Enhanced the biomass of *Bacillus subtilis* cultured in industrial wastewater through statistical methodologies

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During the processes of canning *Agaricus bisporus* (*A. bisporus*), larges of the water-soluble components of the mushroom were dissolved into the processed hot water. The aim of this study was to make use of the *A. bisporus* industrial wastewater to produce the feed additives. In this study, the *Bacillus subtilis* (*B. subtilis*) culture condition in the *A. bisporus* industrial wastewater was optimized. The suitable range of each culture condition was determined using single-factor method. The most significant factors for biomass of *B. subtilis* were screened using Plackett-Burman Design. The center area of the three factors was determined using the steepest ascent experiment. Then the 3-level-3-factor Box-Behnken design was applied to conduct response surface analysis to obtain the optimal culture condition and predict the maximum biomass. Evaluation of the experimental results signified that the optimum conditions for maximum biomass of *B. subtilis* (2.68 ± 0.06×10^8 Objects/mL (Obj/mL)) in 250 mL flask were culture temperature of 32.3℃, inoculum dose of 7.9%, wastewater concentration of 0.7%, shaking speed of 150 revolutions per minute (rpm), loaded liquid of 90 mL, initial pH of 6.5, and culture time of 24 h. The biomass of *B. subtilis* under optimized conditions was also far higher than that of the feed additives' standard in China. Therefore, it is feasible to use *A. bisporus* industrial wastewater as the natural medium for *B. subtilis* to produce the feed additives.

Keywords: *Bacillus subtilis*, feed additives, industrial wastewater, response surface method.

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Introduction

China is a big country of livestock, poultry, and aquaculture with broad animal antimicrobial production and use. Animal antimicrobials play an important role in preventing and controlling animal diseases, improving breeding efficiency, and ensuring effective supply of aquatic products for livestock and poultry. However, the situation of bacterial resistance is becoming more and more serious due to several factors such as the unstandardized market order and unreasonable breeding links. The increase of bacteria resistance rate leads to the decrease of the therapeutic effect of veterinary antimicrobial agents, and forces the increase of the dosage in breeding links, which exacerbate the side effects of veterinary antimicrobial agents and the risk of excessive residue, seriously threat the quality and safety of aquatic products and public health, and bring hidden dangers to human and animal health. The microbial feed additive developed by

probiotics can solve this problem by gradually replacing antibiotics with this natural, non-toxic side effects, safe and reliable, clean, and pollution-free product.

The United States Food and Drug Administration (FDA) and the Association of American Feed Control Officials (AAFCO) published 44 microbial strains that can be directly used in feed. The Ministry of Agriculture of China published the Feed Additives Catalogue in 2013, which identify 34 microbial strains that can feed animals directly with *B. subtilis* being included in the catalogue.

B. subtilis is a facultative aerobic gram-positive bacterium widely distributed in nature with straight rod-shaped and sized of (0.8-1.2) µm × (1.5-4.0) µm. It can form endospores and capsule, mesophytic or near mesophytic spores, and can move using peripheral flagella. It has simple nutrition demand, and therefore, grows and reproduces fast. *B. subtilis* can withstand the changes of temperature, pressure, acid and base environment during feed processing. It demonstrates high survival rate [1]. *B. subtilis* does not consume the nutrition component of feed during feed storage and shows highly adaptable to the environment and resistant to stress. Therefore, it can be stored for a long time to make it more favorable for application in feed. *B. subtilis* consumes a large amount of free oxygen after entering the intestine, which forms a microenvironment beneficial to anaerobic growth, produces substances that have antagonistic effects with pathogenic bacteria, and then, regulates the intestinal microecological balance. It can secrete many enzymes such as lipase and cellulase, then promote the digestion of nutrients in feed, improve intestinal morphology, and promote the absorption of nutrients. *B. subtilis* can produce nutrients to reduce the intestinal pH and improve nutrient availability. It can improve antioxidant capacity of body and relieve oxidative stress, enhance immune function, reduce intestinal inflammation, reduce harmful gas production, and improve the culture environment. Therefore, *B. subtilis* has become one of the most widely used microbial additives in feed.

A. bisporus is the fourth abundant mushroom species worldwide, accounting for 15% of global total mushroom production (34×10^6 t edible mushrooms). Because of the short preservation period, its main form of sale is pot storage. During the processes of canning *A. bisporus*, the softening tissue must be pre-cooked in order to passivate the polyphenol oxidase causing browning. China is the top largest producer of the genus and produces a large amount of industrial wastewater every year [2]. During the process, some water-soluble components of the mushroom were dissolved into the processed hot water including polysaccharides, proteins, free amino acids, mannitol, nucleotides, inorganic ions, and so on. According to the reports, there are 0.04% of amino acid nitrogen, 0.17% of total sugar, 0.65% of protein, and 0.08% of reducing sugar in the industrial wastewater [3], which can provide abundant carbon, nitrogen, and inorganic salts for plant and microbial growth [4, 5]. However, the wastewater from mushroom industries contains high chemical oxygen demand (COD) and biochemical oxygen demand (BOD) contents with the COD contents 13.07 times higher (540.29 g/L) than that of national three-level emission standard [6]. If the wastewater is not treated and used well, it will cause damage to the ecological environment and waste of resources. Therefore, in this study, the wastewater resource of *A. bisporus* was used as the natural liquid medium of *B. subtilis* to produce the standard feed microbial additives, which may broad the downstream outlets for the *A. bisporus* processing industry and provide a feasible scheme for the green, healthy, and sustainable development of animal husbandry.

Materials and methods

Preparation of seed suspension

A colony of activated *B. subtilis* (ACCC 01055, Agricultural Culture Collection of China, Beijing, China) was taken and seeded into 100 mL of seed

Table 1. Factors and levels assessed in the one factor at a time experiment.

