

CYCLOSPORIN A PRODUCTION BY GENETICALLY IMPROVED FUSANTS OF *TOLYPOCLADIUM INFLATUM*

Khattab A.A.¹ and I.S Abd-Elsalam^{2*}

¹Genetic & Cytology Dept., National Research Center, Dokki, Cairo, Egypt.

²Natural and Microbial Products Chemistry Dept., National Research Center, Dokki, Cairo, Egypt.

Article Received on
17 Aug 2015,

Revised on 10 Sept 2015,
Accepted on 04 Oct 2015,

***Correspondence for
Author**

I. S. Abd-Elsalam

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

ABSTRACT

Cyclosporin A (CsA) is an important immunosuppressive drug used in organ and bone marrow transplantation. The production of CsA is accomplished by some imperfect fungi especially *Tolypocladium inflatum* strains by submerged fermentation. Protoplast fusion had been applied to increase the productivity of CsA. Through antifungal and heavy metal resistance markers, only two superior mutants were forced to protoplasts and intra-specific protoplast fusion between them was achieved. The results showed that the most fusants used showed remarkable higher productivity of CsA than the original strain. The superior fusant was F-11 which produced 92.70 mg/L compared to the original strain which produced 55.75 mg/L.

KEYWORDS: *Tolypocladium*, CyclosporinA, protoplast fusion.

INTRODUCTION

Cyclosporins are a group of closely related cyclic undecapeptides produced as secondary metabolites by some strains of fungi imperfecti, *Cylindrocarpum lucidum* and *Tolypocladium inflatum* Gams both were isolated from soil samples.^[1,2] The fungus *Tolypocladium inflatum* is of major biotechnological interest due to its ability to synthesize different cyclosporins. Cyclosporin A is the most important molecule of this group, with unique immunosuppressive properties. Cs A was introduced into clinical use in the late 1970s to reduce graft rejection after organ transplantation, a property based upon interference of the agent with lymphokine biosynthesis.^[3] CsA is a drug with broad spectrum pharmacological properties including antifungal.^[1,4] antiparasitic, anti-inflammatory.^[5] and immunosuppressive activity.^[3] For more

than a decade CsA has been used as a potent immunosuppressant drug due to its beneficial effect on patients after kidney transplantation.^[2] Also, CsA plays a role in reversing multi-drug resistance in several types of cancer.^[6] It is in clinical use world wide under the trade name of Sandimmun. On the other hand, CsA has been described as a potent agent in the treatment of HIV.^[7,8]

Today submerged fermentation process dominates as the preferred method for most commercial compounds principally because sterilization and process control are easier to be engineered in these systems. In order to obtain and maintain a competitive economic position for new and existing fermentation process, it is often necessary to find means for increasing the yields of the fermentation product. In this situation the mutation and protoplast fusion approaches so far has found most extensive use for industrial organisms.^[9,10,11,12,13] The aim of this study was to select the high CsA producing fusants, through the protoplast fusion protocol.

MATERIALS AND METHODS

Microorganisms: The microorganism used in the present work, *T. inflatum* ATCC 34291 was a gift from Universitate Hannover, Microbiology institute (Germany) in a lyophilized form. The fungal strain was maintained on (Malt extract 2.5% yeast extract 0.4%, agar 2%) (MY medium), stored at 4°C and were monthly regenerated. The superior mutants: UV 15/2; UV 15/8; 20/6 and 40/4 are obtained as described by.^[13] The fungal strains were maintained on (Malt extract 2.5% yeast extract 0.4%, agar 2%) (MY medium), stored at 4°C and were monthly regenerated.

Chemicals: The authentic CsA was provided by Sigma Company. All the other chemicals were laboratory reagents obtained from Merck and the solvents used are HPLC grade.

