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انتاج السيكلوسبورين أ باستخدام فطر الفيوزاريوم أوكسيسبورم تحت تحسين الظروف احصائيا

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الملخص العربي

عقار السيكلوسبورين هو عقار مثبط للمناعة يستخدم في علاج مرضي نقل الأعضاء وبعض الأمراض المناعية.في هذه الدراسة يتم انتاج السيكلوسبورين باستخدام فطر الفيوزاريوم أوكسيسبورم ويجري دراسة تأثير عشر عوامل بيئية (pH,Incubation period ,volume of medium) وعوامل تغذية (glucose conc.,Corn steap liquor conc.,L-valine conc.,L-valine addition time.,Glycine conc.,Glycine addition time,Kcl conc) علي انتاج السيكلوسبورين باستخدام برامج احصائية علي مستويين ،الأول هو (Plackett-Burman) والذي نتج عنه التأثير الإيجابي الواضح لزيادة فترة التخمر وتركيز حمض الجليسين علي انتاج السيكلوسبورين وكذلك التأثير السلبي لزيادة الرقم اليوني ،لذا فقد تم دراسة هذين العاملين:١- تركيز الجليسين علي ٣ مستويات (٤,٥,٦ جم / لتر) و٢- قترة التخمر علي ثلاثة مستويات(١١,١٣,١٥ يوم) علي مستويين الأول بالتحكم في الرقم الهيدروجيني والثاني بدون تحكم به باستخدام برنامج (central composite rotatable design) .فوجد أنه علي مستوي المعاملات التي تم التحكم في رقمها الهيدروجيني كان لزيادة الجليسين تأثير سلبي بينما لزيادة فترة التخمر تأثير ايجابي ،أما علي مستوي المعاملات التي لم يتم التحكم في رقمها الهيدروجيني فوجد أن زيادة كلا من تركيز الجليسين وفترة التخمر يزيد من انتاج السيكلوسبورين. وكان أفضل انتاج للسيكلوسبورين هو ٦٥٤

CYCLOSPORINE A PRODUCTION BY *FUSARIUM OXYSPORUM* UNDER STATISTICALLY OPTIMIZED SUBMERGED CONDITIONS

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ABSTRACT: *An optimization strategy based on statistical designs was employed to improve the production of cyclosporine A (CyA) by Fusarium oxysporum in a submerged culture. A 2-level Plackett-Burman design was used to screen the most effective environmental and nutritional parameters on CyA production. Among the ten tested parameters, glycine concentration and incubation time were selected, owing to their significant positive effect on CyA production. A central composite rotatable design was adopted to acquire the best process parameters on both buffered & unbuffered conditions. Optimal combination of the major media constituents for CyA production were evaluated using statistical analysis soft wore (SAS) resulting in a maximum CyA yield of 654.76 mg/l .*

Key wards : *Cyclosporine A, Fusarium Oxysporum, Plackett – Burman design ,CYA production ,*

INTRODUCTION

Cyclosporine A is a neutral lipophilic cyclic polypeptide (consists of 11 amino acids) which is the main components of 25 naturally occurring cyclosprines having the substitutions of amino acids in positions 1,2,4,5,7 and 11 (Traber *et al*., 1989). It was initially developed as an antifungal in the early 1970s (Borel and Kis 1991). It exhibits a variety of biological activities, including anti-inflammatory and antiparasitic properties (Dreyfuss *et al*., 1976). It is used for the treatment of organ transplantation, autoimmune diseases like rheumatoid arthritis, uveitis, bronchial asthma, and inflammatory bowel patients owing to its superior T-cell specificity and low level of myelotoxicity (Kahan 1984 and Shindler 1985).

Although the enzymatic production of cyclosporine has already been established and proven (Billich and Zocher., 1987), submerged fermentation production is normally used, owing to the complexity of enzymatic synthesis. Many attempts have also been made to optimize CyA production, including strain improvement (Agathos and Parekh 1990), solid state fermentation (Survase *et al*.,2009-a), immobilization (Survase *et al*.,2010) ,addition of amino acids and statistical design approach (Abdel – Fattah *et al*., 2007).

