

Full Paper

Studies on the application of immobilization technique for the production of cyclosporin A by a local strain of *Aspergillus terreus*

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The formation of cyclosporin A (Cy A) by immobilized spores and mycelia of *Aspergillus terreus* was investigated. Different carriers were tested as immobilizing carriers, whereby Ca-alginate was selected for further experimentation. The role of alginate concentration, biomass weight, pH value of the cultivation medium, repeated utilization of the immobilized fungus as well as the supplementation of different amino acid precursors were studied. Best Cy A outputs were attained with Ca-alginate 3% (w/v), mycelial weight 15% (w/v), pH 4.5 and four repeated cycles. Similarly, the Cy A productivity was markedly accelerated in the presence of L-valine and L-valine and L-leucine mixture.

Key Words—*Aspergillus terreus*; cyclosporin A; immobilization; production

Introduction

The production of cyclosporin A (an effective immunosuppressant in organ transplantation and in the treatment of auto-immune diseases) by *Tolypocladium inflatum* free cells using both shaker flasks and stirred tank fermentors was described by Dreyfuss et al. (1976), and Koble and Traber (1982). More recently, Chun and Agathos (1989) studied the immobilization of *T. inflatum* conidia into porous celite beads for Cy A production. The yield of immobilized cell culture was up to three-fold higher than for the free cells system. In addition, Lee and Agathos (1991) and Traber (1998) compared the physiological and environmental effects of Cy A production by suspended and immobilized cells of *T. inflatum*, and found that there was a significant difference in precursor flow between the immobi-

lized and free cell systems particularly in the presence of L-valine.

In a previous communication (Sallam et al., 2003), we selected a local isolate of *A. terreus* as a new Cy A producing fungus. The fermentation of Cy A was investigated under different fermentation and nutritional conditions. The productivity was optimized using the submerged free mycelia of *A. terreus*. However, in the present study we aimed at investigating the capacity of *A. terreus* to produce Cy A adopting the cell immobilization technique. Advances in understanding and control of the fermentation of Cy A are actually necessary in order to arrive at more effective production process.

Materials and Methods

Microorganism. *A. terreus* used in this investigation was obtained from the Center of Cultures of the Microbial and Natural Products Chemistry Department, National Research Center (NRC), Cairo, Egypt. The

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complete identification of this strain was described by the authors in the previous communication (Sallam et al., 2003). Cy A authentic was imported from Sigma Company, USA. All other chemicals used in the current work were laboratory reagents purchased from Merck and the organic solvents were HPLC grade except for butyl acetate.

Color reagents: Iodine vapor obtained from crystalline iodine.

Solvent system: Acetone/*n*-hexane (1/1, v/v).

Maintenance of the microorganism. It was maintained on agar slants at 4°C using MY medium consisting of malt extract 2% and yeast extract 0.4% (w/v); pH 5.3 (Agathos et al., 1986).

Inoculum preparation. A 250 ml Erlenmeyer flask containing 50 ml of MY medium was inoculated with a 5 ml spore suspension (9.3×10^3 spores/ml) and incubated on a rotary shaker at 200 rpm for 48 h at $28 \pm 2^\circ\text{C}$.

Immobilization techniques.

Spores propagation: A series of agar slopes of MY medium was inoculated by the pure organism. The inoculated slants were then incubated for 7 days at 32°C. The spores were then harvested by the addition of 5 ml sterile distilled water to each slant and gentle scraping of the agar surface under aseptic conditions.

Spores immobilization:

i) Using alginate carrier—Aliquots of the spore suspension (9.3×10^3 spores/ml) were added to 3% sodium alginate solution. The alginate spore mixtures were added dropwise to sterile 2% calcium chloride solution, whereby fine pellets of diameters ranging from 3–5 mm were obtained (Chun and Agathos, 1989).

ii) Using celite carrier—The method adopted by Gbewonyo and Wang (1983), Chun and Agathos (1989) was used. The celite beads were washed with distilled water several times and heated in a furnace overnight at 600°C, then autoclaved for 1 h and allowed to dry at 121°C for 30 min. The spore suspensions (prepared as described before) were added to the dry celite beads in ratio 2:1 (v/v), stirred at 200 rpm for 2 h, decanted with the supernatant to get the celite-spore mixture. Finally aliquots of sterile semisynthetic medium (SSM) consisting of (g%, w/v) glucose 5, peptone 1, KH_2PO_4 0.5, and KCl 0.25, pH 5.3 were added aseptically to the immobilized celite beads dispensed in 250 ml flasks. The inoculated beads were incubated at 150 rpm for 10 days at

$28 \pm 2^\circ\text{C}$.

iii) Using silica gel S carrier—The procedure of immobilization using silica gel S was similar to that described previously for celite carrier.

