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EVALUATION OF THE INFLUENCE OF METAL IONS ON THE SURVIVAL, ACTIVITY, AND STABILITY OF *BACILLUS* SPP.

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ABSTRACT

Backgrounds: *Bacillus renowned for their probiotic benefits, including resilience in the gastrointestinal environment and compatibility with antibiotic therapies with high-density cultivation through medium optimization has employed various statistical experimental designs but which not emphasized increasing spore yield frequently neglecting the functional properties of spores.* ***Objectives:*** *The purpose of the study is to determine the types of metals and metal ion concentrations that affect Bacillus subtilis M70, Bacillus clausii M31 (isolated in a laboratory in Vietnam), with the goal of enhancing its function, stability, and cell density in an optimal culture metal ion.* ***Materials and methods:*** *B. subtilis M70, B. clausii M31 have growth under conditions: 37°C, 200 rpm, for 18-24 hrs. Bacillus were cultured on media with varying ingredients of metal ion. A Design of Experiments (DOE) was designed in JMP Pro software (14th edition) applied. Impacts of 5 metal ions on total survival, viability efficiency, and activity of Bacillus spp. in cultures medium were examined.* ***Results:*** *$MgSO_4$ and $MnSO_4$ concentrations on Bacillus spore formation. Stability and production efficiency of Bacillus spores at $CaSO_4$ and $FeSO_4$ along with controlled agitation and aeration rates, maximal viability and stability were observed. High spore densities of approximately 2.06 log10 CFU/mL and significant sporulation efficiency, antibacterial ring 2.83cm and survival about 75% in pH 3 in 4 hours.* ***Conclusion:*** *Optimization of media constituents and culture parameters led to potential spore efficiency for Bacillus in fermentative systems, reduced nutrient requirements, increased number spores, and strengthened spore activity.*

Keywords: *Bacillus, spore, survival, activity, viability, JMP pro.*

I. INTRODUCTION

In addition to the gastrointestinal tract, probiotics have shown promise in other conditions outside their natural niche such as immune modulation and reduced infections. Living bacteria which can provide health benefits when present in large quantities of the body - known as probiotics [1]. A unique benefit of *Bacillus* species is their ability to make spores, which are heat-stable during the process of production as a probiotic drying. Most bacterial species have been found to survive lyophilization at -20°C under vacuum conditions, although this increases the cost of production [2]. *Bacillus subtilis*, *Bacillus clausii*, *Bacillus cereus*, *Bacillus coagulans*, and *Bacillus licheniformis* are among the species under study. Heat-stable *Bacillus* spores provide an advantage over non-spore-forming species such as *Lactobacillus* spp., as they allow goods to be maintained at room temperature in a dry state without losing viability. *B.subtilis* and *B.clausii* were intensively studied as a model organism in cellular and molecular biology and plays an important role in commercial applications due to its ability to produce enzymes, antibiotics, and other bioactive substances.

This advantage is reinforced by research indicating that more than 10% of implanted *B. clausii* spores may germinate, spread, and regenerate spores [3]. These spores can be found in a variety of settings, including dirt, straw, muck, and the intestines of insects, animals, and humans. Numerous research have shown that *Bacilli* can help manage digestive issues, constipation, and irritable bowel syndrome [3], [4].

The goal of this research is to find the best metal ions for cultivating the laboratory-isolated *B. subtilis* M70 and *B. clausii* M31 strains in order to achieve high survival rates, dense spore populations, and powerful spore activity. The ultimate goal is to leverage these findings to build a consistent source of raw materials and generate probiotics for use in food and medicinal applications.

II. MATERIALS AND METHODS

2.1. Microorganisms and Culture Conditions

B. subtilis M70 and *B. clausii* M31 strains were isolated from a laboratory in Vietnam. The reference train was *Lactobacillus acidophilus* ATCC 4356. Inoculum cultures were grown in Luria-Bertani (*LB*) broth (*Himedia, India*). Subsequently, cultures were transferred to production medium and incubated in a shaking incubator under specific conditions: 37°C, 200 rpm, for 18-24 hrs. The composition of the culture medium was optimized to maximize total cell density and spore production.

