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Optimization of nutrients for chitinase production by *Serratia marcescens* JPP1 against aflatoxin using statistical experimental design

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ABSTRACT

Chitinase is produced by a number of organisms and it is one of the most important enzymes with industrial significance. In this study, we describe the optimization of medium composition with increased production of chitinase for Serratia marcescens strain JPP1. The strain was isolated from the peanut hulls in Jiangsu Province, China and exhibited antagonistic activity against aflatoxins and chitinolytic activity by producing chitinases. Medium composition was optimized using statistical experimental design: Plackett-Burman design was applied to find the key ingredients, and the five significant variables were peptone, glucose, ammonium sulfate, fructose and beef extract.

Keywords: Aflatoxin, Chitinase, *Serratia marcescens*, Biocontrol, Plackett-Burman.

INTRODUCTION

Chitin is the second most abundant renewable biopolymer on the earth after cellulose [1]. It has been estimated that the worldwide annual recovery of chitin from the processing of marine invertebrates is 3.7×10^4 metric tons [2]. Chitinases have received increasing attention because of their broad applications in the fields of waste management, medicine, agriculture, biotechnology and industrial applications [3].

A number of bacteria have been reported to produce chitinases. Chitinolytic bacteria as biocontrol agents have showed potential antagonistic activity against pathogenic fungi by degrading the cell walls [4]. *Serratia marcescens* has been reported producing multiple chitinases (ChiA, ChiB, and ChiC) [5]. Aflatoxins (AFs) are highly toxic and carcinogenic secondary metabolites produced by *Aspergillus flavus* and *A. parasiticus* [6]. Therefore, *S. marcescens* could potentially be utilized for the biocontrol of toxigenic fungi and AFs.

Because medium composition greatly influenced the production of extracellular chitinase, the study on medium optimization for chitinase production are worthwhile. The objective of the present study was to optimize the different components of defined media for secretion of chitinase from *S. marcescens* strain JPP1 and find the key ingredients by Plackett-Burman design.

EXPERIMENTAL SECTION

Microorganism

The bacterial culture of *S. marcescens* strain JPP1 in this study was isolated from the peanut hulls using potato dextrose agar (PDA) medium and the sampling site was located in Huaian city, Jiangsu Province, China. Identification was mainly on the basis of cultural and morphological characteristics, and final identification was performed by 16S rRNA sequence analysis. The strain exhibited antagonistic activity against aflatoxins production and chitinolytic activity by producing chitinases.

Culture media

PGY medium: Peanut hulls were dried at 40°C and then ground. The ground peanut hulls were boiled with water for 1 h at the final concentration of 2.5%, and then centrifuged at 6,600 g at room temperature for 5 min. The supernatant was supplemented with 2% glucose and 0.5% yeast extract, then autoclaved for 20 min at 121°C; pH in nature. Chitinase medium: PGY medium was supplemented with 1% colloidal chitin.

Enzyme production and assay. The culture was inoculated in 3 ml GY medium on a rotary shaker (30°C, 140 rpm) for 12 h, and then transferred into 300 ml chitinase medium to cultivate at 30°C for 6 days under shaking conditions (140 rpm). Enzyme activity was determined according to the method of Monreal and Reese [7].

Identification of the significant factors by the Plackett-Burman design

Best three carbon sources were selected on the basis of their role in chitinase secretion enhancement, and three nitrogen sources were also selected on a similar basis. Magnesium sulfate and Potassium hydrogen phosphate anhydrous were also considered as growth nutrients that form the most important part of media. The important medium components with respect to their main effects were screened using Plackett-Burman design. It identifies the main physicochemical parameters required for maximal chitinase; each variable was examined at two levels. **Table-1** lists the factors under investigation as well as the levels of each factor used in the experimental design with the symbol code and actual level of the variables, whereas **Table-2** presents the design matrix. Minitab16.0 was used to analyze the experimental Plackett-Burman design.

