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Research Article

Application of A Rational Feeding Strategy to Increase the Cell Density of Avian *Pasteurella multocida*

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Abstract

Avian *Pasteurella multocida* infection, can cause serious economic losses to the poultry industry every year. The inactivated vaccines of avian *Pasteurella multocida* are used to prevent infection. Increasing the cell density of avian *Pasteurella multocida* is the key to the application of these inactivated vaccines. This can be achieved by controlling the feeding strategy. This study aimed to achieve high-cell-density cultivation of avian *Pasteurella multocida* by applying an appropriate medium and a rational feeding strategy based on viable cell growth and dissolved oxygen level variation during a fermentation process. An optimized medium suited for the growth of avian *P. multocida* was used. Meanwhile, besides the online real-time determination of viable cell density, the concentration of glucose was maintained at 1.5 g/L using a glucose-stat feeding strategy after 2 h. The selected nutrient mixture, including yeast extract, tryptone, betaine, V_{B1}, and V_H, was fed using a dissolved oxygen feedback feeding strategy after 4 h. As a result, the viable cell density and cell count of avian *P. multocida* under optimized conditions were increased to OD_{600}: 9.38 and 4.58×10^{10} CFU/mL, which were higher by 7.27 and 7.26 times than those under the original conditions, respectively.

Keywords: Avian *Pasteurella multocida,* Cell density, Feeding strategy, Medium, Online viable cell monitoring

Introduction

Pasteurella multocida, a Gram-negative bacterium, is a zoonotic pathogen [1,2]. Fowl cholera, caused by avian *P. multocida*, is a highly contagious disease among various domestic and wild avian species. It is associated with high mortality and morbidity, resulting in significant economic losses in global livestock production [3]. Antibiotics used to treat diseases caused by avian *P. multocida* often result in the existence of antibiotic residues in animal-derived products, eventually leading to antibiotic resistance [4,5]. Vaccination is a successful method of controlling avian *P. multocida* infection, but vaccine application and disease control are limited by low fermentation concentration and high production costs [6,7]. The inactivated vaccines of avian *P. multocida* have been proven effective in preventing avian *P. multocida* infection [8]. Increasing the cell density of avian *P. multocida* can reduce vaccine production costs and improve vaccine efficacy.

Appropriate concentrations of nutrients in the fermentation medium could satisfy the requirement of cell growth and increase cell density [9]. Optimizing the medium components via a Box-Behnken design, the cell count of *Lactobacillus plantarum* Y44 was increased to 3.363×10^{10} CFU/mL, which was higher by 6.11 times than that in the MRS medium [10]. Meanwhile, based on the characteristics of bacterial growth, the addition of specific nutrient components could further increase the abundance of bacteria. Further, 20 mM histidine was added to the basal medium, which greatly enhanced the growth of *gdhA* derivative *P. multocida* B:2 mutant by approximately 19 times compared with that in the control culture [11]. Thiamine and biotin had the highest bioavailability from midexponential to late-exponential phase of *Streptococcus thermophilus* MN-ZLW-002, media containing trace elements can be useful for high-density cultures of bacteria [12]. Betaine can act as a stress

protectant, methyl donor, or enzyme stabilizer *in vitro* [13], the maximum L-threonine production of 127.3 g/L and glucose conversion percentage of 58.12% was obtained fed by *Escherichia coli* with the glucose solution containing 2 g/L betaine hydrochloride, which increased by 14.5 and 6.87% more compared to that of the control [14].

In the present study, appropriate nutrient components were selected for the growth of avian *P. multocida*. Meanwhile, the time point of feeding was detected rationally based on online viable cell monitoring. An appropriate feeding strategy was used to maintain glucose concentration and feed nutrient mixture, leading to an increase in the cell density of avian *P. multocida.*

Material and Methods

Strain and Media

The strain of avian *P. multocida* used in this study was obtained from the China Veterinary Culture Collection Center (Collection Number: CVCC44802).

