**Optimisation of isoamylase production in *Escherichia coli* under different nutrient limitation conditions**

**Running title: Isoamylase production in *E. coli***

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**Abbreviations**

*E. coli Escherichia coli*; IA, Isoamylase; N nitrogen; P phosphorus; Mg magnesium; S sulphur; C carbon; MWR Modified Wilms & Reuss synthetic medium; MOPS 3-(N-morpholino)-propanesulphonic acid; RAMOS Respiration activity monitoring system; OTR oxygen transfer rate; CTR CO2 dioxide release rate; GP960 Growth Profiler 960; DO dissolved oxygen; IPTG Isopropyl - β - D-thiogalactoside; OUR oxygen uptake rate; CER CO2 evolution rate; HPLC High-performance liquid chromatography

**Abstract**

Isoamylase (IA), a starch debranching enzyme that cleaves α-1,6-D-glycosidic bonds in amylose, is used in various industries. However, its low expression in *Escherichia coli* BL21 (DE3) limits its application, necessitating fermentation optimisation. Limiting carbon (C) and other nutrients can optimise bacterial growth for better IA expression.

Herein, using *E. coli* expressing IA-mCherry as the research object, we explored whether low concentrations of the nutrients nitrogen (N), phosphorus (P), magnesium (Mg) and sulphur (S) have a limiting effect on bacterial growth. We subsequently investigated differences in expression under restricted nutrient conditions, and performed batch fermentation experiments under various conditions to determine optimal nutrient limitation conditions.

Low concentrations of N and P had a limiting effect on bacterial growth. IA expression in the P-limited group was increased by ~10% but limiting N had no influence. Combinations of C and P restriction during fed-batch fermentation showed that the total yield was 73.73% higher under C excess and P-limited conditions than under other combinations, while corresponding expression per unit biomass was 24.42% higher. The results revealed that P restriction plays a key role in increasing IA production in *E. coli*.

**KEYWORDS**: Isoamylase production, Fermentation optimisation, Nutrient limitation, Phosphorus restriction, Fed-batch fermentation

**1 | INTRODUCTION**

Starch debranching enzymes are an important class of glycosidases that can be divided into pullulanase type I (EC 3.2.1.41), pullulanase type II (EC 3.2.1.41) and isoamylase (IA; E.C.3.2.1.68; glycogen-6-glucanohydrolse).[1] Pullulanase type I enzymes can hydrolyse α-1,6-glycosidic bonds in pullulan and amylopectin, while pullulanase type II can also hydrolyse α-1,4-glycosidic bonds, making them more widely used in starch processing. IA specifically acts on α-1,6-glycosidic bonds in amylopectin and glycogen. Because IA has the ability to hydrolyse amylopectin into amylose, it is widely used in food, feed, detergent and other industries.[2] IA is a debranching enzyme that hydrolyses α-1,6-glucosidic linkages in amylopectin, glycogen and related oligosaccharides.[3] It has been isolated from various microbial sources including *Pseudomonas* sp., *Flavobacterium odoratum* and potato tubers.[4–6] The molecular weight ranges from 78,000 to 95,000 Da with an isoelectric point between 4.4 and 8.7.[5,6] IA exhibits optimal activity at pH 3−6 and temperatures of 45−50°C, depending on the source and presence of calcium ions.[4–6] It completely debranches amylopectin and glycogen but does not hydrolyse pullulan. The high debranching efficiency and transglycosylation activity make it valuable in various industries including food, feed and detergent production.[3]

IA has potential applications in various industries but heterologous expression of IA genes in *Escherichia coli* remains challenging, despite being studied using various approaches. Lin[7] reported that the IA gene from *Pseudomonas amyloderamosa* expressed in *E. coli* resulted in intracellular insoluble aggregates requiring solubilisation and refolding. Abe[8] successfully expressed the *Flavobacterium odoratum* IA gene in *E. coli*, with the enzyme being excreted into the culture medium. Jung[9] demonstrated that heterologous protein expression levels can vary significantly among different *E. coli* strains, with some strains producing up to 500 times more protein than others. They also observed that low protein production was often due to inefficient translation rather than transcription. These findings highlight the complexity of expressing foreign proteins in *E. coli* and the importance of optimising expression conditions.

*E. coli* is widely used for recombinant protein expression due to numerous advantages including rapid growth to high cell density, low-cost media requirements, and the ability to target proteins to specific subcellular locations.[10] A short life cycle, ease of genetic manipulation, and well-understood cell biology make it an ideal host.[11] This bacterium can accumulate proteins up to 20% of total cellular protein and translocate them from cytoplasm to periplasm, making it attractive for large-scale production.[12] In addition, *E. coli* offers a wide range of molecular tools and expression vectors, allowing researchers to optimise protein production strategies.[13] However, achieving optimal production of foreign proteins using *E. coli* remains challenging.

Various methods have been developed to improve heterologous protein expression in *E. coli*. These include using mutant host strains with reduced protease activity and rare codon tRNAs[10], optimising vector and host selection, and employing fusion tags and chaperones to enhance protein solubility. Metabolic engineering, strong promoters, and novel vector elements have also been utilised to increase expression levels. Fermentation optimisation can also be employed to increase product expression.

