratio was increased by 25 %, which was mainly the result of 50–60 % reduction of Met-TP and fMet-TP ratios, although a slightly higher final Glu-TP ratio at the end of fermentation for the new process was observed (Table 2). Therefore, the fed-batch fermentation conditions had a significant impact on the TP variant profiles. In comparison to the standard process, the new process with a lower protein production rate (Table 3) resulted in significant reduction of Met-TP and fMet-TP formation, although the new process had a 12-h-longer induction duration. It is proven that our hypothesis works well for the reduction of methionylation.

TP titer, cell biomass, and specific production yield

The new process with a lower metabolic and protein production rate not only improved the TP N-terminal variant profile but also improved the final TP titer, cell biomass, and specific production yield. The TP titer for the new process continued to increase with a much longer protein production duration than the standard process (Fig. 3b), which led to an improvement of the final TP titer by 85 % (Table 3). The final specific TP yield was also improved from 210 to 330 mg TP/g DCW (Table 3), which is

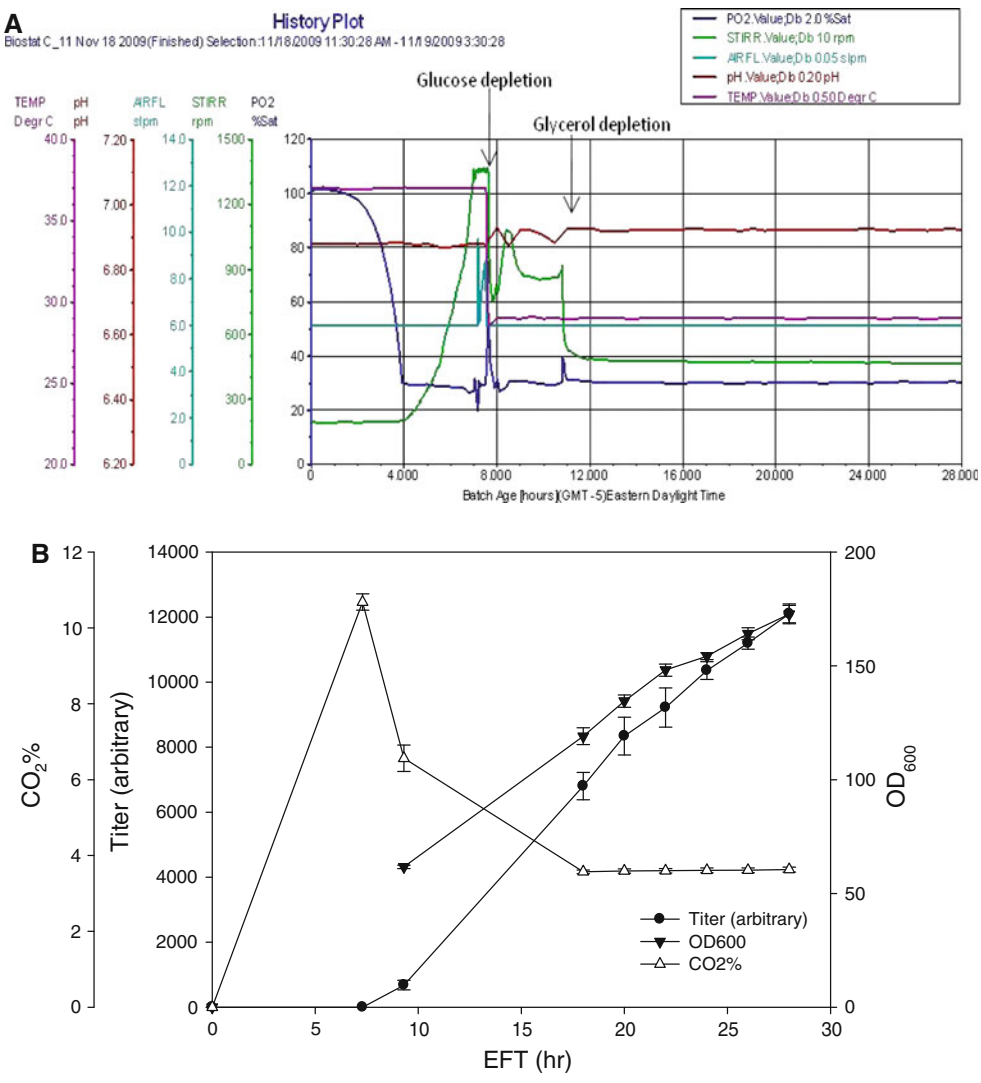

Fig. 3 The fermentation profiles for the new process in 10-l fermentors. **a** Online monitoring data using B Braun MFCS/win software for DO% (PO_2), agitation rate (STIRR), air flow rate (AIRFL), pH, and temperature (TEMP), only one 10-l fermentor run was displayed as an example, while the other two 10-l fermentor runs were similar. **b** Offline measurement data for OD_{600}, TP titer (arbitrary), and out gas CO_2% (v/v), values are reported as average ± standard deviation ($n = 3$)

