Electronic Journal of Biotechnology 48 (2020) 13–22

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

Improving the yield of *Anoectochilus roxburghii* by *Bacillus licheniformis* cultured in *Agaricus bisporus* industrial wastewater

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ARTICLE INFO

Article history:
Received 23 April 2020
Accepted 20 August 2020
Available online 3 September 2020

Keywords:
Agaricus bisporus
Anoectochilus roxburghii
Bacillus licheniformis
Box–Behnken
Flow cytometry
Industrial wastewater
Microbial fertilizer
Plackett–Burman
Response surface methodology
Wastewater

ABSTRACT

Background: There is a large amount of industrial wastewater produced by the mushroom industry during the canning processing each year, which could provide abundant carbon, nitrogen and inorganic salts for microbial growth. The aim of this study was to optimize the culture conditions for *Bacillus licheniformis* cultured in the *Agaricus bisporus* industrial wastewater to produce the agricultural microbial fertilizer.
Results: In this work, the maximal biomass of *B. licheniformis* could be obtained under the following culture conditions: 33.7°C, pH 7.0, 221 rpm shaking speed, 0.5% wastewater, 2 (v:v, %) inoculum dose, loading liquid of 60 mL/250 mL and a culture time of 24 h, and the average experimental value obtained was $1.35 \pm 0.04 \times 10^9$ Obj/mL, which was within the 95% confidence interval of the predicted model ($1.29–1.38 \times 10^9$ Obj/mL), and met the national microbial fertilizers' standard in China. Furthermore, the field experiment results showed that the fermentation broth of *B. licheniformis* could significantly improve the yield of *Anoectochilus roxburghii*.
Conclusions: Agaricus bisporus industrial wastewater can be used to produce agricultural microbial fertilizer.
How to cite: Huang J, Huang A, Lu L, et al. Improving the yield of Anoectochilus roxburghii by Bacillus licheniformis cultured in Agaricus bisporus industrial wastewater. Electron J Biotechnol 2020;48. https://doi.org/10.1016/j.ejbt.2020.08.002.

1. Introduction

Bacillus licheniformis, which belongs to the *Bacillus* genus of the *Bacillaceae* family, is a type of gram-positive probiotic widely distributed in nature. *B. licheniformis* can express catalase, oxidase and contact enzyme, and produce endophyte spores [1]. *B. licheniformis* has various favorable characteristics, including simple nutrient requirements, strong resistance to adverse environments, high heat resistance, high-yield production of various enzymes, inhibition of pathogenic bacteria and innocuity safety [2,3,4,5,6,7]. These characteristics allow *B. licheniformis* to be broadly used in various industries such as food processing [8,9], biological medicine [10], livestock maintenance [11,12,13], production of aquatic products [14,15,16], crop cultivation [17,18], re-utilization of wastes [19,20,21,22] and oil production [23].

Anoectochilus roxburghii, a traditional Chinese medicinal herb with high medicinal and ornamental value, contains several bioactive compounds, such as kinsenoside, polysaccharides, flavonoids, and glycosides, and has been commonly used for treatment of liver disease, diabetes, tumors, hyperlipemia and rheumatoid arthritis [24,25,26].

According to data obtained from the Food and Agriculture Organization (FAO), *A. bisporus*, *Lentinus edodes* and parts of wild fungus, viz., *Tricholoma matsutake*, are the main trade products in the edible mushroom sector. Due to the short storage period of fresh edible mushrooms, international trade mainly involves canned mushroom products, resulting in a large amount of industrial wastewater produced by the mushroom industry during the canning processing each year [27]. Industrial wastes are of great interest as substrates in the production of value-added products, as people seek to reduce cost, while managing the waste in an economical and environmentally-friendly manner. Microorganism fermentation of industrial wastes has gained more and more attention because of the abundance, availability, and rich carbon and nitrogen content of these

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

https://doi.org/10.1016/j.ejbt.2020.08.002

wastes. According to previous reports, industrial wastewater contains 0.65% protein, 0.17% total sugar, 0.08% reducing sugar, and 0.04% amino acid nitrogen [28,29], which could provide sufficient nutrients for microorganisms [30,31] or plants [32,33].

Therefore, we chose *B. licheniformis*, which displays simple nutritional requirements and high application rate, to treat the industrial wastewater generated by *A. bisporus* processing, turning waste into microbial fertilizer. Through this, we can reduce the burden of businesses on the environment.

The combined use of Plackett–Burman design and Box–Behnken response surface experiment is a rapid and convenient experimental optimization method which has been broadly applied for microbial fermentation culture and material extraction. For optimization of microorganism culture conditions, the orthogonal test design method was also chosen, however it could not provide the functional relationship between area factor and response value, or visual images. This problem could be solved by combining Plackett–Burman with Box–Behnken response surface experiment. The Plackett–Burman experimental design method is an important statistical method that can rapidly screen out the significant influential factors from various factors using fewer experiments, and can provide a basis for further optimization of the experiment. The Box–Behnken response surface method can establish the related mathematical models and regression equations, produce corresponding response surface diagram, and effectively analyze the interaction effects between significant factors [34,35,36,37]. Therefore, we optimized the culture condition of *B. licheniformis* using *A. bisporus* industrial wastewater with Plackett–Burman combined with Box–Behnken response surface method in this study.

2. Materials and methods

2.1. Strains, culture medium, and culture conditions

B. licheniformis (GIM1.863) was purchased from the Guangdong Culture Collection Center.

For seed medium, 0.2 g KH_2PO_4, 0.8 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaSO_4 \cdot 2H_2O$, 0.5 mg $Na_2MoO_4 \cdot 2H_2O$, 0.5 g Yeast extract, 20 g mannitol, and 0.5 mg $FeCl_3$ were dissolved in 1 L distilled water, then adjusted to pH 7.2 and stored after sterilization (121°C, 15 min).

