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Comparative Effect of Amino Acids in the Production of Cyclosporin by Solid and Submerged Fermentations

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Abstract: Cyclosporin aids in the differentiation and proliferation of T-cytotoxic lymphocytes that are the main cause of the rejection of organ transplantations. Fungi belonging to the Class Deuteromycetes, *Tolypocladium inflatum* (ATCC 34921) in submerged fermentations, accomplish the production of cyclosporin. In this study, a comparison was made in the yields of cyclosporin in both the solid and submerged fermentations (SmF) with and without the addition of amino acids. A 40% increased yield in cyclosporin was obtained with SSF when compared to the SmF without the addition of amino acids. Though the incubation periods were higher in SSF, an increased yield was achieved with the addition of L-valine (221.6 mg kg^{-1}), L-leucine (206.4 mg kg^{-1}) and L-α-Aminobutyric acid (204.9 mg kg^{-1}).

Key words: Cyclosporin, *Tolypocladium inflatum*, solid state fermentation, submerged fermentation, amino acids-valine, leucine, aminobutyric acid

INTRODUCTION

Cyclosporin is a member of a group of cyclic undecapetides with anti-inflammatory, immunosuppressive, antifungal and antiparasitic properties (Sallam *et al*., 2005). In addition to the immunosuppressive property, cyclosporin has additional medicinal applications such as role in reversing multidrug resistance in several types of cancers (Derks and Madris, 2001). Cyclosporin is the strongest immunosuppressant discovered so far; it also overcame many of the risk factors associated with azathioprine and is relatively non-toxic to bone marrow (Upton, 2001; Borel, 2002; Gharavi *et al*., 2004).

Cyclosporins are generally produced by *Tolypocladium inflatum* in submerged fermentation (SmF) process and are reported to have low yield (Robinson *et al*., 2001). To increase the product yield different approaches were made. One such attempt was the addition of amino acid in the fermenting medium. Addition of L-valine has been previously shown to have promising effects on the formation of cyclosporin in SSF and SSF (Lee and Agathos, 1989; Sekar and Balaraman, 1996; Nisha *et al*., 2005). In addition to L-valine, other forms of valine namely, D-valine, DL-valine, Norvaline and Provaline and other essential amino acids like L-leucine, L-methionine, L-threonine, L-tyrosine, L-isoleucine, L-phenylalanine, L-lysine and L-α-aminobutyric acid were added to both the processes to obtain an enhanced yield of cyclosporin. While a number of reports on SmF are available, very little is known in Solid State Fermentation (SSF) on the production of cyclosporin. In the present investigation, a comparative study on the production of cyclosporin in SmF and SSF were made with and without addition of amino acids.

MATERIALS AND METHODS

Tolypocladium inflatum ATCC 34921 was obtained from American Type Culture Collection, Rockville Md, USA. The lyophilized culture was revived on Sabourad Dextrose Agar (SDA). The culture upon revival was maintained on Malt Yeast Agar (MY) slants and was used. Conidial suspension was produced on Malt Yeast Agar (MYA) slants as described by Lee and Agathos (1989). Authentic cyclosporin standards (99% purity) were obtained from M/s Sandoz, Hanover, NJ for comparison and evaluation. All the chemicals and biochemicals used in this investigation were of analytical grade purchased from M/s Aldrich Chemicals.

Submerged fermentation (SmF): Shake flask fermentations were carried out on MY broth as described by Agathos and Lee (1993). The flasks were inoculated with the conidial suspension (10^8 mL^{-1}) and were incubated on a rotary shaker (250 rpm) at 26°C for 2 weeks.

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Solid-State Fermentation (SSF): SSF was carried out as per standard protocols (Tengerdy, 1985; Pandey, 1991). Conidial suspensions (10^8 cm^{-2}) were inoculated to the SSF flasks and were incubated in slanting position. The flasks were incubated up to six weeks with periodical mixing (every 24 h) and samples were withdrawn every week. The initial moisture content of the SSF flasks was set at 40%.

Addition of amino acids: Amino acids were incorporated initially into both the media containing SmF and SSF flasks. Amino acids used in this study were L-valine, D-valine, DL-valine, Norvaline, Provaline, L-leucine, L-methionine, L-threonine, L-tyrosine, L-isoleucine, L-phenylalanine, L-lysine and L-α-aminobutyric acid. The concentration of amino acid used was 0.4% of the substrate (w/w).