The objectives of the present study were to evaluate the significance of different culture conditions and specific physical parameters on CyA production by *F.oxysporum*. Most of the previous studies were carried out using *Tolypocladium* species, in this study , *Fusarium oxysporum* was used as the producing organism.

MATERIALS AND METHODS

Microorganism

The *F.oxysporum* was delivered as a slant from plant pathology institute/National plant agricultural center. It was maintained

on malt – yeast extract medium (MY medium) obtained by Agathos *et al*. (1986) which consists of (g/l): malt extract 20, yeast extract 4, agar 20, at pH 5.3.

Cultivation conditions

The inoculum was prepared using MY agar plates cultivated with the fungus and incubated at 27°C for 7 days. Flasks were inoculated each with a 5mm fungal disc.

Analysis

Sample preparation and determination of cell dry weight.

Samples, in the form of unit flask were taken at different fermentation times in a Falcon centrifugation tube and centrifuged at 5,000 rpm for 5 min. The centrifuged cells were washed with distilled water and dried to a constant weight at 120^{o}C.

Glucose determination.

The glucose was enzymatically determined using the glucose oxidase-glucose peroxidase method (Diamond Diagnostics, Cairo, Egypt), and the intensity of the developed color was determined at 500 nm using a spectrophotometer (Pharmacia Biotech, Cambridge, England).

Extraction of CyA .

The CyA extraction was carried out according to the method of Abdel–Fattah *et al*. (2007). Equal volumes of ethyl acetate were added to each fermentation flask, and then shaken at 27°C, 2000 rpm for 20h. The samples were centrifuged at 10,000 rpm for 10 min., and then the ethyl acetate layer was removed, dried and dissolved in methanol HPLC grade.

Quantification of CyA.

The quantification of CyA was carried out by HPLC according to the modified method of Abdel–Fattah *et al*. (2007), where a hyperclone 5 µ C8 (250×24 mm) column was used with a mobile phase composed of acetonitrile: water: phosphoric acid (700:300:0.1) pumped at a flow rate of 1 ml/min (Agilant 1200 UV detector) at 60°C. Samples (10 µl) containing 0-100 mg/l CyA were used. The method was calibrated using Sandimmune ampoules.

Statistical designs

Plackett-Burman design. For screening purposes, various medium components and culture parameters were evaluated. Based on a Plackett-Burman factorial design, each factor was examined at 2 levels: -1 for the low level, and 1 for the high level (Plackett and Burman 1946). This design was especially practical in the case of a large number of factors and when it was unclear which settings are likely to be nearer to the optimum responses (Strobel and Sullivan 1999) and for screening medium components with respect to their main effects and not their interaction effects (Plackett and Burman 1944). Table 1 represents the media components , as well as the levels of each factor used in the experimental design, whereas Table 2 represents the design matrix.

Table 1. Media components and testing levels for the Plackett- Burman experiment.

Media component	Component	Low level	High level
Glucose concentration	X1	20 (g/l)	50(g/l)
Kcl concentration	X2	1.5 (g/l)	3(g/l)
Corn steap liquor concentration	X3	10 (ml/l)	20(ml/l)
L-valine concentration	X4	1(g/l)	4(g/l)
Time of addition of L-valine	X5	Zero	48 hrs
Glycine concentration	X6	1(g/l)	4(g/l)
Time of addition of glycine	X7	Zero	48 hrs

Volume of medium	X8	50 ml	125 ml
pH	X9	4.5	6.5
Incubation period	X10	7	14

Table 2: The investigated factors for the production of cyA as well as the levels of each factor used in the experimental design (Plackett-Burman Design).