Under nutrient limitation conditions such as phosphorus (P) source,[14] nitrogen (N) source[15] and metal elements,[16] bacterial metabolism varies. It is of great significance to optimise the expression of foreign proteins from the perspective of nutrient limitation. *E. coli* employs different translation strategies under nutrient limitation to maintain protein production rates. Under P limitation, ribosome abundance is reduced, while N limitation leads to slower translation elongation, and carbon (C) limitation results in inactive ribosomes.[17] These adaptations allow *E. coli* to balance growth and prepare for nutrient upshifts. Gene expression analysis revealed that 9.8% of genes are differentially expressed between glucose and ammonia-limited cultures, particularly those associated with metabolism, transport and the cell envelope.[18] *E. coli* can scavenge limited nutrients by upregulating corresponding transport and assimilation genes. Nutrient limitation strategies have been applied to enhance the production of valuable compounds such as (R)-3-hydroxybutyrate. In a recombinant *E. coli* strain, ammonium or P-limited fed-batch processes were used to control flux from acetyl-CoA to 3-hydroxybutyrate, with P limitation achieving the highest volumetric productivity of 1.5 g L−1 h−1.[19]

In this study, with the help of high-throughput equipment, we used nutrient limitation to optimise the fermentation of *E. coli* BL21 (DE3) expressing fluorescently labelled IA, and applied the findings to fed-batch fermentation.

**2 | MATERIALS AND METHODS**

**2.1 | Microorganisms and media**

**The *E. coli* BL21 (DE3) strain expressing IA-mCherry was obtained from the Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences.**

**The culture media used in the experimental procedures included LB medium, Minimal M9 medium, MDG mineral medium, Modified Wilms & Reuss (MWR) synthetic medium, and a proprietary composite medium.** **All media utilised glucose as the C source, with an additional adjustment to pH 7 using 6 M NaOH, and addition of 1 g/L ampicillin antibiotic prior to inoculation.**

**LB medium consisted of 10 g/L yeast extract, 10 g/L tryptone and 5 g/L glycerol.**

**Minimal M9 medium**[20] **consisted of 1.32 g/L (NH4)2SO4, 0.50 g/L NaCl, 14.33 g/L Na2HPO4·12H2O, 2.72 g/L KH2PO4, 0.25 g/L MgSO4·7H2O, 0.3 g/L thiamine and 1 mL/L trace metals (stock solution comprising 50.01 g/L FeCl3·6H2O, 11.10 g/L CaCl2, 1.18 g/L MnSO4·H2O, 2.01 g/L ZnSO4·7H2O, 1.67 g/L CoCl2·6H2O, 1.75 g/L CuSO4·5H2O, 0.05 g/L EDTA).**

**MDG mineral medium**[21] **consisted of 8.95 g/L Na2HPO4·12H2O, 3.40 g/L KH2PO4, 2.67 g/L NH4Cl, 0.71 g/L Na2SO4, 2.5 g/L aspartate, 0.49 g/L MgSO4·7H2O, 1 mL/L trace metals (stock solution consists of 2.7 g/L** FeCl3·6H2O, 0.44 g/L CaCl2, 0.40 g/L MnCl2·4H2O, 0.58 g/L ZnSO4·7H2O, 0.095 g/L CoCl2·6H2O , 0.068 g/L CuCl2·2H2O, 0.095 g/L NiCl2·6H2O, 0.082 g/L Na2MoO4, 0.069 g/L Na2SeO3, 0.025 g/L H3BO3).

**MWR medium**[22] **consisted of 5 g/L (NH4)2SO4, 0.5 g/L NH4Cl, 3 g/L K2HPO4, 2 g/L Na2SO4, 0.5 g/L MgSO4•7H2O, 41.85 g/L 3-(N-morpholino)-propanesulphonic acid (MOPS), 0.01 g/L thiamine hydrochloride and 1 mL/L trace metals (0.54 g/L ZnSO4•7H2O, 0.48 g/L CuSO4•5H2O, 0.3 g/L MnSO4•H2O, 0.54 g/L CoCl2•6H2O, 41.76 g/L FeCl3•6H2O, 1.98 g/L CaCl2•2H2O, 33.39 g/L Na2EDTA [Titriplex III]). The pH was adjusted to 7.0 with NaOH.**

**2.2 | Cultivation**

2.2.1 | Respiration activity monitoring system **(RAMOS) cultivation**

The RAMOS device is utilised to measure the oxygen transfer rate (OTR) and CO2 dioxide release rate (CTR) during shake flask fermentation, thereby enabling analysis of microbial growth.[23]

Before inoculation a gas leaking test was conducted. During cultivation, eight RAMOS shake flasks were used, with duplicate flasks for each condition. Four controls were prepared using 20 mL of M9, MDG, MWR and confidential complex medium, with a glucose concentration of 12 g/L and a pH of 7. Upon inoculation, 100 μL of bacterial glycerol stock was added to each of the four synthetic media and cultures were incubated at 37°C with shaking at 300 rpm. After 24 h of cultivation the OD600, pH, glucose concentration and fluorescence intensity were measured.

2.2.2 | **GP960 cultivation**

A Growth Profiler 960 (GP960; Enzyscreen, Herenweg, The Netherlands) was used to record the growth of microorganisms in microtiter plates, employing a 96-well flat-bottom microtiter plate format. EnzyScreen Image software was employed to capture and process time-lapse photos to generate green values, which were correlated with cellular biomass measured as OD600 (Figure S2). Due to its high-throughput cultivation capabilities, this system can be applied for laboratory-directed evolution,[24] medium optimisation[25] and strain screening,[26] among other applications.

Bacteria were pre-cultured using LB medium, washed three times with ultra-pure water, and inoculated into MWR microtiter plates to avoid affecting subsequent experiments. Each well was filled with 200 μL of culture and incubated at 37℃ and 250 rpm. The MWR culture medium had a glucose concentration of 12 g/L and a pH of 7.

