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OPTIMIZATION OF FERMENTATION MEDIUM FOR SPORE PRODUCTION OF *Paenibacillus polymyxa* IN937a AND ITS ANTIFUNGAL ACTIVITY

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SUMMARY

In this study, we optimized the fermentation medium for spore production of *Paenibacillus polymyxa* IN937a. Seven factors including molasses, glucose, magnesium sulfate, potassium pyrophosphate, yeast extract, zinc sulfate, and ammonium sulfate are selected as the basis for the screening of factors affecting the spore production of *P. polymyxa* IN937a by the Plackett-Burman experiment. Based on the analysis of the Plackett-Burman matrix, the result showed that yeast extract, molasses, and ammonium sulfate were the three main impact factors ($P < 0.05$), which affected the yield of *P. polymyxa* IN937a spores. Then, the optimum combination of the three factors was subsequently optimized by the response surface methodology (RSM) using Box-Behnken design to increase the spore production in *P. polymyxa* IN937a fermentation. The obtained results by RSM predicted that maximum spore density of *P. polymyxa* IN937a was 6.606×10^9 spore/mL after 48 hours of the experiment when the appropriate medium for the spore production of *P. polymyxa* IN937a included yeast extract 14.44 g/L, molasses 19.14 g/L, and ammonium sulfate 0.20 g/L. In addition, the antifungal activity of *P. polymyxa* IN937a was also tested in this study. The preliminary results of *in vitro* antifungal activity indicated that *P. polymyxa* IN937a had a good inhibition on the growth of two phytopathogenic fungal strains *Sclerotium rolfsii* and *Rhizoctonia solani*. These results could be used for further research on the fermentation of *P. polymyxa* IN937a on a pilot scale to obtain the optimal number of spores for use in the development of biological crop protection products.

Keywords: Box Behnken, fermentation, Paenibacillus polymyxa IN937a, Plackett-Burman, response surface methodology, spore production

INTRODUCTION

Plant growth-promoting rhizobacteria (PGPR) are bacteria that freely distributed in the soil, living around or on the root surface, symbiosis inside the roots, directly or indirectly involved in the development of plants through the production and secretion of various chemicals

around the root zone. Research related to the use of these strains of microorganisms for the production and application in the development of eco-friendly and biological plant protection products are an important issue that has been a concern in many countries for years (Sivasakthi *et al.,* 2014; Riaz *et al.,* 2021). Outstanding PGPR strains include species of the following genus: *Arthrobacter, Azoarcus, Azospirillum, Bacillus, Burkholderia, Enterobacter, Gluconacetobacter, Herbaspirillum, Klebsiella, Paenibacillus, Pseudomonas, Serratia*...

One of the most studied PGPR is *Paenibacillus polymyxa*, also known as *Bacillus polymyxa* (Ash *et al.,* 1991). This is a gram-positive, rod-shaped bacterium with nitrogen fixation, found in soil, tree roots, and marine sediments (Timmusk *et al.,* 1999). *P. polymyxa* has many important properties that have been reported such as nitrogen fixation (Heulin *et al.,* 2006), the potential for antibiotic production (Rosado, Seldin, 1993), soil phosphorus solubilization (Singh, Singh, 1993) and the ability to enhance soil porosity (Gouzou *et al.,* 1993). Based on the above properties, *P. polymyxa* can antagonize harmful phytopathogenic microorganisms by penetrating and creating biological membranes around plant roots. In addition, it also helps to produce nitrogen-fixing hormone that facilitates the development of other beneficial microorganisms in the soil, increases soil porosity and humus and participates directly and indirectly in plant growth. Therefore, in the current sustainable agriculture, *P. polymyxa* has an important role in the ecosystem and is a potential bacterial strain in industrial processes that plant protection products.

In this study, we reported on the optimization of fermentation medium in detail for spore production of *P. polyxyma* IN937a through applying Response Surface Methodology (RSM) with Box-Behnken Design (BBD). In addition, we also carried out a preliminary test of antifungal activity of *P. polymyxa* IN937a against *Sclerotium rolfsii* and *Rhizoctonia solani*.

MATERIALS AND METHODS

Microorganism

The strain of *P. polymyxa* IN937a was provided by Dr. Chang Ho Chung at Jeonju Biomaterial Institute, Korea. The fungal used in the present study were *S. rolfsii* and *R. solani*, which were provided by R&D Center of Bioactive Compounds, Vietnam Institute of Industrial Chemistry (VIIC).

