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Full Length Research Paper

Improvement of acid protease production by a mixed culture of *Aspergillus niger* and *Aspergillus oryzae* using solid-state fermentation technique

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The synthesis of acid protease by *Aspergillus oryzae* AS3042 was enhanced significantly with the mixed culture of *Aspergillus niger* SL-09 using solid-state fermentation technique. The influence of carbon sources, nitrogen sources and the addition of phytic acid on acid protease production were investigated. The enzyme production varied depending on the nitrogen source. Apart from that, it was found that the enzyme activities formed in the mixed culture were enhanced dramatically by the addition of phytic acid to the medium. The response surface methodology (RSM) was used to optimize the medium composition for the production of acid protease and by this method the enzyme activity was enhanced near 5-fold higher than that obtained in the basal medium.

Key words: Acid protease, *Aspergillus niger*, *Aspergillus oryzae*, solid-state culture, optimization, response surface methodology (RSM).

INTRODUCTION

Proteases are used extensively in detergent, leather, pharmaceutical and food industries (Rao et al., 1998). Acid proteases (E.C.3.4.23.) are endopeptidases, with molecular masses in the range 30 to 45 kDa, which depend on aspartic acid residues for their catalytic activity and show maximal activity at low pH. Acid proteases find application in the production of seasoning materials, protein hydrolysates, fermentation of soy sauce and as digestive aids (Rao et al., 1998). Filamentous fungi are exploited for the production of industrial enzymes due to their ability to grow on solid substrate and produce a wide range of extracellular enzymes. Among them, *Aspergillus oryzae*, as generally regarded as safe (GRAS) status, is widely used and much work has been done to enhance the activities of the acid proteases from this fungus in solid-state fermentation, such as mutagenesis, optimization of the culture condition, identifying the genes which are coding for the acid proteases, among others (Datta, 1992; Vishwanatha et al., 2009; Vishwanatha et al., 2010; Katsuya et al., 1993; Hideyuki et al., 2002).

However, the reported activity levels of the acid proteases are still poor (Tari et al., 2006; Tremacoldi et al., 2004; Villegas et al., 1993; Lee et al., 2010) and it was reported that, proteases production by fungi was inducible, therefore, to further enhance the acid proteases synthesis and decrease substrate inhibition, it is necessary it decrease the fermentable protein hydrolysates in the medium. In this article, *Aspergillus oryzae* AS3042 and *Aspergillus niger* SL-9 were co-cultured in the submerged cultivation system for acid protease production. RSM, an efficient experimental strategy was used to optimize the media composition for increasing the acid proteases production.

MATERIALS AND METHODS

Microorganisms and media

A. oryzae AS3042, an active producer of acid proteases which was used in carrying out the primary experiments, was isolated from the soil. *A. niger* SL-09 were kindly presented by Dr. Ge, which was

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Table 1. Range and levels of experimental variables.

Factor	Level of factor		
	-1	0	1
Wheat bran (X_1, g)	2	4	6
Soybean flour (X_2, g)	4	6	8
Phytic acid (X_3, g/kg)	2	4	6

described previously (Ge et al., 2009). Both strains were grown on potato agar (Shen et al., 1999) slants at 30°C for 3 days and then stored at 4°C until use and were subcultured every month. Wheat bran and soybean flour were purchased from local market of Wuxi, China and were of commercial grade. Solid-state fermentation was carried out in 250 ml Erlenmeyer flasks which contained basal medium: wheat bran 4.0 g, soybean flour 6.0 g, distilled water 10 g, without pH control. All other chemicals used were of analytical grade.

Cultivation

Erlenmeyer flasks containing 20 g substrate were autoclaved at 121°C for 30 min and inoculated with 1 ml spore suspension containing 10^6 spores/ml of *A. oryzae* AS3042. The flasks were incubated at 30°C and 90% relative humidity (RH). The activity of the acid protease was checked at intervals of 24 h during 180 h of fermentation. Samples (10 g) of fermented material were mixed with 10 ml distilled water and pressed in a manual press; filtered through Whatman no. 1 filter paper and the liquid extract were kept at 4°C for enzymatic assays.

