# Optimization of culture conditions of *Sporosarcina pasteurii* using the response surface method

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**The use of industrial wastes as substrates in the production of value-added products is of great interest in efforts to reduce cost, while managing the waste economically and in an environmentally friendly manner. In this paper, *Sporosarcina pasteurii* are used in the treatment of *Agaricus bisporus* industrial wastewater to produce agricultural microbial fertilizer. The culture conditions, in which *S. pasteurii* was used to treat *A. bisporus* industrial wastewater as the natural medium, were optimized using the Plackett-Burman and Box-behnken response surface methods. The total number of *S. pasteurii* in the fermentation broth was analyzed using multispectral imaging flow cytometry. First, using the Plackett-Burman experimental design method, the six factors of industrial wastewater including the concentration, initial pH, loading volume, inoculum size, rotation speed, and culture temperature were experimentally investigated. Three significant factors were found to affect the total number of viable *S. pasteurii* bacteria: culture temperature, concentration, and inoculum amount, all of which were determined by the steepest climbing experiment in order to determine the suitable horizontal interval of the three significant factors. The Box-Behnken response surface method was then utilized to optimize the interaction among the three main factors. The total number of viable *S. pasteurii* was set as the response surface value and the three main significant factors were the independent variables. Multivariate linear regression and binomial equation fitting were carried out to obtain the optimal culture conditions: *A. bisporus* industrial wastewater solubility of 1%, initial pH of 7.0, inoculum size of 8%, loading volume of 90 mL/250 mL, rotation speed of 150 rpm, culture temperature of 30℃, and a culture time of 24 h. The average experimental value was 1.52±0.04×10$^8$ Obj/mL and reached approximately 98.70% of the model's optimum predicted value (1.54×10$^8$ Obj/mL), which illustrates the validity of the model. The main indicator of the total number of living bacteria was also far higher than that of the agricultural microbial fertilizers' standard, in which the effective number of living bacterium must reach 0.2×10$^8$ Obj/mL. Therefore, the results of this study highlight the potential of using *A. bisporus* industrial wastewater as the natural medium for *S. pasteurii* in the production of microbial fertilizer.**

**Keywords:** *Sporosarcina pasteurii*; *Agaricus bisporus* industrial wastewater; response surface method; multispectral imaging flow cytometry; microbial fertilizer.

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## Introduction

Due to the rapid development of the economy, urbanization, and increasing population sizes, the crop demand is growing. Improvements to crop yield through the addition of fertilizers is common practice, however, the frequent use of fertilizers and pesticides in agricultural

production results in soil degradation problems including soil acidification and salinization, imbalance or loss of elements in the soil, and soil pollution [1-4]. These issues are becoming increasingly more prominent, contributing to severe ecological and environmental problems. Currently, there is a strong focus on food safety, however, agricultural development and the prevention of soil degradation-related issues should be a parallel focus. It is imperative that efforts are made to protect the ecological environment and improve both the quality and safety of crops. Therefore, microbial fertilizers have been recently gaining more attention. Their use could have numerous benefits including: improving the organic matter and content of various trace elements in soil through the life activities of the microorganisms [5, 6], increasing the supply of plant nutrients or promoting plant growth and yield [7], improving the physical and chemical structure of the soil [8, 9], inhibiting the proliferation of pathogenic bacteria [10], and contributing to the formation of a suitable environment for crop growth as well as helping to improve crop resistance to disease and stress [11, 12], thus improving the overall quality of agricultural products as well as the agroecological environment [13].

*S. pasteurii*, also called *Bacillus pasteurii*, is a gram-positive bacterium that is broadly distributed in soil, compost, and sewage [14]. It can produce a highly active urease, and is able to decompose urea from nitrogenous fertilizer into ammonia [15], which is one of the sources of the nutrient nitrogen absorbed by plants and plays an important role in nitrogen cycling and improving soil fertility.

*A. bisporus* is a type of healthy edible fungus, high in protein, low in fat, low energy, highly nutritious, and has been shown having beneficial medical uses. The main processing method for *A. bisporus* is canning because overcomes the short preservation period of *A. bisporus*. During the canning process, water-soluble nutrients including proteins, amino acids, soluble polysaccharide, nucleotides, and mineral ions dissolve in the hot water [16-18], which is enough to provide necessary carbon and nitrogen sources for microbial or plant growth [19-21]. However, it is always discarded as wastewater during the canning process. Hence, *A. bisporus* industrial wastewater was used as the natural liquid medium for *S. pasteurii*, which not only reduces the pollution of resources and the environment, but also reduces cost, and serves as an environmental protection measure to turn waste into something useful. Therefore, we can make full use of the *A. bisporus* industrial wastewater to culture *S. pasteurii* to make it into a microbial fertilizer. In this study, we lay the theoretical foundation for the comprehensive utilization of *A. bisporus* industrial wastewater and the development of microbial fertilizer.