Inoculum's preparation: Erlenmeyer flasks 250 ml containing 50 ml of MY medium (Malt extract 2, yeast extract 0.4%) pH 5.3 was inoculated with 5ml spore suspension (9.3×10^3 spores /ml). It was incubated a rotary shaker at 200 rpm at 27° C, for 72 hrs.^[13,14]

Production process: According to the method described by.^[14] Erlenmeyer flasks 250 ml containing 100 ml of the following medium (glucose 5%, peptone 1%, potassium dihydrogen phosphate 0.5%, potassium chloride 0.25% was inoculated with 2ml of 72 hrs aged inoculum and shaken at 200 rpm at 27°C.

Labeling of the parental mutants for fusion: The original strains and the higher producing mutants were streaked on the surface of plates containing MY medium supplemented with antifungal and heavy metal agents. The plates were incubated at 28 °C for 6-days and the colonies which exhibited resistance to specific antifungal and/or heavy metal agents were retested to ensure its stability.

Protoplasts preparation: A volume (200 ml) of complete medium (5% maltose, 1%Bacto-tryptone, 0.5% KH_2PO_4 , 0.25% KCl , pH 5.6) was inoculated with 10^9 conidia and incubated on a rotary shaker (200 rpm) for 72 hrs at 28 °C. The mycelium was harvested and incubated with protoplast buffer (0.8M NaCl , 0.02M $\text{MgSO}_4 \cdot \text{H}_2\text{O}$, pH 7.5) containing 50 mM dithiothreitol. Protoplasts were generated by incubating the mycelium with Novozyme 234 (Novo Industry, AIS, Bagsvaerd, Denmark) at a final concentration of 10 mg/ml in protoplast buffer, shaking at 100 rpm for 2 h at 28 °C. Protoplasts were collected by low speed centrifugation and washed twice in protoplast buffer. The protoplasts were readily detected microscopically according to.^[15]

Protoplast fusion and fusants detection: Protoplasts from two strains were mixed and centrifuged at 2000 rpm for 5 min at 4°C. The biomass was then re-suspended in 2 ml of protoplast buffer containing 20 % (w/v) of polyethylene glycol 6000 and incubated at 30°C for 30 min. The fusion suspensions were plated onto osmotic MY medium with protoplast buffer which containing specific antifungal and/or heavy metal agents; 0.01 % (w/v) α -amino butyric acid ; 0.1 (v/v) Triton-x 100 as a restriction factor for radial colonies growth at 28°C for 6 days. The colonies developed on the surface of the plates were considered as fusants.

Qualitative analysis of cyclosporin A: Cyclosporin A was qualitatively detected according to the method described by.^[16,17] where the methanolic solution of the tested materials as well as the authentic sample were individually applied to TLC plate and the developing system consists of n-hexane: acetone (1:1, v/v). The TLC profile was colored in comparison with the standard CsA sample by color agent iodine vapor. CsA presents in the sample was identified in comparison with standard CsA sample through the specific RF value.

Quantitative analysis of cyclosporin A: High pressure liquid chromatography (HPLC) was adopted to determine CsA. A water Pye HPLC system (waters, Milford, MA, USA) equipped with reversed phase C-8 Nova-Pak column (150+3.9 mm, 10 μm packing, waters) was used.

The procedure was followed according to the method described by [14], where 5 μ l of CsA firstly injected for determination of its RT, then another 5 μ l from sample was applied.

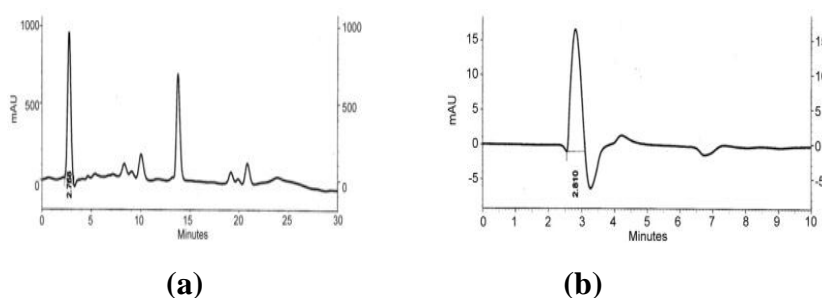


Fig. (1) HPLC analysis of (a) fermentation sample (b) authentic cyclosporine A

The mobile phase consists of acetonitrile /methanol/water (42.5:20:37.5 v/v/v) with flow rate 1ml/min and the column was maintained at 72°C with approximately pressure 1200ib/in2. CsA was determined using detector (waters, 486) at 210nm wave length.

