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Full Length Research Paper

Production enhancement of Cyclosporin 'A' by *Aspergillus terreus* through mutation

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Cyclosporin 'A' is an important immunosuppresive drug used in organ and bone marrow transplantations. Nine isolates of *Aspergillus terreus* was used for the production of Cyclosporin 'A' by submerged fermentation in this study. The highest drug yielding isolate FCBP-58 was subjected to both physical and chemical mutation to increase the biosynthetic capabilities of Cyclosporin 'A'. In this study, mutation was carried out by ultraviolet radiation (254 nm) and alkylating agent ethylmethane sulphonate (EMS). UV 5 min time treatment was proved to be the best treatment because most of the resulted mutants showed enhancement in drug production. Chemical treatment of 200 µg/ml produced best mutants, among which MC 3.2 showed maximum increment of 885% in Cyclosporine 'A' production when compared to the parental isolate.

Key words: Cyclosporin 'A', *Aspergillus terreus*, UV radiation, EMS.

INTRODUCTION

Cyclosporin 'A' is a fungal secondary metabolite that is produced non-ribosomally from a multi-functional enzyme template, cyclosporin synthetase, by the filamentous fungus, *Tolypocladium inflatum*. It possesses anti-inflammatory, immunosuppressive, antifungal and antiparasitic properties. It showed antifungal activity against *Aspergillus fresenii*, *Aspergillus juponicus*, *Aspergillus niger, Cryptococcus neoformans, candida* sp, *Trichophyton mentagrophytes, Trichophyton tonsurans, Trichophyton violaceum* and *Fusarium* sp. (Bhosale et al. 2008). Cyclosporin 'A' is in clinical use worldwide under the trade name of SANDIMMUN®.

Today, submerged fermentation process dominates as the preferred method for the production of most commercial compounds, principally because sterilization and processes control are easier to be engineered in these systems. *T. inflatum* has been used for a large-scale production of cyclosporin 'A' by submerged fermentation. Sallam et al., (2003) first time worked on a local isolate of *Aspergillus terreus* for the production of cyclosporin 'A' by submerged fermentation. Cyclosporin formation is also reported for *Cylindrocarpon* spp, *Fusarium* spp. *Tolypocladium geodes*, *Trichoderma virile, Neocosmospora vasinfecta, Isaria spp, Verticellium* spp, *Acremonium* spp, *Beauveria nivea* (Khedkar et al., 2007). Proper organism, medium, aeration, agitation, antifoam, pH control and so forth are associated with the accumulation of high yield of the fermentation products like cyclosporin 'A'. These considerations may well not provide a suitable increase in the overall product yield for fermentation. In this situation, the mutation approach has been extensively used in industrial organisms for the high production of Cyclosporin 'A'.

The aim of the present study is to enhance Cyclosporin 'A' production by selecting high drug yielding mutant strain through UV and chemical treatment, that is, ethylmethane sulphonate (EMS). The UV radiation has been recommended as a mutagen of first choice. The ratio of mutation to lethality is usually high and is a relatively safe mutagen. The intrastrand cyclobutan pyrimidine dimer is the predominant DNA lesion reported to be produced by UV radiation. EMS is an alkylating agent and is known to produce random mutations in genetic material by nucleotide substitution, specifically by

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Abbreviations: EMS, Ethylmethane sulphonate; **FCBP,** fungal culture bank of Pakistan; **MEA,** malt extract agar; **MY,** malt-yeast.

Table 1. Nine strains of *Aspergillus terreus* and their source of isolation.

Strain	Source
FCBP-58	Soil, canal bank LHR
FCBP-113	Air micro flora, FSD
FCBP-119	Air micro flora. P.U.LHR
FCBP-122	*Dalbergia sisso* root
FCBP-148	Air micro flora. P.U.LHR
FCBP-168	Air micro flora. P.U.LHR
FCBP-196	Air micro flora. P.U.LHR
FCBP-536	Air micro flora. P.U.LHR
FCBP-652	Soil, LHR

Where, LHR = Lahore; FSD = Faisalabad; P.U. = Punjab University.

guanine alkylation.

MATERIALS AND MATHODS

Microorganism

Different strains of *A. terreus* used in this investigation are recorded in Table 1. These strains were obtained from first fungal culture bank of Pakistan (FCBP).

Chemicals

For confirmation, authentic Cyclosporin 'A' was imported from Sigma-Aldrich, Fluka (Cat. No. 30024) and Sandimmun Neoral® capsule (100 mg) was obtained from Novartis. Organic solvents were high-performance liquid chromatography (HPLC) grade except butyl acetate.

Selection of high yielding strain

Nine isolates of *A. terreus* were tested for their production capability of Cyclosporin 'A' using the following protocol.