Level	Factors					
	Wastewater concentration (%)	Initial pH	Inoculum dose (*v:v*, %)	Culture temperature (°C)	Shaking speed (rpm)	Loaded liquid (mL/250 mL)
1	0.5	5.0	1.0	24	50	30
2	1.0	5.5	2.0	28	100	60
3	2.0	6.0	4.0	32	150	90
4	4.0	6.5	8.0	36	200	120
5	8.0	7.0	16.0	40	250	150
6	16.0	7.5				
7		8.0				

medium (Beijing Land Bridge Technology Co., Ltd, Beijing, China) (5 g of NaCl, 30 g of Beef extract, and 5 g of Pepton in 1 L distilled water at pH 7.0-7.2), and then, cultured in shaking table at 30℃, 150 rpm, for 24 h.

Growth curve

The total biomass of *B. subtilis* in culture medium was determined by FlowSight Flow Cytometry (Millipore, St. Louis, MO, USA) at intervals of two hours, and continuously tested for 48 h. The growth curves were drawn with continuous culture time and total biological volume as the horizontal and vertical coordinates, respectively.

One factor at a time experiment

The one factor at a time experiment was executed to estimate the optimal range of each influencing factor. All the factors that affect the biomass of *B. subtilis* cultured in the mushroom wastewater of *A. bisporus* collected from Fujian Keren biotechnology Co., Ltd. (Zhangzhou City, Fujian Province, China) including industrial wastewater solubility, inoculum dose, shaking speed, loaded liquid, initial pH, and culture temperature were investigated in this study. The factors and levels in the one factor at a time experiment were selected as described in the Table 1.

Determination of significant variables using Plackett-Burman design

The advantage the Plackett-Burman design is that it can accurately and quickly examine the interactions between factors and main effects with a quite low resource consumption. The Plackett-Burman design was applied to screen the main factors that significantly influenced the total number of living *B. subtilis*. Based on the results of single factor experiment and growth curve of *B. subtilis*, we conducted the Plackett-Burman design with 12 experiments and 6 factors with others as blank items. Each selected factor included high (+) and low (-) levels in experimental design, in which low level was the highest level in single factor experiment, and the high level is the 1.25 times of low level [7].

Determination of steepest ascent path

Based on the Plackett-Burman results, the ascent direction of each factor was determined by their effect values. The changing gradient of experimental value was the changing step. We further confirmed the optimal range of three main factors, and then, rapidly reached the best area. Through this method, we establish the effective model [8]. According to the steepest ascent test design, the final ranges of the fermentation conditions needed to attain the most biomass of *B. subtilis* were determined.

Box-Behnken design and response surface analysis

According to the principle of response surface method, with the best values obtained from the path of steepest ascent for the factors utilized as the center point of the response surface, we performed a response analysis of the critical factors. For a 3-level-3-factor Box-Behnken design at the center, 17 experiments were

conducted to optimize the critical factors using *Bacillus subtilis* biomass as response value and three main effect factors as independent variable model to determine the influence of each factor on *Bacillus subtilis* biomass and to simulate the optimal combination of culture conditions [9].

Validation of the test results

To verify the reliability of the experimental model, three parallel experiments were performed according to the optimal fermentation conditions identified through Box–Benhnken design experiments. The resulting values were averaged to obtain the final results [10].

Detection of total biomass of B. subtilis

As described by Ou *et al*. [11, 12], the fermentation broth was diluted 10-fold with PBS and 1 mL of the diluted solution was mixed and incubated with 3 µL of LIVE/DEAD™ BacLight™ Bacterial Viability Kit (ThermoFisher, Waltham, MA, USA) staining reagent in the dark for 30 min. The total biomass of *B. subtilis* in the fermentation broth was quantified by FlowSight flow cytometry. PBS was used as a flow sheath and a 480 nm wavelength laser was used to collect fluorescence signals and images.

Statistical analysis

All the experiments were repeated at least three times in this study. All data were subjected to statistical analysis using Design-Expert V.12.0.1.0 (Stat-Ease, Inc., Minneapolis, MN, USA) and IBM SPSS Statistics V 19.0 (IBM, Ammon, New York, USA). The data were expressed as mean ± SD. Graphs were plotted using a computer program GraphPad Prism 8.2.0 (GraphPad Software, San Diego, CA, USA).

Results and discussion

Detection of total biomass using multi-dimensional panoramic flow cytometry

Each point on Figure 1A represented a bacterium or an object with three areas, in which red and green were dead and living bacteria, respectively, while black was inanimate particle. SYTO 9 stain is a high-affinity fluorescent DNA dye. It can permeate cell membranes of both prokaryotic and eukaryotic cells by passive diffusion and bind to the cells' DNA to exhibit enhanced green fluorescence when excited at 488 nm wavelength. SYTO 9 stain can be used to specifically stain both live and dead Gram-positive and Gram-negative bacteria. Propidium iodide (PI) is a DNA dye often used for identification of dead cells with red fluorescence because PI can only pass through the incomplete cell wall. Therefore, both SYTO 9 and PI were used at the same culture. The living *B. subtilis* with complete cell membrane can only be stained by SYTO 9 and show green fluorescence, while dead *B. subtilis* with destructed cell membrane can be bound by both SYTO 9 and PI, and demonstrated both green and red fluorescence. Nucleic acid free particles have only open field signals. In Figure 1B, only SYTO 9 field showed green fluorescence representing living *B. subtilis*, while in Figure 1C, the SYTO 9 field showed green fluorescence and PI field showed red fluorescence, representing no living *B. subtilis*. The open-field image with no SYTO 9 and PI fluorescence signal in Figure 1D suggested that there were no nucleic acid as inanimate particles. Therefore, we could calculate the total biomass number of living *B. subtilis* according to the total number in green area.

