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Medium Optimization for Spore Production of a Straw-Cellulose Degrading Actinomyces Strain under Solid-State Fermentation Using Response Surface Method

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Abstract: The strains capable of degrading cellulose have attracted much interest because of their applications in straw resource utilization in solid-state fermentation (SSF). However, achieving high spore production in SSF is rarely reported. The production of spores from *Streptomyces griseorubens* JSD-1 was investigated in shaker-flask cultivation in this study. The optimal carbon, organic nitrogen and inorganic nitrogen sources were sucrose, yeast extract and urea, respectively. Plackett–Burman design (PBD) was adopted to determine the key medium components, and the concentration levels of three components (urea, NaCl, $MgSO_4 \cdot 7H_2O$) were optimized with the steepest ascent path and central composite design (CCD), achieving 1.72×10^9 CFU/g of spore production. Under the optimal conditions (urea 2.718% *w/v*, NaCl 0.0697% *w/v*, $MgSO_4 \cdot 7H_2O$ 0.06956% *w/v*), the practical value of spore production was 1.69×10^9 CFU/g. The determination coefficient (R^2) was 0.9498, which ensures an adequate credibility of the model.

Keywords: optimization; Plackett–Burman design; spore production; central composite design

1. Introduction

Straw has been considered a potential resource for organic fertilizer and energy sources although it used to be recognized as an agricultural byproduct [1]. However, resource utilization of straw is infrequent under current processing conditions. Open-field straw burning is still the predominant straw disposal method due to labor shortages and the high manual cost of collection, which has caused a huge waste of resources and air pollution [2]. Efficient utilization of straw resources will ensure environmental sustainability and, coupled with promoting a less hazardous atmosphere, will lead to economic and social development in a sustainable direction [3]. Bassani [4] and Wu [5] reported that auto-hydrolysis or enzymatic pretreatment are methods that can be used to recover cellulose and antioxidant compounds from straw. In addition, straw composting or incorporation into soil could be a promising alternative, where the actions of microbial enzymes transform the lignocellulose component of the straw into compost. Nevertheless, it generally needs more time to decompose and impoverishes the soil easily. In recent years, screening and identification of isolated cellulose-degrading microorganisms have attracted much interest. Until now, the inoculants have been principally bacteria

and fungi, but there is less research specifically on the effect of the degradation of lignocellulose polymers under actinomycete inoculation [6–8].

In our laboratory, a cellulose-degrading actinomycete *Streptomyces griseorubens* JSD-1, capable of effectively degrading rice straw, was successfully isolated from soil and rotten straw [9]. A previous study found that this strain can secrete extracellular cellulase, hemicellulase, ligninase and pectinase at the same time; within 10 days the degradation rate of rice straw reaches 88% [10]. Degraded straw can be used as fertilizer to return to the field. The strain also has a robust inhibitory effect on the activity of pathogenic bacteria such as *Escherichia coli* and *Staphylococcus aureus* [11].

For the straw-cellulose degradation by this strain, a great quantity of spores is demanded. Solid-state fermentation (SSF) has the advantages of low cost, simple process equipment, less pollution, and low energy consumption compared to liquid-state modality [12–15]. SSF also performs much better in the production of thermostable *exo*-polygalacturonase and laccase compared to other methods [16,17]. Additionally, SSF produces live inoculants which have strong activity even under a longstanding storage and are convenient to transport [18,19]. However, media components deeply affect the spore production in fermentation and their interplay performs a crucial role in the productivity of spores [20]. Thus, optimization is required.

Generally, mutual influences between various variables were too intricate to quantify in the solid fermentation process. Traditional methods such as the one-variable-at-a-time method are weak in capturing the interaction of various factors. Response surface methodology (RSM), utilizing a complete quadratic polynomial to demonstrate the relationships, is a regularly used and efficient biotechnology optimization method. A central composite design (CCD) in RSM, a statistical approach that fully considers the interaction and influence between variables, has been utilized, which is widely applied in media conditions [21–23] and enzyme production of media components [24–26].

