MODIFICATION OF C AND N SOURCES FOR ENHANCED PRODUCTION OF CYCLOSPORIN ‘A’ BY ASPERGILLUS TERREUS

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ABSTRACT

Most of the studies regarding cyclosporin ‘A’ production through fungi concentrate around Tolypocladium inflatum. This is mainly due to lower reported production of this drug in other fungi. The present study was therefore conducted to explore indigenous isolates of Aspergillus terreus for synthesis of this drug and defining a production medium for obtaining high yield of cyclosporin ‘A’. For this purpose carbon and nitrogen sources were optimized for the selected best strain of A. terreus. Overall results depicted that the best cyclosporin ‘A’ yield from selected Aspergillus terreus (FCBP58) could be obtained by using production medium containing glucose 10% as carbon source and peptone 0.5% as nitrogen source. This modification in production medium enhanced drug synthesis by selected fungi significantly. The production capabilities when compared with biomass of fungi there was found no relationship between the two confirming that the medium modification increased overall drug synthesis powers of the fungi.

Key words: Aspergillus terreus, cyclosporin ‘A’, carbon, nitrogen.

INTRODUCTION

Secondary metabolites of filamentous fungi are of extreme interest to humankind due to their pharmaceutical and/or toxic properties (6). Cyclosporin ‘A’ is a main product of secondary metabolism of fungal species originally identified as strains of Trichoderma polysporum (12) but currently classified as belonging to the species Tolypocladium inflatum. Other soil inhabiting filamentous fungi like Fusarium solani (26) Neocosmospora vasinfecta (24) and Fusarium oxysporum (13) have also been reported to produce lower levels of cyclosporins. Recently, different strains of A. terreus have been identified as new producers of cyclosporine ‘A’ (27, 28).

Cyclosporin ‘A’ is a nonpolar cyclic peptide of eleven amino acids with a molecular weight of 1202.6. It exhibits a narrow spectrum of antifungal activity and in addition has immunosuppressant properties (12). This is a strong and selective immunosuppressant in transplant surgery inhibiting the rejection of allogeneic grafts and is also a very promising drug against autoimmune and parasitic diseases (8). In addition this is applied in reversing multidrug resistance in several types of cancers (11).

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The drug is produced by submerged culturing of aerobic filamentous fungi (17). Workers have also isolated the enzyme fraction from T. inflatum extracts (5, 18). Fermentation conditions (21) and nutrient medium have already been optimized for the production of cyclosporin ‘A’ by T. inflatum. Some workers obtained best production of the drug from T. inflatum by exploiting sorbose in their fermentation media (2, 15) while others found glucose and yeast extract to be best carbon and nitrogen sources (16). Investigation by Sallam et al. (28) for cyclosporin ‘A’ production by A. terreus revealed that glucose, bactopeptone, pH 5.3 incubated with 2% standard inoculum of 48 hour age, shaken at 200 rpm for 10 days are the best fermentation condition.

Cyclosporin ‘A’ production was significantly influenced by the addition of amino acids (16, 19). The external addition of L-leucine and L-valine during exponential growth phase highly increased the production of cyclosporin ‘A’ (1, 3). The productivity from T. inflatum was markedly increased by utilizing immobilized fungus (20, 22) in the presence of L-valine (9, 10). Sallam et al. (27) investigated the formation of cyclosporin ‘A’ by immobilizing the spores and mycelium of A. terreus and found L-valine to increase the production. The current study is conducted to detect high yielding strains of A. terreus among several local isolates and enhance the production powers of local isolate by altering carbon and nitrogen sources in nutritional medium.

MATERIALS AND METHODS

Procurement of fungal strains

Nine strains of A. terreus were obtained from the First Fungal Culture Bank of Pakistan (FCBP). The strains were preserved on Malt Extract Agar (MEA) at 4 °C and were provided in the form of slants in culture tubes. The strains were revived on MEA plates (malt extract 2 %, agar 2 %, pH 6.5) and the inoculated plates were incubated at 27±1 °C for 4-5 days. The strain numbers and their origins of isolation are mentioned in Table 1.