2.2. Enumeration of Viable Cells and Spores

Cell growth was assessed by enumerating viable cells and spores in the culture medium. Samples were collected under aseptic conditions. Viable cells and spores were quantified using the serial plating technique on LB agar. To differentiate spores, a heat treatment step at 65°C for 15 mins was employed to eliminate vegetative cells. Spore counts were determined by plating and incubating samples for 18 hrs at 37°C. The number of vegetative cells was calculated as $\boldsymbol{N2\ (\log CFU/ml) = N - N1}$

The serial plating technique on LB agar was used to determine colony-forming units (CFU). Total cells and spores (N); the total number of spores (N1); the number of vegetative cells (N2)

2.3. Impact of Culture Conditions on Spore Formation

B.subtilis M70 and *B.clausii* M31 were cultured in media with varying compositions in separate conical flasks. pH adjustments were made using 6 N NaOH and 3 N HCl. Samples were collected at regular intervals over 24 hrs, and optical densities were measured at 600-610 nm using a UV-160A spectrophotometer (*Japan*) to assess spore survival and viability.

Spore activity was evaluated through:

+ **Acid Tolerance Test**: Spores of *B. subtilis* M70 and *B. clausii* M31 were incubated in PBS at pH 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 (adjusted with 5N HCl) at 37°C. Samples were collected at 0, 1, 2, 3, and 4 hrs, diluted, and plated on agar medium. The number of surviving colonies (CFU/mL) was counted, and the survival rate was calculated using the formula:

$$\% \text{ Survival} = (X/Y) \times 100\%$$

Where as: X is the number of colonies after treatment. Y is the initial number of colonies

+ Antimicrobial Resistance: *Bacillus* culture supernatants were added to wells on agar plates containing *S. aureus* and incubated at 37°C for 24 hrs. The diameter of the inhibition zone (cm) around the well was measured to assess antibacterial activity.
The diameter of inhibition zones (mm) around the wells was quantified.

2.4. Statistical Optimization of Media Components

Significant components of the production medium influencing cell growth were identified using a Design of Experiments (DOE) approach. Optimal concentrations of these components were determined using three-center point assays. Statistical analyses were conducted using JMP Pro software (*Version 17, USA*).

2.5. Statistical Analysis

Experimental design, regression analysis, and graphical examination of data were performed using JMP statistical software (USA). Analysis of variance (ANOVA) and LSD multiple comparison tests were conducted at a 95% confidence level. Total cell and spore densities (CFU/mL) were logarithmically transformed to stabilize variance. These methods provided comprehensive insights into the growth, spore formation, and optimal culture conditions of *B. subtilis* M70 and *B. clausii* M31 under various experimental setups.

III. RESULTS

3.1. Assessment of culture medium constituents to facilitate the growth of Bacillus

The results of individual and interactive effects of these factors were evaluated using a design of experiments approach. A series of 20 experiments were conducted to systematically investigate the influence of various factors (X1-X5) on the growth of *Bacillus*, using the statistical JMP design approach.

In the second run formulation, the following components were tested: 0.1% $MgSO_4$, 0.025% $MnSO_4$, 0.05% $FeSO_4$, 0.05% $CaSO_4$, and 5.0% yeast extract. This combination yielded a significant number of cells that formed thick spores observable under a microscope. The activity of the spore suspension was tested and found to be within the inhibitory zone of 2.7 cm to 2.9 cm for both *B. clausii* and *B. subtilis* strains, respectively.

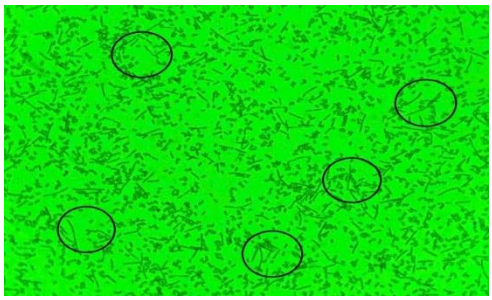
3.2. Medium optimization

The optimization of the culture medium for *Bacillus* growth was performed using the JMP software. The growth of *Bacillus* in the optimal medium was increased similarly compared to that in the initial culture medium. Our findings reveal that glucose, magnesium, manganese, calcium, and iron exert positive effects on the growth of *Bacillus*, while other factors display negative correlations. For spore production, significant positive effects were observed for the factors $MgSO_4.7H_2O$ (X_2) and $MnSO_4.4H_2O$ (X_3). These findings underscore the necessity of elevating the levels of these two factors to enhance the biomass yield of *Bacillus* spores. Furthermore, with regard to strengthen sporulation activity, the significance of compounds such as $FeSO_4$ and $CaSO_4$ in substantially promoting sporulation and reinforcing spore activity has been demonstrated [5]. Screening input variable medium components: (w/v) X_1: 0-0.2 % of $MgSO_4.7H_2O$, X_2: 0-0.05 % of $MnSO_4.4H_20$, X_3: 0-0.1 % $CaSO_4$, X_4: 0-0.1 % $FeSO_4$; X_5: $ZnSO_4$ 0-0.1 %.
**Code for output variable component concentrations:

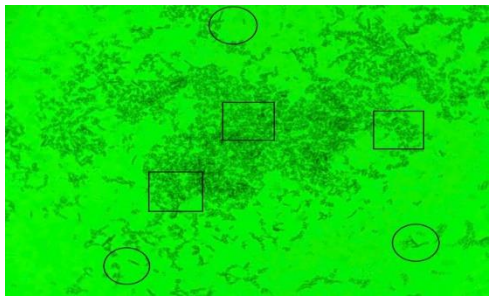
Y_1: Total cell density of *Bacillus* in 1 mL suspension (log10 CFU/ mL); Y_2: Number of spores *Bacillus* in 1 mL suspension (log10 CFU/ mL); Y_3: Activity of spores is calculated

by inhibition zone (cm) of *Bacillus* spores suspension was accessed for their antibacterial using agar well diffusion method. The assessment of antibacterial efficacy for the isolated *Bacillus* strains involved the measurement of the growth inhibition zone diameter against various pathogenic bacteria (*S.aureus* ATCC 6538) within an agar plate environment supplemented with probiotics. The antibacterial activity of *Bacillus* spores against *S. aureus* ATCC 6538 was assessed using the agar well diffusion method. Antimicrobial compounds, including bacteriocins and lipopeptides (e.g., surfactin, iturin, fengycin), diffuse from the well into the surrounding medium, creating a concentration gradient [4], [6]. When these compounds reach an effective concentration, they inhibit *S. aureus* growth, forming a clear inhibition zone [4]. This experiment evaluates the probiotic potential of *Bacillus* strains by determining their ability to suppress pathogenic bacteria. It also examines how culture medium composition influences sporulation and antimicrobial activity. By optimizing the medium to enhance spore formation and bioactivity, the study aims to develop probiotic formulations with improved efficacy.

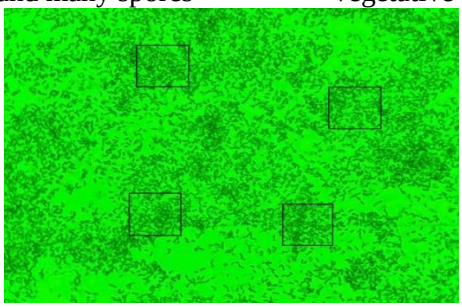
Following the expansion of the *Bacillus* culture, to gain further insights into its microscopic characteristics, slides of the cultured bacteria are prepared and examined under an electron microscope at 40X magnification, as depicted in *Figure 1*. The images reveal the morphology, viability, and number density of *B. subtilis M70* observed at 100X magnification under the optical microscope.



a. The fermented biomass solution comprises primarily vegetative cells and many spores



b. The fermented biomass solution has few vegetative cells but many spores



c. The fermented biomass solution totally transforms to spores

Figure 1. Relative concentration of the main growth factors (%)

Note: circle: vegetable cell; square: spore

The results of antibacterial activity of *B. subtilis* M70, *B. clausii* M31 strains using well diffusion method. The inhibition zone (mm) of *B. subtilis* M70, *B. clausii* M31 and Reference strain (*L. acidophilus)* with *S. aureus* ATCC 6538 was 13.16 ± 0.21; 13.50 ± 0.32; 12.43 ± 0.20 (mm), respectively. The assessment of antibacterial efficacy for *B. subtilis* M70 and *B. clausii* M31 strains involved the measurement of the growth inhibition zone diameter against various pathogenic bacteria within an agar plate environment supplemented with

probiotics. The findings presented that *Bacillus* M31 exhibits superior effectiveness in restraining the proliferation than control strain. Inhibition zones of *B.subtilis* M70 were smaller than *B. clausii* M31 and larger than *L. acidophilus* with *S. aureus* ATCC 6538.

