Investigations into the Microbial Production of Cyclosporin A

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in

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by

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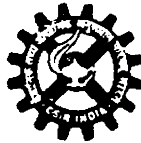
to

reasonable probabilities,
fantastic possibilities
&
the distinctions between them.

Dr. Ashok Pandey
Project Leader



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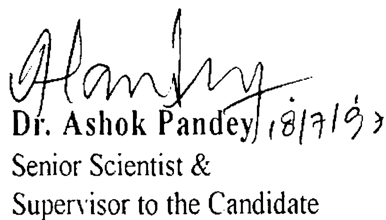
CERTIFICATE

This is to Certify that this thesis entitled **Investigations into the Microbial Production of Cyclosporin A** incorporates results of original research work done on the subject by **Shri. Balakrishnan. K.** Regional Research Laboratory. Trivandrum during the study period and that the thesis has not formed the basis for the award of any Degree. Diploma. Associateship. Fellowship or other similar title.

The thesis represents the original work carried out by the candidate under my guidance.



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STATEMENT

I hereby declare that the matter embodied in this thesis entitled **Investigations into the microbial production of Cyclosporin A.** is the result of investigations carried out by me in the Biotechnology unit of Regional Research Laboratory (CSIR). Trivandrum under the supervision of Dr. Ashok Pandey. Scientist. RRL. Trivandrum. In keeping with the general practice of reporting scientific observations. due acknowledgments have been made wherever the work described is based on the findings of other investigators.

Balakrishnan. K

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As I reach the end of the crescendo among many nosey parkers, infallible & otherwise I should be grateful to the uncommon commoner, a visage of the vintage. As the connoisseur too favoured no volte-face, I quote Lord Byron

> "To mingle with the universe and feel....
> .....what I can ne'er express, yet cannot conceal".

Balakrishnan. K

CONTENTS

A. **INTRODUCTORY REMARKS**	1
A.1. General Introduction and Objectives	1-3
A.2. Review of Literature	4-27
B. **EXPERIMENTAL DETAILS**	28
B.1. Isolation, Identification and Characterization of a Microbial Strain Producing Bioactive Metabolite.	28-37
B.2. Iron Requirements and Siderophore Search in the Producing Organism.	38-42
B.3. Submerged Fermentation of *T. inflatum* for the production of Cyclosporin A	43-48
B.4. Solid State Fermentation of *T. inflatum* for the Production of Cyclosporin A	49-59
B.5. Cyclosporin A Production in Specially Treated Solid Substrates	60-64
B.6. Immobilization Studies	65
B.6.1. Studies on the Special Properties of Gel Beads Used for the Fungal Immobilization	65-70
B.6.2. Cyclosporin A Production by Immobilized *T. inflatum*	71-78
B.7. Yield Improvement Trials and Selection of Morphological Varients	79-83
B.8 . Purification Studies	84
B.8.1 Partial Purification of the Enzymes Involved in the Biosynthesis of Cyclosporin A	84-90
B.8.2. Purification of Cyclosporin A from the Fermentation Broth	91-94
C. **SUMMARY AND CONCLUSIONS**	95-98
D. **BIBLIOGRAPHY**	99-106

A: INTRODUCTORY REMARKS

A.1: General Introduction

Rummaging of the literature reveals the scuba diving of modern biotechnology industry from laboratory curiosity to multibillion commercial activity fortifying scores of biotechnology derived products and recipes that are either already on the market or in various stages of development and commercialization. While the thrust is on high value/low volume therapeutics, biotechnology is all set to ushering in a new era in the history of man kind making a far reaching impact through its uncanny ability that is widely recognized (Nisbet and Porter 1989; Omura 1992; Yarborough *et al.* 1993).

The practice of fermentation attains imperial status when it comes to the production of molecules which are too complex to be manufactured by conventional chemical processes. While fermentation industry embarks on its second century of operation, production of a masquerade of speciality compounds having novel implications and greater activities on therapeutic sector becomes the glitter and glint (Omura 1992)

In recent times, the product profile by fermentations is altered so enormously that a need arises to categorize them into commodity chemicals and speciality chemicals. The break-through in the use of microbial secondary metabolites for better health care resulted in certain supreme compounds exhibiting enthralling functions viz. inhibitors of wide range of crucial enzymes, anticholestrenemics, immunomodulators, vasodialators, anticancers, etc.

This work envisaged many vital aspects of process optimization concerning different routes of fermentations of a bioactive microbial metabolite employing a locally isolated microbial strain with special emphasis on characterization of the producing organism, media design, biochemical principles of product biosynthesis, bioreactor development, yield improvement strategies and product purification protocals. Since the locally isolated fungal strain turned out to be *Tolypocladium inflatum* B-58, the potential producer of cyclosporin A(CsA), efforts were directed towards the development of a bioprocess for the production of this new generation immunosuppressive compound.

Various attempts to elicit the immune response as a means to induce resistance to infectious diseases and the knowledge on the involvement of the defective regulations of body's

natural defence mechanisms on a significant number of illnesses heralded the beginning of a new approach in medical immunology-modulating of the immune response (Stahelin 1986). Such a mechanism was of utmost importance in all corrective surgeries involving organ transplants and tissue grafts. In order to circumvent the problems of rejection, the major hurdle in all organ transplantation trials, it was imperative to treat the recipient with the immonosuppressive drugs. Thus the search for the immunosuppressive drugs attained significance and immunosuppression went on to become one of the cynosure disciplines of medical immunology.

Till 1980, there was a dearth of patient-friendly immunosuppressant exhibiting specific site of action. Most of them inhibited the proliferation of immunocompetent cells also by blocking cellular divisions nonspecifically (Beveridge 1986).

CsA was the first of the new generation immunosuppresant with a pharmacological specificity and without the undesirable cytotoxicity (Borel 1981a). This new generation immunosuppressant was found to be specifically acting at an early stage of T lymphocyte activation without damaging the phagocytic activity and migratory capacity of the reticuloendothelial system (Paavoven *et al.* 1981; Mc Intosh and Thomson 1980). Since this compound worked meticulously towards the gradual acceptance of the graft by the receiver in many transplant surgeries, several thousands of patients were benefited in diverse transplant centres world wide.

CsA and its minor analogues represent a group of cyclic oligopeptides produced as secondary metabolites by the fungi *T.inflatum* and *Cylindrocarpen lucidium* (Dreyfuss *et al.* 1976). Only scant information is available in the public domain on the bioprocess aspects of CsA production. Though it has been successfully utilised for over decade in preventing allograft rejection or in achieving prolonged graft survival, it is not being manufactured by any firm in India. The drug has already produced superior results in kidney, liver, heart and lung transplantations and in skin grafting. It is employed even for the prevention of "graft vs host reactions" (GVHR) occuring in bone marrow transplantations. Besides it exhibits antiparasitic, antiinflammatory and antifungal properties. But details on the production process is scarse and at present the entire requirement of this drug in India is met through import.

Hence, an attempt was made to produce CsA through various routes viz. submerged fermentation (SmF), solid state fermentation (SSF), immobilization, etc. employing the indigenously isolated strain of *T.inflatum* B-58.

Objectives of the present study include:

1. to isolate and characterize a microbial culture producing bioactive compound.

2. to optimise the growth and activity of the culture including the detection of the compound of interest.

3. to evaluate the alternative routes of fermentation viz. SSF, Immobilization, etc.

4. to study the yield improvement trials and the subsequent selection of the mutants of interest and the role of precursors in enhanced product biosynthesis.

5. to study the purification strategies concerning the product and the enzymes of the biosynthetic machinery and their subsequent characterization

A.2 : Review of Literature

A.2.1 : ***Prelude:*** Contemporary antibiotic research is characeterised by advanced screening procedures aiming specifically not only at antibacterials or antifungals but at speciality compounds like enzyme inhibitors, antitumor drugs, immunomodulators etc. New sources of rare microorganisms are currently under exploitation. Development of microbial products with new medicinal activities that were not even thought of earlier but have greater rammifactions in terms of patient care has become a much sought after resaerch arena spear headed by the success story of small molecular weight immunomodulators. Long accepted as the best way to prevent or treat rejection in patients undergoing organ transplantation, immunosuppressive therapy has been attracting increased attention. Among the various compounds exhibiting a discriminatory action on the activation of helper lymphocytes sans any undesirable side effects associated with many conventional immunosuppressants.

Cyclosporin, by demonstrating for the first time the feasibility of drugs to effect an immunopharmacologic approach to the modulation of the immune response became the prototype of a new generation of immunosuppressive agents earmarking a decisive step forward in transplantation therapy. This enabled CsA, the main component of the family of natural cyclosporins, to become the drug of choice in majority of transplantation centres all over the globe.

There has also been a considerable interest in the prospective value of CsA in antiinflammatory, antiparasitic and antifungal activities. Basketful of accolades were poured on its utility as a tool to study the mechanism of action of the immune system and many such conundrums of basic research. It is also noteworthy to have a look at the impact that this particular drug is having in the field of immunopharmacology to have an idea of the far reaching impact, the more smart immunosuppressants of the future will hopefully have.