| Strain no. | Origin of isolation                |
|------------|-----------------------------------|
| FCBP58     | Soil, canal bank Lahore           |
| FCBP113    | Air mycoflora, NIAB               |
| FCBP119    | Air microflora, Punjab university Lahore |
| FCBP122    | Dalbergia sissoo root             |
| FCBP148    | Air microflora, Punjab university Lahore |
| FCBP168    | Air microflora, Punjab university Lahore |
| FCBP196    | Air microflora, Punjab university Lahore |
| FCBP536    | Air microflora, Punjab university Lahore |
| FCBP652    | Soil, Lahore                      |

Seed inoculum preparation

Seed inoculum was prepared in Malt Yeast extract (MY) medium (malt extract 2 %, yeast extract 0.4 %, initial pH of 5.7). A 0.8 cm disk of five days old strain culture was inoculated in sterilized medium and incubated on orbital shaker at 200 rpm for 72 hours at 30±2 °C (7).

Cultivation

According to the method of Agathos et al. (2) the flasks containing 50mL of production medium (glucose 5 %, peptone 1 %, KH₂PO₄ 0.5 %, KCl 0.25 %, pH 5.3 as designed by Sallam et al. (28) were inoculated with 5 mL of prepared seed inoculum (i.e. 10 % v/v). The inoculated flasks were incubated on orbital shaker at 200 rpm at 30±2 °C for 10 days.

Extraction of cyclosporin ‘A’

The extraction of cyclosporin ‘A’ was performed by using n-butyl in equal quantity in the medium and flasks were incubated at 200 rpm and 30±2 °C for 24 hours. Two distinguish immiscible layers of top organic phase and bottom aqueous phase were formed containing extraction solution and medium respectively. The organic phase was carefully separated by using separating funnel and evaporated under
vacuum till dryness at 40 °C. The dried sample was weighed and dissolved in 30 mL methanol.

**Biomass harvest**

The biomass was harvested by filtering aqueous layer of cultivation medium containing fungal pellets using Whatman filter paper No.1. The biomass was dried in oven overnight at 40 °C and was weighed.

**Analysis and Estimation of cyclosporin ‘A’**

**Antifungal bioassay:** The antifungal bioassay was performed against *Aspergillus niger* FCBP74 isolated from air mycoflora, Punjab University, Lahore by well method. The inhibition zone was measured by measuring diameter from eight different sides and taking the mean.

**High performance liquid chromatography (HPLC):**

High performance liquid chromatography (HPLC) was done for detection and estimation of cyclosporin ‘A’. Hitachi HPLC system equipped with UV-VIS detector (L-2420) and pump (L-2130) was used for detection and estimation of cyclosporine ‘A’ under the following operating conditions: Mobile phase consisted of acetonitrile: methanol: water (42.5: 20: 37.5), Flow rate of 0.8 mL/min, C18 column, wavelength of 215 nm. 20 µl of diluted samples and standard samples were injected in the HPLC system. The standards were 100 mg capsules of Sandimmun Neoral® (Novartis) and ≥ 98.5 % pure authentic sample of cyclosporin ‘A’ purchased from Fluka Analytical, Japan. The following formula was used to determine cyclosporin ‘A’ level in crude extracts (23).

\[
\% \text{ cyclosporin 'A' by weight} = \frac{A_s W_r V_r}{A_r W_s V_s} \times 100
\]

Where, As is peak area of sample, Ar is peak area of reference, Wr is weight of reference material in grams, Ws is weight of sample in grams, Vs is volume of sample, Vr is volume of reference material. The areas of sample peaks and of reference peak were calculated from the chromatograms obtained by HPLC program LaChrom Elite.

**Optimization of medium composition**

The selected *A. terreus* FCBP58 strain was grown on different medium to achieve an increased yield of cyclosporin ‘A’. Previously used cultivation medium containing glucose 5 %, peptone 1 %, KH₂PO₄ 0.5 %, KCl 0.25 % and initial pH of 5.3 as designed by Sallam *et al.* 2003 was altered by using different carbon and nitrogen sources.