Growth curve of *B. subtilis*

The growth curve of *B. subtilis* is a typical S curve showed in Figure 2. The duration from 0 h to 6 h was the lag phase. The bacteria were in the adaptation to the environment to prepare for the later proliferation. The duration from 6 h to 12 h was logarithmic growth period with a geometric series growth, in which the number of live bacteria rapidly increased and reached the maximum growth rate. The total biomass of *B. subtilis* remained stable between 12 h and 22 h, which suggested that this period was the best period for fermentation. After 22 h, the total biological mass was slightly decreased over time due to nutrient depletion, accumulation of harmful metabolites, and competition among

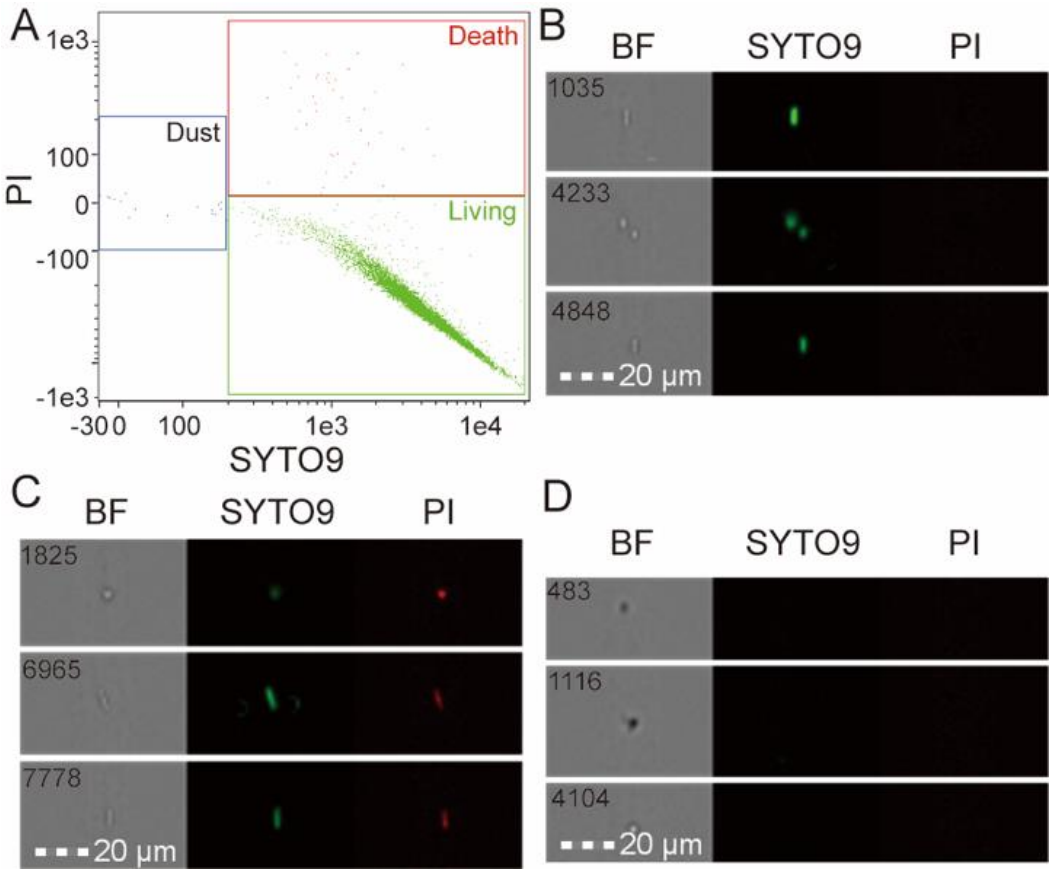

Figure 1. Living and dead *B. subtilis* based on multispectral imaging flow cytometry.

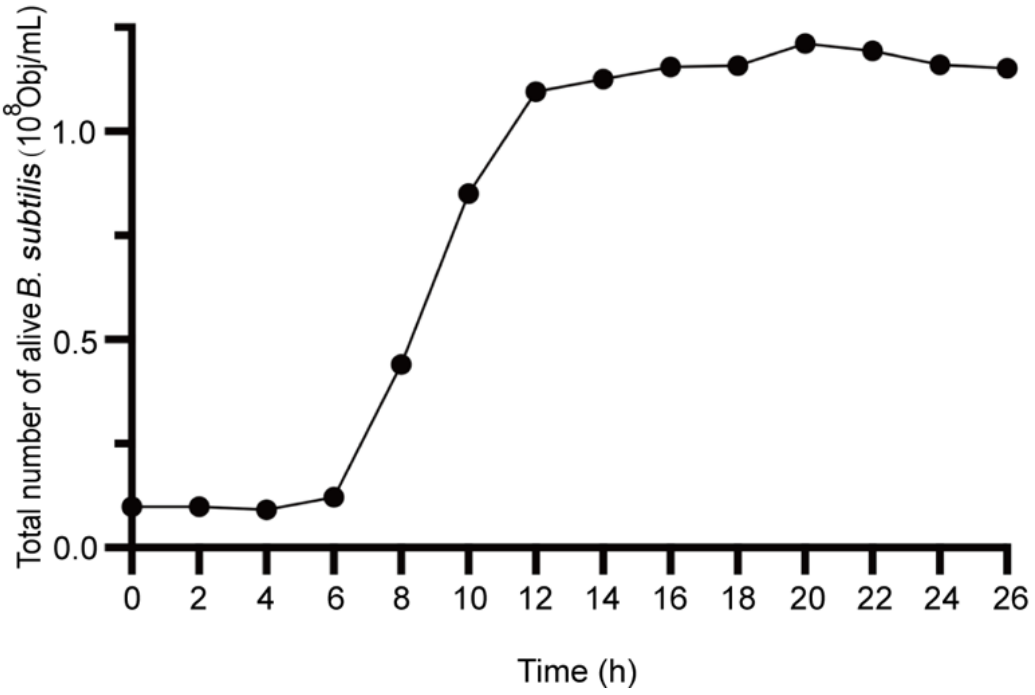

Figure 2. Growth curve of *B. subtilis*.

