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Development of Medium for Griseofulvin Production: Part I. Screening of Medium Constituents Using the Plackett-Burman Experimental Design

VENKATA DASU, V., T. PANDA*, AND M. CHIDAMBARAM

Biotechnology Research Centre, Department of Chemical Engineering, Indian Institute of Technology-Madras, Chennai - 600 036, India

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Abstract The Plackett-Burman experimental design was employed to evaluate the relative importance of medium constituents of each medium for enhanced griseofulvin production by *Penicillium griseofulvum* MTCC 1898 and *Penicillium griseofulvum* MTCC 2004. It was found that the medium constituents, sucrose, K_2HPO_4, $NaNO_3$, and $FeSO_4 \cdot 7H_2O$, significantly influenced the griseofulvin production by *Penicillium griseofulvum* MTCC 1898. In the case of *Penicillium griseofulvum* MTCC 2004, lactose, glucose, and $MnSO_4 \cdot H_2O$ significantly influenced the griseofulvin production.

Key words: *Penicillium griseofulvum*, griseofulvin, screening, Plackett-Burman experimental design

Griseofulvin is a less toxic systemic antifungal antibiotic. It is used for the treatment of mycotic diseases of skin, hair, and ring worm infections. This antibiotic is also effective against fungal diseases of plants, but its cost seems to be high for wider agricultural applications [10]. Griseofulvin is a fungistatic compound active against dermatophytic fungi having a chitinous cell wall. This drug has no effect on other fungi, yeast, actinomycetes, protozoa, and bacteria [2]. Griseofulvin was first isolated from *Penicillium griseofulvum* Dierckx [8]. Extensive literature is available on griseofulvin production by *Penicillium* species [1, 6-7, 11, 13], however *Penicillium griseofuvum* is found to be the best strain for griseofulvin production [1, 13].

The development of a medium for maximum production of any metabolite is a key step in industrial scale fermentation. The screening and optimization of medium constituents that significant influence the production have been done for the development of medium. However, medium optimization is generally a time-consuming and labour-intensive process.

*Corresponding author
Phone: 91-44-2578217; Fax: 91-44-2570509;
E-mail: tpanda@hotmail.com

In order to achieve the maximum yield of griseofulvin, experiments were performed with three different media on *Penicillium griseofulvum* MTCC 1898 and *Penicillium griseofulvum* MTCC 2004 for the selection of medium. It was found that medium II for *Penicillium griseofulvum* MTCC 1898 and medium I for *Penicillium griseofulvum* MTCC 2004 were most suitable for maximum production of griseofulvin (unpublished results, data not given). Further studies had been carried out with the above selected media. The Plackett-Burman experimental design proved to be a valuable tool for the rapid evaluation of the effects of the various medium constituents, in spite of ignoring the optimal quantity of constituents required in the medium. Krishnan *et al.* [5] applied the Plackett-Burman experimental design to screen the medium components for higher lactic acid production. Kern *et al.* [4] have studied the effects of two nitrogen sources, yeast extract and NH_4NO_3, on the growth of xylose-fermenting *Candida tenuis* as well as on the production of aldose reductase and xylitol dehydrogenase using Plackett-Burman design. It was found that yeast extract was the significant component for growth as well as the production of aldose reductase and xylitol dehydrogenase. The objective of the present investigation is to screen the media constituents that are significantly influencing the griseofulvin production by *Penicillium griseofulvum* MTCC 1898 and *Penicillium griseofulvum* MTCC 2004. The Plackett-Burman experimental design was used to evaluate the relative importance of various constituents of each medium for higher production of griseofulvin.

MATERIALS AND METHODS

Organisms

Penicillium griseofulvum MTCC 1898 and *Penicillium griseofulvum* MTCC 2004 were obtained from the Institute of Microbial Technology, Chandigarh, India.

Culture Maintenance

Penicillium griseofulvum MTCC 1898 was maintained on agar slants containing (g/l) sucrose, 30.0; yeast extract, 5.0; K_2HPO_4, 1.0; $NaNO_3$, 3; KCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01. *Penicillium griseofulvum* MTCC 2004 was maintained on agar slants containing (g/l) sucrose, 30.0; K_2HPO_4, 1.0; $NaNO_3$, 2; KCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; $ZnSO_4 \cdot 7H_2O$, 0.01; and $CuSO_4 \cdot 5H_2O$, 0.005. The organisms were subcultured every month and then preserved at 4±1°C.