To explore the concentrations of N, P, Mg and S as nutritional components, different gradient conditions were set up (Table S1). Taking N as an example, components containing N in the original MWR medium include 5 g/L (NH4)2SO4 and 0.5 g/L NH4Cl (with a nitrogen element concentration of 1191 mg/L). To set different nitrogen concentrations, the concentrations of NH4Cl and (NH4)2SO4 in the MWR medium were adjusted proportionally. For instance, to achieve an N concentration of 595.5 mg/L, the concentrations were set to 2.5 g/L (NH4)2SO4 and 0.25 g/L NH4Cl. Since changing the N concentration also altered the S concentration being investigated, to avoid an interactive effect between changes in S and N concentrations, Na2SO4 was additionally supplemented. Under the 595.5 mg/L N concentration condition, 606.6 mg/L of S was lost, so an additional 2.687 g/L Na2SO4 was added. The P source was modified using K2HPO4 with no supplementary component. The Mg source was modified with MgSO4·7H2O and the supplementary component was Na2SO4. The S source was modified with (NH4)2SO4 and MgSO4·7H2O, with supplementary components NH4Cl and MgCl2·6H2O.

2.2.3 | **BioLector cultivation**

A BioLector (Beckman Coulter Inc., Brea, CA, USA) is a high-throughput cultivation system similar to the GP960. In addition to monitoring biomass online, it can also measure pH values, dissolved oxygen (DO) and fluorescence (product quantity) online. The detection parameters used in the experiments are shown in Table S3. Moreover, because the culture microplates are pre-calibrated before leaving the factory, there is no need to establish additional standard curves for accurate measurements. To explore the differences in product levels under different N and P sources, the concentrations of N and P sources in the MWR medium were adjusted. The final OD600 and fluorescence values were analysed using the Pandas library in Python (https://pandas.pydata.org/).

The culture medium used was MWR medium. The adjustment method for the concentrations of each element was the same as that for the GP960 system. The cultivation conditions were 37°C and 1000 rpm. Aeration was set at 30 mL/min. The culture volume per well was 1000 μL, and the concentration gradient conditions for each element are shown in Table S2. To avoid the impact of the seed liquid on the experimental design, the bacterial liquid was washed twice with ultra-pure water before inoculation. The initial sugar concentration was set at 10 g/L. Isopropyl - β - D-thiogalactoside (IPTG , 100 μM) was used for induction when cells were in the exponential phase.

2.2.4 | **DASGIP cultivation**

The DASGIP bioreactor system (Eppendorf, Wesseling-Berzdorf, Germany) was used to compare four different restriction condition combinations of C-source and P-source to optimise the fermentation process of *E. coli* expressing IA enzyme (Figure S1).

The initial medium for DASGIP culture was MWR medium with adjusted glucose and P concentrations, which ensured that C and P were consumed at the same time at the end of batch fermentation. The feed medium was glucose and K2HPO4, and the specific concentration ratios are listed in Table S4. To avoid the influence of nutrients contained in the residual LB of the inoculated seed, a two-stage feeding strategy was devised. Initially, the culture was grown in LB medium, then inoculated (1% inoculum) into the RAMOS containing MWR medium, and the DASGIP fed-batch fermentation experiment was carried out with another 1% inoculum. The exhaust gas was analysed using an EXTREL MAX300-CAT Laboratory Mass Spectrometer (Process Insights, Suzhou, China). Data from the DASGIP system and the off-gas mass spectrometer were transferred to the PostgreSQL database (https://www.postgresql.org/) via Python scripts. Python was also used to calculate the initial feeding rate and feeding rate exponent during the feeding phase.

The LB culture conditions were 37°C, 220 rpm, 100 mL and a 1% inoculation rate. The RAMOS culture conditions were 40 mL, 37°C, 280 rpm and a 1% inoculation rate. The entire DASGIP cultivation process conditions were pH 6.8, 37°C, aeration flow rate 2.5 L/min, gauge pressure maintained at 0.01 MPa, and agitation and DO cascade-controlled to ensure DO remained >50%. The initial glucose concentration for the DASGIP batch fermentation phase was 5 g/L. After the rebound of DO, the culture was induced with 100 μM IPTG during feeding. The initial feeding rate and feeding rate exponent for the DASGIP feed start were calculated from the data obtained during the batch fermentation phase (Equation 3). The DASGIP fermentation process was terminated when the offline measured fluorescence values no longer tended to stabilise.

2.2.5 | **Calculation of CER and OUR**

**The numpy and pandas libraries were used for calculations. The formulae for calculating the oxygen uptake rate (OUR) and the** CO2 **evolution rate (CER) were as follows:**

**where is the air flow rate introduced into the fermenter with units in litres per hour (L/h);**

**is the molar volume with one mole of gas occupying a volume of 22.4 litres at standard temperature and pressure, with units in mmol/L;**

**is the total volume of the fermentation liquid with units in L;**

**andare the volume fractions of and in the inlet air, respectively; and**

**and are the volume fractions of and in the exhaust gas, respectively.**

2.2.9| **Calculation of initial feeding rate and feeding rate exponent**

After the rebound of DO in the DASGIP system, calculations were performed to obtain the critical feeding rate and feeding rate exponent (Equations 3 to 10) for exponential feeding cultivation. By controlling the C/P ratio in the feed solution and the feed rate, the microorganisms can be maintained under different C/P conditions post-feeding. Table S4 presents the agreed symbols and control strategies under various C and P source conditions. The initial feed rate is equal to the critical feed rate, which represents the theoretical substrate consumption of the biomass at the current level of biomass.

where is the feeding rate with units in grams per hour (g/h);

is the initial feed rate of each fermentation tank with units in grams of feed solution per hour (g/h);

is the feeding rate exponent with units in 1/h; and

t is the duration of fermentation feed supplementation with units in h.

where represents different parallel experimental groups; is the critical feed rate for the th group with units in g/h;

is the maximum CO2 evolution rate during the batch fermentation phase with units in mmol/L/h;

is the volume of the fermentation broth during the batch fermentation phase with units in L; is the yield of CO2 from glucose during the batch fermentation phase with units of mmol CO2/g glucose;

is the mass concentration of glucose in the feed solution with units in g/g;

is the initial time of the batch fermentation phase with units in h;

is the end time of the batch fermentation phase with units in h;

is the glucose concentration at time with units in g/L; and is the glucose concentration at time with units in g/L.

