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# Application of Factorial Experimental Designs for Optimization of Cyclosporin A Production by *Tolypocladium inflatum* in Submerged Culture

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**Abstract** A sequential optimization strategy based on statistical experimental designs was employed to enhance the production of cyclosporin A (CyA) by *Tolypocladium inflatum* DSMZ 915 in a submerged culture. A 2-level Plackett-Burman design was used to screen the bioprocess parameters significantly influencing CyA production. Among the 11 variables tested, sucrose, ammonium sulfate, and soluble starch were selected, owing to their significant positive effect on CyA production. A response surface methodology (RSM) involving a 3-level Box-Behnken design was adopted to acquire the best process conditions. Thus, a polynomial model was created to correlate the relationship between the three variables and the CyA yield, and the optimal combination of the major media constituents for cyclosporin A production, evaluated using the nonlinear optimization algorithm of EXCEL-*Solver*, was as follows (g/l): sucrose, 20; starch, 20; and ammonium sulfate, 10. The predicted optimum CyA yield was 113 mg/l, which was 2-fold the amount obtained with the basal medium. Experimental verification of the predicted model resulted in a CyA yield of 110 mg/l, representing 97% of the theoretically calculated yield.

**Keywords:** Cyclosporin A, *Tolypocladium inflatum*, optimization, factorial designs

The cyclic undecapeptide cyclosporin A, an immunosuppressive with a weak, narrow spectrum of antifungal activity, is produced nonribosomally from a multifunctional enzyme template (cyclosporin synthetase) by the filamentous fungus *Tolypocladium inflatum* Gams [11] and some other rare actinomycetes [15]. Cyclosporin contains unusual amino acids (*e.g.*, butenyl dimethyl threonine, 2-amino-butyric acid, and D-alanine) at positions 1, 2, and 8, respectively [24]. In addition to a potential immunosuppressive effect, CyA is a drug with broad-spectrum pharmacological properties, including antifungal, antiparasitic, and antiinflammatory activities [19]. The history of CyA has already been reviewed [17], and the enzyme well-researched, purified, and cloned [5, 8, 13, 22, 23, 25].

However, even though the enzymatic production of cyclosporin has already been established and proven [5], submerged fermentation production is normally used, owing to the complexity involved in enzymatic synthesis.

In addition to the use of recombinant DNA technology for strain improvement, many attempts have also been made to optimize CyA production, including immobilization [19] and solid state fermentation [14]. In addition, one practical method for improving yield is a statistical design approach, which has been extensively applied over the last decade with many enzymes, secondary metabolites, and biotechnology products [1, 2, 18].

Nonetheless, it would seem that optimizing the medium constituents for the production of CyA in a submerged culture has not yet been carried out based on a numerical modeling approach.

Accordingly, the objectives of the present study were to evaluate the significance of different culture conditions on CyA production by *T. inflatum* DSMZ 915, followed by optimizing the titer of the identified significant variables and creating a polynomial model equation to correlate the relationship between the variables and the cyclosporin yield. Finally, the model was assessed experimentally to verify its accuracy.

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## MATERIALS AND METHODS

### Microorganism

The *T. inflatum* (DSMZ 915) was delivered as a lyophilized culture from DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany). The spores were obtained from a sporulation agar (ISP medium), and then spore suspensions were prepared after 10 days of cultivation and maintained in 50% glycerol at -80°C. The viability of the deep-frozen cultures was periodically controlled by plating them onto an ISP2 agar (malt extract 10 g/l; yeast extract, 4 g/l; glucose, 4 g/l; agar, 20 g/l; pH 7.2) and counting the colonies formed.

