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Application of Plackett-Burman factorial design to improve citrinin production in *Monascus ruber* batch cultures

Abdulaziz Q. M. AL-SARRANI[1] and Moustafa Y. M. EL-NAGGAR[2,*]

[1] *Biology Department, Microbiology Division, Faculty of Science, Taibah University, P.O. Box 344, Al-Madinah Al-Munawarah, Kingdom of Saudi Arabia*
[2] *Botany Department, Microbiology Division, Faculty of Science, Alexandria University, Moharram Bay 21511, Alexandria, Egypt*

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ABSTRACT. A fungal strain previously isolated from a hot sauce of red pepper putatively identified as *Monascus ruber* was investigated for its potential to produce an antibacterial substance in batch cultures. This fungus was grown in three liquid media. The highest antibacterial activity was recorded for the synthetic medium No. 2. The antibacterial substance was extracted, purified and identified as the mycotoxin citrinin. Plackett-Burman experimental design was applied to optimize the components of medium No. 2 in an attempt to improve citrinin production for non-food applications. As a result a medium of the following formula was predicted to be near the optimum for producing an extracellular citrinin in the culture filtrate of *M. ruber* (g/l): glucose, 45; $NaNO_3$, 3.75; KH_2PO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.1 and $ZnSO_4 \cdot 7H_2O$, 0.01; $MnSO_4 \cdot H_2O$, 0.006 and $FeSO_4 \cdot 7H_2O$, 0.003. The improvement of the antibacterial activity amounted to 1.75-fold. On the other hand, the dry weight of the fungus in the optimized medium was 1.86 times the figure recorded for the basal setting. The HPLC determination of citrinin concentration in the culture supernatant amounted to 220 mg/l and 400 mg/l before and after medium optimization, respectively.

Keywords: Batch cultures; Citrinin; *Monascus ruber*; Optimization; Plackett-Burman experimental design.

INTRODUCTION

Monascus is an ascomycetous fungus discovered by van Tieghem (1884) traditionally used for the production of food colouring, fermented foods and beverages (Martinkova and Patakova, 1999). Other components of *Monascus* pigments have anti-inflammatory activity and have been reported to suppress skin cancer caused by tumour promoters in experimental animals (Yasukawa et al., 1994 and 1996), in addition to their clinical benefits in treating high blood pressure in humans (Kushiro et al., 1996) In Saudi Arabia, *Monascus ruber* has been isolated from the hot sauce of red pepper (Al-Sarrani, 1998). In Greece, the same fungus has been isolated from the brine of thermally-processed green olives of the Conservolea variety (Panagou et al., 2003). It was shown that *M. ruber* is able to produce the mycotoxin citrinin (Blanc et al., 1995a). This mycotoxin has antibiotic activities against Gram-positive bacteria, but its nephrotoxic properties (Blanc et al., 1995a) have limited its use as an antibiotic for therapeutic purposes. Consequently, the production of citrinin together with the red pigments rules out the use of *M. ruber* as a producer of natural colourants for food technology (Blanc et al., 1995a; Hajjaj et al., 1997).

Nutritional and fermentation conditions have a great influence on antibacterial activity (El-Naggar et al., 2001). The application of statistically based experimental designs to optimize fermentation media is an efficient approach to studying the effects of several factors and to improve product yields. The conventional practice of single factor optimization by maintaining other factors involved at an unspecified constant level does not depict the combined effect of all factors involved (Elibol, 2004). Factorial design considers the statistical interaction between variables to obtain a maximum of inferences for minimum of tests, reducing process variability, time of development and overall costs.

This work is meant to optimize the fermentation medium composition of *Monascus ruber* for citrinin (Figure 1) production to be used for toxicological studies and as a reference for analytical purposes. Factors affecting these objectives were evaluated by the application of a two-level factorial Plackett-Burman design (Plackett and Burman, 1946).

*Corresponding author: E-mail: Moustafa64@Yahoo.com

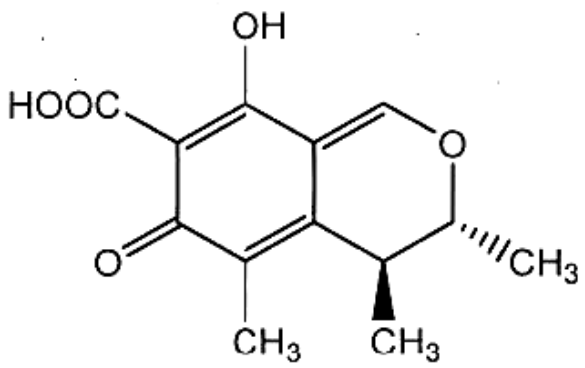

Figure 1. Structure of citrinin.