A.2.2 ***Discovery:*** Scientists at Sandoz Laboratory, Switzerland discovered cyclosporins in 1970 (Borel 1982). While attempting to identify new antifungal agents, crude extracts from two strains of fungi imperfecti *C.lucidium* BOOTH and *T.inflatum* GAMS demonstrated a narrow spectrum of activity *in vitro* mainly against clinically irrelevant organisms. However, this antifungal activity was accompanied by a very low degree of toxicity which lead to the revaluation of the latent activity of the compound. Immunosuppressive abilities revealed in pharmacological

screenings conducted later stressed the point that microbial metabolites exhibit versatile bioactivities and it is high time to effectively utilize more specific compound based assays to unleash an array of compounds with special bioactivities.

In 1973, cyclosporin was purified from the fungal extract of *T.inflatum* and in 1975 structural analysis was established (Wenger 1982). Synthetic CsA having identical biological spectrum of activity as that of fungal CsA became a reality in 1980 (Wenger 1984).

Immunosuppressants available till 1978, viz. cyclophosphoride, methotrexate, azothioprine and corticosteroids were found to be lacking pharmacological specificity as indicated by their general cytotoxicity. They were found blocking cellular division nonspecifically and thereby inhibiting the proliferation of the immunocompetent cells also (Beveridge 1986). Early studies of Borel and coworkers (Borel *et al.* 1976; 1977) established the selective immunosuppressive role of CsA acting only on definite subpopulation of immunocompetent cells. It was found to be free of myelotoxicity and was not impairing the proliferation of hemopoietic stem cells (Borel 1981a; von Wartburg and Traber 1986). Its use in transplantation surgery became attractive by dint of it not damaging the phagocytic activity and migratory capacity of the reticuloendothelial system (Mc Intosh and Thomson 1980). CsA was first used clinically in 1978 (Powels *et al.* 1978; Clane *et al.* 1978) and it soon became an imperative component in all transplantion protocals.

A.2.3 *Cyclosporin family :* Cyclosporin of molecular formula $C_{62}H_{111}N_{11}O_{12}$ represent a class of neutral hydrophobic cyclic undecapeptides differ in their amino acid composition. Table A.1 shows the list of the natural cyclosporins (Traber *et al.* 1977a; 1977b; 1982).

The unique aminoacid at position 1 was previously unknown. It was found to have the structure (4R)-4-(KE)-2-butenyl-4, N-dimethyl-L-threonine and abbreviated as MeBmt Synthesis of MeBmt as an intermediate in cyclosporin synthesis was reported (Wenger 1985; Ewans and Weber 1986) All chiral amino acids in cyclosporin belong to the L-series with the exception of D-alanine. Of the 11 amino acids seven are N-methylated and hence contributed a lipophilic character to the molecule.

A.2.4 *Structure of CsA :* CsA having a molecular weight of 1202 is the major product of all the natural cyclosporins (Traber *et al.* 1987) Except the first amino acid all the other 10 are previously known aliphatic amino acids. All have the S-configuration of the natural L-amino acids except for the D-alanine in position 8.

Metabolite	amino acid composition 1	2	3	4	5	6	7	8	9	10	11	Molecular Formula
CyA	C_9	Abu	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal	$C_{62}H_{111}N_{11}O_{12}$
CyB	C_9	Ala	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal	$C_{61}H_{109}N_{11}O_{12}$
CyC	C_9	Thr	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal	$C_{62}H_{111}N_{11}O_{13}$
CyD	C_9	Val	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal	$C_{63}H_{113}N_{11}O_{12}$
CyE	C_9	Abu	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	Val	$C_{61}H_{109}N_{11}O_{12}$
CyF	desoxy-C_9	Abu	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal	$C_{62}H_{111}N_{11}O_{11}$
CyG	C_9	Nva	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal	$C_{63}H_{113}N_{11}O_{12}$
CyH	C_9	Abu	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	D-MeVal	$C_{62}H_{111}N_{11}O_{12}$
CyI	C_9	Val	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	Leu	MeVal	$C_{62}H_{111}N_{11}O_{12}$
CyK	desoxy-C_9	Val	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal	$C_{63}H_{113}N_{11}O_{11}$
CyL	N-desmethyl-C_9	Abu	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal	$C_{61}H_{109}N_{11}O_{12}$
CyM	C_9	Nva	Sar	MeLeu	Nva	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal	$C_{63}H_{113}N_{11}O_{12}$
CyN	C_9	Nva	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	Leu	MeVal	$C_{62}H_{111}N_{11}O_{12}$
CyO	MeLeu	Nva	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal	$C_{60}H_{108}N_{11}O_{11}$
CyP	N-desmethyl-C_9	Thr	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal	$C_{61}H_{109}N_{11}O_{13}$
CyQ	C_9	Abu	Sar	Val	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal	$C_{60}H_{107}N_{11}O_{12}$
CyR	C_9	Abu	Sar	MeLeu	Val	Leu(?)	Ala	D-Ala	MeLeu	Leu(?)	MeVal	$C_{60}H_{109}N_{11}O_{12}$
CyS	C_9	Thr	Sar	Val	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal	$C_{60}H_{107}N_{11}O_{13}$
CyT	C_9	Abu	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	Leu	MeVal	$C_{61}H_{109}N_{11}O_{12}$
CyU	C_9	Abu	Sar	MeLeu	Val	Leu	Ala	D-Ala	MeLeu	MeLeu	MeVal	$C_{61}H_{109}N_{11}O_{12}$
CyV	C_9	Abu	Sar	MeLeu	Val	MeLeu	Abu	D-Ala	MeLeu	MeLeu	MeVal	$C_{63}H_{113}N_{11}O_{12}$
CyW	C_9	Thr	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	Val	$C_{61}H_{109}N_{11}O_{13}$
CyX	C_9	Nvs	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	Leu	MeLeu	MeVal	$C_{62}H_{113}N_{11}O_{12}$
CyY	C_9	Nva	Sar	MeLeu	Val	Leu	Ala	D-Ala	MeLeu	MeLeu	MeVal	$C_{62}H_{111}N_{11}O_{12}$
CyZ	MeAminooctanoic	Abu	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal	$C_{61}H_{111}N_{11}O_{11}$

Table A.1. Amino Acid Composition of CsA to Z

C9 = McBmt

Cy = Cyclosporin

Structure of CsA (Fig. A.1) was well established by chemical degradation and X-ray crystallographic analysis (Ruegger *et al.* 1976). Amino acid residues at position 1-6 of the backbone adopt antiparallel β-pleated sheet conformation. Only 4 amide (NH) groups are available for H-bond formation since the remaining 7-N atoms are methylated. These H-bonds contributed significantly to the rigidity of the skeleton

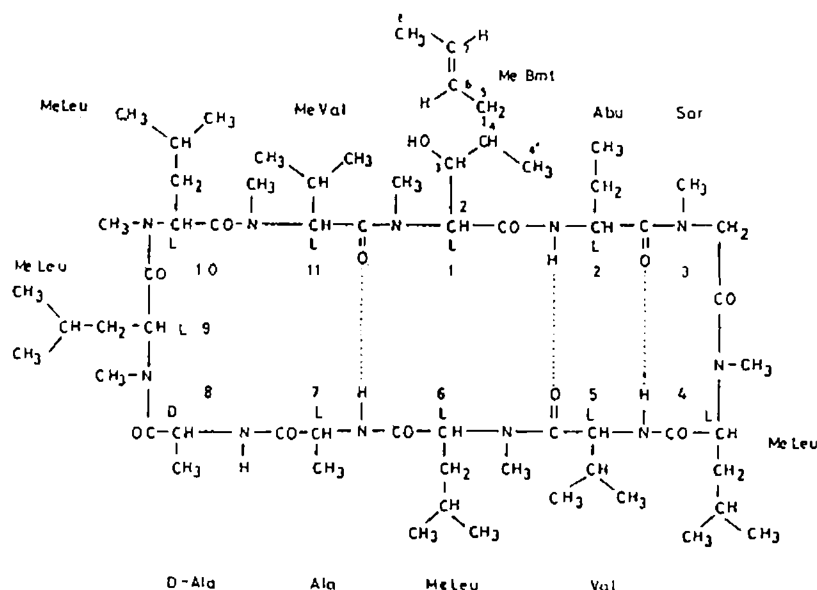


Fig. A.1 : Structural Formula of CsA

A.2.5 ***New analogues of cyclosporins :*** The cource of cyclosporin biosysnthesis is reported to be strongly influence an exogenous addition of amino acid precursor (Traber *et al.* 1989; Lee and Agathos 1989). The specific incorporation achieved by the addition of Dl-α-ally glycine to the medium resulted in the production of (ally gly 2) CsA. Exogenous supply of L-β-cyclohexylalanine led to the production of (Me cyclohexyl ala) CsA . The D-alanine in position 8 when substituted by D-serine new (D-ser 8) analogs of CsA. C, D and G as well as (Ally gly 2) CsA with high immunosuppressive property were produced (Traber *et al.* 1989) Lawen *et al.* (1994) recently reported the biosynthesis of ring extend cyclosporins. Due to the introduction of β-alanine into position 7 or 8 of the ring instead of the α-alanines, the 33 membered ring of the cyclo undecapeptide becomes a 34 membered ring of CsA. Both β ala (7)) CsA and (β ala (8)) CsA showed impressive immunosuppressive activity.