Mycelium immobilization. The mycelium entrapment in calcium alginate beads was carried out according to Maddox et al. (1981), Schmuader et al. (1991) and Schlosser et al. (1993). Samples of the wet mycelia (10 g) were added to sterile sodium alginate solution. The alginate-mycelia mixture was added dropwise to 2% calcium chloride solution to give spherical beads (3–5 mm diameter). A number of these beads (100) were transferred to 250 ml flask containing SSM (100 ml) and allowed to grow on a rotary incubator shaker at 150 rpm for 10 days at $28 \pm 2^\circ\text{C}$.

Semi-continuous fermentation. At the end of each fermentation cycle the calcium alginate beads were aseptically separated from the fermentation broth by filtration using a sterile Büchner funnel. The beads were washed twice with sterile water and then resuspended in a 250 ml flask containing 100 ml of sterile SSM. The sequence of fermentation, bead separation and recultivation was repeated.

Estimation of the fungus dry weight (biomass yield). At the end of the incubation period the beads were separated and suspended in phosphate buffer, and shaken for 2 h at 200 rpm. The mycelia were separated by filtration, washed and then oven dried till constant weight at 90°C.

Cyclosporin A extraction and analysis. The method of extraction and analysis described by Agathos et al. (1986) was adopted. Equal volumes of butyl acetate were added to the harvested fermentation treatment, and stirred at 200 rpm for 24 h at 30°C. The organic layer was separated and evaporated under vacuum till dryness. The dried extract was dissolved in the least amount of methanol, and a 5 µl sample was used for Cy A determination using the HPLC analysis technique (PYE HPLC system, Waters, Milliford System, USA), under the following conditions: mobile phase consists of a mixture of acetonitrile : methanol : water (42.5 : 20 : 37.5, v/v), flow rate 0.8 ml/min, column of 15 cm with 2 mm i.d., packed with 2 µm spherisorb C_8 maintained at room temperature and an operating pressure of 1,200 lb/inch². The detector was set at 210 nm and 0.02 attenuation units full scale. The data obtained was identical with that of an authentic sample of cyclosporin A analyzed under the same conditions. The

other cyclosporins have been detected but focus has been only imposed on cyclosporin A (Sallam et al., 2003).

Estimation of Cy A productivity. The volumetric production of Cy A was estimated by using Millenium Chromatography Manager Software (Water Milford). The concentration of Cy A is referred to as the volumetric production of Cy A. The specific production was calculated by dividing the volumetric production by the biomass yield as previously described by Sallam et al. (2003).

Results and Discussion

Selection of the carrier

Different entrapping substances (celite, silica gel S and sodium alginate) were used for the immobilization of *A. terreus* spores. The results indicated that the type of the entrapping carrier affects the yield of Cy A. The celite gave a relatively high yield, as compared to the other carriers (Table 1). Jones et al. (1986) and Chun and Agathos (1989) recommended the suitability of celite for the production of Cy A by *T. inflatum* spores. Although, sodium alginate gave comparatively lower Cy A output (34.6 mg/L), it was selected to complete the subsequent experiments since it was the first trial to use the sodium alginate carrier in Cy A production.

The effect of different alginate concentrations (1–5%) on the production of Cy A by the fungus mycelia and spores was illustrated in Fig. 1. The data revealed that the maximum yield of Cy A was obtained at an alginate concentration 3% using the immobilized mycelia. Many investigators selected 3% alginate as a suitable matrix for the immobilization process (Abd-Elsalam, 1990; Bihari et al., 1984). This concentration of alginate seems to be important for the viability and enzymatic activity of the immobilized cells. Also, this result suggested the suitability of pore size, bead stability and optimum oxygen diffusion at this concentration (3% sodium alginate). On the other hand, concentrations below 3% showed low stability and higher concentrations showed reduced diffusion rate affecting the activity of the entrapped cells (Kopp and Rhem, 1983; Ohlson et al., 1980).

