SOYBEAN LECITHIN ENHANCED CELLULASE PRODUCTION BY *PENICILLIUM OXALICUM* JG IN A SCALED-UP BIOCONVERSION PROCESS

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The present research focuses on the production of cellulase from *Penicillium oxalicum* JG by optimizing the bioconversion process parameters and investigates the effects of different surfactants on the submerged fermentation process. Initially, cellulase production in shake flasks resulted in a cellulase activity of 0.22 FPU/mL. The optimization of process parameters increased the cellulase yield 1.7-fold and resulted in maximum cellulase activity of 0.366 FPU/mL within 7 days, with wheat bran and soybean meal selected as carbon source and nitrogen source at an initial pH of 1.5. Furthermore, the addition of soybean lecithin significantly enhanced cellulase production 2.19-fold and resulted in a maximum cellulase activity of 0.482 FPU/mL. The cellulase production was successfully scaled up to a 5 L stirred-tank bioreactor, the maximum cellulase activity reached 0.662 FPU/mL, while the addition of the soybean lecithin significantly enhanced the cellulase activity, reaching 1.08 FPU/mL. A similar cellulase titer can be obtained with shortened cultivation time from 7 to 3 days. The addition of low-cost soybean lecithin provides a great potential for its application in cellulase production.

Keywords: cellulase, *Penicillium oxalicum* JG, lecithin, bioreactor

INTRODUCTION

As agricultural and forest by-products, biodegradable lignocellulosic wastes from plants contain 40-50% cellulose and can be regarded as a potentially sustainable carbon source for renewable biofuels.[1] The key step for the utilization of cellulose is its enzymatic hydrolysis into monomeric sugars and subsequent conversion into valuable fine chemicals and energy.[2,3] Cellulase is a multi-enzyme complex that is mainly composed of endo-glucanase, exo-glucanase and β-glucosidase (BGL) acting synergistically during enzymatic hydrolysis. At present, almost all commercially available cellulases are mainly derived from mutant strains of *Trichoderma reesei*. However, the lower ratio between BGL activity and filter paper cellulase (FPase) activity of *T. reesei* results in feedback inhibition from higher accumulation of cellobiose during hydrolysis. Also, *T. reesei* might have obvious disadvantages on some important parameters, such as protein production level and cellulase hydrolytic performance per unit of activity or milligram of protein.[4,5] Therefore, other filamentous fungi, including *Penicillium*, *Aspergillus*, *Chrysosporium* and *Acremonium* species, have been tested as alternatives to *T. reesei* for the production of cellulases.[6] Several strains of *Penicillium* spp. have been reported to produce a better cellulase titer, higher BGL/FPA (FPase activity), and other necessary enzymatic activities.[7]

To enhance enzyme production and decrease the cost of bioconversion, the process parameters should be optimized for achieving a maximal enzyme-producing potential. The response surface methodology (RSM) method has been successfully employed for optimizing bioprocess parameters to enhance enzymatic hydrolysis, enzyme production *etc*.[8,9] Surfactants also have a wide range of applications for research and for the industrial production of cellulases.[10-12] They can enhance cellulose degradation and improve the

efficiency of cellulase production and stability.[13-15] However, most chemically synthesized surfactants, such as Tween-20, Tween-80, Triton X-100 and polyethylene glycol (PEG), are not biodegradable. Biosurfactant rhamnolipid can noticeably increase cellulase production and activities, but its high cost limits its application.[16,17] Lecithin is a natural biodegradable surfactant and additive, which has the advantages of amphiphilicity, low toxicity and low cost. It is widely used in the food and pharmaceutical industries,[18] but little information is available on its application for large-scale cellulase production.

In addition, even though cellulase is critical for cellulose degradation, its utilization might be restricted because of the high crystallinity of the cellulose structure, its compactness, insolubility in water and lack of adsorption to cellulase. At an industrial level, the cellulose substrate is usually pretreated by acid-base or steam explosion methods to make the structure loose and dispersed. This study optimized the fermentation process parameters with regard to growth substrates and initial pH using RSM, and discussed the influences of surfactant addition and acidification pretreatment on the enzyme production. The optimized process of cellulase production was further tested in a scaled-up bioreactor.

