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OPTIMIZATION OF FERMENTATION CONDITIONS FOR *BACILLUS VELEZENSIS* STRAIN TO ACHIEVE HIGH-CELL-DENSITY CULTURE

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ABSTRACT

Bacillus velezensis strain exhibits notable potential for mycotoxin degradation. However, its industrial use is restricted by low cell density during fermentation. This study optimized medium composition and key environmental parameters to achieve high-cell-density fermentation of *B. velezensis* strain, using OD_{600} as the evaluation index. Single-factor experiments were conducted to screen carbon and nitrogen sources and to determine optimal temperature, initial pH, liquid volume, and inoculum size. Sorbitol was the most effective carbon source, with an optimal concentration of 40g/L, while 10g/L soybean peptone enhanced biomass accumulation. The optimal fermentation conditions were 37°C, an initial pH of 7.0, a 40% medium volume, and a 3% inoculum size. Under these conditions, *B. velezensis* reached an OD_{600} of 1.257 after 12h, representing a 52.36% increase compared with the baseline. This optimized, low-cost fermentation process provides a practical basis for pilot-scale production and industrial application of *B. velezensis* strain as a biological control agent.

***Keywords*:** *Bacillus velezensis*; Fermentation optimization; Single-factor experiment.

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1. INTRODUCTION

Due to the rapid development and utilization of microbial resources, the genus *Bacillus* has become a research hotspot due to its unique biological characteristics. These Gram-positive bacteria produce stress-resistant spores that enable survival under high temperatures, drought and extreme pH. (Smirnov et al., 2024). Additionally, they harbor diverse secondary metabolite gene clusters that encode antibacterial agents, enzymes, and plant growth regulators. Therefore, *Bacillus* species are indispensable in agricultural, industrial, and medical applications (Suárez-Bautista et al., 2024).

Bacillus velezensis is a type of Gram-positive bacterium with both aerobic and facultatively anaerobic metabolic capabilities. As a typical plant-associated strain, it possesses a core characteristic: the ability to form highly thermotolerant spores, which confer it a significant survival advantage in complex environments. As an important functional strain of the genus *Bacillus*, this bacterium not only inherits the typical stress resistance of the genus, being able to tolerate various environmental stresses such as high temperature and drought but also possesses a strong ability to synthesize a variety of secondary metabolites (Liu et al., 2021; 2025). Studies have confirmed that it can efficiently produce multiple functional enzymes, including lactonases and oxidoreductases (Wang et al., 2023). These bioactive substances provide a solid foundation for its applications in fields such as plant growth regulation and ecological environment improvement, making it a highly potent functional strain in microbial resource development.

Despite its promise, industrial application of microbial strains is often limited by fermentation efficiency. The optimization of fermentation conditions directly influences biomass accumulation and active metabolite production (Pournejati & Karbalaei-Heidari, 2020; van Niekerk and Pott, 2023; Hu et al., 2024; Shi et al., 2024), thereby affecting production costs and market competitiveness (Zhou et al., 2023; Wu et al., 2024). Carbon and nitrogen sources are the primary growth substrates, and their type and ratio not only determine nutrient supply efficiency but also regulate secondary metabolism via mechanisms such as carbon catabolite repression (Yang et al., 2018). or instance, adjusting the carbon-to-nitrogen ratio from 100:0.5 to 100:1.5 can increase *B. velezensis* biomass by 1.8-fold, while a deviation of 1 unit can cause a 20–40% fluctuation in metabolite yield (Liang et al., 2017). Initial pH affects intracellular metabolism by influencing cell membrane permeability and enzyme activity (Wu et al., 2019). For *B. velezensis* FJ17-4, an initial pH of 6.5–7.0 yields an OD_{600} peak of 1.24, whereas a 0.5-unit deviation reduces membrane permeability by 12% and ATP synthase activity by 18%, extending the fermentation cycle by 4h (Wen-Zhi et al. 2013). Fermentation temperature is a critical parameter that directly affects cellular energy metabolism,

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growth rate, and enzyme activity. For example, at 30°C, the specific growth rate reaches 0.92h^{-1}, whereas even a 2°C deviation can reduce this rate by 35–42%, and metabolite production may vary by up to 27-fold. In addition to temperature, factors such as dissolved oxygen, inoculum size, and fermentation time also exert significant influence on fermentation performance through their combined and synergistic effects. (Klausmann et al., 2021; Tang et al., 2024; Wang et al., 2024; Wen et al., 2025). Therefore, establishing a robust fermentation system is essential for industrializing the strain.

