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Selection of nutrient parameters for endoglucanase production from rice bran by *Penicillium* sp. using Plackett–Burman design

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Abstract

Cellulose is the most abundant renewable agricultural material on earth. Endoglucanase is well known for bioconversion of cellulose by cleaving intramolecular β-1,4-glycosidic bond randomly to useful products such as oligomers and reducing sugar. Various substrates/byproducts and microbial cultures have been used successfully in solid state fermentation (SSF) for endoglucanase production. Plackett-Burman design is well established and widely used for screening important medium components affecting the production of valuable microbial products. The aim of this study was to use Plackett-Burman design for screening of significant medium components for endoglucanase production. The significance of fourteen medium components was determined. The experiments were conducted at 30°C for 4 days with 70% initial moisture content. Initial pH at 7.0 and 10^6 spores/g rice bran of fungal inoculum were allowed in SSF under static condition with 5.0 g rice bran as a substrate. From the results, the highest endoglucanase activity was 17.35 units/g rice bran. Eight components namely yeast extract, KH_2PO_4, peptone, $(NH_4)_2SO_4$, carboxymethyl cellulose (CMC), cellobiose, $ZnSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot H_2O$ revealed a significant influence on endoglucanase production. However, only five components were selected for further optimization since cellobiose, $ZnSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot H_2O$ provided negative effect on endoglucanase production by *Penicillium* sp.

Keywords: endoglucanase, Plackett-Burman design, solid state fermentation, *Penicillium* sp.

1. Introduction

Agricultural materials are major renewable resources on earth since they are produced in large amounts worldwide (1). Cellulose is the most abundant agricultural material which is mainly degraded by cellulases, the enzymes well known for releasing valuable products including glucose and other reducing sugars from cellulosic wastes. Cellulases can be divided into 3 groups based on their activity: endoglucanase (EC 3.2.1.4), exoglucanase or cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). Endoglucanase randomly breaks down intramolecular β-1,4-glycosidic bond of cellulose, resulting in shorter polymer chains such as oligomers and reducing sugars, which will be further hydrolyzed by exoglucanase and β-glucosidase (2).

When entire hydrolysis processes take place, glucose (an important substrate for the fermentation of numerous valuable products) is produced.

Endoglucanase has broad applications in cellulose recycling as well as industrial and commercial purposes (3). This enzyme is mainly produced by fungi. Microorganisms mostly reported as producers of endoglucanase and other two cellulases were members of the genera *Trichoderma, Aspergillus*, *Fusarium, Penicillium* and *Humicola* such as *Trichoderma reesei, T. harzianum, T. viride, Aspergillus niger*, etc. (4). Most processes of endoglucanase production involved solid state fermentation (SSF) rather than submerged fermentation (5). Since commercial endoglucanase is still relatively high cost (6), strategies for effective bioproduction of endoglucanase is highly required.

Plackett-Burman design is a statistical design proposed by Plackett and Burman in 1946. It works at two levels, low and high. This design estimates linear effects of all factors for a given number of observations. It had long been applied for screening of medium components which significantly influence the production of valuable fermentation products. With Plackett-Burman design, the number of experiments needed to be conducted can be decreased since only significant parameters are selected. However, few studies on the application of Plackett-Burman design for screening of medium components for endoglucanase production by *Penicillium* spp. have been reported (7).

The aim of this study was to use Plackett-Burman design for screening of significant medium components for endoglucanase production by *Penicillium* sp.

2. Materials and Methods

Microorganism

The fungus strain SM3, eventually identified as *Penicillium* sp., was used in this study. It was isolated from soil samples collected from Silpakorn University, Nakhon Pathom, Thailand. The culture was maintained on Potato Dextrose Agar (PDA) slant at 4°C with monthly sub-culturing.

Materials

Rice bran was obtained from a local market in Nakhon Pathom, Thailand. Yeast extract was purchased from Lab M Limited, UK. KH_2PO_4 was purchased from RANKEM; RFCL Limited, India. $MgSO_4 \cdot 7H_2O$ was provided by Fisher Chemicals; Fisher Scientific, UK. $CaCl_2$ was obtained from Chem X; UCARE pharma solution, USA. Other chemicals were purchased from Fluka chemika, Switzerland and UNIVAR; Ajax Finechem, Australia.