Seed Culture Medium

The composition of the medium used for the development of seed culture was the same as described for culture maintenance, with glucose 10 g/l being the additional constituent added for both the strains. Five-hundred-ml Erlenmeyer flasks containing 100 ml of inoculum medium were autoclaved at 121°C for 20 min. The initial pH of the media was adjusted to 6.5 before sterilization. The seed culture medium was inoculated with 1 ml of spore suspension (in sterile distilled water) containing 1.8×10^7 spores. The age of organisms in slant growth, seed age, and inoculum level were maintained as reported by Venkata Dasu and Panda [16].

Cultivation Medium and Culture Conditions

The composition of the production medium was the same as the culture maintenance medium for *Penicillium griseofulvum* MTCC 1898. The production medium containing (in g/l) corn steep liquor, 1.0; glucose, 11.0; lactose, 28.0; KH_2PO_4, 0.5; $NaNO_3$, 3; KCl, 1.0; $MgSO_4 \cdot 7H_2O$, 0.25; $MnSO_4 \cdot H_2O$, 0.02; $ZnSO_4 \cdot 7H_2O$, 0.044; and $CaCO_3 \cdot 2H_2O$, 1.0 was used for *Penicillium griseofulvum* MTCC 2004. The initial pH of the medium was adjusted to 6.5 before sterilization. One hundred ml of sterile medium in a 500-ml Erlenmeyer flask was inoculated with 12% (v/v) and 9% (v/v) of seed culture for *Penicillium griseofulvum* MTCC 1898 and *Penicillium griseofulvum* MTCC 2004, respectively. The culture was incubated on a rotatory shaker at 160 rpm and at 30°C. Samples were withdrawn at regular intervals of 24 h and assayed for griseofulvin [3].

Experimental Design and Methodology

The Plackett-Burman experimental design was applied to evaluate the relative importance of various constituents of both media for enhanced griseofulvin production [9]. It is a two-level factorial design, in k variables, where the number of design points, N, is equal to k+1. These designs are available only when N is a multiple of 4.

The Plackett-Burman screening design is based on the probabilistic model:

$$Y=\beta_0+\Sigma\beta_i x_i \quad (1)$$

where

Table 1. Variables and their levels employed in the Plackett-Burman design for the screening of medium constituents by *Penicillium griseofulvum* MTCC 1898.

Variable	Medium constituents	Levels (g/l)	
		+	-
A	Sucrose	37.5	30.0
B	Yeast extract	6.25	5.0
C	K_2HPO_4	1.25	1.0
D	$NaNO_3$	3.75	3.0
E	KCl	0.625	0.5
F	$MgSO_4 \cdot 7H_2O$	0.625	0.5
G	$FeSO_4 \cdot 7H_2O$	0.0125	0.01

Y=predicted response
β_0=offset term
β_i=linear term
x_i=independent variable

with the assumption that there is no interaction among the variables. Each medium constituent is treated as a variable. Each variable is represented at two levels; a high level denoted by (+) and a low level designated by (-). The high level (+) represents 125% of the unoptimized medium (low level (-)). In the earlier reports on fungal fermentation, 1.25 times values showed appreciable results [14, 15]. The variables and their levels employed in the Plackett-Burman experimental design for the screening of medium constituents by *Penicillium griseofulvum* MTCC 1898 and *Penicillium griseofulvum* MTCC 2004 are shown in Tables 1 and 2, respectively.

According to the Plackett-Burman screening design, 7 variables in 8 runs were used to evaluate the important variables that influence the griseofulvin production by *Penicillium griseofulvum* MTCC 1898. Similarly, 11 variables with one dummy variable in 12 runs was used for *Penicillium griseofulvum* MTCC 2004. The Hadamard matrices for 8 and 12 runs (experimental plan) are shown in Tables 3 and

Table 2. Variables and their levels employed in the Plackett-Burman design for the screening of medium constituents by *Penicillium griseofulvum* MTCC 2004.

Variable	Constituent	Levels (g/l)	
		+	-
A	CSL	1.25	1.0
B	Glucose	13.75	11.0
C	Lactose	35.0	28.0
D	$NaNO_3$	3.75	3.0
E	KH_2PO_4	0.625	0.5
F	$MgSO_4 \cdot 7H_2O$	0.3125	0.25
G	KCl	1.25	1.0
H	$MnSO_4 \cdot H_2O$	0.025	0.02
I	$ZnSO_4 \cdot 7H_2O$	0.055	0.044
J	$CaCl_2 \cdot 2H_2O$	1.25	1.0
K	dummy	-	-

Table 3. Hadamard matrix and griseofulvin production for screening of medium components for griseofulvin production by *Penicillium griseofulvum* MTCC 1898.
Maximum griseofulvin production was achieved on the 8th day of fermentation.