Three sets of media were tested for production of cyclosporin ‘A’. In the first set, six carbon sources i.e. glycerol, glucose, maltose, fructose, sucrose and cellulose were used in four different concentrations of 1, 2, 5 and 10 % (w/v) in replacement of glucose 5 %. In the second set of production medium, Peptone, trypton and casamino acids in concentrations of 0.5, 1.0, 1.5 and 2.0 % (w/v) were used in place of peptone 1 %. Four different amino acids (asparagine, tyrosine, valine and leucine) in concentrations of 0.1, 0.2, 0.4 and 0.6 % (w/v) were supplemented to the medium in the third set.

A 5mL of prepared seed inoculum was inoculated in the prepared cultivate media of different sets and incubated on orbital shaker at 200 rpm and 30±2 °C for 10 days. The extraction, analysis and estimation of cyclosporin ‘A’ was performed by the same procedure as described above for the selection of best yielding strain.

**RESULTS AND DISCUSSION**

**Screening of fungal strains**

Present study was conducted to design a production media most conducive for cyclosporin ‘A’ production using *A. terreus* strain. In the first step nine isolates of the selected fungal species were checked for the drug production. The strains isolated from soil i.e., FCBP652, FCBP122 and FCBP58
produced maximum quantities of cyclosporin ‘A’. The highest production reached to 62.4 µg/mL by FCBP58 (Fig. 1) as shown in Table 2. This quantity was much lower than 105.5 mg/L recorded by Dreyfuss et al. (12) and Agathos et al. (2), and 183 mg/L recorded by Balakrishan and Pandey, (3) by most studied fungal strain T. inflatum. Sallam et al. (28) also recorded higher productivity of 86.57 mg/L by an isolate of A. terreus. The project was therefore planned to enhance the production potential of FCBP58 for increased drug production.

**Table 2. Cyclosporin ‘A’ production by nine different Aspergillus terreus strains.**

| Strains   | Peak area  | dry weight | % Cyclosporin | wt. of | wt. of |
|-----------|------------|------------|---------------|--------|--------|
| FCBP58   | 9,585,692  | 32         | 9.76          | 3.12   | *62.4 ± 2.89 |
| FCBP113  | 5,190,782  | 22         | 7.69          | 1.69   | 33.38 ± 1.16 |
| FCBP119  | 1,290,593  | 36         | 1.13          | 0.30   | 6.0 ± 1.85  |
| FCBP122  | 8,447,103  | 25         | 11.04         | 2.76   | 55.2 ± 2.6  |
| FCBP148  | 8,586,308  | 27         | 10.43         | 2.81   | 56.2 ± 1.85 |
| FCBP168  | 5,031,317  | 33         | 4.95          | 1.63   | 32.6 ± 1.5  |
| FCBP196  | 2,327,129  | 27         | 2.77          | 0.74   | 14.80 ± 0.75 |
| FCBP536  | 6,042,242  | 40         | 4.95          | 1.98   | 39.60 ± 2.08 |
| FCBP652  | 7,857,211  | 30         | 8.51          | 2.55   | 51 ± 1.74   |

**Confirmation of cyclosporin ‘A’**

Cyclosporin-related metabolites are reported to have a broad spectrum of antifungal activity and a narrow spectrum of activity against bacterial cultures (25). The harvested mixture assumed to have cyclosporins showed strong antifungal activity against A. niger when tested through well method. The inhibition zone of restricted growth of A. niger was 1.15 cm (Fig. 2).

When run through HPLC, a peak appeared between 2.7 and 2.8 min (Fig. 5), which was confirmed as cyclosporin ‘A’ when compared with Sandimmun Neoral® capsules containing 100 mg cyclosporin ‘A’ as active ingredient and pure cyclosporine ‘A’ as authentic drug supplied by Fluka analytical, Japan. Sandimmun Neoral® capsules showed a clear peak at 2.768 min (Fig. 3) whereas authentic compound recorded at 2.81 min (Fig. 4).
**Figure 3.** HPLC chromatogram of Sandimmun Neoral\textsuperscript{®} capsule (Novartis) 100mg cyclosporin as active ingredient extracted at 215nm. Peak at 2.7min is of cyclosporin ‘A’.

