Acta Pharmaceutica Turcica
46: 197 - 204 (2004)

Kinetic Studies on the Growth and Cyclosporin a Production by a Local Isolate of *Fusarium oxysporum*, NRC

H.A. El-Refai, I.S.*Abd-Elsalam and L.A.Sallam

Natural and Microbial Products Chemistry Department, National Research Center, Dokki, Cairo, Egypt.

Abstract

The kinetics of cyclosporin A (Cy A) production by *F. oxysporum* NRC were studied in both shaking flask and bioreactor (5L capacity) cultures. Both the volumetric and specific production values of Cy A were higher by about 73.5% and 57.1% in case of adopting the bioreactor culture technique, respectively. In addition, more vigorous fungus growth was observed with the bioreactor culture. On glucose limitation, the fungal biomass yield nearly remained almost constant, while Cy A production was stimulated under the same conditions, wherein maximum Cy A yield (349.2 mg/l) was obtained after 10 days incubation.

Key words : cyclosporine A production, *Fusarium oxysporum*

Introduction

Cyclosporin A is a cyclic undecapeptide with anti-inflammatory, immunosuppressive, antifungal, and antiparasitic properties (Borel, 1986). It is used in transplantation surgery and in the treatment of some autoimmune diseases (Povlsen *et al.*, 1989).

Cy A was produced by the fungus *Beauveria nivea* (previously designated *Trichoderma polysporum*, *Tolypocladium inflatum* and *T. niveum*) as the main components of 25 naturally occurring cyclosporins which have substitutions of amino acids in positions 1, 2, 4, 5, 7 and 11 and/or contain unmethylated peptide bonds in positions 1, 4, 6, 9, 10 or 11. Beside these naturally occurring cyclosporins, some cyclosporins differing in positions 1, 2 and 8 from Cy A could be produced by feeding amino acid precursors to the fungus culture (Traber *et al.*, 1989).

The biosynthesis of Cy A is also likely to proceed by non ribosomal process as indicated by the cyclic structure and the presence of several unusual amino acids in this compound. Cy A is a secondary metabolite obtained mainly during the stationary phase and at the start of the decline phase of growth (Agathos *et al.*, 1986 and Abd-Elsalam, 2000).

Cy A productivity depends mainly on the constitution of the fermentation medium specially the nature and the level of carbon and nitrogen sources (Agathos *et al.*, 1986). The role of some physiological parameters on Cy A by *Aspergillus terreus* was also investigated by Sallam *et al.* (2003).

The present investigation aims to describe a kinetic model for Cy A production in relation to the fungus biomass yield adopting both the culture flask and bench top bioreactor techniques.

* *Corresponding author e-mail: abdelsalom@hotmail.com*

Materials and Methods

Chemicals
The authentic Cy A was provided by Sigma Company. All chemicals used in the current work were analytical grade which obtained from Merck. The solvents used are HPLC grade except butylacetate.

Microorganism
The local culture of *Fusarium oxysporum* NRC was obtained from the Natural and Microbial Products Chemistry Department, National Research Center (NRC), it was selected according to previously done screening experiments.

Maintenance of the microorganism
The experimental fungus was maintained on the malt-yeast extract medium (MY medium) showed by Agathos *et al.* (1986) which consists of (g/l): malt extract 20, yeast extract 4, agar 20, pH 5.3.

Production medium
The medium used for cultivation was composed of g/l: glucose 50; peptone 10; KH_2PO_4 5; KCl 2.5, pH 5.3.

Inoculum preparation
Erlenmeyer flasks (250 ml) each contains 100 ml of MY medium was inoculated by pure culture of *F. oxysporum* and inoculated at 200 rpm, 27 °C for 72 h (Koble and Traber, 1982). Specific volume of the obtained growth was used as standard inoculum.

Cultivation conditions
Using flasks: According to the method described by Agathos et al. (1986), Erlenmeyer flasks (250 ml) containing 100 ml portions of the production medium were inoculated by 2 ml of the previously prepared inoculum and incubated in rotary shaker at 200 rpm, at 27 °C for 10 days.
Using bioreactor: The fermentation process was performed in Biflo fermentor (NW Brunswick Scientific, Edison, N.J., USA) which adopted for bench-scale cultivation, under the following conditions: liquid volume, 3 liter, inoculum level 2% (v/v), agitation rate 300 rpm, aeration 1 v/v/min. the temperature was controlled at 27 °C and the pH value of the medium was automatically controlled at 5.3 by the addition of NaOH (0.1 N) or HCl (0.1 N). Under these conditions, growth was found to be filamentous with no apparent clumping. Samples (25 ml) were daily collected for analysis.