Given that microbial growth is unlimited at the exponential batch phase, the specific growth rate () and specific CO2 evolution rate are both constant, and the exponential growth of *E. coli* can be expressed as

where is the *E. coli* concentration and is the specific release rate of .

Then,

and where the ln value of CER during the exponential batch phase is linearly correlated with the batch time, regression between ln(CER) and batch time t gives a linear equation between these two variables, assuming the regressed model constants are k and b ( and b in Eqn. 10). Thus, we can use this relationship to determine the feeding rate exponent ( in Eqn. 3).

After determining the initial feeding rate, the feeding exponent needs to be determined. By controlling the excess/limitation of the feeding amount, the bacteria can be placed under different limiting conditions (Table S4).

where is the feeding exponent with units in 1/h;

is the maximum specific growth rate in the batch fermentation stage with units in 1/h; and

k and b are the slope and intercept of the linear fitting of ln(CER) in the batch fermentation stage, respectively.

**2.3 | Offline analysis**

**2.3.1 | Quantification of biomass**

**The fermentation broth was diluted to an appropriate concentration for measurement of OD600 using a UV-1900i spectrophotometer** (**Shimadzu**, Suzhou, China)**. OD represents the measured absorbance value multiplied by the dilution factor.**

**2.3.2 | Quantification of PO4+**

**Quantification of P was conducted using a Malachite Green Phosphate Detection Kit (Beyotime Corporation, Jiangsu, China)** **following the manual instructions. The standard curve for P determination is shown in Figure S3.**

**2.3.3 | Measurement of glucose concentration**

**Samples were first centrifuged at 10,625 g for 10 min, followed by filtration through a 0.2 μm filter membrane and appropriate dilution for analysis. Determination of glucose concentration in samples was performed by High-performance liquid chromatography (HPLC) using a Chromeleon 7 system equipped with an Aminex HPX-87H column (Bio-Rad Corporation, CA, USA). The detection conditions consisted of a mobile phase of 5 mM sulphuric acid (300 μL added to 1 L of water), a flow rate of 0.6 mL/min, a column temperature of 50°C, an injection volume of 5 μL, a detection time of 30 min, and an IDEX RefractoMax521 detector (IDEX Corporation, Illinois, USA). Finally, peak heights were converted to glucose concentrations using the standard calibration curve** **shown in Figure S4.**

**2.3.4 | IA-mCherry measurement**

**Fluorescence detection was employed using a SpectraMax iD5 system to quantify the product in the sample. A 100 μL volume of sample was used for detection with an excitation wavelength of 550 nm and an emission wavelength of 615 nm. The fluorescence value was converted to enzyme activity using the fluorescence-enzyme activity standard curve shown in Figure S5.**

**3 | RESULTS**

**3.1 | Screening the optimal RAMOS culture medium**

In pursuit of the optimal synthetic medium for *E. coli* cultivation, a comparative analysis of different culture media was conducted using the RAMOS device. When *E. coli* expresses the byproduct acetic acid, a low solution pH can affect metabolic activity and inhibit bacterial growth. Therefore, selecting a medium with sufficient pH buffering capacity is crucial. Furthermore, complex media contain components such as yeast extract and peptone with unclear compositions, making it difficult to precisely control nutrient-limiting conditions. Synthetic media, with their defined compositions, allow for more accurate quantitative analysis. In addition, complex media may contain lactose analogs, potentially leading to increased leaky expression in *E. coli*. To select the optimal medium, we screened three synthetic media and one complex medium. We cultured *E. coli* cells in each medium and compared offline measurements at the end of the culture and respiratory curves during the process. The MWR and MDG media exhibited no residual glucose and achieved higher final OD values (Figure 1A). This can be attributed to their relatively stronger pH buffering capacity (Figure 1A). The OTR curve also shows an earlier decline for M9, likely due to its insufficient pH buffering capacity. In terms of gene expression, MWR medium displayed lower levels than MDG when IPTG was not used (Figure 1A). This is likely because the primary buffering component of MWR, MOPS, provides a more stable pH environment. Based on these findings, MWR medium was selected for further studies.

图表

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**FIGURE 1 RAMOS results for diff**erent synthetic media. (A) pH, Glc, K2HPO4 and mCherry at the end of the culture. (B) OTR curves for RAMOS with different culture media.

**3.2 | Determining limiting conditions for the main nutrients**

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**FIGURE 2** Growth results for *E. coli* under different element concentrations. (A) OD curves as a function of time at different P/N/Mg/S concentrations. (B) Maximum OD value curves achieved during the entire process at different P/N/Mg/S concentrations. P/N/Mg/S are phosphorus, nitrogen, magnesium and sulphur, respectively.

Limiting concentrations for each of the four main nutrient element sources (i.e. N, P, S and Mg) were determined by varying their initial concentration in the start medium when culturing *E. coli* in GP960. The concentrations of components containing N, P, Mg and S in MWR medium were adjusted (Table S1), and the process OD values measured by GP960 were analysed. By comparing the OD curves under different concentration conditions (Figure 2A), we found that the growth of bacteria under different P, N, and Mg concentrations varied. Comparison of the final OD values at the end of the process (Figure 2B) showed when lower than a specific concentration (0.082 g/L for P-source and 0.6 g/L for N-source ), the final OD value at the end of the process was linearly dependent on the corresponding concentration. However, no linear dependency was observed for Mg-source or S-source. This demonstrated that both P-source and N-source were critical for *E. coli* growth. P is a component of cell membranes and nucleic acids, while N is a component of proteins, bases and many other molecules. Therefore, low concentrations of these two elements can significantly limit the growth of *E. coli*.