Extraction of cyclosporin A: Equal volume of butyl acetate (100 ml) was add to the fermentation medium 100 ml and stirred at 200 rpm at 27° C for 24. The organic layer was separated under vacuum, then dissolved in methanol and undergo HPLC analysis.

RESULTS AND DISCUSSION

Cyclosporin A is a hydrophobic cyclic undecapeptide produced by some fungal strains belonging to *T. inflatum*. Unlike other immunosuppressant agent for hindering the patients ability to combat infection, CsA is an important immunosuppressant drug used in organ transplantation. Protoplast fusion method had been applied to increase the biosynthesis of CsA.

Protoplast fusion and cyclosporin A productivity: Protoplast fusion was the main protocol for exchanging genetic material between two cell type and subsequently obtaining the new gene recombinants towards the isolation of higher cyclosporin A producing fusant(s).

Detection of selective markers

In order to investigate the effect of intra-specific protoplast fusion on cyclosporin A production, four superior mutants and the original strain were selected to determine their antifungal and heavy metals resistance or sensitivity as the main selective markers during fusants detection.

Table (1) presents the selected strains response to five antifungal and four heavy metals agents. Results showed that, the original strain was resistant to one antifungal (M) and sensitive to one heavy metal (Se). On the other hand, all the tested mutants exhibited different responses.

The obtained results indicated that, mutant No. 15/2 showed G, N, Cd, Mc, and Se resistance. The mutant No. 15/8 showed the highest level of resistance, since it was resistant to seven agents as follows: G, M, N, As, Cd, Mc, and Se while it was sensitive to B and C agents. The mutant No. 20/6 was resistant to G, M, N, As, Cd and Se. Finally, the mutant No. 40/4 was sensitive to all agents except for As agent.

Table (1): Response of the superior mutants for cyclosporin A production against five antifungal and four heavy metals agents.

Mutant No.	CsA productivity (mg/L)	Antifungal agents					Heavy metals			
		B	C	G	M	N	As	Cd	Mc	Se
-	-									
Original st.	55.75	-	-	-	+	-	+	+	+	-
UV-10/2	78.50	-	-	+	-	+	-	+	+	+
UV-15/2	88.35	-	-	+	-	+	+	+	+	+
EMS-20/6	85.52	-	-	+	+	+	+	+	-	+
EMS-40/4	92.30	-	-	-	-	-	+	-	-	-

B = Benomyl (20 µg/ml), C = Cyclohexamide (250 µg/ml),
 G = Griseofulvin (400 µg/ml),
 Cd = Cadmium (250 ppm), Mc = Merck Chloride (75 ppm),
 Se = Selenium (75 ppm), + = Good growth (Resistant),
 — = No growth (Sensitive).

The above different responses of the superior mutants were used as the selective markers during the detection of fusants after protoplast fusion. As mentioned before under materials and methods, the mycelia of each of the two selected mutants were force to protoplasting.

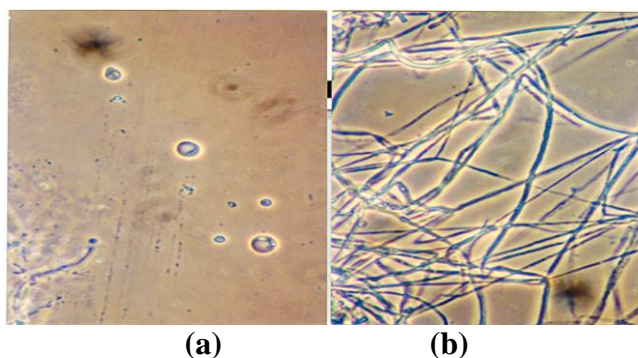


Fig.(2): Photomicrographs of *T. inflatum* mutant protoplasts (a) in comparison with its mycelia (b).

