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Optimization of solid-state fermentation conditions for the production of cellulase and its hydrolytic potentials by *Trichoderma virride* Sn-9106

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Trichoderma viride **Sn-9106 with high cellulase activity was used to produce enzyme on residues of Chinese herbs as substrate in solid state fermentation. Residues of Chinese herbs and peptone were found to be the best combination of carbon and nitrogen source for the production of cellulase. The nutrient composition of medium was optimized using response surface methodology. A fractional factorial design (3^3) was applied to elucidate the nutrient medium components that significantly affect cellulase production. The concentration of peptone and wheat bran in the medium was a significant factor. The composition of nutrient fermentation medium optimized with response surface methodology was in g/L: wheat bran, 19.8, peptone, 2.06 and KH_2PO_4, 2.9. Compared to the original medium, the cellulase activity increased from 3.8 to 7.5 IU/mL.**

Key words: cellulase, *Trichoderma viride* Sn-9106, response surface methodology (RSM), solid state fermentation (SSF), residues of Chinese herbs (RCH).

INTRODUCTION

In recent years, one of the most important biotechnological applications is the conversion of lignocellulosics wastes into products of commercial interest such as bioethanol (Den Haan et al., 2007; Lynd et al., 2005). Cellulase is responsible for the hydrolytic cleavage of β-glycosidic bonds in cellulose and plays a critical role in the processing of lignocellulosics. It is a complex made up of three classes of enzymes: exoglucanase, endoglucanase and β-glucosidase (Chandrasekharaiah et al., 2012; Salahuddin et al., 2012). Cellulase is produced by two methods: submerged fermentation and solid-state fermentation (SSF). Compared to submerged fermentation, SSF has high productivity and its cost is low. Furthermore, the sub-strates used in solid-state fermentation are always industrial and agricultural wastes, which can reduce the fermentation cost (Jecu, 2000; Mekala et al., 2008; Zhao et al., 2010). In China, 13 million tons of residues from the Chinese herbs are produced annually. This abundant but low value resource contains about 70% hydrolysable cellulose

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Abbreviations: RSM, response surface methodology; **SSF,** solid-state fermentation; **RCH,** residues of Chinese herbs.

Table 1. Factors and coded values of RSM.

Variables (% w/w)	Coded	Range and levels		
		-1	0	+1
Wheat bran	X_1	15	20	25
peptone	X_2	1.5	2	2.5
KH_2PO_4	X_3	0.25	0.3	0.35

and hemicellulose, crude protein and trace element (Li et al., 2010). However, 90% residues of Chinese herbs (RCH) litter the environment and constitute waste problem (Xu et al., 2007). The use of Chinese herbs residues as the basis of the cultivation media, for decreasing costs of energy production and meeting the increased awareness in energy conservation and recycling is a matter of great interest. Although the production of cellulase using various nutrients as substrates by microorganisms has been reported (Khaleel and Gilna, 2011; Gamarra et al., 2010), researches are seldom done on production of cellulase using RCH as substrate. The aim of the present study was to demonstrate the optimization of SSF conditions with RCH for the production of cellulase and its hydrolytic potentials by *Trichoderma virride*.

MATERIALS AND METHODS

Microorganisms

T. virride Sn-9106 was isolated from soil samples collected from the soils in Dongling Mountain in Shenyang, China. Identification of isolates was carried out by the method of Barnett (1960) and Domch et al., (1980). Liquid inoculum medium (per liter, used in mycelium culture) consisted of 5.0 g of wheat bran, 2.0 g of peptone, 1 g of KH_2PO_4, 1.0 g of $CaCl_2$, and 6.0 g of glucose.

Substrate treatment

Residue from China herb was kindly provided by the Liaoning Benxi third medicine co., LTD. This material was thoroughly washed, dried and milled to 20 mm particle size. It contained 39% cellulose, 20% lignin, 28% hemicellulose, 7.5% extractives, 3.5% ash and 2% protein, on a dry-wt basis.