The strain was cultured in the TSB medium containing (in g/L) glucose 2.5, tryptone 17.0, soy peptone 3.0, K_2HPO_4 2.5, and NaCl 5.0.

The four kinds of media for avian *P. multocida* fermentation in *Table 1*. The five kinds of nutrient mixture solutions used as the feed medium during avian *P. multocida* fermentation in *Table 2*.

Culture Conditions

A sample of 100-mL seed medium in a 500-mL baffled flask was inoculated with a single colony of avian *P. multocida* and cultivated at 37°C by shaking at 160 rpm for 8 h. This culture grown in the baffled flask was then inoculated aseptically (5% *v*/*v*) into 3 L of fermentation medium in a 5-L fermenter (Biotech 2012, Shanghai Bailun Biotech Co., Ltd., Shanghai, China). The temperature and pH were controlled at 37°C and 7.0, respectively, whereas the DO level was maintained at 30% by adjusting the agitation and aeration rates. We maintained the concentration of glucose at the stated level and fed the nutrient mixture solution using the DO feedback feeding strategy [15] to meet the experimental requirements.

Analytical Methods

The viable cell density of avian *P. multocida* was assessed by combining cell capacitance determination with spectrophotometric absorbance reading (OD_{600}). The cell capacitance was determined using an online viable cell monitoring system (DN 12-120, Switzerland), in a high-frequency electric field, living cells are treated as tiny capacitors, according to the principle of capacitance measurement a capacitance probe is used to measure the charge carried by the viable cell count. The viable cell count was determined by a flat colony counting method, dilution of the culture solution to the Appropriate concentration and apply to glass garden with 0.1 mL, repeat three times; calculated from the equation between capacitance and the viable cell count. The glucose concentration was determined using a glucose biosensor (SBA-40E; Biology Institute of Shandong Academy of Sciences, China), glucose oxidation to generate H_2O_2, H_2O_2 contact with the electrode generates a current, the current signal is proportional to the concentration of glucose, used to indirectly determine the concentration of glucose (Glucose + O_2 + H_2O $\xrightarrow{\text{Glucose Oxidase}}$ Gluconic acid + H_2O_2).

Table 1. *The media for avian P. multocida fermentation*

Media	Components of Media (g/L)
Medium I	glucose 5.0, yeast extract 5.0, tryptone 10.0
Medium II	glucose 5.0, yeast extract 5.0, tryptone 10.0, $(NH_4)_2SO_4$ 4.0, $MgSO_4$ 1.0, KH_2PO_4 3.0
Medium III	glucose 5.0, yeast extract 5.0, tryptone 10.0, $(NH_4)_2SO_4$ 4.0, $MgSO_4$ 1.0, KH_2PO_4 3.0, citric acid 3.0, KCl 2.0
Medium IV	glucose 5.0, yeast extract 5.0, tryptone 10.0, $(NH_4)_2SO_4$ 4.0, $MgSO_4$ 1.0, KH_2PO_4 3.0, citric acid 3.0, KCl 2.0, betaine 2.0, V_{B1} 0.025, V_H 0.01.

Table 2. *The nutrient mixture solutions for avian P. multocida fermentation*

Supply Strategy	Components of Nutrient Mixture Solution (g/L)
Strategy I	yeast extract 5.0 and tryptone 10.0
Strategy II	yeast extract 5.0, tryptone 10.0, $(NH_4)_2SO_4$ 4.0, $MgSO_4$ 1.0, KH_2PO_4 3.0
Strategy III	yeast extract 5.0, tryptone 10.0, $(NH_4)_2SO_4$ 4.0, $MgSO_4$ 1.0, KH_2PO_4 3.0, citric acid 3.0, and KCl 2.0
Strategy IV	yeast extract 5.0, tryptone 10.0, $(NH_4)_2SO_4$ 4.0, $MgSO_4$ 1.0, KH_2PO_4 3.0, citric acid 3.0, KCl 2.0, betaine 2.0, V_{B1} 0.025, and V_H 0.01
Strategy V	yeast extract 5.0, tryptone 10.0, betaine 2.0, V_{B1} 0.025, and V_H 0.01