Run #	Variables/Levels							Griseofulvin production (g/l)
	A	B	C	D	E	F	G	
1	+	+	+	–	+	–	–	0.79
2	–	+	+	+	–	+	–	0.61
3	–	–	+	+	+	–	+	0.53
4	+	–	–	+	+	+	–	0.78
5	–	+	–	–	+	+	+	0.70
6	+	–	+	–	–	+	+	0.75
7	+	+	–	+	–	–	+	0.73
8	–	–	–	–	–	–	–	0.74

A - Sucrose C - K_2HPO_4 E - KCl G - $FeSO_4 \cdot 7H_2O$
B - Yeast extract D - $NaNO_3$ F - $MgSO_4 \cdot 7H_2O$

Table 4. Hadamard matrix for screening of medium components for griseofulvin production by *Penicillium griseofulvum* MTCC 2004.
Maximum griseofulvin production was achieved on the 7th day of fermentation.

Run #	Levels/Variables											Griseofulvin production (g/l)
	A	B	C	D	E	F	G	H	I	J	K	
1	+	+	–	+	+	+	–	–	–	+	–	0.41
2	–	+	+	–	+	+	+	–	–	–	+	0.51
3	+	–	+	+	–	+	+	+	–	–	–	0.38
4	–	+	–	+	+	–	+	+	+	–	–	0.47
5	–	–	+	–	+	+	–	+	+	+	–	0.47
6	–	–	–	+	–	+	+	–	+	+	+	0.30
7	+	–	–	–	+	–	+	+	–	+	+	0.31
8	+	+	–	–	–	+	–	+	+	–	+	0.51
9	+	+	+	–	–	–	+	–	+	+	–	0.49
10	–	+	+	+	–	–	–	+	–	+	+	0.51
11	+	–	+	+	+	–	–	–	+	–	+	0.44
12	–	–	–	–	–	–	–	–	–	–	–	0.39

A - CSL (corn steep liquor) E - KH_2PO_4 I - $ZnSO_4 \cdot 7H_2O$
B - Glucose F - $MgSO_4 \cdot 7H_2O$ J - $CaCO_3 \cdot 2H_2O$
C - Lactose G - KCl K - Dummy variable
D - $NaNO_3$ H - $MnSO_4 \cdot H_2O$

4 for *Penicillium griseofulvum* MTCC 1898 and *Penicillium griseofulvum* MTCC 2004, respectively. All experiments were performed in duplicate and the average of the maximum griseofulvin yield was taken as the response.

The effect of each variable on the response for *Penicillium griseofulvum* MTCC 1898 was given by:

$$\text{Effect}=\text{Responses at (+)}/4-\text{Responses at (–)}/4 \quad (1)$$

Similarly, The effect of each variable on the response for *Penicillium griseofulvum* MTCC 2004 was given by:

$$\text{Effect}=\text{Response at (+)}/6-\text{Response at (–)}/6 \quad (2)$$

The data was analyzed by Design Expert version 2.05.

Results and Discussion

The arrangement of variable levels in a Hadamard matrix according to the Plackett-Burman experimental plan along with corresponding response (griseofulvin yield) are given in Table 3 and Table 4 for *Penicillium griseofulvum* MTCC 1898 and *Penicillium griseofulvum* MTCC 2004, respectively. The variables designated A through G represents medium constituents (7 variables, in 8 runs) for *Penicillium griseofulvum* MTCC 1898, and A through K for *Penicillium griseofulvum* MTCC 2004 (11 variables with 1 dummy, in 12 runs). It is pertinent to mention that 11 variables in 12 experiments have been used to develop a medium for xanthan production [12].

The effect of each variable on response, the model coefficients, t values, probability due to chance, and confidence levels obtained by analyzing the experimental data for *Penicillium griseofulvum* MTCC 1898 and for *Penicillium griseofulvum* MTCC 2004 are given in Tables 5 and 6, respectively. It was found that the variables B, E, and F have confidence levels below 65% and hence were

Table 5. Variables as medium constituents, their effects, coefficients, sum of squares, t-values, probability due to chance, and confidence levels obtained by analyzing the experimental data for the Plackett-Burman design used for screening of medium components for griseofulvin production by *Penicillium griseofulvum* MTCC 1898.

Variable (x_i)	Effect (E_{xi})	Coefficient (β_i)	Sum of squares	t for Ho parameter=0	Probability >|t|	Confidence level (%)
Mean	–	–	–	27.693	0.023	–
A	0.1152	0.05758	0.02652	1.8773	0.2090	79.10
B	0.0064	0.00320	0.00008	0.1043	0.3860	61.40
C	-0.06595	-0.03298	0.00869	-1.0792	0.2897	71.03
D	-0.08145	-0.04073	0.01327	-1.3279	0.2644	73.56
E	-0.01240	-0.00620	0.00030	-0.2021	0.3760	62.40
F	0.0123	0.00615	0.00030	0.2009	0.3770	62.30
G	-0.0509	-0.02545	0.00518	-0.8298	0.3142	68.50

A - Sucrose B - Yeast extract C - K_2HPO_4 D - $NaNO_3$ E - KCl F - $MgSO_4 \cdot 7H_2O$ G - $FeSO_4 \cdot 7H_2O$

Table 6. Variables as medium constituents, their effects, coefficients, sum of squares, t-values, probability due to chance, and confidence levels obtained by analyzing the experimental data for the Plackett-Burman design used for screening of medium components for griseofulvin production by *Penicillium griseofulvum* MTCC 2004.