EXPERIMENTAL

Fungal strain and culture conditions

Penicillium oxalicum JG was maintained and sub-cultured on potato dextrose (PDA) plates (agar 20 g/L) at 28±1 °C and the culture sporulated fully after 5 days.[19] Spores were obtained by dislodging them from the surface of fully grown *Penicillium oxalicum* JG using a PDA slant into sterile 0.05% Tween-80 solution. Spores were counted on a haemocytometer and 10^8 spores/100 mL were inoculated into the shake flasks for enzyme production.

Analytical grade polyethylene glycol (PEG), rhamnolipid and soybean lecithin were purchased from J&K Scientific Ltd. (China).

Enzyme production in shake flasks

The medium used for initial shake-flask fermentation was composed of (per litre): 30 g of wheat bran, 3 g of soybean meal, 2 g of KCl, 2 g of KH_2PO_4. Enzyme production was carried out in 250-mL Erlenmeyer flasks, which were incubated at 28 °C under shaking at 200 rpm for 7 days.

Enzyme activity assay

FPase activity (FPA) was assayed by incubating the diluted enzyme (0.2 mL) with 1.8 mL HAc-NaAc buffer (0.1 M, pH 4.8), containing Whatman No. 1 filter paper (50 mg, 1 x 6 cm). The reaction mixture was incubated at 50 °C for 60 min. BGL activity was estimated using PNPG (*p*-nitrophenyl-D-glucopyranoside) as substrate. The total of the assay mixture (1 mL), consisting of 0.8 mL of PNPG (1 mg/mL) and 0.2 mL of suitably diluted enzyme, was incubated at 50 °C for 30 min. The p-nitrophenol liberated was measured at 410 nm after adding 2 mL of sodium carbonate (2%). The total reducing sugars as glucose equivalents were analyzed by the 3,5-dinitrosalicylic acid method, as described by Miller (1959).[20]

One unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of glucose or p-nitrophenol produced from the appropriate substrates per min of crude filtrate under standard assay conditions.

Statistical optimization of conditions for cellulase production using RSM

The second order Box–Behnken design (BBD) was employed for optimizing the conditions for cellulase production.[21] The concentrations of wheat bran (%, w/v) and soybean meal (%, w/v) and the initial pH were selected as independent variables at three different levels based on the results of the single-factor analysis method (not shown). Design-Expert software version 8.0 (Stat Ease Inc., Minneapolis, MN, USA) was used to build and analyze the experimental design by subjecting the data to the analysis of variance (ANOVA), determining the estimated effects and interactions, and fitting the regression equation to the data, as described by Saini *et al*.[22] A second-order polynomial (Eq. (1)) was used to describe the effects of variables on the response:

$$Y_1=b_0 +b_1A+b_2B+ b_3C+b_{11}A_2 +b_{22}B_2+b_{33}C_2 +b_{12}AB+ b_{13}AC+b_{23}BC \quad (1)$$

where Y_1 is the response variable, b_0 is a constant term, b_1, b_2 and b_3 are the monomial coefficients; b_{11}, b_{22} and b_{33} are the squared coefficients; b_{12}, b_{13} and b_{23} are the interaction coefficients.

Surfactant addition tests

For investigating the effect of surfactant addition on enzyme production, several surfactants, including EDTA-2Na (2 g/L), SDS (2 g/L), PEG (5 g/L), rhamnolipid (1 g/L) and lecithin (5 g/L), were added to the optimized medium. Enzyme production was carried out in 250-mL Erlenmeyer flasks, which were incubated at 28 °C under shaking at 200 rpm for 7 days.

Pretreatment of wheat bran

The pretreatment of cellulose is one of the most important factors that affect the catalytic efficiency of cellulase. Therefore, wheat bran was pretreated with 12 g/L lecithin and 12 g/L lecithin with muriatic acid for 3

hours. Then, the flasks were incubated at different temperatures (28 °C, 36 °C, 40 °C), separately, under shaking at 200 rpm for 144 hours, for determining the optimum temperature of the hydrolysis process. Each FPA and the total reducing sugars were assayed.