图示

描述已自动生成**FIGURE 3** Process curves for *E. coli* under different N concentrations. (A) pH curves at different timepoints. (B) Growth curves of *E. coli* at different timepoints. (C) mCherry expression curves at different timepoints. (D) Unit biomass expression levels at different timepoints. (E) Final Biomss and mCherry levels per unit biomass at different N-source concentrations.

High-throughput fermentations under different N-source and P-source concentrations were carried out in a BioLector XT system that monitors online fluorescence during the fermentation process. Using this instrument, the optimum concentration for these two nutrient sources can be determined with high efficiency.

The results revealed a significant influence of N-source concentration on both bacterial growth and fluorescence intensity; as N-source concentration increased, bacterial growth also increased (Figure 3B), leading to a corresponding enhancement in mCherry production (Figure 3C). Notably, while the mCherry yield per unit biomass exhibited a remarkable increase with decreasing N-source concentration (Figure 3D), overall product levels remained low due to substantial growth suppression (Figure 3E). This observation demonstrates that maximising mCherry expression per unit biomass does not guarantee the highest overall product accumulation. The results presented in Figure 3E show a general correlation between final biomass and final mCherry levels, suggesting that employing N-limiting strategies to enhance product expression may not be a viable approach. To achieve the highest product concentrations, it is crucial to avoid subjecting the bacteria to N-source-induced growth inhibition. Therefore, it is not suitable to apply the N-source limitation strategy to the fed-batch fermentation process. However, it is also important to acknowledge that varying N-source concentrations can lead to substantial differences in initial pH (Figure 3A). With increasing N-source concentration, the initial pH increased from 6.4 to 6.8, introducing a degree of variability in the induction timing of IPTG addition for individual bacteria at the same timepoint. This variation likely stems from the sole adjustment of the MOPS component during pH regulation, and could contribute to the observed decline in final mCherry levels with increasing N-source concentration.

图示

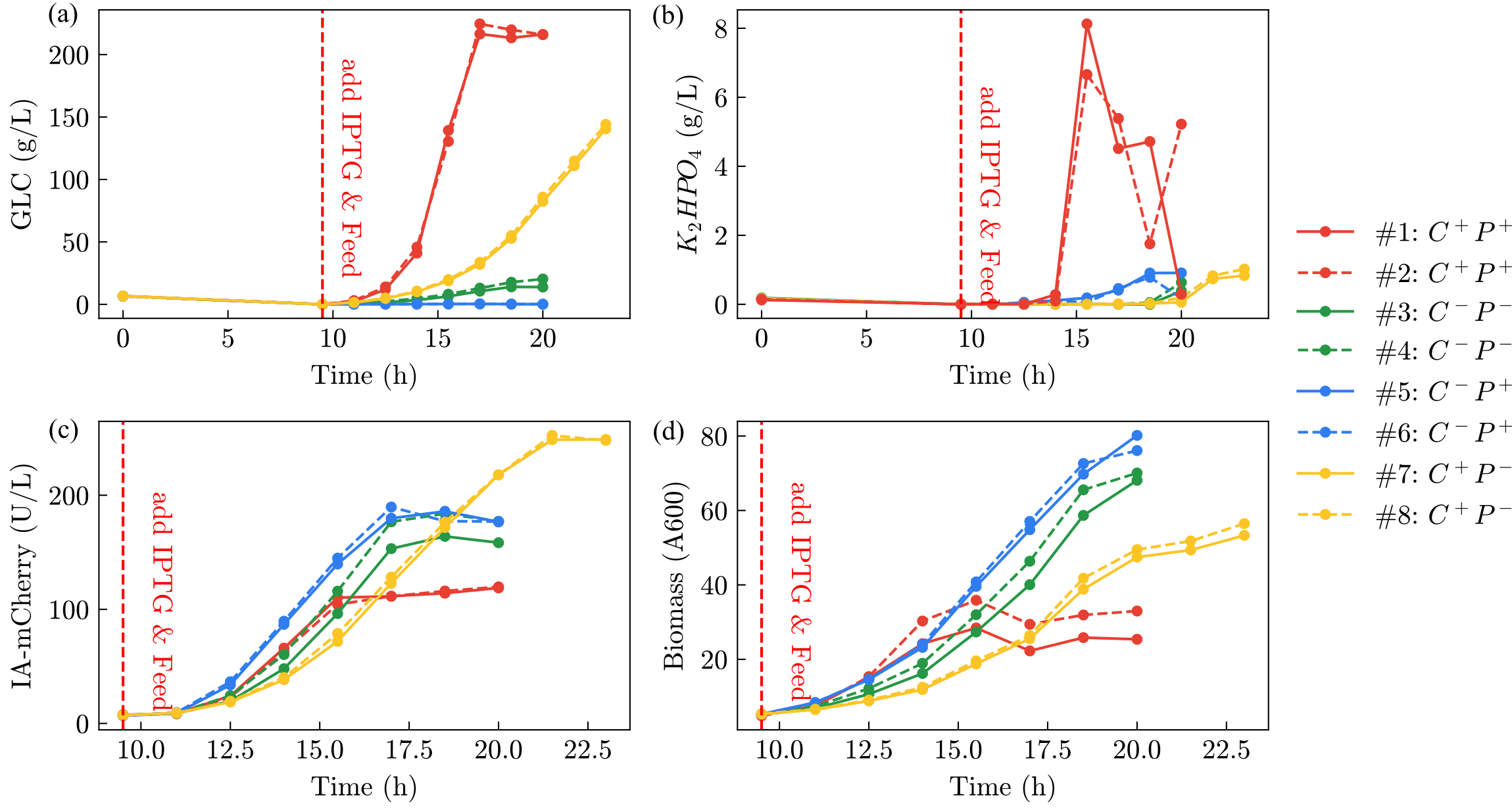
描述已自动生成**FIGURE 4** Process curves for *E. coli* under different P concentrations. (A) pH curves at different timepoints. (B) Growth curves of *E. coli* at different timepoints. (C) mCherry expression curves at different timepoints. (D) Unit biomass expression levels at different timepoints. (E) Final Biomss and mCherry levels per unit biomass at different P-source concentrations.