In the present study, CCD was adopted to optimize the culture medium of SSF for the growth of spore production of *Streptomyces griseus* JSD-1 in this research. The carbon and nitrogen sources suitable for the solid fermentation process of JSD-1 were first screened out. In the next step, the primary variables affecting the performance of the fermentation in terms of spore production as a function of the levels of carbon sources, nitrogen sources and inorganic salts (KNO_3, NaCl, K_2HPO_4, $MgSO_4{\cdot}7H_2O$, $FeSO_4{\cdot}7H_2O$) and the contents themselves were investigated.

2. Materials and Methods

2.1. Strain and Chemicals

Streptomyces griseorubens JSD-1, CGMCC No.5706, which was isolated from the rotten rice straw in the soil and preserved in our laboratory, was used in this study. The isolated strain was suspended in 20% glycerol and stored at −80 °C until further use. All chemicals used in the research were obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

2.2. Medium and Culture Condition

Inoculation medium: the thawed strains were inoculated into Gao's solid medium which contains starch 20 g, KNO_3 1 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, NaCl 0.5 g, K_2HPO_4 0.5 g, $FeSO_4{\cdot}7H_2O$ 0.01 g, agar15~20 g, pH 7.3~7.5 and cultured in a 32 °C incubator for 72 h.

The preparation of spore suspension: a loop of conidia was harvested from the inoculation medium surface by inoculation loop and inoculated into 150 mL liquid of Gao's medium in 500 mL Erlenmeyer flasks and incubated for 72 h at 32 °C with shaking at 180 rpm on the shaker. It was used as the spore seed liquid and adjusted to approximately 10^6 spores mL^{-1}.

Solid-state fermentation: each 250 mL Erlenmeyer flask contained 20 g fermentation substrate of peat soil which were passed through a 20-mesh sieve. Another 2% *w/w* rice husk was added to the fermentation system to improve the aeration of the substrate. The initial moisture content of 60% was reached. After the fermentation substrate cooled to ambient temperature, 10% inoculation volume

of the spore suspension was inoculated in the flasks with a sterilized pipette and then well stirred. In total, 70% of the final moisture content was held. The spore production of *Streptomyces griseorubens* JSD-1 was calculated after 7 days of cultivation at 32 °C which was flipped every 24 h.

The supplement of carbon sources and nitrogen sources: six sorts of supplementary carbon sources (starch, maltose, glucose, galactose, millet flour and sucrose) at a concentration of 2% *w/v*, five sorts of supplementary organic nitrogen sources (yeast extract, peanut meal, arginine, peptone and soybean powder) and five sorts of supplementary inorganic nitrogen sources (NH_4HCO_3, NH_4Cl urea, $(NH_4)_2SO_4$ and KNO_3) at a concentration of 1% *w/v* were added into the solid substrate.

2.3. Analytical Methods

A 5 g sample was measured from dried solid substrate and then mixed with 95 mL sterile water in a shaker for 30 min at 250 rpm and 35 °C, to separate the spores from the fermentation substrate completely. The obtained spore liquid was diluted in a gradient and then coated in 100 µL of the diluent on the solid plates. Spore production was expressed as spores per g colony-forming unit. All the experiments were performed in triplicate.

2.4. Plackett–Burman Design (PBD)

Plackett–Burman design is an effective mean which can recognize significant factors affecting the corresponding variables from numerous factors in the fermentation [27,28]. The effects of the medium component of carbon source, organic nitrogen source and inorganic nitrogen source, NaCl, K_2HPO_4, $MgSO_4{\cdot}7H_2O$ and $FeSO_4{\cdot}7H_2O$ on spore production were investigated by PBD. Each independent variable was devised at a low (−1) and high (+1) level (Table 1). The PBD was fitted based on the following first-order polynomial equation:

$$y = \alpha_0 + \Sigma \alpha_i x_i \quad (1)$$

where y is the dependent variable, α_0 is the intercept of the model, α_i is the coefficient and x_i is the independent variable.

Table 1. Plackett–Burman design (PBD).