Analysis
Glucose estimation: The residual glucose content was estimated by glucose oxidase method described by Dahlguist (1961).
Growth estimation: At the end of the incubation period the fungal growth was separated, by filtration, washed and air dried, then oven dried till constant weight at 90 °C and the average of the obtained weights was recorded.
Extraction of Cy A: Equal volume of butyl acetate (100 ml) was added to the fermentation medium and shaked at 200 rpm, at 27 °C for 24 h. The organic layer was separated and dried under vacuum (test material), then dissolved in methanol and undergo the necessary chromatographic analysis.

Cy A analysis

Qualitative estimation: Cy A content was qualitatively identified by the TLC technique. Silica gel plate was prepared and the test sample was resolved by the solvent system (acetone : n-hexane, 1 : 1 v/v). The developed plates were subjected to iodine vapour as colour reagent, wherein the Cy A acquired a faint brown colour as compared with the authentic (Agathos et al., 1986).

Quantitative estimation: High pressure liquid chromatography (HPLC) was adopted to determine Cy A. A Water Pye HPLC system (Waters, Milford, MA, USA) equipped with a reversed phase C_8 NOVA-PAK column (150 x 3.9 mm, 10 μm packing, Waters) was used. The procedure described by Agathos et al. (1986) was followed ; 5 μl of Cy A solution firstly injected for determination of its Rt, then another 5 μl sample was injected for analysis. The mobile phase consists of acetonitrile: methanol: water (42.5 : 20 : 37.5), flow rate 1 ml/min and C_8 column was maintained at 72 °C with operating pressure of 1200 lb/in^2. Cy A was determined using detector (Waters, 486) at 210 nm wave length. The concentration of Cy A calculated by this procedure is referred to be as volumetric production. The specific Cy A production was also calculated by dividing the volumetric production by the biomass yield.

Results presentation

All results given in this work were arithmetic means of at least three different replicates. The experimental values of the same treatment showed difference exceed 5% were routinely discarded and the treatment were repeated again. All data in this work were statistically analyzed by ANOVA test.

Results

Growth and Cy A productivities using the shaked flask culture technique

The cultivation of *F. oxysporum* NRC was carried out in shaking flasks to investigate the kinetics of Cy A production in relation to the fungus growth.

As shown in Fig. 1, the fungus growth could be differentiated into three phases. During phase I (the first 10 days) which represents the accelerating phase of growth, the biomass yields were steadily enhanced and the most vigorous growth was maintained after 10 days. Similarly, increasing amounts of glucose were consumed during the same period which was also accompanied by the formation of elevating amounts of Cy A.

The stationary phase of growth (Phase II, 10-12 days) was characterized by achieving more or less similar growth yields, with consumption of more amounts of glucose and formation of the highest Cy A yield.

During the third phase (12-15 days) of growth, glucose was completely depleted from the fermentation medium wherein both the fungus biomass yield and Cy A productivity were clearly retarded.

In accordance with our results, Koble and Traber (1982) and Agathos *et al.* (1986) had reported that Cy A obtained its maximum volume at the stationary phase of growth.

The relation between the fungus growth (biomass yield) and the amount of consumed glucose [$Y_{x/s}$] was studied. The data illustrated in Fig. 2 revealed that the maximum [$Y_{x/s}$] value was obtained at the end of phase I (the time of glucose limitation). This value remained more or less constant during the second phase of growth, and started to decrease during the decline phase of growth due to cell lysis.

On the other hand, the value of [$Y_{p/s}$] which represents the amount of Cy A produced per 1 g of the consumed glucose reached maximum after 12 day fermentation. Obviously, this good Cy A

output was obtained after glucose limitation. This indicates that the biosynthesis of Cy A was not terminated by glucose limitation.

Growth and Cy A production relations using laboratory fermentor

The kinetic of the fungus growth and Cy A production was studied adopting batch cultivation of *F. oxysporum* in a laboratory fermentor. The results illustrated in Fig. 3, assessed the existence of the three different growth phases previously shown on using the shaking flask technique. At the end of the first phase (7 days) biomass yield reached maximal value. Thereafter (7-10 days) the fungus growth yields remained constant (stationary phase). The elongation of the fermentation period to 12 days was accompanied by a marked decrease of the fungus growth yield (decline phase).

On the other hand, Cy A reached its maximal value (349.2 mg/l) at the 10^{th} day of incubation then it started to decrease gradually.

The yield coefficients [$Y_{x/s}$], [$Y_{p/x}$] and [$Y_{p/s}$] were calculated during the different growth phases. The results given in fig 4 clearly show that the biomass yield, based on the consumed glucose [$Y_{x/s}$], reached its maximal value during the stationary phase. Thereafter, the growth yield was gradually decreased. Further consumption of glucose seems to be correlated to Cy A biosynthesis rather than for fungal cell proliferation. The values of the Cy A yield coefficient [$Y_{p/x}$], were increased gradually during the cultivation process and reached maximal value after 10 days fermentation.