## Materials and methods

### Strains and culture media

*S. pasteurii* (GIM1.803) was purchased from the Guangdong Culture Collection Center (Guangzhou, China). *A. bisporus* industrial wastewater was collected from Fujian Keren biotechnology Co., Ltd (Longhai, Fujian, China), filtered to remove impurities. After a specific double-effect concentration was achieved, it was then reserved after sterilization.

Fermentation liquid medium was obtained from a specified amount of *A. bisporus* industrial wastewater. Both the concentration and the pH of the solution were then adjusted to their optimal values; the solution was stored and then sterilized (121℃, 15 min) to reserve. Seed medium was made by adding 15.0 g of tryptone, 5.0 g of soy peptone, 5.0 g of NaCl, 20.0 g of urea, and 10 mL of 500 mg/L $MnSO_4$ into 1 L of distilled water. The solution was then heated to dissolve all the compounds. The pH was adjusted to 7.1-7.5 after cooling down. The medium was sterilized at 121℃ for 15 min.

### Strain activation

Freeze-dried bacteria were first activated then dissolved in 0.3 to 0.5 mL of sterile water, seeded

onto the slope, cultured in an incubator at 30℃ for 48 h, and then stored at 4℃.

**Preparation of seed suspension**

A ring of activated *S. pasteurii* was taken and seeded into 100 mL of seed medium, and then, cultured on a shaking table at 30℃, 150 rpm, for 48 h.

**Growth curve of *S. pasteurii***

$1.0\times10^9$ Obj of *S. pasteurii* was inoculated into 100 mL of liquid culture medium, cultured at 30℃, 150 rpm. Every two hours, 0.1 mL of the solution was removed under aseptic conditions and diluted at a ten-fold gradient dilution with PBS. 1 mL of the dilution solution was then mixed with 3 μL of the dyeing reagent, LIVE/DEAD Baclight$^{Tm}$ (Molecular Probes, ThermoFisher, Eugene, OR, USA), which was then placed in the dark for 30 min. The biomass of *S. pasteurii* was then measured using multispectral imaging flow cytometry (FlowSight, Merck Millipore, Darmstadt, Germany). The growth curves were drawn, in which the culture time and the biomass of *S. pasteurii* were horizontal and vertical, respectively [22].

**Plackett-Burman experimental design**

The Plackett-Burman design method could quickly screen out the main factor from multiple factors using the fewest number of experiments [23]. To investigate the effects of different culture conditions on the number of living *S. pasteurii*, we utilized the Plackett-Burman experimental design with the number of experiments being N=12 and 6 factors. The letters A, B, C, D, E, and F were used to represent the concentration of the processing wastewater, initial pH value, culture temperature, rotation speed, inoculum amount, and loading volume, respectively. Each factor included a high (+) and low (-) level in the experimental design, in which the high level was approximately 1.25 times greater than the low level [24], and the total number of living bacteria was counted using flow cytometry [25].

**Steepest ascent design**

According to the results of the Plackett-Burman experiment, the ascent direction of each factor was determined by their effect values and the changing gradient of the experimental values was the changing step. We then further confirmed the optimal range for the three main factors, and then rapidly reached the best area; only in this way we were able to establish the effective response surface equation [26].

**Box-Behnken response surface design**

Based on the results of the Plackett-Burman experiment and the steepest ascent experiment, we confirmed the experimental factors and the level of the Box-Behnken experiment. We conducted three-factor and three-level experiments, in which the total number of living bacteria was the response value, and the three main factors were arguments, confirming the effect of each factor on the total number of living bacteria, and determining the optimal combination of fermentation conditions [23, 27].

**Verification of experimental design**

Three parallel experiments were conducted using the optimal culture conditions, from which we obtained the average in order to verify that the results were consistent with the theoretical value from the response surface method, and whether the model was reliable, so that the final optimal conditions could be determined [27].

**Quantification of total number of living bacteria**

As described by Robertson and Ou *et al*. [28, 29], the quantification of the total number of living *S. pasteurii* in industrial wastewater broth was determined using multispectral imaging flow cytometry.

**Statistical analysis**

All the experiments in this study were carried out in triplicate. The 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 were used to analyse the data. All the data are expressed as X ± S.

## Results and discussion

### Growth curve of *S. pasteurii*

The growth curve of *S. pasteurii* was characterized by a typical "S" type curve, as shown in Figure 1. The lag phase was from 0 h to 4 h, and the logarithmic growth phase was from 4 h to 14 h, in which the bacteria grew at a geometric progression and the number of living bacteria increased rapidly, attaining the maximum growth rate. The total number of *S. pasteurii* was stable, which suggested that this period was the optimal time for fermentation culture. After 28 h, the total number of living bacteria showed a slight decrease due to nutrient depletion, and the accumulation of harmful metabolites, resulting in competition among the *S. pasteurii*.