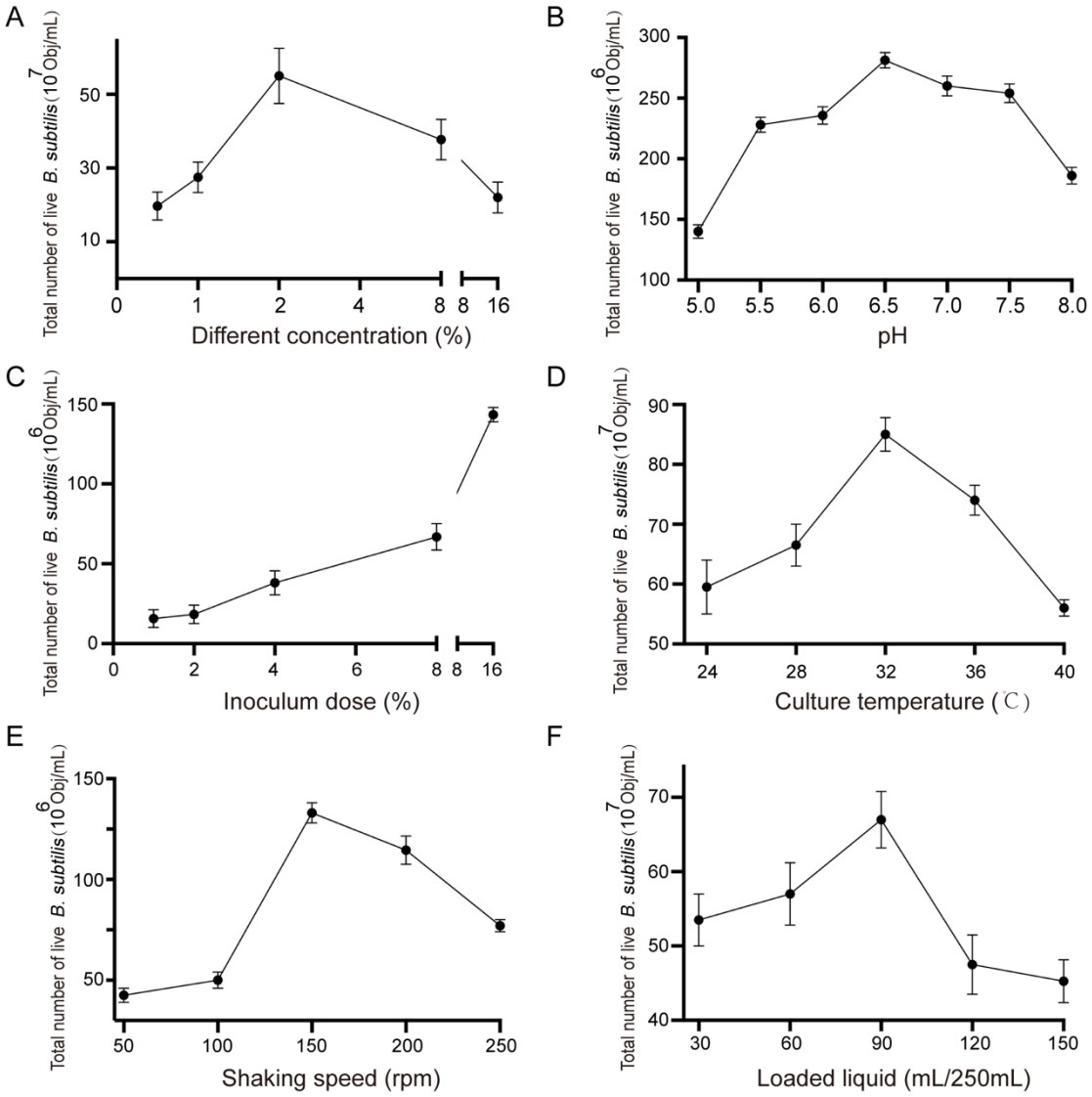

Figure 3. Results of the one-factor-at-a-time experiments. Effects of (A) wastewater concentration, (B) initial pH, (C) inoculum dose, (D) culture temperature, (E) shaking speed, and (F) loaded liquid on biomass of *B. subtilis*.

individuals of *B. subtilis*.

Single-factor tests

The total number of living *B. subtilis* increased first, and then, decreased following the increasing of processing wastewater (Figure 3A). When the concentration of wastewater was lower than 1%, the low nutrient content in processing wastewater cannot meet the needs of *B. subtilis* growth, the total biomass was at a low level. When the concentration of wastewater was 2%, the total number of living bacteria was up to the maximum ($5.50\pm0.07\times10^8$ Obj/mL). When concentration was higher than 8%, although the nutrient was sufficient, there was high osmotic pressure, which affected the absorption of nutrients by cells. Therefore, the total biomass significantly decreased. Accordingly, the suitable concentration of wastewater was between 2% and 8% for *B. subtilis*. *B. subtilis* maintained a high total biomass when the initial pH range was wide (5.5-7.5) (Figure 3B). When the pH was 6.5, the total biomass of *B. subtilis* attained the maximum

