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*Full Length Research Paper*

# Cellulase production by *Aspergillus* sp. on rice grass (*Spartina* spp.) under solid-state fermentation

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**In this study, the feasibility of using rice grass (*Spartina* spp.) as the main substrate for cellulase production by a newly isolated strain *Aspergillus* sp. SEMCC-3.248 under solid-state fermentation was evaluated. The optimized medium and culture conditions were: rice grass (0.8-0.9 mm) 2.5 g, Wheat bran 1.5 g, 4 ml of nutrient solution ($(NH_4)_2SO_4$ 14 g/L, $KH_2PO_4$ 2 g/L, $CaCl_2$ 4 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L, soluble starch 2.62 g/L, peptone 1.51 g/L), 1 ml of spore suspension ($1\times10^7$ spores/ml), initial moisture content 70%, initial pH 5.0, incubation temperature 32°C and fermentation period 5 days. Under these conditions, the cellulase activity could reach 1.14 FPIU/gds.**

**Key words:** Cellulase, isolation, identification, solid-state fermentation, *Spartina* spp., *Aspergillus* sp.

## INTRODUCTION

Rice grass (*Spartina* spp.) was a kind of serious invasive species which causing extensive damage to natural salt marsh ecosystems in coastal areas at present in Asia, Australia, New Zealand and North America (Hacker et al., 2001). In recent years, the rice grass also spread to the Yellow River delta in Shandong Province of China, mainly distribute in the area between the Xianhe Town and the fifth stake of the Dongying City, the bayou of small limpid river, and the tide land of Wudichajian, and the distribution area was over 700 $hm^2$. So it is very necessary to find a reasonable method to make full use of these plants. It has been reported that the rice grass could be used for power generation by gas-heat-electricity triple cogeneration method or for feed production (Dong et al., 2007). However, the large-scale utilization of rice grass is limited due to the high salt content.

Cellulase have the capability of hydrolyzing cellulose into fermentable sugars such as glucose (Ladisch et al., 1981), which can be used for producing many useful products such as ethanol, biofuel and other useful chemicals from the cellulosic feedstocks (Bhat, 2000; Peng and Chen, 2008; Tomás-Pejó et al., 2009). Cellulase could be produced by many lignocelluloytic feed stocks such as straws, bagasse, wheat bran, corn stover, corncob, and etc. (Xia and Cen, 1999; Romero et al., 1999; Camassola and Dillon, 2009). Rice grass was known as one of the fastest growing plants and a kind of alien species, and the large-scale outbreak of it leads to many environmental problems in coastal areas of China. However, because of the large amount of biomass and rich nutrient, these plants might be used in many areas. The mold fungi *Aspergillus* spp. are considered could be widely used for amylase, protease and cellulase production (Feng et al., 2007), and some of them could improve the nutritional quality of food and feed by fermentation (Hong et al., 2004). And there was no data about celluluase production from rice grass with *Aspergillus* sp. reported at present. In this study, rice grass was firstly used as the main substrate for cellulase production in solid-state fermentation by the strain

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*Aspergillus* sp. SEMCC-3.248. The production of cellulase was up to 1.14 FPIU/g dry solid, which provide a new method for rice grass treatment or utilization.

## MATERIALS AND METHODS

Rice grass, collected from Xianhe Town, Dongying City, Shandong Province of China, which were dried at 80°C, crushed and sieved to an average sizes of 0.8-0.9 mm. Reagents used in this study were of analytical grade and obtained locally.

Basal medium: Rice grass 3 g, wheat bran 1 g, initial pH 6.0, nutrient salt solution $(NH_4)_2SO_4$ 14 g/L, $KH_2PO_4$ 2 g/L, $CaCl_2$ 4 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L) 4 ml, and the initial moisture content was adjusted to 75% with distilled water.

### Nutriention content analyses

Crude protein, cellulose, hemicellulose, lignin, ash, fat and moisture content were analyzed according to the methods described in the literature (Nielsen, 1998).

### Cellulase-producing strain

Strain *Aspergillus* sp. SEMCC-3.248, stored on a potato dextrose agar (PDA) medium, containing potato extract 20% (w/v), glucose 20 g/L, agar 20 g/L ) slant at 4°C before use.