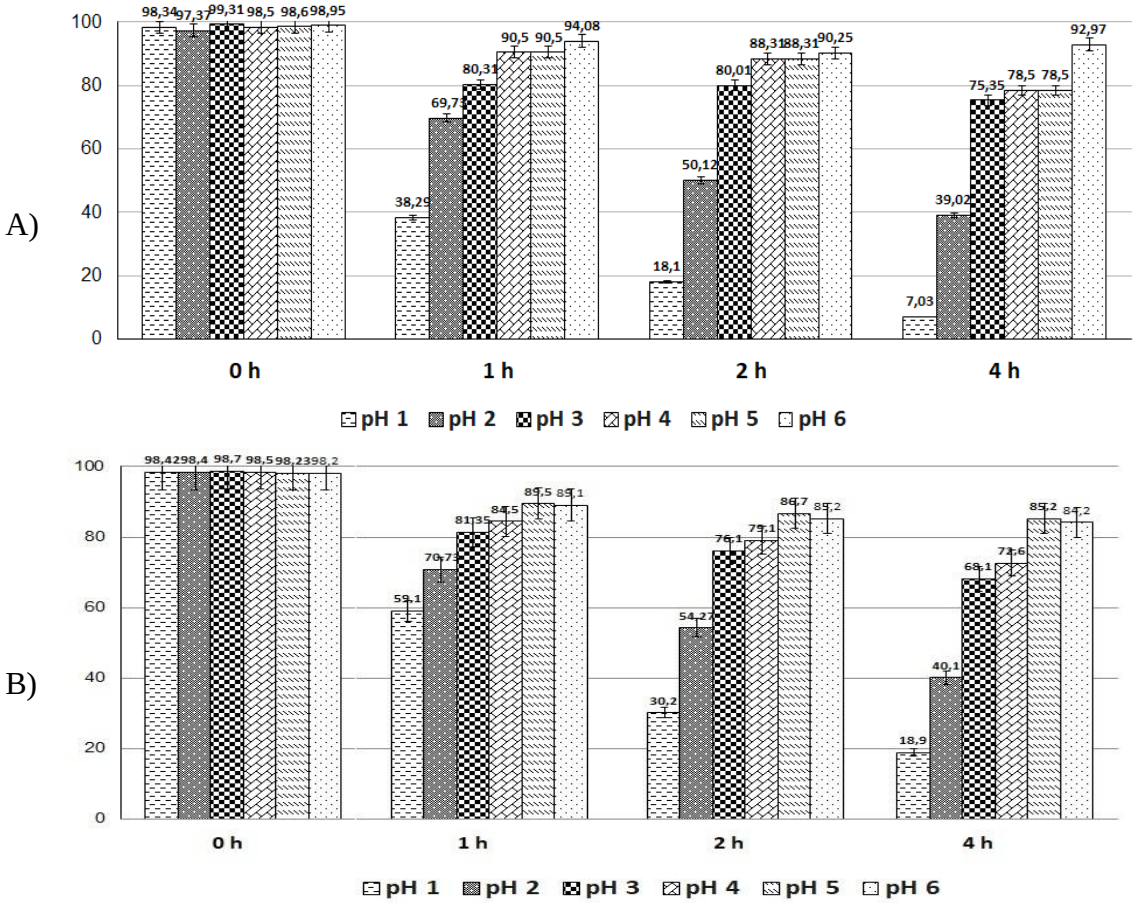


Figure 2. Survival of *B. subtilis* M70 and *B. clausii* M31 in different pH conditions
Note: A) *B. subtilis* M70; B) *B. clausii* M31

As shown in Figure 2A and B, both B. subtilis M70 and B. clausii M31 demonstrated high stability and survival rates under harsh acidic conditions. Notably, they maintained an excellent survival rate (>75%) at pH 3 for 4 hours and exhibited considerable tolerance at pH 1-2 (>50% survival at pH 2 for 2 hours). These results confirm the significant potential of M70 and M31 for use in oral probiotics, ensuring their survival through the gastric passage to exert beneficial effects in the intestine.

3.3. Model verification and evaluation of modified medium

Verification of the optimal medium and assessment of modified media at anticipated values. To corroborate the predictions made by mathematical optimization, the spore cell density and sporulation efficiency of *B.clausii* M31 were determined through an independent trial conducted in culture flasks. This experiment was replicated independently twice. To validate the optimization outcomes, an experiment was conducted using the nutrient levels optimized by the model. Spore cell density and sporulation efficiencies reaching 2.45 log10 CFU/mL and 2.16 log10 CFU/mL, respectively, were achieved. This alignment between experimental and predicted values confirms the validity of the model.

The model's variance analysis (Table 1) showed strong significance, with low probability values (Pmodel < 0.05). The model also fit the data well, as indicated by the p-value, which were the most relevant model terms in this model. The model's RSquare was found to be ~0.99 confirming its reliability.