A.2.6 ***Biological spectrum of activity :*** Cyclosporins exhibit a narrow spectrum of antibiotic activity. Strains of some mucorales, ascomycetes and fungi imperfecti like *Curvularia lunata, Neurospora crassa,* etc show differing sensitivity. In sensitive yeasts it leads to simple growth rate reduction and in other cases inhibition becomes frequently evident as deformation and branching of the growing hyphal tips (Dreyfuss *et al.* 1976).

In spite of their low scope as an antifungal antibiotic, they possess immunosuppressive and antiphalogistic actions (Borel *et al.* 1976), which make them as potential candidates for transplantation therapy. The organs successfully grafted under cyclosporin treatment include nerve (Gulati and Zalewski 1982), muscle cells (Walt *et al.* 1981) lung (Beveridge 1983), small bowel (Craddock *et al.* 1983), islet cells (Reece *et al.* 1982) cornea (Hunter *et al.*1976), skin (Balaraman *et al.* 1991), heart (Reitz and Stinson 1982) and liver (Starzl *et al.* 1982). CsA has become established as the agent of choice in the control of human kidney and heart allograft rejection and has contributed significantly to the improved outcome of human kidney, heart and pancreas transplantations (Morris 1981). The very low myelotoxicity associated with these compounds makes them the better candidates than many conventional drugs (Britton and Palacios 1982).

Antiparasitic effect of CsA was noticed against murine schistosomiasis (Bueding *et al.* 1981) malaria (Thommen 1981), toxoplasmosis (Mack and Mcleod 1984) and filariasis (Bout *et al.*1984). Cyclosporins were also employed successfully either preventively or therapeutically against many diseases related to autoimmune disorder (Williams 1993). Weiser and Matha (1988) studied the insecticidal activity of cyclosporin on mosquito larvae. Cyclosporins were used in many hair tonic preparations along with vitamin E or their organic esters.

The role of cyclosporin in cancer chemotherapy has been viewed with caution and interest. Though cyclosporin could potentiate the effect of some cytostatic drugs in both tumour and normal cells, any form of immunosuppression of sufficient duration and intensity could lead to the development of certain forms of cancer (Penn 1982; 1986).

Since cyclosporin exhibits a large spectrum of pharmacological activities (see Fig. A.2) such as supression of antibody and cell-mediated response, inhibition of chronic inflammatory reactions, a number of antiparasitic actions and reversal of multidrug resistance, the search for a powerful derivative exhibiting only one of these effects and largely without the others has relentlessly been pursued.

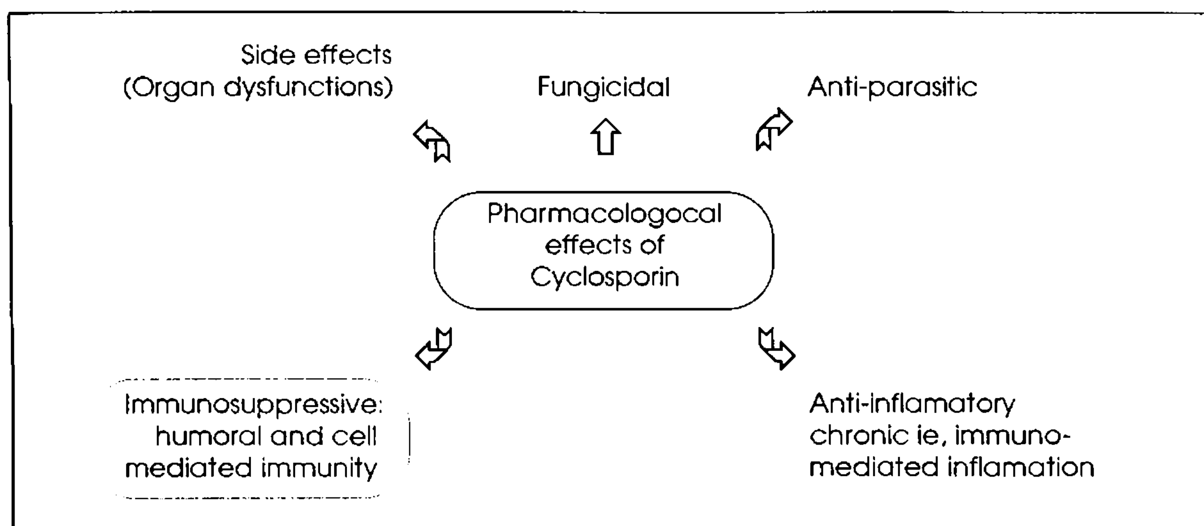


Fig. A.2. Pharmacological Actions of Cyclosporin

A.2.7 ***Structure-activity relationships :*** Cyclosporins (A to Z) have been tested for antifungal activity as well as in many *in vitro* and *in vivo* assays for immunosuppressive activity (Borel *et al.* 1976; 1977; Wiesinger and Borel 1979).

A review of the immunopharamacological data indicates the necessity of intact structure of amino acid 1 (MeBmt) for the high immunosuppressive effects. Replacement of these units by N-methyl-L-leucine (CsO) or N-methyl-2-amino octanoic acid (CsZ) together with the absence of hydroxyl function (CsF & K) caused a relative loss of activity. Also, the olefinic double bond in MeBmt is reported to be of importance since dihydro derivatives are generally less active in producing immunosuppressive effects. Though MeBmt is intimately involved in the biological activity, a larger portion of the cyclosporin structure involving the amino acid 1, 2, 3, 9, 10 and 11 which are clustered on the surface of the molecule was proved to be playing the lead role in controlling the biological activity of the molecule.

Structural variation in the side chain of amino acid 2 were tolerated to some extent. Regarding the length and shape of the alkyl residue CsA (α-amino butyric acid) and CsG (norvaline) showed about equal activity, whereas a shortened or branched alkyl group like in CsB (alanine) and CsD (valine) lead to a slight decrease in the activity. CsC with a polar threonine in position 2 exhibited a strong immunosuppressive activity.

Table A.2.
Immunosuppressive activity of cyclosporin analogues

Analogue of cyclosporin	Activity#	Natural (N)/ Synthetic (S) product
Cyclosporin	***	N
(Me Thr[1]) cyclosporin	*	S
(Thr[2]) cyclosporin	***	N
(Ser[2]) cyclosporin	**	S
(Ala[2]) cyclosporin	**	N
(Val[2]) cyclosporin	**	N
(n Val[2]) cyclosporin	***	N
(D-Pro[2]) cyclosporin	*	S
(D-MeVal") cyclosporin	*	N
(Meleu") cyclosporin	*	S
(O-acetyl) cyclosporin	*	
3'-Desoxy- cyclosporin	*	N
Dihydro- cyclosporin	**	
H-MeBmt-OH	*	S

*** *Potent immunosuppressive activity* ** *Intermediate activity* * *Little or no activity*

Modification in positions 5 and 7 resulted in only minor changes in the biological activities as was evident from the replacement of L-norvaline (CsM) and alanine at position 7 by α-abu (CsV). In contrast, N-demethylated congeners in general, possess little or no significant immunosuppressive activity, as was seen in CsE and W lacking methylation of valine in position 11. In both the cases, an additional hydrogen bond between the amide portion of L-valine and the carbonyl group of D-valine resulted in considerable conformational changes in the open loop resulting in a drastic distortion of the ring and complete loss of immunosuppressive activity. These results indicate that a molecular geometry as in CsA or a closely congruent backbone conformation is intimately associated in the immunosuppressive activity. Table A.2 shows the immunosuppressive activity of many synthetic and natural analogues of cyclosporin (Traber *et al.* 1977a; 1977b; 1982; Ruegger *et al.* 1976; Lawen *et al.* 1994).

A.2.8 *Mode of action :* The most interesting of all the pharmacological effects that cyclosporin exerts is the selective modulation of defined subpopulations of immunocomponent

cells (Borel 1981b). Early studies have already revealed the sparing effect of CsA on haemopoietic tissues and on leukocyte functions, except those of lymphocytes. The proper reversibility of immunosuppressive activity both *in vivo* and *in vitro* demonstrates that it is not due to a lymphocytotoxic action such as an antimitotic effect (Borel *et al.* 1976; Wiesigner and Borel 1979). The compound is known mainly to inhibit the expression of helper function, ie the

Table A.3

Step in the conversion or resting T cells (T) to activated T cells (T')

Reactions	Cyclosporin sensitivity
Reaction A : Primary activation (1) T ☞ T' (2)	+
Reaction B : Clonal expansion T' ☞ nT' (2)	-
Reaction C : Triggering of lymphokine release (1) T' ☞ L	+
Reaction D : Cytotoxic effector function T'c = expression of cytotoxicity Borel and Lalfery (1983)	-

(1) signal 1, when antigen binds to a receptor on T cell surface

(2) signal 2, by co-stimulator activity such as IL-1 etc.

capacity of the T cells to sysnthesise and release IL-2 Interleukin-2 or T cell growth factor (Bunjes *et al.* 1981; Dos and Shevach 1982; Hess *et al.* 1982a,b) Although CsA affects production of several other lymphokines (Horowitz *et al.* 1984; Palcios 1985; Reem *et al.* 1983; Thomson *et al.* 1983), it does not interfere with their effects on target lymphocytes such as IL2 dependent T cell proliferation (Hess 1985; Lafferty 1983), or on the phagocytic migratory and monokine release activity of granulocytes or macrophages (Thomson *et al.* 1983; Bunjes *et al.* 1982; Drath and Kahan 1984; Janco and English 1983)

The step in the transformation of resting T cells (T) into activated cells (T') with receptor for IL-2 and the functions of these T' cells such as the production of lymphokines have different sensitivities to CsA (Table A.3) (Borel and Ryffel; 1985).