Enzyme production in a 5-L stirred-tank bioreactor

The medium used for enzyme production in a 5-L stirred-tank bioreactor (HG-1010-5L, Beijing Baiwan Electronic Technology, China) had a similar composition, as described above. Sterilization of the bioreactor vessel with enzyme production medium was performed in an autoclave at 121 °C for 30 min. The temperature was maintained at 28 °C, and the pH was controlled at 2.0 by addition of 1 M HCl or 1 M NaOH. Spores were cultivated in seed medium for 48 h, and inoculated into the fermentation broth at the rate of 10^9 spores/L and airflow of 1 vvm. Pitch blade impellers were used to maintain an agitation speed of 200. Antifoam (2%, v/v) was autoclaved separately and approximately 0.5 mL was added manually during frothing. Sampling was performed every 12 h for analysis of cellulase activities. Extracellular enzyme was extracted by centrifugation of the sample at 8000 rpm for 10 min.

RESULTS AND DISCUSSION

Statistical optimization of cellulase production under submerged fermentation (SmF) using RSM

Penicillium oxalicum JG initially produced maximum FPA of 0.22 FPU/mL. Considering the cellulase activities need to be enhanced further for biofuel applications, RSM was adopted for further optimization at a shake flask level for improving the cellulase enzyme production. Design Expert (version 8.0) was employed for designing the experimental layout with a total of 17 experiments according to the Box-Behnken design (BBD), with three different variables at three different levels, namely, lower, middle and upper (-1, 0, +1). Table 1 shows the different independent variables and the cellulase activity (FPU/mL) responses. The data obtained from the BBD matrix yielded the following regression equation (Eq. (2)), which represents the empirical relationship between cellulase activity (FPU/mL) and the significant test variables in coded form:

$$Y = +0.31 + 0.05A + 8.750X10^{-3}\,B - 0.011C - 0.018AB + 0.015AC + 1.000X10^{-3}\,BC - 0.048A^2 - 0.028B^2 - 2.550X10^{-3}\,C^2$$

where the coded variables were: Y = cellulase activity (FPU/mL); A = wheat bran concentration (%, w/v); B = soybean meal concentration (%, w/v) and C = pH.

The results for cellulase production predicted by the Design Expert software were fitted to a second-order model. The model was found 'significant' with P value < 0.0001 and a 'non-significant' lack of fit value (Table 2). ANOVA of the model for cellulase production showed validity of the predictions in terms of p-value (<0.0001) and the coefficient of determination (R2) value of 0.9805, the adjustment coefficient (adjusted R2) of 0.9554. Contour plots for cellulase (FPU/mL) production were generated by using the coefficients of the polynomial equation. In this case, factors A (wheat bran concentration), B (soybean meal concentration), and C (pH) and the interactions between AB and AC were significant model terms.

Three-dimensional response surfaces (Fig. 1) were plotted for each dependent variable, considering the interaction between two most important independent variables. Figure 1a shows the interaction of wheat bran concentration and soybean meal concentration to the cellulase (FPU/mL). At a certain pH, cellulase (FPU/mL) increased with increasing concentration of wheat bran up to 45-48 g/L, then a downward trend occurred with higher concentration of wheat bran. The concentrations of wheat bran and soybean meal determine the C/N ratio of the medium, which affects strain growth and enzyme production. Figure 1b depicts the interaction of wheat bran concentration and pH. Cellulase production increased with increasing concentration of wheat bran at lower pH (Fig. 1b), but the interactions between soybean meal concentration and pH have less effect on the response values (result was not given). Based on the ANOVA analysis, the influence of the above three factors on the response values was as follows: A (carbon source) > C (pH) > B (nitrogen source). Therefore, the optimal culture conditions for maximum cellulase production of 0.384 FPU/mL were predicted as: 46.9 g/L wheat bran and 6.33 g/L soybean meal and initial fermentation pH of 1.5 with 2.0 g/L KH_2PO_4 and 2.0 g/L KCl. Furthermore, BGL activity increased by 1.08 IU/mL, while it was 0.806 IU/mL initially. Several strains of *Penicillium* spp. have been reported to produce comparable or even better cellulase titer, along with higher BGL, as well as other necessary enzyme activities.[23] The validation experiment performed in a shake flask under these conditions resulted in cellulase activity of 0.366 FPU/mL in 7 days. It represented

a 1.7-fold increase in cellulase enzyme activity when compared to the cellulase production under non-optimized conditions. Previous studies reported that process optimization resulted in 4.7-fold enhanced cellulase activities from *T. reesei* RUT C-30 in solid state fermentation and 1.6-fold (84%) enhanced cellulase activities from *Penicillium sp*. CLF-S.[24,25] This proved an overall good agreement between the experimentally determined (observed) response and the optimum response predicted through the model.

