Optimization of solid-state fermentation conditions for the production of cellulase and its hydrolytic potentials by *Trichoderma viride* 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 KH₂PO₄, 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 (Chandrasekharaiyah 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.
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 Dornch 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$_2$PO$_4$, 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-slate 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$^\circ$C for 2 days before use. The inoculated flasks were shaken at 30±2$^\circ$C and 150 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$_2$PO$_4$ (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$_2$PO$_4$ 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 $\Delta X_i$ 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:

\[
Y = \alpha + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{2} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=1}^{3} \beta_{ij} X_i X_j
\]

Where, $\beta_0$ was an intercept; $\beta_i$, first-order model coefficient; $\beta_{ii}$, quadratic coefficient for the variable; $\beta_{ij}$, interaction coefficient for the interaction of variables $i$ and $j$, and $\beta_i$ and $\beta_{ii}$ were evaluated by

**Table 1. Factors and coded values of RSM.**

<table>
<thead>
<tr>
<th>Variables (% w/w)</th>
<th>Coded</th>
<th>Range and levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat bran</td>
<td>$X_1$</td>
<td>15  20  25</td>
</tr>
<tr>
<td>peptone</td>
<td>$X_2$</td>
<td>1.5  2  2.5</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>$X_3$</td>
<td>0.25 0.3 0.35</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Enzyme activities (IU/g dry-weight substrate)</th>
<th>Fermentation days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Endo-glucanase</td>
<td>2.32</td>
</tr>
<tr>
<td>Filter-paper cellulase</td>
<td>1.23</td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>1.37</td>
</tr>
</tbody>
</table>

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

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. viride* 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$_2$PO$_4$ 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. viride* 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$_2$PO$_4$. 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$_2$PO$_4$, which showed the maximum activities of the enzymes at concentration of 0.25%. Fig. 2

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$_2$PO$_4$ 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$_2$PO$_4$ also have significant interaction. However, the interaction between peptone and KH$_2$PO$_4$ was insignificant.

We can learn that the response surface has a maximum point. The maximum FPase production by *T. viride* Sn-9106 was obtained in the optimized medium when the initial concentration of wheat bran, peptone and
KH$_2$PO$_4$ 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
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$_2$PO$_4$ 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$_2$PO$_4$ on FPase production. 

C. Effect of interaction between the peptone and KH$_2$PO$_4$ on cellulase FPase production.
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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