No.	PH	C.S.L	Incubation time	Kcl	Glucose	Volumeof medium	L-Valine	Timeof addition	Glycine conc.	Time.of addition
1-	7	20	14	3	50	125	4	48	4	48
2-	4	20	7	1.5	50	125	4	48	1	48
3-	4	10	14	1.5	20	125	4	48	4	0
4-	7	20	14	3	20	125	1	48	1	0
5-	7	10	7	1.5	20	125	4	0	1	48
6-	4	10	7	3	50	50	1	48	1	0
7-	7	10	7	3	20	50	4	48	4	48
8-	7	20	7	3	20	125	1	0	1	0
9-	7	20	7	1.5	50	50	1	48	4	48
10-	4	10	14	3	50	125	1	48	1	48
11-	4	20	7	1.5	20	50	4	48	1	0
12-	4	20	14	1.5	20	125	1	0	4	48
13-	4	10	14	3	20	50	4	0	1	48
14-	4	20	14	3	50	50	4	0	4	0
15-	7	10	14	1.5	20	50	1	48	4	0
16-	7	10	7	3	50	125	4	0	4	0
17-	4	20	7	3	20	50	1	0	4	48
18-	7	20	14	1.5	50	50	4	0	1	0
19-	4	10	7	1.5	50	125	1	0	4	0
20-	7	10	14	1.5	50	50	1	0	1	48

Superior optimization stage: RSM:

A central composite rotatable design (CCRD) for 2 independent variables was used to obtain the combination of values that optimizes the response within the region of three dimensional observation spaces, which allows one to design a minimal number of experiments. The independent variables selected for the optimization were (glycine concentration and incubation period). Table 3 illustrates the factors investigated, whereas Table 4 (a and b) showes the actual values of independent variables on buffered and unbuffered levels respectively.

RESULTS

An optimization strategy was applied, where the first stage dealt with determining the nutritional and environmental factors affecting CyA production by *F.oxysporum* using Plackett-Burman experimental design. The second stage involved more details for the most effective parameters obtained from the first stage and effect of their interaction using central composite rotatable design (CCRD).

Table 3: Factors investigated using CCRD

Media component	Level
Glycine concentration	1,4 (g/l)
Incubation period	11,13,15 days

Table 4 a: Testing levels for the CCRD(buffered level).

Fermentation time(x1)	Glycine conc.(x2)
13	6
11	6
15	5
15	6
13	5
13	4
13	5
11	4
15	4
11	5

b: Testing levels for the CCRD(Unbuffered level)

Fermentation time (x1)	Glycine conc.(x2)
13	6
11	6
11	4
15	6
13	5
15	5
13	4

11	5
13	5
15	4

Plackett-Burman experimental design:

A Plackett-Burman experimental design was applied to reflect the relative importance of applying ten culture and nutritional factors (variables) at two different levels for the medium optimization . Table 5 represents the design matrix and results of different trials (response) in mg/l. A Plackett-Burman design is a 2-level design, where each variable is tested at a low (-1) and high (+1) level. On analyzing the regression coefficients for the ten variables, the following three variables; incubation period, glycine and corn steap liquor concentrations had positive effects on the CyA production, whereas increasing pH values and L-valine addition time had negative effects. The other tested variables (kcl conc, l-valine conc., glucose conc., volume of medium and addition time of glycine) had a lower effect on the CyA production, Table 6.

The most significant variables, namely glycine conc., incubation period were then tested for optimization using CCRD, on two designs; buffered and unbuffered.

Glycine was used in the following conc. (4,5,6 g/l), incubation period was tested at 3 levels(11,13,15 days) , whereas the variables with a less significant effects were fixed at their minimum values.

Table 5: The Plackett-Burman Design design matrix and results of different trials in mg/l.

No.	PH	C.S.L	Incubation time	Kcl	Glucose	Volumeof medium	L-Valine	Timeof addition	Glycine conc.	Time.of addition	CyA yield(mg/l)
1-	7	20	14	3	50	125	4	48	4	48	22.4
2-	4	20	7	1.5	50	125	4	48	1	48	15.1
3-	4	10	14	1.5	20	125	4	48	4	0	143.4
4-	7	20	14	3	20	125	1	48	1	0	4.6
5-	7	10	7	1.5	20	125	4	0	1	48	5.4
6-	4	10	7	3	50	50	1	48	1	0	10.01
7-	7	10	7	3	20	50	4	48	4	48	14.2
8-	7	20	7	3	20	125	1	0	1	0	2.5
9-	7	20	7	1.5	50	50	1	48	4	48	8.8
10-	4	10	14	3	50	125	1	48	1	48	55.3
11-	4	20	7	1.5	20	50	4	48	1	0	6.9
12-	4	20	14	1.5	20	125	1	0	4	48	50
13-	4	10	14	3	20	50	4	0	1	48	7.8
14-	4	20	14	3	50	50	4	0	4	0	351.1
15-	7	10	14	1.5	20	50	1	48	4	0	2.8
16-	7	10	7	3	50	125	4	0	4	0	1.0