Table 1
Three level Box–Behnken design and the experimental response of dependent variable cellulase activity (FPU/mL) of *P. oxalicum* JG*

Run no.	Wheat bran concentration (g/L)	Soybean meal concentration (g/L)	pH	Cellulase activity (FPU/mL)
1	42(0)	6(0)	2.25(0)	0.311
2	42(0)	6(0)	2.25(0)	0.310
3	42(0)	8(1)	1.50(-1)	0.295
4	42(0)	6(0)	2.25(0)	0.300
5	28(-1)	8(1)	2.25(0)	0.209
6	28(-1)	6(0)	3.00(1)	0.176
7	28(-1)	4(-1)	2.25(0)	0.163
8	42(0)	4(-1)	3.00(1)	0.263
9	42(0)	8(1)	2.25(0)	0.290
10	56(1)	8(1)	2.25(0)	0.271
11	42(0)	4(-1)	1.50(-1)	0.272
12	56(1)	4(-1)	2.25(0)	0.297
13	56(1)	6(0)	3.00(1)	0.309
14	56(1)	6(0)	1.50(-1)	0.315
15	42(0)	6(0)	2.25(0)	0.320
16	28(-1)	6(0)	1.50(-1)	0.242
17	42(0)	6(0)	2.25(0)	0.312

Experimental cellulase activity (FPU/mL) is the mean of triplicate experiments;
*Values in parentheses represent the levels of independent variables in coded form

Table 2
ANOVA for response surface quadratic model for cellulase production using *P. oxalicum* JG

Source	Sum of squares	Degree of freedom (df)	Mean square	F-value	P-value Prob>F
Model	0.03800	9	0.0041	39.05	<0.0001**
A	0.02000	1	0.0200	188.87	<0.0001**
B	0.00061	1	0.0006	5.73	0.0480*
C	0.00092	1	0.0009	8.64	0.0217*
AB	0.00129	1	0.0013	12.12	0.0103*
AC	0.00090	1	0.0009	8.41	0.0230*
A^2	0.00952	1	0.0095	89.01	0.0001**
B^2	0.00331	1	0.0033	30.97	0.0008*
Residual	0.00074	7	0.0001		
Lack of fit	0.00005	3	0.0001	3.58	0.1249
Pure error	0.0002032	4	0.00005		
Total	0.38	16			

Values of "Prob > F" less than 0.0500 indicate model terms are significant; *significant; **highly significant

Table 3
Effect of surfactant on cellulase activity by P. oxalicum JG

Surfactants	FPA (FPU/mL)	BGL activity (IU/mL)
Not added	0.366 ± 0.017	1.081 ± 0.012
EDTA-2Na	0.167 ± 0.011	0.218 ± 0.009
SDS	0.181 ± 0.020	0.436 ± 0.018
PEG	0.396 ± 0.009	0.982 ± 0.010
Lecithin	0.482 ± 0.021	1.197 ± 0.015
Rhamnolipid	0.464 ± 0.014	1.243 ± 0.020