Variable (x_i)	Effect (E_{xi})	Coefficient (β_i)	Sum of squares	t for Ho parameter=0	Probability >|t|	Confidence level (%)
Mean	–	–	–	41.082	0.0006	–
A	-0.02007	-0.01003	0.00121	-0.585	0.3095	69.05
B	0.10080	-0.05040	0.03048	2.936	0.1873	81.27
C	0.06853	-0.03427	0.01409	1.996	0.2362	76.38
D	-0.03027	-0.01513	0.00275	-0.8817	0.2942	70.58
E	0.00223	0.00112	0.00002	0.0650	0.3366	66.34
F	-0.00363	-0.00182	0.00004	-0.1057	0.3345	66.55
G	-0.04510	-0.02255	0.00610	-1.3137	0.3156	68.44
H	0.01613	0.00807	0.00078	0.4698	0.2417	75.83
I	0.02960	0.01480	0.00263	0.8622	0.2951	70.49
J	-0.03757	-0.01878	0.00423	-1.0943	0.2830	71.49

A - CSL (corn steep liquor) C - Lactose E - KH_2PO_4 G - KCl I - $ZnSO_4 \cdot 7H_2O$ K - Dummy variable
B - Glucose D - $NaNO_3$ F - $MgSO_4 \cdot 7H_2O$ H - $MnSO_4 \cdot H_2O$ J - $CaCO_3 \cdot 2H_2O$.

considered insignificant for griseofulvin production by *Penicillium griseofulvum* MTCC 1898 (Table 5) [14-15]. The rest of the variables, i.e., A, C, D, and G, which represent sucrose, K_2HPO_4, $NaNO_3$, and $FeSO_4 \cdot 7H_2O$, respectively, having confidence levels above 65%, were considered to significantly influence griseofulvin production by *Penicillium griseofulvum* MTCC 1898. In the case of *Penicillium griseofulvum* MTCC 2004, the variables B, C, and H represents glucose, lactose, and $MnSO_4 \cdot H_2O$, respectively, and having confidence levels above 75%, were considered to significantly influence griseofulvin production (Table 6). With respect to griseofulvin production, the role of various variables can not be properly justified (Tables 3 and 4) as there are random changes observed. Therefore, the screening is based on the confidence level (Tables 5 and 6). Similar critical phenomenon has been observed in the *Trichoderma* systems [14-15]. So, it is very difficult to propose a suitable explanation in this case. Hence, sucrose, K_2HPO_4, $NaNO_3$, and $FeSO_4 \cdot 7H_2O$ were considered most significant constituents for griseofulvin production by *Penicillium griseofulvum* MTCC 1898. For *Penicillium griseofulvum* MTCC 2004, lactose, glucose, and $MnSO_4 \cdot H_2O$ were most significant on griseofulvin production.

Thus, equation (1) on substitution of coefficients is as follows:

$$Y=0.07048+0.05758\,A+0.0032\,B-0.03298\,C -0.04073\,D-0.0062\,E+0.00615\,F-0.02545\,G \quad (3)$$

$$Y=0.43282-0.01003\,A+0.0504\,B+0.03426\,C -0.01513\,D+0.00112\,E-0.00182\,F-0.02255\,G +0.00806\,H+0.01480\,I-0.01878\,J-0.00172\,K \quad (4)$$

Equations (3) and (4) represent the effect of each variable on griseofulvin production by *Penicillium griseofulvum* MTCC 1898 and *Penicillium griseofulvum* MTCC 2004, respectively. The variables found to be significant in the Plackett-Burman screening experiment were then grouped in a central composite design plan, as given in a separate paper [17], to find the optimal level of medium components for griseofulvin production. The medium constituents sucrose, K_2HPO_4, $NaNO_3$, and $FeSO_4 \cdot 7H_2O$ were screened for griseofulvin production by *Penicillium griseofulvum* MTCC 1898. For *Penicillium griseofulvum* MTCC 2004, lactose, glucose, and $MnSO_4 \cdot H_2O$ were screened.

CONCLUSIONS

The screening of medium constituents were carried out successfully by using Plackett-Burman experimental design.

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