Although previous studies have investigated *B. velezensis* fermentation, substantial strain-specific differences in genetic background and metabolism exist (Gari and Abdella, 2023), As a result, optimal conditions established for one strain cannot be directly applied to another. In this study, shake-flask experiments were performed to evaluate the effects of carbon and nitrogen source types and concentrations on the growth of the target *B. velezensis* strain, with the aim of determining an optimal fermentation medium. Single-factor experiments were then used to assess the influence of liquid volume, temperature, inoculum size, and initial pH, ultimately identifying the most suitable conditions for high-density cultivation. This work provides a foundation for industrial fermentation and supports the development of microbial agents based on this *B. velezensis* strain.

2. MATERIALS AND METHODS

2.1 Strain and Experimental Reagents

B. velezensis strain was isolated previously in our laboratory and identified by 16S rRNA gene sequencing. Agar was purchased from Beijing Solarbio Science & Technology Co., Ltd. Luria-Bertani (LB) medium, beef extract, yeast extract, peptone, tryptone, and soybean peptone were all supplied by Guangdong Huankai Microbial Science & Technology Co., Ltd. Fructose, glucose, sucrose, corn starch, and sorbitol were provided by Aladdin Reagent (Shanghai) Co., Ltd.

2.2 Composition of LB Medium

LB medium contained 10g/L tryptone, 5g/L yeast extract, and 10g/L NaCl. For LB agar medium, 15g/L agar was added prior to autoclaving at 121°C for 20min.

2.3 Experimental Methods

2.3.1 Preparation of Strain Culture

A pure culture of *B. velezensis* strain was streaked onto an LB agar plate using a sterile inoculating loop and incubated at 37°C for 24h to obtain isolated colonies. A single colony was transferred to 5mL LB broth and incubated at 37°C and 180rpm for 12h. This pre-culture was then transferred to 100mL fresh LB broth and grown under the same conditions until OD_{600} reached 0.8±0.05, as measured by a microplate reader (Thermo Scientific Multiskan FC). This culture served as the inoculum for all subsequent experiments.

2.3.2 Growth Curve Analysis

The culture of *B. velezensis* strain was inoculated into 50mL of LB broth at 1% (v/v) inoculum. The culture was incubated at 37°C at 180rpm. At 2h intervals, 200µL samples were taken and measured OD_{600} against a sterile LB blank. Measurements were performed in triplicate, and the mean values were used to construct the growth curve.

2.3.3 Screening and Optimization of Carbon Source Type and Dosage

Different carbon sources (30g/L) were added to LB medium, including monosaccharides (fructose, glucose), disaccharides (sucrose, lactose), polysaccharides (corn starch), and the sugar alcohol sorbitol. The medium pH was adjusted to 6.8–7.2, and the basic medium served as the control. The *B. velezensis* culture was inoculated into each medium at 2% and incubated at 37°C and 180rpm for 12h. After incubation, samples were collected and OD_{600} values were measured to assess the effect of each carbon source on strain growth and to identify the optimal carbon source. For the selected optimal carbon source, concentration gradients of 10, 20, 30, 40, 50, and 60g/L were prepared. OD_{600} values were measured under each concentration to evaluate the effect of carbon source levels on strain growth and to determine the optimal addition amount.

2.3.4 Optimization of Nitrogen Source and Dosage

In the basic medium supplemented with the optimal carbon source concentration, different nitrogen sources (beef extract, yeast extract, peptone, tryptone, and soybean peptone) were added at 3g/L to replace the original nitrogen source. The pH was adjusted to 6.8–7.2 and the medium without added nitrogen served as the control. The *B. velezensis* seed culture was inoculated into each medium at 2% and incubated at 37°C and 180 rpm for 12h. After incubation, OD_{600} values were measured to identify the optimal nitrogen source for strain growth. For the selected optimal nitrogen source, concentration gradients of 5, 10, 15, 20, and 25g/L were prepared. OD_{600} values were measured at each concentration to evaluate the effect of nitrogen source levels on strain growth and to determine the

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optimal concentration. The medium supplemented with the optimal nitrogen and carbon sources was used for subsequent experiments.