Inoculum and solid-state fermentation

T. virride Sn-9106 was used for cellulase production (Chen et al., 2012). It was grown on potato/dextrose/agar slants. Spores were washed from a 3-day agar-slant culture with 10 ml sterile distilled water and 2 ml of the suspension (10^6 spores/ml) was added to 250 ml shake-flasks, each containing 100 ml liquid inoculum medium. The inoculated flasks were incubated at 30±2°C and 150 rpm as a source of mycelia inoculum for SSF for 2 days before use.

Fermentations were carried out with pan bioreactor containing 20 g (dry-wt basis) of RCH as fermentation medium. The mixtures were autoclaved at 126°C for 40 min. Then, each pan bioreactor was inoculated with 1% (w/v) mycelium of Sn-9106. The nutrient elements (in g/L) of fermentation medium were calculated by weight of polysaccharide (cellulose and hemicellulose) content of residue and added to the substrate. The moisture content of the substrate after inoculation was about 75% (dry-wt basis) and the final pH was adjusted to 5.4. The fermentation was maintained for 72 h on the conditions of 30±2°C temperature; duplicate pan bioreactors were set up for each experimental variation.

Enzyme extraction

According to each gram of initial substrate weight, 100 ml distilled water was used to dispense the fermented moldy pith. The dispensed pith was shaken at 30±2°C and 130 rpm for 1 h. The mixture was filtered through nylon cloth of 200 mesh. The pH of the collected solution was measured before it was centrifuged. The supernatant was assayed for cellulase activity .

Assay of enzyme activity

Filter paper activity (FPA) was determined according to the method of the International Union of Pure and Applied Chemistry (IUPAC) and expressed as international units (IU). One IU of cellulase activity is the amount of enzyme that forms 1 µmol glucose (reducing sugars as glucose) per minute during the hydrolysis reaction. Reducing sugar was determined by the dinitrosalicylic acid (DNS) method (Ghose, 1987).

Optimization of enzyme production with SSF

Single factor experiment

Several single factors of nutrient elements influencing enzyme production were optimized. The effect of wheat bran (5% to 40%), peptone (0.5% to 4%) and KH_2PO_4 (0.1% to 0.4%) on cellulase synthesis was determined by growing the organism in SSF. The methods used in fermentation process experiment and enzyme assay are described above.

RSM for medium optimization

The optimal experiments for wheat bran, peptone and KH_2PO_4 supply were undertaken using the response surface methodology, in which MATLAB was used. A 3×3 fractional factorial design was employed to optimize medium components. The factors and levels used are shown in Table 1.

The responses were analyzed using MATLAB 14.0 software. In developing the regression equation, the test factors were coded according to the Equation:

$$x_i = (X_i - X_0) / \Delta X_i$$

Where, x_i was the coded value of the independent variable; X_i was the actual value of the independent variable; X_0 was the actual value of the independent variable at the central point and ΔX_i was the steep change value. A quadratic polynomial regression model was assumed to predict both Y responses. The model response of Y was expressed as:

$$Y = \square_0 + \sum_{i=1}^{3} \beta i Xi + \sum_{i}^{3} \beta ii\ X2i + \sum_{i=1}^{2} \quad \sum_{j=i+1}^{3} \beta ijXi\ Xj$$

Where, β_0 was an intercept; β_I, first-order model coefficient; β_{ii}, quadratic coefficient for the variable; β_{ij}, interaction coefficient for the interaction of variables I and j, and β_I and β_φ were evaluated by

Table 2. Cellulase production by *T. viride* in solid-sate fermentation of residue from Chinese herb carried out in pot fermenters.

Enzyme activities (IU/g dry-weight substrate)	Fermentation days			
	2	3	4	5
Endo-glucanase	2.32	10.07	9.87	9.52
Filter-paper cellulase	1.23	3.87	3.88	3.83
β-Glucosidase	1.37	4.45	4.89	4.23

the coefficient of determination (R^2) and the SPSS.

Contour plots were developed using the fitted quadratic polynomial equations obtained by keeping one of the independent variables at a constant value and changing the levels of the other two variables.

Hydrolysis experiments

In this study, hydrolysis efficiency was defined as cellulose and hemi-cellulose conversion efficiency. Pre-treated straw mixture was centrifuged at 10 *g* for 25 min to separate the supernatant and solids parts. TS contents of solids were adjusted to 10% by mixing supernatant and solids part. The hydrolysis experiments were conducted in 100 ml reaction system containing 10 g concentrated straw at 20 FPU/g cellulase. During the hydrolysis, the temperature was kept at 50±2°C, the revolution was kept at 250 rpm and the pH was maintained at 5.0 by pH controller.