For the fermentation liquid medium, *A. bisporus* industrial wastewater was collected from Fujian Keren biotechnology Co., Ltd., filtered to remove impurities, processed through double-effect concentration, adjusted to the required concentration and pH, sterilized (121°C, 15 min) and stored.

2.2. Strain activation

Freeze-dried bacteria were first activated, dissolved in 0.3–0.5 mL sterile water, seeded onto the slope and cultured in an incubator at 28–30°C for 24–48 h, and then stored in 4°C.

Table 1
Factors and levels assessed in single-factor tests.

Level	Factor					
	Concentration (%)	pH	Inoculum dose (*v:v*, %)	Temperature (°C)	Shaking speed (rpm)	Loaded liquid (mL/250 mL)
1	0.0625	5.0	0.25	24	50	30
2	0.125	5.5	0.5	28	100	60
3	0.25	6.0	1.0	32	150	90
4	0.5	6.5	2.0	36	200	120
5	1.0	7.0	4.0	40	250	150
6	2.0	7.5	8.0			
7	4.0	8.0				
8	8.0					

2.3. Preparation of seed suspension

A colony of activated *B. licheniformis* was seeded into 100 mL medium, and then cultured in a shaking incubator at 30°C and 150 rpm for 24 h.

2.4. Single-factor test

In the single-factor tests, as previously described by Huang et al. [38], a fixed culture time of 24 h was used, and the remaining factors (wastewater solubility, initial pH, inoculum dose, culture temperature, shaking speed, and loaded liquid) were tested, as shown in Table 1.

2.5. Plackett–Burman experimental design

The Plackett–Burman design is an effective two-level test design that can identify factors that have a significant impact on the results using fewer trials, thus improving test efficiency and avoiding waste of test resources. This confers the advantage of being able to examine the main effects and interaction effects between factors quickly and accurately with low resource consumption. The Plackett–Burman design was chosen to select the main significant factors that influenced the total number of living *B. licheniformis*. Based on the results of our single-factor experiments, we conducted the Plackett–Burman experimental design with 12 experiments and 6 factors. Each selected factor included high (+) and low (-) levels in the experimental design, in which the low level was the highest level in a single-factor experiment, and the high level was 1.25 times the low level [39].

2.6. Determination of the path of steepest ascent

We confirmed the optimal range of three main factors according to their effect values in the Plackett–Burman experiment. Then, the three factors rapidly reached the best area relying on the path of steepest ascent test. At last, three factors were combined to establish the most effective model to attain the highest biomass of *B. licheniformis* [40].

2.7. Box–Behnken experimental design

According to the principle underlying the response surface method, the optimal value derived for each factor from the path of steepest ascent determination was used as the center point of the response surface, and a response analysis of the key factors was then carried out. Based on the results of Plackett–Burman experiment and steepest ascent experiment, we confirmed the experimental factor and level in a Box–Behnken experiment, and conducted three-factor and three-level analysis using Design-Expert software. A model using the total number of living bacteria as the response value, and main three factors as arguments were established to investigate the effect of each factor on the total number of living bacteria and to determine the optimal combination of culture conditions [41,42].

2.8. Design of verification experiment

To test the reliability of the models in predicting optimal responses and in accordance with the optimization results obtained from Box–Behnken design with the desirability function, verification experiments were carried out at the determined levels [42].

2.9. Quantification of total number of living bacteria

As described by Robertson et al. [43] and Ou et al. [44], quantification of the total number of living *B. licheniformis* in the industrial wastewater broth was analyzed using multispectral imaging flow cytometry.

2.10. Field test

B. licheniformis was cultured under the confirmed optimal culture conditions, and then used for field tests. We selected the tissue culture seedlings of *Anoectochilus roxburghii* that grew well and were of a similar height after 10 d in open air to transplant into a plain soil seedling tray with the same fertility at a depth of 2 cm. Each seedling hole had 2 plants, and the trays were randomly divided into 4 groups, in which the first group was considered as control group and was treated with the same amount of water but no fertilizer; the second group was treated with fermentation broth once after 7 d of transplanting survival; the third group was treated with fermentation broth at 7 d and 30 d of transplanting survival; the fourth group was treated with fermentation broth at 7 d, 30 d and 60 d of transplanting survival. The fermentation broth amount was added at a dose of 0.5 kg/tray each time. During the test, the temperature was 25 ± 2°C, light intensity was 1500–2000 Lx, light time was 11 h/d, and water was added to maintain the humidity of soil. After 90 d, the *A. roxburghii* was obtained to measure their individual height, output and leaf area. Each group was analyzed in triplicate.

2.11. Statistical analysis

In this study, all experiments were performed in triplicate, and data were analyzed 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) software. All data are shown as $\bar{x} \pm s$.

3. Results and discussion

3.1. Detection of total number of living bacteria using multispectral imaging flow cytometry

In Fig. 1A, each point represents a bacterium or an object, and the plot has two areas, an upper red one showing dead bacteria, and lower green one showing living bacteria. SYTO 9 is able to pass through the cell membrane by passive diffusion, and binds to the DNA in living and dead bacteria. SYTO 9 glows green when excited with 488 nm light. Conversely, PI can only pass through the incomplete cell wall and stain dead cells. PI glows red under 488 nm light. Therefore, SYTO 9 and PI could be used at the same time, and living bacteria with a complete cell membrane can only be stained with SYTO 9 and will display green fluorescence. Those with a disrupted cell membrane can be stained by both SYTO 9 and PI, so dead *B. licheniformis* will display green and red fluorescence [45,46]. In Fig. 1B, the SYTO 9 field shows green fluorescence and PI field shows red fluorescence. Using these differential fluorescence properties, we were able to calculate the total number of living *B. licheniformis*.