2.3.5 Single-Factor Optimization Experiment of Fermentation Conditions

2.3.5.1 Effect of Liquid Loading Volume on the Growth of B. velezensis strain: Conical flasks were filled with the optimized medium at different liquid loading volumes (20, 30, 40, 50, 60, and 70%). The *B. velezensis* culture was inoculated into each flask at 2% and incubated at 37°C and 180rpm for 12h. After incubation, OD_{600} values were measured to evaluate the effect of liquid loading volume on strain growth.

2.3.5.2 Effect of Temperature on the Growth of B. velezensis strain: Conical flasks were filled with the optimized medium at a 20% liquid loading volume. The *B. velezensis* culture was inoculated into each flask at 2%. After inoculation, the flasks were incubated at 180 rpm and set at different temperatures (22, 27, 32, 37, 42, 47, and 52°C) for 12h. OD_{600} values were then measured to assess the effect of culture temperature on strain growth.

2.3.5.3 Effect of Medium pH on the Growth of B. velezensis strain: The initial pH of the optimized medium was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 using 1mM NaOH or 1mM HCl. The pH-adjusted medium was transferred to conical flasks at a 20% liquid loading volume. The *B. velezensis* culture was inoculated into each flask at 2% and incubated at 37°C and 180rpm for 12h. OD_{600} values were then measured to evaluate the effect of different initial pH levels on strain growth.

2.3.5.4 Effect of Inoculation Amount on the Growth of B. velezensis strain: Conical flasks were filled with the optimized medium at a 20% liquid loading volume. The *B. velezensis* culture was inoculated into the flasks at 1, 2, 3, 4, 5, and 6%. The cultures were incubated at 37 °C and 180rpm for 12 h. After incubation, OD_{600} values were measured to evaluate the effect of different inoculation amounts on strain growth.

2.4 Statistics and Analysis

Microsoft Excel (2019 version) and SPSS (version 26) were used for data analysis, and Origin (2021 version) was used for graphing. One-way analysis of variance (ANOVA) and Duncan's multiple range test were applied to compare the data and determine significant differences between means ($P < 0.5$).

3. RESULTS AND DISCUSSION

3.1. Growth Curve of *B. velezensis* Strain

The growth curve of *B. velezensis* in LB medium showed the typical four stages of bacterial growth (Fig. 1). During 0–5 h, the strain remained in the lag phase, and the OD value increased slowly as the cells adapted to the new environment and initiated metabolic activity. From 5–12h, the culture entered the logarithmic phase, and the OD value rose rapidly from 0.267 to 1.010, indicating active metabolism, rapid cell division, and substantial biomass accumulation. The stationary phase occurred between 12–24h, during which the OD value stabilized, reaching a maximum of 1.021. This stabilization suggested that nutrients became limiting and that cell division and cell death reached equilibrium. After 24h, the culture entered the death phase, evidenced by a decline in OD value as cell lysis increased and the number of viable cells decreased. The growth pattern of *B. velezensis* observed in this study was consistent with that reported for *B. velezensis* FZB42 in LB medium (Chen et al., 2007), with both strains displaying a short lag phase and an extended logarithmic phase, reflecting strong environmental adaptability and rapid proliferation.

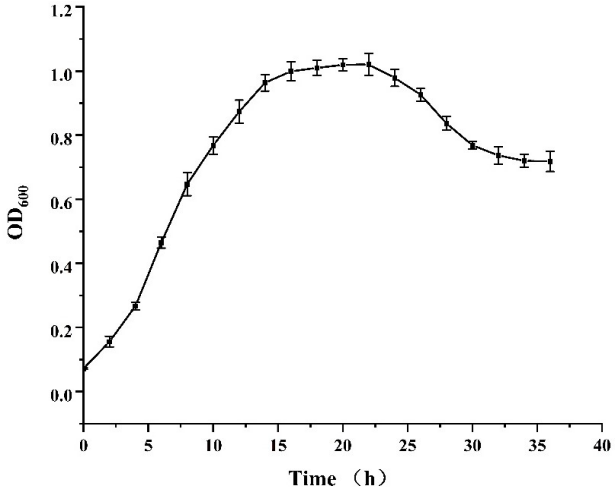

Fig. 1: Growth curve of *B. velezensis* strain in LB medium at 37 °C and 180 rpm (OD_{600}).