Analytical methods: pH, sugar and biomass determinations were carried out as per the standard method (Tengerdy, 1985; Pandey, 1991). Extraction and detection of cyclosporin were carried out as described by Lee and Agathos (1991). Cyclosporin in the fermentation medium were analyzed (Agathos *et al*., 1987) by HPLC equipped with C8 column at 60°C with acetonitrile water (70:30) containing 0.01% orthophosphoric acid as mobile phase with a UV detector at 210 nm to confirm the detection of cyclosporin in the fermentation medium using authentic cyclosporin as standard.

RESULTS AND DISCUSSION

Production of cyclosporin in SmF: Sugar concentration and the pH of the medium continued to decline with the increase in the incubation period. However, the biomass concentration and cyclosporin production continued to increase (Table 1). The final cyclosporin titer in SmF was observed to be 128.6 mg L^{-1} after the completion of two weeks of incubation.

Production of cyclosporin in SSF: An appreciable level of cyclosporin production was observed after two weeks of incubation and the maximum production (176.3 mg kg^{-1} of Cyclosporin) was noticed at the fourth week of incubation. However, the yield declined beyond four weeks of incubation. The cyclosporin yield in SSF when compared to the SmF was found to be higher by 40% (Fig. 1).

SSF has already been successfully used for the production of enzymes and secondary metabolites (Pandey, 1991; Robinson *et al*., 2001). These products are associated with the stationary phase of microbial growth and are produced on an industrial scale for use in agriculture and the treatment of diseases (Robinson *et al*., 2001; Hoppert *et al*., 2001). Many of these secondary metabolites are still produced by Submerged fermentations (SmF) even though production by this method has been shown to be less efficient as far as the yield is concerned compared to the SSF (Agathos and Lee, 1993). SSF process had shown to produce more stable product, requiring less energy in small fermenters, with easier downstream processing measurement. The results we have obtained indicate that fungi *Tolypocladium inflatum* growing under SSF conditions are more capable of further scale up of the process.

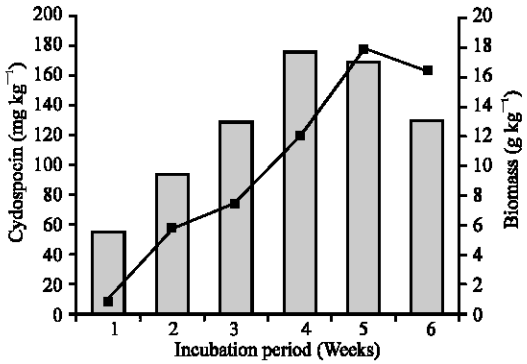

Fig. 1: Cyclosporin and biomass production at different incubation periods in Solid State Fermentation (SSF) using *Tolypocladium inflatum* (ATCC 34921)

Table 1: Cyclosporin production and various fermentation parameters analyzed in submerged fermentation (SmF) at different incubation periods by *Tolypocladium inflatum* (ATCC 34921)

Incubation period (days)	pH	Biomass (g L^{-1})	Sugar (%)	Cyclosporin (mg L^{-1})
0	5.3	Nil	5.42	Nil
3	5.1	2.17	3.96	Nil
5	4.9	3.06	3.08	29.8
7	4.2	5.94	2.63	66.4
9	4.0	6.81	2.11	97.3
12	4.1	6.49	1.94	110.1
14	4.0	6.54	0.99	128.6

SD ±38.94: SE ±0.55 cyclosporin yields, The values are average of three replicates

Effect of addition of amino acid on cyclosporin production: Cyclosporin, the main product consisting of 11 amino acids (Fig. 2) is critically affected by the addition of exogenous amino acids, which are members of the cyclosporin ring (Lee and Agathos, 1991; Agathos and Lee, 1993). The supplemented amino acids are modifying the endogenous amino acid pool of the producer fungus *Tolypocladium inflatum* and directing the biosynthesis of cyclosporin as precursors (Hoppert *et al*., 2001).