**Figure 4.** HPLC chromatogram of authentic cyclosporin ‘A’ purchased from Fluka analytical, Japan. The chromatogram extracted at 215nm is showing cyclosporin ‘A’ peak at 2.8min.
Effect of carbon source

Six different carbon sources were tried in this study to augment the drug production (Table 3). Highest tested concentration was 10% at which when glucose was added, it proved to be the most effective carbon source significantly increasing cyclosporin ‘A’ production up to 358.5 µg/mL (Fig. 5B). This shows that A. terreus can utilize this sugar most efficiently as primary source as it is a hexose monosaccharide and is a primary source of energy for most of the living organisms including fungi. Balakrishnan and Pandey (3) stated that by using T. inflatum the best yield of cyclosporin ‘A’ can be obtained on a medium consisting of 20 g/L glucose. In a contemporary study Balaraman and Mathew (4) obtained maximum cyclosporin ‘A’ production with medium containing 8% glucose. In a research conducted by Sallam et al. (28) on A. terreus, the medium containing 50 g/L glucose produced a maximum yield (86.77 mg/L) of cyclosporin ‘A’.

Beside glucose, cellulose also proved a fairly good carbon source in case of A. terreus, when used in concentration of 2%, it increased cyclosporin ‘A’ production from 62.4 µg/mL to 235 µg/mL (Table 3). Increase in concentration to 5% result in 143.6% increase in cyclosporin ‘A’ production. However it was lower in comparison to the treatment in which cellulose 2% was used. Although cellulose is a complex carbohydrate (polysaccharide), but A. terreus is known to produce extracellular endo-β-1, 4-glucanase, exo-β-1, 4-glucanase with high levels of β-glucosidase and has the ability to utilize cellulose as carbon source. Previous studies also show increase in fungal biomass with increase of carbon sources (29).

Effect of nitrogen sources

Peptone, trypton and casamino acids was used individually and then in combinations to check the productivity of cyclosporin ‘A’ (Table 4). These three compounds are amino acid mixtures. Peptone when checked at lower concentration of 0.5%, increased drug production spectacularly by 765.4% (Fig. 5C). Peptones are derived from animal milk or meat digested by proteolytic digestion and contains small peptides along with fats, metals, salts, vitamins and many other biological compounds. As peptone can provide vitamins and metals along with amino acids it proved best supplement for drug enhancement in comparison to others.
### Table 3. Effect of different carbon sources and their concentrations on the production of cyclosporin A by Aspergillus terreus FCBP58.

| Treatment | Peak area | Weight of dry biomass (g/50mL) | Weight of dry extract (mg/50mL) | % | mg/50mL | µg/mL | Increase/ decrease in production | % increase/ decrease in production |
|-----------|-----------|---------------------------------|---------------------------------|---|---------|-------|----------------------------------|-----------------------------------|
| Cellulose 1% | 666,767 | 0.66 | 53 | 0.41 | 0.22 | 4.40 ± 0.6 | -58 | -93.0 |
| Cellulose 2% | 35,868,578 | 0.97 | 50 | 3.82 | 2.7 | 4.40 ± 9.5 | -34 | -87.5 |
| Cellulose 5% | 23,291,007 | 2.07 | 64 | 1.32 | 1.5 | 4.40 ± 2.7 | -34 | -87.5 |
| Cellulose 10% | 1,197,291 | 8.17 | 14 | 2.7 | 0.39 | 7.8 ± 0.5 | -54 | -86.5 |

### Table 4. Effect of different nitrogen sources and their concentrations on the production of cyclosporin A by Aspergillus terreus FCBP58.

| Treatment | Peak area | Weight of dry biomass (g/50mL) | Weight of dry extract (mg/50mL) | % | mg/50mL | µg/mL | Increase/ decrease in production | % increase/ decrease in production |
|-----------|-----------|---------------------------------|---------------------------------|---|---------|-------|----------------------------------|-----------------------------------|
| Glucose 1% | 5,562,128 | 0.26 | 17 | 10.76 | 1.83 | 36.6 ± 1.2 | -25.9 | -63.9 |
| Glucose 2% | 3,047,128 | 0.45 | 42 | 2.4 | 1.008 | 20.16 ± 0.7 | -42.2 | -67.7 |
| Glucose 5% | 8,076,613 | 0.84 | 49 | 5.38 | 2.63 | 52.60 ± 2.0 | -9.8 | -15.0 |
| Glucose 10% | 54,475,949 | 1.18 | 49 | 36.59 | 17.9 | 358.50 ± 7.5 | 296.1 | 473.7 |