3.2. Optimal Fermentation Carbon Source and Concentration for *B. velezensis* Strain

The type and concentration of carbon sources influence the growth rate and final cell density of *B. velezensis* through mechanisms such as carbon catabolite repression (CCR) (Guo et al., 2010). Therefore, determining the optimal carbon source type and concentration is essential for achieving high-density cultures. Different carbon sources

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were added to the basic medium, and OD_{600} values were measured after 12h of fermentation (Fig. 2A). Four carbon sources promoted the growth of *B. velezensis* compared with the control. Sorbitol yielded the highest OD_{600} (1.137±0.020), 33.76% higher than the control, followed by corn starch (OD_{600} = 1.029±0.031). In contrast, fructose and glucose inhibited strain growth, likely due to the strain's metabolic pathways and carbon utilization efficiency. Sorbitol, a sugar alcohol, corn starch, a polysaccharide, and lactose, a disaccharide, are metabolized slowly, avoiding carbon catabolite repression and facilitating strain growth. Ruiz-García et al. (2005) reported that the type of strain of *B. velezensis* prefers slow-metabolized carbon sources such as sugar alcohols to avoid CCR caused by glucose. This agrees with the current results, where sorbitol was optimal and glucose inhibited growth. Rapidly metabolized sugars like glucose and fructose can trigger CCR and metabolic imbalance, suppressing proliferation. These findings suggest that *B. velezensis* prefers non-monosaccharide carbon sources. Sorbitol was therefore selected as the optimal carbon source.

As shown in Fig. 2B, increasing sorbitol concentrations first enhanced and then reduced OD_{600}. The maximum OD_{600} (1.142±0.017) was observed at 40g/L. At lower concentrations, metabolic enzymes efficiently supported growth, increasing OD_{600}. Beyond 40g/L, OD_{600} declined, likely due to enzyme saturation, high osmotic pressure from excess sorbitol, and accumulation of metabolic by-products, all of which inhibited growth. This trend aligns with previous findings in *B. velezensis* FZB42, where maximum biomass (OD_{600} = 1.250) occurred at 40g/L sorbitol, and higher concentrations reduced growth due to osmotic stress (Xu et al., 2021). Therefore, 40g/L was determined to be the optimal sorbitol concentration for *B. velezensis* growth.

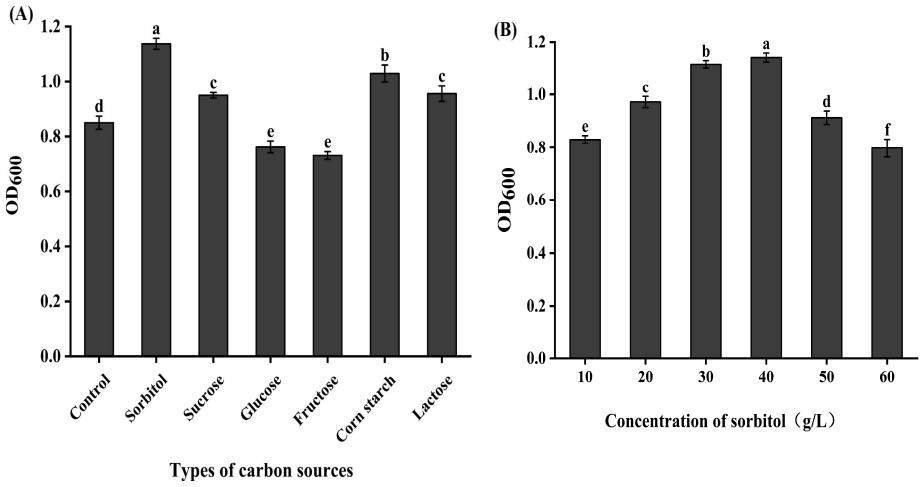

Fig. 2: Optimization of carbon source type and concentration for *B. velezensis* strain growth (A) Effect of different carbon sources on *B. velezensis* strain growth. (B) Effect of sorbitol concentration on *B. velezensis* strain growth.