Samples were taken aseptically after 72 h. The released glucose and xylose were determined on HPLC analysis with an Aminex HPX-87H column (Bio-Rad Laboratories) operating at 50°C and a flow rate of 0.6 ml 4 mM H_2PO_4 min^{-1}, using the refractive index detector. Cellulose and hemicellulose contents before and after hydrolysis were analyzed by strong acid analysis.

RESULTS AND DISCUSSION

Enzyme production by residue from Chinese herb of *T. virride*

RCH contains phenolic compounds which can restrain the growth of fungi (Xu et al., 2007; Yang et al., 2009); thus, an anti-phenol strain, *T. virride* Sn-9106 was isolated to produce cellulase. The formation of various enzymes in RCH cultures, under the conditions of SSF is shown in Table 2. Enzyme production appeared to be growth-associated. Maximum endo-glucanase, FP-cellulase and β-glucosidase activities were reached on day 3 of SSF, whereas their activity did not continue to rise after that.

Optimization of enzyme production by RSM

T. virride Sn-9106 was further investigated for cellulase production with RCH containing different concentration of wheat bran, nitrogen sources and inorganic salts in SSF. Peptone and KH_2PO_4 were selected as the main nitrogen source and inorganic salts; also wheat bran was added for optimizing cellulase production.

As shown in Figure 1, when the wheat bran was at 20%, the *T. virride* Sn-9106 produced a maximal FPase activity of 4.74 IU/g (Figure 1A). Application of peptone to mixed medium also induced activities of the enzymes to increase. The top activity was observed at 1.5% of peptone when FPU had activity of 6.45 IU/g (Figure 1B). We tested the growth and enzyme release activity using KH_2PO_4. Our result shows a pattern of activities for both enzymes similar to those supplied with wheat bran and peptone (Figure 1C). Also, the change in activities of FPU was dependent on the concentrations of KH_2PO_4, which showed the maximum activities of the enzymes at concentration of 0.25%.Fig.2 B

To get insight into the interaction with the three factors and to see the multiple capability of inducing cellulase, a combination experiment was designed with 3 factors × 3 levels (Table 3).

Then, SPSS was applied to get ideal second-degree polynomial regression models of FPase activity.

$$Y=7.16-0.023\ X_1+0.435\ X_2-0.025\ X_3-0.498\ X_1\ X_2+0.168\ X_1\ X_3+0.198\ X_2\ X_3-1.229\ X_1^2+1.859\ X_2^2-1.504\ X_3^2$$

The results shown in Tables 3 to 5 show that the model for FPase production was significant ($p=0.0180<0.0500$) with a satisfactory value of coefficient of determination, R^2 Ad (0.813423). This indicated that 81.34% of the variability in the response could be explained by the second-order model equation given above. Probability value for the lack of fit (LOF) was 0.0502, which was not significant. The results showed that this model is appropriate.

The resulting response surface showed the effect of wheat bran, peptone and KH_2PO_4 concentration on the FPase production (Figure 2). Because the shape of contour could reflect the instance, elliptical contour means strong interaction. This result demonstrate that there is a significant interaction between wheat bran and peptone. Wheat bran and KH_2PO_4 also have significant interaction. However, the interaction between peptone and KH_2PO_4 was insignificant.

We can learn that the response surface has a maximum point. The maximum FPase production by *T. virride* Sn-9106 was obtained in the optimized medium when the initial concentration of wheat bran, peptone and

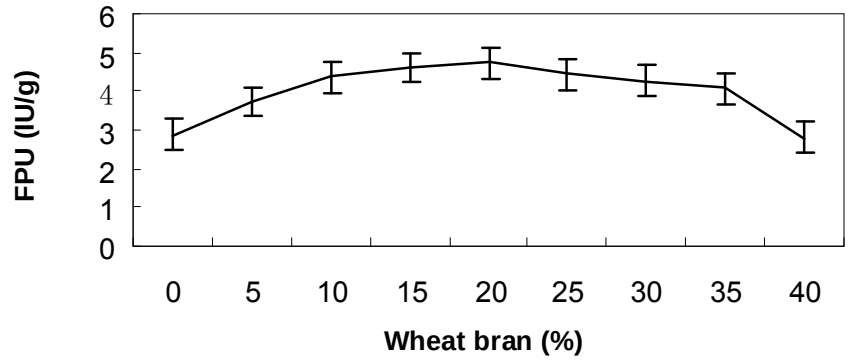

Figure 1A. Influence of wheat bran on FPase production of *T.virride* Sn-9106.