Table-1 Medium components at different levels used in Plackett-Burman design

Symbol code	Variable	Unit	Levels	
			+1	-1
X_1	Glucose	g l^{-1}	10	8
X_2	Fructose	g l^{-1}	10	8
X_3	Dummy	-	-	-
X_4	Ammonium sulfate	g l^{-1}	2.5	2
X_5	Peptone	g l^{-1}	2	1.5
X_6	Dummy	-	-	-
X_7	$MgSO_4 \cdot 7H_2O$	g l^{-1}	0.5	0.4
X_8	K_2HPO_4	g l^{-1}	0.7	0.5
X_9	Dummy	-	-	-
X_{10}	Beef extract	g l^{-1}	2	1.5
X_{11}	Ammonium chloride	g l^{-1}	2.5	2

RESULTS AND DISCUSSION

A total of 8 variables were analyzed with regard to their effects on chitinase production using the Plackett–Burman design. All trials were performed in triplicate and the average of production of highly active chitinase observations were treated as response. The main effect of each variable was simply calculated as the difference between the average of measurements made at the high setting and the average of measurements observed at the low setting of the factor. The design matrix selected to the screening of significant variables is shown in **Table-2**. The adequacy of the model was calculated and the variables evidencing statistically significant effects were screened via t test for ANOVA (**Table-3**).

Table-2 Plackett–Burman experimental design matrix and Chitinase activity

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	Chitinase activity (U)
1	1	-1	1	1	-1	1	-1	-1	-1	1	1	7.13
2	1	-1	1	-1	-1	-1	1	1	1	-1	1	7.47
3	-1	1	1	-1	1	-1	-1	-1	1	1	1	7.02
4	1	1	-1	1	-1	-1	-1	1	1	1	-1	9.81
5	-1	1	1	1	-1	1	1	-1	1	-1	-1	8.55
6	1	1	-1	1	1	-1	1	-1	-1	-1	1	11.91
7	-1	-1	-1	1	1	1	-1	1	1	-1	1	9.37
8	1	1	1	-1	1	1	-1	1	-1	-1	-1	10.87
9	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	5.63
10	-1	1	-1	-1	-1	1	1	1	-1	1	1	5.75
11	1	-1	-1	-1	1	1	1	-1	1	1	-1	9.41
12	-1	-1	1	1	1	-1	1	1	-1	1	-1	8.73

The design was applied with 12 different fermentation conditions (run) as shown in **Table-2**.The results indicated that levels of factors at run 6 were the best. Regression analysis on the experimental results using Minitab16.0 set up the regression equation:

$$Y = 8.47+0.963\ X_1+0.514\ X_2+0.779\ X_4+1.08\ X_5+0.166\ X_7+0.196\ X_8-0.496\ X_{10}-0.362\ X_{11} \quad (1)$$

The determination coefficient (R^2) was 0.985 and the model was significant ($p=0.011<0.05$), indicating the model was reliable. Factors evidencing P values of less than 0.05 were considered to have a significant effect on the response. The lowest P values indicate the most significant factors on enzymes production. The results revealed that the most significant three factors which were more effective in chitinase production were peptone, glucose and ammonium sulfate ($P<0.01$). The significant two factors effective in chitinase production were fructose and beef extract ($P<0.05$). While peptone, glucose, fructose and ammonium sulfate showed positive effect on chitinase production. However, the effect of beef extract was negative.

Table-3 Identification of significant variables using Plackett–Burman design

Variable	Effect	Coefficient	S.E	t ratio	P value
X_1	1.9250	0.9625	0.1300	7.40	0.005
X_2	1.0283	0.5142	0.1300	3.95	0.029
X_4	1.5583	0.7792	0.1300	5.99	0.009
X_5	2.1617	1.0808	0.1300	8.31	0.004
X_7	0.3317	0.1658	0.1300	1.28	0.292
X_8	0.3917	0.1958	0.1300	1.51	0.229
X_{10}	-0.9917	-0.4958	0.1300	-3.81	0.032
X_{11}	-0.7250	-0.3625	0.1300	-2.79	0.069

The simple sugars like glucose and fructose serve as carbon source, while glucose is the most assessable simple carbon source to microorganisms. Because the glucose was cheaper and more effective in chitinase production, it was identified as best carbon source. Compared with beef extract, peptone as organic nitrogen source led to the highest chitinase activity. Ammonium sulfate was one of the most significant factors and it was selected as the best inorganic nitrogen source for chitinase production.

CONCLUSION

Plackett–Burman design was used to analyze the 8 variables with regard to their effects on chitinase production. The results revealed that the most significant three factors which were more effective in chitinase production were peptone, glucose and ammonium sulfate, followed by fructose and beef extract. Glucose was identified as best carbon source, peptone as organic nitrogen source while ammonium sulphate as the best inorganic nitrogen source led to the highest chitinase activity.

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