3.3. Optimal Fermentation Nitrogen Source and Concentration for *B. velezensis* Strain

Nitrogen sources are essential for microbial cell structure and act as limiting factors for bacterial density and metabolic activity. They influence the synthesis of amino acids, coenzymes, and other key biomolecules, thereby affecting strain growth rate (Zhao et al., 2022). Based on the optimal carbon source study, the fermentation medium was supplemented with 40g/L sorbitol to maintain consistency with the previously determined optimal carbon source (adjust to "corn starch" if originally intended for subsequent nitrogen source screening). OD_{600} values after fermentation are shown in Fig. 3A. *B. velezensis* displayed a stronger preference for organic nitrogen sources than inorganic ones. Among the inorganic sources, urea resulted in the lowest OD_{600} (0.901±0.022), likely because urea must first be hydrolyzed into ammonia by urease. This process requires additional energy for enzyme expression, reducing growth efficiency and biomass accumulation. In contrast, soybean peptone, a plant protein hydrolysate, provides a wide range of nutrients, including amino acids, vitamins, and growth factors. When soybean peptone was used, the strain exhibited the highest growth, with an OD_{600} of 1.167±0.017. Yeast extract was the next most effective, yielding an OD_{600} of 1.136±0.026. Therefore, soybean peptone was selected as the optimal nitrogen source.

The effect of different soybean peptone concentrations on strain growth is shown in Fig. 3B. OD_{600} initially increased slightly with increasing concentrations and then decreased at higher levels. At 5g/L and 10g/L, OD_{600} values were 1.182±0.012 and 1.187±0.016, respectively, indicating high biomass. However, further increases in soybean peptone concentration caused a continuous decline in OD_{600}, reaching 0.992±0.029 at 25g/L. This decline is likely due to an imbalance in the carbon-to-nitrogen ratio at high nitrogen concentrations, which limits

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available carbon skeletons for biosynthesis. Additionally, excess nitrogen may accumulate as NH_4^+, inhibiting growth. These results are consistent with the findings of Jiang et al. (2023), who reported that OD_{600} reached 1.19 at 10g/L soybean peptone, while concentrations above 20g/L led to NH_4^+ accumulation, pH rise to 8.2, and a 22% decrease in biomass (Jiang et al., 2023). Consequently, the optimal soybean peptone concentration for *B. velezensis* growth was determined to be 10g/L.

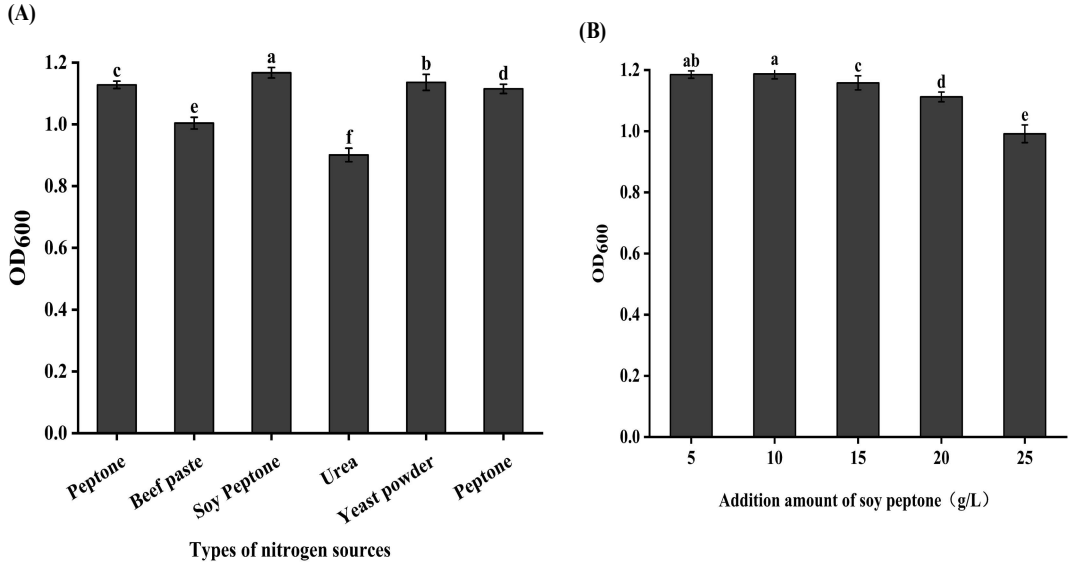

Fig. 3: Optimization of nitrogen source type and concentration for *B. velezensis* strain growth. (A) Effect of different nitrogen sources on *B. velezensis* strain growth. (B) Effect of different soy-peptone concentrations on the growth of *B. velezensis* strain.