Production of Cy A by immobilized mycelia as affected by the incubation period

The results presented in Fig. 2 showed that the im-

Table 1. Effect of different carriers on the production of Cy A by immobilized *A. terreus* spores.

Entrapping carrier	Biomass yield (g/L)	Cy A productivity	
		Volumetric production (mg/L)	Specific production (mg/g biomass)
Celite (mesh 60–180)	2.533	45.13	17.83
Silica gel S (mesh 70–120)	2.324	36.25	15.62
Sodium alginate	2.700	34.60	12.81

The spore suspension (9.3×10^3 spores/ml). Sodium alginate 3% (w/v, g%). Celite : mycelia or spores = (2 : 1, v/v). Silica gel : mycelia or spores = (2 : 1, v/v). Volumetric production: mg Cy A output/1 L of fermentation medium. Specific production: mg Cy A output/1 g dry weight. Biomass yield: dry weight of fungus growth/1 L medium.

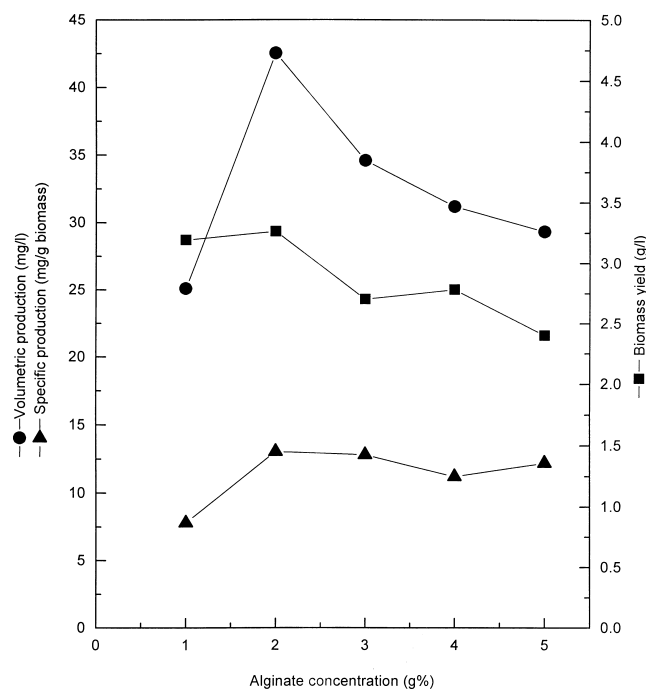


Fig. 1. Effect of different alginate concentrations on the production of Cy A using immobilized *A. terreus* spores.

mobilized mycelia produced 61.5 mg/L Cy A after 12 days fermentation.

Effect of the initial pH values of the medium

Better Cy A productivity (97.9 mg/L) was maintained with the fungal mycelium entrapped in alginate using the fermentation medium adjusted to pH 4.3. On the

Table 2. Effect of different initial pH values on the production of Cy A using the immobilized mycelia of *A. terreus*.

Initial pH value	Final pH value	Biomass yield (g/L)	Cy A productivity	
			Volumetric production (mg/L)	Specific production (mg/g biomass)
3.3	3.2	3.43	55.72	16.48
4.3	3.5	3.11	97.92	31.48
5.3	3.4	3.22	62.50	19.40
6.3	4.5	3.32	59.13	17.81
7.3	4.7	3.45	40.54	11.75

Sodium alginate: 3%. Mycelium weight: 10 g. rpm: 150 at $28 \pm 2^\circ\text{C}$. Fermentation period: 12 days.