In contrast to the effects of N-source, the influence of P-source concentration on cell density and mCherry production appeared to be significant only at particularly low P-source concentrations (0.02−0.06 g/L) within the investigated range. The minimal variation in P-source concentration resulted in correspondingly small changes in initial pH, with a difference of only 0.1. Moreover, based on the results from different P-source concentrations, it was observed that a low P-source concentration (0.02−0.04 g/L) clearly led to higher protein production per individual bacterium (Figure 4D), resulting in a higher specific mCherry value. However, once the specific mCherry concentration exceeded 0.05 g/L, the mCherry quantity per unit of bacterium no longer changed. It is noteworthy that under conditions of low P-source concentration, although bacterial growth is limited, the final mCherry concentration is paradoxically higher. This suggests the feasibility of employing a P-limited strategy in fed-batch fermentation processes.

In summary, P-source limitation, but not N-source limitation, is a crucial factor in enhancing the expression capacity of bacterial enzymes. When the P-source concentration was 0.02−0.04 g/L, there was an increase in specific mCherry production per cell and the total product yield.

**3.3 | Optimisation of fed-batch fermentation by combining different C-source and P-source conditions**

In order to explore the effects of different C and P sources on the expression of IA-mCherry in *E. coli* under fed-batch fermentation, four different C and P source fed-batch fermentation conditions were designed (Table S4) to assess differences in product expression. Data for the batch fermentation stage were collected and results calculated using a python script to determine the initial feeding rate ( in Eqn. 3) and feeding exponent (in Eqn. 10). By comparing the lnCER curves under four different conditions (Figure S6), it was revealed that the average maximum specific growth rate in the batch fermentation stage was ~0.67 h−1. To create the specific design conditions (C+P+, C−P−, C−P+ and C+P−), feeding exponent () was determined (Table S4). For all four conditions = 0.7 h−1 was used, which is larger than mu\_max used to create C+P+, while for other conditions = 0.3 h−1 was used. The same initial glucose and phosphate concentrations, inoculation size and culture parameters were used in the batch fermentation stage for all conditions. The similar specific growth rates for each group confirmed the similarity of the batch fermentation stage. We obtained the specific growth rate from the batch fermentation stage data, and finally determined the feeding exponent (Table S4).



**FIGURE 5** Fed-batch fermentation process offline data. (A) Glucose concentration curve. (B) *E. coli* growth process curve at different timepoints. (C) Product accumulation process curve at different timepoints. (D) *E. coli* growth and product accumulation at the fermentation endpoint under different P concentrations.

After completing the fed-batch fermentation experiments under four different C and P conditions, the substrate, product and biomass during the fermentation process were analysed. By comparing the glucose concentration curves (Figure 5A) it was revealed that under C excess conditions ( and ), the glucose concentration indeed accumulated to excess (>20 g/L) after feeding. However, with excess P-source present in the feed medium, the C-source concentration barely needed to be controlled to a limited level, while limiting P-source conditions could preserve C-source limitation. Therefore, under the maximum glucose concentration did not exceed 0.4 g/L over the whole fermentation process, while under the glucose concentration continued to accumulate throughout the process to reach 20.29 g/L at the end of fermentation.

Different P-source concentration conditions were tested and the results are shown in Figure 5B. Under P excess conditions ( and ), the P-source concentration accumulated to excess and reached 6−7 g/L during the process. Meanwhile, under P-source limiting conditions (and ), the concentration remained below 0.05 g/L (<0.08 g/L, determined as the critical P-source concentration). The IA-mCherry signal profiles in Figure 5C show that the final product concentration for the conditions was 60.72% higher than the average of the other groups, indicating that a P-source-limiting but C-source excess strategy can promote the IA production. However, the conditions did not show significant differences compared with other groups. Figure 5D shows the biomass profiles for all four conditions, and the growth of *E. coli* under conditions was significantly inhibited, due to the high acetate produced under excess glucose. It is interesting that combining and produced less biomass after the same duration but more IA. Phosphate limitation under glucose-sufficient conditions increased the accumulation of the IA-mCherry product.

Comparison of total enzyme activity profiles (Figure S7A) showed that conditions yielded the highest values among all four conditions. Total enzyme activity under conditions was 73.73% higher than the average of other groups at the end of fermentation (Figure S7A), while enzyme activity per unit volume was 24.42% higher than that of other groups (Figure 5C). We also compared the profiles of product per biomass, which denotes the capacity of cells during the process. The results also showed that conditions gave the highest value throughout and especially at the end of the process. In addition, unit cell expression under and conditions was higher than for the other two groups. Under the same C excess conditions, unit cell expression of IA under conditions was slightly higher than that of the group. This shows that P limitation further increased unit cell expression based on C excess.

**4 | DISCUSSION AND CONCLUSION**

Here, we embarked on a systematic investigation of the influence of P limitation on *E. coli* BL21 (DE3) cell growth and mCherry expression by varying the components of synthetic growth media. Interestingly, under conditions of C sufficiency and P limitation, *E. coli* exhibited an enhanced capacity to express IA.