Growth medium and inoculum preparation

The strain was maintained on TSB medium (tryptone 17 g/L, papaic digest soybean meal 3 g/L, glucose 2.5 g/L, K_2HPO_4 2.5 g/L, NaCl 5 g/L, pH 7). A 150 mL Erlenmeyer flasks containing 30 mL of medium were incubated at 37^{o}C in a rotatory shaker at 200 rpm for 24 h to prepare the inoculum with a final optical density (OD) of approximately 0.5. Then, a 10% inoculum was added to a 150 mL flask containing 30 mL of defined medium with varying concentrations of compositions. Finally, the fermentation was incubated at 35 - 37^{o}C in rotary flasks at 200 rpm for 48 h.

Selection of significant variables by Plackett–Burman design

Table 1. The variables at different levels used for the spore production of *P. polymyxa* IN937a using Plackett–Burman design.

Variables	Unit	Symbol code	Level if variables	
			Low (-1)	High (+1)
Molasses	g/L	X_1	10	20
Glucose	g/L	X_2	10	20
$MgSO_4.7H_2O$	g/L	X_3	0.3	0.5
K_2HPO_4	g/L	X_4	4	6
Yeast extract	g/L	X_5	5	15
$ZnSO_4.7H_2O$	g/L	X_6	0.01	0.05
$(NH_4)_2SO_4$	g/L	X_7	0.1	0.25

The selection of significant variables for spore production of *P. polymyxa* IN937a was carried out by Plackett-Burman matrix design with 07 variables in 12 runs to assess the impact of these variables on the fermentation. Each independent variable was investigated at two

code levels: -1 and +1 (Plackett, Burman, 1946). The variables were as follows: molasses, glucose, $MgSO_4.7H_2O$, K_2HPO_4, yeast extract, $ZnSO_4.7H_2O$, and $(NH_4)_2SO_4$ (Table 1). Plackett-Burman matrix design was shown in Table 2. The data were processed and analyzed by software "Design Expert® 10.0.8" (Stat- Ease Inc., Minneapolis, USA).

Table 2. Twelve-trial Plackett-Burman design matrix for seven variables with actual values along with the observed and predicted spore production of *P. polymyxa* IN937a.

Run order	Code level							Spore production (10^9 spore/mL)	
	X_1*	X_2	X_3	X_4	X_5	X_6	X_7	Observed	Predicted
1	-1	+1	+1	-1	+1	+1	+1	3.9	4.35
2	-1	-1	-1	-1	-1	-1	-1	0.7	1.20
3	-1	+1	+1	+1	-1	-1	-1	0.9	0.22
4	-1	-1	-1	+1	-1	+1	+1	3.3	3.12
5	+1	-1	+1	+1	+1	-1	-1	4.5	5.13
6	-1	-1	+1	-1	+1	+1	-1	3.7	3.25
7	+1	+1	-1	-1	-1	+1	-1	3.8	4.12
8	+1	-1	+1	+1	-1	+1	+1	5.1	5.28
9	-1	+1	-1	+1	+1	-1	+1	3.7	4.07
10	+1	+1	+1	-1	-1	-1	+1	4.6	4.47
11	+1	-1	-1	-1	+1	-1	+1	7.9	7.22
12	+1	+1	-1	+1	+1	+1	-1	6.2	5.88

*X_1: molasses; X_2: glucose; X_3: $MgSO_4.7H_2O$; X_4: K_2HPO_4; X_5: yeast extract; X_6: $ZnSO_4.7H_2O$; X_7: ammonium sulfate

Optimization by response surface methodology with the Box–Behnken design

Based on a single-factor experiment for spore production of *P. polymyxa* IN937a, proper ranges of yeast extract (5, 10, and 15 g/L), molasses (10, 15, and 20 g/L), and ammonium sulfate (0.1, 0.175, and 0.25 h) were preliminarily determined. The process parameters were used to optimize the medium fermentation for spore production of *P. polymyxa* IN937a based on RSM following BBD. The significant and independent variables utilized were as follows: yeast extract, molasses, and ammonium sulfate, each of which was assessed at three levels as -1, 0, +1, as is shown in Table 3. The BBD with 3 variables, three levels, and 15 runs and the experimental points used according to this design are shown in Table 4. The second-order regression equation obtained from this design was applied to represent the spore production of *P. polymyxa* IN937a during the fermentation as a function of the independent variables as follows:

$$Y = B_0 + B_1 \times X_1 + B_2 \times X_2 + B_3 \times X_3 + B_{12} \times X_1 \times X_2 + B_{13} \times X_1 \times X_3 + B_{23} \times X_2 \times X_3 + B_{11} \times X_1^2 + B_{22} \times X_2^2 + B_{33} \times X_3^2 \quad (1)$$