Thus cyclosporin reversibly affects the early steps immediately following antigen or mitogen recognition, leaving the T cells in the resting phase of the cell cycle, unable to transform and release lymphokines and fail to induce and maintain an immune repsonse. CsA not only prevents a primary immunisation but may also terminate the ongoing immune reaction such as in autoimmune relapse (Borel *et al.* 1976; Borel 1981b; Deed *et al.* 1980; Ryffel *et al.*1980). This therapeutic as opposed to preventive effect, may be most likely to occur through inhibition of continued lymphokine release as well as through suppression of further activation and recruitment of effector cells. The concentrative and biphasic uptake of CsA in the cytoplasm, largely due to high affinity is now known to be because of a cytosolic drug binding protein, consisting of a single homogenous polypeptide chain of about 117 amino acids, cyclophilin (Handschumacher *et al.* 1984). Colombani *et al.* (1985) demonstrated that CsA binds to calmodulin, another ubiquitous cytosolic protein and inhibits calmodulin dependant enzymatic activation.

Cyclophilins were initially detected by their ability to bind cyclosporins and were recognised as the cellular target protein of CsA (Handschumacher, *et al.*1984). They posses a cis/trans peptidyl-prolyl isomerase (PPIase) activity and hence involved in protein folding (Gething and Sambrook 1992; Stamnes *et al.* 1991; Lodish and Kong 1991). They have been detected in various species of bacteria, mammals, insects, plants and fungi (Fisher 1994; Schreiber 1991). Cyclophilins can be classified into different families according to their size and location in cell compartments and many play a crucial role in signal transduction especially in T cell signalling (Bierer *et al.* 1990).

In human serum, the hydrophobic molecule is exclussively associated with lipoproteins as was seen in the case of many lipophilic drugs (Bickel 1975; Danon and Chen 1979) insecticide (Maliwal and Guthrie 1982) and amphiphilic lipid. About 80% of cyclosporin is in the cytosol, largely due to a cytosolic drug binding protien, cyclophilin, a highly conserved protein encountered in eukaryotic cells (Filipovick *et al.* 1979; Kolestsky *et al.* 1986; Haendler *et al.* 1987; Danielson 1988; Tropschung 1988; Foxwell 1988) and is identical to peptidylprolyl cis-trans isomerase (Yakahashi *et al.* 1989; Fisher *et al.* 1984; 1989; Kyo *et al.* 1994), a novel enzyme that accelerates the slow refolding phase of certain proteins in vitro (Freidman 1989; Lang *et al.* 1987; Lang and Schimid 1987). In cyclosporin resistant mutants of *Neurospora crassa* and *Saccharomyces cerevisiae*, the cyclophilin was either lost completely or had lost its ability to bind cyclosporin.

Specific inhibition by CsA of m-RNA synthesis encoding for some lymphokines was observed (Kronke *et al.* 1984; Elliot *et al.* 1984; Piperno *et al.* 1984). It suppressed synthesis

of IL-2, α-interferon, B cell and cytolytic T cell-stimulating factors by blocking the induction of active lymphokine m-RNA. In constrast, total protein sysntheis was unaffected suggesting that it can selectively inhibit cellular functions without affecting other consecutive processes.

From the different studies now it can be deduced that CsA binds to the drug binding protein which is not identical with the antigen recognition site or T cell receptor. Upon internalisation it is concentrated in the cytoplasm where it is bound to cyclophilin. In association with the receptors in the cystol it penetrates into the nucleus (Karin *et al.* 1984), where it inhibits specifically the transcription of m-RNA coding for lyphokines, but not for the bulk of other proteins (Piperno *et al.* 1984). This might be the crucial CsA sensitive step, because addition of the drug beyond this stage (2 to 4 hours after stimulation) remains ineffective, for instance, the subsequent translation and protein (lyphokine) sysntheis proceed normally (Kronke *et al.* 1984).

It is now proved that the cyclosporin A-cyclophilin (CsA-CyP) complex inhibits the protein phosphates calcineurin and interferes with cellular signal transduction pathway (Schreiber 1992; Foor *et al.* 1992; Luan *et al.* 1994). When acts with calcineurin, a serine / threonine phosphatase (CsA-CyP) complex lead to the suppression of the dephosphorylation of the transcription factor NF-AT (nuclear factor of activated T cells), which regulates IL-2 transcription in T-cells (Liu *et al.* 1991; Flangan *et al.* 1991) (Fig. A.3).

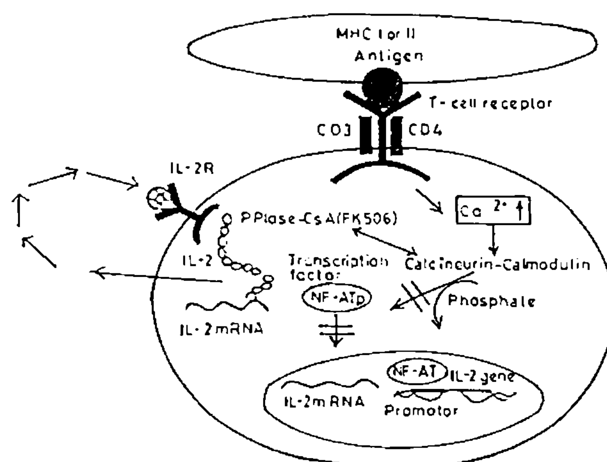


Fig. A.3 : Hypothetical Mechanism for the Suppression of Antigen-Stimulated Clonal T-Cell Expansion by CsA

A.2.9 *Pharmacokinetics and metabolism*

CsA is highly soluble in less polar solvents (Cavank and Sucker 1986). The intestinal lymphatic absorption does not play a major role in rats (Ueda *et al.* 1983). The kinetics of drug absorption are not affected by the presence of food in the gastrointestinal tract, but absorption is diminished by approximately 25%. The absorption which is around 30 to 40% under optimal conditions, is markedly depressed by gastrointestinal dissorder (Keown *et al.* 1985). Following intravenous administration, high concentration may be achieved rapidly in the central blood compartment. Absorption from the intramuscular administration is unreliable and this route is seldom employed now in human (Keown *et al.* 1985). Continued therapy appears to lead to progressive saturation of peripheral compartment necessitating a reduction in dosage to avoid excessive concentration within the central or blood compartment.

CsA is distributed approximately 20% in the plasma and 70% in the erythrocytes (Lemaire and Tillment 1982; Niederberger *et al.* 1983). At concentration above 500 ng/ml, there is a sharp decrease in the fraction bound to the erythrocytes and a corresponding increase in the plasma level. The distribution of CsA between plasma and erythrocytes is temperature dependant (Hows and Smith 1983; Wenk *et al.* 1983). In erythrocytes, it is not bound to carbonic anhydrase and moderatily bound to heamoglobin (Nussbaumer 1984). Binding on leukocytes is less when compared to erythrocyte binding (Ryffel *et al.* 1980; Le Grure *et al.* 1982). Binding to plasma proteins is independant of the concentration but varies with temperature with 80% of CsA found to be associated with lipoproteins. High and low density lipoproteins account for about 80% of plasma binding.

Pancreas, adrenal glands and to a relatively lesser extent the liver contain high concentrations of CsA (Atkinson *et al.* 1983; Reid *et al.* 1983). Brain contain very low levels (Atkinson *et al.* 1983; Boland *et al.* 1984) indicating that only little crosses the blood brain barrier. CsA was also detected in the milk of lactating women on cyclosporin therapy (Lewis *et al.* 1983). Fat contains higher concentrations as the drug is lipophilic.

CsA is mainly metabolized in the liver (about 99%) and is eliminated through the bile (Sheets and Mason 1984; Freeman *et al.* 1984). The degradation is NADPH-dependent. All metabolites contained the intact cyclic oligopeptide structure of the parent compound and were sufficiently water soluble. The metabolites are present in considerably lower plasma concentration than the parent molecule. (Eood *et al.* 1983).

Metabolism is almost exclussively by series of cytochrome P-450 dependant reactions in the liver, resulting in atleast 14 metabolites. Lemaire *et al.*(1986) chemically characterised about 24 metabolites isolated from urine and bile. Many of the drugs known to interact with CsA

are either inducers, inhibitors or substrates for the metabolic enzymes of centrilobular hepatocytes and intestinal mucosa (Murray *et al.* 1988; Watkins *et al.* 1988). The degradation products have only very low immunosuppressive activity and hence may not contribute to the overall immunosuppressive activity of CsA but they are devoid of nephrotoxic potential, the major side effect associated with the parent compound. Administration of aroclor 1254 and keto cenazole which increases the metabolism resulted in reduced nephrotoxicity.