17-	4	20	7	3	20	50	1	0	4	48	107.1
18-	7	20	14	1.5	50	50	4	0	1	0	95.2
19-	4	10	7	1.5	50	125	1	0	4	0	20.2
20-	7	10	14	1.5	50	50	1	0	1	48	197.6

Table 6 : Sorted Parameter Estimates

Term	Estimate	Std Error	t Ratio	t Ratio
inc time(7,14)	0.1038	0.042629	2.43	
l-valine time(1,2)	-0.001842	0.000888	-2.07	
ph(4.5,6.5)	-0.0726	0.042629	-1.70	
glycine(1,4)	0.0422	0.028419	1.48	
corn steap liquor(1,2)	0.0552	0.042629	1.29	
kcl(1.5,3)	-0.0355	0.042629	-0.83	
l-valine(1,4)	-0.015133	0.028419	-0.53	
glycine time (1,2)	0.0004729	0.000888	0.53	
volume of medium(50,125)	-0.0044	0.042629	-0.10	
g;ucose(35,60)	-0.0043	0.042629	-0.10	

Results of central composite rotatable design (CCRD):

The combined effect of two independent variables A: glycine conc. (g/l); B: incubation period for the production of CyA was examined using RSM. The experimental values of yields of CyA are given in Table 7.

Two central composite designs (buffered and unbuffered) for the two independent variables were used to obtain combination of the variables that optimizes the CyA production.

On buffered condition, increased glycine conc. had a significant negative effect, incubation time had a less significant positive effect while their interaction had a less significant negative effect Table 8-a

On the other hand, unbuffered condition showed different results where, incubation time had a high significant positive effect; glycine conc. had a less significant positive effect while their interaction showed the least significant positive effect Table 8-b.

DISCUSSION:

PH effect:

Changes in external pH affect many cellular processes such as the regulation of the biosynthesis of secondary metabolites (Chang *et al*., 1991; Datta and Kothary, 1993; Sole *et al*., 1994). The biosynthesis of CyA is greatly affected by the pH value of the medium (Sallam *et al*.,2003) .The results of this research reflects the importance of pH on CyA production as the producing flasks were mainly those which started with medium having low pH values table 9 , this result agrees with (Margaritis and Chahal ; 1989),(Sallam *et al*.,2003), (Survase *et al*., 2009-b), (Chun and Agathos; 1989) and (Manuela *et al.,*1996) .In this study the low starting pH was optimum for the growth of *F.oxysporum* , according to (Gangadhara *et al*.,2010) who stated that the most suitable pH level for growth of fungus was 5.0 and 6.0.

Wang *et al.,* (2011) stated that pH plays an important role in *Xenorhabdus nematophila* YL001 fermentation process. Cell concentration and antibiotic activity profiles had a similar trend in response to initial pH, our results are in accordance with these results as there is an obvious relation between the pH and the dry weight obtained

specially in buffered flasks used within central composite experiments, data is shown in table 10 a and b. It was very exciting to know that the the increased CyA production in the case of the controlled pH culture resulted from a higher cell mass rather than an increase in cell productivity (El Enshasy *et al*. 2008).

Table 7.a: Testing levels for the CCRD(buffered level).

Fermentation time(x1)	Glycine conc.(x2)	CyA yield(mg/l)
13	6	4.1
11	6	5.45
15	5	238.09
15	6	9.11
13	5	12.16
13	4	10.3
13	5	7.4
11	4	416.67
15	4	654.76
11	5	7.12

b: Testing levels for the CCRD(Unbuffered level) .

