ARTICLE IN PRESS

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
x (x): x-x, 2026
DOI: 10.9775/kvfd.2026.36550

RESEARCH ARTICLE

Improvement of IBDV VP2 Protein Expression in *Escherichia coli* Through a Staged pH Control Strategy

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How to cite this article?

Fu Q, Zhao X, Sun Y, Meng W, Lin C, Miao L, Wang W, Cheng L: Improvement of IBDV VP2 Protein Expression in *Escherichia coli* Through a Staged pH Control Strategy. *Kafkas Univ Vet Fak Derg*, 2026 (Article in Press). DOI: 10.9775/kvfd.2026.36550

Article ID: KVFD-2026-36550
Received: 13.03.2026
Accepted: 23.06.2026
Published Online: 08.07.2026

Abstract

Infectious bursal disease (IBD), caused by infectious bursal disease virus (IBDV), severely endangers the global poultry industry. The VP2 subunit vaccine represents a primary strategy for the prevention and control of IBD. *Escherichia coli* is widely used for recombinant VP2 production; however, acetate overflow during fermentation severely inhibits cell growth and target protein synthesis. Additionally, conventional constant-pH strategies and empirical pH regulation approaches fail to achieve a balance between high cell density, low by-product accumulation, and efficient expression of active VP2 protein. This study aimed to investigate the effects of various ions from pH regulators on the growth of the engineered strain *E. coli* 0125. Aqueous ammonia ($NH_3 \cdot H_2O$) was identified as the optimal regulator. Based on real-time monitoring of dissolved oxygen (DO) dynamics, the optimal pH for strain growth was determined to be 7.2. Subsequently, nine staged pH control strategies were established to optimize the fermentation process. The optimal protocol was to maintain the pH at 7.2 during the initial 0-6 h of fermentation, followed by adjustment to pH 6.8 during 6-12 h using $NH_3 \cdot H_2O$. Under these conditions, the wet cell concentration reached 28.37 g/L, the specific VP2 titer increased to 96 U/g, and acetate content was maintained at a low level of 2.19 g/L ($P<0.05$). Compared with the group maintained at pH 7.0 using sodium hydroxide, the total VP2 titer of the optimized group increased by 2.95 times. This refined staged pH control strategy effectively resolves the contradictions in *E. coli* fermentation and provides a practical technical approach for the large-scale industrial production of IBDV VP2 subunit vaccines.

Keywords: Acetate, *Escherichia coli*, IBDV, pH regulation, VP2 protein

Introduction

Infectious bursal disease (IBD) is a highly contagious and immunosuppressive disease in poultry caused by the infectious bursal disease virus (IBDV). It leads to increased mortality, growth retardation, and secondary infections in chickens, and even compromises the efficacy of other vaccinations, causing enormous economic losses to the global poultry industry [1,2]. Vaccination remains the primary strategy for the prevention and control of IBD. Compared with traditional live vaccines, VP2-based subunit vaccines exhibit superior biosafety and immunogenicity, and have emerged as a major focus in the research and development of IBDV vaccines [3]. The expression level and biological activity of the VP2 protein directly determine vaccine efficacy and industrialization potential. Therefore, achieving high-efficiency heterologous expression of VP2 is essential for promoting the industrial application of VP2 subunit vaccines.

As a classic heterologous expression host, *Escherichia coli* possesses several advantages, including a clear genetic background, low cultivation cost, and fast proliferation, and is commonly used for VP2 protein production. Nevertheless, the low yields and poor biological activity of recombinant VP2 remain major obstacles restricting its large-scale industrial production. Protein expression is jointly affected by intrinsic properties of the expression system and external fermentation parameters. Among various process factors, pH plays a decisive role in regulating bacterial metabolism and recombinant protein synthesis.

pH is a vital environmental factor in microbial fermentation. It modulates cell growth, membrane permeability, and gene expression profiles, reshapes intracellular metabolic flux to control by-product formation, and ultimately affects the synthesis efficiency of heterologous proteins

[4,5]. Acetate is a major inhibitory by-product generated during *E. coli* cultivation and has adverse impacts on both cell growth and recombinant protein expression [6]. At low concentrations, acetate can reduce respiratory activity and glucose consumption, and even trigger cell autolysis. When the acetate concentration exceeds 40 mmol/L, recombinant protein expression is significantly suppressed, with genetically engineered strains exhibiting high sensitivity to acetate stress [4,7]. The toxic mechanism of acetate is mainly attributed to protonated acetate penetrating cell membranes and decreasing intracellular pH. Cells consume large amounts of ATP to extrude hydrogen ions for pH homeostasis, forming futile energy cycles and disrupting normal metabolism, thereby drastically inhibiting the accumulation of target proteins [4,8].

In *E. coli*, acetate is mainly synthesized through two metabolic pathways. The AckA–Pta pathway is the major route of acetate production, in which excess carbon sources drive the conversion of acetyl-coenzyme A (acetyl-CoA) into acetate [9]. In addition, the PoxB pathway can be activated under conditions of low oxygen and high glucose, catalyzing the oxidation of pyruvate to generate additional acetate [10]. Previous studies have demonstrated that knockout of acetate-synthesizing genes, including *ackA*, *pta*, and *poxB*, induces impaired cell growth, suggesting that genetic modification is not a feasible approach for relieving acetate accumulation [9,11]. In contrast, precise pH regulation offers a promising approach to optimize carbon flux distribution and reduce by-product synthesis. By influencing membrane properties and enzyme activities, pH stabilizes intracellular acid–base homeostasis, improving plasmid stability and protein folding efficiency [12,13]. Furthermore, pH also modulates key glycolytic enzymes to optimize carbon distribution and reduce futile metabolism, ultimately creating a favorable intracellular environment for heterologous protein expression [5,9].

pH regulation and staged pH control are commonly used for optimizing *E. coli* fermentation. However, the selection of pH regulators has received little attention; improper pH regulators tend to accumulate inhibitory ions in the system and induce metabolic disorders, leading to reduced yield and unstable product quality. In addition, single-factor experiments cannot analyze interactive effects among variables and fail to achieve global process optimization, limiting their value for industrial-scale production [14]. Given the high toxicity and cost of isopropyl-β-D-thiogalactoside, lactose was selected as a safer and more economical alternative inducer in this study.