### Genomic DNA extraction

Samples used for DNA extraction were collected by liquid state fermentation in the shake flask, and the medium composition was: glucose 20 g/L, potato extract 20% (w/v), pH 5.0. Strain SEMCC-3.248 was incubated in a 250 ml Erlenmeyer flask containing of 50 ml medium (sterilized at 121°C for 30 min) at 30°C on a reciprocal shaker at 180 rpm for 48 h. The fresh mycelia were precipitated by centrifugation at 10,000 g, 4°C for 15 min. Genomic DNA was isolated as described by Paavanen-Huhtala et al. (1999).

### Polymerase chain reaction (PCR) amplification and sequencing

The 18S rDNA was amplified using the universal primer pairs of NS1 (5'-GTAGT CATAT GCTTG TCTC-3')/NS8 (5'-TCCGC AGGTT CACCT ACGGA-3') (White et al., 1990). 50 µl of reaction mixture for PCR amplification was prepared with 1× PCR buffer ($Mg^{2+}$ free), 2.5 m mol/L $MgCl_2$, 200 µ mol/L of each deoxynucleotide triphosphate (dNTPs), 2.5 U *Taq* DNA polymerateand 200 n mol/L of each NS1/NS8 primer. The amplifications were performed in a ThermoHybaid PCR Sprint Thermal Cycler (Thermo Electron, USA). The PCR reaction detailes were as follows: 4 min at 94°C for initial denaturation; 45 s at 94°C for denaturation, 1 min at 50°C for annealing of 18S rDNA, 1 min 45 s at 72°C for extension with total 30 cycles of amplification, and 10 min at final extension. The 18S rDNA were sequenced by Shanghai Shengon, China.

### Sequence alignments and analyses

BLAST (http://www.ncbi.nlm.nih.gov/BLAST) searches were used to find homologous sequences. The corresponding sequences of representative species were selected for phylogenetic analyses. Preliminary multiple alignments were conducted using Clustal W version 1.83 for the datasets. Alignment adjustments were made by BioEdit version 7.0.1. Distance trees were generated based on neighbor-joining methods using Mega version 3.1, Tree topologies were estimated by bootstrap analyses with 1000 replicates.

### Cellulase production by solid-state fermentation (SSF)

Strain SEMCC-3.248 was cultured on PDA slants in the dark at 28°C for 7 days. Inoculum was prepared by suspending the spores from a PDA plates with 10 ml sterile distilled water containing 0.1% Tween 80, which contained $1\times10^7$ spores/ml. The SSF experiments were conducted in 250 ml Erlenmeyer flasks containing 4 g of substrate moistened with 12 ml of nutrient salt solution. After sterilized at 121°C for 30 min, the flasks were cooled and inoculated with 1 ml spore suspension and incubated at 30°C for cellulase production. After fermentation, the medium of the flasks were collected for cellulase activity determination.

Various process parameters were optimized by conventional methods for maximal cellulase production as follows: incubation temperature (24, 26, 28, 30, 32, 34 and 36°C), initial total moisture content (55, 60, 65, 70, 75, 80 and 85%), initial pH (3.0-9.0) were varied. Supplemental carbon sources (lactose, fructose, glucose and maltose, potato starch and soluble starch) and nitrogen sources (peptone, urea, $NaNO_3$, soybean meal, corn steep powder and $NH_4NO_3$) were also studied at different levels, three replicates were prepared for each treatment, and an uninoculated flask served as control. The best additional nitrogen source and carbon source were selected for further optimization by response surface methodology with central composite design.

### Experimental design

In order to obtain the best combination of peptone and soluble starch, (response surface methodology (RSM) were used to obtain the optimum level. The experimental design and results were listed in Table 1.

### Enzyme extraction and assay

Cellulase was extracted by suspending the fermented substrate with 5-fold of distilled water and mixing it for 1 h at 300 rpm. And then the crude enzyme was further extracted by centrifugation (10,000 g, 20 min). The total cellulase activity (Filter Paper Unit, FPU) was measured by the standard filter paper assay (Afolabi, 1997). The filter paper enzyme activity (FPA) was expressed as FPIU/g of dry substrate (FPIU/gds). One International Unit (IU) of enzyme activity is defined as the amount of enzyme required to liberate 1 µmol of product per min at 50°C.