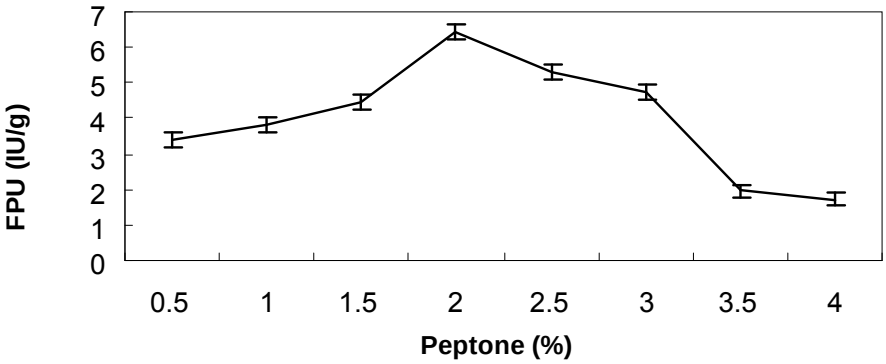

Figure 1B. Influence of peptone on FPase production of *T.virride* Sn-9106.

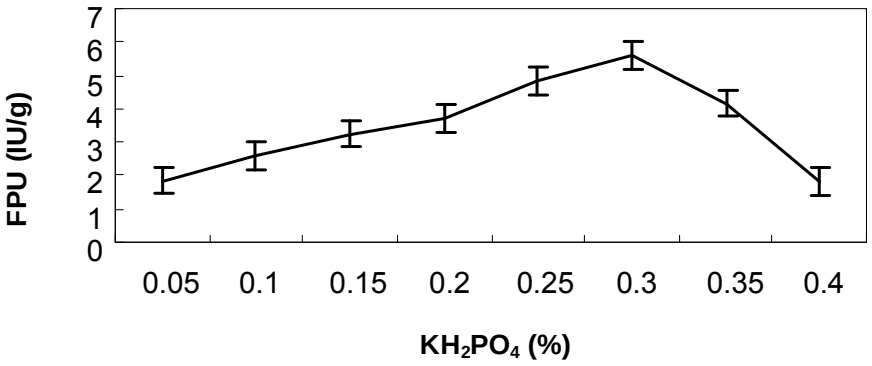

Figure 1C. Influence of KH_2PO_4 on FPase production of *T.virride* Sn-9106.

KH_2PO_4 was 19.8, 2.06 and 2.9% respectively. The maximum response predicted from the model was 7.5 IU/g. Repeated experiments were performed to verify the predicted optimum. The result from replications 7.4 IU/g was coincident with the predicted value and the model was proven to be adequate. Compared with the original medium, the FPase activity of *T. virride* Sn-9106 increased from 3.8 to 7.5 IU/g.

Enzymatic hydrolysis and conversion experiment

In this study, enzymatic hydrolysis of pre-treated straw mixture with TS content of 10% was studied. Two different cellulases, produced by *T. virride* Sn-9106 and Cellulast+Novozyme 188 (Purchased from Novozyme) were compared.

Figure 3 shows that the highest cellulose conversion

Table 3. Experimental design and results of RSM.

Number	X_1	X_2	X_3	Measured value	Y
1	-1	-1	0	6.16	4.9375
2	1	-1	0	5.9	6.1625
3	-1	1	0	7.7	7.4375
4	1	1	0	3.84	5.0625
5	-1	0	-1	6.8	7.7225
6	1	0	-1	8.05	7.4875
7	-1	0	1	7.35	7.9125
8	1	0	1	7.92	6.9975
9	0	-1	-1	7.09	7.39
10	0	1	-1	7.2	6.54
11	0	-1	1	5.03	5.69
12	0	1	1	8.24	7.94
13	0	0	0	12.99	12.60333
14	0	0	0	12.45	12.60333
15	0	0	0	12.37	12.60333

Table 4. Model Summary Statistics.