Dynamic feedback control integrated with metabolic flux analysis and intelligent algorithms has emerged as an advanced strategy for fermentation optimization [15]. Nevertheless, it demands sophisticated equipment and high operational costs. In this study, we optimized the staged pH process for VP2 protein expression, including selecting proper pH regulators through ionic effect evaluation, identifying the optimal growth pH using dissolved oxygen (DO) curves, and constructing multi-gradient staged pH control regimes. The resulting pH control strategy enabled high-level VP2 production in *E. coli* 0125, mitigating the limitations of constant-pH fermentation and achieving high cell density, low acetic acid content, and high yield of active VP2. The methodologies developed for pH regulator screening and pH parameter determination provide a practical approach for optimizing recombinant protein fermentation processes.

Material and Methods

Ethical Statement

Ethical approval was not required for this study.

Bacterial Strain

The engineered strain *E. coli* 0125 for VP2 protein expression was constructed by transforming competent *E. coli* BL21 (DE3) cells with the recombinant plasmid pET28a-rVP2 harboring the VP2-encoding gene. This strain is preserved in the Culture Collection Center of the Shandong Binzhou Animal Science and Veterinary Research Institute. The expression vector pET28a is a medium-to-low copy-number plasmid.

Culture Media

Seed Medium

The seed medium consisted of yeast extract (5.0 g/L), peptone (10.0 g/L), sodium chloride (NaCl; 10.0 g/L), and glucose (5.0 g/L).

Fermentation Medium

The fermentation medium contained glycerol (10 g/L), yeast extract (5 g/L), peptone (2 g/L), ammonium sulfate [$(NH_4)_2SO_4$; 4 g/L], potassium dihydrogen phosphate (KH_2PO_4; 3 g/L), potassium chloride (KCl; 1.5 g/L), magnesium sulfate ($MgSO_4$; 2 g/L), citric acid (2 g/L), betaine (1.5 g/L), ferrous sulfate ($FeSO_4$; 0.5 g/L), thiamine (0.05 g/L), biotin (0.02 g/L), and trimethylglycine (2.0 g/L). Additional inorganic salts were supplemented into the fermentation medium according to experimental designs.

Culture Conditions

Seed Culture

A single colony of *E. coli* 0125 was picked, inoculated into a 100-mL Erlenmeyer flask containing 50 mL of the seed medium, and cultured at 37°C with shaking at 180 r/min for 12 h.

Shake-flask Culture

The seed culture was inoculated into a 500-mL Erlenmeyer flask containing 100 mL of the fermentation medium at an inoculum size of 2%, followed by cultivation at 37°C and 180 r/min for 12 h.

10-L Fermenter Culture

The seed culture was inoculated into a 10-L fermenter (GRJ-10; Zhenjiang Green Bioengineering, China) containing 6 L of the fermentation medium at an inoculum size of 2%. The temperature was maintained at 37°C, and DO was maintained above >20% by adjusting the stirring speed and the aeration rate. The pH was kept at the preset value throughout fermentation as required. When the optical density at 600 nm reached 2.0, lactose was added to a final concentration of 10 g/L to induce recombinant VP2 production. Once the initial carbon source was depleted, glycerol was supplied through a DO-feedback-controlled feeding strategy.

Staged pH Regulation Strategy

Based on the effects of different pH conditions on VP2 protein expression observed in this study, and with reference to the design method reported by Zhao et al. [5], nine two-stage pH control strategies were designed and evaluated. Different pH values were applied during the early culture stage to determine the optimal conditions for strain growth, and a series of pH gradients were arranged for the late stage to evaluate their effects on acetic acid production and VP2 expression. The detailed parameters for each group are shown in *Table 1*.

Determination of Fermentation Parameters

Real-time monitoring of temperature, pH, and DO was performed using the built-in electrodes of the fermenter.

Fermentation broth samples were appropriately diluted, and the absorbance at 600 nm was measured to assess cell growth. The cells were harvested by centrifugation at 8000 g for 10 min at 4°C. The wet cell weight (g/L) was determined immediately.

Table 1. *Design of pH regulation strategy*

Strategy No.	First-stage pH and Time	Second-stage pH and Time
I	7.2 (0–8 h)	7.0 (8–12 h)
II	7.2 (0–8 h)	6.8 (8–12 h)
III	7.2 (0–8 h)	6.5 (8–12 h)
IV	7.2 (0–6 h)	7.0 (6–12 h)
V	7.2 (0–6 h)	6.8 (6–12 h)
VI	7.2 (0–6 h)	6.5 (6–12 h)
VII	7.2 (0–4 h)	7.0(4–12 h)
VIII	7.2 (0–4 h)	6.8 (4–12 h)
IX	7.2 (0–4 h)	6.5 (4–12 h)

Acetate concentration was determined by high-performance liquid chromatography (HPLC). The HPLC system consisted of an Agilent 1260 HPLC instrument (Agilent Technologies, CA, USA) equipped with an Aminex HPX-87H column (Bio-Rad, CA, USA) and a refractive index detector. Fermentation samples were centrifuged at 12.000*g* for 3 min prior to detection. The supernatant was diluted 10 times and filtered through a microporous membrane. The mobile phase was 0.005 M sulfuric acid (H_2SO_4) at a constant flow rate of 0.5 mL/min.