## RESULTS AND DISCUSSION

### Main nutrient ingredients of the rice grass

As shown in Table 2, the cellulose and hemicellulose were about 60% of the dried rice grass, and the protein content was also higher than wheat straw or corn stover, which were both lower than 5% reported by Hu et al. (2008).

So it is suitable for cellulase production. pH of the rice grass was usually at 8.9-9.0, so the medium pH should be adjusted when it was used as the main cellulose substrate.

**Table 1.** Experimental design and results of the central composite design.

| Run no. | $X_1$ (Peptone) | $X_2$ (Soluble starch) | Observed cellulase activity (FPIU/gds) |
|---|---|---|---|
| 1 | 1 (1.8) | 1 (3.0) | 0.84±0.12 |
| 2 | 1 (1.8) | -1 (2.0) | 0.61±0.09 |
| 3 | -1 (1.2) | 1 (3.0) | 0.79±0.04 |
| 4 | -1 (1.2) | -1 (2.0) | 0.71±0.07 |
| 5 | 0 (1.5) | 0 (2.5) | 1.06±0.11 |
| 6 | 0 (1.5) | 0 (2.5) | 1.03±0.05 |
| 7 | 0 (1.5) | 0 (2.5) | 1.04±0.07 |
| 8 | 0 (1.5) | -1.41 (1.8) | 0.49±0.06 |
| 9 | 0 (1.5) | 1.41 (3.2) | 0.77±0.13 |
| 10 | -1.41 (1.1) | 0 (2.5) | 0.68±0.12 |
| 11 | 1.41 (1.9) | 0 (2.5) | 0.74±0.10 |

* The data in the brackets is the coded factor levels.

**Table 2.** Main nutrient ingredients of the dried rice grass.

| Crude protein (%) | Hemicellulose (%) | Lignin (%) | Crude cellulose (%) | Crude fat (%) | Crude ash (%) | Total sugar (%) | Moisture content (%) |
|---|---|---|---|---|---|---|---|
| 10.4 | 22.1 | 13.2 | 34.9 | 2.1 | 11.7 | 10.8 | 2.3 |

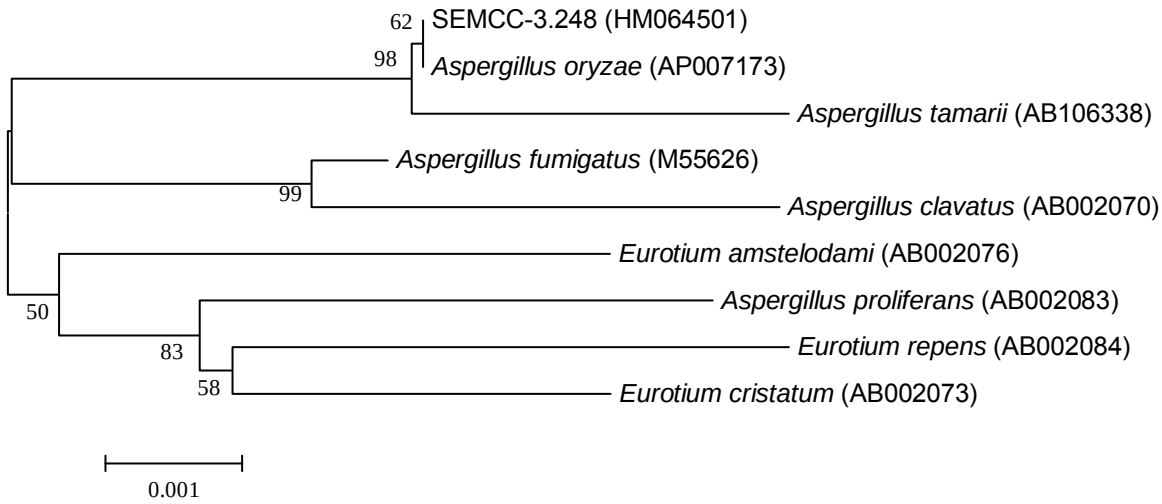


**Figure 1.** A Neighbor-joinging tree analysis of the strain SEMCC-3.248 18S rDNA.

## 18S rDNA analyses

A 1770 bp sequence was amplified from the genome DNA with the NS1/NS8 as primers, and was submitted to GenBank (HM064501). Three strains belonging to *Aspergillus* genus were closely related to the strain SEMCC-3.248, which on the 62%-bootstrap-supported branch with *Aspergillus oryzae* (AP007173). According to the results of morphology and 18S rDNA analyses, the strain SEMCC-3.248 should be *Aspergillus* sp. (Figure 1).