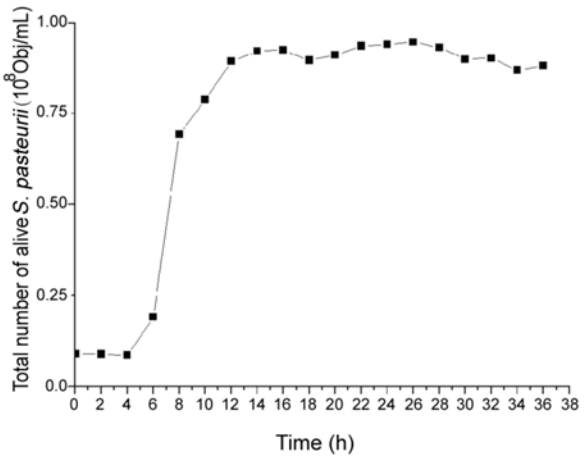


**Figure 1.** Growth curve of *S. pasteurii*.

### Detection of total number of living *S. pasteurii* using multispectral imaging flow cytometry

In Figure 2A, each point represents a bacterium, or an object comprised of two areas, where the green area indicated living bacteria and the blue area showed particulate matter. SYTO9 glowed green light at 488 nm, and PI glowed red at 488 nm. SYTO9 is able to permeate the cell membrane through passive diffusion and binds the bacterial DNA of both live and dead bacteria. Cell membrane permeability is altered after a cell has died and PI only permeates an incomplete cell wall and is used to stain the dead cells. The simultaneous use of both SYTO9 and PI resulted in the visualization of a green light for the bacteria with a complete cell membrane, whereas green and red lights were visualized for bacteria with an incomplete cell membrane. Particles such as dust or unknown solids have no nucleus, so they are not stained by SYTO9 and PI [30, 31]. In summary, living bacteria had a green fluorescence, dead bacteria had both green and red fluorescence, and non-biological objects, such as particles or other impurities, only had BF figures. In Figure 2B, the SYTO9 field has a green fluorescence whereas the PI field is dark, representing living bacteria. In Figure 2C, the SYTO9 field does not have any green fluorescence and the PI field is dark, the BF showed objects, which represents particles. This afforded us the ability to calculate the total number of living bacteria.