Results are presented as the mean (n=3) ± SD

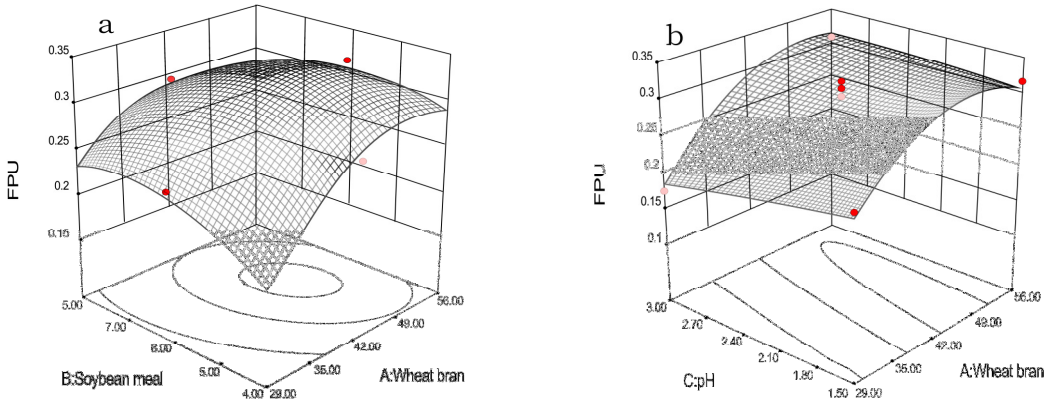

Figure 1: Three-dimensional response surface plots showing (a) the effect of wheat bran and soybean meal concentration and (b) the effect of wheat bran and initial pH on cellulase production (FPU/mL) by *P. oxalicum* JG under submerged fermentation (other factors in the plots were at their respective middle levels)

Lecithin addition effect on cellulase production

The addition of each surfactant, including EDTA-2Na, SDS, PEG, rhamnolipid and lecithin, showed diverse effects on the cellulase production from *Penicillium oxalicum* JG (Table 3). The EDTA-2Na and SDS additions showed obvious inhibitory effects on FPA and BGL activity, while PEG addition performed a promoting effect on FPA. As expected, it was concluded in this study that rhamnolipid promoted the cellulase production to the maximal value of 0.464 FPU/mL, while BGL activity increased by 1.243 IU/mL. This is consistent with the observations from several previous studies that rhamnolipid promotes the cellulase activities.[26,27] Wang *et al*. also observed that the stimulatory effects of rhamnolipid are superior to those of Triton X-100 on cellulase activities from *Penicillium expansum*.[28] It must also be mentioned that soybean lecithin addition increased the FPA to its highest observed value of 0.482 FPU/mL, which was 33.3% higher than the optimized RSM value and 119% higher than basal medium, BGL activity also increased by 10.7% (1.197 IU/mL). Lecithin is also a zwitterionic biosurfactant; J. W. Park noted that cellulase can easily combine with the functional groups of amphiphilic substances to improve the enzyme stability and solubility.[29] Considering a series of advantages of lecithin, such as its low cost, accessibility and purity, the addition of lecithin has promising potential for application in the industrial production of cellulase by *P. oxalicum*.

Efficiency of pretreatment with lecithin addition on the reducing sugar production

Previous data have shown that the physicochemical pretreatment of cellulose is beneficial to cellulase production.[30] Acidic conditions can promote the combination of surfactant with the substrate and improve the efficiency of degradation, thus further improving the enzyme activity.[31] Pretreatment of wheat brans with soybean lecithin under acidic and non-acidic conditions was carried out, using only the addition of soybean lecithin as control. As shown in Figure 2a, the yield of reducing sugar produced by wheat bran with acidic pretreatment was about

5 times higher than that obtained without acid pretreatment.

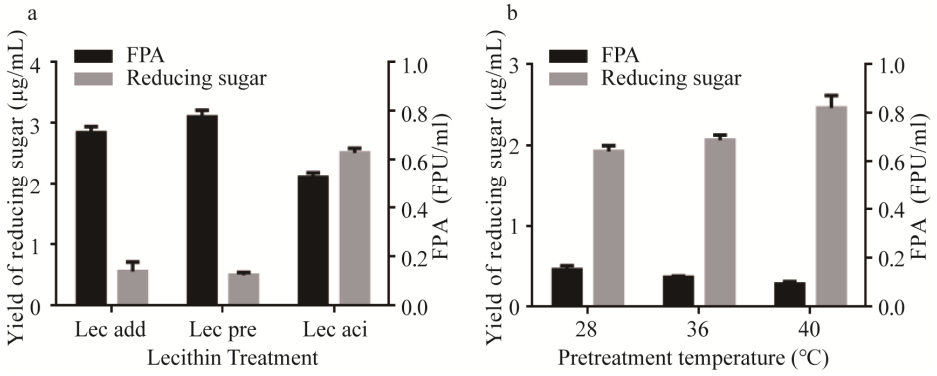

Figure 2: Effect of pretreatments on wheat bran cellulose degradation; a) effect of lecithin treatment (Lec add: lecithin addition, Lec pre: lecithin pretreatment, Lec aci: lecithin acidic pretreatment), b) effect of pretreatment temperature