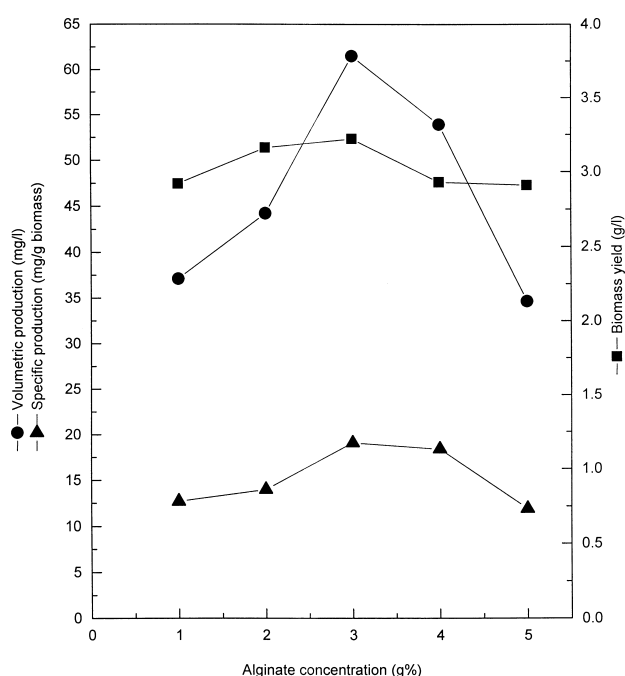


Fig. 2. Effect of different alginate concentrations on the production of Cy A using *A. terreus* immobilized mycelia.

other hand, Chun and Agathos (1989) and Manuela et al. (1996) obtained high amounts of Cy A by adjusting the pH value to 5.6 using *T. inflatum* mycelia immobilized on celite.

Role of mycelium weight (Table 3)

The study of the effect of different mycelium weights on the production of Cy A by the immobilized mycelia indicated that the best yield Cy A (105.7 mg/L) was obtained on using 20 g of the fungus mycelia/100 ml medium.

Table 3. Effect of different immobilized mycelium weights of *A. terreus* on the production of Cy A.

Mycelium weight (g%)	Biomass yield (g/L)	Cy A productivity	
		Volumetric production (mg/L)	Specific production (mg/g biomass)
2	2.35	33.24	14.14
5	2.74	45.92	16.75
10	3.11	97.92	31.48
15	4.14	97.34	23.51
20	4.53	105.70	23.33

Semi-continuous production of Cy A by the immobilized mycelium of *A. terreus*

One of the most important advantages of the cell immobilization technique is the availability of repeated use of the immobilized cells. In the present experiment, the work was extended to carry out the Cy A production process in a semi-continuous form. This schedule was repeated for 4 cycles. Table 4 clearly showed that immobilized cells could be repeatedly used for 4 different cycles with a maximum yield 97.7 mg/L at the first cycle. The total Cy A production in all cycles reached 264.15 mg/L. These results supported the suitability of sodium alginate as an entrapping matrix for cell immobilization.

Role of L-valine

In the previous communication, Sallam et al. (2003) recorded the stimulatory effect of L-val supplement on the formation of Cy A by free *A. terreus* mycelium. It was intended to test its effect on the Cy A productivity

Table 4. Semi-continuous production of Cy A using the immobilized cells of *A. terreus*.

Number of cycles	Biomass yield (g/L)	Cy A productivity	
		Volumetric production (mg/L)	Specific production (mg/g biomass)
1	—	97.92	1.1
2	—	68.55	1.4
3	—	55.23	1.5
4	3.1	42.45	1.5
Total		264.15	5.5

The fermentation process was carried out by using 3% alginate concentration, mycelium weight 10 g% and growth was continued at 150 rev/min for 12 days at 27°C.

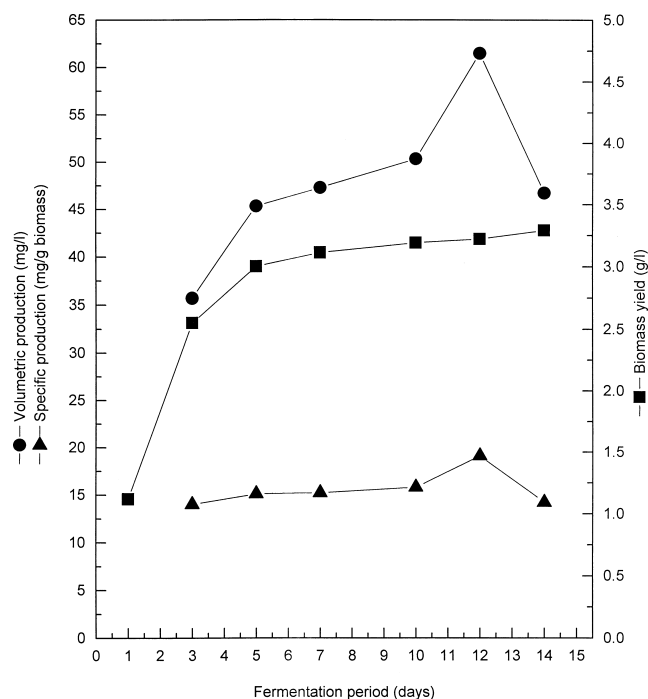


Fig. 3. Effect of different fermentation periods on the production of Cy A by *A. terreus* immobilized mycelia.

by the mycelium immobilized on Ca-alginate carrier. L-Val (2 g/L) was added at different time intervals. The obtained results (Table 5) revealed that the addition of L-val increased the Cy A production. Chun and Agathos (1991) stated that L-val must be added during or after the exponential growth phase to achieve the maximum production of Cy A (160 mg/L) by the immobilized spores of *T. inflatum*.