Effect of the inoculated *A. niger* SL-09 on enzyme production and growth performance of *A. oryzae* AS3042

Previous study found that the production of acid protease was inducible, therefore, to enhance the enzyme activity, a 1 ml of spore suspension of *A. niger* SL-09 (with spore of 10^6/ml) from the slant was inoculated to the enzyme-producing medium to exhaust the protease synthesis. The culture without the inoculation of *A. niger* SL-09 was used as a control.

Acid proteases production by *A. oryzae* AS3042 in medium containing phytic acid

In order to determine the effect of the addition of phytic acid to the medium on the production of acid proteases using *A. oryzae* AS3042, phytic acid was added to the basal medium in the concentration of 5 g/kg and the medium without phytic acid was used as a control. In order to investigate whether the addition of phytic acid to the medium was essential for the enhanced enzyme activities of acid proteasess, the phytic acid was added to the basal medium at different times (at the beginning and in 12-h intervals).
To determine whether the regulation of acid proteases synthesis in *A. oryzae* AS3042 occurs at transcription or translation level, actinomycin (a repressor of transcription) and cycloheximide (a repressor of translation) (Turner et al., 2002) were added to the basal and phytic acid-containing media after 24 h of fermentation with the concentration of 100 and 50 µg/g, respectively, after which the samples were periodically withdraw at the beginning and after 6 h intervals for the analysis of the enzyme activities.

Effect of additional nutrients on acid protease production

To determine the effects of various nutrients in the medium on acid proteases production, 4 g of various carbon sources, including wheat bran, glucose, sucrose and soluble starch were added separately to the basal medium. Meanwhile, 6 g of nitrogen sources including peptone, yeast extract, corn steep liquor, soybean flour, urea, $NH_4H_2PO_4$, $(NH_4)_2SO_4$ and NH_4Cl were also added to the basal medium to investigate their influence on the enzyme production. To study the effect of mineral salt solution on acid protease production in the medium, 5 ml of salt solution was added to the basal medium. The mineral salt solution comprised (in g/l): K_2HPO_4 1.0, $MgSO_4 \cdot 7H_2O$ 5.0, $CaCl_2 \cdot 2H_2O$ 1.0, $FeSO_4 \cdot 7H_2O$ 0.05 and $ZnSO_4 \cdot 7H_2O$ 0.05.

Analytical methods

The extracellular acid protease activity was determined using 2% hemoglobin (acid denatured) in glycine-HCl (pH 3.2, 0.1 M) as substrate, as reported earlier (Tello-Solis et al., 1994). In short, activity was determined by incubating 1 ml substrate with 400 µl of appropriately diluted enzyme solution for 10 min at 55°C. Enzyme activity was arrested by the addition of 2 ml of 5% TCA. The unhydrolyzed protein was removed by filtration (Whatman no. 1). Absorbance of the supernatant was measured at 280 nm. One unit (U) is defined to be the amount of enzyme that produced an increase in absorbance of 0.001/min under the state's conditions. Protein concentration was determined by Lowry's method (Lowry et al., 1951) using bovine serum albumin as the standard. The pH was measured by pH-meter. The free amine acid in the medium was determined by amino acid analyzer (Beckman 121MB). Biomass concentration determination in SSF processes was carried out by erghosterol determination method (Enric et al., 2005). Under this experimental condition, the erghosterol concentration in the mycelium was 4.89 mg/g.

Statistical analysis

The software package SAS version 8.0 was used for the design of RSM for optimization of medium composition and calculation of significant differences between groups of values and correlation coefficients as described by Ge et al. (2008). A three factors-levels experimental design was employed and the lowest and highest concentrations of the selected ingredients in the media were: wheat bran 2.0 and 6.0 g, soybean flour 4.0 and 8.0 g, phytic acid 2 and 6 g/kg, respectively (Table 1).