Inoculum and cultivation

Fully sporulated culture was used for an inoculum preparation. Five ml of sterilized distilled water supplemented with 0.1% (v/v) Tween 80 were added into agar slants containing the required culture to make spore suspensions approximately 10^8 spores/ml. This spore suspension was used as an inoculum for endoglucanase production.

Solid state fermentation (SSF) of endoglucanase was carried out in 250 ml Erlenmeyer flasks that contained 5.0 g of rice bran as a substrate, 1 ml of spore suspension and 10.7 ml of medium solution comprising twenty combinations of ingredients designed in Table 2. The value of high and low levels (designated as (+) and (-) respectively) of each variable was described in Table 1. Under the mentioned compositions, initial moisture content of fermentation medium was around 70% and the inoculum size was approximately 10^6 spores/g rice

bran. Then, the medium pH was adjusted to 7.0 and fermentation was allowed under a static condition at 30°C for 4 days.

Endoglucanase assay

The extraction of endoglucanase was performed at 4 days by adding 50 ml of sodium citrate buffer (50 mM, pH 4.8) into each flask. Then, the flasks were kept at 30°C for 30 min under stirring condition (200 rpm). The slurry was filtered through Whatman no. 1 filter membrane and centrifuged at 10,000 rpm for 15 min at 4°C. The collected extract was used for endoglucanase assay.

Endoglucanase activity was measured following the method described by Ghose (8) which determined the amounts of reducing sugars released from CMC. One ml of the extract was incubated with 1 ml of 2% CMC in sodium citrate buffer (50 mM, pH 4.8) at 50°C for 30 min. The reducing sugar produced was assayed by dinitrosalicylic acid (DNS) method (9) using D-glucose as a standard. One unit (IU) of endoglucanase activity was defined as the amount of enzyme which liberates 1 μmol of glucose equivalent per minute under the specified condition.

Screening of important components using Plackett-Burman design

In this study, Plackett-Burman design was used to determine the importance of fourteen medium components on endoglucanase production by *Penicillium* sp. when rice bran was used as a substrate. The components were applied at two levels, low and high. The low level was designated as (-) while the high level was designated as (+). The amount of each level for each variable studied was presented in Table 1. According to Plackett-Burman design, twenty combinations of medium components were organized (10) (Table 2). All trials were performed in triplicate. Five dummy variables were included to increase the reliability of the results.

Table 1. Nutrient concentrations at low and high levels in Plackett-Burman design for endoglucanase production.

Variable No.	Variables with designate		Low level (-)	High level (+)	Unit
1	X1	Yeast extract	0.1	1.50	% w/v
2	X2	KH_2PO_4	0.0	0.20	% w/v
3	X3	$CaCl_2$	0.0	0.03	% w/v
4	X4	$MgSO_4 \cdot 7H_2O$	0.0	0.03	% w/v
5	X5	Peptone	0.0	0.08	% w/v
6	X6	Urea	0.0	0.03	% w/v
7	X7	$(NH_4)_2SO_4$	0.0	0.14	% w/v
8	X8	CMC	0.0	1.00	% w/v
9	X9	Cellobiose	0.0	0.05	% w/v
10	X10	$FeSO_4 \cdot 7H_2O$	0.0	0.0005	% w/v
11	X11	$CoCl_2$	0.0	0.0020	% w/v
12	X12	$MnSO_4 \cdot H_2O$	0.0	0.0002	% w/v
13	X13	$ZnSO_4 \cdot 7H_2O$	0.0	0.0001	% w/v
14	X14	Tween 80	0.0	0.20	% v/v

2. Results and Discussion

Endoglucanase activities of *Penicillium* sp. from different combinations of medium compositions were revealed in Table 2. The highest endoglucanase activity at 17.35 units/g rice bran was obtained when the medium components were supplied at the levels revealed in medium solution 6. However, the medium solution which provided the lowest endoglucanase activity was not the solution 20, although it contained no medium ingredient other than rice bran. The results indicated that rice bran alone provided some necessary nutrients needed by the fungus and that nutritional requirement of the fungus was not very complex. On the other hand, the addition of improper types and amounts of medium components could lead to an adverse effect on endoglucanase production. From Table 2, the medium solution 15 showed the lowest endoglucanase activity although it consisted of many nutrient ingredients. Thus, the selection of significant medium components for endoglucanase production by an efficient method is necessary.