3.2. Single-factor tests

The total number of living *B. licheniformis* increased first and then decreased with increasing concentration of wastewater (Fig. 2A). When the concentration of wastewater was below 0.5%, the total number of living bacteria was low. When the concentration of wastewater was increased to 0.5%, the total number of living bacteria reached its maximum (0.85 ± 0.05 × 10^8 Obj/mL). When the concentration was between 0.5% and 1%, there was a slight, but statistically insignificant decline. When the concentration was higher than 1%, the total number of living bacteria significantly decreased. Therefore, the optimal concentration of wastewater was between 0.5% and 1% for *B. licheniformis*. As shown in Fig. 2B, the total number of living *B. licheniformis* increased first and then decreased following an increase in the pH of wastewater. When the initial pH of wastewater was below 6.5, the total number of living *B. licheniformis* increased as the pH increased. When the pH was 6.5, the total number of living bacteria reached its maximum (1.22 ± 0.03 × 10^8 Obj/mL), however, when the initial pH was above 6.5, the total number of living *B. licheniformis* declined, indicating that the optimal initial pH of wastewater was 6.0–7.0 for *B. licheniformis*. In Fig. 2C, when the inoculum dose was lower than 2%, the total number of living *B. licheniformis* increased as the inoculum dose increased. When the inoculum dose was 2%, the total number of living bacteria peaked (1.22 ± 0.08 × 10^8 Obj/mL). At inoculum doses higher than 2%, however, the total number of living *B. licheniformis* began to decline. These results indicate that for *B. licheniformis*, the optimal inoculum dose was 2–8%. The total number of living *B. licheniformis* increased and then decreased with increasing culture temperature. When the temperature was 32°C, the total number of living bacteria reached its maximum (0.56 ± 0.01 × 10^8 Obj/mL), indicating that the optimal

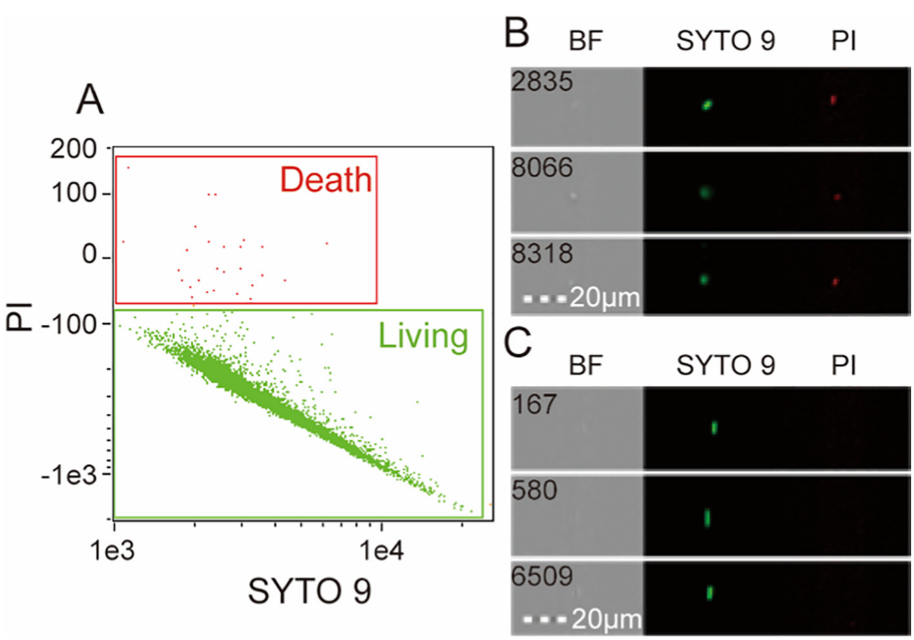

Fig. 1. Live and dead *B. licheniformis*, determined by multispectral imaging flow cytometry.

temperature was 28–36°C for *B. licheniformis*. As shown in Fig. 2E, the total number of living bacteria increased and then decreased with increasing shaking speed. When the shaking speed was 150 rpm, the total number of living bacteria reached its peak ($1.21 \pm 0.02 \times 10^8$ Obj/mL), suggesting that the optimal shaking speed for *B. licheniformis* was 100–150 rpm. Finally, we analyzed the effect of increasing the amount of loaded liquid used (Fig. 2F). We observed an increase, followed by a decrease, in the total number of living bacteria with increasing amounts of loaded liquid. When loaded liquid was 60 mL per 250 mL, the total number of living bacteria reached its maximum ($1.53 \pm 0.08 \times 10^8$ Obj/mL), therefore, the optimal loaded liquid amount was 30–90 mL per 250 mL for *B. licheniformis*.

3.3. Plackett–Burman design

We analyzed six variables using the Plackett–Burman experimental design, as this method can be useful for determining the key influential factors on the experimental response. The total number of living *B. licheniformis* was selected as the observed response to determine the effects of the variables studied. The matrix and results of Plackett–Burman design are shown in Table 2, and the contribution of the screened variables is shown in Fig. 2. Our results indicate that the culture temperature had the highest effect on the biomass of *B. licheniformis*, followed by pH and shaking speed. Compared to the other fermentation parameters tested, these three factors were determined to have the greatest influence on the biomass of *B. licheniformis* and, hence, were selected for further optimization.