Preparation of inoculums

Standard inoculum from malt extract agar (MEA) plates was introduced into 250 ml Erlenmeyer flask containing 50 ml of malt-yeast (MY) medium of the following composition: Malt extract, 2%; yeast extract, 0.4% (w/v), pH 5.7. The seed inoculum was incubated on orbital shaker incubator at 200 rpm for 72 h at 28 ± 1°C (Borel et al., 1977).

Cultivation

According to the method of Agathos et al., (1986), 5 ml of seed inoculum were introduced into 250 ml Erlenmeyer flask containing 50 ml of production medium having the following composition: glucose, 5%; peptone, 1%; KH_2PO_4, 0.5%; KCL, 0.25% (w/v), at pH 5.3.The fermentation was continued at 28 ± 1°C, 200 rpm for 10 days.

Cyclosporin 'A' extraction and analysis

Equal volume of butyl acetate was added to harvest fermentation medium that was 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 weighed and dissolved in methanol. The cyclosporin 'A' level in the crude extract was analyzed by HPLC. HPLC was carried out using Hitachi system consisting of L-2100/2130 pump, L-2420 UV-VIS Detector with a detection span from 190 to 900 nm. The analysis was done using a C_{18} column with a 5 µm particle size, and acetonitrile : methanol : water (42.5:20:37.5, v/v) as mobile phase at a flow rate of 0.8 ml min^{-1} with UV detection at 215 nm. The data obtained was matched with those of standard samples of cyclosporin 'A' analyzed under the same conditions for the confirmation of cyclosporin peak.

Measurement of fungal biomass

After the extraction of organic layer, remaining media that contained fungal pellets was filtered through pre-weighed whattman filter paper for the estimation of fungal dry biomass.

Antifungal activity

As the compound is known for its antifungal properties, the filtrate was checked against *A. niger* which was isolated from air mycoflora and preserved under reference no. FCBP74 for its affectivity.

A. niger was inoculated on MEA media plates with the help of needle. Wells of 0.8 mm diameter were punched into these plates by using sterile cork borer and 50 µl of purified cyclosporin 'A' by several runs of HPLC was added to each well. Plates were incubated at 30°C for 5 days and the inhibition zone of fungal growth around the well was observed. The radius of inhibition zone was measured in cm at eight different positions and mean value was taken as measure of inhibition zone.

Production enhancement of Cyclosporin 'A'

In this study, the selected isolate of *A. terreus* was exposed to UV light (254 nm) and ethyl methane sulphonate to increase the chance of mutation for increasing yield of Cyclosporin 'A'.

UV mutation

Malt extract agar media (2%) of pH 6.5 was used to subculture the selected isolate of *A. terreus*. A conidial suspension from 5 days old culture of *A. terreus* was prepared in sterile distilled water, filtered through cheese cloth and adjusted to a concentration of 10^5 conidia/ml. With respect to UV treatment, 5 ml of conidial suspension was poured into each of the 12 Petri plates with the help of a micropippet. Plates were irradiated by short wave UV radiation of 254 nm in germicidal UV lamp. The distance of UV lamp above the suspension was 20 cm and the time of irradiation varied from 5-60 min with regular interval of 5 min.

Growth of mutant colonies: For the growth of mutant colonies, 300 ml of minimal media containing glucose, 2%; KH_2PO_4, 0.04%; $MgSO_4$. $7H_2O$, 0.005%; KCL, 0.01%; agar, 1.5% (w/v), was prepared and 25 µl of irradiated conidial suspension from each treatment was separately inoculated into minimal media plates. The suspension was spread with the help of spreader and the plates were incubated for two days at 30°C.

Separation and selection of UV induced mutants: After two days, mutant colonies grown on minimal media were separated by inoculating each colony on MEA plates. The inoculated plates were incubated at 30°C for five days.
After five days, colony diameter of mutant colonies were measured and compared with the colony diameter of parental strain of *A. terreus*. The mutants that have increased colony diameter than parental one were selected. The mutated strains were nominated according to the conditions of radiation, that is, time. After fermentation, measurement of biomass, Cyclosporin 'A' extraction and analysis was carried out as described earlier.

Chemical mutation

Chemical mutation was done by 99% pure EMS. Four concentrations of EMS, that is, 50, 100, 200 and 300 µg/ml were prepared. For chemical treatment, 20 ml of conidial suspension (10^5 conidia/ml) of selected strain of *A. terreus* was prepared. Conidial suspension of 5 ml was added into each 5 ml of respective concentration of EMS with the help of micropippet in falcon tubes and heated in water bath at 37°C for 30 min. The tubes were centrifuged at 3,000 rpm for 2 min. After centrifugation, supernatant was discarded and 1% saline water was added in the falcon tubes containing pellets and centrifuged at 3,000 rpm for 60 s. The centrifugation step was repeated two times with saline H_2O. After that the mutated conidia were suspended in 2 ml of 1% saline H_2O.