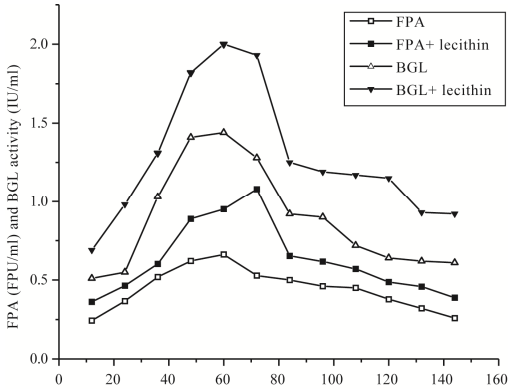

Figure 3: Cellulase production by *P. oxalicum* JG in a bioreactor under optimized conditions (enzyme activity after adding lecithin)

The reason might be that the acidification pretreatment can loosen the structure of cellulose molecules, so that the surfactant enables the cellulase to approach the surface of the cellulose molecule, then adapts to perform the degradation function, and increases the yield of reducing sugar. However, the repressive effect of reducing sugar products results in lower cellulase activity.

The effect of pretreatment temperature was also studied by performing the pretreatment of wheat bran with lecithin at 28 °C, 36 °C and 40 °C for 3 h, followed by the reducing sugar assay. The production of reducing sugar increased gradually with increasing temperature. The maximum yield of reducing sugar (2.78 μg/mL) was observed at 40 °C, but the enzymatic activities declined gradually because of the repression from glucose production (Fig. 2b).

Cellulase production in a bioreactor

Penicillium oxalicum is a highly aerobic filamentous fungus, and a bioreactor can better supply the desired pH and dissolved oxygen requirements for *Penicillium* growth. Therefore, based on the RSM-optimized liquid fermentation, a combination of pretreatments of wheat bran, with or without the addition of soybean lecithin, was used for scaled-up fermentation in a 5-L stirred-tank bioreactor. Figure 3 shows that FPA started increasing after 12 h, the maximum value of 1.08 FPU/mL was reached at 72 h with soybean lecithin addition, while without soybean lecithin addition, the value of 0.662 FPU/mL was obtained. This fermentation is significantly higher and faster than the cellulase activity of 0.35 FPU/mL reported for *P. funiculosum* with the duration of 170-230 h for optimum enzyme

production.[32] The cellulase activities obtained in this study were higher than those reported recently for *P. pinophilum* (0.18 FPU/mL), *C. cubensis* (0.39 FPU/mL) and *A. awamori* (0.42 FPU/mL).[33,34] BGL activities also started increasing after 12 h and continued till 60 h, reaching a maximum of 2.00 IU/mL with soybean lecithin addition, and a value of 1.441 IU/mL without soybean lecithin. Therefore, adding lecithin can obviously improve cellulase production. Moreover, the use of a reactor revealed a decrease in incubation time from 144 h in shaking flasks to 60-72 h to obtain similar cellulase enzyme titers. Even so, there is room for improvement in the enzyme yields, comparable to previous reports of 4.5 FPU/mL, employing a mutant strain *P. janthinellum* EMS-UV-8.[35]

Compared with the optimized shake flask fermentation, with soybean lecithin addition, the maximum FPA and BGL increased by 224% and 160%, respectively. However, the value of cellulase activity began to drop after 72 h of submerged fermentation, and the rapid enzyme production led to a shortage of the lignocellulose substrate; the alternative process of continuous feeding remains a subject for further exploration. Future work will also focus on enhancing the BGL/FPA ratio to reduce enzyme inhibition caused by lesser cellobiose accumulation during hydrolysis.

CONCLUSION

Soybean lecithin proved its efficiency in cellulase production by *P. oxalicum* JG. When scaled-up to a 5 L stirred-tank bioreactor, the addition of soybean lecithin significantly enhanced the cellulase production 4.9-fold, while reducing the incubation time from 7 to 3 days to obtain similar cellulase enzyme titers. As an inexpensive, accessible and biodegradable additive, soybean lecithin appears to be lucrative by further optimizing the culture and nutritional parameters to improve the cellulase production. Therefore, the soybean lecithin proves to be a good candidate for biomass hydrolysis for bioethanol applications.

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