3.4. Determination of the path of steepest ascent

The positive or negative effect of each parameter can be seen in Fig. 3, where the parameters are color coded by their effect. pH, shown in orange, had a positive effect, indicating that the chosen value should be gradually increased. Temperature and shaking speed

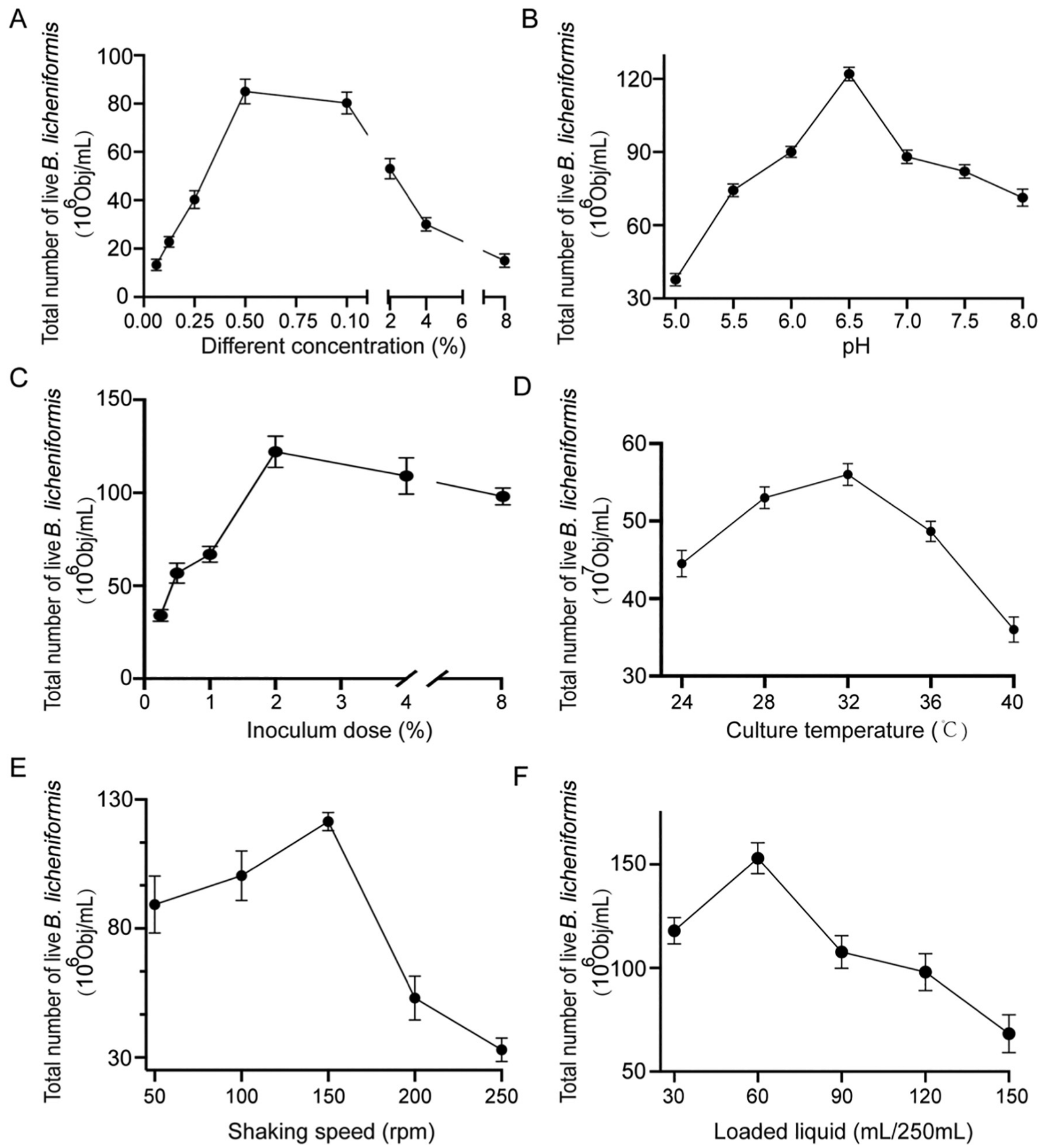

Fig. 2. Single-factor test results. Effect of (A) wastewater concentration, (B) initial pH, (C) inoculum dose, (D) culture temperature, (E) shaking speed, and (F) loaded liquid amount on total number of live *B. licheniformis*.

Table 2
Experimental design and results of Plackett–Burman design.

Run	Code												
	A-Solubility (%)		B-pH		C-Inoculum dose (%)		D-Temperature (°C)		E-Shaking speed (rpm)		F-Loaded liquid (mL/250 mL)		The total biomass of *B. licheniformis* (10^8Obj/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	0.625	1	8.0	1	2.5	-1	32	-1	150	-1	60	3.94 ± 0.04
2	-1	0.5	1	8.0	-1	2.0	-1	32	1	180	-1	60	3.87 ± 0.06
3	-1	0.5	1	8.0	1	2.5	-1	32	-1	150	-1	60	3.94 ± 0.05
4	-1	0.5	-1	6.5	-1	2.0	-1	32	-1	150	-1	60	3.34 ± 0.04
5	1	0.625	-1	6.5	1	2.5	-1	32	1	180	-1	60	1.66 ± 0.92
6	1	0.625	-1	6.5	1	2.5	1	40	-1	150	1	75	1.58 ± 0.93
7	1	0.625	1	8.0	-1	2.0	1	40	-1	150	1	75	2.62 ± 0.97
8	-1	0.5	-1	6.5	1	2.5	1	40	1	180	1	75	1.32 ± 0.69
9	1	0.625	1	8.0	-1	2.0	1	40	1	180	1	75	2.00 ± 0.47
10	1	0.625	-1	6.5	-1	2.0	-1	32	1	180	-1	60	2.98 ± 0.48
11	-1	0.5	-1	6.5	-1	2.0	1	40	-1	150	1	75	2.17 ± 0.06
12	-1	0.5	1	8.0	1	2.5	1	40	1	180	1	75	1.99 ± 0.03