Run	Variables											Spore Production ($\times 10^8$ CFU/g)
	A	B	C	D	E	F	G	H [a]	I [a]	J [a]	K [a]	
1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	12.77 ± 0.62
2	1	−1	−1	−1	1	−1	1	1	−1	1	1	8.79 ± 1.48
3	−1	1	1	−1	1	1	1	−1	−1	−1	1	13.57 ± 0.86
4	1	1	−1	−1	−1	1	−1	1	1	−1	1	1.73 ± 0.11
5	−1	−1	−1	1	−1	1	1	−1	1	1	1	5.43 ± 0.66
6	1	1	−1	1	1	1	−1	−1	−1	1	−1	1.48 ± 0.26
7	−1	1	1	1	−1	−1	−1	1	−1	1	1	9.33 ± 0.54
8	−1	1	−1	1	1	−1	1	1	1	−1	−1	7.33 ± 0.24
9	−1	−1	1	−1	1	1	−1	1	1	1	−1	13.15 ± 1.24
10	1	−1	1	1	1	−1	−1	−1	1	−1	1	12.79 ± 0.78
11	1	1	1	−1	−1	−1	1	−1	1	1	−1	16.47 ± 0.60
12	1	−1	1	1	−1	1	1	1	−1	−1	−1	10.57 ± 0.42

[a] Represents a dummy variable.

2.5. The Path of Steepest Ascent

Results acquired from Equation (1) indicated the direction and step length of the path of steepest ascent design. High value (+1) was selected if the effect value of the variable was positive, and low value (−1) was selected if the effect value of the variable was negative [29] in this experiment. Increment

was a direct ratio to coefficients α_i of Equation (1) while simultaneously the experimental status should also be considered. The experiments were carried out along the steepest ascent path until the response no longer increased; this point was considered to be close to the optimal point and could then be identified as the center point to optimize [30] and proceed to subsequent optimization (Table 2).

Table 2. The designs of the path of steepest ascent.

Run	C. Urea (*w*/*v*)	D. NaCl (*w*/*v*)	F. $MgSO_4 \cdot 7H_2O$ (*w*/*v*)	Spore Production ($\times 10^8$ CFU/g)
1	2.2%	0.09%	0.09%	11.67 ± 0.59
2	2.4%	0.08%	0.08%	15.70 ± 0.91
3	2.6%	0.07%	0.07%	16.95 ± 0.90
4	2.8%	0.06%	0.06%	15.17 ± 0.57
5	3.0%	0.05%	0.05%	10.57 ± 0.52
6	3.2%	0.04%	0.04%	8.51 ± 0.35

2.6. Central Composite Design

Central composite design in RSM is a commonly used optimization method. To determine the optimal sporulation medium for *Streptomyces griseorubens* JSD-1 in the SSF process, a rotating CCD was chosen for modeling and optimizing, which could establish a model capable of predicting a constant variance at the design center equidistant point to improve prediction accuracy. The response value of y was analyzed by multiple regression to fit the following quadratic polynomial model:

$$y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ii} x_i^2 + \Sigma\beta_{ij} x_i x_j \quad (2)$$

where y is the predicted value of spore production, x_i and x_j are the coded independent variables affecting y, β_0 is an intercept, and β_i, β_{ii} and β_{ij} are the coefficients of the i,j-th linear, quadratic and interactive terms, respectively.

Three-factor CCD with five coding levels (Table 3) was adopted to optimize seven variables. The terminal level of axial points was chosen to make the design rotatable. The axial point can be expressed by the equation

$$\alpha = 2^{k/4} \quad (3)$$

where α and k are the axial point and the number of significant variables, respectively.

Table 3. Independent variables and experimental levels of central composite design (CCD).

Significant Variables		Levels (*w*/*v*)				
Code	Terms	−1.68 (−α)	−1	0	+1	1.68 (+α)
C	Urea	2.264%	2.400%	2.600%	2.800%	2.936%
D	NaCl	0.053%	0.060%	0.070%	0.080%	0.087%
F	$MgSO_4 \cdot 7H_2O$	0.053%	0.060%	0.070%	0.080%	0.087%

2.7. Statistical Analysis

All experiments were performed in at least triplicate biological repeats, unless otherwise stated, with data presented as means ± SD. Design expert version 11.0.4 (Stat-Ease Inc., Minneapolis, MN, USA) was adopted in the PBD and CCD experiments. p-values less than 0.05 implied factors which are significant at the probability level of 95%.