Table 2 TP N-terminal variant profiles for standard and new processes in 10-l fermentors

Process type	TP%	Met-TP%	fMet-TP%	Glu-TP%	PhoGlu-TP%
Standard	62.6 ± 0.6	23.5 ± 0.2	5.0 ± 0.6	5.1 ± 0.1	2.5 ± 0.1
New	76.7 ± 0.2	9.6 ± 0.1	2.5 ± 0.1	8.7 ± 0.2	2.2 ± 0.2

The samples at EFT 16 h were collected and assayed for the standard process and at EFT 28 h for the new process. Values are reported as mean ± standard deviation ($n = 3$)

Table 3 Final TP titer (arbitrary unit mass per liter), specific TP production yield (mg TP/g dry cell weight), TP production rate (arbitrary unit mass per liter per hour), and productivities (arbitrary unit mass per liter per hour) for standard and new processes in 10-l fermentors

Process type	Final TP titer	Specific TP production yield	TP production rate	Fermentation productivity	Manufacturing productivity
Standard	6,540 ± 490	210 ± 13	957 ± 5	409 ± 31	48 ± 4
New	12,100 ± 306	330 ± 20	601 ± 23	432 ± 11	82 ± 2

The final titers and specific production yields were measured at the end of fermentation at 16 h for the standard process and at 28 h for the new process. The TP production rates were calculated from EFT ~ 8–14 h for the standard process and from EFT ~ 9–28 h for the new process. The fermentation productivities were calculated based on the total fermentation time 16 h for the standard process and 28 h for the new process. The manufacturing productivities were calculated based on the total fermentation time plus 5 days of additional manufacturing cycle time. Values are reported as mean ± standard deviation ($n = 3$)

Table 4 Comparison of CERs during the induction phase for standard and new processes ($n = 3$)

	EFT (h)	CO_2% (v/v)	Culture volume (l)	CER (mmol/l/h)	CER per fermentor (mmol/h)
Standard process	11	8.10 ± 0.04	7.8 ± 0.05	155 ± 1.5	1,206 ± 6.5
	16	7.29 ± 0.10	10.3 ± 0.06	105 ± 1.9	1,084 ± 15.1
New process	18	3.57 ± 0.05	7.1 ± 0.00	74 ± 1.1	529 ± 7.6
	28	3.63 ± 0.07	8.1 ± 0.06	66 ± 0.8	538 ± 9.7

comparable to the highest specific protein yield of 350 mg/g DCW [15]. The cell biomass increased to a higher OD_{600} ~ 170 for the new process (Fig. 3b) than the standard process (Fig. 2b).

It was found that the final culture volume was reduced by 15–20 % and the titer was improved by 19 % when 1.5× concentrated standard feed medium was used (data not shown), suggesting that concentrating feed could be a simple and effective strategy to improve volumetric titer. Therefore, the titer increase for the new process would also be partially contributed by using the concentrated new feed with a lower final culture volume at harvest time point (Table 4).

Productivities

Since the TP production rate was lower and production duration was longer for the new process than the standard process, the volumetric productivities were evaluated for the overall efficiency of the new process (Table 3). The fermentation productivity is defined as the arbitrary volumetric titer divided by total fermentation time. *E. coli* fermentation times are relatively short (usually around 1 day) compared to the manufacturing cycle time which includes fermentor harvest, cleaning, GMP validation, media preparation, fermentation, recovery, and purification. This cycle time varies from one to another GMP facility, but we considered that 5 days would be a reasonable estimation in addition to the fermentation time. Therefore, the concept of manufacturing productivity was introduced for evaluation of manufacturing efficiency here. The manufacturing productivity is defined as the volumetric titer divided by the total production cycle time. There was only <10 % improvement of the fermentation productivity for the new process, but the manufacturing productivity for the new process was improved by 71 %, which was closer to 85 % improvement for the final titer (Table 3). There was only 12-h longer fermentation duration for the new process than the standard process, which is much less than the total manufacturing cycle time. Therefore, the new process is better than the standard process for effective TP manufacturing. Since the manufacturing cycle time may vary from one facility to another and may be impacted by resources available, it would be difficult to define the manufacturing cycle time. However, in most cases, the increase in final volumetric titer approximates the increase in manufacturing productivity for *E. coli* fermentations. Therefore, the final titer would be more

convenient than the productivities to evaluate the improvement and efficiency of therapeutic protein manufacturing processes for *E. coli* fermentations.