Source	Standard Deviation	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	3.007071	0.01667	0.25151	0.46262	147.9487	
2FI	3.42252	0.073595	0.62121	1.22904	225.4754	
Quadratic	1.161061	0.933365	0.813423	0.03523	104.7174	Suggested
Cubic	0.337244	0.997751	0.984259		+	Aliased

Table 5. Anova results for cellulase production obtained from RSM.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	94.41	9	10.49035	7.781795	0.0180	significant
A-A	0.66	1	0.66125	0.490518	0.5149	
B-B	0.98	1	0.98	0.726969	0.4328	
C-C	0.045	1	0.045	0.033381	0.8622	
AB	3.24	1	3.24	2.403448	0.1818	
AC	0.12	1	0.1156	0.085753	0.7814	
BC	2.4	1	2.4025	1.782186	0.2394	
A2	33.94	1	33.93601	25.1739	0.0040	
B2	49.78	1	49.7765	36.92445	0.0017	
C2	15.39	1	15.39103	11.41714	0.0197	
Residual	6.74	5	1.348063			
Lack of Fit	6.51	3	2.17095	19.08807	0.0502	not significant
Pure Error	0.23	2	0.113733			
Cor Total	101.15	14				

obtained was 86.3% at the enzyme loading of 20 FPU/g-cellulose, which is quite comparable with commonly cellulose conversion of 92.5% at commercial enzyme loading of 20 FPU/g-cellulose.

Previous study revealed that *Trichoderma* can grow on solid substrates, such as corn straw (Wang et al., 2005;