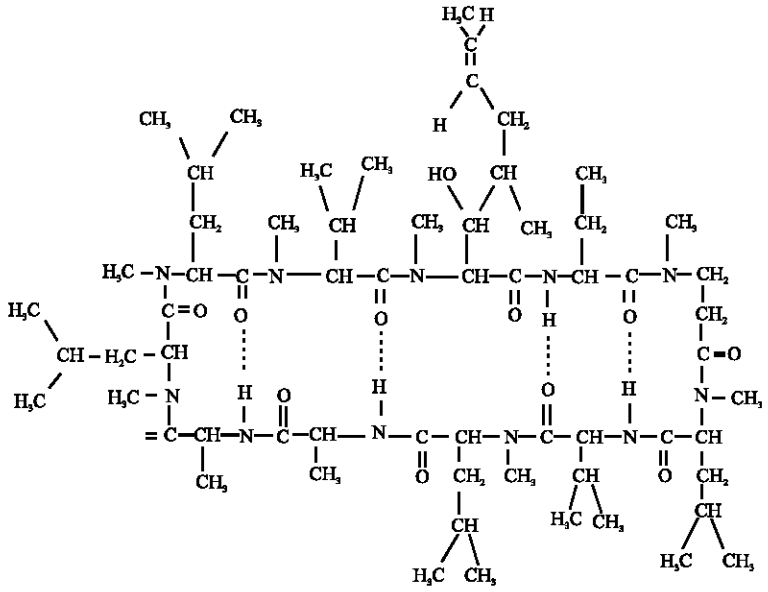

Fig. 2: Chemical structure of cyclosporin comprising of 11 amino acids

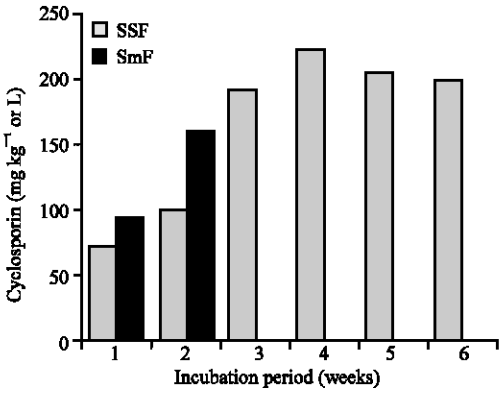

Fig. 3: Comparative cyclosporin production in Submerged (SmF) and Solid State Fermentations (SSF) using *Tolypocladium inflatum* (ATCC 34921)

Table 2: A comparative effect of amino acid addition in both Submerged (SmF) and Solid State Fermentations (SSF) for the production of cyclosporin using *Tolypocladium inflatum* (ATCC 34921)

	SmF		SSF	
Amino acids	Biomass (g L^{-1})	Cyclosporin (mg L^{-1})	Biomass (g kg^{-1})	Cyclosporin (mg kg^{-1})
L-valine	12.9	159.3	20.3	221.6
D-valine	10.8	131.8	18.2	148.4
DL-valine	7.8	102.3	18.9	110.2
Norvaline	6.8	96.4	16.6	77.2
Provaline	9.1	104.8	17.3	110.3
L-leucine	9.4	149.1	20.6	206.4
L-methionine	8.6	99.1	17.2	108.7
L-threonine	6.9	84.9	11.6	94.0
L-tyrosine	6.7	74.8	14.3	87.2
L-isoleucine	5.0	66.4	11.0	67.6
L-penylalanine	7.0	91.2	19.1	111.6
L-lysine	7.2	90.3	19.7	189.5
L-α ABA*	7.8	138.6	16.8	204.9

*: L-α ABA-Aminobutyric acid SD (n = 3); Cyclosporin titers were determined at 14 days for SmF and 28 days for SSF

Enhancement of cyclosporin production by exogenous supply of amino acids is known in SmF (Lee and Agathos, 1989). The amino acids used in this study are the constituents of the cyclosporin ring and we have observed that the exogenous supply of amino acids significantly increases the production of cyclosporin. The effect of different amino acids on the production of cyclosporin and cell biomass concentration in both SmF and SSF process are shown in Table 2. L-valine, L-leucine and L-α-aminobutyric acid had a significant effect on cyclosporin production in both the processes showing a rise of 26, 17 and 16%, respectively in SSF whereas, there is no remarkable enhancement in the production of cyclosporin upon the addition of other amino acids tested. Present result complies with the results of Sekar and Balaraman (1996) in the production of cyclosporin in SSF.

The constituent amino acids of cyclosporin ring, L-valine, L-leucine and L-α-aminobutyric acid (Fig. 2) has also been found to enhance the production. This could be attributed to the direct link between the cyclosporin productivity and the magnitude of the intracellular pool of amino acids (Lee and Agathos, 1991). The results obtained on a comparative account prove that SSF cyclosporin yields were higher than that of SmF yields (Fig. 3). The results also indicate that the periods of incubation were shorter in SmF when compared to SSF. However, SSF yields were appreciable (by 40%)

considering the SmF cyclosporin yields. The cyclosporin production obtained in SmF is in agreement with the results reported by Nisha *et al.* (2005).

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