IB recovery quality and yield

During the process development for TP production, we found that the fermentation conditions could significantly impact downstream processing. For example, if we harvested the *E. coli* culture broth for the standard process at EFT 20 h instead of the normal harvest time point of EFT 16 h, the quality of recovered IBs decreased because of increasing impurity. Thus, IB harvest experiments using the same protocol described in the method section were performed with the broth samples generated from both the standard and new fermentation processes. The recovered IBs had similar purity ~80 % and amounts of insolubles ~12 % for both processes. However, the TP recovery yield for IBs purified from the new process broth was 85 ± 0.3 % ($n = 2$), which were higher than 66 ± 0.2 % ($n = 2$) harvested from the standard process broth. This was probably due to a higher specific TP production yield in the final culture broth for the new process (Table 3). The final fermentation titer was improved by ~85 % for the new process (Table 3). Adding both improvements together, ~105 % more TP recovered in the purified IBs from per liter of fermentation broth for the new process than the standard process. Thus, based on the IB recovery results, we conclude that the new process is better than the standard process, too.

Metabolic and protein production rates

The online fermentation profile results (Figs. 2a, 3a) such as agitation rates indicated that the metabolic rates during the protein production for the new process were lower than the standard process after glucose depletion by lowering induction temperature and feed rates. As directly calculated in Table 3, the TP protein production rate for the new process was significantly lower than the standard process.

The CO_2% (v/v) in the out gas and CER results also indicated that the new process had lower metabolic rates than the standard process. CO_2% for the standard process reached maximum at EFT 6–8 h, and decreased to 7–8 % after glycerol depletion at EFT ~ 8.5 h (Fig. 3b; Table 4). The decrease of CO_2% was mainly due to less carbon source available after the glycerol depletion. A similar CO_2% trend for the new process before glucose depletion was observed, but decreased to much lower at ~3.5 % after glycerol depletion (Fig. 3b; Table 4). The CER values during the induction for the new process were also significantly lower than those for the standard process (Table 4).

It should be mentioned that the new process was more stable than the standard process during the induction phase after glycerol depletion. This was supported by the agitation profiles and total CER values per fermentor. Although the air flow rates were the same at 6 slpm during the induction phase for both standard and new processes, to maintain 30 % DO the agitation rates decreased from ~900 to ~650 rpm for the standard process (Fig. 2a), while the agitation rates were kept stable at ~480 rpm for the new process (Fig. 3a). Since the CER values were calculated based on the culture volume and the culture volumes were different for the new and standard processes (Table 4), the total CER per fermentor would be more appropriate for the evaluation of stableness of different processes. The total CER per fermentor for the standard process decreased during the induction, while the total CER per fermentor was kept stable at ~530 mmol/h for the new process (Table 4). In addition, the TP titer and cell biomass profiles also indicated that the new process was more stable than the standard process (Figs. 2b, 3b).

Conclusions

We demonstrated a method for significantly reducing unauthentic methionylated protein ratio while improving protein titer in auto-induction of a therapeutic protein expression in IBs by *E. coli* BL21 during fed-batch fermentations in 10-l fermentors. The specific production yield and IB recovery yield were also improved. The strategy was to lower metabolic and protein production rates by reducing feed rates and auto-induction temperature simultaneously while increasing induction duration. The fermentation profiles for both standard and new processes were discussed in detail. The new process was more stable and easier for operation without a need to determine IPTG addition timing. Although the TP fermentation productivity for the new process was only slightly increased, the manufacturing productivity was significantly improved, since the manufacturing cycle time is usually much longer than the fermentation time for therapeutic protein production by *E. coli*. This strategy should be applied to other therapeutic protein production in IBs using *E. coli* for controlling N-terminal variant profile while increasing titer during fed-batch fermentations.

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