3. Results and Discussion

3.1. Effect of Different Carbon and Nitrogen Sources

The effects of different carbon and nitrogen sources on spore production under peat soil substrate were determined. As shown in Figure 1, the maximum spore production (8.06×10^8 CFU/g, 4.48×10^8 CFU/g, 2.00×10^8 CFU/g) was observed when sucrose, arginine and urea served as the carbon sources, organic nitrogen sources and inorganic nitrogen sources, respectively.

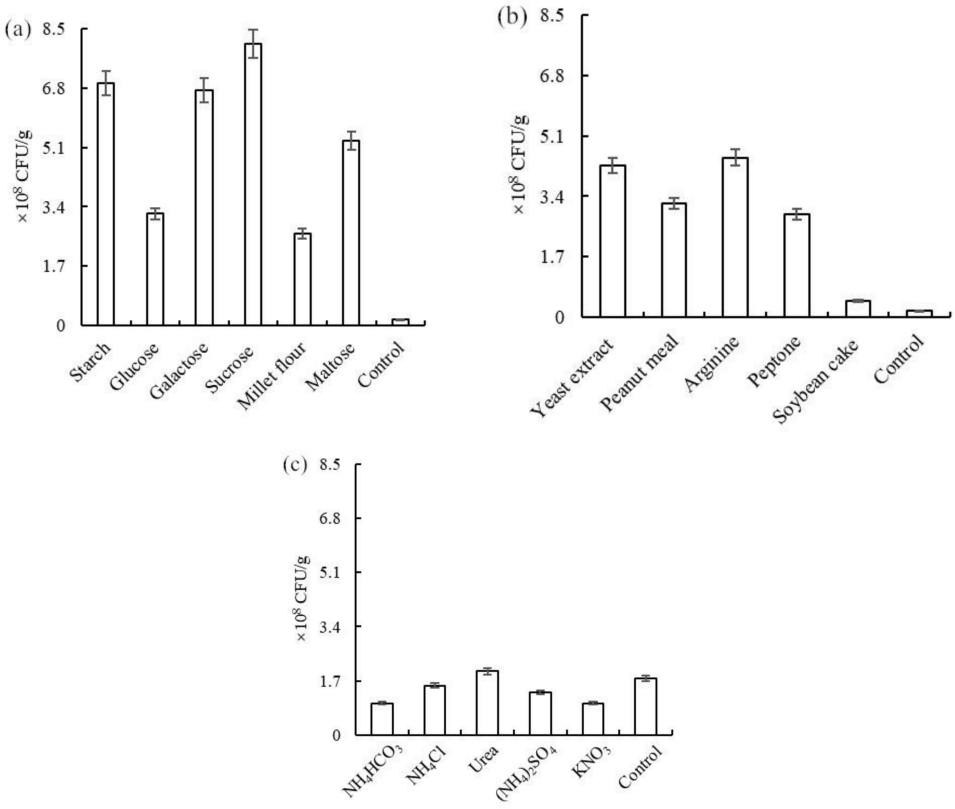

Figure 1. Effects of various carbon sources and nitrogen sources on spore productions (**a**–**c**). The spore production of different kinds of carbon sources (**a**), organic nitrogen sources (**b**) and inorganic nitrogen sources (**c**) serve as the supplementary nutrient sources, respectively.

As shown in Figure 1, sucrose performs better than other carbon sources. The reason may be that in the intricate fermentation environment, sucrose which was not metabolized by the *streptomycete* itself performed a pivotal role beside counterpoising osmotic pressure between cytoplasm and the surrounding environment [31], which was important for nutrient acquisition in SSF. Figure 1a also shows that the sucrose induced sporulation, whereas the glucose did not, which agrees with the results of Ajdari et al. [32].

Among the five organic and inorganic nitrogen sources, the arginine and urea show a good fit with the fermentation system (Figure 1b–c). However, considering the high cost of arginine and the small difference with significance ($p = 0.8092$) compared to yeast extract (Figure 1b), the latter was chosen as the better organic nitrogen source.