## Time course of the cellulase production

Time course of the cellulase production was shown in Figure 2. The enzyme activity reached the maximum at 120 h, and then began to decrease. So the incubation period was 5 days in the follow experiments.

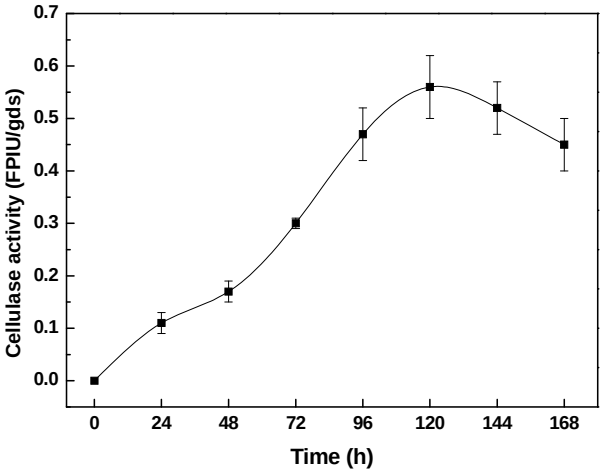


**Figure 2.** Time course of the cellulase production.

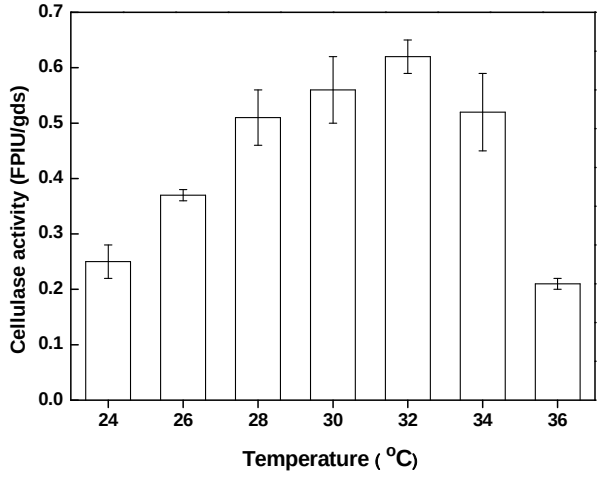


**Figure 3.** Effect of temperature on the cellulase production.

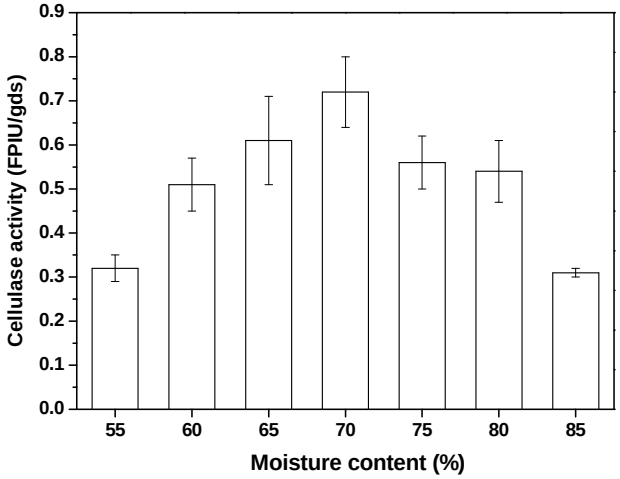


**Figure 4.** Effect of initial moisture content on the production of cellulose.

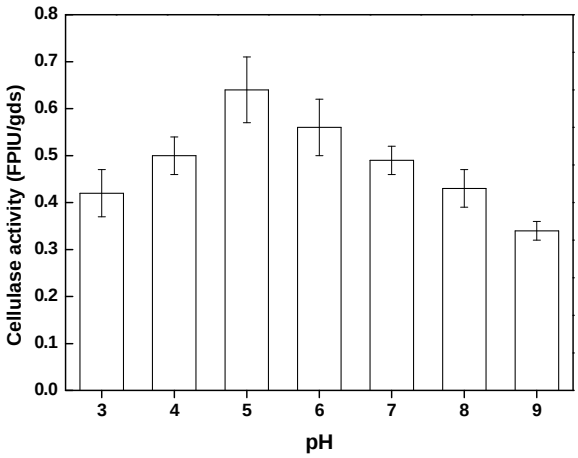


**Figure 5.** Effect of different pH levels on the cellulase production.

## Effect of different incubation temperature on the cellulase production

As shown in Figure 3, cellulase production was significantly influenced by incubation temperature ($P$<0.05). When the temperature was at 32°C, the cellulase activity reached maximum. So the optimal incubation temperature was 32°C.

## Effect of different moisture contents on the cellulase production

Seven moisture levels ranging from 55 to 85% were selected to study their effect on cellulase production, it was found that the moisture contents were