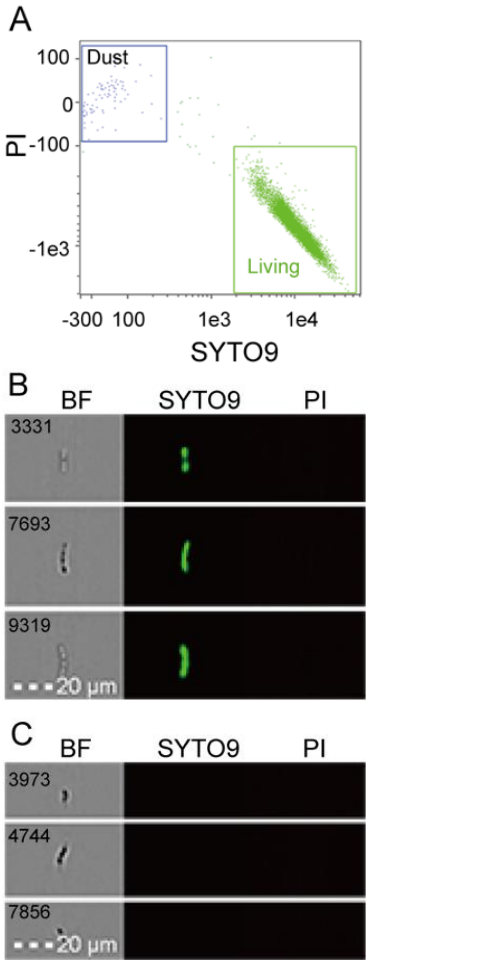


**Figure 2.** Live and dead *S. pasteurii* based on multispectral imaging flow cytometry.

**Table 1.** Plackett-Burman experimental design and results.

| Code / No. | A Solubility (%) | | B pH | | C Inoculum dose (%) | | D Temperature (°C) | | E Shaking speed (rpm) | | F Loaded liquid (mL/250 mL) | | The total number of live *S. pasteurii* ($10^7$ 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 | 0.625 | 1 | 8.7 | 1 | 10 | -1 | 30 | -1 | 150 | -1 | 90 | 0.75±0.02 |
| 2 | -1 | 0.5 | -1 | 7.0 | 1 | 10 | 1 | 37 | 1 | 180 | -1 | 90 | 0.39±0.03 |
| 3 | 1 | 0.625 | 1 | 8.7 | -1 | 8 | 1 | 37 | 1 | 180 | 1 | 110 | 0.25±0.01 |
| 4 | -1 | 0.5 | 1 | 8.7 | 1 | 10 | 1 | 37 | 1 | 180 | -1 | 90 | 0.38±0.04 |
| 5 | 1 | 0.625 | -1 | 7.0 | 1 | 10 | -1 | 30 | 1 | 180 | 1 | 110 | 0.43±0.03 |
| 6 | 1 | 0.625 | -1 | 7.0 | -1 | 8 | -1 | 30 | 1 | 180 | -1 | 90 | 0.52±0.02 |
| 7 | -1 | 0.5 | -1 | 7.0 | -1 | 8 | -1 | 30 | -1 | 150 | -1 | 90 | 0.26±0.03 |
| 8 | -1 | 0.5 | 1 | 8.7 | -1 | 8 | -1 | 30 | 1 | 180 | 1 | 110 | 0.19±0.04 |
| 9 | 1 | 0.625 | -1 | 7.0 | 1 | 10 | 1 | 37 | -1 | 150 | 1 | 110 | 0.21±0.03 |
| 10 | 1 | 0.625 | 1 | 8.7 | -1 | 8 | 1 | 37 | -1 | 150 | -1 | 90 | 0.57±0.04 |
| 11 | -1 | 0.5 | -1 | 7.0 | -1 | 8 | 1 | 37 | -1 | 150 | 1 | 110 | 0±0.00 |
| 12 | -1 | 0.5 | 1 | 8.7 | 1 | 10 | -1 | 30 | -1 | 150 | 1 | 110 | 0.22±0.02 |

**Three significant main factors conformed by Plackett-Burman experiment**

The experimental design and results of Plackett-Burman are shown in Table 1. As shown in Figure 3, the effect of the different factors on the total number of viable *S. pasteurii*, from the largest effect to the weakest, was as follows: D > A > C > F > B > E; culture temperature, industrial wastewater solution, inoculum dose, loading volume, initial pH, rotation speed, respectively. The three factors with the most significant influence on the total number of viable bacteria include culture temperature (D), concentration (A), and inoculation amount (C), therefore they were considered as the key significant factors [32] and were used in the additional steepest ascent and response surface experiments.

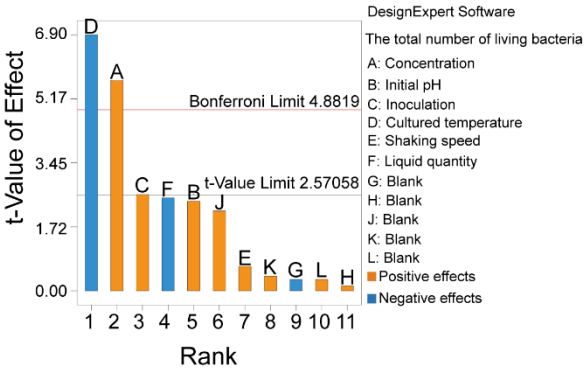


**Figure 3.** Pareto chart of each factor standard effect on the number of living *S. pasteurii*.

**Results of Steepest ascent design**

According to the color of each factor shown in Figure 3, we estimated the positive or negative effect on the biomass of *S. pasteurii*. The concentration and inoculum amount appeared in orange, indicating an overall positive effect, and the value increased gradually; while the culture temperature appeared blue, indicating an overall negative effect, and the value decreased gradually [19, 33]. Since the other factors did not show a significant effect on the total number of living bacteria, their values were chosen according to their individual effect value as loading volume of 90 mL/250 mL, pH of 7.0, and the rotation speed at 150 rpm. The steepest ascent design is shown in Table 2. We observed that the total number of living *S. pasteurii* bacteria increased and then decreased following decreases in the culture temperature and increasing the concentration and inoculum amount. The maximum total number of living bacteria ($4.44±0.05×10^7$ Obj/mL) was achieved when the culture temperature was 30°C with the concentration of 0.5%, and the inoculum amount of 4%. Hence, we conducted a response surface design, choosing each argument in No. 3 of Table 2 as the center point [20].