Detection and Activity Definition of VP2 Protein

Preservation of the three-dimensional structure of the VP2 protein is critical for its immunogenicity. The agar gel precipitation (AGP) assay can preserve native protein conformation and ensure specific recognition of functional epitopes by antibodies. Accordingly, VP2 antigen titer was determined by AGP in accordance with the national standard GB/T 19167-2020 *Diagnostic Techniques for Infectious Bursal Disease* [16]. To improve the accuracy of endpoint judgment, continuous gradient dilution was adopted in addition to the conventional two-fold serial dilution. The highest dilution that produced clear, specific precipitation lines was recorded as the antigen titer of the sample.

The specific VP2 titer (U/g) was defined as the VP2 activity per gram of wet cells, which directly reflected the functional expression level of recombinant VP2. Compared with total protein quantification, this indicator provides a more objective evaluation of the effects of fermentation conditions on VP2 biological activity. The total VP2 titer was calculated as follows:

Total VP2 titer per unit volume (U/L) = Wet cell weight (g/L) × Specific VP2 titer (U/g)

Statistical Analysis

Data were expressed as mean ± standard deviation. All statistical analyses were performed using R software, version 4.5.1. Data normality and homogeneity of variance were assessed using the Shapiro-Wilk test and Levene's test, respectively. One-way analysis of variance was performed for data satisfying the assumptions of parametric tests. The Kruskal-Wallis *H* test was used for nonparametric analysis. Multiple comparisons were conducted using Duncan's new multiple range test using the agricolae package. Differences were considered statistically significant at $P<0.05$.

Results

Influence of Inorganic Salt Cations on the Growth of the *E. coli* 0125 Strain

The effects of different inorganic salt cations on the growth of *E. coli* 0125 are shown in *Fig. 1-A*. NaCl supplementation

at concentrations of 0-125 mmol/L did not significantly affect cell density (P>0.05), indicating that Na^+ had neither growth-promoting nor inhibitory effects on the strain. In contrast, ammonium chloride (NH_4Cl) and KCl supplementation significantly increased cell density (P<0.05) in a concentration-dependent manner *(Fig. 1-B, C)*. The maximum cell density was observed at 100 mmol/L for both NH_4Cl and KCl, indicating their strongest growth-promoting effect at this concentration. As Cl^- was present in all three salts, its contribution to the observed strain growth could be excluded. Collectively, these results confirmed that NH_4^+ and K^+ significantly promoted the growth of *E. coli* 0125, whereas Na^+ had no such effect.

Influence of Inorganic Salt Anions on the Growth of Strain *E. coli* 0125

The effects of different anions on the growth of *E. coli* 0125 were further evaluated. As shown in *Fig. 2*, supplementation with sodium nitrate ($NaNO_3$) and sodium sulfate (Na_2SO_4) significantly increased cell density in a concentration-dependent manner (P<0.05). For both salts, the maximum cell density was achieved at 100 mmol/L. Combined with the results obtained for NaCl (*Fig. 1-A*), the three treatments shared the same cation (Na^+), which had previously been shown to exert no significant effect on strain growth. Therefore, the contribution of the cation could be excluded, and the effects of the respective anions were evaluated. The results demonstrated that Cl^- had no significant influence on cell growth (P>0.05), whereas both NO_3^- and SO_4^{2-} significantly promoted cell proliferation of *E. coli* 0125 (P<0.05).

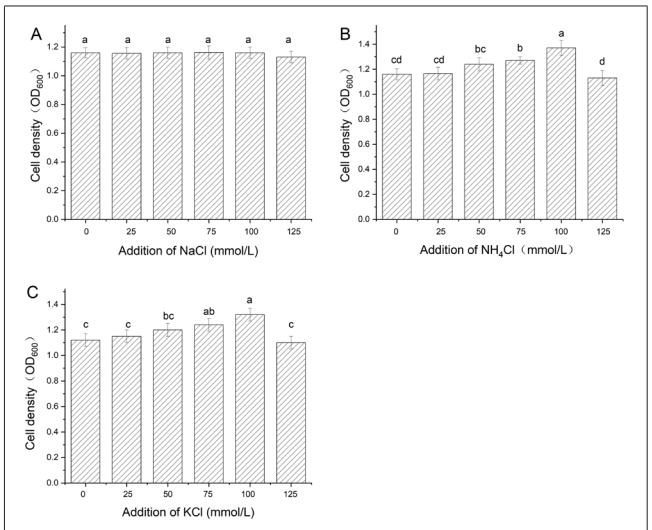

Fig 1. Effects of different concentrations of NaCl, NH_4Cl, and KCl on the growth of E. coli 0125. **(A)** NaCl addition; **(B)** NH_4Cl addition; and **(C)** KCl addition. Data are presented as mean ± standard deviation of three independent biological replicates (n = 3). Different lowercase letters above the bars denote significant differences (P<0.05)

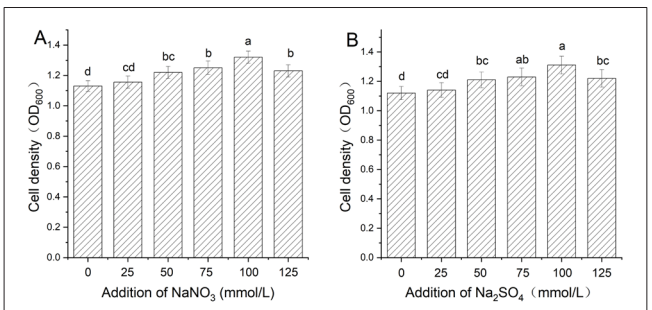

Fig 2. Effects of different concentrations of $NaNO_3$ and Na_2SO_4 on the growth of E. coli 0125. **(A)** $NaNO_3$ addition and **(B)** Na_2SO_4 addition. Data are presented as mean ± standard deviation of three independent biological replicates (n = 3). Different lowercase letters above the bars denote significant differences (P<0.05)