Result

The Relationship of Between Capacitance Value, Viable Cell Density and Cell Count for Avian *P. multocida*

The relations among capacitance value, cell density, and viable cell counts during the logarithmic phase of avian *P. multocida* cultivation are displayed in *Fig. 1*. The cell count and capacitance showed a good linear relationship (y=8.0597x; y: cell count OD_{600}; x: capacitance pF/cm; R^2=0.9922); the cell count and viable cell density showed a good linear relationship (y=1.9241x; y: cell count OD_{600}; x: viable cell density (x10^{10}CFU/mL); R^2=0.9934); the capacitance and viable cell density showed a good linear relationship (y=4.3469x; y: capacitance pF/cm; x: viable cell density (x10^{10}CFU/mL); R^2=0.9906). The results showed a good linear relationship between capacitance value, viable cell density and cell count. The viable cell density during avian *P. multocida* cultivation, thus, could be calculated based on viable cell density via this equation.

Avian *P. multocida* Fermentation with Different Media

The cell capacitance and glucose concentration with different media in avian *P. multocida* fermentation are displayed in *Fig. 1*. The type of medium not only affected cell growth according to cell capacitance but also impacted glucose consumption. The cell capacitance with medium IV was the highest compared with that with other media. The maximum cell capacitance with medium IV was 2.0 h, indicating that the cells of avian *P. multocida* did not grow after 2.0 h. The glucose consumption with medium IV was the maximum, which was caused by higher cell density. The glucose concentration was 1.0 g/L after cultivation for 2.0 h.

Viable Cell Density and Cell Count

The viable cell density and cell count with different media during avian *P. multocida* fermentation are presented in *Fig. 2*. The viable cell density and cell count increased with an increase in the amount of nutrients in the media, which was in accordance with the cell capacitance shown in *Fig. 1*. The viable cell density (2.58, OD_{600}) and cell count ($1.26^{x}10^{10}$ CFU/mL) with medium IV were the highest, which were higher by 2.01 and 2.03 times than those with medium I.

Avian *P. multocida* Fermentation with Different Glucose Concentrations

Fig. 3 shows the cell capacitance, viable cell density, and cell count with different glucose concentrations after 2.0

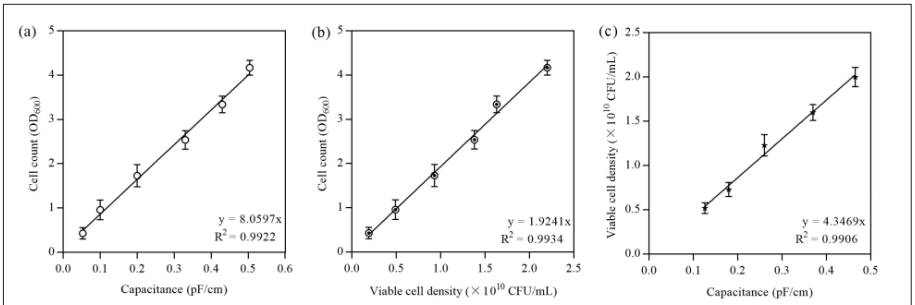

Fig 1. Relationship between capacitance and cell density and between viable cell density and cell count. (a) Linear relationship between capacitance and cell count. (b) Linear relationship of between viable cell density and cell count, (c) Linear relationship of between capacitance and viable cell density