Cyclosporin A production after protoplast fusion

The cross was carried out using protoplasts of the superior mutants (15/2 and 20/6) which varying in their M and Mc responses. Table (2) presents the cyclosporine A productivity of the parental and fuants strains which obtained after the above cross at the level of intra-specific protoplast fusion. The results in table (2) show that, ten out of the 13 fusants were higher cyclosporin A producers than both parents. Six of the above ten fusants proved to be higher cyclosporin A producers than their higher producer parent. The excellent fusant in this cross was F-11 which produced 66.28 percent cyclosporine A more than the original strain and at the same time represents 4.92 percent more than the higher parent(15/2) as described by.^[18,19,25]

Table (2): Cs A production using some fusants obtained after intra-protoplast fusion.

Isolate No.	Biomass (g/L)	Volumetric productivity (mg/L)	Specific productivity (mg/g)
Original st.	3.5	55.75	15.80
P1 15/2	3.4	88.35	25.04
P2 20/6	3.5	85.52	24.24
F-1	3.6	85.93	24.35
F-2	3.4	89.90	25.48
F-3	3.7	87.95	24.93
F-4	3.5	86.57	24.53
F-5	3.4	81.57	23.12
F-6	3.3	84.56	23.96
F-7	3.5	88.32	25.03
F-8	3.6	92.30	26.16
F-9	3.4	89.71	25.42
F-10	3.4	90.34	25.60
F-11	3.7	92.70	26.27
F-12	3.5	79.50	22.53
F-13	3.4	90.58	25.67

In general, it could be concluded that, intra-specific protoplast fusion proved to be an effective tool to improve cyclosporine A production. Since the enhancement of cyclosporine A productivity reached up to 66.28 percents higher than the original strain. Therefore, protoplast fusion have been used successfully to enhancement the productivity of fungal metabolites and enzymes.

The recombinants strains can be obtained by this technique.^[20,21,12,22] However,^[23,24] obtained similar results, when they fused protoplasts between two different strains of *T. inflatum* to improve the production of cyclosporine A and got recombinants produced Cyclosporin 2.8 times more than the parent strain, *T. inflatum* PTCC 5252, and 2.3 times more than *T. inflatum* PTCC 5253.

CONCLUSION

The application of mutagenesis and protoplast fusion protocols are useful for production of the superior genetically improved strains of *T. inflatum*, which led to produced high levels of cyclosporin A compared to the original strain. Therefore, the optimization and maximizing its production for high yields of CsA using the above protocols are recommended.

REFERENCES

1. Dreyfuss, M.; Harni E.; Hoffman H.; Kobel H.; Pache W. and Tscherta H. Cyclosporin-A and C, new metabolites from *Trichoderma polysporum*. Eur J Appl Microbiol, 1976; 45: 1223-1228.
2. Borel JF, Feurer C, Gubler HU, Steahelin H. Biological effects of cyclosporin A: a new antilymphocytic agent. Agents Actions, 1976; 6: 468-475.
3. Rahacek, Z. and De-Xiu Z. The biochemistry of cyclosporin formation-a review. Process Biochem., 1991; 26: 157-166.
4. Weiser, J., and Matha V. The insecticidal activity of Cyclosporin- A on mosquito larvae. J. Invert. Pathol., 1988; 5: 92-93.
5. Stiller, C. R. and Opelz G. Should Cyclosporin be continued indefinitely? Transplant Proc., 1991; 23: 36-40.
6. Derks, P. M. and Madrid S. M. The *Aspergillus niger* CYPA gene encodes a cyclophilin that mediates sensitivity to the immunosuppressant cyclosporin A. Mol. Gent. Genomics, 2001; 206: 527-536.
7. YoshiKawa, H. and Seebach S. Lymphotropic delivery of cyclosporin A by inter a muscular injection of biodegradable microspheres in mice. Biol. Pharm. Bull., 1996; 19: 1527-1529.
8. Sharmila, K. Thillamarani, A, Rlogesh, A., Sathishkumar,S.Kalaishsel, M., production of cyclosporine A by saprophytic filamentous fungus *Fusarium oxysporium* . International journal of pharmacy and pharmaceutical sciences, 2012; 4(4): 149-153.