significantly influenced the cellulase production ($P$<0.05) (Figure 4), and the highest cellulase activity was attained when the initial moisture level was 70%. Either low or high moisture content would decrease the cellulase production.

## Effect of different pH on the cellulase production

The effect of pH on the cellulase yield was shown in Figure 5. pH was important for cellulase production by the strain SEMCC-3.248. pH should be essential for cellulase production when the rice grass power was used as the main substrate. The highest cellulase activity was observed when the strain SEMCC-3.248 was cultured at pH 5 (Figure 5).

## Effect of different rice grass/wheatbran ratio (RG/WB) on the cellulase production

Wheat bran could improve cellulase production (Xu, et

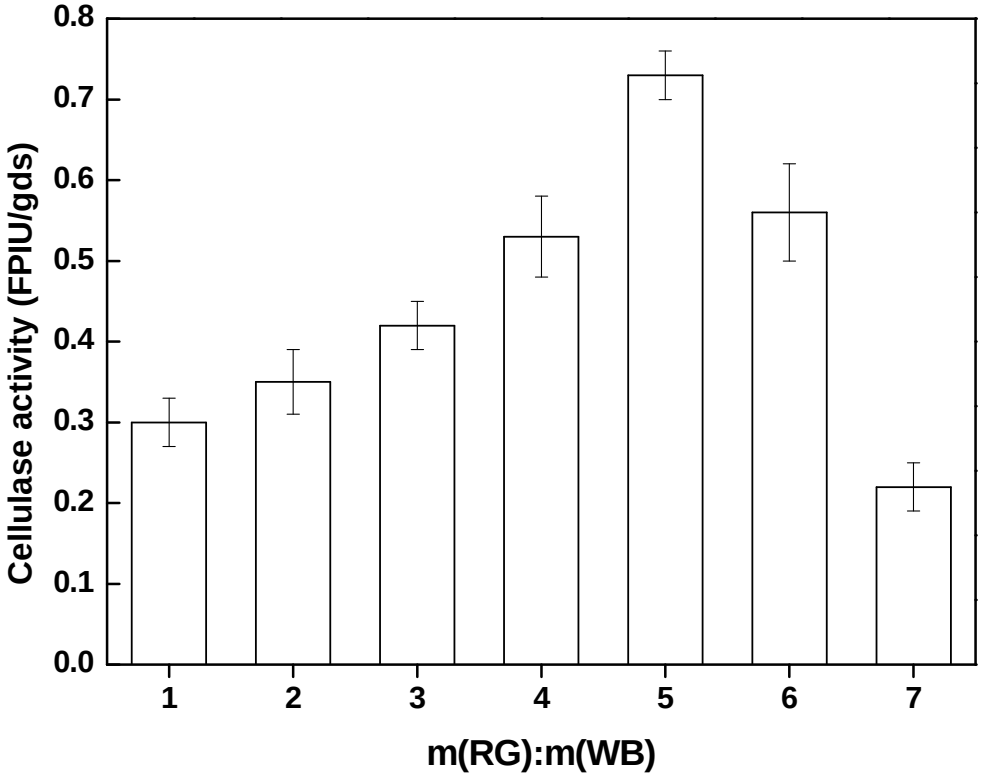


**Figure 6.** Effect of RG/WB ratio on the cellulase production. (RG/WB ratio 1: 0.5/3.5; 2: 1/3; 3: 1.5/2.5; 4: 2/2; 5: 2.5/1.5; 6: 3/1; 7: 3.5/0.5).

al., 2005), but superfluous of wheat bran could cause the substrate to agglomerate, which results in insufficient aeration and bad heat transfer. Effect of RG/WB ratio was shown in Figure 6. When the ratio was 2.5/1.5, highest cellulase activity was observed.