**Box-Behnken response surface experimental design and results analysis**

**Table 2.** Experimental design of steepest ascent and corresponding results.

| Run | Culture temperature (°C) | Solubility (%) | Inoculum dose (*v:v*, %) | The total number of live *S. pasteurii* ($10^7$Obj/mL) |
|---|---|---|---|---|
| 1 | 38 | 0.125 | 1 | 0.17±0.04 |
| 2 | 34 | 0.25 | 2 | 0.30±0.02 |
| 3 | 30 | 0.5 | 4 | 4.44±0.05 |
| 4 | 26 | 1 | 8 | 3.73±0.04 |
| 5 | 22 | 2 | 10 | 0.45±0.03 |
| 6 | 18 | 4 | 12 | 0.31±0.03 |

**Table 3.** Experimental design and results of Box–Behnken design.

| Run | $X_1$-Temperature (℃) | | $X_2$-Solution (%) | | $X_3$- Inoculum dose (*v:v*,%) | | The total number of live *S. pasteurii* ($10^7$Obj/mL) |
|---|---|---|---|---|---|---|---|
| | Code level | Real level | Code level | Real level | Code level | Real level | |
| 1 | 1 | 34 | 1 | 1 | 0 | 4 | 1.62±0.02 |
| 2 | -1 | 26 | 0 | 0.5 | -1 | 2 | 0.31±0.03 |
| 3 | 0 | 30 | -1 | 0.25 | -1 | 2 | 0.25±0.01 |
| 4 | 0 | 30 | 0 | 0.5 | 0 | 4 | 1.65±0.01 |
| 5 | 0 | 30 | 0 | 0.5 | 0 | 4 | 1.69±0.04 |
| 6 | -1 | 26 | 0 | 0.5 | 1 | 8 | 1.66±0.02 |
| 7 | 1 | 34 | 0 | 0.5 | -1 | 2 | 0.39±0.02 |
| 8 | 1 | 34 | 0 | 0.5 | 1 | 8 | 1.39±0.02 |
| 9 | 1 | 34 | -1 | 0.25 | 0 | 4 | 0.57±0.03 |
| 10 | 0 | 30 | 0 | 0.5 | 0 | 4 | 1.90±0.02 |
| 11 | 0 | 30 | -1 | 0.25 | 1 | 8 | 1.45±0.01 |
| 12 | 0 | 30 | 1 | 1 | 1 | 8 | 1.48±0.5 |
| 13 | -1 | 26 | 1 | 1 | 0 | 4 | 0.56±0.02 |
| 14 | 0 | 30 | 0 | 0.5 | 0 | 4 | 1.77±0.04 |
| 15 | 0 | 30 | 0 | 0.5 | 0 | 4 | 1.73±0.02 |
| 16 | 0 | 30 | 1 | 1 | -1 | 2 | 0.32±0.03 |
| 17 | -1 | 26 | -1 | 0.25 | 0 | 4 | 1.46±0.01 |

We confirmed the optimal response range for the three significant factors according to the steepest ascent results. The three-factor and three-level central response surface design was carried out using the culture temperature (X1), concentration (X2), and inoculum amount (X3) as arguments, and the total number of living bacteria (Y) as the response value. The experimental design and results are shown in Table 3.

We conducted a multiple regression analysis on the response surface results using the Design-Expert V. 12.0.1.0 software. After fitting the regression model, we obtained a regression equation of the effects of the experimental factors to the response value, $Y = 1.97 + 0.076X_1 + 0.031X_2 + 0.99X_3 + 0.47X_1X_2 - 0.084X_1X_3 - 0.012X_2X_3 - 0.32X_1^2 - 0.44X_2^2 - 1.06X_3^2$, in which $X_1$ was the culture temperature, $X_2$ was the concentration, $X_3$ was the inoculum amount, and Y was the total number of living *S. pasteurii*. Positive and negative values of each coefficient represented the impact direction. The absolute value of the coefficient reflected the degree of the effect of each variable to the experimental results.

**Table 4.** Analysis results of regression and variance.

| Source of variance | quadratic sum | df | Mean square | *F* | *Pr>F* | Significance |
|---|---|---|---|---|---|---|
| Model | 6.04 | 9 | 0.67 | 84.37 | < 0.0001 | ** |
| $X_1$ | 0.044 | 1 | 0.044 | 5.54 | 0.0507 | |
| $X_2$ | 0.007813 | 1 | 0.007813 | 0.98 | 0.3548 | |
| $X_3$ | 3.62 | 1 | 3.62 | 454.28 | < 0.0001 | ** |
| $X_1 X_2$ | 0.94 | 1 | 0.94 | 117.97 | < 0.0001 | ** |
| $X_1 X_3$ | 0.022 | 1 | 0.022 | 2.76 | 0.1405 | |
| $X_2 X_3$ | 0.00049 | 1 | 0.00049 | 0.062 | 0.8112 | |
| $X_1^2$ | 0.42 | 1 | 0.42 | 53.03 | 0.0002 | ** |
| $X_2^2$ | 0.6 | 1 | 0.6 | 75.78 | < 0.0001 | ** |
| $X_3^2$ | 1.9 | 1 | 1.9 | 238.10 | < 0.0001 | ** |
| Residual | 0.056 | 7 | 0.00796 | | | |
| Missing items | 0.019 | 3 | 0.00628 | 0.68 | 0.6082 | |
| Pure error | 0.037 | 4 | 0.00922 | | | |
| Total difference | 6.10 | 16 | | | | |
| $R^2$=0.9909, $R^2_{Adj}$=0.9791, $R^2_{pred}$=0.9444, C.V.= 7.51% | | | | | | |