(2.82±0.06×10^8 Obj/mL). Figure 3C demonstrated that the total biomass of *B. subtilis* showed a rising trend following the increasing of inoculum dose. However, if the amount of inoculation was used for the purpose of increasing the total amount of organisms, it does not fit the aims and perspective significance of using mushroom industrial wastewater. For the application perspective and cost, the suitable inoculum dose was between 4% and 8% for *B. subtilis*. Figure 3D showed that the total biomass of *B. subtilis* increased first, and then, decreased following the rising of culture temperature. When the temperature was 32℃, the total biomass of *B. subtilis* attained the maximum (8.50±0.03×10^8 Obj/mL). The suitable temperature was between 32℃ to 36℃ for *B. subtilis*. With the increasing of shaking speed, the total biomass of *B. subtilis* showed an increased and then decreased trend (Figure 3E). When shaking speed was 150 rpm, the total biomass of *B. subtilis* attained the maximum (1.33±0.05×10^8 Obj/mL). Therefore, the suitable shaking speed was between 150 and 200 rpm for *B. subtilis*. As shown in Figure 3F, with the increasing of loaded liquid, the total biomass of *B. subtilis* showed an increased and then decreased trend. When loaded liquid was 90 mL/250 mL, the total biomass of *B. subtilis* attained the maximum (6.70±0.04×10^8 Obj/mL). Therefore, the suitable loaded liquid was between 60 and 90 mL/250 mL for *B. subtilis*.

Screening out the most effective factors by Plackett-Burman design

The Plackett–Burman design and results based on the results of one-factor-at-a-time were shown in Table 2. Five dummy variables in 12 experiments were investigated to obtain the standard error. Figure 4 showed the contributions of the variables screened. Among them, the culture temperature exhibited the most significant impact on the biomass of *B. subtilis*, followed by inoculum dose, wastewater solution, shaking speed, loaded liquid, and initial pH. The first three factors had the largest importance in affecting the biomass of *B. subtilis*, and therefore, they were subjected to further optimization [13].

Identification of approximate ranges of key factors for the optimal fermentation conditions by determining the path of steepest ascent

Based on the pareto chart analysis for each factor standard effect on the biomass of *B. subtilis*, the path of steepest ascent was then utilized to identify the most suitable direction for changing the variable ranges. According to the color in Figure 4, the negative or positive effect of each factor was determined. Among which, the concentration with orange color represented positive effect and should be gradually increased, while the temperature and inoculum amount showed blue color representing negative effect and should be gradually decreased. The other factors showed lower effect than top three factors. Therefore, we chose their lower level in Plackett–Burman experiments as the shaking speed 150 rpm, loaded liquid 90 mL/250 mL, and pH 6.5. The steepest ascent design was shown in Table 3. According to the results of steepest ascent experiment, we observed that the total biomass of *B. subtilis* increased first, and then, decreased following the decreasing of inoculum amount and culture temperature, and increasing of concentration. The total biomass of *B. subtilis* attained the maximum (1.06±0.09×10^8 Obj/mL) when the culture temperature was 32℃, inoculum amount was 8%, concentration was 2%. Hence, we conducted response surface design choosing 32℃ of temperature, 8% inoculum amount, and 2% of concentration as the centre point [5].

Determination of the best fermentation conditions using Box-Behnken Design and response surface method

A three-level and three-factor Box-Behnken design was applied to analyse the interactions of the three factors and to determine the best fermentation conditions. The Box-Behnken design and results were shown in Table 4. Experimental design and data analysis were performed by using Design-Expert. The response of the total biomass of *B. subtilis* was studied. The quadratic multiple regression equations of three critical variables were obtained as:

Table 2. Plackett–Burman experimental design and results.

Code / Run	Solubility (%)		pH		Inoculum dose (*v:v*, %)		Culture temperature (°C)		Shaking speed (rpm)		Loaded liquid (mL/250 mL)		The total biomass of *B. subtilis* (10^8 Obj/mL)
	Code level	Real level	Code level	Real level	Code level	Real level	Code level	Real level	Code level	Real level	Code level	Real level	
1	1	2.5	1	8.1	1	10.0	-1	32	-1	150	-1	90	0.77±0.01
2	-1	2.0	-1	6.5	1	10.0	-1	32	1	187	1	112	0.46±0.04
3	1	2.5	1	8.1	-1	8.0	1	40	1	187	1	112	0.82±0.25
4	-1	2.0	1	8.1	1	10.0	-1	32	1	187	1	112	0.41±0.02
5	1	2.5	-1	6.5	1	10.0	1	40	1	187	-1	90	0.14±0.01
6	1	2.5	-1	6.5	-1	8.0	-1	32	1	187	-1	90	1.40±0.23
7	-1	2.0	-1	6.5	-1	8.0	-1	32	-1	150	-1	90	1.47±0.16
8	-1	2.0	1	8.1	-1	8.0	1	40	1	187	-1	90	0.20±0.02
9	1	2.5	-1	6.5	1	10.0	1	40	-1	150	1	112	0.12±0.02
10	1	2.5	1	8.1	-1	8.0	-1	32	-1	150	1	112	1.80±0.22
11	-1	2.0	-1	6.5	-1	8.0	1	40	-1	150	1	112	0.45±0.01
12	-1	2.0	1	8.1	1	10.0	1	40	-1	150	-1	90	0.32±0.01