### Cultivation Conditions

The inoculum was prepared using the inoculate spore suspension in a 250-ml Erlenmeyer flask containing 50 ml of a DSMZ 90 medium (malt extract, 30 g/l; Bactopeptone, 3 g/l; pH 5.7) and incubated on an incubatory shaker at 27°C and 200 rpm for 48 h, and then used for inoculating the production medium at a 5% v/v ratio. The CyA production step was also performed in a 250-ml Erlenmeyer flask with a working volume of 50 ml. The basal semisynthetic medium (SSM) used for the CyA production had the following composition (g/l): glucose, 10; sucrose, 20; starch, 20; yeast extract, 10; $(NH_4)_2SO_4$, 10; $KH_2PO_4$, 1; $MnSO_4$, 1 and pH 5.9. The inoculated flasks were shaken at 200 rpm on a rotary shaker (New Brunswick, Edison, NJ, U.S.A.) with an eccentricity of 2.0 cm, and the cultivation temperature was 27°C.

### Analysis

**Sample Preparation and Determination of Cell Dry Weight.** Samples, in the form of 2 flasks with 50 ml of broth in each or 10 ml of broth (in the case of bioreactor cultures), were taken at different times during the cell cultivation in a preweighed Falcon centrifugation tube (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) and centrifuged at 3,000 rpm for 20 min. A small fraction of the supernatant was frozen at -20°C and left for a glucose and carbohydrate analysis. Meanwhile, the centrifuged cells were washed twice with distilled water, centrifuged, and the centrifugal tubes dried to a constant weight at 80°C.

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

**Total Carbohydrates Determination.** The total carbohydrates were determined spectrophotometrically according to the method of Dubois *et al.* [10]. The samples were heated with sulfuric acid to hydrolyze the polysaccharides and hydrate the monosaccharides to form furfural from pentoses and hydroxymethylfurfural from hexoses. The solutions of furfural and hydroxyfurfural were then treated with a phenol reagent to produce a colored compound that was measured spectrophotometrically using a Novaspec II spectrophotometer (Pharmacia Biotech., Cambridge, England).

**Extraction of CyA from Culture.** The CyA extraction from the culture broth was carried out according to the method of Agathos *et al.* [3] with some modifications, where 50 ml of ethyl acetate was added to the fermentation flasks, which were then shaken at 27°C, 200 rpm, for 20 h. Thereafter, the samples were centrifuged at 4,000 rpm for 30 min to separate the organic and aqueous layers, and then the ethyl acetate layer was removed and filtered using a bacterial filter.

**Quantification of CyA.** The quantification of CyA was carried out by HPLC according to the modified method of Kreuzig [12], 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 2 ml/min (pump model 168 solvent module; Beckman, Fullerton, CA, U.S.A.) at 60°C. Samples (20 μl) containing 0–100 mg/l CyA were used. The product was detected based on the UV absorbance (variable wavelength photoarray detector model 168; Beckman) at 214 nm. The method was calibrated using standard CyA obtained from Sigma, U.S.A.

### 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 [16]. This design is especially practical in the case of a large number of factors and when it is unclear which settings are likely to be nearer to the optimum responses [21]. Table 1 illustrates the factors investigated,

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

| Media component | Component | Low level (-1) (g/l) | High level (+1) (g/l) |
|---|---|---|---|
| Glucose | $X_1$ | 0 | 10 |
| Sucrose | $X_2$ | 0 | 10 |
| Starch | $X_3$ | 0 | 10 |
| Peptone | $X_4$ | 0 | 10 |
| Yeast extract | $X_5$ | 0 | 10 |
| $(NH_4)_2SO_4$ | $X_6$ | 0 | 1 |
| $KH_2PO_4$ | $X_7$ | 1 | 5 |
| $MnSO_4$ | $X_8$ | 0 | 1 |
| $ZnSO_4$ | $X_9$ | 0 | 1 |
| $CoCl_2$ | $X_{10}$ | 0 | 1 |
| KCl | $X_{11}$ | 0 | 1 |

as well as the levels of each factor used in the experimental design, whereas Table 2 represents the design matrix.

The Plackett-Burman experimental design was based on the following first-order model:

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

where Y is the response (CyA yield), $\beta_0$ is the model intercept, $\beta_i$ is the linear coefficient, and $x_i$ is the level of the independent variable. This model does not describe the interaction among the factors, but is used to screen and evaluate the important factors that influence the response. In the present work, 11 assigned variables were screened in 12 experimental trials. All the experiments were carried out in triplicate and the averages of the CyA volumetric yield taken as the response.