Screening of the Optimal pH Regulator for the *E. coli* 0125 Strain

Based on the cation screening results *(Fig. 1-B, C)*, NH_4^+ and K^+ were identified as growth-promoting ions. Therefore, aqueous ammonia ($NH_3{\cdot}H_2O$) and potassium hydroxide (KOH) were selected as candidate pH regulators, and sodium hydroxide (NaOH) served as the control. As shown in *Fig. 3-A*, the maximum cell density in the NaOH group was 19.23 g/L, whereas that in the $NH_3{\cdot}H_2O$ and KOH groups increased to 22.76 g/L (18.47% increase) and 21.85 g/L (13.62% increase), respectively. Moreover, the specific VP2 titer in both the $NH_3{\cdot}H_2O$ and KOH groups reached 64 U/g, which was significantly higher than that in the NaOH group (48 U/g; P<0.05; *Fig. 3-B)*. Notably, when $NH_3{\cdot}H_2O$ was used for pH control, the strain entered the stationary phase in approximately 8 h *(Fig. 3-A)*.

Identification of the Optimal Growth pH Using DO Profiles

The optimal growth pH of *E. coli* 0125 was determined by monitoring DO dynamics. Because hydrochloric acid (HCl) and NaOH exerted no obvious adverse effects on strain growth, pH adjustment was conducted after the strain entered the stationary phase. First, the pH was reduced to 6.0 using HCl within 10 min, and then

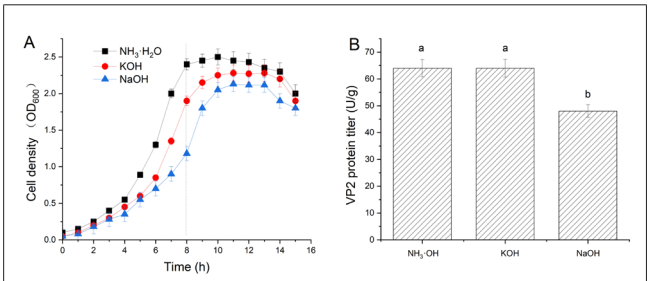

Fig 3. Validation of the optimal pH regulator and determination of the stationary phase for E. coli 0125. **(A)** Time course of cell density under different pH regulators. **(B)** Specific VP2 titer. Data are presented as mean ± standard deviation of three independent biological replicates (n = 3). Different lowercase letters denote significant differences (P<0.05)

gradually increased to 9.0 using NaOH over a period of 30 min. The corresponding DO profiles are presented in *Fig. 4*.

As the pH increased from 6.0 to 7.2, the DO content decreased continuously, suggesting an increase in the oxygen consumption rate and cellular metabolic activity. The minimum DO value was observed at pH 7.2, which corresponded to the highest oxygen consumption rate, vigorous respiratory metabolism, and strong physiological activity of the strain. When pH exceeded 7.2, the DO concentration rebounded, indicating a reduction in oxygen uptake and a decline in metabolic activity. Based on the principle that the DO trough corresponds to the maximum oxygen consumption rate, pH 7.2 was identified as the optimal growth pH for *E. coli* 0125.

Determination of the Optimal Growth pH Value for the *E. coli* 0125 Strain

The effects of constant-pH control on cell growth, VP2 expression, and acetate accumulation were further evaluated *(Fig. 5)*. As shown in *Fig. 5-A*, the highest wet cell weight (25.62 g/L) was achieved at pH 7.2, exceeding the values observed at pH 6.8, 7.0, and 7.4 by 26.08%, 12.57%, and 19.55%, respectively (P<0.05). The specific VP2 titer reached 80 U/g at both pH 7.0 and pH 7.2, which was significantly higher than the values observed at pH 6.8 and pH 7.4 (P<0.05; *Fig. 5-B)*. However, acetate accumulation was also the highest at pH 7.2, reaching 2.49 g/L, which was 1.20-, 1.11-, and 1.15-fold higher than that measured at pH 6.8, 7.0, and 7.4, respectively (P<0.05; *Fig. 5-C)*.

Collectively, the findings revealed that high cell density at pH 7.2 was accompanied by severe acetate overflow.

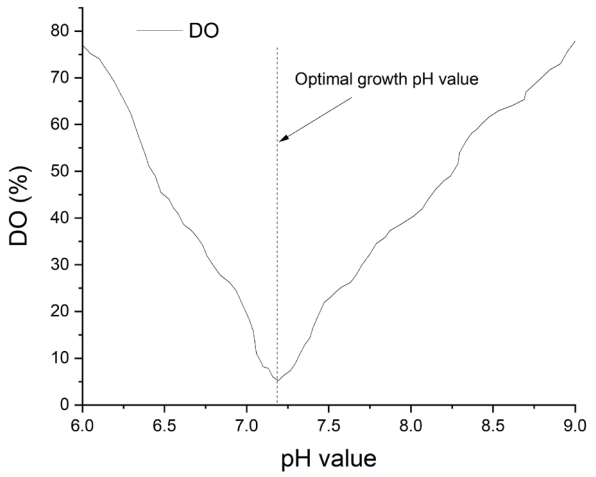

Fig 4. Changes in DO levels of E. coli 0125 during the stationary phase under varying pH conditions. The dashed line marks the optimal growth pH value (7.2). Note: The curve shows a schematic representation of the overall trend