**Note:** * means significance; ** means extreme significance.

The *Pr* of the regression model > *F* value and < 0.0001, shown in Table 4, suggested that the linear relationship between the dependent variable and the total independent variable is very significant when the regression equation was used to describe the relationship between each factor and the response value. This experimental method was reliable. The *Pr* of $X_3$, $X_1X_2$, $X_1^2$, $X_2^2$, and $X_3^2$ were all larger than the F value and all less than 0.05, which suggested that the culture temperature, concentration, and inoculum amount showed significant effects in the model. The *Pr* of the missing items > F value (0.6082) > 0.05, indicating no significant difference and suggesting there were no missing items in this model, no abnormal value in this data, and no need to introduce the higher order terms. The correlation coefficient of the regression model ($R^2$) was 0.9909; adjusting coefficient of determination ($R^2_{Adj}$) was 0.9791. The similarity of the two values suggested that measured value of the total number of living bacteria fit well with the predicted value and could be used to predict the fermentation of *S. pasteurii*. The coefficient of variation (C.V.) = 7.51% < 10% also supported the credibility of the experiment [34].

**Response surface and contour display analysis**

According to the fitting quadratic multiple regression equations, one of the culture temperatures, concentrations, and the inoculum amounts in the model was fixed separately at the 0 level. We then used it to obtain the interaction results of the other factors, such as the response surface and the contour display figures of their interaction effects on the total number of viable bacteria, shown in Figure 4.

We found that the surface of this response surface was steep and red, as shown in Figure 4, which indicated that the culture temperature and the industrial wastewater solution had obvious effects on the biomass of *S. pasteurii*, and additionally, this response surface contained a red vertex that indicating the predicted value that would achieve the highest value. When $X_3$, (inoculation dose) was set at the 0 level and $X_1$ (culture temperature) was fixed, the total number of living bacteria first increased, then decreased following the increase of $X_2$ (industrial