**Box-Behnken Design.** To describe the nature of the response surface in the experimental region, a Box-Behnken design [6] was applied. The factors with the highest confidence levels were prescribed at three levels, coded −1, 0, and +1 for the low, middle, and high concentrations (or values), respectively. Table 3 represents the design matrix for 15 trials, along with the natural values for the three factors (g/l). To predict the optimal point, a second-order polynomial function was fitted to correlate the relationship between the independent variables and the response (CyA yield). The equation for the three factors was as follows:

$$Y=\beta_0+\beta_1 X_1+\beta_2 X_2+\beta_3 X_3+\beta_{12} X_1X_2+\beta_{13} X_1X_3 +\beta_{23} X_2X_3+\beta_{11} X_1^2+\beta_{22} X_2^2+\beta_{33} X_3^2 \quad (2)$$

where Y is the predicted response; $\beta_0$ is the model constant; $X_1$, $X_2$, and $X_3$ are the independent variables; $\beta_1$, $\beta_2$, and $\beta_3$ are the linear coefficients; $\beta_{12}$, $\beta_{13}$, and $\beta_{23}$ are the cross-product coefficients; and $\beta_{11}$, $\beta_{22}$, and $\beta_{33}$ are the quadratic coefficients. Microsoft Excel 97 was used for a regression analysis of the experimental data obtained. The quality of the fit of the polynomial model equation was expressed by the coefficient of determination $R^2$. The experiments were performed in triplicate and the mean values are given.

**Statistical Analysis of Data.** The CyA yield data were subjected to multiple linear regressions using Microsoft Excel 97 to estimate the *t*-values, *P*-values, and confidence levels as a percent expression of the *P*-value. The optimal value for CyA was estimated using the *Solver* function of the Microsoft Excel tools.

## RESULTS

### Optimization of CyA Production Based on Multifactorial Experiments

A sequential optimization strategy was applied, where the first phase dealt with culture screening and identifying the nutritional factors affecting CyA production by *T. inflatum* DSMZ 915. Once the significant factors affecting CyA production were determined, the second phase involved ascertaining the combination that produced the maximum CyA production yield.

### Evaluation of Factors Affecting CyA Production

In the first phase, a Plackett-Burman experimental design was applied to reflect the relative importance of various fermentation factors [16]. For this optimization process, 11 different factors (variables) at 2 different levels were selected for the medium constitution, as shown in Table 1. Table 2 presents 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. The main effect of each variable on CyA production was estimated as the difference between the averages of the measurements at the high (+1) and low (−1) levels of that factor. The data shown in Table 2 illustrates a wide variation from 0.01 to 99 mg/l of CyA yield, thereby reflecting the importance of the medium

**Table 2.** Randomized Plackett-Burman experimental design for evaluating factors influencing CyA production by *Tolypocladium inflatum* DSMZ 915.