Where Y is the predicted response; B_0 is constant; B_i are linear coefficients; B_{ij} are cross-product coefficients; and B_{ii} are quadratic coefficients. The input and independent variables are yeast extract (X_1), molasses (X_2), and ammonium sulfate (X_3). The coefficients of the equation were calculated by using Design Expert software version 10.0.8 (Stat-Ease, Inc, USA). The significance of each coefficient was verified by the P-value at a 95% confidence level. The P-values of less than 0.05 were statistically significant. The 2D contour plots and 3D surface response curves were created by varying two variables while the experiment range and holding the other variable.

In vitro evaluation of antifungal activity of *P. polymyxa* IN937a against two phytopathogenic fungi

The antifungal activity of *P. polymyxa* IN937a was tested on Petri plates with PDA medium (potato 250 g/L, glucose 15 g/L, agar 15 g/L, pH 6.8 - 7.0). The medium was sterilized at 121ºC under 1 atm. The inoculating loop was dipped in the fermentation solution of *P. polymyxa* IN937a obtained at the optimum condition, then bacterial strain was spread on the surface of PDA medium in straight lines or squares to observe the ability to inhibit *S. rolfsii* and *R. solani*. The treated Petri dishes were inoculated with a mycelial plug in the center and allowed to grow at 25 – 28 °C for 1 - 2 days. Qualitative antifungal activity of bacteria based on the mycelial growth of the test sample compared with the negative control (PDA medium containing no bacteria *P. polymyxa* IN937a). The mycelial growth of the two fungal strains after 1 and 2 days were observed and photographed.

Table 3. Experimental codes, ranges, and levels of the three independent variables for response surface methodological experiment.

Variables	Unit	Symbol code	Level		
			-1	0	+1
Yeast extract	g/L	X_1	5	10	15
Molasses	g/L	X_2	10	15	20
Ammonium sulfate	g/L	X_3	0.1	0.175	0.25

Table 4. Central composite design matrix for the experimental design and predicted responses for spore production of *P. polymyxa* IN937a.

Run order	Code levels			Spore production (10^9 spore/mL)	
	X_1	X_2	X_3	Observed	Predicted
1	-1	-1	0	3.7	3.700
2	+1	-1	0	4.9	5.025
3	-1	+1	0	5.5	5.375
4	+1	+1	0	6.5	6.500
5	-1	0	-1	3.5	3.612
6	+1	0	-1	5.3	5.287
7	-1	0	+1	5.4	5.412
8	+1	0	+1	6.3	6.187
9	0	-1	-1	3.6	3.487
10	0	+1	-1	5.1	5.112
11	0	-1	+1	4.9	4.887
12	0	+1	+1	6.3	6.412
13	0	0	0	5.9	5.833
14	0	0	0	5.8	5.833
15	0	0	0	5.8	5.833

RESULTS AND DISCUSSION

Screening of significant variables for spore production of *P. polymyxa* IN937a using Plackett–Burman design

Using a Plackett–Burman design, a total of seven variables were analyzed regarding their effects on spore production of *P. polymyxa* IN937a (Table 1). The design matrix chosen for the screening of significant variables for spore production and corresponding reactions was shown in Table 2. The Plackett-Burman matrix