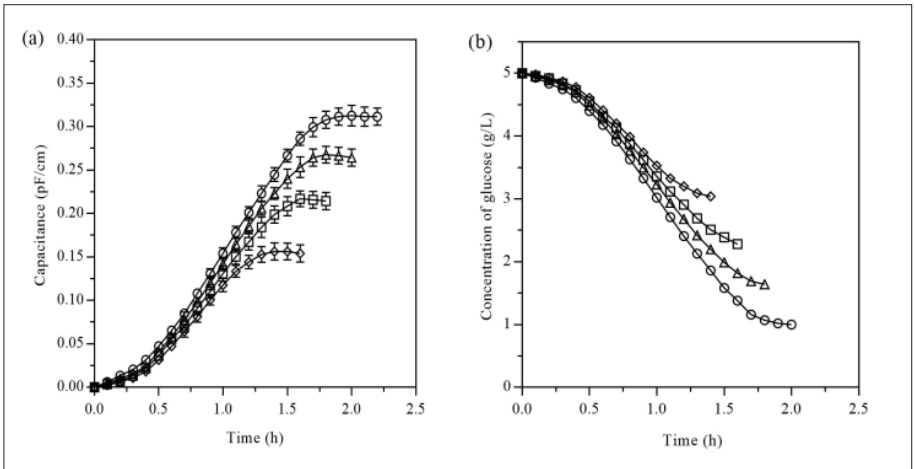

Fig 2. Cell capacitance (a) and glucose concentration (b). All values are presented as the mean±SD

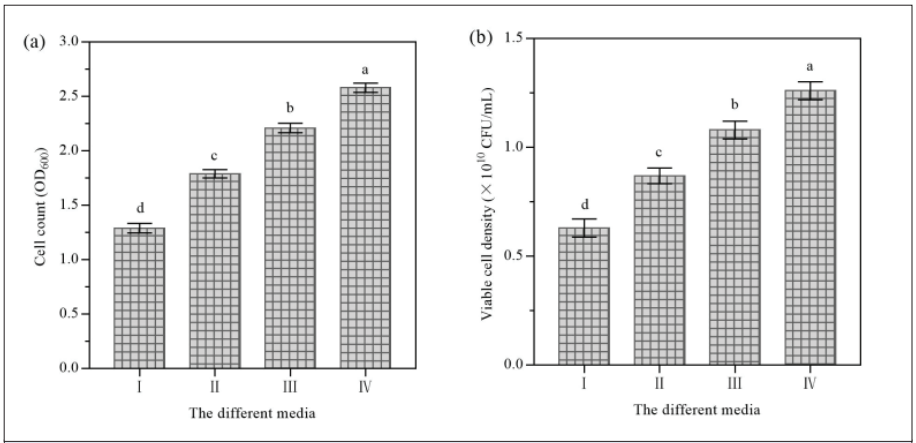

Fig 3. Effect of different media on cell count (a) and viable cell density (b) in avian *P. multocida* fermentation. All values are presented as the mean±SD. Different letters indicate significant difference (P<0.05)

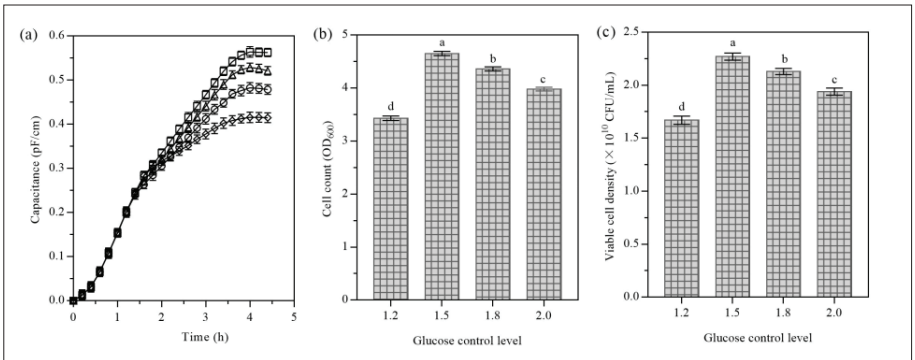

Fig 4. Effect of glucose control strategy on cell capacitance (a), cell count (b) and viable cell density (c) in avian *P. multocida* fermentation. All values are presented as the mean±SD. Different letters indicatesignificant difference (P<0.05)