Comparison of the fungus growth and Cy A productivities of F. oxysporum grown on shaking flasks and lab fermentor

The differences between the results recorded with shaking flask and the bioreactor techniques were summarized in Table 1. Obviously, more vigorous growth yield was achieved upon using the laboratory fermentor. Thus 38% increase in the fungus dry weight was recorded with the fermentor treatment. This may be due to the agitation and aeration facilities which permit higher oxygen transfer rate as compared to the flask culture technique. Similarly Taguchi (1971), Schump and Dectwer (1979) reported that an adequate amount of dissolved oxygen must be always available in the medium to achieve good fungus growth yields. Moreover this was also accompanied by the formation of relatively high Cy A yield.

On the other hand, complete consumption of glucose was correlated by a significant decrease in Cy A productivity in both cultivation techniques.

Table 1. Kinetic parameters of cell growth and Cy A production by *F. oxysporum* adopting both shaking flask and bioreactor fermentation techniques.

Parameter	Type of fermentation	
	Shaking flask	Fermentor
X_{max} [g/l]	3.4	5.5
P_{max} [g/l]	92.75	349.2
μ [mg/g dry wt.]	27.26	63.49
$Y_{X/S}$ [g/g]	0.074	0.2
$Y_{P/X}$ [g/g]	27.27	63.49
$Y_{P/S}$ [g/g]	1.85	6.98

X_{max} : maximal biomass dry weight
P_{max} : maximum cyclosporin A production
μ : Specific cyclosporin A production
$Y_{X/S}$: g fungal dry weight / g consumed glucose
$Y_{P/X}$: g cyclosporin A production / g fungal dry weight
$Y_{P/S}$: mg cyclosporin A production / g consumed glucose

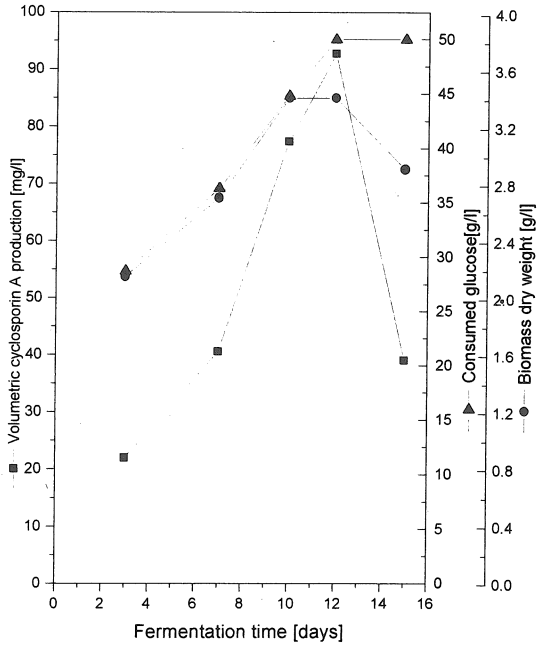

Fig 1. The biomass yield and Cy A production by *Fusarium oxysporum*, NRC in shaked flasks.

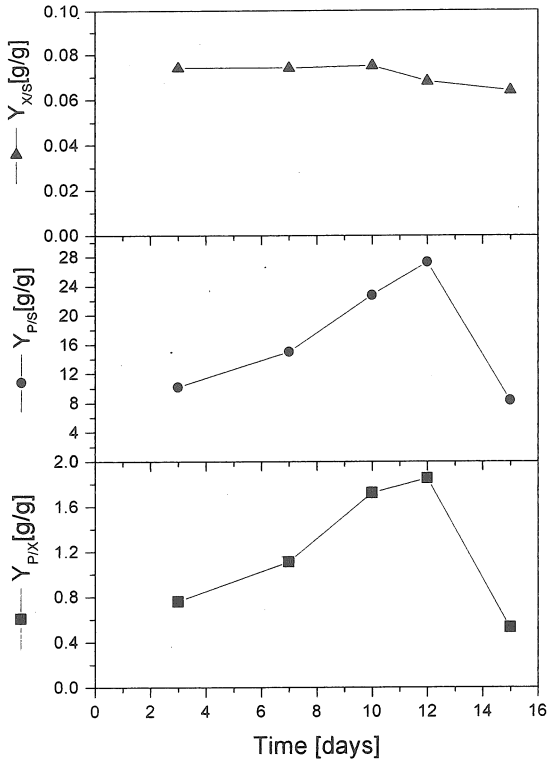

Fig 2. Different yield coefficients of *F. oxysporum* grown in shaked flasks.