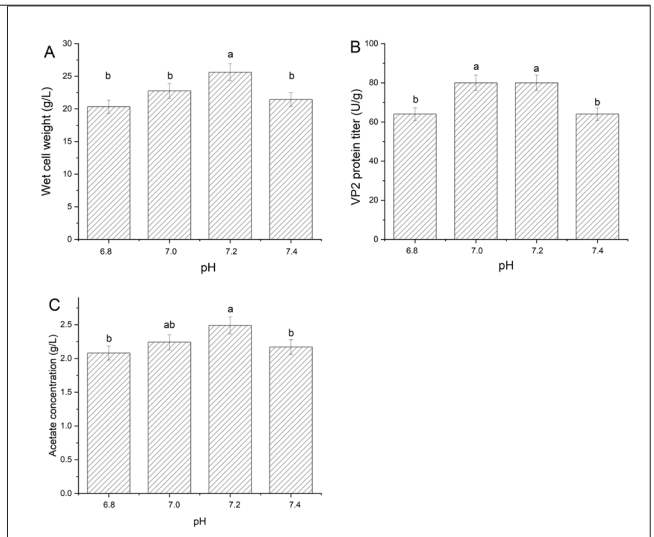

Fig 5. Effects of different constant-pH control levels on E. coli 0125 fermentation. **(A)** Wet cell weight, **(B)** specific VP2 titer, and **(C)** acetate concentration. Data are presented as mean ± standard deviation of three independent biological replicates (n = 3). Different lowercase letters denote significant differences (P<0.05)

Therefore, a single constant-pH strategy is insufficient to simultaneously balance cell growth, recombinant protein expression, and by-product control. These results further highlight the necessity of developing a staged pH regulation strategy for this process.

Influence of Various Staged pH Control Strategies on VP2 Protein Expression

The effects of nine staged pH regulation strategies on cell growth, VP2 expression, and acetate accumulation were evaluated *(Fig. 6)*. Significant differences in all three indicators were observed among the groups (P<0.05).

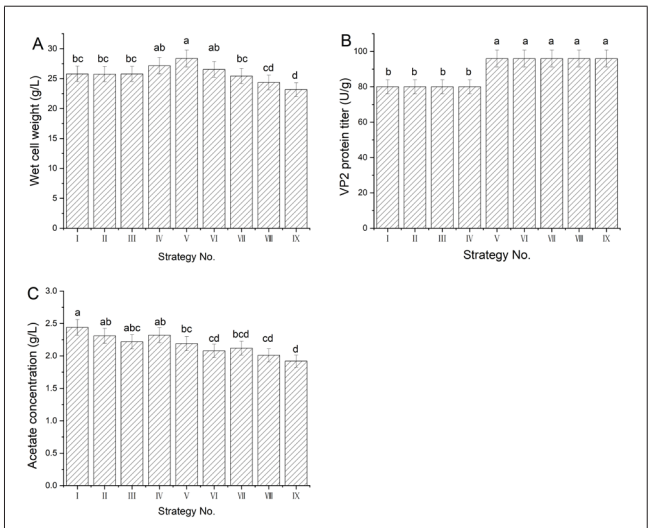

Fig 6. Effects of different staged pH control strategies on E. coli 0125 fermentation and VP2 protein expression. **(A)** Wet cell weight, **(B)** specific VP2 titer, and **(C)** acetate concentration. Data are presented as mean ± standard deviation of three independent biological replicates (n = 3). Different lowercase letters denote significant differences (P<0.05)

Group V exhibited the best comprehensive performance. It achieved the highest wet cell weight of 28.37 g/L, which was significantly higher than most constant-pH groups *(Fig. 6-A)*. The specific VP2 titer reached 96 U/g, whereas acetate concentration remained at a relatively low level of 2.19 g/L *(Fig. 6-B, C)*.

Further analysis showed that maintaining pH at 7.2 during the first 6 h resulted in significantly higher cell density compared with the 4 or 8 h initial periods ($P<0.05$). Moreover, the lowest acetate accumulation was observed in groups that shifted to a lower pH in the second stage, which effectively reduced overflow metabolism. Lower acetate levels contributed to improved VP2 activity, as the specific titer in Groups V-IX reached 96 U/g, significantly higher than that in Groups I-IV ($P<0.05$).

Discussion

As a critical environmental factor, pH modulates the growth, carbon metabolism distribution, and heterologous protein production of *E. coli*. Dynamic changes in pH can significantly affect intracellular metabolism, by-product formation, and protein folding, thereby playing a vital role in the optimization of recombinant protein fermentation. Focusing on the fermentation characteristics of recombinant IBDV VP2 protein, this study systematically carried out three sets of experiments: screening of ionic pH regulators, determination of baseline pH based on DO profiles, and multi-gradient optimization of staged pH control. Based on the findings, a tailored pH regulation system was established for the engineered strain *E. coli* 0125 and VP2 antigen production. This strategy successfully addresses the dilemma of conventional constant-pH fermentation, which often fails to simultaneously achieve robust cell growth, minimize acetate accumulation, and maintain high-activity protein synthesis. The findings provide valuable technical references for the industrial fermentation of VP2 subunit vaccines.

In fermentation systems, inorganic anions and cations introduced through pH regulators can modulate cellular metabolism by changing membrane permeability, intracellular enzyme activity, and environmental osmotic pressure. Such ionic effects are essential considerations when selecting acid–base regulators [17,18]. To eliminate interference from mixed ions, we adopted a single-variable design with paired fixed ions to separately evaluate the effects of different cations and anions on strain growth *(Fig. 1; Fig. 2)*. The results showed that Na^+ exerted no significant influence on cell proliferation, whereas NH_4^+, K^+, NO_3^-, and SO_4^{2-} significantly promoted cell growth. Based on these findings, $NH_3{\cdot}H_2O$ and KOH were selected as optimal pH regulators for subsequent tests. Compared with NaOH, $NH_3{\cdot}H_2O$ supplies an additional nitrogen source while regulating pH, making it more widely applicable in industrial fermentation [19]. Shen et al. [20] also reported that $NH_3{\cdot}H_2O$ stabilized pH and provided supplemental nitrogen during amylase production by *Bacillus*. The results of this study further validated this advantage: the cell density in the $NH_3{\cdot}H_2O$ group increased by 18.36% relative to the NaOH group, and the specific VP2 titer reached 80 U/g. These findings highlight the practical significance of rational regulator selection for industrial production and indicate that focusing merely on pH values while ignoring ionic effects will lead to mismatched process parameters in practical manufacturing.