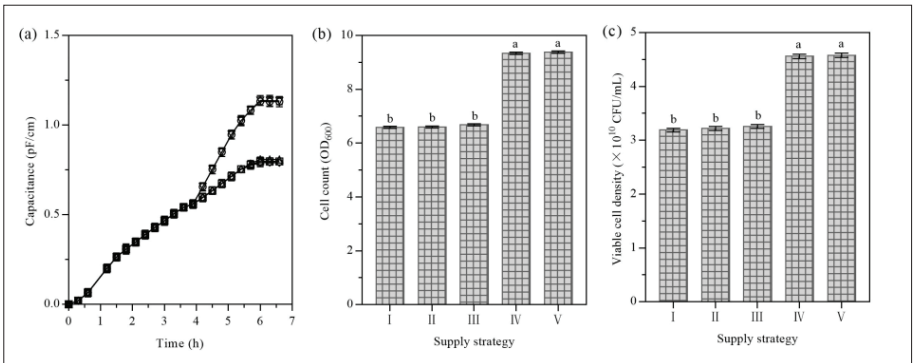

Fig 5. Effect of feed nutrient mixture solutions on cell capacitance (a), cell count (b) and viable cell density (c) in avian *P. multocida* fermentation. All values are presented as the mean±SD. Different letters indicate significant difference (P<0.05)

h using the glucose-stat feeding strategy. The glucose concentration controlled at more than 1.0 g/L could extend the growth period of avian *P. multocida* and increase its viable cell density and cell count, but the viable cell density and cell count decreased with glucose concentration more than 1.5 g/L. The maximum cell capacitance was achieved at a glucose concentration of 1.5 g/L, and the DO level increased suddenly at the same time. The viable cell density and cell count obtained at the aforementioned glucose concentration were the highest, which were 4.65 (OD_{600}) and 2.27×10^{10} CFU/mL, respectively *(Fig. 4)*.

Avian *P. multocida* Fermentation with Different Feed Mixture Solutions

The avian *P. multocida* fermentation with different nutrient mixture solutions after 4.0 h using the DO feedback feeding strategy is displayed in *Fig. 5*. The nutrient mixture solutions supplied after 4.0 h also extended the

cell growth period, leading to an improvement in the viable cell density and cell count. The viable cell density and cell count with the feed nutrient mixture I were 6.53 (OD_{600}) and 3.19×10^{10} CFU/mL. The viable cell density and cell count with feed nutrient mixture II and mixture III were not significant different from those with feed nutrient mixture I. The viable cell density and cell count with feed nutrient mixture IV increased by 43.03% and 42.95% compared with that with feed nutrient mixture I. The viable cell density and cell count with feed nutrient mixture V were 9.38 (OD_{600}) and 4.58×10^{10} CFU/mL, respectively, which were not significantly different from those with feed nutrient mixture IV.

Discussion

This study showed that the growth of bacteria was affected by fermentation medium and cultivation conditions. The form of the medium was the basis for fermentation [16]. Higher cell density and cell count were obtained with the enriched medium compared with different media for avian *P. multocida* fermentation; the highest cell density and count were achieved with medium IV. During *Streptococcus suis* fermentation, the cell density increased by 15.37% on increasing the concentration of nitrogen source [17].

The key nutrient compounds were essential for cell growth and enhancing cell density in the medium [18]. As shown in *Fig. 5*, yeast extract, tryptone, betaine, V_{B1}, and V_H increased the cell density and viable cell count. The hydrolyzed yeast components contained in yeast extract efficiently supplied the TCA cycle, improving oxidative metabolism and increasing Chinese hamster ovary cell density by 70% [19]. Adding an appropriate amount of V_H improved the cell viability of *Corynebacterium glutamicum*, thus increasing the yield and productivity of the product [20]. When betaine was fed into the fermentation culture medium, the metabolic flux entering into the pentose phosphate pathway and the biosynthesis route of L-threonine increased by 57.3% and 10.1%, respectively, increasing the production of L-threonine by 14.5% [21].