## Effect of additional nitrogen sources and carbon sources on cellulase production

Studies of supplementation of nitrogen and carbon sources were shown in Tables 3 and 4, respectively. Addition of soluble starch and peptone could obtain the highest cellulase production. So the two factors were selected for further optimization.

## Optimization of the selected nitrogen and carbon source

The experimental results were analyzed by ANOVA and central composite design was fitted with the second-order polynomial equation (Table 5):

$$Y=1.0433+0.0044X_1+0.0882X_2+0.0375X_1X_2-0.1498X_1^2-0.1898X_2^2$$

Where $Y$ is the response factor (cellulase production, FPIU/gds) and $X_1$, $X_2$ represent peptone and soluble starch, respectively. The analysis of variance and regression for the cellulase production was shown in Table 6. The model $F$-value of 25.52 implies the model is significant. There is only 0.14% chance that a "Model $F$-value" this large could occur due to noise. The fit of the model was checked by the coefficient of determination $R^2$, which was calculated to be 0.9623, indicating that about 96% of the variability in the response could be explained by this model. It was considered as very high correlation when the $R^2$-value was higher than 0.9 (Li et al., 2005). The statistical significance of the model equation was evaluated by the $F$-test for ANOVA. The $P$-value was also very low (0.0014) indicating the significance of the model. The coefficient of variation ($CV$) indicates the degree of precision with which the treatments are compared. A lower $CV$ means a higher reliability of the experiment. The relatively lower value of $CV$ (6.35%) demonstrated that the performed experiments were reliable. The lack of fit $P$-value of 0.0543 implied the lack of fit is not significant relative to the pure error.

The response surface curves are plotted to explain the interaction of the variables and to determine the optimum level of each variable for maximum response. The

**Table 3.** Effect of additional nitrogen sources on cellulase production.

| Nitrogen sources | Content (g/L) | Cellulase activity (FPIU/gds) |
|---|---|---|
| Urea | 1.5 | 0.82±0.16 |
| $NaNO_3$ | 1.5 | 0.71±0.11 |
| Peptone | 1.5 | 0.87±0.19 |
| Soybean meal | 1.5 | 0.74±0.15 |
| Cornsteep powder | 1.5 | 0.79±0.19 |
| $NH_4NO_3$ | 1.5 | 0.64±0.08 |
| Control | | 0.76±0.10 |

**Table 4.** Effect of additional carbon sources on cellulase production.

| Carbon sources | Content (g/L) | Cellulase activity (FPIU/gds) |
|---|---|---|
| Glucose | 2.5 | 0.64±0.10 |
| Fructose | 2.5 | 0.68±0.07 |
| Potato starch | 2.5 | 0.84±0.21 |
| Soluble starch | 2.5 | 0.87±0.15 |
| Lactose | 2.5 | 0.54±0.19 |
| Maltose | 2.5 | 0.74±0.09 |
| Control | | 0.76±0.10 |

**Table 5.** Parameter estimates of the model.

| Term | Estimate | Std Error | *t*-value | *P*-value |
|---|---|---|---|---|
| Intercept | 1.0433 | 0.0292 | 35.75 | <0.001* |
| $X_1$ | 0.0044 | 0.0179 | 0.24 | 0.8171 |
| $X_2$ | 0.0882 | 0.0179 | 4.94 | 0.0043* |
| $X_1X_2$ | 0.0375 | 0.0253 | 1.48 | 0.1980 |
| $X_1^2$ | -0.1498 | 0.0213 | -7.04 | 0.0009* |
| $X_2^2$ | -0.1898 | 0.0213 | -8.92 | 0.0003 |

*Statistically significant at the 95% confidence level.