Following the selection of appropriate pH regulators, online DO monitoring was employed to identify the favorable growth pH for the strain. Previous studies have shown that stable agitation and aeration conditions allow oxygen consumption rate to indicate cellular respiratory activity, and DO troughs usually coincide with high physiological performance [21,22]. In this study, aeration, stirring speed, and tank pressure were maintained at constant levels during the stationary phase of the VP2 expression strain to ensure a stable oxygen supply. Under these conditions, DO profiles were considered to indirectly reflect cell growth. Therefore, the pH corresponding to peak oxygen consumption was selected as the optimal growth pH, which was determined to be 7.2 *(Fig. 4)*. Regulators with little impact on cell growth were used in this study. Validation results revealed that biomass at pH 7.2 increased by 26.08% compared with that at pH 6.8 *(Fig. 5-A)*. This rapid screening technique has been patented [23]. Nevertheless, its applicability is limited, and it is primarily suitable for pH condition screening of aerobic microorganisms.

However, the pH conditions that favor optimal growth also present obvious disadvantages. A high growth rate accelerates glycolysis and triggers acetyl-CoA overflow, leading to massive acetate accumulation. The acetate concentration reached 2.49 g/L at pH 7.2, which was 1.2-fold higher than that at pH 6.8 *(Fig. 5-C)*. Excess acetate further inhibited heterologous protein expression [21,22], consistent with previous studies [24,25]. These results highlight an inherent contradiction between the optimal pH for cell growth and that for target protein synthesis. Consequently, a single constant-pH strategy is unable to simultaneously achieve high cell density, low by-product formation, and high-activity protein production, which is the core motivation for developing the staged pH strategy in this study.

Microorganisms usually require distinct pH conditions for cell growth and product synthesis. Staged dynamic pH regulation, in which pH is dynamically adjusted to meet the stage-specific physiological demands, has emerged as a classic strategy to balance cell proliferation and product

formation [26]. Mital et al. [27] reported that weakly alkaline conditions suppressed cell growth but improved soluble expression of intracellular alcohol dehydrogenase. Guo et al. [28] applied a two-stage differentiated pH strategy in mixed-strain fermentation and achieved a substantial increase in ethanol yield. Both studies demonstrated the superiority of staged pH control. For *E. coli*, the conventional optimal growth pH ranges from 7.0 to 7.5. However, this pH range induces excessive carbon flux and severe acetate overflow, and also increases plasmid loss and reduces recombinant protein stability [29]. Therefore, maintaining the optimal growth pH in the early phase to ensure cell proliferation, followed by a shift to weakly acidic pH in the middle and late phases to reduce acetate accumulation, is a feasible strategy for VP2 production.

Based on the effects of different pH values on VP2 fermentation, nine staged pH control modes were established in this study *(Table 1; Fig. 6)*. As pH 7.2 was identified as the optimal condition for strain growth, it was used throughout the early fermentation stage. Acetic acid content increased with rising pH during cultivation, while favorable fermentation performance was achieved at pH 6.5, 6.8, and 7.0. Therefore, these values were selected for pH regulation in the late stage. Rapid cell proliferation is known to accelerate acetic acid synthesis. Accordingly, three duration gradients (0-4 h, 0-6 h, and 0-8 h) were designed to represent different phases of exponential growth. Although the specific cell growth rate was not measured in this study, staged pH adjustment was employed to regulate cell growth status, so as to reduce acetic acid accumulation and improve VP2 production. The results demonstrated that the retention time of pH 7.2 in the early stage significantly affected the final biomass yield. Maintaining pH 7.2 for the first 6 h resulted in satisfactory biomass accumulation, whereas lowering pH at 4 h in advance caused acid stress and inhibited cell growth. Shortening the duration of initial high pH combined with low pH regulation in the later stage effectively reduced the total acetic acid production *(Fig. 6-C)*. Group IX exhibited the lowest acetic acid content, which was 22.89% lower than that at constant pH 7.2 conditions. A negative correlation was observed between acetic acid accumulation and VP2-specific potency, and Groups VI-IX achieved a specific potency of 96 U/g *(Fig. 6-B)*. Thus, a two-stage pH control strategy could simultaneously achieve high-density culture and efficient VP2 synthesis. Previous studies have demonstrated that mild acidic conditions can reshape carbon metabolism, alleviate glucose overflow metabolism in *E. coli*, and restrict acetic acid formation without severely interfering with primary metabolic pathways [30,31]. These observations were highly consistent with the findings of this study. Specifically, the results of this study demonstrated that pH 6.5 could inhibit acetic acid accumulation more effectively compared with neutral pH. Therefore, appropriately decreasing pH during the middle and late fermentation stages represents an efficient approach to relieve overflow metabolism and increase recombinant protein yield. This approach provides a valuable reference for by-product control in other fermentation processes of recombinant proteins expressed by *E. coli*.

Unlike conventional studies that focus solely on optimizing a single pH value or adopting fixed staged control regimes, this study combined the screening of pH regulators with the stage-dependent physiological characteristics of cells to develop a novel staged pH control process. This process does not require molecular modifications such as gene knockout, thereby avoiding growth defects caused by strain engineering. It has a lower technical threshold and is easier to popularize in practical production.