RESULTS

Enzyme production in the mixed culture

Figure 1 shows the acid protease production in the mixed culture of *A. oryzae* AS3042 and *A. niger* SL-09. It was found that the maximum acid protease activity of 2.75×10^5 U/ml was obtained, which was over 2-fold higher than that of the control. Further studies found that, in the mixed culture, the inoculation of *A. niger* SL-09 decreased the free total amine acid concentration significantly, which was lower than 1 from 5 g/l at 24 h of the culture. Therefore, the high performances in the co-cultured system may attribute to the synergetic effect between both strains.

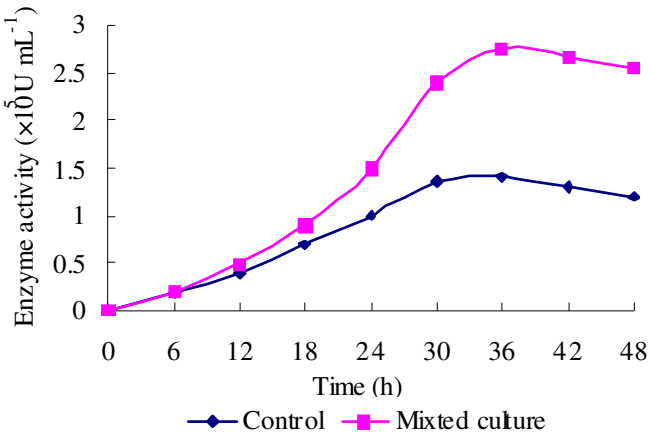

Figure 1. Acid protease production in mixed culture and the control. The experiments were incubated at 30°C. ~~°C for 48 h with mixed culture of *A. oryzae* AS3042 and *A. niger* SL-09 in the basal medium.~~

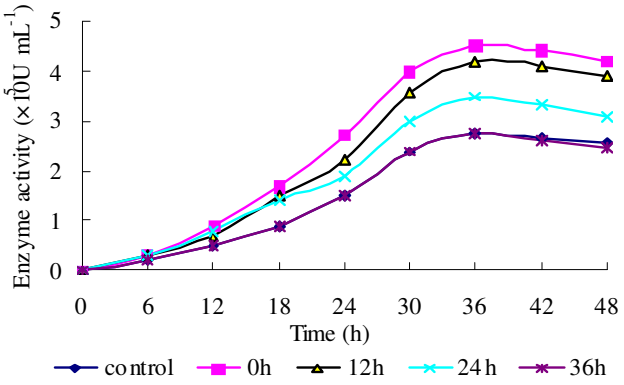

Figure 2. Effect of the addition of phytic acid at the beginning and after 12 h intervals of culture on acid protease production. The experiments were incubated at 30°C for 48 h for *A. oryzae* AS3042 in the basal medium with addition of phytic acid at various time.

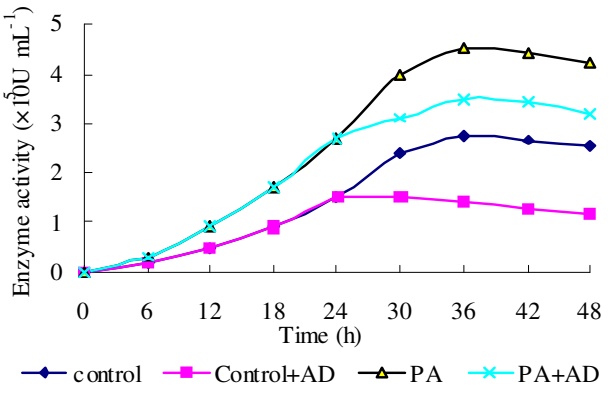

Figure 3. Effect of actinomycin D (AD) on the acid protease production in the control and phytic acid (PA) containing medium by the mixed culture. The experiments were incubated at 30°C for 48 h with mixed culture of *A. oryzae* AS3042 and *A. niger* SL-09.