Growth of mutant colonies: For the growth of mutant colonies 300 ml of minimal media containing glucose, 2%; KH_2PO_4, 0.04%; $MgSO_4.7H_2O$, 0.005%; KCL, 0.01%g; agar, 1.5%; Triton X-100, 0.1%; L-Sorbose, 0.4% (w/v) was prepared and 25 µl of mutated conidial suspension from each treatment was separately inoculated in minimal media plates. The suspension was spread with the help of spreader and plates were left in incubator for two days at 30°C.

Separation and selection of mutant colonies: After two days, mutants were separated and selected according to the criteria as described earlier for UV mutant selection. Chemical treated mutants were named according to the concentration of EMS used.
After chemical treatment, selected mutants were subjected to fermentation process as described earlier. After fermentation, biomass measurement, Cyclosporin 'A' extraction and analysis was carried out as previously mentioned.

Estimation of Cyclosporin 'A'

The following equation was used for the estimation of cyclosporin through chromatograms (Minutza et al., 2009).

$$\%\text{By weight} = \frac{\text{Area of Sample Peak}}{\text{Area of Ref Peak}} \times \frac{\text{Weight of Ref Material}}{\text{Weight of sample}} \times \frac{\text{Volume of sample}}{\text{Volume of ref}} \times \frac{100}{1}$$

Data shown in various tables are mean of three replicates of various tested parameters and are presented as means ± standard error.

RESULTS AND DISCUSSION

Screening of fungal strain for drug production

Nine strains of *A. terreus* were tested for their potential to produce Cyclosporin 'A'. The harvested liquid assumed to have mixture of Cyclosporins was confirmed first through antifungal bioassay against *A. niger* and then by comparing the retention time of their peaks with authentic compounds. Cyclosporin-related metabolites are reported to have a broad spectrum of antifungal activity and a narrow spectrum of activity against bacterial cultures (Ramachandran et al., 2006). The mixture assumed to have Cyclosporins showed strong antifungal activity against *A. niger* when tested through well method. The average diameter of inhibition zone was 1.15 cm, which was calculated after measuring diameter of zone of inhibition at eight different dimensions and then taking its mean. When run through HPLC, a peak appeared between 2.7 and 2.8, which was confirmed as Cyclosporin 'A' when compared with Sandimmun Neoral® capsules containing 100 mg Cyclosporin 'A' as active ingredient and pure Cyclosporin 'A' as authentic drug supplied by Fluka analytical, Japan. Sandimmun Neoral® capsules showed a clear peak at 2.768 whereas authentic compound gave its peak at 2.81 (Figures 1 and 2). Quantitative determination of Cyclosporin 'A' revealed that maximum yield of (62.4 µg/ml) was obtained by FCBP-58, so was selected for further studies (Table 2).

This quantity was found much lower to that recorded by earlier workers in the most studied fungal strain, that is, *T. inflatum.* Dreyfuss et al., (1976) and Agathos et al., (1986) found a very good yield of 105.5mg/L and Balakrishnan and Pandey (1996) showed that *T. inflatum* can produce higher levels of Cyclosporin 'A' up to 183 mg/L. Sallam et al., (2003) revealed that maximum yield of 95.13 mg/L can be obtained by *T. inflatum* and *A. terreus* can produced comparably less quantity of the drug. They recorded a production of 86.57 mg/L by an isolate of *A. terreus*. The production capability of our isolate was 27.8% lower than this report. The project was therefore planned to enhance the production potential of the strain FCBP-58 for increased drug production.

UV mutation

The over production of desired metabolite can be achieved by the genetic removal of feed back control, which is possible through mutation. The UV radiation has been recommended as a mutagen of first choice. Ultraviolet wavelength is absorbed by pyrimidines, especially thymine. When the energy is absorbed, the ring structure becomes unstable and often leads to the formation of thymine-thymine dimmers. If the thymines are in opposite strands, the chromosomes will break when trying to replicate, but more often, the thymine dimmers form from adjacent thymines in the same strand. The T:T dimmers do not have normal base pairing properties, so when DNA tries to replicate, the wrong base may be inserted. Ultraviolet radiation of 254 nm was used for mutagenesis in this study. The exposure of parental cultures towards UV light was increased from 5