All fermentation experiments in this study were performed in triplicate. The experimental data showed minimal variation and consistent trends across replicates, indicating excellent batch-to-batch repeatability and stability of the established staged pH strategy. For industrial applications, this strategy relies solely on conventional acid-base feeding, ensuring compatibility with existing equipment while minimizing material costs, thereby meeting the requirements of large-scale vaccine production. In terms of scale-up potential, the core principle of "promoting cell growth at the early stage and restraining by-product formation at the late stage" is consistent with the general metabolic characteristics of high-density *E. coli* fermentation, thereby facilitating gradual process scale-up.

In conclusion, this study established a refined, physiology-driven staged pH control strategy for the high-yield production of active IBDV VP2. Unlike empirical and universal pH control methods, this study innovatively integrated ionic effect evaluation with real-time DO monitoring, achieving coordinated optimization of cell growth, reduction of acetic acid overflow, and synthesis of functional antigens. Owing to its favorable stability, ease of implementation, and potential for industrial scale-up, the proposed process provides a practical and reliable approach for industrial fermentation of IBDV VP2 subunit vaccines.

Declarations

Availability of Data and Materials: The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgments: We thank all authors for their significant contributions to this study.

Financial Support: This study was funded by the Young Science

and Technology Rising Star Program, Binzhou (QMX2023003), and the Natural Science Foundation of Shandong Province (ZR2023MC196).

Conflicts of Interest: The authors declare no conflicts of interest.

Declaration of Generative Artificial Intelligence (AI): The authors confirm that no generative AI created the manuscript text, tables or figures. AI tools were only used for linguistic revision.

Author Contributions: Q.F. and Y.S.: Data curation, data analysis, methodology, writing - original draft preparation. X.Z. and W.M.: Supervision, writing - review. L.M. and W.W.: Supervision, writing - review and editing. C.L. and L.C.: Conceptualization, experimental design, supervision, writing – review and editing.