3.3. Results of Single-Factor Fermentation Optimization Experiments

3.3.1. Optimal Inoculation Amount for *B. velezensis* strain Fermentation: In microbial fermentation, the inoculation amount determines the initial bacterial density, which affects nutrient and oxygen consumption rates as well as the formation of metabolic products. A higher inoculation amount accelerates the strain's entry into the logarithmic growth phase, but insufficient dissolved oxygen in the early stage can negatively impact later fermentation. Conversely, a lower inoculation amount delays bacterial growth and reduces production efficiency. Therefore, evaluating the effect of different inoculation amounts on *B. velezensis* growth is essential for determining the optimal seed solution dosage for achieving high bacterial density. As shown in Fig. 4A, OD_{600} first increased and then decreased with increasing inoculation amounts. The highest OD_{600} (1.227±0.021) was observed at 3% inoculation. When the inoculation amount exceeded 3%, OD_{600} decreased by 12.9%–27.1%, likely due to rapid nutrient and oxygen depletion, which inhibited growth. These results indicate that the inoculum amount should not be too high, and the optimal inoculum for *B. velezensis* is 3%.

3.3.2. Optimal Initial pH for B. velezensis strain Fermentation: The initial pH of the fermentation broth is a critical factor affecting bacterial growth, as overly acidic or alkaline conditions can impair enzyme activity and inhibit microbial metabolism (Le Marc et al., 2021). To investigate the effect of initial pH on growth, shake-flask cultivation of *B. velezensis* was performed with pH values ranging from 4 to 10. As shown in Fig. 4B, OD_{600} first increased and then decreased with rising initial pH, reaching a maximum of 1.200±0.029 at pH 7.0. Although the strain remained viable under extreme pH conditions (pH 4.0 or 9.0), growth and metabolism were inhibited, resulting in lower OD_{600} values. The strain preferred a near-neutral environment: when pH exceeded 7.0, OD_{600} declined sharply, and at pH 10, OD_{600} dropped to 0.161±0.036, indicating near inactivation. These results are consistent with previous studies showing that *B. velezensis* exhibits maximum membrane permeability and enzyme activity at pH 7.0, while pH ≥ 9.0 reduces OD_{600} by 60% (Awofiranye et al., 2020). *B. velezensis* tolerates moderately acidic or alkaline conditions, but the optimal initial pH for growth is 7.

3.3.3. Optimal Fermentation Temperature for B. velezensis strain: Temperature influences strain growth by affecting enzyme activity and energy conversion efficiency: low temperatures slow growth, while excessively high temperatures may reduce viability. Determining the optimal fermentation temperature is essential for scale-up production. To evaluate this, *B. velezensis* was cultured at 22, 27, 32, 37, 42, 47, and 52°C. As shown in Fig. 4C,

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temperature significantly affected growth. OD_{600} increased from 22°C to 37°C, reaching a maximum of 1.198±0.015 at 37°C. Beyond 37°C, OD_{600} declined, dropping to 0.705±0.021 at 42°C, and growth was severely inhibited at 52°C, with OD_{600} only 37.3% of that at 37°C. The strain was still able to grow slowly at 42–52°C, possibly due to spore formation under heat stress. These results are consistent with Ashraf et al., who reported that *B. velezensis* achieved its maximum specific growth rate at 37°C, with a 45% decrease at 42°C (Rather et al., 2021). *B. velezensis* exhibits moderate heat tolerance, and the optimal fermentation temperature is 37°C.