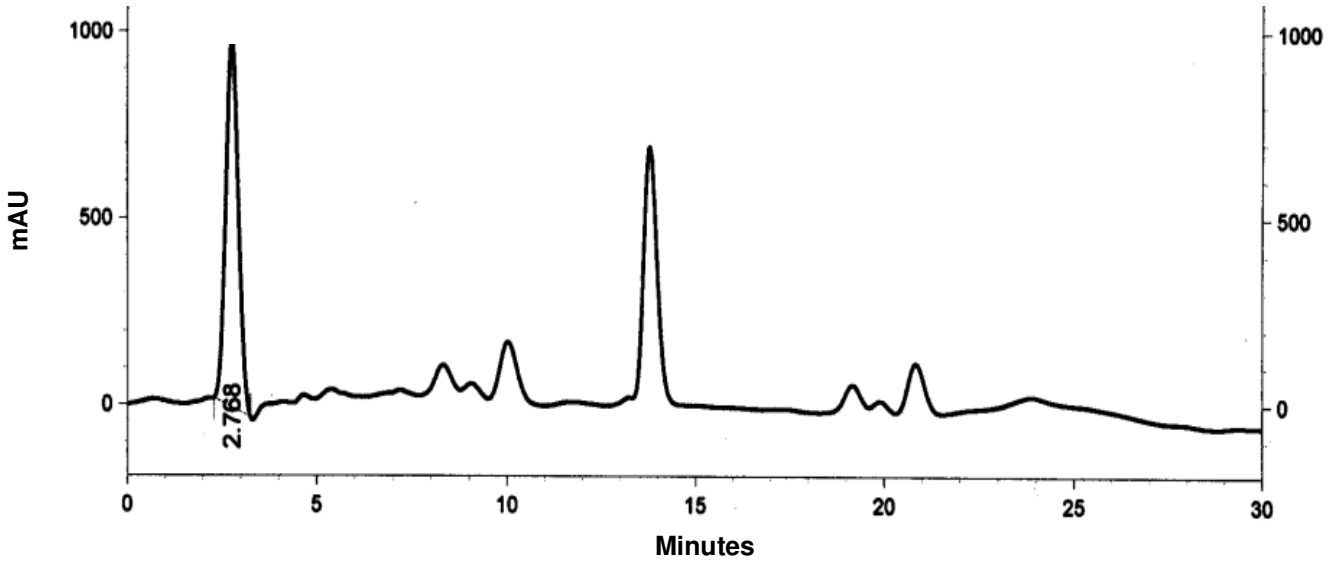

Figure 1. HPLC chromatogram of Sandimmun Neoral® capsule extracted at 215 nm. Peak at 2.7 is of Cyclosporin 'A'.

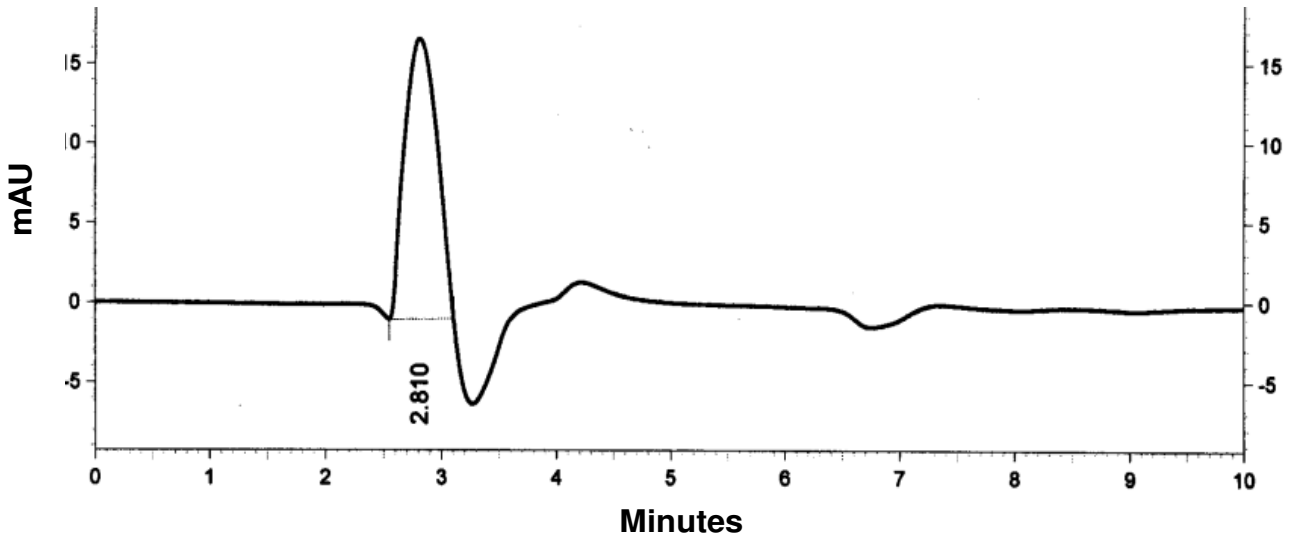

Figure 2. HPLC chromatogram of authentic Cyclosporin 'A' purchase from Fluka, Japan. The chromatogram extracted at 215 nm is showing Cyclosporin 'A' peak at 2.8.

to 60 min with regular five minutes interval. Increase in time of exposure resulted in decreased number of survivals and complete death was recorded in the last treatment (Table 3).