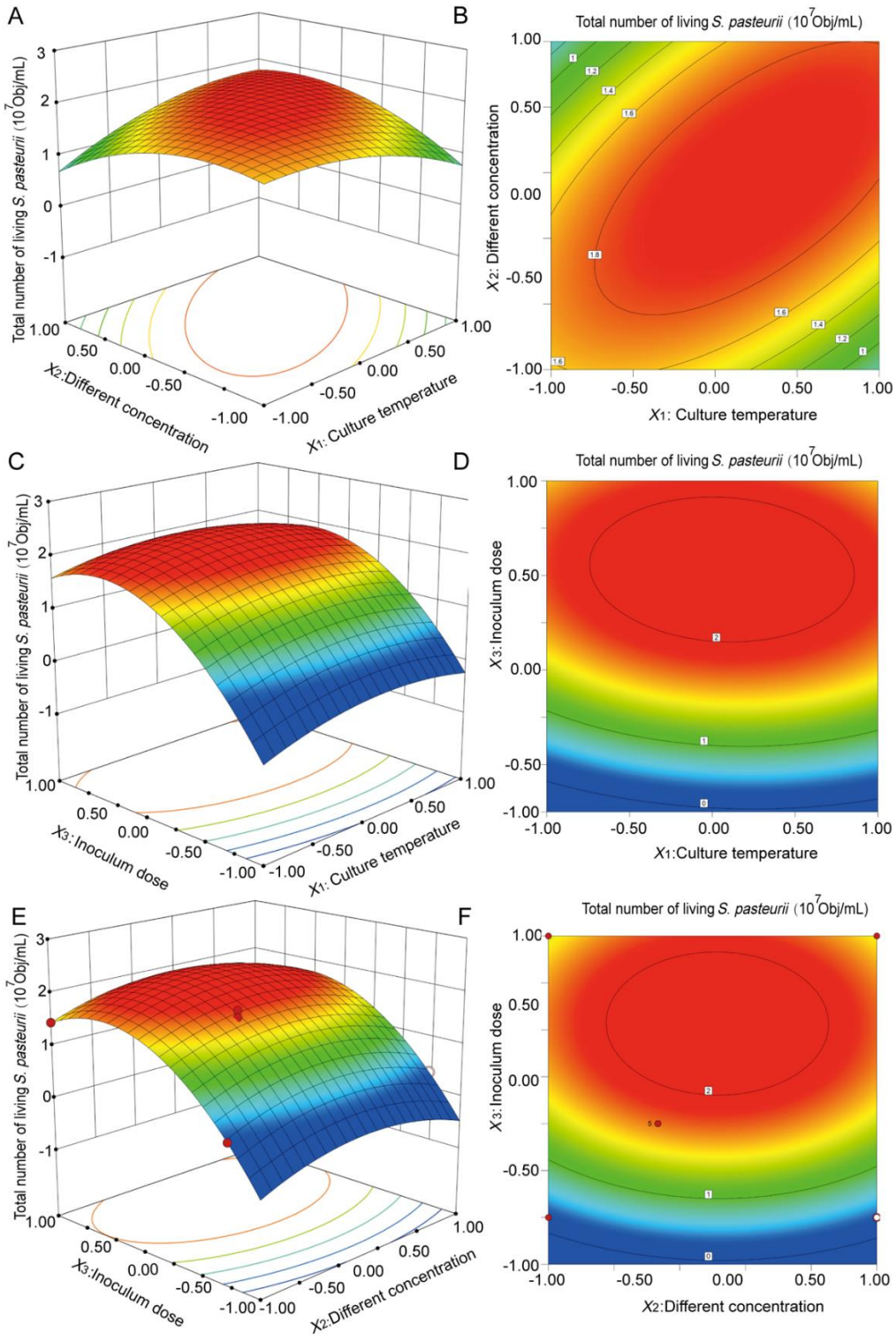


**Figure 4.** The effect of cross interaction among culture temperature, different concentration, and inoculum dose on total number of alive *S. pasteurii*. **A.** Response surface plot (3D) of effects of interaction between culture temperature and different concentration on total number of alive *S. pasteurii*; **B.** Contour line (2D) of effects of interaction between culture temperature and culture temperature on total number of alive *S. pasteurii;* **C.** Response surface plot (3D) of effects of interaction between culture temperature and inoculum dose on total number of alive *S. pasteurii*; **D.** Contour line (2D) of effects of interaction between culture temperature and inoculum dose on total number of alive *S. pasteurii*; **E.** Response surface plot (3D) of effects of interaction between different concentration and inoculum dose on total number of alive *S. pasteurii*; **F.** Contour line (2D) of effects of interaction between different concentration and inoculum dose on total number of alive *S. pasteurii*.

wastewater concentration). When $X_3$ (inoculation) was set at the 0 level and $X_2$ (industrial wastewater concentration) was fixed, the total number of living bacteria first increased and then decreased, following the increasing of $X_1$ (culture temperature). This suggested that a culture temperature that was either too low or too high, along with the industrial wastewater concentration, was not beneficial to the culture of *S. pasteurii*. As shown in Figure 4B, we observed that the contour display was oval, which revealed that the culture temperature and the inoculum amount had a significant interaction effect on the number of living *S. pasteurii*.

The surface of this response surface was steep and the color of the surface gradually changed from blue to red as the horizontal coordinate goes from -1 to 1, shown in Figure 4C. This result indicated that the culture temperature and the inoculated dose had obvious effects on the total number of viable bacteria. When $X_2$ (industrial wastewater concentration) was at the 0 level and $X_1$ (culture temperature) was fixed, the total number of living bacteria first increased and then decreased following increase of $X_3$ (inoculation). When $X_2$ (industrial wastewater concentration) was at the 0 level and $X_3$ (inoculation) was fixed, the total number of viable bacteria first increased and then decreased following increase of $X_1$ (culture temperature). This result suggested that at culture temperatures that were too high or too low culture temperature, the inoculation is no longer beneficial to the culture of *S. pasteurii*. The contour display was observed to be oval, as shown in Figure 4D, which suggested that the culture temperature and the inoculum amount had a significant interaction effect on the biomass of *S. pasteurii*.

The surface of this response surface was observed to be steep, as shown in Figure 4E, and the color of the surface changed gradually from blue to red as the horizontal coordinate goes from -1 to 1. This result indicates that the industrial wastewater concentration and the amount inoculated had obvious effects on the total number of live *S. pasteurii*. As shown in Figure 4F, we observed that the contour display was oval, which suggested that the industrial wastewater concentration and the inoculated amount had a significant interaction effect on the number of viable bacteria. Additionally, we observed a red vertex in the contour line that suggested that range of the predicted value was within the interval, indicating that the optimal designed condition was within the range of the experimental setting value.

**Verification test**

The optimal conditions were determined according to the established regression model and parameter optimal analysis of the response surface and contour display with the culture temperature at 30°C, the concentration of 1%, inoculum amount of 8%, the loading volume at 90 mL/250 mL, the pH of 7.0, and the rotation speed at 150 rpm. The predicted total number of living *S. pasteurii* was $1.54\times10^8$ Obj/mL. We conducted verification experiments in triplicate using the optimal culture conditions and obtained the experimental total number of living bacteria as $1.52\pm0.04\times10^8$ Obj/mL after culturing for 24 h. The experimental result was the 98.7% of the predicted theoretical value and within the 95% confidence interval of model. The experimental results fitted well with the regression model, which indicated that it was feasible to use the response surface method to optimize the fermentation conditions of *S. pasteurii* using *A. bisporus* industrial wastewater under the shaking flask culture conditions.