Effect of phytic acid on enzyme activities

When phytic acid was added to the basal medium, the extracellular enzyme activities increased from 2.75×10^5 in the basal medium to 4.51×10^5 U/ml. The addition of phytic acid to the medium has very little effect on the cell growth. These observations lead to the conclusion that the acid protease is an inducible enzyme, rather than a constitutive one, which is similar to previous work reported by Vishwanatha et al. (2010).

As shown in Figure 2, only in the case of phytic acid supplementation at the beginning and after 12 h of the culture, enzyme activities of more than 4.0×10^5 U/ml for acid protease was obtained. The supplementation of phytic acid after 24 h of the beginning of the fermentation process led to an increase in enzyme activity to 3.50×10^5, but after 36 h it had very little effect on the acid proteases production. These observations indicated that the action of phytic acid on acid proteases production was almost complete within 36 h.

Effect of phytic acid on the regulation of acid proteases synthesis in *A. oryzae* AS3042

With the addition of actinomycin D, acid proteases biosynthesis in the basal medium was reduced dramatically and the synthesis of the enzyme nearly ceased. However, in the medium containing phytic acid, the acid proteases biosynthesis continued up to 24 h after the addition of actinomycin D and the acid proteases activity was increased up to 3.5×10^5 U/ml (Figure 3). This observation suggested that the amount of mRNA available for translation in the system of the medium containing phytic acid were higher than that in the system of basal medium. When cycloheximide was added instead of actinomycin D, acid proteases production decreased markedly in both basal and phytic acid containing media (data not presented).

The mentioned observation suggested that the regulation of acid protease synthesis in *A. oryzae* AS3042 occurs at both transcriptional and translational level and the enzyme synthesis was provoked by the addition of phytic acid at transcriptional level.

Effect of the additional nutrients on acid protease production

As presented in Figure 4, the wheat bran showed maximum enzyme yield, followed by soluble starch. Meanwhile, as the wheat bran was cheaper and locally abundantly available, it should be chosen as carbon source for acid proteases production.

Among the tested nitrogen sources, peptone was found

to be the best, followed by soybean flour and yeast extract (Figure 5). However, as the soybean flour was cheaper and locally abundantly available, it was chosen

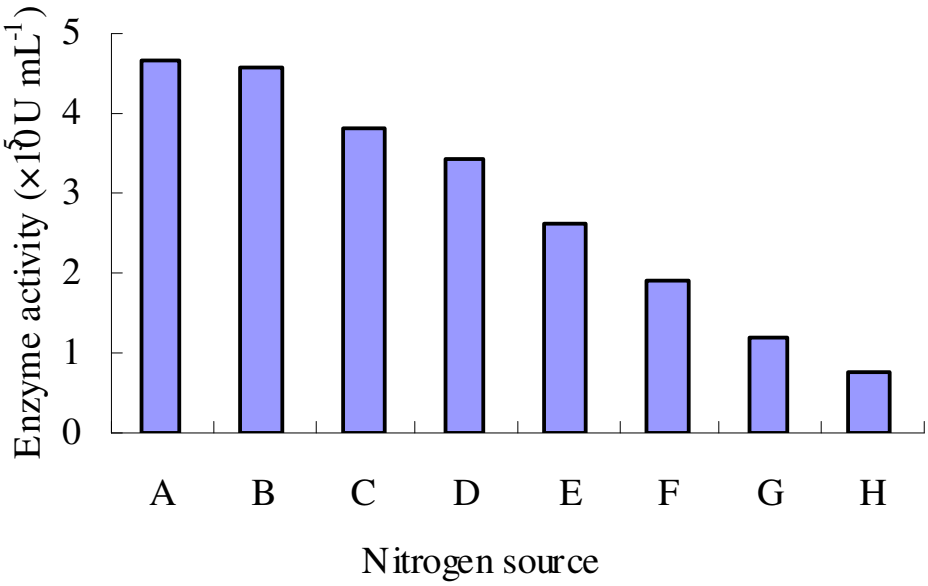

Figure 5. Effect of nitrogen sources on acid proteases production in the mixed culture. A, peptone; B, soybean flour; C, yeast extract; D, corn steep liquor; E, urea; F, $NH_4H_2PO_4$; G, $(NH_4)_2SO_4$; H, NH_4Cl. The nitrogen sources were added to the media the same (6 g).