Mutants with desired higher drug production were recorded in the very first treatment of five minutes exposure. Among all UV treated mutants, MU 1.3 produced highest amount of Cyclosporin 'A' (Figure 3a) whereas, an increase in Cyclosporin 'A' production was also recorded in MU 1.2, 1.6, 1.8, 1.10, 1.11, 1.14, MU 3.6 and MU 5.3. These mutants were obtained after 5, 15 and 25 min of UV treatment (Table 4). Remaining UV mutant's undergone negative mutation and produced Cyclosporin 'A' much lower than parental *A. terreus* FCBP-58.

Previous studies also show successful use of UV mutagenesis in increase of biosynthetic capabilities of fungi for Cyclosporin 'A'. Gharavi et al., (2004) used UV radiation for higher production of Cyclosporin 'A' in *T. inflatum* (DSM 915). In their study, mutation was carried

Table 2. Cyclosporin 'A' production from nine isolates of *Aspergillus terreus.*

Strain	Peak area	% of Cyclosporin 'A'	Wt. of Cyclosporin 'A' (µg/ml)
58	9,585,692	9.76	62.4
113	5,190,782	7.69	33.38
119	1,290,593	1.13	6.0
122	8,447,103	11.04	55.2
148	8,586,308	10.43	56.2
168	5,031,317	4.95	32.6
196	2,327,129	2.77	14.80
536	6,042,242	4.95	39.60
652	7,857,211	8.51	51

Table 3. Number of survivals, activity range and % improvement in Cyclosporin 'A' production from each UV treatment.

UV treatment (min)	Number of survivals	No. of selected survivals	Activity range (µg/ml)	Improvement (%)
5	90	15	2.6 - 298.4	379
10	75	15	0.0016 - 62.2	-0.32
15	69	10	0.010 - 118.4	-8
20	60	10	3.4 - 29	-54
25	50	10	0.003 - 150.8	145
30	39	7	0.005 - 15.8	-75
35	36	7	0.014 - 30	-52
40	30	5	3.6 - 10.02	-84
45	21	2	1.46 - 5.4	-91.3
50	12	2	4.4 - 5.6	-91
55	6	1	7	-89
60	No growth	No growth	-	-

The survivals were selected on the base of increased growth in comparison to the wild type strain.

out by UV light and auxotroph dependent on α-aminobutyric acid was prepared which changed cell's metabolism and increased the biosynthesis of Cyclosporin 'A'.

Chemical mutation

The most potent chemical mutagens are alkylating agents e.g. EMS and the nitroso compounds such as N-methyl-N-nitro-N-nitrosoguanidine. In this study EMS was used. This chemical is known to produce random mutations in genetic material by nucleotide substitution, specifically by guanine alkylation. The ethyl group of EMS reacts with guanine in DNA, forming the abnormal base O-6-ethylguanine. During DNA replication, DNA polymerases that catalyze the process frequently place thymine, instead of cytosine opposite O-6-ethylguanine. Following subsequent rounds of replication, the original G:C base pair can become an A:T pair.

Four different concentrations of EMS were used for mutagenesis. The number of survivals with greater Cyclosporin 'A' biosynthesis than parental one was recorded similar in all treatments (Table 5). Mutants resulted from 50, 100 and 300 µg/ml of EMS concentration do not produce higher Cyclosporin 'A' except MC 2.6, MC 2.7 and MC 4.5 that showed enhanced drug production by 201, 542 and 661%, respectively, than parental strain.

Chemical mutation resulted by the dose of 200 µg/ml showed constant increase in all resulted mutants (Table 6). The highest increment when checked in all mutants was recorded in the above-mentioned treatment of EMS where MC3.2 showed an increase of 885% in drug production (Figure 3b). This increase was almost double to that recorded in top most drugs producing UV mutant MU1.3. A similar observation was made by Mala et al., (2001), where after exposing the spores of *A. niger* to UV and nitrous acid mutagenesis, the nitrous acid mutant produced enhanced lipase production while the UV