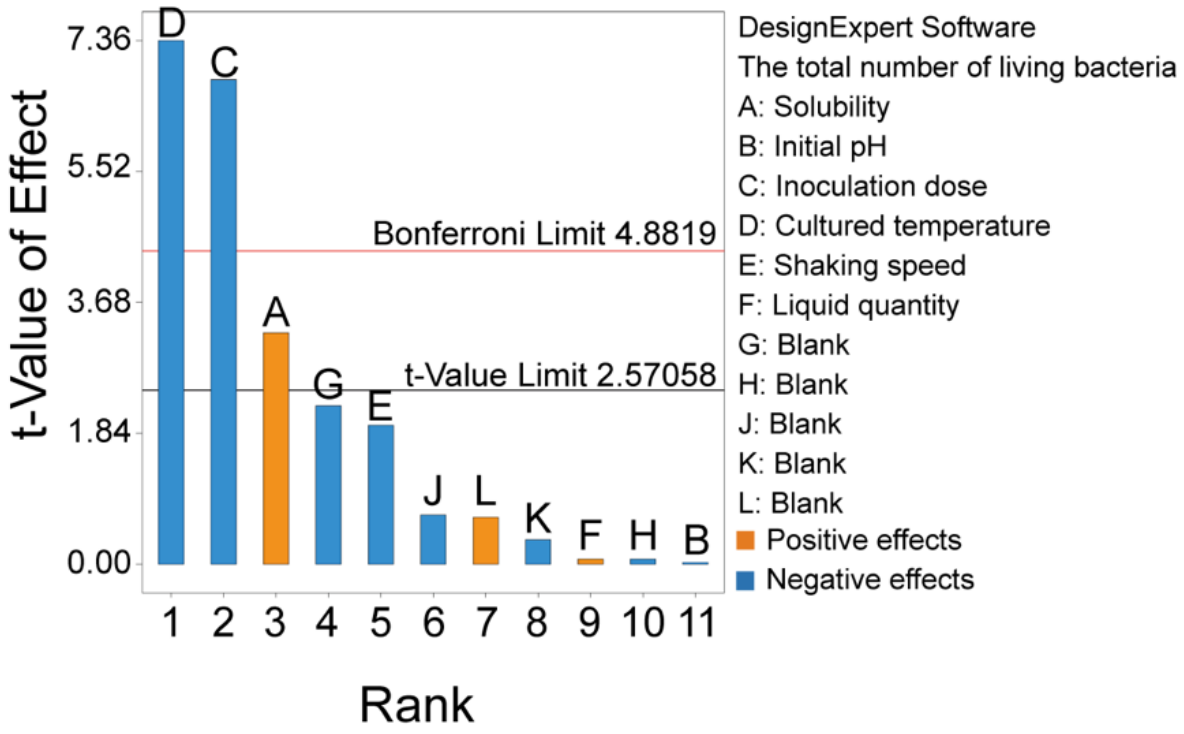

Figure 4. Pareto chart of each factor standard effect on the biomass of *B. subtilis*.

$Y = 253.29 - 20.76X_1 - 4.19X_2 + 36.13X_3 + 9.37X_1X_2 - 43.16X_1X_3 - 48.77X_2X_3 - 93.99X_1^2 - 80.49X_2^2 - 77.80X_3^2$

in which Y was the total biomass of *B. subtilis*, X_1 was culture temperature, X_2 was inoculum dose, X_3 was wastewater concentration. The optimal

Table 3. Design and results of steepest ascent experiment.

Run	Culture temperature (°C)	Inoculum dose (*v:v*,%)	Wastewater solution (%)	The total biomass of *B. subtilis*（10^7Obj/mL）
1	40	12	0.5	2.73±0.06
2	36	10	1	8.27±0.05
3	32	8	2	10.63±0.92
4	28	6	4	8.60±0.11
5	24	4	8	0.13±0.05

Table 4. Experimental design and results of Box-Behnken design.

Run	X_1- Culture temperature (℃）		X_2- Inoculum dose (*v:v*, %)		X_3- Concentration (%)		The total biomass of *B. subtilis* (10^8Obj/mL)
	Code level	Real level	Code level	Real level	Code level	Real level	
1	0	32	0	8	0	2	2.30±0.12
2	0	32	0	8	0	2	2.17±0.18
3	0	32	1	10	1	12	0.92±0.08
4	1	36	1	10	0	2	0.81±0.01
5	0	32	-1	6	1	4	2.05±0.15
6	0	32	1	10	-1	1	0.79±0.07
7	1	36	-1	6	0	2	0.27±0.04
8	0	32	-1	6	-1	1	0.04±0.01
9	1	36	0	8	-1	1	0.83±0.04
10	-1	28	-1	6	0	2	0.55±0.02
11	0	32	0	8	0	2	2.43±0.09
12	0	32	0	8	0	2	2.21±0.25
13	-1	28	0	8	1	4	1.66±0.08
14	-1	28	1	10	0	2	0.71±0.11
15	-1	28	0	8	-1	1	0.43±0.09
16	1	24	0	8	1	4	0.35±0.05
17	0	32	0	8	0	2	2.54±0.15

culture conditions of *B. subtilis* were simulated and predicted by this quadratic multiple regression equation.

The relationship between the total biomass of *B. subtilis* and the variables was assessed using ANOVA (Table 5), which indicated the significance of the regression coefficient combined with *p* value. The *F*-value 25.18 indicated that it was significant. The occurring rate of this *F*-value due to noise was only 0.02%. The Lack of Fit *F*-value 3.83 indicated that it was not significant. The occurring rate of the Lack of Fit *F*-value due to noise was 11.35%. The Non-significant lack of fit was good, and the model will be fit.

The regression equation binomial coefficients X_1^2, X_2^2, and X_3^2 were very dramatic, implying that the response value change (i.e. the total biomass of *B. subtilis*) was complicated. The influence of the factors on the total biomass of *B. subtilis* did

Table 5. ANOVA results of quadratic model for the total biomass of *B. subtilis*.