The concentration of glucose affected cell growth, and a higher cell density of avian *P. multocida* was achieved at a glucose concentration of 1.5 g/L after 2.0 h. When the concentration of glucose was less than 1.5 g/L, the glucose uptake rate of cells was not adequate for the growth of avian *P. multocida* [22,23]. However, the synthesis of by-products inhibited the growth of *E. coli* [24]. High glucose concentrations can increase the osmotic pressure of the culture medium, which is detrimental to microbial growth, thus, the growth of avian *P. multocida* was inhibited at a glucose concentration of more than 1.5 g/L. Glucose concentration was a key parameter for the biosynthesis of desired products by *E. coli* [25]. The flux and accumulation of acetate during L-tryptophan production were reduced by genetically modifying a strain that produced L-tryptophan and implementing glucose feedback feeding. The flux of tryptophan formation and yield of L-tryptophan increased by 21.36% and 35.81%, respectively [26].

The viable cell sensor was a reliable online biomass monitoring tool for Gram-positive and Gram-negative bacteria [27]. Applying a rational feeding method was an effective strategy for high-cell-density cultivation of bacteria [28]. Based on the selection of feeding time by online viable cell monitoring, suitable nutrients were fed using the appropriate feeding strategy after 2 or 4 h, leading to high-cell-density cultivation of avian *P. multocida*. The viable cell sensor and electronic nose in ethanol fermentation is used to monitor the cell density of *Saccharomyces cerevisiae* B1, a dynamic feeding strategy of glucose was applied, and the ethanol concentration, productivity, and yield were enhanced by 15.4%, 15.9%, and 9.0%, respectively [28]. Real-time monitoring and pH regulation strategies were implemented. The total viable count of *Clostridium butyricum* was 3.32 times higher than that in the control group, and the sporulation rate escalated from 73.9% to 96.6% [29]. In *Glaesserella parasuis* (*G. parasuis* formerly *Haemophilus parasuis*) fermentation, based on online viable cell monitoring, combined with the relationship between nicotinamide adenine dinucleotide (NAD) consumption and cell growth, the feeding concentration of NAD was adjusted to 30 mg/L. The viable cell count of *G. parasuis* increased to 1.57×10^{10} CFU/mL, which was higher by 8.26 times compared with that obtained in tryptic soy broth (TSB) medium [30]. Real-time monitoring allows for a more comprehensive understanding of the bacterial fermentation process. The development and application of effective real-time and online sensors plays an important role in optimizing biological processes to improve product concentration, productivity, and yield [30], thus providing a more effective feeding strategy for bacterial fermentation culture.

In this study, an appropriate medium was selected for the fermentation of avian *P. multocida*. Meanwhile, using online real-time monitoring and DO-level variations, the glucose concentration was maintained at 1.5 g/L after 2 h and a nutrient mixture containing yeast extract, tryptone, betaine, V_{B1}, and V_H was fed after 4 h using the DO feedback feeding strategy. Thus, the viable cell density and cell count of avian *P. multocida* under the optimized conditions increased to OD_{600}: 9.38 and 4.58×10^{10} CFU/mL, which were higher by 7.27 and 7.26 times than those under the original conditions, respectively. This experiment provides a reference for the high-density fermentation culture of avian *P. multocida* and lays the foundation for the preparation of inactivated vaccine. Simultaneously, the research methods presented in this study provide a

theoretical foundation and technical approach for the high-density cultivation of other auxotroph bacteria, only the media components and media replenishment strategies were investigated and the effect of fermentation process parameters on avian *P. multocida* was neglected.

Declarations

Availability of Data and Materials: The authors declare that the data and materials are available on request from the corresponding author (L.C.).

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Competing Interests: The authors declared that there is no conflict of interest.

Declaration of Generative Artificial Intelligence (AI): The article and/or tables and figures were not written/created by AI and AI-assisted technologies.

Ethical Statement: This study does not require the approval of the local ethics committee for animal experiments.

Authors' Contributions: YS and YB performed the experiments, analysed the results, and drafted the manuscript. XZ, LG and WW assisted in the experimental design and summarized the experimental results. QF, NT and CW put forward valuable suggestions for the revision and improvement of the paper. XX, JL and LC conceived and designed the study, revised the manuscript and funded the study. All authors have read and agreed to the published version of the manuscript.

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