Fermentation time(x1)	Glycine conc.(x2)	CyA yield(mg/l)
13	6	9.4
11	6	5.2
11	4	3.5
15	6	315.48
13	5	2.01
15	5	535.71
13	4	1.09
11	5	7.3
13	5	2.8
15	4	9.4

Table 8 a: Sorted Paramete restimates(buffered)

Term	Estimate	Std Error	t Ratio	t Ratio	Prob>|t|
X2	-169.6667	66.20737	-2.56		0.0625
X1*X1	193.21429	106.168	1.82		0.1429
X1(11,15)	76	66.20737	1.15		0.3150
X2*X2	90.214286	106.168	0.85		0.4433
X1*X2	-62	81.08714	-0.76		0.4871

b: Sorted Paramete rEstimates(unbuffered)

Term	Estimate	Std Error	t Ratio	t Ratio	Prob>|t|
X1(11,15)	135.5	55.16462	2.46		0.0700
X1*X1	152	88.46019	1.72		0.1609

Term	Estimate	Std Error	t Ratio	t Ratio	Prob>|t|
X2*X2	-100.5	88.46019	-1.14		0.3194
X1*X2	71.25	67.56259	1.05		0.3511
X2(4,6)	53	55.16462	0.96		0.3911

Table 9: Relation between starting pH, dry weight and CyA conc.(mg/l) for Plackett burmann design.

No.	Incubation time-days	Initial pH	Glucose	Dry weight(g/l)	CyA yield(mg/l)
1	14	7	50	18.4	22.4
2	7	4	50	6.24	15.1
3	14	4	20	5.2	143.4
4	14	7	20	8.2	4.6
5	7	7	20	6.4	5.4
6	7	4	50	11	10.01
7	7	7	20	8.8	14.2
8	7	7	20	13.12	2.5
9	7	7	50	23	8.8
10	14	4	50	11.44	55.3
11	7	4	20	20	6.9
12	14	4	20	9.44	50
13	14	4	20	6.2	7.8
14	14	4	50	20.2	351.1
15	14	7	20	21	2.8
16	7	7	50	6.08	1.0
17	7	4	20	13.4	107.1
18	14	7	50	20.2	95.2
19	7	4	50	8.16	20.2
20	14	7	50	5.8	197.6

Table 10-a: Relation between dry weight(g/l) , fermentation time & CyA conc.(m g/l) for CCRD design (buffered level)

Fermentation time(x1)	Glycine conc.(x2)	Yield(mg/l)	Dry weight(g/l)
13	6	4.1	1.16
11	6	5.45	0.89
15	5	238.09	0.99
15	6	9.11	1.18
13	5	12.16	1.24
13	4	10.3	0.77

13	5	7.4	1.24
11	4	416.67	1.045
15	4	654.76	0.73
11	5	7.12	1.22

b: Relation between dry weight(g/l) , fermentation time & CyA conc.(mg/l) for CCRD design (unbuffered level).

Fermentation time(x1)	Glycine conc.(x2)	Yield(mg/l)	Dry weight(mg/l)
13	6	9.4	0.68
11	6	5.2	0.56
11	4	3.5	0.62
15	6	315.48	0.57
13	5	2.01	0.78
15	5	535.71	0.45
13	4	1.09	0.82
11	5	7.3	0.5
13	5	2.8	0.78
15	4	9.4	0.65

Table 9 shows no obvious relationship between initial pH and dry weight, but there is a relation between dry weight and initial glucose concentration.

From table 10 a and b, by comparison there is obvious relationship between the use of buffering system and both CyA production and dry weight conc. which is an indicator for growth.

It was also noted that the final pH of producing media was greater than that of their starting pH mainly, this result agree with (Aarino and Agathos;1990).

The lower final concentration of CyA in unbuffered systems may be due to its degradation which was higher in the pH-uncontrolled culture (El Enshasy *et al*. 2008).

Glycine concentration:

This research dealt with the effect of addition of both L-valine and glycine and also with the effect of their addition time. Results revealed that glycine concentration is more effective on CyA production than the previously mentioned parameters. The result of glycine agree with that obtained by (Lee and Agathos;1989) who stated also that glycine is a member of the CyA molecule, which may affect the drug production by assuming one or more roles such as precursor, inducer, and/or developmental regulator. Some amino acids may act as inducers which must be added in exponential growth phase to manifest their ability to enhance secondary metabolite production. It is possible that these amino acids may direct cell development in a manner favoring secondary metabolite production by affecting transcription of secondary metabolite genes during vegetative cell growth (Grafe , 1982).