are shown in blue, representing a negative effect, and indicating that the chosen value should be gradually decreased [30,47]. The other factors tested showed lower effects than these top three factors, so we used their lower levels from the Plackett–Burman experiments (wastewater concentration: 0.5%; inoculum amount: 2%; loaded liquid: 60 mL/250 mL). The steepest ascent design is shown in Table 3. Using this experimental design, we observed that the total number of living *B. licheniformis* bacteria increased and then decreased with decreasing culture temperature and inoculum amount, and increasing pH. The total number of living *B. licheniformis* reached its maximum (7.12 ± 0.08 × 10^8 Obj/mL) under the following culture conditions: 32°C, pH 6.5, shaking speed 200 rpm, 0.5% wastewater and 4% inoculum. According to the effect values of the three factors in the path of steepest ascent test, the change distance and climbing direction can be determined. Hence, we chose 32°C, pH 6.5 and 200 rpm shaking speed as the center point when conducting our response surface design [27].

3.5. Box–Behnken experimental design

We confirmed optimal response levels of three important factors according to the steepest ascent results. We conducted the three-factor and three-level central response surface design using culture temperature, pH and shaking speed as the arguments, and total number of living bacteria as the response value. The experimental design is shown in Table 4.

We conducted multiple regression analysis on Box–Behnken results using Design-Expert software. After fitting the regression model, we

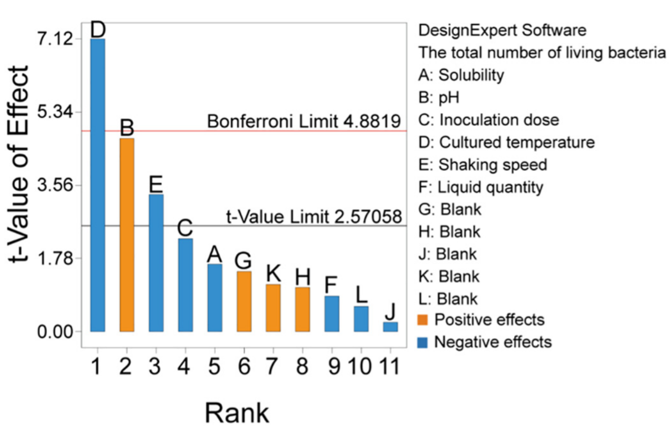

Fig. 3. Pareto chart of the standard effect of each factor on the total number of live *B. licheniformis*.

obtained a regression equation of the effects of the experimental factors on the response value, as shown below:

$$Y = 11.81 + 0.46X_1 + 0.71X_2 + 1.46X_3 + 0.12X_1X_2 + 1.64X_1X_3 - 0.35X_2X_3 - 1.43{X_1}^2 + 0.46{X_2}^2 - 2.18{X_3}^2$$

In this equation, X_1 is the culture temperature, X_2 is the pH, X_3 is the shaking speed and Y is the total number of living *B. licheniformis* bacteria. Positive and negative values of each coefficient represent the impact direction of each argument on the total number of living *B. licheniformis*, and the absolute value of each coefficient reflects the degree of its effect. The *F*-value of the model was 72.27, which indicated that the terms in the model had a significant effect on the response, and a *p* value <0.0001 for the regression model indicated that the linear relationship between dependent variable and all independent variables was highly significant (Table 5). Together, these values confirm that the experimental method was reliable.

The *p* values for X_1, X_2, X_3, X_1 X_3, X_1^2, X_2^2 and X_3^2 were all less than 0.05, suggesting that culture temperature, pH, and shaking speed, as well as the effects caused by the interaction of these factors, had significant effects on the model. The *p* value of the factors that were not included was 0.1215, indicating no significant effect. Overall, this suggests there were no missing items in this model, no abnormal values in the data, and no need to introduce the higher order terms, indicating that the model was sound. The predicted R^2 was 0.9894 and the adjusting coefficient of determination (R^2_{Adj}) was 0.9757, suggesting that the measured value of total living *B. licheniformis* fit well with the predicted value, and the model could theoretically be used to predict *B. licheniformis* growth in culture. A low CV value (3.05%) revealed that the deviations between the experimental and predicted values were low, indicating that our model displayed not only a high degree of precision but also reliability in our experiments. Adequate precision measures the signal-to noise ratio, and a ratio greater than 4 is desirable. In this study, a ratio of 27.2176 indicated adequate precision. All in all, the data of the Box–Behnken experimental design

Table 3
Experimental design and results of steepest ascent design.

Run	Temperature (°C)	pH	Shaking speed (rpm)	The total number of live *B. licheniformis* (10^8 Obj/mL)
1	40	5.5	300	4.37 ± 0.50
2	36	6	250	5.94 ± 0.01
3	32	6.5	200	7.12 ± 0.08
4	28	7	150	7.01 ± 0.07
5	24	7.5	100	5.05 ± 0.04
6	20	8	50	0.72 ± 0.07