Table 1. The results measures of model optimization

Strain	*B. subtilis* M70			*B. clausii* M31		
Response	Measures of model	Training	Validation	Measures of model	Training	Validation
Total cell Y1	RSquare	0.9921	0.9196	RSquare	0.9987	0.9996
	Loglikehood	-31.455	-8.126	Loglikehood	-39.958	-13.541
Sporulation Y2	RSquare	0.9932	0.9825	RSquare	0.9941	0.9868
	Loglikehood	-32.710	-6.134	Loglikehood	-27.758	-4.951
ActivityY3	RSquare	0.9909	0.9537	RSquare	0.9925	0.9462
	Loglikehood	-10.133	0.259	Loglikehood	-18.443	0.187

IV. DISCUSSION

The composition of the culture medium plays a crucial role in microbial growth and sporulation. Our study demonstrates that specific metal ions significantly influence the biomass yield, spore formation, and functional activity of *Bacillus subtilis* M70 and *Bacillus clausii* M31. The optimization of metal ion concentrations led to increased total cell density and enhanced sporulation efficiency, aligning with previous findings on the importance of metal ions in bacterial physiology.

Among the tested metal ions, Ca^{2+} and Mg^{2+} promoted bacterial growth and spore formation. Ca^{2+}, a key structural component of Bacillus spores, accounts for approximately 10% of the spore core, playing a vital role in dehydration and resistance to environmental stress. Additionally, Ca^{2+} interacts with spore surface proteins, enhancing bacterial adhesion to host cells. Similarly, Mg^{2+} contributes to peptidoglycan synthesis, strengthening the bacterial cell wall and improving overall resilience. Our results confirm that optimized concentrations of these ions significantly improved Bacillus biomass yield and sporulation rates.

Conversely, Mn^{2+} and Fe^{2+} primarily influenced bacterial activity rather than cell density. Mn^{2+} is well-documented for its role in oxidative stress resistance and sporulation. In this study, Mn^{2+} supplementation increased spore viability and antibacterial activity. Fe^{2+}, when incorporated at controlled levels, enhanced the inhibitory effect of *Bacillus* against *S. aureus*, as evidenced by a larger inhibition zone (2.83 cm).

To assess the antibacterial potential of *Bacillus* spores, we conducted an agar well diffusion assay using *S. aureus* ATCC 6538 as an indicator strain. The formation of clear inhibition zones around *Bacillus* spores suspensions confirmed their antimicrobial efficacy. The mechanism underlying this inhibitory effect likely involves the production of antimicrobial peptides (AMPs), bacteriocins, or other bioactive compounds that disrupt the integrity of *S.aureus* cell membranes. Additionally, Fe^{2+} may play a role in stimulating the production of these antimicrobial compounds, further enhancing the antibacterial activity of *Bacillus* spores [7], [8].

The inclusion of this antibacterial assessment in our study serves a dual purpose. First, it validates the functional properties of Bacillus spores beyond mere survival and sporulation efficiency, reinforcing their potential as probiotic candidates with pathogen-

inhibiting capabilities. Second, it supports the optimization process by identifying metal ion concentrations that enhance both *Bacillus* viability and antibacterial potency. By integrating antimicrobial efficacy into the culture optimization process, we ensure that Bacillus spores not only survive but also exhibit functional benefits, particularly in probiotic applications.

Additionally, controlled agitation and aeration rates played a crucial role in maximizing spore formation and stability. Proper aeration ensures sufficient oxygen availability, which is essential for aerobic spore development. Our study optimized these parameters alongside metal ion concentrations, achieving high spore densities (~2.06 log CFU/mL) and survival rates (~75% in pH 3 for 4 hrs).

These findings highlight the potential of culture medium optimization in probiotic production. By fine-tuning metal ion concentrations, we achieved improved *Bacillus* spore yield, enhanced antibacterial activity, and increased survival under acidic conditions. This optimization approach can be applied to industrial fermentation processes to develop robust probiotic formulations with enhanced functional properties.

V. CONCLUSION

The best culture medium for *Bacillus* development was identified by screening and optimizing the key components with the neural JMP design approach. Optimizing specific metal ions (Mg, Mn, Ca, Fe) and culture parameters significantly enhanced the spore yield, stability, and bioactivity of *B. subtilis* M70 and *B. clausii* M31. The optimized spores demonstrated high density, potent antibacterial activity (2.83 cm zone), and robust acid tolerance (~75% survival at pH 3 for 4h), confirming their immense potential for oral probiotic development. This result is very encouraging and merits further scaling up, since it considerably contributes to optimizing dose and delivery frequency for a variety of biotechnological applications utilizing *Bacillus* spp.

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