obtained the predicted spore density of *P. polymyxa* IN937a from 0.22 to 7.22 (10^9 spores/mL) and the observed spore density of *P. polymyxa* IN937a from 0.7 to 7.9 (10^9 spores/mL), as is shown in Table 2. This result shows the difference between observed and predicted experimental data of spore density based on seven variables, thereby determining which factors have the most effective on the spore production of *P. polymyxa* IN937a. The F- and P-value of each variable on spore production of *P. polymyxa* IN937a was calculated by software Design Expert® 10.0.8 and shown in Table 5. Three factors with positive and large F-value on the spore production of *P. polymyxa* IN937a were molasses, yeast extract, and ammonium sulfate with a significant P-value < 0.05. Molasses, with a probability P-value of 0.0041, was identified as the most significant factor, followed by yeast extract (P-value = 0.0129), and ammonium sulfate (P-value = 0.0318). The remaining factors, with P-value > 0.05, were neglected. Therefore, in this study, molasses, yeast extract and ammonium sulfate were chosen as significant variables for the experimental design by the surface response methodology with BBD. In the previous optimization study of *P. polymyxa* JSa-9 fermentation medium, Mei Ying *et al*. (2016) selected 4 optimum compositions for spore formation including malt extract, corn extract steep liquor, $CaCO_3$, and $MgCl_2.6H_2O$.

Table 5. Effect, regression coefficient and corresponding F- and P-value for spore production of *P. polymyxa* IN937a in seven variables in Plankett-Burman design.

Variables	Unit	Symbol code	Coefficient	Standard error	F-value	P-value
Molasses	g/L	X_1	1.32	0.22	34.97[a]	0.0041
Glucose	g/L	X_2	-0.17	0.22	0.61[b]	0.4784
$MgSO_4.7H_2O$	g/L	X_3	-0.24	0.22	1.16[b]	0.3415
K_2HPO_4	g/L	X_4	-0.075	0.22	0.11[b]	0.7547
Yeast extract	g/L	X_5	0.96	0.22	18.29[a]	0.0129
$ZnSO_4.7H_2O$	g/L	X_6	0.31	0.22	1.89[b]	0.2408
Ammonium sulfate	g/L	X_7	0.72	0.22	10.47[a]	0.0318

[a]Significant at P < 0.05; [b]Non-significant at P > 0.05

Optimization of significant variables using response surface methodology following a Box–Behnken design

The experiments implemented in this report were targeted toward the construction of a quadratic model including fifteen trials. The design matrix and the corresponding results of RSM (Box-Behnken design) experiments to determine the effects of three independent variables (yeast extract, molasses, and ammonium sulfate) were shown in Table 6, along with the mean predicted values. The ANOVA analysis result of the optimization study showed that the model terms, X_1, X_2, X_3, X_1X_3, X_1^2, X_2^2, X_3^2 were significant (P < 0.05). The effects of yeast extract, molasses, and ammonium sulfate were highly significant (P < 0.001). The interactions between yeast extract and ammonium sulfate were significant, as was shown by the low P-value (0.0199 < 0.05) for the interactive terms. The model F-value of 82.52 indicated that the quadratic model was valid for this study. The F-value for lack of fit was 8.25 (> 0.05) pointed out that the model errors were in the same range as pure errors and the model was adequate to describe the tested data. The P-value for that model (<0.0001) and for lack of fit (0.11) suggested that the obtained experimental data was a good fit with the model.

The regression equation obtained from the ANOVA showed that the R^2 (multiple correlation coefficient) was 0.9933 (a value > 0.75 shows the fitness of the model), which implied that the sample variation of more than 99% was attributed to the variables and only 0.67% of the total variance are not explained by the model. The "adjusted R^2" = 0.9813 consistent with the "predicted R^2" = 0.8999, which confirmed that the model is highly significant. The "adequate precision value" of the present model was 40.69 > 4, and this also suggests that the model can be used to navigate the design space.

Table 6. ANOVA analysis results are optimized for the fermentation medium of significant variables.

Source	Sum of squares	Degree of freedom	Standard error	Mean square	F–value	P-value
Model	13.24	9	0.077	1.47	82.52	< 0.0001
X_1	3.00	1	0.047	3.00	168.29	< 0.0001
X_2	4.96	1	0.047	4.96	278.20	< 0.0001
X_3	3.65	1	0.047	3.65	204.39	< 0.0001
X_1X_2	0.01	1	0.067	0.20	0.56	0.4877
X_1X_3	0.20	1	0.067	0.42	11.36	0.0199
X_2X_3	0.0025	1	0.067	0.16	0.14	0.7234
X_1^2	0.26	1	0.069	0.26	14.72	0.0122
X_2^2	0.64	1	0.069	0.64	35.95	0.0009
X_3^2	0.72	1	0.069	0.72	40.39	0.0014
Lack of fit	0.083	3		0.028	8.25	0.1100
Pure Error	0.00667	2				
Total	13.33	14				