MATERIALS AND METHODS

Microorganism and growth conditions

The filamentous fungus used in this study was previously isolated from the hot sauce of red pepper *Capsium annum* (Al-Sarrani, 1998). This fungus was identified as *Monascus ruber* Tieghem according to the taxonomy proposed by Hawksworth and Pitt (1983). The identification was confirmed by the Commonwealth Mycological Institute, Great Britain (code number IMI 364278). *Monascus ruber* was kept on slants of potato dextrose agar (PDA, Difco Laboratories, Detroit, Mich., USA). Spores were prepared by growing the fungus on PDA slants for 10 days at 30°C. Spores were washed with sterile distilled water and a spore suspension of 2.5×10^7 spores was used to inoculate each of the Erlenmeyer flasks (250 ml) which contained 50 ml culture medium for the production of citrinin. The flasks were then incubated at 30°C, 240 rpm using shaker incubator for 5 days. A time scale experiment was carried out, which revealed that maximum growth of this fungus and mycotoxin production were attained at day 5 (data not shown). In the present work, three fermentation media were originally used to study their effect on the production of citrinin by *M. ruber*:

Medium 1. The synthetic medium classically used for the production of red pigments (Fabre et al., 1993) composed of monosodium glutamate, 5 g; K_2HPO_4, 5 g; KH_2PO_4, 5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2$, 0.5g; $FeSO_4 \cdot 7H_2O$, 0.5 g; $ZnSO_4 \cdot 7H_2O$, 0.01 g, $MnSO_4 \cdot H_2O$, 0.03 g; ethanol, 20 g/l deionized water.

Medium 2. The synthetic medium designated for the production of pigments (El-Naggar et al., 2000) composed of glucose, 40 g; $NaNO_3$, 3 g; KH_2PO_4, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; $FeSO_4 \cdot 7H_2O$, 0.001 g; $ZnSO_4 \cdot 7H_2O$, 0.008 g, $MnSO_4 \cdot H_2O$, 0.003 g/l deionized water.

Medium 3. A modified yeast extract-sucrose (YES) medium, specifically used for the production of fungal toxins (Davis et al., 1975) composed of yeast extract, 40 g and sucrose, 160 g/l deionized water.

The initial pH of each fermentation medium was adjusted to 6.5 with NaOH or HCl.

Antibacterial activity

A culture broth (10 ml) was centrifuged in order to separate the mycelium and the supernatant. The supernatant was concentrated 10-fold and used for the determination of antibacterial activity. Thirty µl were placed in each hole (6 mm of diameter) in the Mueller-Hinton agar medium in a Petri-dish inoculated with 0.1 ml bacterial suspension (3×10^6 cfu/ml) of one of the selected indicator bacterial strains (*Bacillus subtilis* ATCC 6633, *B. cereus* PX MUCOB, *Streptococcus lactis*, or *Pseudomonas fluorescens*). *S. lactis* and *P. fluorescens* are both local isolates from the Hope Hospital, Salford, England. Petri dishes were kept for 2 h in the refrigerator and then incubated for 12 h at 35ºC and the inhibition zone diameter was then measured. This experiment was performed in triplicates and repeated twice, and the mean was considered the response.

Dry weight determination

The fungal biomass (mg/ml) was determined after filtration the sample culture through pre-weighed membrane filter (45-µm Millipore, Millipore Corp., Beford, Mass., USA) and washed with sterile distilled water. The mycelia were dried at 80ºC to a constant weight.

Plackett-Burman experimental design

The Plackett-Burman experimental design, a fractional factorial design, was used in this work to demonstrate the relative importance of medium components on citrinin production and growth of *M. ruber*. Seven independent variables (Table 1) in eight combinations were organized according to the Plackett-Burman design matrix (Table 2). For each variable, a high (+1) and low (-1) level was tested. All trials were performed in triplicate, and each experiment was repeated twice, with the mean considered the response. The main effect of each variable was determined according to the following equation:

$$E_{xi} = (\Sigma M_{i+} - \Sigma M_{i-}) / N$$

where E_{xi} is the variable main effect, M_{i+} and M_{i-} are the response percentage in trials, in which the independent variable (xi) was present in high and low concentrations, respectively, and N is the half number of trials. Using Microsoft Excel, statistical t-values for equal unpaired samples were calculated for the determination of variable significance.