9. Agathos, S. N. and Parekh R. Enhancement of cyclosporin production in a *Tolypocladium inflatum* strain after epichlorohydrin treatment. J. Biotechnol., 1990; 13: 73-8110.
10. Rehm, H. and Reed G. Mutation. Biotech., 1986; 1: 401-403.
11. EL-Bondkly, A. M. and Khattab A. A. Clavulanic acid and cephamycin improvement in *Streptomyces clavuligerus* by inducing combined resistant mutations and protoplast fusion. Egypt. J. Genet. Cytol., 2004; 33: 217-230.
12. Abdel Salam, I. S. and Khattab A. A. (2005). Enhancement of side chain degradation of β - sitosterol via genetic improvement of *Fusarium* spp. Arab J. Biotech., 8: 201-210.
13. Abdel Salam I.S. and Khattab A. A. (2009). Enhancement of cyclosporine A production by *Tolypocladium inflatum* using some mutagenic agents. Acta Pharmaceutica Scientia, 51: 87-92.
14. Agathos, S.N.; Marshal J.W.; Moraiti C.; Parekh R. and Madhosingh C. Physiological and genetic factors for process development of cyclosporine fermentations. J. Industrial Microbiol., 1986; 1: 39-48.
15. Stimberg N, Walz M, Schorgendorfer K, Kuck U. Electrophoretic karyotyping from *Tolypocladium inflatum* and six related strains allows differentiation of morphologically similar species. Appl Microbiol Biotechnol, 1992; 37: 485-489.
16. Sallam, L.A., El-Refai, A and El-kady,I., Thin layer chromatography of some C21,C19nd C18 steroids. J. Gen. Appl. Microbiol., 1969; 15: 309-315.
17. Sallam,L., El-Refai, A. ,Hamdy. A and Abd-Elsalam, I Role of some fermentation parameters on cyclosporine A production by a new isolate of *Aspergillus terreus*.J .Gen.Appl. Microbiol.. 2003; 49: 321-328.
18. Kathryn, E., Rajani, R.; Cumbie, J; Nonogaki, M. and boyd, A. The genome of *Tolypocladium inflatum*. POLS genet. 2013; 9: 1003.
19. Balke, W.; LinDu; Carter, A., Robert, H. and Bradley, S. Draft genome of tow Sordariomycetes fungi that produce novel secondary metabolites. Genome Announ, 2015; 3: 2-5
20. Kirimura, K.; Yagughi T. and Usami S. Intra-specific protoplast fusion of citric acid-producing strains of *Aspergillus niger*. J. Ferment. Technol., 1986; 64: 473-479.
21. Bawa, S. and Sandhu D. K. Parasexual analysis in *Trichoderma reesei* using protoplast fusion. Fungal Genet., News lett., 1994; 41-43.
22. Lin, C. C.; Hsieh P. C.; Mau J. L. and Teng D. F. Construction of an inter-generic fusants from *Schizosaccharomyces pombe* and *Lentnula edodes* for xylan degradation and polyol production. Enzyme Micro. Technol., 2005; 36: 107-117.

23. Mohammad, R. B.; Masoud F.; Pupak F. and Nasrin M. Protoplast fusion technique in *Tolypocladium inflatum* for increasing Cyclosporine production. Abstracts/Journal of Biotechnology, 2007; 133–187.
24. Balakrishnam, K. and pamedy A. Influence of amino acids on the biosynthesis of cyclosporine A by *Tolypocladium inflatum*. Appl. Microbiol. Biotech., 1996; 6: 800-803.
25. Gharavi, M.; Bahri N. R. and Kloobandi A. Mutation of *Tolypocladium inflatum* DSM 9/5 by radiation for higher production of cyclosporine A. Saudi Pharmaceutical journal, july.2004; 12: 2-3.