Table 2. Plackett-Burman design of 14 independent variables (X1, …, X14) and 5 dummy variables (D1, …., D5) for endoglucanase production by *Penicillium* sp.

Medium solution	X 1	X 2	X 3	X 4	X 5	X 6	X 7	X 8	X 9	X 10	X 11	X 12	X 13	X 14	D 1	D 2	D 3	D 4	D 5	Endo-glucanase Activity (Unit/g rice bran)
1	+	-	+	+	-	-	-	-	+	-	+	-	+	+	+	+	-	-	+	10.67±0.28
2	+	+	-	+	+	-	-	-	-	+	-	+	-	+	+	+	+	-	-	14.00±1.74
3	-	+	+	-	+	+	-	-	-	-	+	-	+	-	+	+	+	+	-	12.21±2.31
4	-	-	+	+	-	+	+	-	-	-	-	+	-	+	-	+	+	+	+	10.42±1.18
5	+	-	-	+	+	-	+	+	-	-	-	-	+	-	+	-	+	+	+	16.22±0.85
6	+	+	-	-	+	+	-	+	+	-	-	-	-	+	-	+	-	+	+	17.35±0.79
7	+	+	+	-	-	+	+	-	+	+	-	-	-	-	+	-	+	-	+	13.22±2.24
8	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-	+	-	+	-	17.18±0.37
9	-	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-	+	-	+	10.49±0.45
10	+	-	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-	+	-	10.75±1.67
11	-	+	-	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-	+	12.74±2.19
12	+	-	+	-	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-	14.57±3.10
13	-	+	-	+	-	+	+	+	+	-	-	+	+	-	+	+	-	-	-	10.32±0.60
14	-	-	+	-	+	-	+	+	+	+	-	-	+	+	-	+	+	-	-	10.82±1.03
15	-	-	-	+	-	+	-	+	+	+	+	-	-	+	+	-	+	+	-	8.89±0.22
16	-	-	-	-	+	-	+	-	+	+	+	+	-	-	+	+	-	+	+	9.27±1.50
17	+	-	-	-	-	+	-	+	-	+	+	+	+	-	-	+	+	-	+	11.36±1.69
18	+	+	-	-	-	-	+	-	+	-	+	+	+	+	-	-	+	+	-	10.71±0.37
19	-	+	+	-	-	-	-	+	-	+	-	+	+	+	+	-	-	+	+	10.89±0.59
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10.38±1.91

Note: The values of endoglucanase activity given above are averages of three experiments. Symbols + and - refer to high and low levels of each variable, respectively.

Significance of each variable was determined. The components were screened at 95% confidence level and the output was analyzed based on effects of the components on endoglucanase activity. Table 3 represented the results of Plackett-Burman experiments with respect to main effect, *t*-value and *p*-value of each component. The *p*-value is considered as a criterion for making a decision whether the component is significant. The *p*-value of less than 0.05 indicates the significance of the parameter on interested response (11).

Out of fourteen medium components tested, eight showed *p*-values smaller than 0.05, implying that they influenced endoglucanase production by *Penicillium* sp. significantly. These included yeast extract, KH_2PO_4, peptone, $(NH_4)_2SO_4$, CMC, cellobiose, $ZnSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot H_2O$ (Table 3). Among the eight components mentioned, cellobiose, $ZnSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot H_2O$ gave negative effects. Therefore, they were eliminated from the list of parameters to be studied in further medium optimization. Other five components showing positive main effects were chosen and the amounts supplied could be increased in future studies. All variables that provided *p*-value of greater than 0.05 were neglected.