Following intravenous administration of the drug, the clearance from the whole blood was found to be 0.37 l/h/kg (Follath *et al.* 1983). In children it was found to be 40% higher than in adults (Burckart *et al.* 1985). Thus higher dose of CsA is required for children than in adults (Burckart *et al.* 1985; Kalare *et al.* 1984). The main route of elimination is through biliary excretion and urinary elimination is not of much significance (Wood *et al.* 1983).

A.2.10 ***Significance of cyclosporin in organ transplantation :*** Cyclosporin was initially shown to inhibit only humoral immunity but later it was found to be effective in suppressing cell mediated immunity too. Thus it became effective in preventing allograft rejection. It has been effective in achieving prolonged graft survival in many species. Both first and second set graft survival could be prolonged under cyclosporin treatment (Deeg *et al.* 1980). It is known to elicit potent immunosuppressive action both *in vivo* and *in vitro* (Borel 1976; Keown *et al.* 1981; White *et al.* 1979). The prevention of rejection of concordant xenografts represents further remarkable feature (Green *et al.* 1982; Homman *et al.* 1981).

Donor-specific immunologic tolerance and clonal detection have been suggested as the mechanism for prolonged allograft survival in patients treated with CsA. *In vitro* evidence, however, indicate a reversible immunosuppressive effect on lymphocytes and graft rejection was noticed immedietely following discontinuance of cyclosporin therapy which shows the lack of tolerance induction with cyclosporin therapy. Ferguson and Fidelus (1978) reported that the presence of CsA in plasma was necessary for its blockage of lymphocytes responsiveness and thus the prevention of allograft rejection.

GVHR likely to arise in bone marrow transplantations could be cured by CsA and often it lead to the repopulation of lymphoid organs and recovery of T-cell dependant immune functions. Beveridge (1983) reviewed the reduction in the number of rejection episodes seen in cyclosporin treated kidney transplanted patients. The drug has produced superior results already in liver (Starzl *et al.* 1982), heart (Reitz and Stinson 1982) and Lung (Beveridge 1983) transplantations.

Thus the introduction of Sandimmune® (cyclosporin) into immunosuppressive therapy has improved the results of organ transplantation with respect to patient survival, graft survival

and the main post transplant complications (Merion *et al.* 1984; von Buren *et al.* 1984). The main advantages include the absence of myelotoxicity, prompt and sustained engraftment, quick heametologic recovery and less time in the hospital. Though cyclosporin is no panacea it can well be considered as one of the potent and specific immunosuppressants having an acceptable incidence of side effects.

A.2.11 ***Significance of cyclosporin in autoimmunity*** : Though infectious diseases had long been fought by eliciting the immune response, later, it was indicated that a significant number of diseases were caused by the body's natural defence mechanisms. Immunosuppressive therapy and other advances in understanding the immune system are thus being used to treat patients with illnesses that were not recognised, as being connected with the immune system even recently. Table A.4 gives a summary of the results of the preventive or therapeutic treatment of cyclosporin in autoimmune animal models (Borel 1983). These results speak of the effectiveness of cyclosporin in animal models and thus offer support for a cautious approach in similar indications in man.

There are a lot of scattered clinical trials conducted with varying degrees of success. It is now felt as if it is imperative to enter into a phase of study in which certain criteria are met viz. 1) the disease should be studied in the phase in which benefit can be detcted, 2) assays that measure the relevant immune response to the disease under study be developed, 3) fool proof clinical study protocals must be developed and 4) randomized control trials arranged. In principle, cyclosporin will be effective against desease in which there is cogent evidence that an abnormality of self regulation of the immune response is present.

Animal Model	Species	Prev/Ther
Adjuvant arthritis (rheumatoid arthritis)	Rat	+/+
Experimental allergic encephalomyelitis (multiple sclerosis)	Rat, guinea pig monkey	+/+[o]
Experimental allergic neuritis (GUILLAIN-BARRE syndrome)	Rat, guinea pig	+/+[o]
Experimental uveitis (human uveitis)	Rat	+/+
Experimental ai myasthenia gravis (myasthenia gravis)	Rat	+/+[o]
Experimental ai thyoiditis (GRAVE's disease: HASHIMOTO's thyroiditis)	Rat	nd/+[o]
Spontaneous ai thyroiditis (HASHIMOTO's thyroiditis)	Chicken	–/nd
Spontaneous ai diabetes (juvenile-onset diabetes Type I)	Rat	+/nd
EMC-induced diabetes (insulin-dependent diabetes)	Mouse	–/–
Murine ai lupus (systemic lupus erythematosus)	NZB/W	+/nd
Murine ai lymphoproliferative disease (SLE, arteritis, arthritis)	MRL/lpr	+/nd

The drug was used either preventively (prev) or therapeutically (ther). The human correlates to the animal models are indicated in parenthesis.
[o] Relapse of symptoms following discontinuation of cyclosporin treatment
ai, autoimmune

Table A.4 : Summary of the Results Obtained with CsA in Experimental Autoimmune Models

A 2.12 ***Antiparasitic effects of CsA:*** Cyclosporin being a selective compound for T cells, attracted the attention of experimental parasitologists due to its usefulness as a probe for dissecting the role of T-cells in immunological responses, particularly in a number of basic pathological phenomena in which the involvement of T-lymphocytes has long been debated. Finally when cylcosporin made to it to several experimental models of parasitic, fungal and viral infections it become all set to exert an impact of its own on fundamental immunology.

It was nevertheless a surprise when the drug was found to have antiparasitic effects against murine schistosomiasis (Bueding *et al.* 1981) and malaria (Thommen 1981). This was significant breakthrough in that these two diseases continued to pose great trouble to mankind throughout the world. Moreover it was of increasing importance in the context of drug resistance exhibited by parasites against many known drugs.

Later, the spectrum of antiparasitic activities of cyclosporin has been found to include toxoplasmosis (Mack and Mcleod 1984) and filariasis (Bout *et al.* 1984.). Trypanosomiasis and giardiasis however remined unaffected (Mc Cabe *et al.* 1985; Belasevic *et al.* 1986). Bout *et al.* (1983) reported the protective effect of this drug against *Schistosoma mansoni* and claimed that the dug rendered the mice resistant to reinfection which was unattainable with other antischistomal drugs such as oxaminigsis. It was confirmed further and corroborate the observation that the drug is most effective against immature stages of the worms during early stages of infection (Bout *et al.* 1984; Nilsson *et al.* 1985).

As the interval between infection and treatment increases, the chemotherapeutic effect was found to be reduced. Drug administration before infection was found to be highly prophylactic (Thomson *et al.* 1986; Bout *et al.* 1986). This might have had an impact on parasite reproduction also (Thomson *et al.* 1986).

Munro and Mc Taren (1990) reported that the schistosomicidal effect was due to a metabolite. It is already established that the antiparasitic activity was independant of immunosuppression (Nickel *et al.* 1982). The schistosomicidal effect of CsA remains longer than its elimination, suggesting that a lipophilic metabolite may have affected the function of a critical metabolite pathway or enzyme system of the parasite.

Suboptimal doses given orally for a few days were effective in preventing infection by *Plasmodium* or in curing already parasitaemic animals infected with *P.berghei*. A clear synergism was exhibited between cyclosporin and pyrimethamine, a drug affecting mainly the schizonts

(Nickel *et al.* 1982). In concentrations between 0.18 and 10 g/ml for 48 hr, it prevented the survival of *P. falciparum*. No cross resistance was found between cyclosporin and chloroquine. According to Loke (1982), depression of immune status exacerbates parasitic infection, But the studies conducted by Nickel *et al.* (1982) and Somasundaram *et al.* (1989) showed that malaria infected mice when treated with cyclosporin recovered from the infection. Since it is a cytotoxic drug it my be concluded that the drug acts directly on the parasite and kills it.

Against *Trichinella spiralin* the drug exerted no effect on the dissemination of the parasite from the gut to the muscles nor did it affect the established muscular larvae (Borel 1983). However, preventive treatment at the time of infection resulted in inhibition of release of new borns by the females and treatment on days 1-14 completely eliminated the adult parasites. A dosage of 15 mg/kg for 5 days was enough to keep the number of microfilaria under check and the female microfilaria recovered from the pleural cavity were all dead in experimental model of cotten rat (Borel 1983).

It is difficult to envisage the use of cyclosporin as an antiparasitic agent. Since there is evidence that the antiparasitic properties are not linked to the immunosuppressive activity, the search for cyclosporin derivative possessing antiparasitic properties but devoid of immunosuppressive activity has become an area of active research.

A.2.13 ***Significance of cyclosporin in basic research:*** Since first used in human medicine in 1978, CsA has been used in multiple lakhs of organ transplant operations conducted with a 1-year survival rate of greater than 90%. Since its discovery, decisive advances have been made towards understanding the biochemical rationale for this drug therapy. The biochemical and structural work initiated by the discovery of this drug is now playing a central role in elucidating general mechanisms of cellular signal transduction (Braun *et al.* 1995).