Various studies have explored P limitation strategies to enhance recombinant protein production in *E. coli*. Huber[27] demonstrated that phosphate limitation at the optimal induction time increased the specific productivity of a fluorescent protein from 22% to 31%. Similarly, Aldor[28] observed upregulation of phosphate starvation proteins and downregulation of ribosomal proteins during phosphate-limited fermentation. Witt[29] found that P in fermentation media could be reduced by up to 70% without negatively affecting biomass or protein yield. Gundinger[30] investigated the phosphate-sensitive phoA expression system and showed that phosphate limitation boosts gene expression but can lead to metabolic breakdown at starvation levels. These findings suggest that carefully controlled phosphate limitation can redirect cellular resources towards protein expression rather than biomass production, potentially improving yields in recombinant protein production processes.

To date, there are no reports that simultaneously consider the effects of C and P concentrations on the expression of exogenous proteins. There is no reported explanation of why P limitation can promote the expression of IA enzyme under C source sufficient conditions. Most previous studies only report the metabolic overflow of *E. coli* under C excess and gene regulation under P limitation. Overflow metabolism in *E. coli* occurs under C-restricted conditions, characterised by acetate excretion despite oxygen availability.[31,32] This phenomenon results from efficient proteome allocation, as respiration requires twice the protein investment per ATP compared to fermentation.[32] The NADH/NAD ratio plays a crucial role in overflow metabolism, with a strong correlation between acetate formation and this redox ratio.[33] Transcriptional regulation, particularly through ArcA, influences the tricarboxylic acid cycle and respiration genes in response to glucose consumption rates.[33] Interestingly, overflow metabolism extends beyond acetate, with various microorganisms excreting a diverse range of central metabolic intermediates and amino acids under C excess conditions.[34] This 'extended' overflow metabolism appears to be a common feature across microbial species and has implications for metabolite balancing and intracellular metabolite quantification.[34] *E. coli* adapts its metabolism under P limitation through various strategies. Under phosphate stress, *E. coli* increases fermentative metabolism despite aerobic conditions.[35] Phosphate limitation leads to increased glucose consumption and acetate production rates.[36] The bacterium employs different translation strategies depending on nutrient limitations, with P limitation resulting in fewer ribosomes to conserve P.[37] As phosphate limitation intensifies, *E. coli* experiences reduced efficiency in ATP formation via respiratory chain activity and ATP synthase complex.[38] This leads to a multi-stage decoupling scenario affecting ATP availability and C usage. Metabolic regulation under phosphate limitation is interconnected with responses to acidic conditions and N limitation through RpoS, RpoD and Pho genes.[36] These adaptations allow *E. coli* to maintain growth under P-limited conditions while preparing for potential nutrient upshifts.

In conclusion, this study effectively demonstrates the potential of using nutrient limitation strategies, especially P limitation, to optimise the fed-batch fermentation process for the production of IA-mCherry by *E. coli* BL21 (DE3). Efficient fermentation process optimisation depends on high-throughput fermentation optimisation equipment. Here, we used GP960 to explore the restrictive conditions of various nutrient sources, RAMOS for rapid screening of culture media, BioLector for rapid identification of online product synthesis capabilities under different conditions, and parallel reactors for rapid optimisation of the culture process. For IA enzyme production by BL21 (DE3), P limitation is beneficial to stimulate the bacteria to synthesise more intracellular IA enzyme, and combining with a sufficient C source can further promote the effect of P limitation. In summary, P-limited-C excess conditions were most conducive for IA enzyme production by *E. coli* BL21 (DE3).

**ACKNOWLEDGMENTS**

This work was financially supported by Tianjin Synthetic Biotechnology Innovation Capacity Improvement Project (TSBICIP-CXRC-033 and TSBICIP-PTJJ-006).

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**DATA AVAILABILITY**

The readers are suggested to contact the corresponding author for the data.

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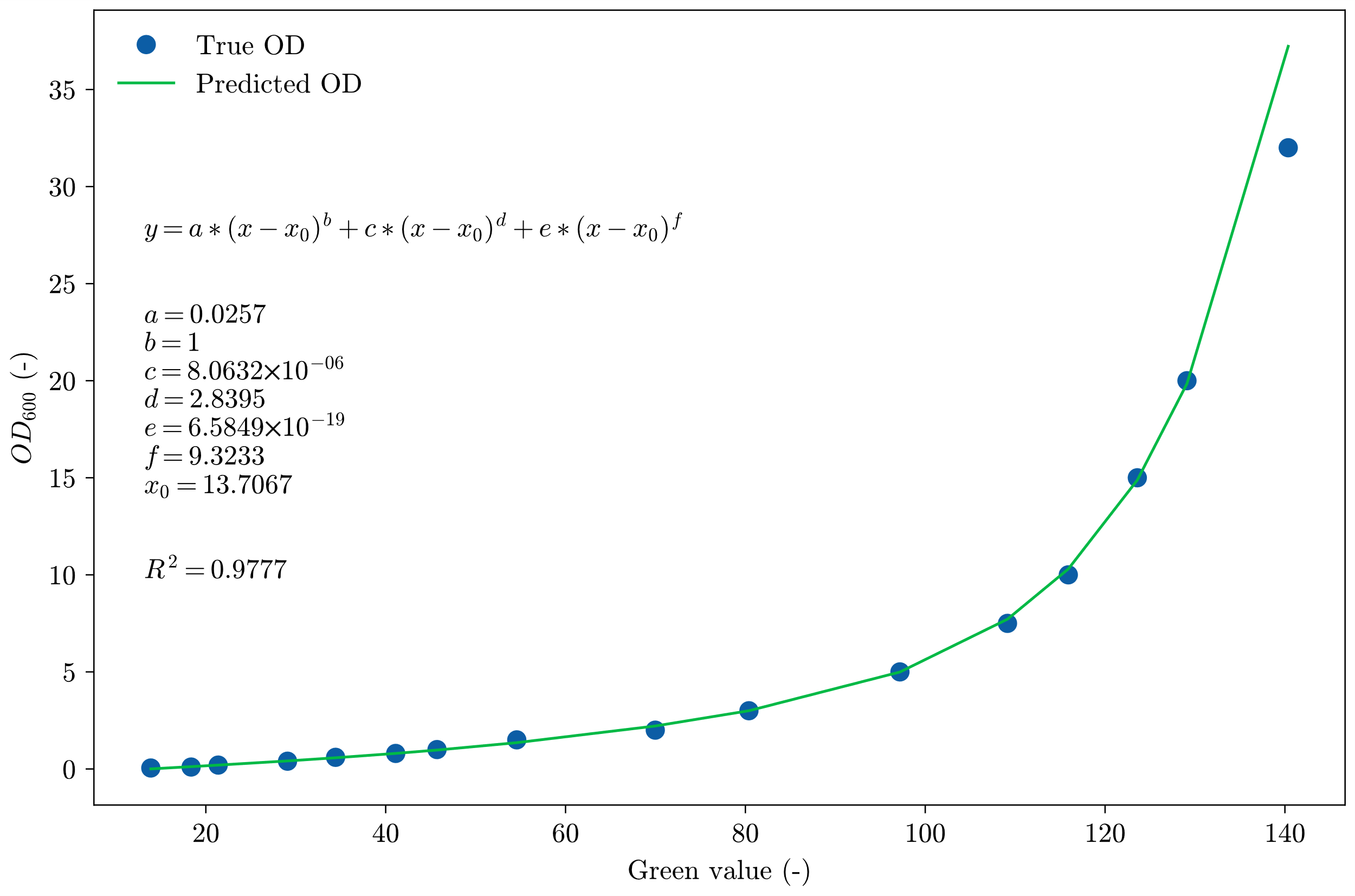
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**Supplemental Figures**