| Trial | Glucose | Sucrose | Starch | Peptone | Yeast extract | $(NH_4)_2SO_4$ | $KH_2PO_4$ | $MnSO_4$ | $ZnSO_4$ | $CoCl_2$ | KCl | Response CyA yield (mg/l) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | −1 | 1 | 1 | −1 | 1 | −1 | −1 | −1 | 1 | 1 | 0.25 |
| 2 | 1 | 1 | 1 | −1 | 1 | 1 | −1 | 1 | −1 | −1 | −1 | 136 |
| 3 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | 3.3 |
| 4 | 1 | 1 | −1 | 1 | −1 | −1 | −1 | 1 | 1 | 1 | −1 | 0.01 |
| 5 | 1 | 1 | −1 | 1 | 1 | −1 | 1 | −1 | −1 | −1 | 1 | 10.5 |
| 6 | −1 | −1 | 1 | 1 | 1 | −1 | 1 | 1 | −1 | 1 | −1 | 0.65 |
| 7 | 1 | −1 | 1 | −1 | −1 | −1 | 1 | 1 | 1 | −1 | 1 | 0.37 |
| 8 | −1 | −1 | −1 | 1 | 1 | 1 | −1 | 1 | 1 | −1 | 1 | 0.22 |
| 9 | −1 | 1 | 1 | −1 | 1 | −1 | −1 | −1 | 1 | 1 | 1 | 0.02 |
| 10 | 1 | −1 | −1 | −1 | 1 | 1 | 1 | −1 | 1 | 1 | −1 | 2.8 |
| 11 | −1 | 1 | 1 | 1 | −1 | 1 | 1 | −1 | 1 | −1 | −1 | 55.85 |
| 12 | −1 | 1 | −1 | −1 | −1 | 1 | 1 | 1 | −1 | 1 | 1 | 0.04 |

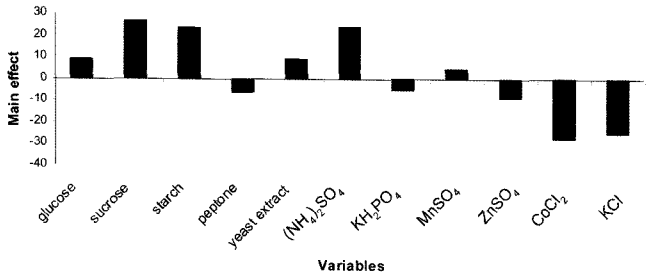


**Fig. 1.** Main effect of media composition on production of CyA by *Tolypocladium inflatum* DSMZ 915.

components for attaining a higher productivity. The main effects of the examined factors on CyA production were calculated and are presented graphically in Fig. 1. When analyzing the regression coefficients for the 11 variables, glucose, sucrose, starch, yeast extract, $(NH_4)_2SO_4$, and $MnSO_4$ all had a positive effect on CyA production, whereas peptone, $KH_2PO_4$, $ZnSO_4$, $CoCl_2$, and KCl had a negative effect. Another more convenient way of presenting the results of a Plackett-Burman design is using a Pareto chart, which displays the magnitude of each estimate; Fig. 2 shows the ranking of the factor estimates in a Pareto chart.

### Statistical Analysis of Plackett-Burman Design

Since a Plackett-Burman design is a 2-level experimental design, it involves a linear polynomial correlation model that describes the correlation between the 11 factors and the CyA yield as follows:

$$Y_{CyA}=17.5+7.5\,X_1+16.2\,X_2+14.7\,X_3-6.3\,X_4+7.5\,X_5 +15\,X_6-5.8\,X_7+5.4\,X_8-7.6\,X_9-16.9\,X_{10}-15.6\,X_{11}$$

To improve the pre-optimization formula for the subsequent optimization step, the most significant variables, namely sucrose, starch, and $(NH_4)_2SO_4$, were fixed at their optimum (high) levels obtained from the Plackett-Burman design, whereas the variables with a negative-effect value were fixed at their (-1) coded values, and the variables with a positive-effect value fixed at their (+1) coded values.

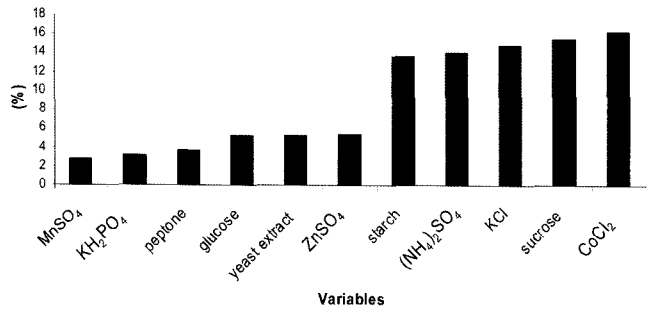


**Fig. 2.** Pareto chart rationalizing the effect of each variable on CyA production by *Tolypocladium inflatum* DSMZ 915.