References

1. **Beshah A, Ahmed A, Dandecha M:** Epidemiology and risk factors of infectious bursal disease: A review. *J Bacteriol Mycol,* 11 (2): 1219, 2024. DOI: 10.26420/jbacteriolmycol.2024.1219
2. **Muneeb JM, Pawaskar DK, Ullah-Rafiqui M, Siddiqui R, Shabir N, Pawaskar NK:** A comprehensive overview of infectious bursal disease, a major threat to poultry health and production. *Int J Adv Biochem Res,* 8, 154-63, 2024. DOI: 10.33545/26174693.2024.v8.i12sc.3092
3. **Ramon G, Legnardi M, Cecchinato M, Cazaban C, Tucciarone CM, Fiorentini L, Gambi L, Mato T, Berto G, Koutoulis K, Franzo G:** Efficacy of live attenuated, vector and immune complex infectious bursal disease virus (IBDV) vaccines in preventing field strain bursa colonization. *Front Vet Sci,* 9:978901, 2022. DOI: 10.3389/fvets.2022.978901
4. **Liu S, Xu J, Zhang W:** Advances and prospects in metabolic engineering of *Escherichia coli* for L-tryptophan production. *World J Microbiol Biotechnol,* 38:22, 2022. DOI: 10.1007/s11274-021-03212-1
5. **Zhao C, Fang H, Wang J, Zhang S, Zhao X, Li Z, Lin C, Shen Z, Cheng L:** Application of fermentation process control to increase l-tryptophan production in *Escherichia coli*. *Biotechnol Prog,* 36:e2944, 2020. DOI: 10.1002/btpr.2944
6. **Sangareddy V, Mallu MR, Matur RV, Shaik FB, Nettem B, Puladas S:** Enhancing *Escherichia coli* cell density and recombinant protein production through the control of acetate accumulation. *3 Biotech,* 15:316, 2025. DOI: 10.1007/s13205-025-04490-4
7. **Zhao C, Cheng L, Wang J, Shen Z, Chen N:** Impact of deletion of the genes encoding acetate kinase on production of L-tryptophan by *Escherichia coli*. *Ann Microbiol,* 66, 261-269, 2016. DOI: 10.1007/s13213-015-1103-4
8. **Zhao C, Cheng L, Xu Q, Wang J, Shen Z, Chen N:** Improvement of the production of L-tryptophan in *Escherichia coli* by application of a dissolved oxygen stage control strategy. *Ann Microbiol,* 66, 843-854, 2016. DOI: 10.1007/s13213-015-1172-4
9. **Schütze A, Benndorf D, Püttker S, Kohrs F, Bettenbrock K:** The impact of *ackA, pta,* and *ackA-pta* mutations on growth, gene expression and protein acetylation in *Escherichia coli* K-12. *Front Microbiol,* 11:233, 2020. DOI: 10.3389/fmicb.2020.00233
10. **Nahku R, Valgepea K, Lahtvee PJ, Erm S, Abner K, Adamberg K, Vilu R:** Specific growth rate dependent transcriptome profiling of *Escherichia coli* K12 MG1655 in accelerostat cultures. *J Biotechnol,* 145 (1): 60-65, 2010. DOI: 10.1016/j.jbiotec.2009.10.007
11. **Ping J, Wang L, Qin Z, Zhou Z, Zhou J:** Synergetic engineering of *Escherichia coli* for efficient production of l-tyrosine. *Synth Syst Biotechnol,* 8, 724-731, 2023. DOI: 10.1016/j.synbio.2023.10.005
12. **Poolman B:** Physicochemical homeostasis in bacteria. *FEMS Microbiol Rev,* 47 (4):fuad033, 2023. DOI: 10.1093/femsre/fuad033
13. **Monterroso B, Margolin W, Boersma AJ, Rivas G, Poolman B, Zorrilla S:** Macromolecular crowding, phase separation, and homeostasis in the orchestration of bacterial cellular functions. *Chem Rev,* 124 (4): 1899-1949, 2024. DOI: 10.1021/acs.chemrev.3c00622
14. **Gupta PK, Edula JR:** Strategies for enhancing product yield: Design of experiments (DOE) for *Escherichia coli* cultivation. **In,** Laranjo M (Ed): Fermentation - Processes, Benefits and Risks. *InTech Open,* 3:15, 2021. DOI: 10.5772/intechopen.99288
15. **Krausch N, Doff-Sotta M, Cannon M, Neubauer P, Bournazou MN:** Deep learning adaptive model predictive control of fed-batch cultivations. *Comp Chem Eng,* 203:109344, 2025. DOI: 10.1016/j.compchemeng.2025.109344
16. **Standardization Administration of the P.R.C. Diagnostic Techniques for Infectious Bursal Disease:** GB/T 19167-2020. Beijing: Standards Press of China, 2020.
17. **Csitári B, Bedics A, Felföldi T, Boros E, Nagy H, Máthé I, Székely AJ:** Anion-type modulates the effect of salt stress on saline lake bacteria. *Extremophiles,* 26 (1):12, 2022. DOI: 10.1007/s00792-022-01260-5
18. **Kim S, Jeong CH, Kim JC, Lee M, Jeong SG, Kim HM, Park HW:** Optimizing divalent cation supplementation to enhance the production of the kimchi starter strain *Latilactobacillus curvatus* WiKim0094. *J Microbiol Biotechnol,* 35:e2505011, 2025. DOI: 10.4014/jmb.2505.05011
19. **Abadli M, Dewasme L, Tebbani S, Dumur D, Wouwer AV:** Experimental validation of a nonlinear model predictive controller regulating the acetate concentration in fed-batch *Escherichia coli* BL21(DE3) cultures. *Adv Cont Appl,* 4:e95, 2022. DOI: 10.1002/adc2.95
20. **Shen P, Niu D, Permau K, Tian, K, Singh S, Wang Z:** Exploitation of ammonia-inducible promoters for enzyme overexpression in *Bacillus licheniformis. J Ind Microbiol Biotechnol,* 48 (5-6):kuab037, 2021. DOI: 10.1093/jimb/kuab037
21. **Wu C, Qu J, Zhang H, Gao M, Zhu L, Zhan X:** New two-stage pH combined with dissolved oxygen control strategy for cyclic β-1,2 glucans synthesis. *Appl Microbiol Biotechnol,* 107, 2235-2247, 2023. DOI: 10.1007/s00253-023-12463-x
22. **Liu E, Wilkins M:** Process optimization and scale-up production of fungal aryl alcohol oxidase from genetically modified *Aspergillus nidulans* in stirred-tank bioreactor. *Biores Technol,* 315:123792, 2020. DOI: 10.1016/j.biortech.2020.123792
23. **Shandong Binzhou Animal Husbandry and Veterinary Research Institute, Shandong Lvdu Biotechnology Co., Ltd:** Method for rapidly screening optimal pH regulator and determining optimal growth pH of aerobic microorganisms. Chinese Patent CN107129934B, 2020.
24. **Candry P, Radić L, Favere J, Carvajal-Arroyo J, Rabaey K, Ganigué R:** Mildly acidic pH selects for chain elongation to caproic acid over alternative pathways during lactic acid fermentation. *Water Res,* 186:116396, 2020. DOI: 10.1016/j.watres.2020.116396
25. **Li S, Meng X, Zhang S, Zhang Y, Yang R, Wang D, Yang Y, Liu P, Kang J:** A unified intracellular pH landscape with SITE-pHorin: A quantum-entanglement-enhanced pH probe. *Sci China Life Sci,* 69, 239-257, 2026. DOI: 10.1007/s11427-025-2971-5
26. **Bühlmann C, Mickan B, Tait S, Batstone D, Mercer G, Bahri P:** Lactic acid from mixed food waste fermentation using an adapted inoculum: Influence of pH and temperature regulation on yield and product spectrum. *J Clean Prod,* 373:133716, 2022. DOI: 10.1016/j.jclepro.2022.133716
27. **Mital S, Christie G, Alcasabas A, Mellor R, Dikicioglu D:** pH-modulated soluble expression of alcohol dehydrogenases in *Escherichia coli* using adaptive laboratory evolution. *Trend Biotechnol,* 43 (11): 2904-2925, 2025. DOI: 10.1016/j.tibtech.2025.07.013
28. **Guo X, Huang Y, Tang Y, Xia A, Zhu X, Zhu X, Liao Q:** Two-step pH regulating ethanol production through continuous CO/CO_2 gas fermentation by mixed bacteria from rabbit faeces. *Renewable Energy,* 228 (C), 2024. DOI: 10.1016/j.renene.2024.120564
29. **Manavalan T, Bi J, Naulchan K, Bich Van NT, Chua XY, Yow R, Lee SY, Ming Chew LJ, Ottenheim C, Sugii S, Lakshmanan M, Zhou W, Arumugam P, Wong FT, Weingarten M, Ang EL:** Hyper-expression of functional human basic fibroblast growth factor 2 in *Escherichia coli*. *Protein Expr Purif,* 240:106894. DOI: 10.1016/j.pep.2026.106894
30. **Nasrin F, Azad DAK, Hasan MR, Kader DMM, Rahman BGMS, Hasan CMM:** Adaptive responses in the metabolism of *Escherichia coli*

in view of gene expressions under aerobic and micro-aerobic condition. *Am J Microbiol Res,* 7 (3): 91-97, 2019. DOI: 10.12691/ajmr-7-3-4

31. **Orr J, Christensen D, Wolfe A, Rao C:** Extracellular acidic pH inhibits acetate consumption by decreasing gene transcription of the tricarboxylic acid cycle and the glyoxylate shunt. *J Bacteriol,* 201 (2):e00410-18, 2018. DOI: 10.1128/JB.00410-18