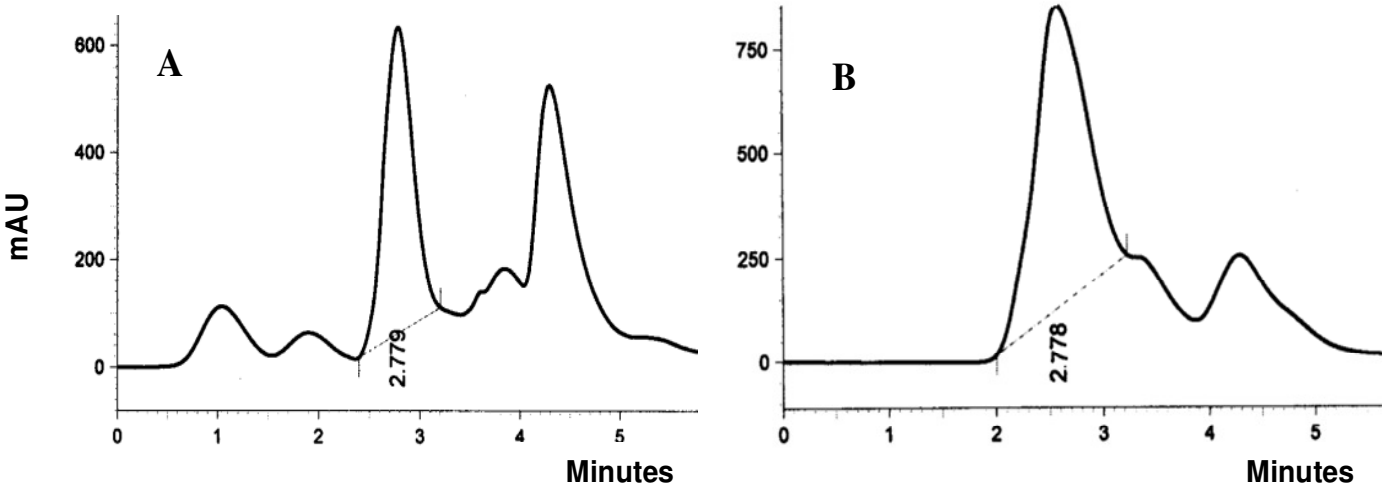

Figure 3. HPLC chromatograms showing production of Cyclosporin 'A' by best drug yielding mutants of *A. terreus* 58 after UV and chemical treatment; **A:** MU 1.3; **B:** MC 3.2.

Table 4. Production details of highest Cyclosporin 'A' producing mutants obtained after 5, 15 and 25 min of UV treatment.

UV treatment (min)	Mutant	Peak area	Sample dry weight (mg)	% by weight	Cyclosporin 'A' production		
					Amount (µg/ml)	Increase or decrease (µg/ml)	Increase or decrease (%)
5	MU 1.2	20059262	40	16.5	132 ± 3.79	69.6	112
	MU 1.3	45936414	26	57.4	298.4 ±5.4	236	379
	MU 1.6	12845616	43	9.72	83.4 ± 2.02	21	33
	MU 1.8	12017245	46	8.31	76.4 ± 1.8	14	22.4
	MU 1.10	12491636	45	9.04	81.2 ± 1.88	18.8	30.1
	MU1.11	20487324	33	20.25	133.6 ± 4.2	712	114
15	MU 3.6	18210301	26	22.8	118.4 ± 2.32	56	90
20	MU 5.3	23244864	23	32.8	151 ± 4.37	88.4	145

Table 5. Number of survivals, activity range and % improvement in Cyclosporin 'A' production from each chemical treatment.

Chemical treatment (µg/ml)	Number of survivals	No. of selected survivals	Activity range (µg/ml)	Improvement (%)
50	80	10	5.2-9.46	-85
100	75	10	6.52-400.6	542
200	68	10	78.5-615	885
300	60	10	6.3-475	661

mutants did not.

Drug production versus fungal biomass

Cyclosporin 'A' production does not seems to be linked with increase in fungal biomass. In both UV and chemical mutation, highest drug producing mutants showed variation of both low and high amount of fungal dry biomass (Figure 4).

Drug production versus extract color

The color of the final extract containing cyclosporins showed variation from light yellow to reddish black. However, the darkness of color did not seem to correspond with any increment in amount of Cyclosporin

Table 6. Production details of highest Cyclosporin 'A' producing mutant *obtained* after 100, 200 and 300 µg/ml EMS treatment.