The use of CsA as a biochemical tool to study the signal transduction pathway in T cells has lead to the discovery of a first family of immunosuppressant-binding proteins (immunophilins), the cylcophilins(CyP). The formation of CsA-CyP complex and the subsequent specific interaction of this complex with the serine/threonine phosphatase, calcineurin (CN) are considered to be the key steps in the cascade of events that result in the desired immunosuppression. Knowledge of the conformation of the CsA-CyP-CN ternary complex is of significant biomedical interest, because mimics of this could provide drugs with improved pharmacological profiles (Braun *et al.* 1995).

Following the discovery of cyclophilins, FK binding proteins (FKBPs), the relevant receptors for FK 506 (a macrolide showing similar immunosuppressive action as that of CsA) and rapamycin (RAPA) (another macrolide with a different immunosuppressive mechanism) were detected (Harding *et al.* 1984; Schreiber 1991). A series of experimental evidences were made available to prove the specificity of CsA-CyP and FK506-FKBP to the enzyme calcineurin (Clipstone and Crabtree 1992; O'keefe *et al.* 1992; Schreiber and Crabtree 1992; Mc Caffrey *et al.* 1993.

A.2.14 ***Methods to measure cyclosporin levels :*** Monitoring the concentration of CsA is often critical to the patient management by dint of the adverse side effect and extremely individual metabolic variability of the drug. Several methods have been developed for the analysis of cyclosporin in plasma and/or blood. Radioimmunoassay (Donatsch *et al.* 1981), now available as a kit was extensively used during the clinical development of this drug. But it showed cross reactivity with some of the circulating metabolites of cyclosporin. Later, an MPLC method based on gradient elution was developed (Niederberger *et al.* 1980). The technical complexity of this method encouraged several investigators to reinvestigate the method (Lawrence and Allwood 1980; Sawchuk and Cartier 1981; Nussbaumer *et al.* 1982; Leyland *et al.* 1982; Kahan *et al.* 1982; Yee *et al.* 1982; Carruthers et al 1983). Nisha *et al.* (1992) studied the variations in the retention behaviour of cyclosporins A, B and C in the culture broth with respect to mobile phase composition, temperature, stationary phase and UV detection wavelength. They were well separated with a C8 column (7.5 cm x 4.6 mm) at 60°C using acetonitrile-water (50 : 50) containing 0.01% of orthophosphoric acid at a flow rate of 1 ml/mm with UV detection at 202 mm. Interface of closely eluting peaks was avoided as good separation of structurally similar molecules achieved. This is an economical method as the solvent consumption was less. Though HPLC has long been considered as the "gold standard" method for monitoring CsA concentration now-a-days monoclonal antibody based assays have been successfully used for CsA estimation (Jones and Brune 1993).

A.2.15 ***Microbiology of cyclosporin production :*** The fungal genus *Tolypocladium*, first described by Gams (1971), belongs to the class fungi imperfecti, occurring in soil or litter habitats. Of the nine species accepted in *Tolypocladium*, four are pathogens of terrestrial invertebrates, and one is often isolated from mycomycete sporangia. These are characterized by white, hyaline or bright colored, relatively slow growing cottony colonies (Bisselt 1983; Samson and Soares 1984. *Tolypocladium* is often characterized as a *Beauveria* like genus (von Arx 1986), although this is currently a point of contention (Samson *et al.* 1988).

The initial description of CsA & C as metabolites of *T.polysporum* was in 1976. Cyclosporin production by *T.inflatum* was examined by Agathos *et al.* (1987). Sorbose (3% w/v) gave the highest final cyclosporin titre (105.5 mg/l). Increasing the concentration of carbon sources (sorbose and maltose) was reported to be successful in achieving volumetric production. Genetic studies intended to develop mutants for increased production and for obtaining novel variants of cyclosporins lead to the isolation viable protoplasts of *T.inflatum*.

Chun and Agathos (1989) reported the production of pink pigment along with cyclosporin by *T.inflatum*. Yellow pigment production also was reported (Weiser and Matha 1988). In *T.inflatum* many colony types viz. morphologically normal white, red and orange and morphologically diverse tiny brown colonies were noticed (Aarino and Agathos 1990). The colour, final pH of the broth and product formation efficiency were different with different colonies and the same was attributed to the difference in the primary metabloic pathways operating among the variants.

The relationship between spore inoculam density and culture morphology was well established (Metz and Kossen 1977) and high spore densities was found favouring higher cyclosporin (Dreyfuss *et al.* 1976). Of the complex nitrogen sources, casamino acid produced the most constant data and the cleanest extract. The favourable carbon sources for many strains of *T.inflatum* were described by Agathos *et al.* (1987) and Zhao *et al.* (1991). The various steps involved in the isolation and purification of cyclosporin from the fermented samples is given Fig. A.4.

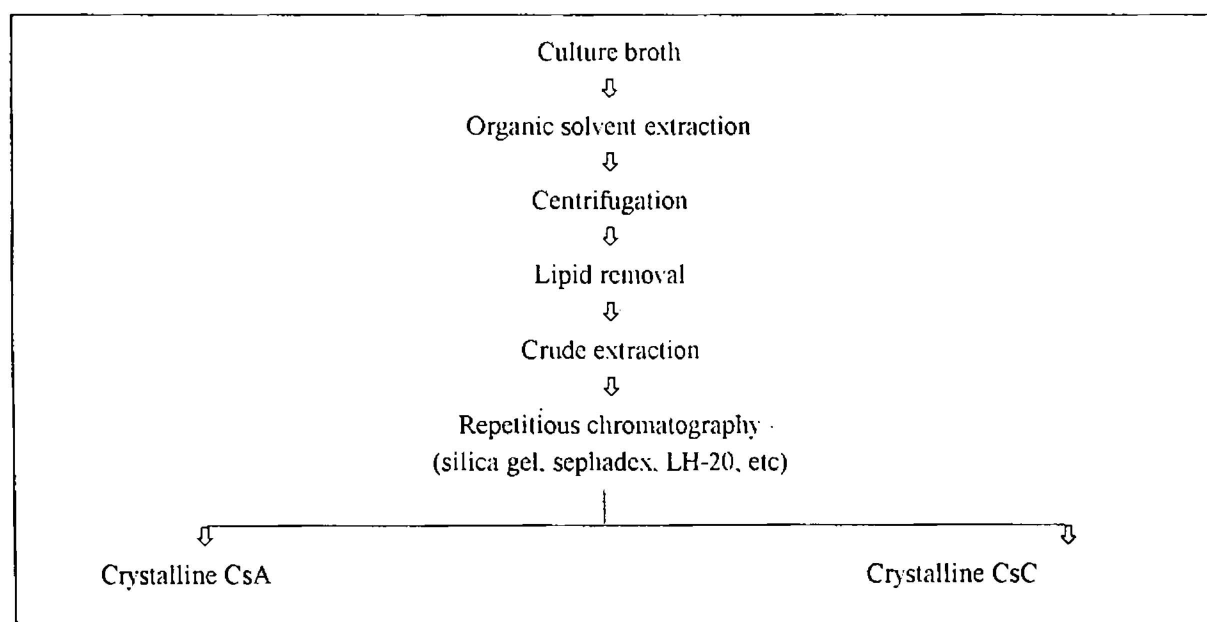


Fig. A.4. Isolation & Purification Scheme for CsA Production

Cyclosporin production by *Beauveria nivea* found to be maximum (170 mg/l) after 8 days of fermentaion in fructose based medium (Margritis and Chalal 1989). Nakajama *et al.* (1989) reported the production of cyclosporin by many species of fungi belonging to the genus *Neocosmospora*. The novel immunosuppressant of molecular formula $C_{62}H_{111}N_{11}O_{13}$, Fr 901459 isolated from the fermentaion broth of *Stachybotrys chartarum* is a member of the cyclosporin family (Kazhutoshi *et al.* 1993). However, it is structurally distinct from any other cyclosporins discovered so far in that leucine is present in position 5 instead of valine. It exhibited relatively good immunosuppression.

A.2.16 ***Production of cyclosporin on solid substrates :*** SSF has not yet been fully utilized for the production of biopharmaceuticals. A process for the lab scale production of cyclosporin on wheat bran supplemented with trace elements and precursor amino acids has recently been initiated (Pandey *et al.* 1995). Efforts have already been initiated for the commercial production of cycolsporin through SSF route. The recent resurgence of interest in SSF and its exploitation to a variety of products are bound to result in further wide-scale exploitation of SSF for the production of small volume-high value products like cyclosporin.

A.2.17 ***Effect of precursors on the synthesis of cyclosporins :*** The biosynthesis of naturally occuring cyclosporins can be directed by externally supplying the corresponding precursor (Kobel and Traber 1982). Addition of DL-amino butyric acid (8 g/l) led to the exclusive production of CsA. Addition of nor valine (8 g/l) resulted in the accumulation of CsG. L-threonine (8 g/l) led to 5 fold increase in total cyclosporin level with a specific yield of 59% CsA and 41% CsC. 5.7 fold increase in the yield of total cyclosporins was noted with the addition of L-valine (8 g/l) with a specific yield of 43% CsA, 20% CsC and 37% CsD. D-valine had no stimulatory effect on the production. L-leucine was also a strong enhancer of drug production in synthetic media (Lee and Agathos 1989). Experiments with different times of addition of L-valine indicated that the amino acid may need to be present in the exponential growth phase for optimal production. In contrast, exogenous L-methionine and sarcosine lowered the production of CsA.