**Table 3.** Box-Behnken factorial experimental design, representing response of CyA yield as influenced by sucrose, starch, and $(NH_4)_2SO_4$.

| Trial | Sucrose (g/l) | Starch (g/l) | $(NH_4)_2SO_4$ (g/l) | CyA yield (mg/l) |
|---|---|---|---|---|
| 1 | -1 (10)[a] | 1 (20)[a] | 0 (5)[a] | 66.99 |
| 2 | -1 (10) | 0 (15) | -1 (1) | 67.218 |
| 3 | 0 (15) | 1 (10) | -1 (1) | 49.05 |
| 4 | -1 (10) | 0 (15) | 1 (10) | 74.73 |
| 5 | 1 (20) | 0 (15) | -1 (1) | 61.17 |
| 6 | 0 (15) | -1 (10) | 1 (10) | 90.89 |
| 7 | -1 (10) | -1 (10) | 0 (5) | 90.7 |
| 8 | 1 (20) | -1 (10) | 0 (5) | 96.634 |
| 9 | 1 (20) | 0 (15) | 1 (10) | 96.99 |
| 10 | 0 (15) | 0 (15) | 0 (5) | 99.624 |
| 11 | 0 (15) | -1 (10) | -1 (1) | 81.569 |
| 12 | 1 (20) | 1 (20) | 0 (5) | 99.54 |
| 13 | 0 (15) | 0 (15) | 0 (5) | 97.71 |
| 14 | 0 (15) | 1 (20) | 1 (10) | 105.357 |
| 15 | 0 (15) | 0 (15) | 0 (5) | 102.299 |

[a]Natural units of variables are in parentheses.

### Optimization of Culture Conditions Using Box-Behnken Design

To identify the optimum response region for CyA production, the significant independent variables [sucrose, $X_1$; starch, $X_2$; and $(NH_4)_2SO_4$, $X_3$] were further explored at three levels. Table 3 presents the design matrix for the variables, given in both coded and natural units, plus the experimental CyA yield results.

To predict the optimal point, within the experimental constraints, a second-order polynomial function was fitted to the experimental CyA yield results (nonlinear optimization algorithm):

$$Y_{CyA}=99.87+6.837X_1-4.853X_2+13.616X_3 +6.654X_1X_2+7.077X_1X_3+11.754X_2X_3 -9.046\,X_1^2-2.364X_2^2-45.803X_3^2$$

where, $X_1$, $X_2$, and $X_3$ are sucrose, starch, and $(NH_4)_2SO_4$, respectively. On the model level, the correlation measures for estimating the regression equation are the multiple correlation coefficient R and determination coefficient $R^2$. The closer the value of R is to 1, the better the correlation between the measured and the predicted values. In this experiment, the value of R was 0.98 for CyA production, indicating a high degree of correlation between the experimental and predicted values. The value of the determination coefficient $R^2$ was 0.968 for CyA production, representing a measure of the model.

The optimal levels for the three components, as obtained from the maximum point of the polynomial model, were estimated using the *Solver* function of the Microsoft Excel tools, and found to be (g/l): sucrose, 20; starch, 20; and $(NH_4)_2SO_4$, 10; with a predicted yield of 113.747 mg/l.