Table 2. Experimental design and results of RSM.

Run	X_1	X_2	X_3	Enzyme activity/(U/ml)
1	0	-1	-1	2.19
2	0	-1	1	3.11
3	0	1	-1	3.18
4	0	1	1	5.44
5	-1	0	-1	2.95
6	-1	0	1	4.51
7	1	0	-1	4.43
8	1	0	1	5.44
9	-1	-1	-1	0.77
10	-1	1	1	2.87
11	1	-1	-1	3.26
12	1	1	1	6.22
13	0	0	0	5.82
14	0	0	0	6.01
15	0	0	0	5.91

as nitrogen source for acid proteases production. These results also show that, all inorganic nitrogen sources chosen in the experiment were not favorable for enzyme production. Studies also indicated that, enzyme activities were not enhanced significantly by the addition of mineral salt solution (data not presented). Therefore, among all the supplementary nutrients, phytic acid, wheat bran and soybean flour were found to be superior for higher enzyme production. Subsequently, experiments were conducted for optimization of these selected nutrients employing RSM.

Optimization of medium ingredients for acid proteases production using RSM

The average acid proteases activity obtained after 5 days of fermentation in 15 experiments of the chosen experimental design and results are shown in Tables 2 and 3. The factorial analysis of variance indicated that the

concentration of wheat bran (X1), soybean flour (X2), phytic acid (X3) and the second power of soybean flour (X22) all met the significant level of 0.05 of enzyme production by *A. oryzae* AS3042. A linear regression

Table 3. ANOVA of experimental result for enzyme activity.

Source	DF	SS	MS	F	Pr > F
Model	9	36.66	4.07	12.24	0.0066
X1	1	8.51	8.51	25.56	0.0039
X2	1	3.42	3.42	10.28	0.0238
X3	1	4.38	4.38	13.17	0.0151
X1 X2	1	0.25	0.25	0.75	0.427
X1 X2	1	0.076	0.076	0.23	0.6537
X2 X3	1	0.45	0.45	1.35	0.2979
X12	1	0.38	0.38	1.15	0.3332
X22	1	2.57	2.57	7.71	0.039
X32	1	0.82	0.82	2.46	0.1772

DF, Degree of freedom; SS, sum of square; MS, mean of square.