It is also known that glycine may act as a buffering agent which may contribute with the explanation of its positive effect on CyA production

Addition time of l-valine:

Addition of l-valine after the beginning of fermentation had a positive effect on CyA production. That may be due to the role of l-

valine as inducer to increase the transcription of genes for CyA synthetase or other structural genes contributing to CyA synthesis pathway giving the positive effect when added after 18 hrs of the beginning of fermentation (Lee and Agathos;1989).

Effect of corn steap liquor addition:

Corn steep liquor is a by-product of the corn wet-milling industry which may serve either as a supplement to replace extracts, or as the main source of nitrogen and carbon (Liggett and Koffler;1984).In this study it had a positive effect on CyA production which may be attributed to the presence of certain amino acids in the steap water as alanine and l-valine , (Cardinal and Hedrick;1948).

Effect of incubation period:

Incubation period had a positive effect on CyA production i.e increasing of incubation period led to increase in CyA production, this may be related to the use of spore form of fungi as inoculum ,as the spores might be unable to utilize the complex substrate as efficiently as the vegetative mycelia and that effective growth and substrate utilization occurred only after germination of the spores leading to the observed lag time in their growth which had been approved by the studies of Sekar and Balarman.,(1998) who observed the appearance of visible growth of *Tolypocladium* species on day 2 in case of using vegetative mycelium as inoculum compared to their appearance on day 4 in case of using spores as inoculum ,this result agree with that obtained by Dreyfuss *et al*.(1976), (Agathos *et al*.,1986), (Agathos and Parekh ;1990), (Sakamoto *et al*.,1993) and (Sallam *et al*.,2003) , all these results supported the assumption that the optimum fermentation period for CyA production was recoded at a period more than 7 days which is the minimum fermentation period applied in this study. On the contrary, Ismaiel *et al*.(2010) found that the optimum growth period for CyA production was recoded at 7 days , then a decline was obtained in with increasing of the growth period.

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انتاج السيكلوسبورين أ باستخدام فطر الفيوزاريوم أوكسيسبورم تحت تحسين الظروف احصائيا

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الملخص العربي

عقار السيكلوسبورين هو عقار مثبط للمناعة يستخدم في علاج مرضي نقل الأعضاء وبعض الأمراض المناعية.في هذه الدراسة يتم انتاج السيكلوسبورين باستخدام فطر الفيوزاريوم أوكسيسبورم ويجري دراسة تأثير عشر عوامل بيئية (pH,Incubation period ,volume of medium) وعوامل تغذية (glucose conc.,Corn steap liquor conc.,L-valine conc.,L-valine addition time.,Glycine conc.,Glycine addition time,Kcl conc) علي انتاج السيكلوسبورين باستخدام برامج احصائية علي مستويين ،الأول هو (Plackett-Burman) والذي نتج عنه التأثير الإيجابي الواضح لزيادة فترة التخمر وتركيز حمض الجليسين علي انتاج السيكلوسبورين وكذلك التأثير السلبي لزيادة الرقم اليوني ،لذا فقد تم دراسة هذين العاملين:١- تركيز الجليسين علي ٣ مستويات (٤,٥,٦ جم / لتر) و٢- فترة التخمر علي ثلاثة مستويات(١١,١٣,١٥ يوم) علي مستويين الأول بالتحكم في الرقم الهيدروجيني والثاني بدون تحكم به باستخدام برنامج (central composite rotatable design) .فوجد أنه علي مستوي المعاملات التي تم التحكم في رقمها الهيدروجيني كان لزيادة الجليسين تأثير سلبي بينما لزيادة فترة التخمر تأثير ايجابي ،أما علي مستوي المعاملات التي لم يتم التحكم في رقمها الهيدروجيني فوجد أن زيادة كلا من تركيز الجليسين وفترة التخمر يزيد من انتاج السيكلوسبورين. وكان أفضل انتاج للسيكلوسبورين هو ٦٥٤