**Table 6.** Analysis of variance and regression analysis for the cellulase production.

| Source | Sum of squares | Degree of freedom | Mean square | *F*-value | *P>F* |
|---|---|---|---|---|---|
| Model | 0.33 | 5 | 0.065 | 25.52 | 0.0014 |
| Residual | 0.013 | 5 | 0.0025 | | |
| Lack of fit | 0.012 | 3 | 0.0041 | 17.59 | 0.0543 |
| Pure error | 0.0005 | 2 | 0.0002 | | |
| Corrected total | 0.34 | 10 | | | |

$R^2$=0.9623; $R^2_{adj}$=0.9246; *CV*%=6.35.

response surface contour curves are shown in Figure 7. The model predicted the optimal values (Coded) of the variables were $X_1$=0.044, $X_2$=0.24.

Correspondingly, the values of the two variables were 1.51 and 2.62 g/L, respectively. The maximum predicted cellulase production was 1.05 FPIU/gds. In order to confirm the optimized culture conditions, three additional experiments in the Erlenmeyer flasks were performed using the predicted medium composition. The mean value of the cellulase production was 1.14±0.08

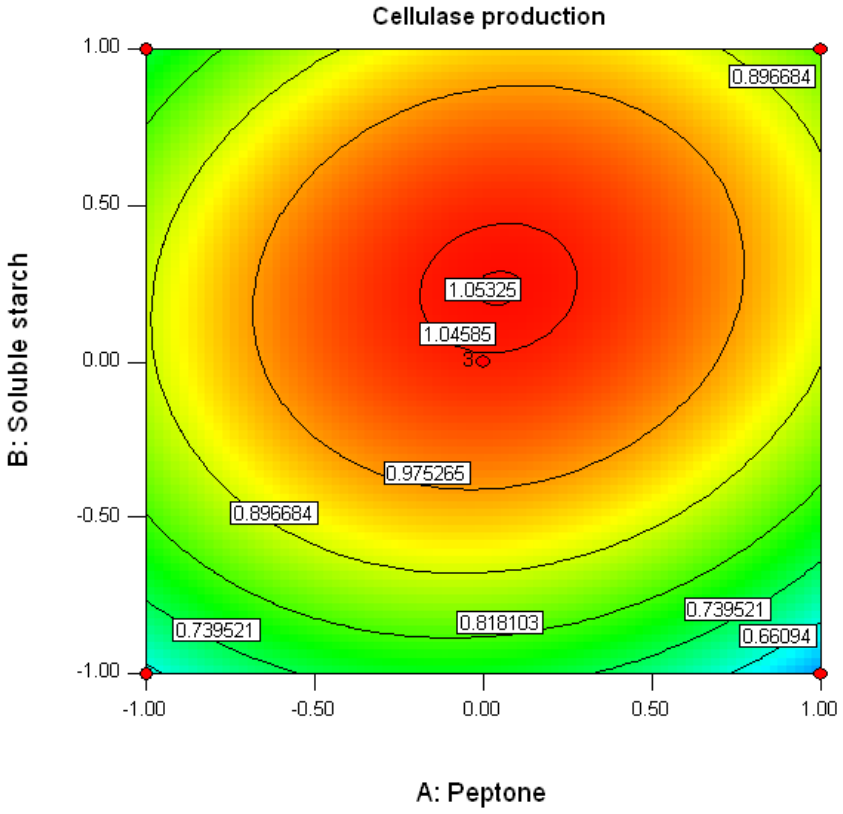


**Figure 7.** Contour plot of the response surface.

FPIU/gds, which agree well with the predicted value. This result demonstrates the validity of the response model.

## Conclusion

In this study, a novel cellulase-producing strain SEMCC-3.248 was isolated from the rice grass, and identify as *Aspergillus* sp. according to 18S rDNA analyses. Then the fermentation conditions for cellulase production were optimized and listed as follows: rice grass (0.8-0.9 mm) 2.5 g, wheat bran 1.5 g, 4 ml of nutrient solution ($(NH_4)_2SO_4$ 14 g/L, $KH_2PO_4$ 2 g/L, $CaCl_2$ 4 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L, soluble starch 2.62 g/L, peptone 1.51 g/L), initial pH 5.0, 1 ml of spore suspension ($1\times10^7$ spores/ml), initial moisture content 70%, incubation temperature 32°C, and fermentation period of 5 days. Under these conditions, the cellulase activity could reach 1.14 FPIU/gds using the strain SEMCC-3.248.

## ACKNOWLEDGEMENTS

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