Yeast extract, a good source of organic nitrogen, expressed an impressive contribution to endoglucanase activity. It comprised various important nutrients such as amino acids and vitamins which enhanced microbial endoglucanase activity (7). The results obtained were in accordance with the work of Jatinder et al. (12) who reported that yeast extract was an appropriate nitrogen source for supporting cellulase activity.

Other nitrogen sources expressing positive impact on endoglucanase activity were peptone and $(NH_4)_2SO_4$. Peptone is associated in many fermentation media. It serves as a pool of carbon, nitrogen and growth factors for microorganisms. The addition of peptone in the range of 0.5-1.0% (w/v) enhanced cellulase production as an extra nitrogen source was supplied to the basal medium (13).

Table 3. Regression analysis output of the Plackett-Burman design for endoglucanase production by *Penicillium* sp.

	Variables	Main effect	Coefficient	SE Coefficient	*t*-value	*p*-value
	Constant		12.1227	0.1956	61.97	0.000
X_1	Yeast extract*	2.9600	1.4800	0.1956	7.57	0.000*
X_2	KH_2PO_4*	1.5767	0.7883	0.1956	4.03	0.000*
X_3	$CaCl_2$	-0.0060	-0.0030	0.1956	-0.02	0.988
X_4	$MgSO_4 \cdot 7H_2O$	0.0913	0.0457	0.1956	0.23	0.816
X_5	Peptone*	1.4387	0.7193	0.1956	3.68	0.001*
X_6	Urea	0.1213	0.0607	0.1956	0.31	0.758
X_7	$(NH_4)_2SO_4$*	0.8493	0.4247	0.1956	2.17	0.035*
X_8	CMC*	1.3720	0.6860	0.1956	3.51	0.001*
X_9	Cellobiose*	-0.7480	-0.8740	0.1956	-4.47	0.000*
X_{10}	$FeSO_4 \cdot 7H_2O$	-0.4227	-0.2113	0.1956	-0.08	0.286
X_{11}	$CoCl_2$	-0.6293	-0.3147	0.1956	-0.61	0.115
X_{12}	$MnSO_4 \cdot H_2O$*	-0.6907	-0.8453	0.1956	-4.32	0.000*
X_{13}	$ZnSO_4 \cdot 7H_2O$*	-0.9093	-0.4547	0.1956	-2.32	0.025*
X_{14}	Tween 80	-0.0327	-0.0163	0.1956	-0.08	0.934

*Variables showed significant effects on endoglucanase production.

$(NH_4)_2SO_4$ is a cost-effective inorganic nitrogen source which is widely used in cellulase production (12). From the report of Long et al. (14), $(NH_4)_2SO_4$ promoted cellulase production by *Penicillium*. The result was in an agreement with Liu and Yang (15) who reported that inorganic nitrogen was preferred for cellulolytic enzyme production.

KH_2PO_4 is a mineral source which is beneficial for fungal growth. From Table 3, KH_2PO_4 gave a positive contribution to endoglucanase activity. The result obtained supported the works of Sehnem et al. (16), Liu and Yang (14) and Han et al. (2) who suggested that mineral sources should be incorporated in fermentation medium for better fungal cellulase production.

Carboxymethyl cellulose (CMC) is a substrate of endoglucanase and is used in standard protocol for endoglucanase activity assay. Ahamed and Vermette (17) reported that CMC was the best carbon source for cellulase production by fungi. Sukumaran et al. (18) and Graminha et al. (19) reported that cellulolytic enzymes are inducible. Therefore, the positive effect of CMC on endoglucanase activity shown in Table 3 was mainly due to a substrate induction mechanism.

In conclusion, Plackett-Burman design was proved as an efficient tool for primary screening of significant nutrient parameters for endoglucanase production by *Penicillium* sp. With Plackett-Burman design, many parameters showing no significant impact can be omitted. This invested less time and cost comparing to conventional method which studies one factor at a time. However, it must be noted that the results reported and discussed hereby were based on the low and high concentrations assigned for each component. If the concentrations were modified, different output might be observed. Thus, appropriate values of low and high levels of each component must be carefully designed.

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