Isolation, purification and characterization of citrinin

The culture supernatant (10 litres) was separated from the mycelia using a Chilspin MSE Fisons centrifuge at 4ºC and 5000 rpm for 15 min. The supernatant was then acidified to pH 5.0 and extracted with ethyl acetate. The aqueous layer was removed and the organic layer was concentrated and applied to a silica gel 60 preparative TLC plate (using ethyl acetate/acetone/water, 4:4:1 v/v/v) as a solvent system. The plates were examined under ultraviolet light at 350 nm according to Blanc et al. (1995a).

Table 1. Experimental range and levels of independent variables in the Plackett-Burman experiment.

Variable	Level (g/l)		
	-1	0	1
Glucose	35.00	40.00	45.00
$NaNO_3$	2.75	3.00	3.75
KH_2PO_4	0.05	0.10	0.2
$MgSO_4 \cdot 7H_2O$	0.025	0.05	0.1
$ZnSO_4 \cdot 7H_2O$	0.006	0.008	0.01
$MnSO_4 \cdot H_2O$	0.001	0.003	0.006
$FeSO_4 \cdot 7H_2O$	0.0008	0.001	0.003

Table 2. The Plackett-Burman design matrix representing the coded values for 7 independent variables.

Trial No.	Factor						
	Glucose	$NaNo_3$	KH_2PO_4	$MgSO_4$	$ZnSO_4$	$MnSO_4$	$FeSO_4$
1	-1	-1	-1	1	1	1	-1
2	1	-1	-1	-1	-1	1	1
3	-1	1	-1	-1	-1	-1	1
4	1	1	-1	-1	1	-1	-1
5	-1	-1	1	1	-1	-1	1
6	1	-1	1	1	1	-1	-1
7	-1	1	1	-1	-1	1	-1
8	1	1	1	1	1	1	1
9	0	0	0	0	0	0	0

0 represents the original concentration of each component in the medium No. 2 before optimization (trial No. 9). -1 represents the low concentration level and +1 represents the high concentration level for each component.

A fluorescent pale yellow spot (R_f=0.6) was detected. The active compound was removed from the plate and dissolved in methanol and again purified with a Hewlett-Packard 1090A HPLC instrument. A Spherisorb C18, 5 µm (25 cm by 4.6 mm) column was eluted with methanol /water (20:80, v/v) at a flow rate of 1.0 ml/min. The concentration of citrinin was also measured in the culture supernatant by the same method to make sure that citrinin was responsible for the antibacterial activity.

Nuclear magnetic resonance (NMR) spectroscopy

^{1}H NMR spectrum (CD_3OD) was recorded on Varian Oxford 300 MHz NMR spectrophotometer.

RESULTS

The antibacterial activity in *M. ruber* culture

After 5 days of *M. ruber* incubation (a time scale experiment revealed that maximum growth of this fungus and mycotoxin production were attained at day 5, data not shown), the antibacterial activity of the crude supernatant of *M. ruber* from the three culture media was determined using *B. subtilis*, *B. cereus*, *S. lactis* and *P. fluorescens* as selected test organisms (Table 3). *Monascus ruber* showed a varied antibacterial activity pattern (measured as inhibition zone diameter in mm) when cultured in media 1, 2 and 3, but medium No. 2 recorded the highest activity. When the concentration of citrinin was estimated in the culture filtrate using HPLC instrument, medium No. 1 recorded 160 mg/l, and medium No. 2 recorded 220 mg/l, compared to 180 mg/l for medium No. 3. Consequently, medium No. 2 will be further optimized using Plackett-Burman experimental design in an attempt to improve citrinin production by *M. ruber* in batch cultures.

Optimization of medium components for citrinin production by *M. ruber*

The application of a complete factorial design would require 2^n experiments if n factors have to be investigated. Thus, seven variables would lead to 128 trials, a huge number. However, using the factorial design without losing information about the main effect of variables could reduce the number of experiments. Seven levels of culture variables were examined in the Plackett-Burman design matrix with 9 different trials. Trial No. 9 represented the original medium composition. The highest citrinin activity

Table 3. Antibacterial activity of the mycotoxin citrinin detected in the *M. ruber* culture supernatant of media 1-3.