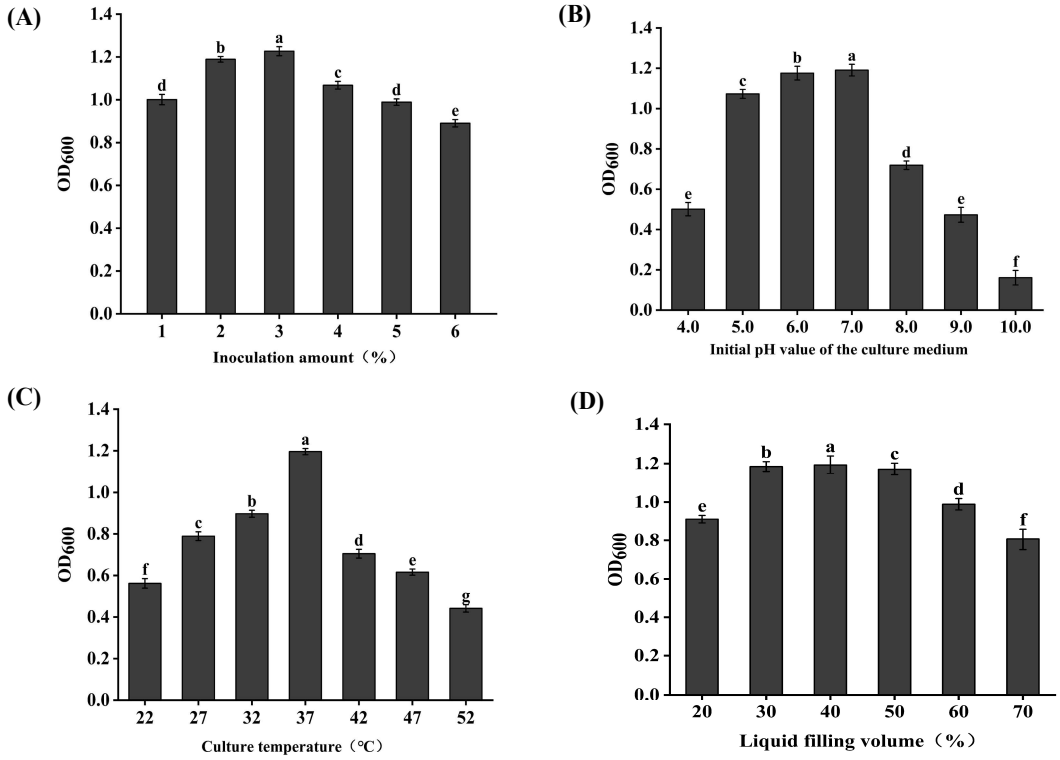

Fig. 4: Single-factor optimization of fermentation parameters for *B. velezensis* strain. (A) Effect of inoculum size on growth. (B) Effect of initial medium pH on growth. (C) Effect of incubation temperature on growth. (D) Effect of liquid-to-flask volume ratio on growth.

3.3.4. Optimal Liquid Loading Volume for B. velezensis strain Fermentation: *B. velezensis* is an aerobic bacterium, and in shake-flask cultivation, the liquid loading volume directly affects dissolved oxygen levels (Li et al., 2024). An appropriate liquid volume ensures sufficient oxygen supply, avoids oxygen limitation caused by excessive liquid, and maintains uniform nutrient distribution. Therefore, optimizing liquid loading volume is important for strain growth and provides guidance for dissolved oxygen conditions in large-scale fermentation. To evaluate its effect, shake-flask fermentation was conducted with liquid loading volumes of 20%, 30%, 40%, 50%, 60%, and 70% of flask volume. As shown in Fig. 4D, OD_{600} increased with liquid volume, reaching a maximum of 1.193±0.046 at 40%. The OD_{600} at 30% loading was the second highest (1.185±0.025), 30.66% higher than at 20%. Beyond 40%, OD_{600} declined, and at 70% loading, it dropped to 0.807±0.055, a 47.83% decrease compared with 40%. This indicates that 40% is the critical liquid loading volume for *B. velezensis*. Insufficient dissolved oxygen likely contributed to the decline in growth at higher volumes (Cusidó et al., 2002) In conclusion, the optimal liquid loading volume for shake-flask fermentation of *B. velezensis* is 30–50%, which can be adjusted based on the required culture volume.

4. CONCLUSION

Single-factor optimization effectively enhanced the OD_{600} of *B. velezensis* strain to 1.257 and identified the optimal fermentation conditions: 40g/L sorbitol, 10g/L soybean peptone, 37 °C, initial pH 7.0, 40% medium filling volume and 3% inoculum. This optimized process is simple, stable, and low-cost, meeting the requirements for high-density culture. Moreover, it can be readily scaled up to 5–50 L fermenters, providing a solid foundation for the economical and large-scale production of *B. velezensis* strain microbial agents.

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Declarations

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Author's Contributions: Lijun Zhang: Conceptualization, methodology, formal analysis, validation, data curation, software, Writing—original draft. Tanvir Ahmad: conceptualization, methodology, validation, Investigation, data curation, writing – review & editing. Zongheng Ru: Writing – review & editing. Yang Liu: project administration, supervision, Investigation, funding acquisition.

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