In this study, we conducted Plackett-Burman experiments, steepest ascent experiments, and Box-Behnken response surface experiments to imitate the optimal conditions of *S. pasteurii* using *A. bisporus* industrial wastewater as a natural culture medium. We identified three main significant factors that influence the growth of *S. pasteurii* according to the Plackett-Burman experiment, which included the culture temperature, concentration, and inoculum amount. Utilizing a combination of the Plackett-Burman design and the Box-Behnken response

surface experiment is a rapid and convenient experimental optimization method and has been broadly applied to microbial fermentation cultures and material extraction [35-38]. For the optimization of microorganism culture conditions, the orthogonal test design method was also chosen, however, it could not obtain the function relationship between the area factor and the response value and visual images. We overcame this issue by combining the Plackett-Burman with Box-Behnken response surface experiments. The Plackett-Burman experimental design method is an important statistical method in the field of statistics and can rapidly identify the significant influential factors from various factors using a fewer number of experiments and provides a basis for further optimizing experiments. The Box-Behnken response surface method can establish the related mathematical models and the regression equations and obtains the corresponding response surface diagram. It can also effectively analyze the interaction effects among significant factors. Finally, we found that the culture temperature, concentration, inoculum amount showed significant effects in the model, in which missing items *Pr* > F value (0.6082) > 0.05, suggesting the model of this experiment had no missing factors and it was feasible.

Multidimensional panoramic flow cytometry could rapidly quantify and differentiate the bacteria by signals from SYTO9 and PI, in which SYTO9 labelled the bacteria with complete and damaged membranes whereas PI only labelled the bacteria with damaged membranes. This allowed us to definitively distinguish between the living and dead bacteria using SYTO9 and PI staining. The traditional plate counting method has cumbersome steps, and when many factors are present in an experimental process, this results in the potential for large experimental errors. Thus, we used flow cytometry to count living bacteria, so that we could not only avoid potential errors of unrelated factors, but it is also less time intensive and more accurately differentiates between the living and dead bacteria. This method is more feasible and convenient than the traditional method. Additionally, compared with real-time fluorescence quantification PCR, the multidimensional panoramic flow cytometry sample is not as easily affected by the reaction conditions, and very visual. Therefore, we selected the multidimensional panoramic flow cytometry to count the total number of living bacteria [39, 40].

We identified the three main significant factors during the growth process of *S. pasteurii* using the Plackett-Burman experiments. These three factors were determined as the culture temperature, concentration, and inoculum amount, and their optimal values were 30℃, 0.5%, and 4%, respectively. According to the principle of Box-Behnken Design, we optimized the three main factors. After the optimization of the response surface, we obtained the optimal conditions for culturing *S. pasteurii* using *A. bisporus* industrial wastewater as the culture temperature at 30℃, concentration of 1%, inoculum amount of 8%, the rotation speed at 150 rpm, the loading volume of 90 mL/250 mL, and the initial pH of 7.0, with a total culture time of 24 h. The total number of living bacteria was $1.52\pm0.04\times10^8$ Obj/mL (N=3) under the optimal conditions, which was closer to the predicted value ($1.54\times10^8$ Obj/mL). This result revealed that it is feasible and effective to use *A. bisporus* industrial wastewater to culture *S. pasteurii*, and the conditions were similar to the study reported by Zhu *et al*. [41].

Currently, *S. pasteurii* has become increasingly more widely used in microbial fertilizers. However, few studies have focused on optimizing their culture conditions. Based on the research method reported by Huang *et al*. [22, 24], in this study, the industrial wastewater of *A. bisporus* was recycled scientifically and effectively, then used as the culture medium for preparing amounts of *S. pasteurii*. These results provide the theoretical basis for the further industrial production of *S. pasteurii* and lay a foundation for the further application of *S. pasteurii* in microbial fertilizers.

## Conclusions

In this paper, we identified the three major factors influencing industrial wastewater including solubility, culture temperature, and the inoculum amount. These factors were identified utilizing the Plackett-Burman design test. The following optimal fermentation conditions of *S. pasteurii* were determined by Box-Behnken design test including concentration of 1%, culture temperature at 30℃, inoculum amount of 8%, agitation speed at 150 rpm, loading volume of 90 mL/250 mL, initial pH of 7.0, and culture time of 24 h. The total number of viable bacteria reached 1.52±0.04 $\times 10^8$ Obj/mL (N=3), which meets the requirements for an agricultural microbial fertilizer. The results of this study highlight the feasibility of using *S. pasteurii* to treat *A. bisporus* industrial wastewater to produce agricultural microbial fertilizer.

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