Microorganism	Inhibition zone diameter in mm		
	Medium 1	Medium 2	Medium 3
B. cereus PX MUCOB	13±1.1	17±1.4	15±1.3
B. subtilis ATCC 6633	13±1.1	16±1.3	14±0.0
S. lactis	10±0.2	12±1.3	11±0.5
P. fluorescens	10±0.1	15±1.5	13±0.3

MUCOB: Manchester University Collection of Bacteria, England.

was recorded for trial No. 8 while the lowest activity was recorded for trials No. 2 and 3. On the other hand, maximum fungal dry weight was also obtained for trial No. 8, and trial No. 1 recorded the lowest fungal biomass (Table 4).

The main effect (percentage) of each variable upon inhibition zone diameter as well as the fungal dry weight was also calculated (Table 5). The main effect figure with a positive sign indicates that the high concentration of this variable is nearly optimum and a negative sign indicates that the low concentration of this variable is nearly optimum. The data obtained showed a range of positive main effect values indicating that the presence of high levels of glucose, $NaNO_3$, KH_2PO_4, $MgSO_4$ and $ZnSO_4$ in the growth medium positively affects citrinin production by *M. ruber*. On the other hand, the presence of $MnSO_4$ and $FeSO_4$ at their lowest levels would result in higher citrinin activity. The presence of high levels of glucose, $MgSO_4$ and $ZnSO_4$ in the medium with low levels of $NaNO_3$, K_2HPO_4, $MnSO_4$ and $FeSO_4$ resulted in an increase in the fungal dry weight.

Statistical analysis (t-values) demonstrated that glucose, $NaNO_3$, K_2HPO_4, had significant positive influences on the citrinin production with main effects of 3.25, 2.25 and 3.75% while $MgSO_4$ and $ZnSO_4$ had significant positive influences on fungal growth with main effects of 3.19 and 8.30%, respectively.

Based on these results obtained from the Plackett-Burman experiment, a medium of the following formula was predicted to be near optimum for producing an extracellular citrinin in the culture filtrate of *M. ruber* (g/l): glucose, 45; $NaNO_3$, 3.75; KH_2PO_4, 0.2; $MgSO_4{\cdot}7H_2O$, 0.1; $ZnSO_4{\cdot}7H_2O$, 0.01; $MnSO_4{\cdot}H_2O$, 0.006 and $FeSO_4{\cdot}7H_2O$, 0.003. Since, the trial No. 8 is a composition of all factors with their maximum dosage in the experiment, care was taken to insure that the predicted formula in trial No. 8 was the optimal one. The optimized composition of this trial was used again as a base for the construction of a new Plackett-Burman matrix, considering concentrations higher than that in trial No. 8. The results obtained from the new Plackett-Burman experiment (data not shown) proved that the formula predicted earlier (trial No. 8) in the first Plackett-Burman experiment was still the optimal one.

In order to evaluate the accuracy of the Plackett-

Table 4. Influence of medium components on citrinin production by and growth of *M. ruber* according to the Plackett-Burman design.

Trial No.	Response (± Standard deviation)		
	Activity	Dry weight (mg/ml)	Citrinin concentration (mg/ml)
1	13±1.3	10.62±0.419	185±5.0
2	10±0.0	1.82±0.088	143±7.0
3	10±0.0	2.44±0.101	143±8.0
4	14±0.0	13.74±0.478	200±9.0
5	12±1.3	3.52±0.049	171±4.0
6	15±1.7	10.77± 0.342	214±9.0
7	13±1.8	1.58± 0.061	185±3.0
8	25±2.5	17.46± 0.193	375±10
9	16±1.2	10.45± 0.021	220±10

B. subtilis ATCC 6633 was used as a test organism. The activity was measured as inhibition zone diameter in mm.

Burman test, a verification experiment was undertaken, in which the predicted optimum levels of independent variables were investigated and compared to the basal setting. The average of inhibition zone diameter and dry weight are shown in Table 6. Citrinin production in the optimized medium expressed as an inhibition zone diameter reached about 28 mm, approximately 1.75-fold that obtained from the basal medium (16 mm). On the other hand, the dry weight of the fungus in the new medium was 1.86 times the figure recorded for the basal medium. HPLC quantification of citrinin by *M. ruber* in the optimized culture supernatant confirmed that an improvement in citrinin production occurred and amounted to 400 mg/ml, 1.8-fold the basal setting before optimization (220 mg/ml).

It was interesting to note the growth pattern in the culture medium, where micropellets (2-4 mm in diameter) were formed in trials 8 and 9 and large pellets (more than 10 mm) were observed for trials 2, 3, 5 and 7. On the other hand, an irregular growth pattern was observed for trials 1, 4 and 6. The colour of culture filtrate was dark yellow for trial No. 8, yellow for 9, and pale yellow for the rest of trials.