Source	Sum of squares	df	Mean squares	*F* value	*p*- value Prob>F	Significance
Model	13,023.43	9	13,023.43	25.18	0.0002	**
X_1	3,274.35	1	3,274.35	6.33	0.0400	*
X_2	133.48	1	133.48	0.2580	0.6271	
X_3	10,440.12	1	10,440.12	20.18	0.0028	**
X_1X_2	351.56	1	351.56	0.6796	0.4369	
X_1X_3	7,866.73	1	7,866.73	15.21	0.0059	**
X_2X_3	10,042.50	1	10,042.50	19.41	0.0031	**
X_1^2	37,194.32	1	37,194.32	71.90	＜0.0001	**
X_2^2	27,276.79	1	27,276.79	52.73	0.0002	**
X_3^2	19,023.51	1	19,023.51	36.78	0.0005	**
Residual	3,620.91	7	517.27			
Lack of fit	2,686.71	3	895.57	3.83	0.1153	
Pure error	934.20	4	233.55			
Cor total	16,644.34	16				
		R^2=0.9700， R^2_{Adj}=0.9315， Adeq precision=12.515				

Note: *siginificant; **extremely significant.

not exhibit a simple linear relationship. Instead, it exhibited an evident surface effect. The *P*-values of X_1 and X_3 were $P < 0.05$, indicating significant linear effects of the total biomass of *B. subtilis*. The *P*-value of X_2 was $P > 0.05$, indicating an insignificant effect. Results also showed that the interactions between X_1X_3 and X_2X_3 were significant.

The "Coefficient of Determination" (R^2) value was 0.9700, indicating a significant correlation between the predicted values using Box-Behnken design model and the corresponding experimental values. In addition, "Adeq precision" revealed a signal-to-noise ratio which was required to be >4.00. In this study, the ratio of 24.911 was adequate enough to verify that this model could be applied to navigate the design space. Thus, the response surface opened facing downwards and the equation had a maximum value. Therefore, the model can be applied to analyse and predict the optimization of culture conditions for *B. subtilis* submerged in industrial wastewater.

The response surface method is a statistical method typically applied to determine the optimal conditions in a multi-factor system. The Box–Behnken center combination design is a method commonly applied to optimize multiple factors with three levels. The response surface graph is a three-dimensional space surface map formed by the response values for each factor (X_1, X_2, X_3). The response surface analysis was carried out by following the fitted regression equation. The contour map and response surface of the interaction between variables were plotted and shown in Figure 5. The profiles of the response surfaces between culture temperature and inoculum dose, culture temperature and different concentration, inoculum dose and different concentration were all convex with an open downward direction (Figure 5A, C, E), indicating a parabolic relationship between the total biomass of *B. subtilis* and the three factors of culture temperature, inoculum dose, and different concentration. The surface of this response surface was steep and changed from -1 to 1 following the horizontal coordinate, and its color changed from green to red, suggesting the three critical fermentation factors had interactive effects on the bacterial growth. Response surface had red vertex, indicating the maximum predicted value of the biomass of *B. subtilis*. The optimal variable values were achieved when moving along the minor and