R^2=0.9933; CV=2.55%; Adj-R^2=0.9813; Pred-R^2=0.8999

The spore production of *P. polymyxa* IN937a can be expressed in terms of the following regression equation: Y (10^9 spore/mL) = -8,07546 + $0.47083\times X_1$ + $0.68917\times X_2$+ $43.48148\times X_2$ - $0.002\times X_1\times X_2$ - $0.6\times X_1\times X_3$ - $0.066667\times X_2\times X_3$ - $0.010667\times X_1^2$ - $0.016667\times X_2^2$ - $78.51852\times X_3^2$

The 3D response surface and 2D contour plots (Figure 1) were generally the graphical representation of the regression equation and clearly evidenced that all the variables exerted a quadratic effect on spore production of *P. polymyxa* IN937a. Each figure showed the effect of two variables on the spore production of *P. polymyxa* IN937a, while the other variable was held at zero level. As is shown in Figure 1A, a linear increase in spore production of *P. polymyxa* IN937a was observed when the concentration of molasses increased gradually to 19 g/L and yeast extract to 13 g/L. From Figure 1B, the *P. polymyxa* IN937a's spore concentration reached the maximum when the yeast extract concentration was between 13-15 g/L and ammonium sulfate in the range of 0.20-0.22 g/L. Similarly, according to Figure 1C, the ammonium sulfate concentration increased gradually to 0.22 g/L and molasses to 19 g/L, the spore concentration of *P. polymyxa* IN937a reached the maximum. From the data obtained using Design Expert® 10.0.8 software and the regression equation, the optimum levels of each variable were identified to be as follows: 14.44 g/L yeast extract, 19.14 g/L molasses and 0.20 g/L ammonium sulfate. The experimental model predicted that the optimum spore production increased to 6.606×10^9 spores/mL after 48 hours. The model was verified when performing experiments with optimal values of three variables for the spore production of *P. polymyxa* IN937a

reached 6.7×10^9 spores/mL. In the previous study, Mei Ying *et al*. (2016) optimized the fermentation medium for *P. polymyxa* JSa-9 to reach a spore concentration of 7.44×10^9 spores/mL.