3.2. Significance Factors for Spore Production

There are many independent variables that have different effects on spore production. It is necessary to identify the influence of the medium component on spore production in the actual situation. PBD is a valid screening method which can determine significant factors affecting the

corresponding variables from a large number of factors [27,28]. As shown in Figure 2a, urea, K_2HPO_4, $FeSO_4{\cdot}7H_2O$ had positive effect while the sucrose, yeast extract, NaCl, $MgSO_4{\cdot}7H_2O$ had a negative effect on spore production according to the parameters, which indicates that the three positive effectors should be increased during the SSF. A suited first-order linear model for spore production was obtained as follows:

$$y = 92.86 - 5.09x_1 - 9.70x_2 + 33.59x_3 - 14.67x_4 + 3.70x_5 - 16.32x_6 + 12.12x_7 \quad (4)$$

where y is the predicted value of spore production ($\times 10^8$ CFU/g), and x_1, x_2, x_3, x_4, x_5, x_6 and x_7 are sucrose (A), yeast extract (B), urea (C), NaCl (D), K_2HPO_4 (E), $MgSO_4{\cdot}7H_2O$ (F) and $FeSO_4{\cdot}7H_2O$ (G), respectively.

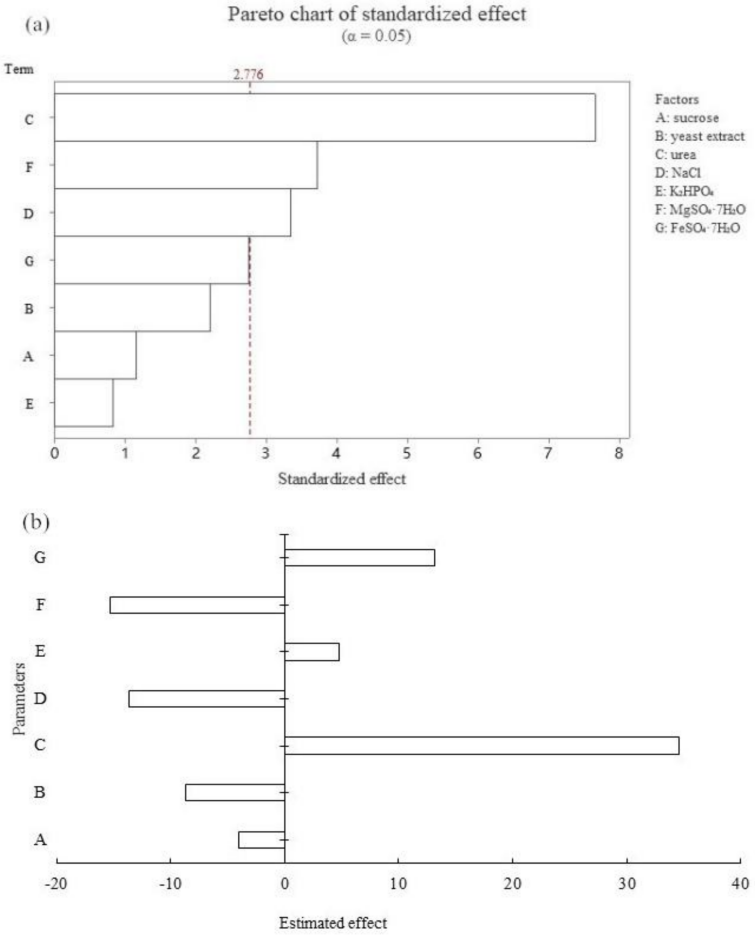

Figure 2. Visualization of the effects of seven variables by PBD. (**a**) Pareto chart by analyzing spore production; (**b**) estimated effects of experimental parameters on spore production.

Furthermore, the *p*-value of urea, NaCl and $MgSO_4{\cdot}7H_2O$ were 0.0016, 0.0205 and 0.0287, respectively, which indicated that the urea has the greatest impact on spore production (Table 4), whereas sucrose, yeast extract, K_2HPO_4 and $FeSO_4{\cdot}7H_2O$ did not significantly influence spore production under the tested levels.

Table 4. The actual value of each variable's level.