**图示

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**FIGURE S1 Experimental procedure for DASGIP fed batch fermentation validation.**



**FIGURE S2** Correlation between green value and OD600 for *E. coli* BL21 (DE3).

**FIGURE S3** Standard curve for phosphate determination.

**FIGURE S4** HPLC glucose standard curve.

**FIGURE S5** Fluorescence and enzyme activity calibration curve per unit volume.

图表, 折线图

描述已自动生成**FIGURE S6** Real-time fitting of maximum specific growth rate during the batch fermentation phase.

图表, 折线图

描述已自动生成**FIGURE S7 Curves of t**otal fluorescence and product expression per unit biomass. (A) Total product. (B) Product per unit cell.

**Supplemental tables**

**TABLE S1** GP960 element concentration gradient design.

| **P element concentration (mg/L)** | **N element concentration (mg/L)** | **Mg element concentration**  **(mg/L)** | | **S element**  **concentration**  **(mg/L)** |
| --- | --- | --- | --- | --- |
| 0.0  38.5  40.3  42.1  44.5  46.8  49.2  52.2  55.7  59.3  63.4  68.2  74.1  80.6  88.9  99.0  111.4  126.9  148.2  177.8  222.3  444.6 | 0.0  220.6  229.0  238.2  248.1  258.9  270.7  283.6  297.7  313.4  330.8  350.3  372.2  397.0  425.3  458.1  496.2  541.4  595.5  661.7  744.4  1191.0 | | 0.0  8.5  8.8  9.1  9.5  9.9  10.3  10.7  11.2  11.7  12.3  13.0  13.7  14.5  15.4  16.4  17.6  19.0  20.5  22.4  24.7  27.4 | 0.0  6.7  6.9  7.2  7.5  7.9  8.2  8.7  9.1  9.6  10.2  10.8  11.5  12.4  13.3  14.4  15.7  17.3  19.2  21.6  24.7  28.8 |

**TABLE S2** Design of nutrient concentration gradients for BioLector.

| **Element** | **Concentration (mg/L)** | | | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| P | 20 | 30 | 40 | 50 | 60 | 70 | 140 | 210 |
| N | 100 | 200 | 300 | 400 | 500 | 1000 | 1500 | 2000 |

**TABLE S3** Optical signals and applied setup for BioLector online monitoring.

|  |  |  |  |
| --- | --- | --- | --- |
| **Optical signal** | **λex [nm]** | **λem [nm]** | **Gain** |
| Biomass  (scattered light) | 620 | - | 1 |
| DO | 505 | 590 | 4 |
| pH | 485 | 530 | 8 |
| mCherry fluorescence | 580 | 610 | 5 |

**Table S4.** DASGIP initial feeding rate and feeding rate exponent calculation results.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Symbol** | **Representative meaning** | **Control strategy** | **Tank Id** |  |  |  |
|  | Excess Carbon and Phosphorus Supplementation |  | #1 | 1.61 | 0.663 | 0.7 |
| #2 | 1.59 | 0.665 | 0.7 |
|  | Carbon Limitation and Phosphorus Limitation |  | #3 | 1.66 | 0.677 | 0.3 |
| #4 | 1.69 | 0.686 | 0.3 |
|  | Carbon Limitation and Excess Phosphorus Supplementation |  | #5 | 1.5 | 0.657 | 0.3 |
| #6 | 1.62 | 0.729 | 0.3 |
|  | Excess Carbon and Phosphorus Supplementation |  | #7 | 1.64 | 0.663 | 0.3 |
| #8 | 1.60 | 0.661 | 0.3 |

The initial feed medium for DASGIP was a modified version of MWR medium, with a Glucose-to-K2HPO4 concentration ratio of ~500:13 g/L (preliminary experimental results indicated a consumption ratio close to this value in the fermentation vessel). The initial feeding rate was determined to be an average of 1.61 g/h, and the feeding exponent was determined to be 0.7 and 0.3 h−1, respectively.