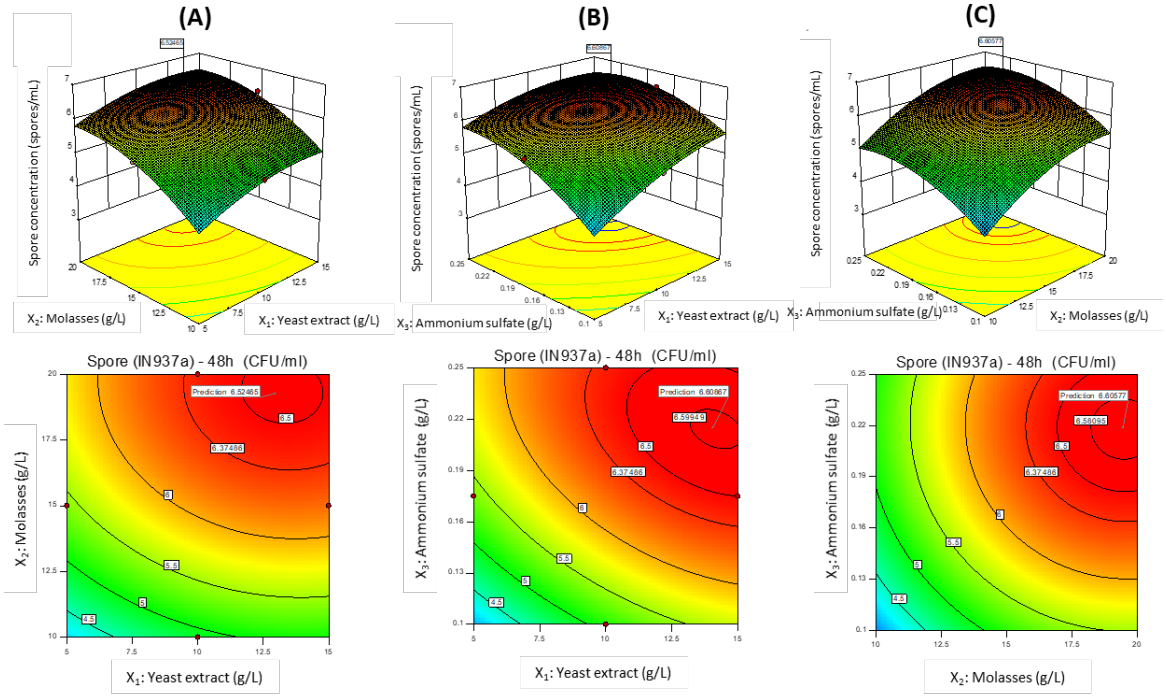

Figure 1. 2D contour and 3D response surface plots representing the effects of process parameters on spore production of *P. polymyxa* IN937a. A, interactive effect of yeast extract (X_1) and molasses (X_2), ammonium sulfate-hold value (X_3) = 0.25 g/L; B, Interactive effect of yeast extract (X_1) and ammonium sulfate (X_3), molasses-hold value (X_2) = 20 g/L; C, Interactive effect of molasses (X_2) and ammonium sulfate (X_3), yeast extract-hold value (X_1) = 13.24 g/L.

Antifungal activity of *P. polymyxa* IN937a

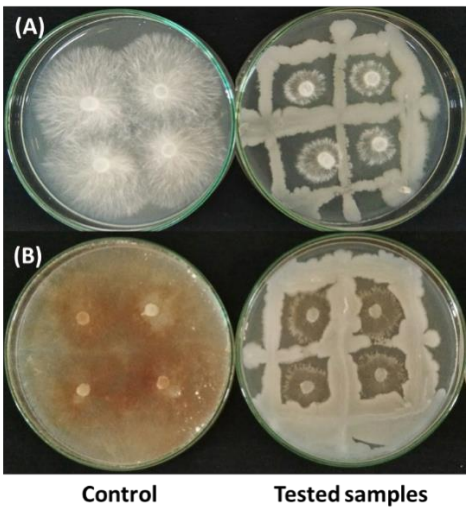

Figure 2. Images of mycelium on Petri dishes of tested samples containing *P. polymyxa* IN937a compared to the negative control 2-day treatment. A, *S. rolfsii*; B, *R. solani.*

In vitro test results of antifungal activity of *P. polymyxa* IN937a against two fungal strains, *S. rolfsii* and *R. solani* after 2-day treatment were shown in Figure 2. Observing the mycelial growth of the negative control compared to the tested sample showed that *P. polymyxa* IN937a was able to inhibit the growth of both *S. rolfsii* and *R. solani* well.

In a previous study, the spore solution of *P. polymyxa* JSa-9 also has an antifungal effect that prevents *Fusarium* wilt on cucumbers and the potential to promote cucumber growth. Accordingly, the spore solution of *P. polymyxa* JSa-9 at a concentration of 7.44×10^9 CFU/mL showed a relative biocontrol efficacy of 69.02% against the growth of *F. wilt* (Mei Ying *et al.*, 2016). The study of Dijksterhuis *et al.* (1999) showed that *P. polymyxa* T129 antagonizes the plant pathogenic fungus *F. oxysporum* in liquid

medium. In another study, five strains of *P. polymyxa* included ShX301, ShX302, ShX303, Hb1, and Hb2 were studied to show the antagonistic activity against spore germination and mycelial growth of *Verticillium dahlia* (Zhang *et al.*, 2018). This is the first study on the antifungal effect of *P. polymyxa* IN937a against *S. rolfsii* and *R. solani* in Vietnam.

CONCLUSION

In the report, we have found the optimization of fermentation medium for spore production from *P. polymyxa* IN937a by surface response methodology. The study screened seven factors and determined three significant factors including yeast extract, molasses, and ammonium sulfate using the Plankett-Burmen matrix design. By surface response methodology with the Box-Behnken design, the optimum composition for spore formation of *P. polymyxa* IN937a was defined as 14.44 g/L yeast extract, 19.14 g/L molasses, and 0.20 g/L ammonium sulfate. The spore production increased to 6.606×10^9 spores/mL after 48 h of incubation. In the *in vitro* bioassay for antifungal activity, *P. polymyxa* IN937a showed a good effect in inhibiting the growth of *S. rolfsii* and *R. solani*. The study results could be used for further research on the fermentation of *P. polymyxa* IN937a on a pilot scale to produce biological crop protection products in the future.

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