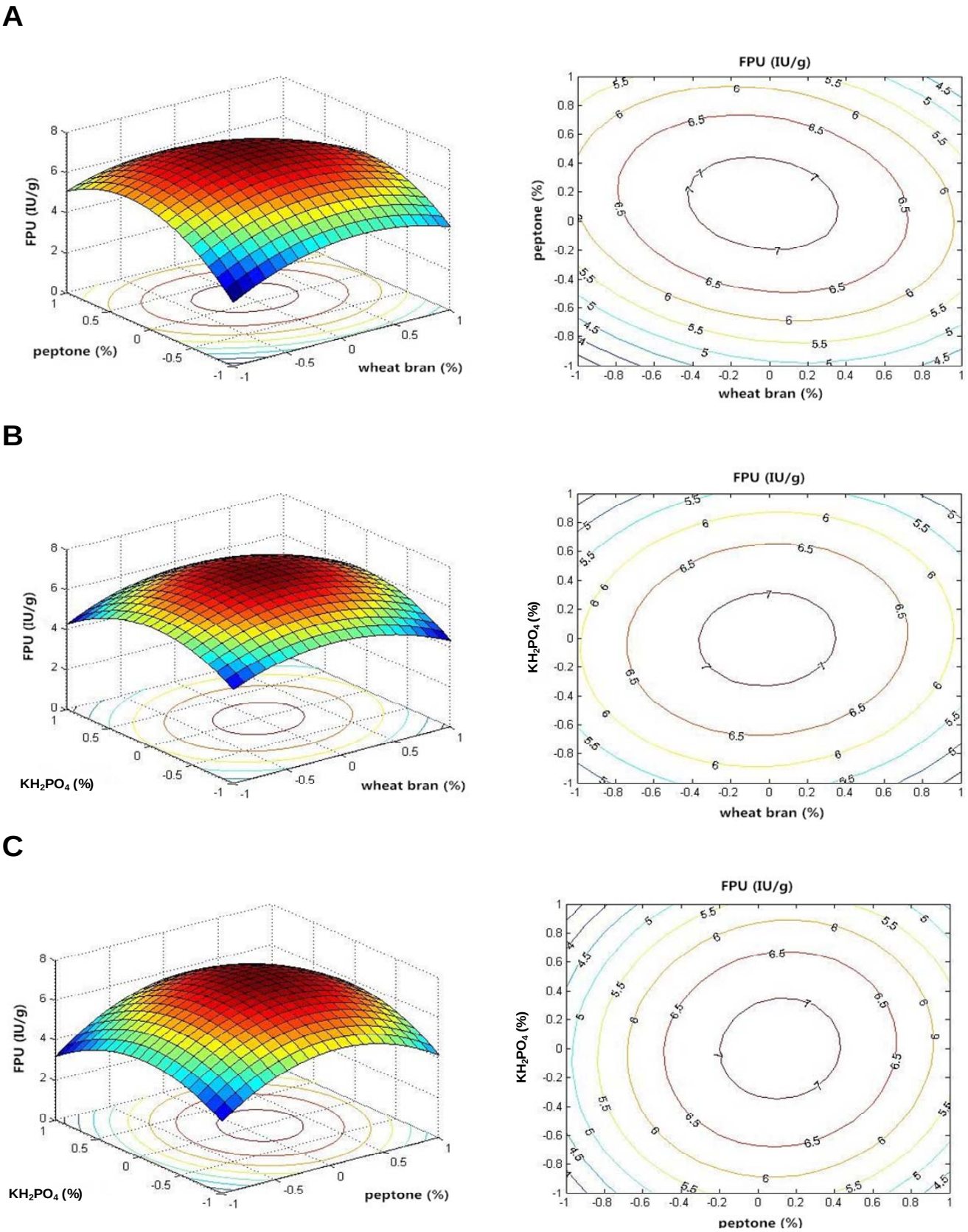

Figure 2. Response surface plot for the effect of wheat bran, peptone and KH_2PO_4 on FPase production. **A.** Effect of interaction between the wheat bran and peptone on FPase production. **B.** Effect of interaction between the wheat bran and KH_2PO_4 on FPase production. **C.** Effect of interaction between the peptone and KH_2PO_4 on cellulase FPase production.

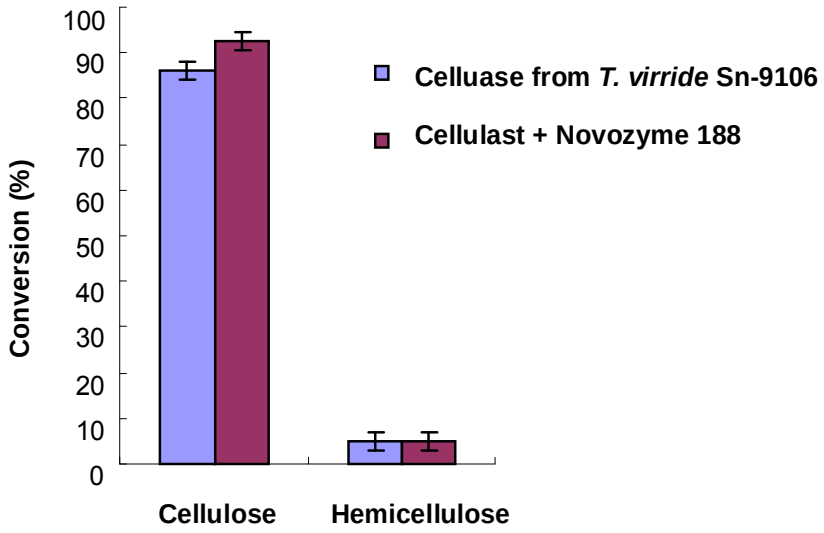

Figure 3. Comparison of hydrolysis efficiency with different enzyme loading.

Wang, 2006), wheat straw (Awafo et al., 1996) as well as sugar cane bagasse (Massadeh et al., 2001; Duenas et al., 1995) to promote cellulase production. But there is little concern on one of the lignocellulose residues-residues of Chinese herbs. In this study, a significant activity of cellulase was produced by the *T. virride* Sn-9106 grown on residues of China herbs. Enzymatic hydrolysis experiment showed that although the percentage of hydrolysis and conversion by *T.virride* Sn-9106 was lower than Celluclast+Novozyme188, a higher conversion was achieved in cellulose hydrolysis by *T. virride* Sn-9106.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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