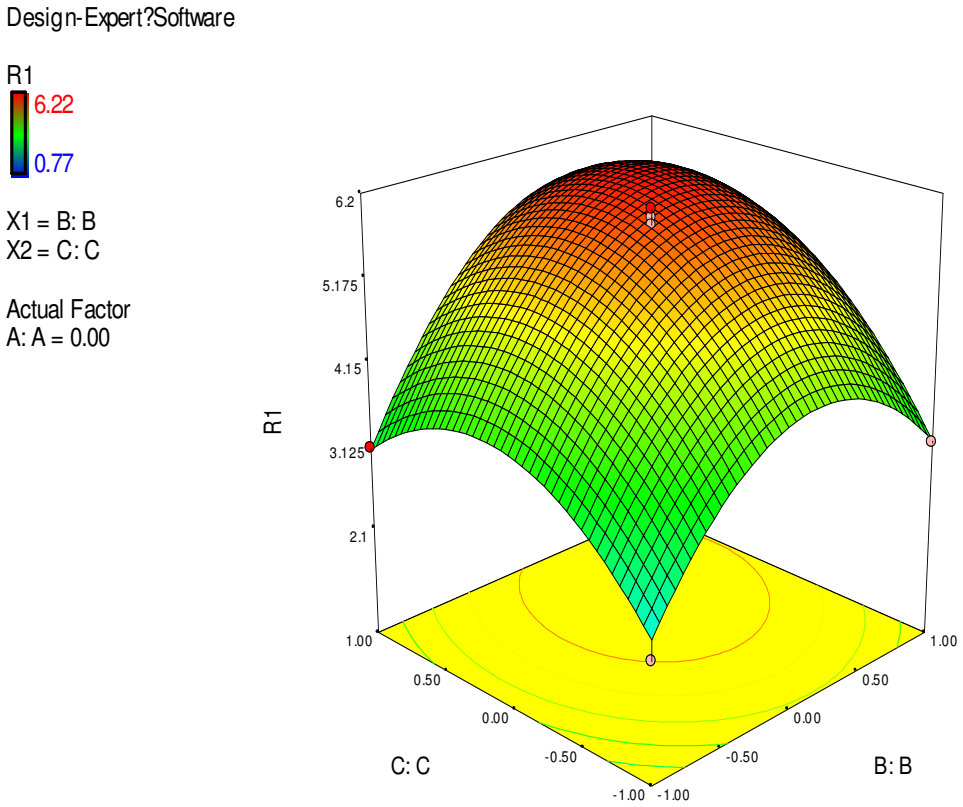

Figure 6. Response surface plot for the effect of soybean flour (X_2) and phytic acid (X_3) on acid protease production (fixed X_1 levels = 0).

equation could be obtained from the regression results of fractional factorial experiment:

Y = 5.91+1.03 X1+0.72 X2+0.66 X3+0.35 X1X2-0.14 X1X3+0.34 X2X3-0.53 X12-1.39 X22-1.05 X32

Where, Y is the measured response of acid proteases activity. The regression coefficients and determination coefficient (R2) for the linear regression model of enzyme production signified that the model was highly significant ($P < 0.01$) and R2 = 0.95.

The resulting response surface shows the effects of soybean flour and phytic acid concentration on enzyme production (Figure 6). From the stated results, the

economically optimized composition of fermentation medium was: wheat bran 4.40 g, soybean flour 6.40 g and phytic acid 4.40 g/kg. The maximum response predicted from the model was 6.29×10^5 U/ml; repeated experiments were performed to verify the predicted optimum. The results from the three replications (6.15×10^5, 6.36×10^5 and 6.28×10^5 U/ml for acid proteases activity) coincided with the predicted value and the model was proven to be adequate. Compared with the enzyme activity obtained in the basal medium (1.4×105 U/ml), the acid proteases activity was enhanced near 5-folder higher.

DISCUSSION

In order to enhance the acid proteases activity from filamentous fungi, much work has been done in solid-state fermentation system. Nevertheless, the low enzyme activity and difficult to scaled up inhibited it to be industrialized. Previous studies found that, mixed culture of *A. niger* and yeast improved the inulinase production of the fungi in the submerged fermentation (SMF) dramatically (Ge et al., 2009). For the first time, *A. oryzae* AS3042 and *A. niger* SL-09 were co-cultured to enhance the acid proteases synthesis. It was found that the inoculation of *A. niger* SL-09 enhanced the enzyme production significantly, which is mainly due to the synergetic effect between both strains. These results signified that mixed culture system was an effective strategy for inducible enzyme production in whatever the SSF or the SMF.

As a result of the data presented in this study, phytic acid is proved to be a very efficient activator for the transcription of mRNA available for acid proteases production. The phytic acid was first used as a significant factor to optimize the composition for acid protease production and the final extracellular enzyme activities in the optimized medium of *A. oryzae* AS3042 were enhanced more than 2-fold than those in the basal medium. Therefore, to further enhance the enzyme activities of acid protease in the solid-state fermentation, phytic acid, as a significant factor, should be taken into account.

RSM is an efficient experiment strategy to seek optimal conditions for a multivariable system. It has been successfully employed for the optimization of the medium composition and operating conditions in many bioprocesses (Ting et al., 2004). In this study, acid protease activity was enhanced to 6.29×10^5 U/ml after 36 h of fermentation using optimized medium, near 5 times higher than those in the basal medium.

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