Finally, the antibacterial substance produced in culture supernatant of *M. ruber* was isolated, purified and characterized by NMR technique. The ^{1}H NMR spectrum (Figure 2) of the isolated antibacterial substance matched the authentic sample of citrinin (Sigma Chemical Co., Mo.) and also matched the ^{1}H NMR trace published by Blanc et al. (1995a).

DISCUSSION

Statistically based experimental designs have proven to be valuable tools in optimizing culture conditions (Zhang et al., 1996; Ooijkaas et al., 1999; El-Helow and El-Ahawany, 1999; El-Helow et al., 2000; Elibol, 2004). One

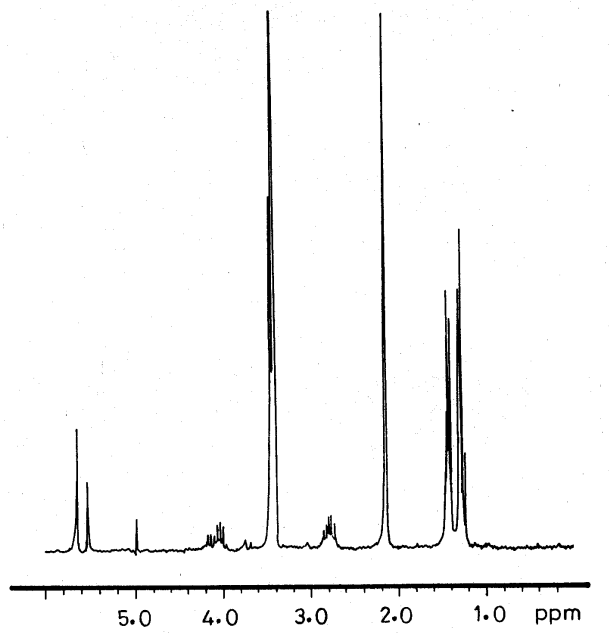

Figure 2. ^{1}H NMR (CD_3OD) spectrum of the mycotoxin citrinin produced by *M. ruber*.

Table 5. The main effect (%) and statistical analysis (t-values) for equal unpaired samples for the determination of variable significance in the Plackett Burman experiment.

Factor	Inhibition Zone diameter			Dry weight		
	Main effect*	t-value	Significance level	Main effect*	t-value	Significance level
Glucose	3.25	2.82	0.01	3.84	0.0034	
$NaNO_3$	2.25	1.385	0.1	-0.37	-0.8448	
KH_2PO_4	3.75	1.4084	0.1	-1.32	-0.4905	
$MgSO_4 \cdot 7H_2O$	4.25	0.0018		3.19	1.8636	0.05
$ZnSO_4 \cdot 7H_2O$	4.75	0.0004		8.30	5.9377	0.005
$MnSO_4 \cdot H_2O$	-7.75	-0.0312		-2.24	-0.2363	
$FeSO_4 \cdot 7H_2O$	-0.25	-0.8709		-5.36	-0.0019	

*Main effect is expressed as the response percentage.

Table 6. Experimental verification of the combined effect of optimized medium on *M. ruber* growth and citrinin production.

Medium No. 2	Inhibition zone diameter (mm)	Citrinin concentration (mg/l)	Dry weight (mg/ml)
Basal setting	16±1.2	220±10	10.45±0.02
Optimized	28±0.9	400±9	19.45±0.12

of the advantages of applying multi-factorial experiments is that such an approach considers the interaction between the non-linear nature of the responses in short experiments (Gresham and Inamine, 1986). In the present study, the Plackett-Burman design has been demonstrated to be an efficient approach to optimizing the medium components affecting the production of citrinin by *M. ruber*.

The optimization results indicated the importance of phosphate for efficient citrinin production. This observation can be interpreted mainly as an effect of phosphate functional groups on medium pH (Bonthrone et al., 2000). Zinc has been shown to be essential for growth of several fungi. One overall effect of zinc in the fungus metabolism is a reduction in the economic coefficient (sugar consumed/weight of fungus). It has been established that the addition of zinc sulphate reduces the biomass (Lin and Demain, 1991). Similarly, for *M. purpureus*, the addition of zinc reduced the economic coefficient from 13.1 to 6.1, indicating that zinc enables the fungus to utilize D-glucose for growth (Johnson and McHan, 1975).