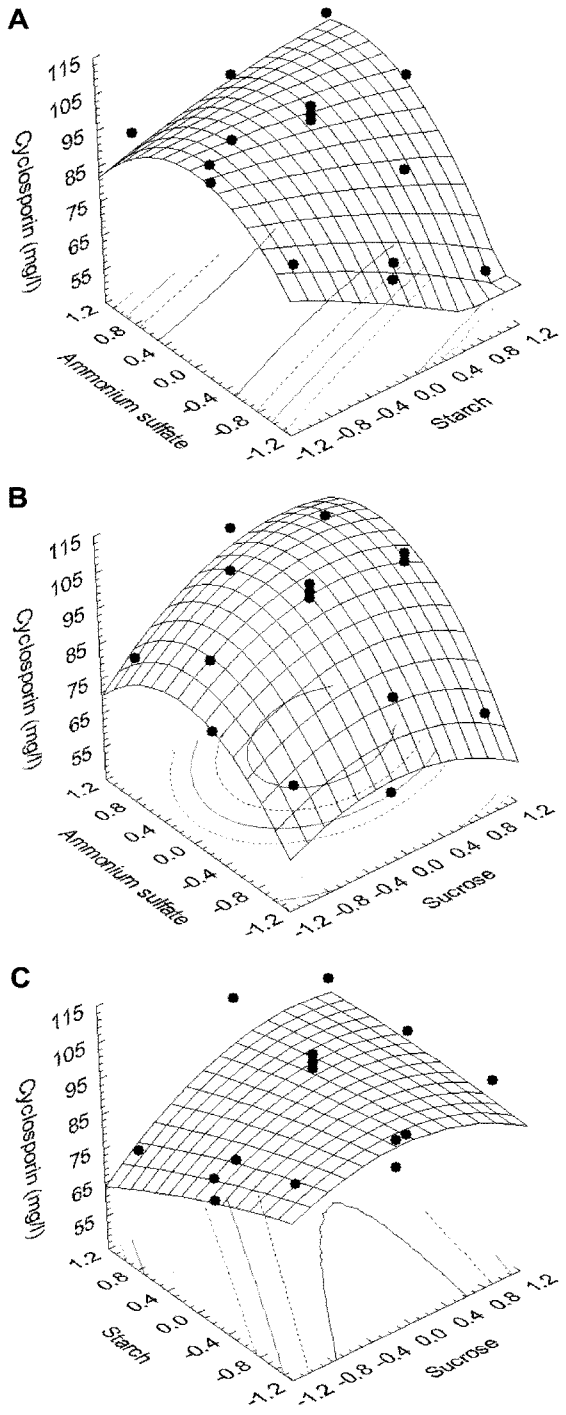


**Fig. 3.** Three-dimensional surface plots showing the relationships between tested significant medium components and cyclosporin A yield (mg/l) by *Tolypocladium inflatum* DSMZ 915 in submerged culture cultivation.
Black dots (●) represent experimental results.

## Verification of Model and Kinetics of Growth

The optimal condition realized from the optimization experiment were verified experimentally and compared with the data calculated from the model. The estimated

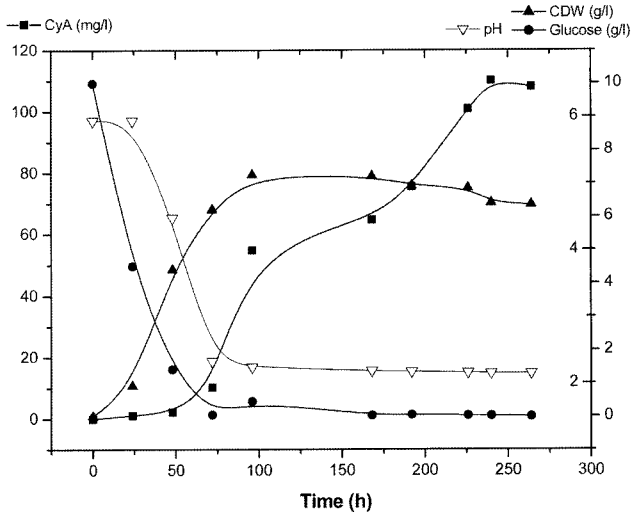


**Fig. 4.** Growth monitoring (▲), glucose consumption (●), pH profile (▽), and cyclosporin A production (■) (mg/l) by *Tolypocladium inflatum* DSMZ 915 in submerged culture using optimized medium.

CyA yield was 110 mg/l, whereas the predicted value from the polynomial model was 113.747 mg/l, thereby confirming the high accuracy of the model at more than 97% under the investigated conditions.