Variables	Levels (*w/v*)		*F*-value	*p*-value	Rank
Code Terms	Low (−1)	High (+1)			
A. Sucrose	2%	4%	1.35	0.3100	6
B. Yeast extract	1%	2%	4.89	0.0915	5
C. Urea	1%	2%	58.64	0.0016	1 **
D. NaCl	0.1%	0.2%	11.18	0.0287	3 *
E. K_2HPO_4	0.1%	0.2%	0.7101	0.4468	7
F. $MgSO_4{\cdot}7H_2O$	0.1%	0.2%	13.85	0.0205	2 *
G. $FeSO_4{\cdot}7H_2O$	0.01%	0.02%	7.64	0.0506	4

** Represents highly significant; * represents significant.

The gradually decreasing pH in the fermentation process of JSD-1 in SSF (data not shown) may account for the positive effects of urea. To maintain a certain neutral pH, urea containing a large number of amino groups was required to provide alkaline ions which is consistent with the findings of Feng et al. [10]. Moreover, the NaCl and $MgSO_4{\cdot}7H_2O$ were shown to be necessary for spore formation in SSF. Therefore, urea, NaCl and $MgSO_4{\cdot}7H_2O$ were chosen to optimize in the next step.

3.3. The Steepest Ascent Path Analysis

In the light of the results of PBD, the coefficient x_3 was positive, while x_4 and x_6 were negative in Equation (4), indicating that an increase in the levels of urea and a decrease in the levels of NaCl and $MgSO_4{\cdot}7H_2O$ could have a positive effect on the spore production of JSD-1. Consequently, the proper direction for altering the levels of the tested variables was ascertained by the steepest ascent path. The other positive effect factors were maintained at a high level (+1), while the negative effect factors were maintained at low level (−1).

The experimental design and corresponding results are presented in Table 2, which shows that the highest spore production was achieved at 16.95×10^8 CFU·g^{-1} at run 3. Accordingly, this point was deliberated close to the maximum spore production region and this combination (Urea 2.6% *w/v*, NaCl 0.07% *w/v*, $MgSO_4{\cdot}7H_2O$ 0.07% *w/v*) was used as the central point of the CCD.

3.4. Optimization of the Medium

The CCD in RSM was adopted to optimize the three significant factors to investigate the optimal level of medium contents and their interaction. The concentrations of those significant factors are presented in Table 3. The experimental results of the CCD were fitted with the following quadratic polynomial equation:

$$\begin{gathered} y = 17.04 + 1.22x_3 + 0.35x_4 \\ +0.35x_6 - 1.55x_3x_4 - 1.83x_3x_6 + 2.7x_4x_6 - 2.18x_3^2 - 5.12x_4^2 - 5.42x_6^2 \end{gathered} \quad (5)$$

where y is the response value, that is, the spore production, and x_3, x_4 and x_6 are coded parameters of urea, NaCl and $MgSO_4{\cdot}7H_2O$, respectively.

The determination coefficient (R^2) and adjusted coefficient of determination ($R_{adj}{}^2$) were employed to assess the goodness of fit of the regression equation. In this case, the determination coefficient (R^2) was 0.9498 and indicated that 94.98% of the variability in the response value could be illustrated by the quadratic model. The adjusted determination coefficient ($R_{adj}{}^2$ = 0.9046) was also high enough to indicate the significance of the model. The relationship between predicted response and experimental results shows that almost all the predicted values were in close agreement with the experimental results (Figure 3).