A.2.18 ***Biosynthesis of cyclosporin :*** The fact that cyclosporin is a partially N-methylated undecapeptide containing many unusual amino acids and that it has a broad pattern of congeners, indicate a non-ribosomal pathway involving multifunctional enzyme as established for other microbial peptides such as Gramicidin S (Kleinkauf and Koischuitz 1978) and Enniatin (Zocher

et al. 1986). Zocher *et al.* (1984) found that short term feeding of the cultures of *T.inflatum* with ^{14}C labelled amino acids lead to selective incorporation of many constituent amino acids to CsA & C. Experiments with L-(14-methyl) methionine demonstrated that all N-methyl groups originate from methionine and a possible mechanism of cyclosporin synthesis was proposed: (1) synthesis to all 11 constituent amino acids (2) activation of each amino acids (3) N-mehylation and peptide bond formation.

A multifunctional protein that is involved in the synthesis of cyclosporin was isolated (Zocher *et al.* 1986) and it was found to be capable of synthesising different cyclosporins *in vitro* in the presence of the constituent amino acids, ATP and S-Adenosyl methionine (methyl donor).

Precursor directed biosynthesis is a useful and efficient way to prepare cyclosporin analogues not encountered in nature. This successful incorporation of constituent and foreign amino acids demonstrate the low specificity of the biosynthesis of cyclosporins which is characteristic for a non-ribosomal biosynthetic pathway directed by multienzyme thiotemplates. Similar results have been encountered for other secondary matabolites (Kutz 1974; Zocher *et al.* 1982; Kobel and Sauglier 1978; Beacco *et al.* 1978).

It is interesting that all peptide and depsi peptide synthetases from fungi do not exhibit sub-unit structure (Kleinkauf and Koischuitz 1978; Peters *et al.* 1988; Liempt 1989). They consists of single polypeptide chains of molecular masses between 250 and 800 KDa and harbour all catalytic activities necessary for peptide formation. Such multienzyme polypeptides represent the counterparts of sub-unit multienzyme complexes of bacteria (Kleinkauf and von Dohernm 1987) in fungi.

Cyclosporin synthetase is polypeptide chain of molecular weight approximately 800 KDa (Lawen and Zocher 1990a). All constituent amino acids activated as thioesters via amino adenylation and carries out specific N-methylation reaction. S-adenosyl-L-methionine serves as methyl group donor. 4'-phosphopantotheine is a prosthetic group of cyclosporin synthetase similar to other peptide synthetases. This ezyme appears to be a large and complex enzymatically active multienzyme polypeptide chain. Schmidt *et al.* (1992) re-evaluated the molecular mass of cyclosporin synthatase by SDS-PAGE and CsCI density gradient centrifugation and with the new molecular mass values obtained from sequencing data. Both methods yielded approximately the same value of about 1.4 MDa.

Dittiomann *et al.* (1994) showed that cyclosporin sysnthesis occur as a single linear undecapeptide precursor. D-alanine at position 8 was found to be a starting amino acid in the biosynthetic process. All the four intermediate peptides of growing peptide chain isolated represent partial sequence of CsA starting with D-alanine. These suggested a step-wise synthesis of a single linear peptide precurosor of CsA.

Lawen and Zocher (1990a) reported that except CsA with D-methyl valine at position 11 all natural cyclosporins were produced by cyclosporin synthetase, which possess rather broad substrate specificity at most of its active sites (Laween and Traber 1993). It catalyses all 40 reaction steps necessary for the biosyunthesis of CsA starting from the unmethylated constituent amino acids (Lawen and Zocher 1990b).

Lawen *et al.* (1994) showed that this enzyme was also capable of introducing a β-alanine into position 8 instead of α-alanines present in the cyclosporin A ring. This lead to 34-membered, ring extended cyclosporins. But the cyclosporin synthetase related energy peptide SDZ 214-103 synthetase (Lawen *et al.* 1991) failed to incorporate either β-alanine into position 7 or β-hydroxy acids into position 8, indicating a higher substrate specificity of this enzyme compared with cyclosporin synthetase (Lawen and Traber 1993). Klienkauf and von Dohren (1990) made a biosynthetic investigation on all types of peptides and depsipeptides from microorganisms. A general mechanism for non-ribosomal multienzymatic peptide synthesis was observed. The functional and structural relationship of these multienzymes indicates that they should be considered as a non-ribosomal system, although the organizational principles are not yet understood.

Recent advances in cloning of multienzyme structures eventully lead to an understanding of the genetic organisation of non-ribosomal templates and may permit their exploitation in peptide synthesis. Equally fascinating are the diverse types of modification reactions found either integrated in multienzymes or on separate enzyme sites. Studies on peptide modifying enzymes will be of considerable use in the expansion of structure-function relationships. Regarding the functional aspects of peptides within the producing organisms, molecular genetic investigation of the regulatory controls of production should be of considerable help in defining their role in nature.

The gene encoding the cyclosporin synthetase has been cloned and sequenced recently (Leitner *et al.* 1994). The DNA has an open reading frame of 45.8 Kb. Weber and Leitner

(1994) recently reported onthe manipulation of this giant gene by DNA mediated transformation. This can be viewed as a first step towards engineering the cyclosporin synthetase gene to enable the production of new cyclosporins or cyclosporin derivatives.

2.19 ***Latest developments in immunosuppression:*** The mechanisms of action of the immunosuppressive drugs CsA, FK 506 and RAPA were found to be strikingly conserved form yeast to human T cells (Kunz and Hall 1993). All the three drugs, although better known for their immunosuppressive properties and clinical applications, are also valuable basic research tools as probes of signelling pathways (Schreiber 1991; Signal and Dumant 1992; Heitman *et al.* 1992). They have been extensively used as biochemical ligands and as agents to select mutants and many of the results thus obtained helped in the identification of new components of signal transduction pathways (Foor *et al.* 1992; Liu *et al.* 1991; Kunz 1993) Table A.5 shows the comparison of the immunosuppressive properties of the three compounds.

FK 506 and RAPA are also naturally occuring secondary metabolites like CsA but they are structurally related to each other but not to CsA (see Table A.6 for comparison of the 3 drugs). These drugs are immunosuppressive because they block T cell activation or proliferation (Schreiber and Crabtree 1992). Activation is the G0-G1 cell cycle transition that occurs in response to antigen binding to the T Cell receptor (TCR). The Ca^{2+}-dependant TCR signal transduction pathway ultimately switches on several genes including those encoding the lymphokine interleukin-2(IL-2) and its receptor. Proliferation is the subsequent G1-S transition that results from autocrine signelling by IL-2. These drugs interfere with intermediate components of these two cell cycle related signal transduction pathways. While structurally unrelated CsA and FK 506 inhibit the TCR signal transduction pathway, RAPA, the FK506 analogue inhibits the IL-2 receptor pathway (Fig. A.5).

Many authors have also proved recently that the use of combinations ofdrugs that have a synergistic effect help to reduce the risk of toxicity. Martin *et al.* (1995) for instance, demonstrated the synergistic relationship between RAPA and CsA which allowed the use of reduced doses of each drug to achieve a therapeutic effect.

These drugs are known to have effect on cells other than T cells including yeast. CsA and FK506 block recovery from G1 arrest induced by mating pheromone (Foor *et al.* 1992) whereas RAPA arrest growth in the G1 phase of the cell cycle(Heitman et al 1992).

Table : A.4

Comparison of three potent immunosuppressants

No.	CsA	FK 506	RAPA
1.	Lipid soluble cyclic undecapeptide	Lipid soluble macrocyclic lactones	Lipid soluble macrocyclic lactones
2.	Molecular mass 12043	822	915
3.	Produced by *T.inflatum*	*Streptomyces tsukubaensis*	*S.hygroscopicus*
4.	Isolated from Norwegian soil sample	From the Tsukuba (N.Japan)	From soil sample of RAPA-Nui Easter Islands
5.	Approved for clinical use in 1983	Under evaluation	Under evaluation
6.	Forms complex with the immunophilin, cyclosphilin	With FKBP	With FKBP
7.	The target for CsA-CyP complex is calcineurin	The target for FK506-FKBP complex is also calcineurin	RAPA-FKBP complexes with a phosphotidylinositol 3-kinase homologue
8.	Inhibits T-cell receptor signal transduction pathway	Same as CsA	Inhibits the IL-2 receptor pathway
9.	Action on yeast cells:Block the recovery of G1 arrest induced by mating pheromone	Same as CsA	Arresting growth in the G1 phase
10.	Inhibition of the proliferation of T lymphocytes inresponse to different stimuli only at higher dossage	Lower dosage	Lower dosage
11.	Time at which complete inhibition of T cell activation possible, 0 to 3 hours after stimulation	0 to 3 hours after stimulation	Effective even after 6 hours of stimulation
12.	Site of inhibitory action in cell cycle, G0 to G1	G0 to G1	Late G1 and early S phases
13.	A narrow spectrum of T & B cells are suppressed	Narrow spectrum	Wider spectrum
14.	Response to cytokine action, receptor antagonist	Receptor antagonist	Functional antagonist
15.	Enhancement of T cell response inhibition when combinations of drugs are used is not concentration dependant.	Concentration dependant between FK506 & RAPA	Concentration dependant between RAPA & FK506

The finding that both cyclophilin and FKBP have PPlase activity and that the immunosuppressants competitively inhibit this activity upon binding lead to an early model that the drug effects are due simply to the loss of PPlase activity. But later this was disproven as some drug analogues were found inhibit PPlase yet fail to supprss the immune response and by the finding in yeast that the cyclophilins and FKBPs are non-essential for life despite being binding proteins for toxic drugs (Heitman *et al.* 1991; Koltin 1991; Wiederrecht 1991) . An alternative and widely accepted model is that the complex (immunosuppressant-immunophilin complex) has a new toxic activity (Heitman *et al.* 1991; Koltin 1991).