Chemical treatment (µg/ml EMS)	Mutant	Peak area	Sample dry weight in mg	% by weight	Cyclosporin 'A' production		
					Amount (µg/ml)	Increase or decrease (µg/ml)	Increase or decrease (%)
100	MC 2.6	28811142	30	31.28	187.6 ± 3.01	125.2	201.1
	MC 2.7	61569766	38	52.72	400.6± 5.8	338.2	542
200	MC3.1	11920391	27	14.54	78.4 ± 1.3	16	26
	MC3.2	97198894	48	64.08	615 ±8	552.6	885
	MC3.3	29631147	34	28.27	192.2 ±3.2	129.8	208
	MC3.4	24755620	42	23.66	160.8 ±1.7	98.4	158
	MC3.5	23439736	55	13.87	152.4 ±1.4	90	144.2
	MC3.6	54511078	30	59.3	354 ±3.5	291.6	467.3
	MC3.7	12377300	30	13.46	80.6±1.83	18.2	29
	MC3.8	56671574	33	55.98	369.4 ±5	307	492
	MC3.1	41601053	44	30.16	265± 3.2	202.6	325
	MC3.1	28856882	43	21.87	188 ±2.9	125.6	201.2
300	MC4.5	74421683	44	53.98	475 ± 2.60	412.6	661

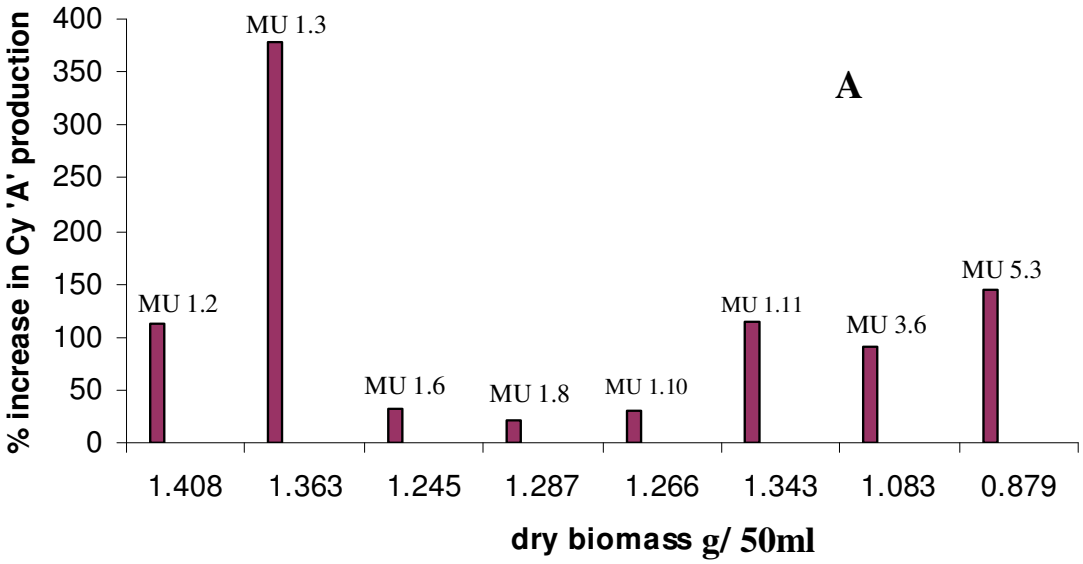

Figure 4. Effect of fungal dry biomass on production of Cyclosporin 'A'. **A:** UV treated mutants with increase drug production. **B:** EMS treated mutants with high production of Cy'A'.

'A'. In UV and chemical treatments, mutants MU1.3 and MC 3.2 with best production gave orange and reddish black colored to final extract. However other mutants with same colored extract did not support drug production.

Drug production versus pellet size

Morphological changes also come with increase in production of desired compound after mutation. In the present study, a variation of pellet size of fungi was recorded in the production media. In comparison to the parental strain, the mutants produce smaller, equal, larger and extra large sized pellet in the production media. Some mutants also showed no pellet production although they produced variable quantities of drug. However, the size of pellet was not found associated with Cyclosporin 'A' production and its quantity. Among the