Different L-val concentrations (1, 2, 4, 6 and 8 g/L)

Table 5. Effect of L-valine addition at different time intervals on the production of Cy A by the immobilized cells of *A. terreus*.

Addition time (days)	Biomass yield (g/L)	Cy A productivity	
		Volumetric production (mg/L)	Specific production (mg/g biomass)
Control ^a	3.00	97.92	31.64
0 ^b	3.10	98.22	31.68
1	3.20	107.20	33.50
2	3.10	114.70	37.00
3	3.18	125.86	39.57
4	3.20	104.22	32.56
5	3.22	100.73	31.28

L-Valine concentration 2 g/L.

^aThe fermentation process was carried out by using 3% alginate concentration, pH 4.3, mycelium weight 10 g%, fermentation period 12 days at 150 rev/min, 27°C without L-val addition.

^bThe fermentation process was achieved as described above and L-val was added at the start of growth.

Table 6. Effect of different L-valine levels on the production of Cy A by the immobilized cells of *A. terreus*.

L-Valine concentration (g/L)	Biomass yield (g/L)	Cy A productivity	
		Volumetric production (mg/L)	Specific production (mg/g biomass)
Control ^a	3.00	97.91	32.63
1	2.90	99.61	34.34
2	3.17	125.86	34.92
4	3.18	144.60	39.56
6	3.34	115.67	24.74
8	3.23	85.70	20.23

^aThe fermentation process was carried out by using 3% alginate concentration, pH 4.3, mycelium weight 10 g%, fermentation period 12 days, 150 rev/min, 27°C without L-valine addition.

L-Valine was added at 3 days of growth.

were tested to investigate their effects on the production process using the immobilized mycelia. The results (Table 6) clearly indicated the enhancement effect of the low concentrations of L-val. The best yield of Cy A (144.6 mg/L) was obtained at the 4 g/L level. In accordance with our results, Chun and Agathos (1991)

Table 7. Effect of different amino acid combinations on the production of Cy A by the immobilized cells of *A. terreus*.

Amino acid combination	Biomass yield (g/L)	Cy A productivity	
		Volumetric production (mg/L)	Specific production (mg/g biomass)
No amino acid supplement	3.00	97.91	32.63
L-Val	3.37	144.60	42.90
L-Leu	3.45	110.33	31.97
L-Val+L-leu	3.32	166.75	50.22
L-Val+L-abu	3.24	136.60	42.16
L-Leu+L-abu	3.33	115.62	34.72

The fermentation process was carried out by using 3% alginate concentration, pH 4.3, mycelium weight 10 g%, fermentation period 12 days, 150 rpm, 27°C.

observed the stimulatory effect of L-val and showed that the best concentration required for maximum Cy A production was 6 g/L on using the immobilized mycelium of *T. inflatum*.

Role of amino acid combinations

The amino acids precursors of Cy A molecule were found to play an important role on its formation when added separately or in combinations. Some amino acids were thus tested as supplements to the fermentation medium. Evidence has been given that the formation of Cy A was markedly stimulated in case of L-val+L-leu treatment (Table 7). Under the latter experimental conditions, Cy A outputs were increased by about 60% as compared with the yield recorded upon using amino acid depleted medium (control treatment).

Conclusion

The main objective of the present communication was to increase the capacity of the local isolate of *A. terreus* to produce Cy A through the application of the cell immobilization technique. The obtained results clearly indicated that Cy A productivity was stimulated particularly upon using the immobilized fungus mycelia. Under the best fermentation conditions, *A. terreus* could afford 166.75 mg/L. The latter Cy A yield was of high magnitude when compared with the yield (99.95 mg/L) given by the free mycelia of the same

fungus which we previously reported (Sallam et al., 2003). These findings supported the conclusion of Chun and Agathos (1989), who reported on the superiority of the immobilized cell culture to free cell systems.

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