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

Run	X_1-Temperature (°C)		X_2-pH		X_3-Shaking speed(rpm)		The total number of live *B. licheniformis* (10^8Obj/mL)
	Code level	Real level	Code level	Real level	Code level	Real level	
1	1	36	0	6.5	-1	150	5.39 ± 0.06
2	0	32	-1	6.0	1	250	10.83 ± 0.76
3	0	32	1	7.0	-1	150	10.06 ± 0.39
4	0	32	-1	6.0	-1	150	7.58 ± 0.09
5	0	32	0	6.5	0	200	11.75 ± 0.37
6	1	36	0	6.5	1	250	11.94 ± 0.10
7	-1	28	1	7.0	0	200	10.80 ± 0.37
8	0	32	0	6.5	0	200	11.97 ± 0.90
9	0	32	0	6.5	0	200	11.97 ± 0.90
10	1	36	1	7.0	0	200	11.96 ± 0.90
11	-1	28	0	6.5	1	250	7.73 ± 0.35
12	0	32	0	6.5	0	200	11.46 ± 0.45
13	0	32	0	6.5	0	200	11.90 ± 0.35
14	-1	28	-1	6.0	0	200	9.96 ± 0.39
15	1	36	-1	6.0	0	200	10.65 ± 0.19
16	-1	28	0	6.5	-1	150	7.74 ± 0.39
17	0	32	1	7.0	1	250	11.91 ± 0.89

Table 5
ANOVA results of quadratic model for the total number of live *B. licheniformis*.

Source	Sum of squares	df	Mean squares	*F* value	*p*-value Prob>F	Significance
Model	64.41	9	7.16	72.27	<0.0001	**
X_1	1.72	1	1.72	17.38	0.0042	**
X_2	4.08	1	4.08	41.16	0.0004	**
X_3	16.94	1	16.94	171.04	<0.0001	**
$X_1 X_2$	0.055	1	0.055	0.56	0.4795	
$X_1 X_3$	10.76	1	10.76	108.65	<0.0001	**
$X_2 X_3$	0.49	1	0.49	4.95	0.0615	
X_1^2	8.63	1	8.63	87.11	<0.0001	**
X_2^2	0.91	1	0.91	9.15	0.0193	*
X_3^2	19.99	1	19.99	201.85	<0.0001	**
Residual	0.69	7	0.099			
Lack of fit	0.51	3	0.17	3.65	0.1215	
Pure error	0.19	4	0.046			
Cor total	65.10	16				

$R^2 = 0.9894$, $R^2_{Adj} = 0.9757$, $R^2_{pred} = 0.8708$, Adequate precision = 27.2176, C.V. = 3.05%
$R^2 = 0.9894$, $R^2_{Adj} = 0.9757$, $R^2_{pred} = 0.8708$, Adequate precision = 27.2176, C.V. = 3.05%.
Note: *, significance; **, extreme significance.

showed that the quadratic model was successfully set up. Therefore, the quadratic model was selected in this optimization study [48].

3.6. Response surface and contour display analysis

Using the fitted quadratic multiple regression equations, temperature, pH and shaking speed were individually fixed at 0 level in the model. This enabled us to determine the interaction results of the other factors and obtain the response surface and contour display figures of their interaction effects on the number of living *B. licheniformis* bacteria (Fig. 4).

We found that the response surface for the interaction between temperature and pH appeared as a saddle with a steep surface and was colored red, suggesting that these factors had significant effects on the total number of living *B. licheniformis*, with its total number always in the higher layer (Fig. 4A). When X_1 (temperature) was 32°C at 0 level and shaking speed was fixed, the total number of living bacteria increased and then decreased with increasing wastewater concentration. Similarly, when X_2 (pH) was 6.5 at 0 level and shaking speed was fixed, the total number of living bacteria increased and then decreased with increasing temperature, which suggests that both too high and too low temperature and pH were not beneficial to the growth of *B. licheniformis*. As shown in Fig. 4B, the red vertex suggests that the range of predicted values was within the interval, meaning that the optimal design condition was within the range of the experimental setting values.

As shown in Fig. 4C, the response surface for the interaction between temperature and shaking speed was steep and resembled an inverted bowl. Its color changed from green to red and it displayed a red vertex, suggesting there was peak predicted value in the model, and the total number of living bacteria could be optimized to reach its maximum. When X_1 (temperature) was 32°C at 0 level and pH was fixed, the total number of living bacteria increased and then decreased with increasing shaking speed. Similarly, when X_3 (shaking speed) was 200 rpm at 0 level and pH was fixed, the total number of living bacteria increased and then decreased with increasing temperature, suggesting that both too high and too low temperature and shaking speed were not beneficial to the culturing of *B. licheniformis*. As shown in Fig. 4D, the contour display was an oval, which suggests that the temperature had a significant interaction effect with shaking speed. The red vertex in the contour display suggests that the range of predicted values was within the interval, indicating that the optimal design condition was within the range of the experimental setting values.

In Fig. 4E, we observed that the response surface for the interaction between pH and shaking speed was steep and resembled a saddle. Its color changed from green to red, revealing that pH and shaking speed had significant effects on the total number of living *B. licheniformis*, and its total number was always in the higher layer. When X_2 (pH) was 6.5 at 0 level and temperature was fixed, the total number of living bacteria increased and then decreased with increasing shaking speed. Similarly, when X_3 (shaking speed) was 200 rpm at 0 level and temperature was fixed, the total number of living bacteria increased and then decreased with increasing pH, suggesting that both too high and too low pH and shaking speed were not beneficial to *B. licheniformis* growth. As shown in Fig. 4F, the red vertex was located in the contour display, suggesting that the range of predicted values was within the interval, meaning that the optimal design condition was within the range of experimental setting values.