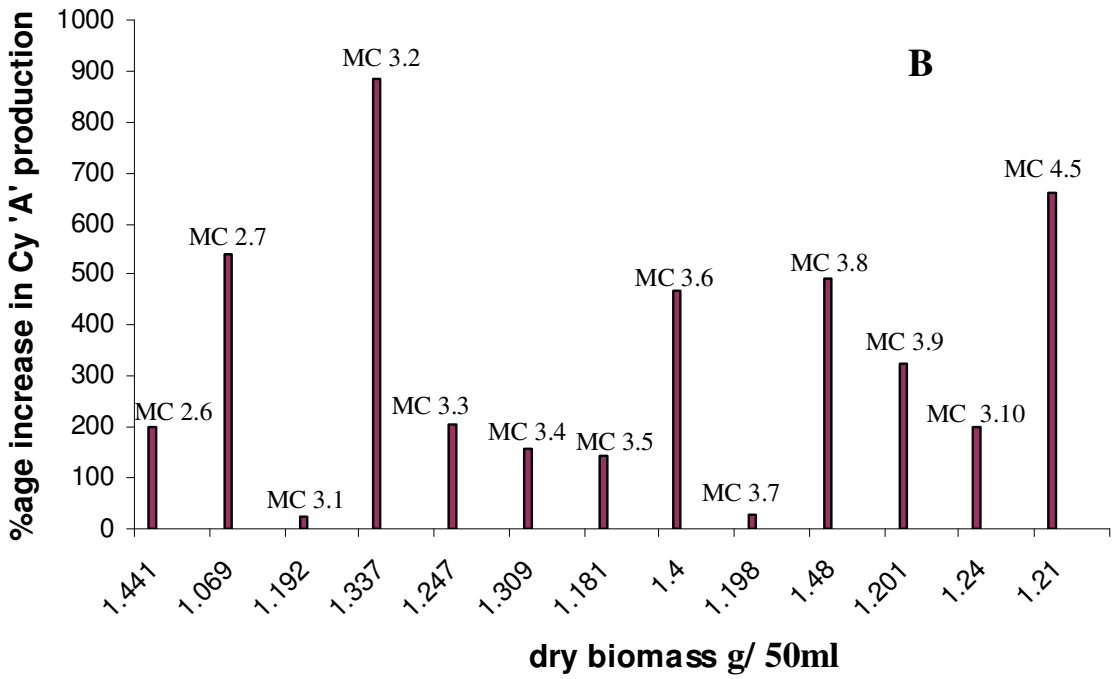

Figure 4. Continue.

UV mutants, the highest drug producing strain showed no change in pellet size when compared to that of the wild type. Contrarily, maximum level of drug producing chemical mutant showed change in pellet size and it was larger than the parental one. Other studies associated with mutagenesis also reports morphological changes like colony size, texture and color difference in both UV and chemical mutants (Chidananda et al., 2008).

Data obtained in this study overall depicts that mutagenic treatments have caused changes in the biochemical characteristics of parental strain that has successfully increased the drug production. However detailed studies are required to precisely point the biochemical and genetic modifications.

REFRENCES

Agathos SN, Marshall JW, Moraiti C, Parekh R, Madhosingh C (1986). Physiological and genetic factors for process development of cyclosporin fermentation. J. Ind. Microbiol. 1: 39-48.

Balakrishnan K, Pandey A (1996). Growth and cyclosporin a production by an indigenously isolated strain of *Tolypocladium inflatum*. Folia Microbiol. 41: 401-406.

Bhosale S, Naik CG, Subrayan P (2008). Process for the isolation of pharmaceutical compound cyclosporin A from fungus. US patent issue feb 26, 2008. http://www.patentstorm.us/patents/7335495/description.html.

Borel JF, Feurer C, Magnee C, Stahelin H (1977). Effect of new anti-lympholytic peptide cyclosporin A in animals. Immunol, 32: 1017-1025.

Chidananda C, Kumar CM, Sattur AP (2008). Strain improvement of Aspergillus niger for the enhanced production of asperenone. Indian J. Microbiol, 48: 274- 278.

Dreyfuss M, Ham E, Hofmann H (1976). cyclosporin A and C. New metabolites from *Trichoderma polysporum*. Eur. J. Appl. Microbiol. 3: p. 125.

Gharavi M, Najafi RB, Kloobandi A (2004). Mutation of *Tolypocladium inflatum* (dsm 915) by UV radiation for higher production of cyclosporin A. Saudi Pharma. J. 12: 2-3.

Khedkar AP, Subramaniyam P, Anand KNS, Ramkrishna M, Tambe SP (2007). Manufacturing and purification cyclosporine A .US Patent issue Feb 13, 2007. http://www.patentstorm.us/patents/7176001.html

Mala JGS, Kamini NR, Puvanakrishnan R (2001). Strain improvement of *Aspergillus niger* for enhanced lipase production. J. Gen. Appl. Microbiol. 47: 181-186.

Minutza L, Itamar K, Michael F (2009). Pharmaceutical composition comprising candesartan cilexetil. US Patent issue Februry 19, 2009. http://www.freepatentsonline.com/y2009/0048316.html.

Ramachandran MM, Balakrishnan K, Cynthiya AD, Sankar M, Panimalar M, Karthikeyan GSR (2006). Screening and isolation of cyclosporine-related compound producing soil fungi from the Western Ghats, Tamil Nadu. Curr. Sci. 92: 6-25.

Sallam LAR, El-Refai AH, Hamdi AA, El-Minofi AH, Abd-Elsalam SI (2003). Role of some fermentation parameters on cyclosporin A production by a new isolate of *A. terreus*. J. Gen. Appl. Microbiol. 49: 321-328.