Note: All the data in table III & IV is compared with cyclosporin A produced by FCBP58 (62.4µg/mL) and given as means ± standard error.
Trypton also enhanced the drug production by 92% and 71.1% when used in 0.5 and 1% concentrations respectively. Although the percentage of enhancement was much lower to that of peptone, but this increase was observed at almost all the tested concentrations. Trypton is commonly used in microbiology to produce Lysogeny broth for the growth of microorganisms and provides a source of amino acids for the growing bacteria. Trypton is similar to casamino acids, both being digests of casein, but casamino acids can be produced by acid hydrolysis and typically only have free amino acids and few peptide chains. Casamino acids in concentration of 0.5% and 1.5% significantly increased cyclosporin ‘A’ production by 69.2% and 60.2% respectively when compared to the value recorded in unmodified production medium.

The fungal production of peptide and depsipeptide antibiotics may be directed and enhanced by amino acid components of the antibiotic molecule. Kobel and Traber (17) reported the direct synthesis of cyclosporin ‘A’ and several analogues in fermentations where the composition and titre of each analogue produced were strongly determined by the kind of externally supplemented amino acid. Accordingly, four amino acids asparagine, leucine, tyrosine and valine were tested for their drug enhancing effect. Valine showed enhancing effect on cyclosporin ‘A’ production, when used in 0.1 and 0.4% concentration (Fig. 5D). Similar results were obtained by Balakrishan and Pandey (3) when they found L-leucine and L-valine as strong inducers of cyclosporin ‘A’. They also noticed that D-valine had no stimulatory effect on drug production. Also the presence of amino acids in the exponential growth phase ensured optimal production, as was indicated in an experiment, in which L-valine was added at different times.

However, in agreement with these workers, an increase in total cyclosporine production was seen, but of considerably higher magnitude. L-valine may have a role of inducer to increase the transcription of genes for cyclosporin ‘A’ synthetase or other structural genes contributing to cyclosporin ‘A’ synthesis in our fungal strain, given the positive effect of the amino acid when added early in the fermentation. Some amino acids may act as inducers which must be added in exponential growth phase to manifest their ability to enhance secondary metabolite production. It is also possible that these amino acids may direct cell development in a manner favouring secondary metabolite production by affecting transcription of secondary metabolite genes during vegetative cell growth (14).

The results showed that the modification of production medium by increasing glucose concentration to 10% as carbon source and addition of lower peptone concentration of 0.5% as nitrogen source along with valine (0.1%) supplementation can significantly increase cyclosporin ‘A’ production.

**Drug production versus fungal biomass**

When increase in fungal biomass produced by selected strain in various modified media was compared to the increment of cyclosporin ‘A’ the drug production was not found directly associated with increase in biomass (Fig. 6-8). Similar results have been reported by earlier workers. Sallam et al. (28) reported that biomass yield and hence the volumetric production of cyclosporin ‘A’ increased linearly when they changed initial pH of the medium from 3.3 to 5.3, however further increase in pH increased biomass of A. terreus but considerably decreased drug production. An exception was observed in case of glucose, when it supplied as a sole carbon source its increase in concentration increased biomass of A. terreus and so the drug production.

**Drug production versus extract colour**

The colour of the final extract containing cyclosporins showed variation from light yellow to dark reddish orange. However, the darkness of colour also did not seem to correspond with any increment in amount of cyclosporin ‘A’. In case of glucose, increase in sugar concentration in production medium increased drug production and also the colour intensity. It is supposed that the final extract is a combination of various cyclosporins and may posses some other compounds. The quantity of these compounds also varies with variation in the treatments. This can affect the colour of final extract that do not directly relates to the increase in cyclosporin ‘A’ production.
Figure 6. Effect of different carbon sources and their concentrations on production of cyclosporin ‘A’ and fungal biomass in production medium (values are mean ± SE of 3 observations).

Figure 7. Effect of different nitrogen sources and their concentrations on production of cyclosporin ‘A’ and fungal biomass in production medium (values are mean ± SE of 3 observations).

Figure 8. Effect of different amino acids and their concentrations on production of cyclosporin ‘A’ and fungal biomass in production medium (values are mean ± SE of 3 observations).
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