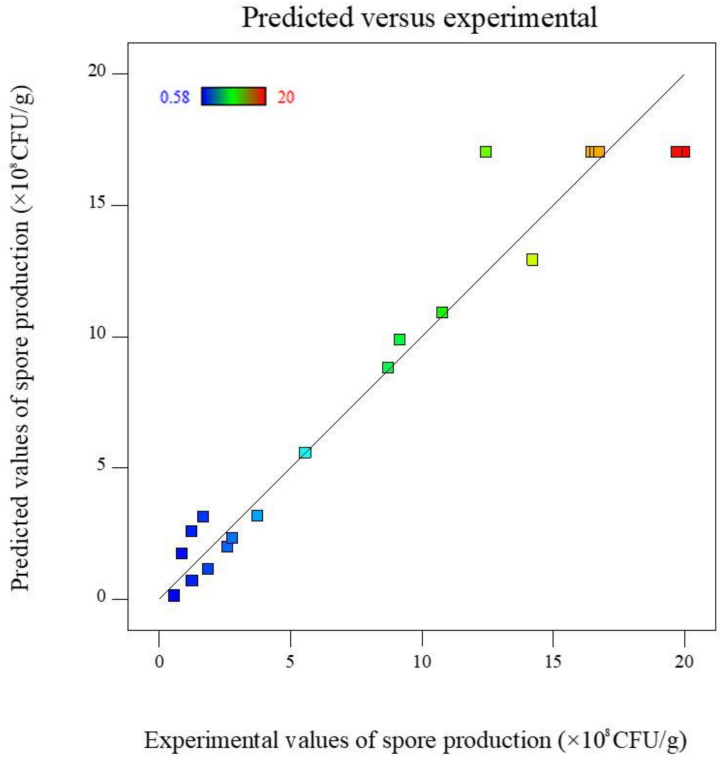

Figure 3. The relevance between experimental and predicted values fitted by the response surface methodology (RSM) model.

The corresponding analysis of variance (ANOVA) for the RSM model of spore production is presented in Table 5 according to the experimental data of CCD. The *F* value is a measure of the variation in the mean value. Generally, the accurate prediction of the experimental results was evaluated by the high calculated *F* value [33]. As shown in Table 5, the high *F* value of 21.01 at a *p*-value of <0.0001 was indicative of the high correlation of the model to the experimental results. The *F* value (21.01) of the model was also much greater (85.67%) than the tabulated *F* value ($F_{9,10}$ = 3.02) at 0.05 level, which demonstrates that the quadratic polynomial of Equation (5) is highly significant.

Table 5. Analysis of variance (ANOVA) for the response quadratic model developed by CCD.

Sources	Sum of Squares	Degree of Freedom	Mean Square	*F*-value	*p*-value
Model	878.41	9	97.57	21.01	<0.0001 *
x_3	20.30	1	20.30	4.38	0.0429 *
x_4	1.67	1	1.67	0.36	0.5637
x_6	1.71	1	1.71	0.37	0.5581
x_3x_4	19.16	1	19.16	4.13	0.0697
x_3x_6	26.72	1	26.72	5.74	0.0375 *
x_4x_6	58.34	1	58.34	12.55	0.0053 *
${x_3}^2$	68.54	1	68.54	14.74	0.0033 *
${x_4}^2$	377.41	1	377.41	81.30	<0.0001 *
${x_6}^2$	422.71	1	422.71	91.03	<0.0001 *
Residual	46.52	10	4.65		
Lack of fit	8.91	5	1.78	0.2368	0.9303
Pure error	37.61	5	7.52		
Cor total	924.66	19			

* Represents significant.

A p-value of less than 0.05 indicates factors that are statistically significant. A lower p-value confirms that the corresponding variable was more significant. In this case, the independent variables (x_3), the interactive terms (x_3x_6, x_4x_6), and all quadratic terms (x_3^2, x_4^2, x_6^2) affected spore production in a significant ($p < 0.05$) manner, while the effects of x_4, x_6 and x_3x_4 were non-significant ($p > 0.05$). The independent variable of x_3 was shown to be significant, which agreed with PBD. Furthermore, the lack-of-fit ($p = 0.9303$) was sufficiently insignificant and indicated that it was not significant relative to the pure error, affirming that the model was sufficient for predicting spore production under any combination of the components.

The interaction of the medium in SSF and the optimum levels of the supplements added into the solid-state medium, which have significant effects on the spore production of JSD-1, were determined by the visualization of the response surface and contour plots. A circular contour plot implies that interactions are insignificant between the corresponding variables, while an elliptical contour plot suggests that the interactions between the selected variables are significant. From the contours of Figure 4, we found that the interactions between urea and NaCl, urea and $MgSO_4{\cdot}7H_2O$, and NaCl and $MgSO_4{\cdot}7H_2O$ were elliptical, implying that the effects of the interactions between each of the two variables are significant.