3.7. Verification test

Using the regression model, as well as optimization of the response surface and contour display, we obtained the ideal conditions for *B. licheniformis* culturing. At 33.7°C, pH 7.0, 221 rpm, 0.5% wastewater, 2% (v:v) inoculum, 60 mL loaded liquid per 250 mL culture, and an incubation time of 24 h, the predicted value of total number of living *B. licheniformis* reached 1.33 × 10^9 Obj/mL. After rounding up the values for the optimal conditions listed above, we conducted a verification test, and obtained an experimental value of 1.35 ± 0.04 × 10^9 Obj/mL (N = 3), attaining a value that was 101.5% of the predicted theoretical value and within the 95% confidence interval of model (1.29–1.38 × 10^9 Obj/mL). This revealed that the experimental

Fig. 4. The effect of cross-interaction between culture temperature, pH and shaking speed on total number of live *B. licheniformis.* (A) Response surface plot (3D) of effects of interaction between culture temperature and pH on total number of live *B. licheniformis*; (B) Contour line (2D) of effects of interaction between culture temperature and pH on total number of live *B. licheniformis;* (C) Response surface plot of effects of interaction between culture temperature and shaking speed on total number of live *B. licheniformis*; (D) Contour line of effects of interaction between culture temperature and shaking speed on total number of live *B. licheniformis*; (E) Response surface plot of effects of interaction between pH and shaking speed on total number of live *B. licheniformis*; (F) Contour line of effects of interaction between pH and shaking speed on total number of live *B. licheniformis*.

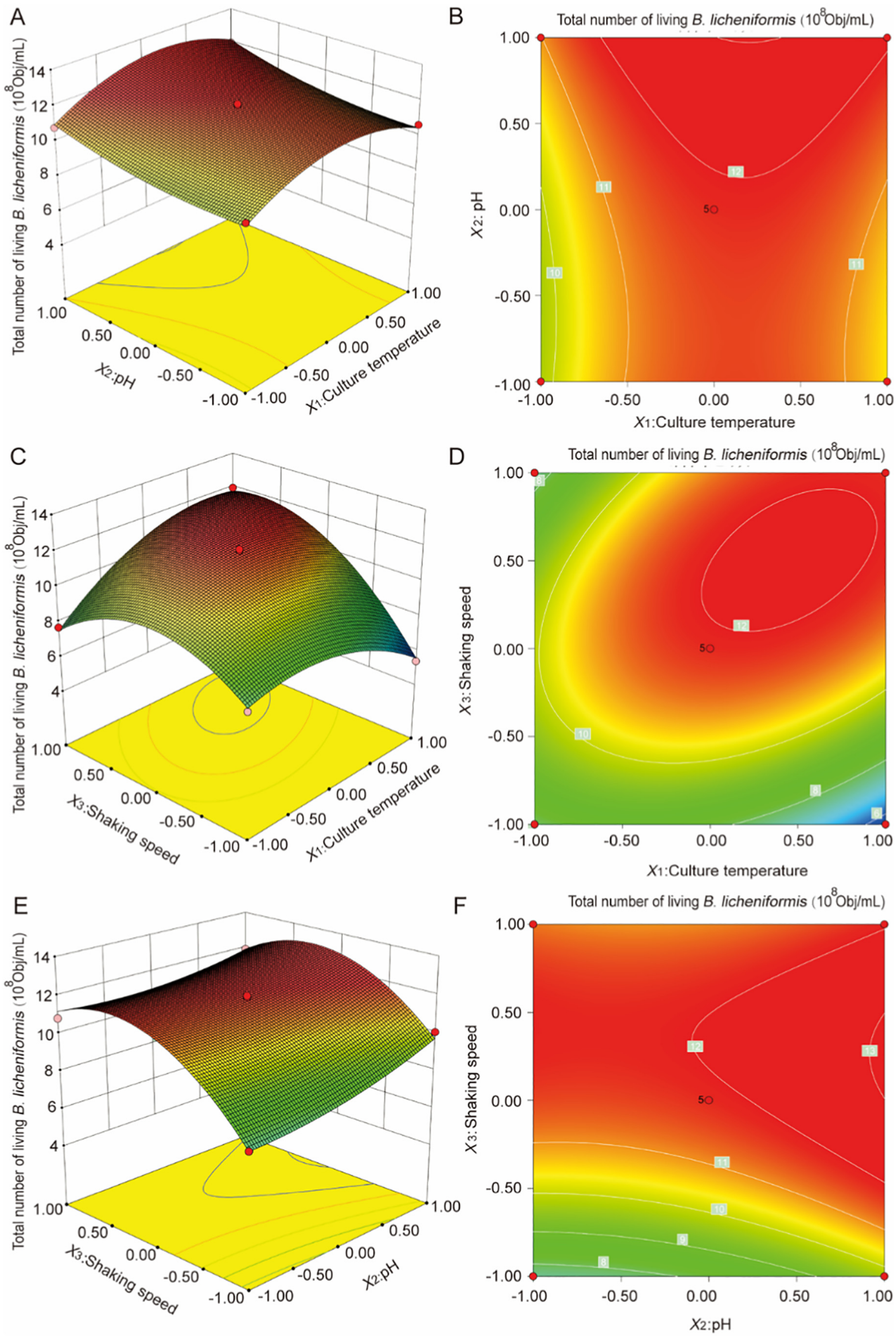
A
Total number of living B. licheniformis (10^8 Obj/mL)
X2:pH
X1:Culture temperature
B
Total number of living B. licheniformis (10^8 Obj/mL)
X2: pH
X1:Culture temperature
C
Total number of living B. licheniformis (10^8 Obj/mL)
X3:Shaking speed
X1:Culture temperature
D
Total number of living B. licheniformis (10^8 Obj/mL)
X3: Shaking speed
X1:Culture temperature
E
Total number of living B. licheniformis (10^8 Obj/mL)
X3:Shaking speed
X2:pH
F
Total number of living B. licheniformis (10^8 Obj/mL)
X3: Shaking speed
X2:pH

Table 6
The effect of fermentation broth on the yield, plant height, and leaf area of *A. roxburghii*.