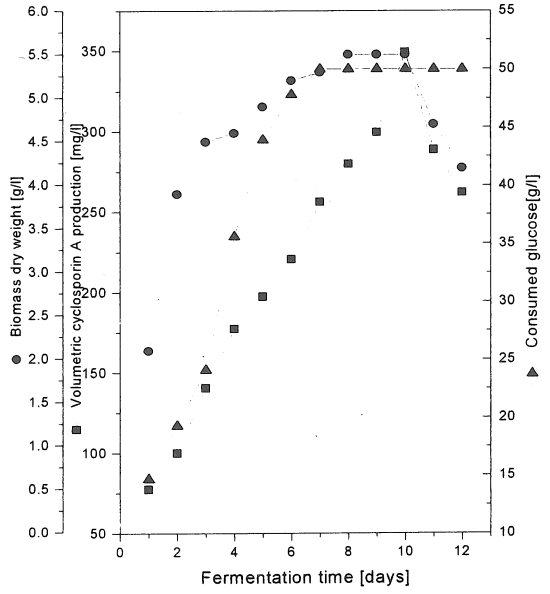

Fig 3. Growth and Cy A production by *F. oxysporum* grown in laboratory fermentor

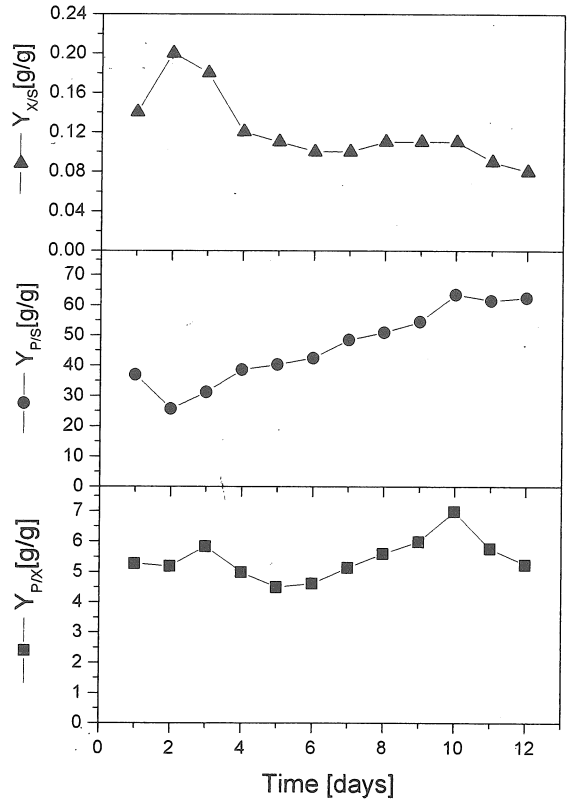

Fig 4. Different yield coefficients of *F. oxysporum* grown in laboratory fermentor.

References

Abd-Elsalam, I.S. (2000). Physiological, biochemical and dynamical studies on cyclosporin A production by some microorganisms. A thesis submitted for Ph. D. degree, Botany Department, Faculty of Science, Tanta University.

Agathos, S.N., Marshall, J.W., Maraiti, C., Parekh, R. and Modhosingh, C. (1986). Physiological and genetic factors for process development of cyclosporin A fermentation. *J. Industrial Microbiol.*, 1: 39-48.

Borel, J.F. (1986). Cyclosporin and its factors. *Prog. Allergy* 38: 927-936.

Dahlguist, A.J. (1961). Determination of maltose and isomeric activities with glucose oxidase reagents. *Biochemistry* 80: 547-549.

Koble, H. and Traber, R. (1982). Directed biosynthesis of cyclosporins. *Eur. J. Appl. Microbiol. Biotechnol.* 14: 237-240.

Povlsen, J., Moller, B., Christiansen, B. and Peterson, C. (1989). Cyclosporin A is a selective immunosuppressive drug. *Tissue Antigens* 33: 4-14.

Sallam, L.A.R., El-Refai, A.H., Hamdy, A.A., El-Minofi, H.A. and Abdelsalam, I.S. (2003). Role of some fermentation parameters on Cy A production by a new isolate of *Aspergillus terreus*. *J. Gen.Applied. Microbiol.* 49: 321-328.

Schump, A. and Dectwert, W.A. (1979). Estimation of O2 and CO2solubilities in fermentation media . *Biotechnol. Bioeng.* 21: 1075 .

Shmidt, G., Riserner, D., Lawen, A. and Klien Kauf, H. (1992). Cyclosporin synthetase is 1.4 Mda multienzyme polypeptides. Revolution of the molecular mass of various peptide synthetases. *FEBS Lett.* 307: 355-360.

Traber, R., Hofmann, H. and Koble, H. (1989). Cyclosporins new analogues by precursor directed biosynthesis. *J. Antibiotic.* 40: 591-597.

Taguchi, H. (1971). The nature of fermentation fluids. *Adv.Biochem.Eng.* 1: 197.

Received: 26.08.2004
Accepted: 02.10.2004