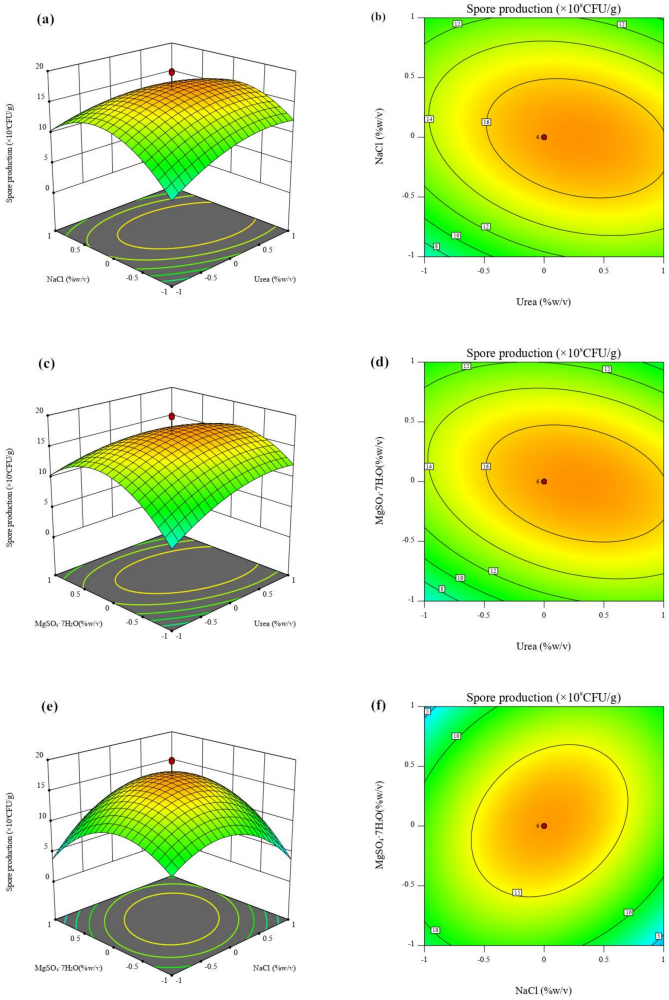

Figure 4. The individual and interactive effects of variables on the spore production of *Streptomyces griseorubens* JSD-1 employing 3D and 2D plots. (**a**,**b**) Effects of NaCl and urea on spore production; (**c**,**d**) effects of $MgSO_4{\cdot}7H_2O$ and urea on spore production; (**e**,**f**) effects of $MgSO_4{\cdot}7H_2O$ and NaCl on spore production.

The three-dimensional RSM plots demonstrated that the maximum spore production should occur with medium levels of urea, NaCl and $MgSO_4 \cdot 7H_2O$. On the basis of the numerical amount optimization, the optimum medium composition for the highest spore production was obtained as follows: $x_1 = 0.294$ (2.718% *w/v*), $x_2 = -0.015$ (0.0697% *w/v*), $x_3 = -0.021$ (0.06956% *w/v*) with the corresponding $y = 1.72 \times 10^9$ CFU/g.

The optimal conditions in SSF experiments which were performed under three replicates were adopted to verify the predicted values. The practical value of response was 1.69×10^9 CFU/g, which confirmed the validity and the utility of the model.

4. Conclusions

The large amounts of waste straw impose an obligation on modern society to follow a sustainable development approach. One of the key elements is the development of biotechnology processes for enabling reuse of waste straw. In the present study, the CCD was adopted to optimize the solid-state medium components for spore production of *Streptomyces griseorubens* JSD-1. The significant factors for spore production, that is, urea, NaCl and $MgSO_4 \cdot 7H_2O$, were identified by the PBD. The path of steepest ascent method and CCD were employed to attempt to approach the optimal area. The optimal supplementary nutrient consisted of urea 2.72% *w/v*, NaCl 0.0697% *w/v* and $MgSO_4 \cdot 7H_2O$ 0.06956% *w/v*. The spore production of 1.72×10^9 CFU/g could be produced in theory and 1.69×10^9 CFU/g in practice under optimal conditions. The results demonstrated that the quadratic polynomial obtained by the CCD performs well in predicting and optimizing the spore production of *Streptomyces griseorubens* JSD-1, which indicates that this model can be used as a reference in subsequent practical productions. Future work should involve increasing production scale by using fed-batch bioreactor processes and the stability of the solid inoculants under large-scale fermentation.

Author Contributions: Conceptualization: H.L., Y.Z. and D.Z. Performed experiments: H.L., X.Z. and C.Z. Collected, analyzed data: H.L. and X.Z. Wrote and edited the paper: H.L., Y.Z., D.Z. and P.Z. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare that they have no conflicts of interest in the study.

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