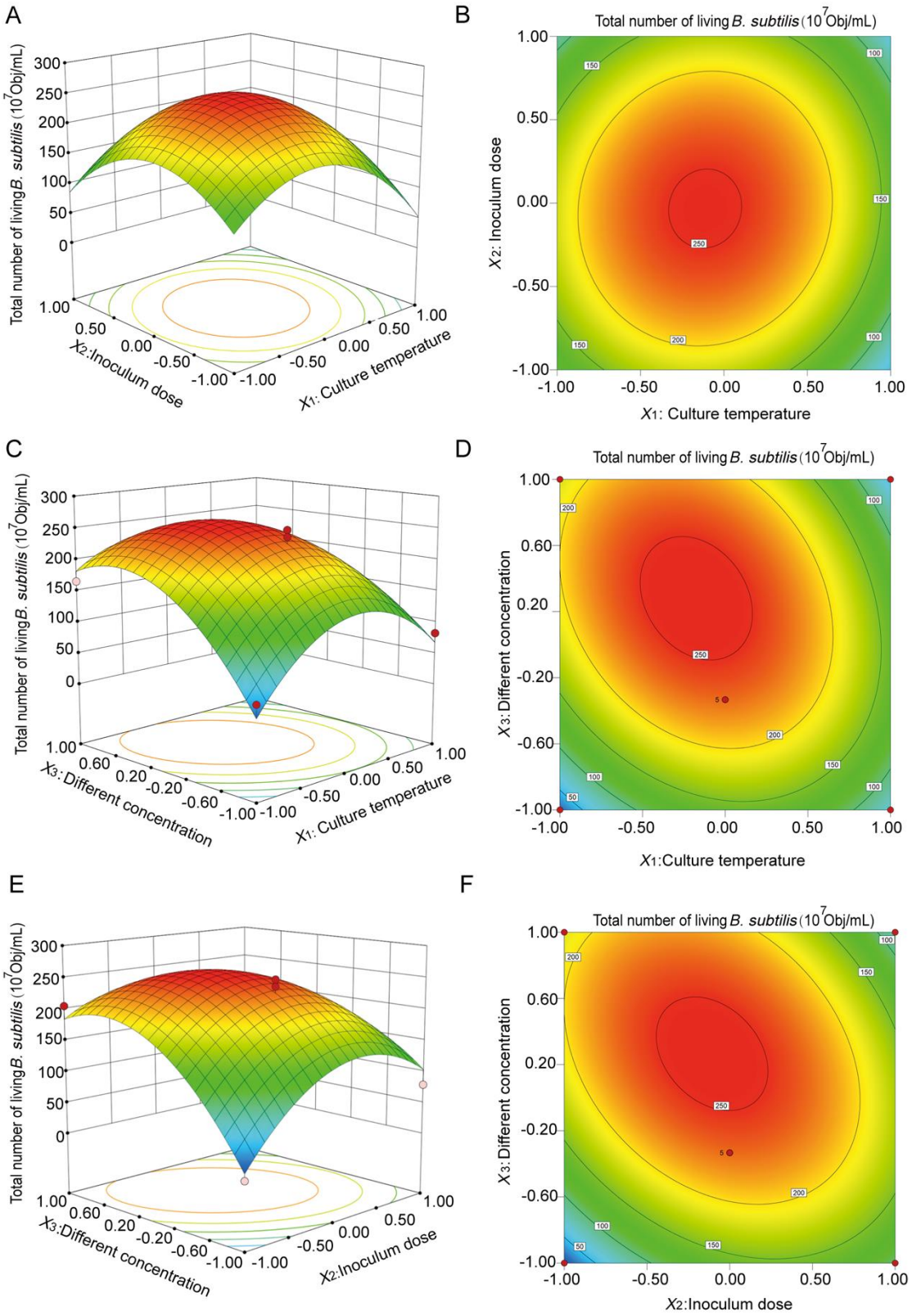

Figure 5. Response surface plots (3D) and contour plots (2D) for the effect of fermentation condition on the total biomass of *B. subtilis*. A and B: effects of culture temperature and inoculum dose; C and D: effects of culture temperature and different concentration; E and F: effects of inoculum dose and different concentration.

major axes, and the response yielded the maximum biomass of *B. subtilis* at the center point. A red vertex was located in the contour display in Figure 5D and F, which suggested that range of predicted value was within the interval, and the optimal designed condition was within the range of experimental setting value. The contour display was oval, which suggested that the temperature had significant interaction effect with inoculum dose and concentration. These results were also demonstrated through canonical analysis of the response surface.

According to the regression model and optimization of response surface and contour display, we obtained the optimal conditions that temperature was 32.3℃, inoculum dose was 7.9%, wastewater concentration was 0.7%, shaking speed was 150 rpm, loaded liquid was 90 mL, culture time was 24 h, and initial pH was 6.5. The predicted value of total number of living *B. subtilis* was 2.61×10^8 Obj/mL.

Experimental verification of theoretical optimum

Regression validation is a critical step of model analysis to evaluate the quantification results of the predicted relationships between variables [14]. Verification experiments at the optimal levels were performed. The total biomass of *B. subtilis* cultured on the optimal conditions reached $2.68 \pm 0.06\times10^8$ Obj/mL (N=3) that was higher than the predicted value (2.61×10^8 Obj/mL) at the 95% confidence interval of the predicted model ($2.26\text{-}2.80\times10^8$ Obj/mL). There was a good match between the predicted and the experimental results under optimal conditions, which validated the response surface method models with good correlation. In addition, the total biomass of *B. subtilis* cultured on the optimal conditions met the national feed additives' standard [15].

In this study, we optimized the submerged fermentation conditions for *B. subtilis* cultured in the *A. bisporus* industrial wastewater for high yield of biomass through combined steepest ascent test, Plackett-Burman Design, Box-Behnken design, and response surface method. The fermentation condition optimization is influenced by many factors with possible interactions among them. The typical single-dimensional studies usually change one independent variable each time while maintain other constants. So that, they often yield inaccurate conclusions, unreliable results, and also lead to frequent interactions between two or more factors. Therefore, applying some reasonable optimization methods and experimental designs in process optimization and condition screening is necessary. In this study, we first utilized a single-factor test to determine the range of culture conditions suitable for *B. subtilis* as the culture time 20-24 h, wastewater concentration 2%-8%, the range of initial pH 5.5-7.5, inoculum dose 4%-8%, culture temperature 32-36℃, shaking speed 150-200 rpm, and the loaded liquid amount 60-90 mL/250 mL. We further screened three most critical factors impacting the total biomass of *B. subtilis* using Plackett-Burman design, and then, we determined the paths of steepest ascent leading to the nearest region of maximum response. The most critical factors identified through Plackett-Burman design were inoculum dose, culture temperature, and wastewater concentrations. The path of steepest ascent was applied to seek proper direction to change the variables based on the main effects on the total biomass of *B. subtilis.* The path of steepest ascent was from the center of the Plackett-Burman design and along the path with increasing wastewater content, while decreasing culture temperature and inoculum dose. It was confirmed that the highest response was at the third step when culture temperature was 32℃, inoculum dose was 8%, wastewater concentration was 2%, which suggested that this point was close to the maximum production response. Taken together, our results showed that Plackett-Burman design could efficiently screen critical factors, and thereby, it had been widely applied in fermentation condition optimization. Although this technique cannot provide exact quantity, it can provide tendency indication about the variables' necessity through few experiments.

The response surface analysis was conducted using three-factor and three-level Box-Behnken design to analyse the interaction of the three factors to obtain the optimal culture conditions and predict the maximum biomass of *B. subtilis*. Response surface method not only determined the optimal levels of the most critical factors, but also well functioned in this process-optimizing practice. Through the optimization, the maximum biomass of *B. subtilis* at $2.68\pm0.06\times10^8$ Obj/mL (N=3) was obtained under the optimal conditions of 32.3℃, 7.9% of inoculum amount, 0.7% of wastewater concentration, 150 rpm of shaking speed, 90 mL of loaded liquid amount, 6.5 of initial pH, and 24 h of culture time.

In the mushroom industry, its cultivation, processing, production process will produce a large number of solid and liquid residues. It will generate great pressure on the environment if not fully utilized these residues. With the improvement of awareness of environmental protection and resource utilization, these abundant and valuable mushroom residues have been used in many different ways such as the extraction of functional molecules with antibacterial, antioxidant, anti-neoplasm, anti-allergic, and immunity enhancement from by-products produced during the processing of *A. bisporus* [16]. Besides, cultivation matrix of *A. bisporus* was used to absorb the heavy metals (lead and cadmium) and degrade organic matter (phenanthrene and polycyclic aromatic hydrocarbons), remedy the soil contaminated by multiple contaminations [17, 18], allowing sewage to be used for agricultural irrigation [19]. In this study, *B. subtilis* produced using processing wastewater of *A. bisporus* had reached the national feed additive standard, and its additional application in animal husbandry and aquatic industry should be further studied.

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