Group	Item		
	Yield (g/plate)	Plant height (cm)	Leaf area (cm^2/plant)
A	121.8 ± 4.2	7.5 ± 0.7	4.12 ± 0.06
B	126.5 ± 3.2	8.6 ± 0.9 *	4.29 ± 0.12
C	159.4 ± 3.1*	9.1 ± 1.4 **	4.34 ± 0.09
D	172.7 ± 3.7**	9.6 ± 1.1 **	4.52 ± 0.14*

Notes: * denotes significant; ** denotes very significant. A: control group treated with water; B: *A. roxburghii* treated with fermentation broth once; C: *A. roxburghii* treated with fermentation broth two times; D: *A. roxburghii* treated with fermentation broth three times.

results fit well with the regression model, and it is feasible to use *B. licheniformis* in *A. bisporus* industrial wastewater at the optimal conditions.

3.8. Field test

As shown in Table 6, the yield, plant height, and leaf area of *A. roxburghii* were significantly improved when treated with the fermentation broth of *B. licheniformis* grown under the optimized conditions. As shown in Fig. 5, the growth status of *A. roxburghii* treated three times with the fermentation broth of *B. licheniformis* (Fig. 5D) showed the best growth compared to controls, as well as those plants that had been treated fewer times.

In this study, the *A. bisporus* industrial wastewater was considered the natural culture medium for *B. licheniformis*, and single factor experimentation was used to confirm the proper level for each factor. We conducted Plackett–Burman, steepest ascent and Box–Behnken response surface analyses to imitate the optimal conditions of *B. licheniformis*, and then conducted a field test using the determined optimal conditions. The single factor experiments confirmed the suitable conditions for *B. licheniformis* (0.5%–1% wastewater, pH 6.0–7.0, 2%–8% inoculum, 28–36°C, 100–150 rpm, and 30–90 mL loaded liquid per 250 mL). Using the Plackett–Burman experiment, we screened out three main significant factors that influenced the growth of *B. licheniformis*, including temperature, pH and shaking speed. Finally, we found the optimal combination of temperature, pH and shaking speed, allowing us to obtain the optimal conditions for *B. licheniformis* culturing (34°C, pH 7.0, 220 rpm, 0.5% wastewater, 2% inoculum amount, 60 mL loaded liquid per 250 mL, and 24 h incubation). We tested these optimal parameters and obtained a total number of living *B. licheniformis* of $1.35 \pm 0.04 \times 10^9$ Obj/mL (N = 3), which was 101.5% of the value predicted by the model. Our field test showed that the fermentation liquid of *B. licheniformis* could significantly improve the plant height, leaf area and output of *A. roxburghii*.

Multidimensional panoramic flow cytometry could rapidly quantify and differentiate living and dead bacteria, as SYTO 9 labeled bacteria that had either a complete or damaged membrane, whereas PI only labeled the bacteria that had a damaged membrane [46]. However, traditional plate counting methods were cumbersome, involved many factors during the experimental processes and resulted in a high degree of experimental error. Therefore, we used flow cytometry to count living bacteria, so that we could work more efficiently and avoid errors. Compared with real-time quantitative PCR, multidimensional panoramic flow cytometry was simple, not easily affected by reaction conditions, and produced visual results. Therefore, we selected multidimensional panoramic flow cytometry to count the total number of living bacteria [49,50].

Wen et al. decided to use *B. licheniformis* when working with wastewater because of its biological adsorption function, as it can absorb 98% of lead ions in wastewater and its maximum absorption amount reached 113.84 mg/g [19]. Sakai et al. used *B. licheniformis* TY7 to treat household garbage to produce thermotolerant l-lactic-acid [20]. Ji et al. [22] combined *B. licheniformis* and chlorella to treat city sewage, and the removal rates of total nitrogen, ammonium, orthophosphate phosphorus and chemical oxygen demand reached 88.82%, 84.98%, 84.87% and 82.25%, respectively. In our study, based on the research reported by Huang et al., the industrial wastewater of *A. bisporus* was recycled in a scientific and effective manner, then it was used as culture medium for *B. licheniformis*, and improved the output of *A. roxburghii* through the application of *B. licheniformis* in microbial fertilizers. Additionally, we have provided a theoretical basis for further industrial production of *B. licheniformis*, and also laid a foundation for its further application in microbial fertilizer.

In conclusion, we determined the following optimal conditions of *B. licheniformis* cultured in the industrial wastewater of *A. bisporus*: 34°C, pH 7.0, 220 rpm, 0.5% waste water, 2% inoculum, 60 mL loaded liquid per 250 mL and 24 h incubation. Under these conditions, we obtained a total *B. licheniformis* biomass of $1.35 \pm 0.04 \times 10^9$ Obj/mL, which was far higher than that of the agricultural microbial fertilizers'

Fig. 5. The growth status of *A. roxburghii*. (A) Control group treated with water. (B) *A. roxburghii* treated with fermentation broth one time. (C) *A. roxburghii* treated with fermentation broth two times. (D) *A. roxburghii* treated with fermentation broth three times.

standard. Furthermore, the fermentation liquid of *B. licheniformis* was able to significantly improve the yield of *A. roxburghii*.

Conflict of interest

The authors declare that there are no conflicts of interest.

Financial support

This study was funded by the Fujian Provincial Department of Science and Technology (no. 2019N0018 and no.2018N0025), the Fujian Provincial Department of Finance (FJDF[2019]0926), the 2019 Fujian Provincial Science and Technology Commissioner Project (no. SKTP19009), the 2020 Fujian Provincial Science and Technology Commissioner Project, the Key Project of Fujian University Youth Natural Foundation (no. JZ160454), Excellent Talents Project of Minnan Normal University of New Century (no. MX13004), and the Education Department of Fujian Province (No. JT180306). All the funders are in the the People's Republic of China.

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