The kinetics of the cell growth and CyA production in the shake flask during cultivation for 11 days are represented in Fig. 4. As shown, the growth curve could be divided into two phases. During the first phase of 96 h, the cells grew exponentially at a rate of 0.088 g/l/h and specific growth rate of 0.028/h, reaching the maximal value of 7.25 g/l. During this phase, glucose was gradually consumed at a volumetric rate of 0.138 g/l/h, and a complete glucose limitation was observed after 72 h. However, glucose was not the only carbon source, as high concentrations of sucrose and starch were also contained in the culture medium. It is also worth noting that, during the growth phase, the pH dropped gradually from 5.9 to 2.33 after 72 h, and then remained more or less constant for the rest of the cultivation time. During the second phase, from 96 h to 240 h, the cell dry weight decreased gradually at a very low rate of about 0.0059 g/l/h, reaching 6.4 g/l at the end of the cultivation time.

The production of CyA started after a lag phase of 48 h and increased gradually during the first phase at a volumetric production rate [Qp] of 1.1 mg/l/h and specific production rate [$q_p$] of about 0.08 mg/g/h, reaching a CyA volumetric production of about 55 mg/l after 96 h. During the second phase, the rate of CyA production was lower at a rate of 0.160 mg/l/h, reaching about 110 mg/l after 240 h, and then remaining more or less constant for the rest of the cultivation time, indicating that the biosynthesis of CyA was not terminated by glucose limitation, owing to the presence of other carbon sources (sucrose and starch)

**Table 4.** Cell growth and cyclosporin A production in optimized medium during batch cultivation of *Tolypocladium inflatum* DSMZ 915 cells in shake flask.

| Time (h) | CyA (mg/l) | C.D.W (g/l) | pH | Glucose (g/l) |
|---|---|---|---|---|
| 0 | 0 | 0 | 5.94 | 10 |
| 24 | 1.1 | 0.91 | 5.94 | 4.51 |
| 48 | 2.2 | 4.4 | 4.48 | 1.41 |
| 72 | 10.3 | 6.2 | 2.33 | 0.05 |
| 96 | 54.9 | 7.25 | 2.24 | 0.45 |
| 168 | 64.7 | 7.2 | 2.18 | 0.03 |
| 192 | 75.5 | 6.92 | 2.17 | 0.05 |
| 226 | 100.786 | 6.85 | 2.16 | 0.03 |
| 240 | 110 | 6.4 | 2.15 | 0.02 |
| 264 | 108 | 6.35 | 2.15 | 0 |

in the cultivation medium. Notwithstanding, the specific CyA production [$Y_{P/X}$], representing the amount in mg of CyA produced per g of glucose consumed, calculated before the cell lysis phase was about 7.58 mg/g.

## DISCUSSION

Since the discovery of its immunosuppressant effect, cyclosporin A has been the main drug used in organ transplantation post-operational therapy, and despite some nepheral toxicity side effects, cyclosporin A is still the most potent immunosuppressant drug used in the United States and Europe.

Whereas many previous studies have attempted to optimize the production of cyclosporin in submerged and solid-state cultures, the present study optimized the medium composition for submerged culture fermentation with a semisynthetic medium using an experimental design strategy, which has apparently never been done before.

In previous studies, a variety of defined media, ranging from complex [9] to synthetic [11], have been employed to elucidate the biosynthetic pathways or regulatory mechanisms involved in cyclosporin fermentation [3]. Other studies have investigated the directed biosynthesis of cyclosporins *via* the supplementation of various pure amino acids to direct the metobolic flux towards specified kinds of cyclosporin.

In contrast, the objective of the present study was to engineer a medium using statistical experimental designs to optimize the production of CyA in a submerged culture of *T. inflatum* DSMZ 915. First, a Plackett-Burman design was implemented to evaluate the culture medium components with several carbon and nitrogen sources, including complex and mineral categories. The results of this study revealed that sucrose and starch were the most promising carbon sources in terms of cyclosporin production, whereas glucose had a less significant effect. Agathos *et al.* [3] previously reported that sucrose and glucose were the favored carbon sources for the volumetric production of cyclosporin. Murthy *et al.* [14] also reported that glucose and sucrose, although not significant variables, had a positive affect on CyA production in the solid-state fermentation of *T. inflatum*. In addition, they reported that Millet and Jowar flour were the most promising medium components for cyclosporin production, whereas soluble starch had an inhibitory effect on CyA yield.