Utilization of the carbon source is an important factor for citrinin production by *M. ruber*. 136 mg/ml was produced in agitated Erlenmeyer flasks containing synthetic medium No. 1, which included ethanol (28 g/l as a carbon source) and 226 mg/ml was produced in the same synthetic medium which contained glucose (45 g/l) as a carbon source (Blanc et al., 1995b). In this work, significant improvement in the production of citrinin by *M. ruber* was achieved and the yield was 400 mg/l when glucose (45 g/l) was used. Although *M. ruber* utilized glucose as a carbon source, other *Monascus* strains grew well on galactose or maltose (Yoshimura et al., 1975; Lin et al., 1992). This result is in agreement with the fact that utilization of carbon source by this genus appears to be strain dependent (Fabre et al., 1993).

Regarding nitrogen source utilization, ammonium chloride has been reported to be a better inorganic nitrogen source than sodium nitrate for biomass and pigment production by *M. purpureus* (Juzlova et al., 1996; Chen and Johns, 1993), but monosodium glutamate and yeast extracts were preferred by other *M. ruber* strains for pigment and citrinin production (Blanc et al., 1995a, b). Although Blanc et al. (1995a) obtained a high citrinin titre (370 mg/l) in *M. ruber* culture that utilized yeast extract as a nitrogen source (YES, medium 3), the present report demonstrates that sodium nitrate (400 mg/ml) is a better nitrogen source than monosodium glutamate (160 mg/l) or yeast extract (180 mg/l) for citrinin production. This is in line with the role of nitrate as a terminal electron acceptor for this aerobic fungus. This again appears to reinforce the strain-dependence concept for nitrogen source utilization. Changes in the nature and concentration of carbon and nitrogen sources and phosphate concentration have been reported to affect polyketide biosynthesis through which citrinin is synthesized (Doull and Vining, 1989). Medium 2 is thus sufficiently improved over the medium 1 and 3 used by the same species (Blanc et al., 1995a) though the reduction in some ingredients and the weight of some others may also provide another biotechnological advantage to the industrial sector. Furthermore, a complete defined medium for citrinin production may provide another advantage for downstream processing.

It was shown that small micropellets formed in trials No. 8 and 9 were related to the highest activity and dry weight, while large pellets observed for trials 2, 3, 5, 7, resulted in the lowest values for both activity and growth. Since the citrinin yield proved to be directly related to the oxygenation conditions (Hajjaj et al., 1999), the large pellets may have reduced the mass and oxygen transfer due to the internal diffusion limitation which in turn lowered citrinin yield.

The results of the present work clearly demonstrate the effectiveness of factorial design in optimizing medium composition and improving the growth of *M. ruber*. Its usefulness in designing an efficient fermentation process for the production of citrinin by *Monascus ruber* to be used for non-food applications has also been proven.

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應用 Plackett-Burman 因子設計以改善 *Monascus ruber* 批次培養時 citrinin 之產量

Abdulaziz Q. M. AL-SARRANI[1] and Moustafa Y. M. EL-NAGGAR[2]

[1] Biology Department, Microbiology Division, Faculty of Science, Taibah University, P.O. Box 344, Al-Madinah Al-Munawarah, Kingdom of Saudi Arabia

[2] Botany Department, Microbiology Division, Faculty of Science, Alexandria University, Moharram Bay 21511, Alexandria, Egypt

先前由紅椒汁單離所得之黴菌定性為 *Monascus ruber* 者，被用來試驗其在批次培養時生產抗菌物質之潛能。此黴以三種液態培養基供試。長在合成培養基 No. 2 者可得最高抗菌活性。該抗菌物質經抽取，純化，及定性為黴毒素 citrinin。Plackett-Burman 實驗設計被用來最適化培養基 No. 2 之成份以求得改善 citrinin 之產量以供非食品應用。結果得出下述配方可得濾液中接近最佳之細胞外生產 citrinin 之條件（克/升）：葡萄糖，45；碳酸鈉，3.75；磷酸二氮鉀，0.2；硫酸鎂（7個水合物），0.1 及硫酸鋅（7個水合物）0.01；硫酸錳，0.006；硫酸鐵，0.003。改善可達 1.75 倍產量。另一方面，黴之乾物量經改善後可達控制組之 1.86 倍。以高性能液態層析法 (HPLC) 測上澄液之 citrinin 濃度，經改善者及控制組分別為：400 及 220 毫克/升。

關鍵詞：批次培養；Citrinin；*Monascus ruber*；最適化；Plackett-Burman 實驗設計。