In the present study, ammonium sulfate was identified as the most potent nitrogen source, although many previous studies have reported that an inorganic nitrogen source is not as competitive as complex ones. In addition, yeast extract was found to have a positive effect on the CyA yield, whereas peptone was inhibitory, as supported by Murthy *et al.* [14]. In support of the present results, Agathos *et al.* [3] reported that the apparent stimulatory effect of ammonium sulfate may signify that $NH_4^+$ serves as the primary nitrogen source for cell growth. As indicated by the growth kinetics and CyA production by *T. inflatum* DSMZ 915 (Fig. 4), CyA was clearly a growth-associated product, supporting the hypothesis of Agathos *et al.* [3]. However, in contradiction to the present results, $ZnSO_4$ and $CoCl_2$ have been shown to have a significant positive effect on CyA production in the solid-state fermentation of a *T. inflatum* mutant strain [14].

On the basis of the calculated *t*-values (Table 2), sucrose, starch, and ammonium sulfate were chosen for further optimization, as these factors had the most significant effect on the CyA production. The variables with a significant negative effect were not included in this optimization experiment, but rather, used at their −1 coded level.

To identify the optimum response region for CyA production, the significant independent variables (sucrose: $X_1$; starch: $X_2$; and ammonium sulfate: $X_3$) were further explored at three levels according to a Box-Behnken design [6], as presented in (Table 3). The resulting polynomial model and correlated three-dimensional graphs (Fig. 3) showed no additivity effect for starch and ammonium sulfate due to significant interaction, where the additivity of a 2-factor effect means that the effect of one factor on the response does not depend on the level of the other factor. In Fig. 3, it is obvious that the maximum CyA production was attained with high levels of sucrose, starch, and ammonium sulfate, where ammonium sulfate had the most significant effect. The curvature shown in Figs. 3A and 3B may have been due to the significance of the ammonium sulfate quadratic effect ($P$=0.0021).

Agathos *et al.* [3] tested the effect of different carbon sources on CyA production, and reported that 3% sorbose was the best concentration and carbon source, giving the highest yield (105.5 mg/l). Similar to the present results, they reported that 2% sucrose yielded 34.3 mg/l of CyA, and also stated that the sequential addition of carbon sources could be advantageous. Therefore, based on this suggestion, the present study used a mixture of carbon sources, where the optimized medium contained glucose,

sucrose, and starch. It is well known that glucose is rapidly metabolized in culture, as seen in Fig. 4, and it yielded 54 mg/l after 72 h. Thereafter, the CyA production continued because of the assimilation of the starch and glucose, yielding 108 mg/l by the end of the fermentation.

It is also worth adding that, although the statistically optimized medium in the present study was able to increase the CyA yield 2-fold, the normal candidates used in industry to optimize production media for secondary metabolites are slowly metabolized nitrogen sources, such as peptonized milk, cotton seed flour, skim milk, and soybean flour. However, these sources can make the product purification steps more complicated, thereby reducing the yield of the purified product, which is why these sources were avoided in the present work.

The CyA production followed a growth-associated modeling pattern during the first 72 h, where glucose was the main metabolized carbon source. When the glucose became depleted and limited, the sucrose and starch were consumed, which resulted in an increase in the CyA volumetric production. However, no further growth occurred during this phase, which has been frequently observed in a wide variety of antibiotic fermentations, including β-lactams and amino-glucosides [7].

Future plans include studying the cell morphology during pH controlled and uncontrolled batch fermentations, as well as monitoring the effect of oxygen availability in cultures, since oxygen causes significant changes to the morphology and physiology related to secondary metabolism in filamentous fungi.

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