It was later proved that CsA-CyP and FK 506-FKBP inhibit the serine/threonine phosphatase activity of calcineurin (either drugs or immunophilins alone were ineffective), a Ca^{2+} dependent enzyme. This selective inhibition agrees well with the known effects of CsA and FK 506 on TCR signal transduction pathway (Schreiber and Crabtree 1992; Signal and Dumont 1992; Heitman *et al.* 1992) . They both block a Ca^{2+} dependent signalling step that normally leads to nuclear transport of a cytoplasmic subunit of NF-AT (Shreiber and Crabtree 1992) . RAPA, on the other hand does not interfere with this step and instead the RAPA-FKBP complex targets a phosphtidylinositol 3-kinase homologue (Schreiber 1992), which is expected to involve in the IL-2 receptor signalling pathway. The currently attractive model for cytoplasm- to-nucleus signalling in the TRC pathway in which CsA is involved is that calcineurin dephosphorylates and thereby allows nuclear translocation of NF-AT (Fig. A.5). This signifies the role of such

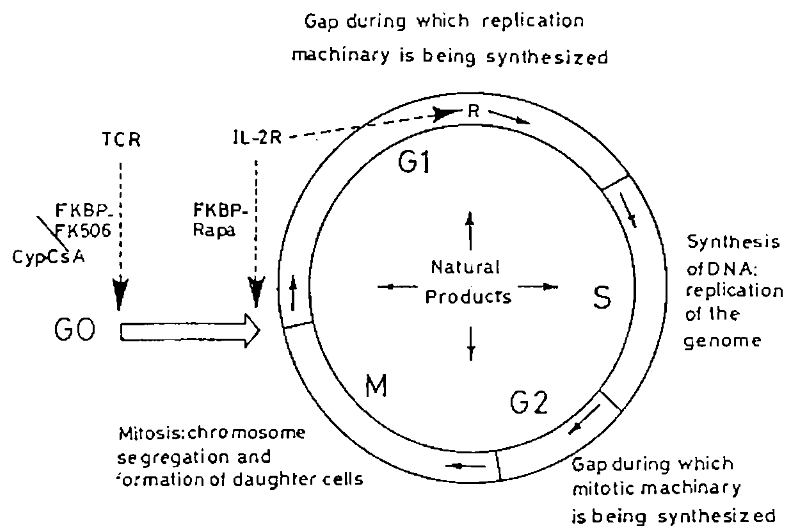


Fig. A.5. **Effectiveness of the Natural Products as Tools to Study the Signalling Phenomenon Guiding the Cell Cycle**

drugs in elucidating many vital steps associated with the cellular regulation, cell cycle and cell-cell interactions. There ability to mediate a precise disruption of the cell cycle progression by the induction of protein-protein heterodimerization event could be exploited to unravel many provocative ideas regarding many concepts in the biochemistry of T cell activation. By performing as pharmacological probes and affinity ligands they bring to light various components constituting the signal cascade inside the cell involving many important targets of therapeutic intervention. In combination with the molecular and cellular studies, the insight gained with these drugs could lead to the better understanding of the cellular signal transduction phenomena associcated with the development of an immune respose.

B: EXPERIMENTAL DETAILS

B.1: Isolation, Identification and Characterization of a Microbial Strain Producing Bioactive Metabolite.

The impact of microbial secondary metabolites on the multifarious activities of our daily life has been convinsingly indicated by the sea change kindled by them in various portfolios viz. increased survival from infections diseases, shortened hospital confinements, reduced work-time losses, highly equiped veterinary medicine, potential upgradations in food and agriculture sectors, improvements in basic research and world wide sales figures. Inspite of the difficulties encountered in screening, the number of bioactive microbial metabolites exhibiting multitudes of unique activities has been on the rise (Omura 1992).

This work encompases the isolation of a strain producing antibiotically active metabolite, identification, characterization and culturing of the producing organism and the successful detection of the product of interest.

Materials and Methods

Isolation : Locally procured soil samples numbered 1 to 200 from diverse humid localities, heaps in gardens, forests with thick plantation etc were subjected to serial dilution and plating (James and Natalie 1983). A known quantity (1g) of each sample was suspended in 10 ml of sterile distilled water held in a test tube. By transferring 1 ml from the previous tube to the 9 ml of sterile distilled water in the successive tubes serial dilutions were performed. This was continued up to 10 dilutions. From each of the maximum diluted samples, 100µl was picked up and plated in order to isolate the diverse microbial strains living in them. The following media were used to prepare the plates (compositions in g/l). All chemicals were supplied by M/s. HiMedia, Bombay, India.

1. Nutrient Agar (pH 7.0)

Peptone	5.0
Meat extract	3.0
Agar	15.0

2. Potato Dextrose Agar (PDA) (pH:5.6)

Potato infusion	200.0
Dextrose	20.0
Agar	15.0

3. ISP-4 (Inorganic Salt Starch Agar) (pH:7.2)

Soluble starch	10.0
K_2PO_4	1.0
$MgSO_4.7H_2O$	1.0
NaCl	1.0
$(NH_4)_2 SO_4$	2.0
$CaCO_3$	2.0
$FeSO_4.7H_2O$	0.001
$MnCl_2.2H_2O$	0.001
$ZnSO_4.7H_2O$	0.001
Agar	20.0

The individual colonies from the plates were picked up after incubation at 30°C for 1-2 days. Each isolated colony was grown in a test tube in the respective medium minus agar for 2 to 4 days by incubating in an orbital shaker (Certomat MO B Braun Biotech International, Germany). Antibiotic activity of each of the isolated colony was examined by the plate diffusion test (Ericsson and Sherris 1971) by applying filter paper discs, soaked in the culture both of each isolated colony, on plates of test organisms viz. *Bacillus* sp. and *Aspergillus niger. Bacillus* plates were prepared using the medium with the following composition (g/1) and pH 7.4.

Yeast extract	3.0
Peptone	5.0
NaCl	5.0
Starch soluble	1.0
Agar	15.0

A. niger plates were prepared with PDA. Colonies which gave positive results alone were selected and isolated as pure colonies in plates holding the respective media. These were then cultured in the orbital shaker as is described above and centrifuged (cooling centrifuge, C24, Remi Instruments, India) at 5000 rpm for 15 minutes followed by extraction of both pellet and broth with methanol. Employing this extract a secondary screening (plate diffusion assay) akin to the earlier described first screening was performed.

Identification : One of the isolates designated as B-58, and showing consistant antifungal activity against *A. niger* , chosen for further investigations, after initial morphological characterization using an optical microscope (Nikkon Optiphot, Japan) was examined using biochemical and chemotaxonomic tests (DSM, Brawnschweig, Germany).

Scanning electron micrograph of the culture was attempted using a scanning electron microscope (Jeol Ltd., Japan). Samples were fixed with 10% gluteraldehyde and mounted on brass stubs followed by coating with a thin layer of gold (100Å) and viewed at an accelerating voltage of 15 KV.

Storage and Inoculum preparation: Seed stock cultures were prepared in 50% glycerol and preserved at -20°C. For short term storage, slants were preserved at 4°C and renewed once a month. Sabouraud dextrose broth/agar (SD agar/broth) supplemented with 0.4% yeast extract was used as the culture maintanance medium and it had the following composition (in g/l) and pH 5.2

Glucose	20.0
Peptone	10.0
(Agar)	20.0

Seed cultures were prepared in this medium by transferring a seed stock culture at 2%(v/v) level. Inocula were prepared in 250 ml conical flasks holding 50 ml SD broth by transferring 2% (v/v) spore suspension (spore concentration 2 x 10^7/ml) in sterile distilled water obtained by scraping the slant with a loop. This was incubated for 24 hours in orbital shaker at 27$\pm$ 1°C with 150 rpm before being used for inoculation.

Fermentation: Fermentation of suspension culture was initiated by adding 2% inoculum to 50 ml SD broth held in 250 ml conical flasks. All the studies were performed using the same medium with necessary substitutions. The flasks were incubated in rotory shaker (180 rpm) at 27 $\pm$ 1°C for 10 days.

To identify the most favoured sugars, various saccharides were employed in place of glucose in SD broth. Inorder to investigate the effect of concentration of carbon source in the medium, the level of glucose in SD broth was altered between 0.5 to 5 g/l. A pH range of 2-8 was tested to find the suitable pH at which this culture thrives best. For this, pH of the media was altered using KOH (1N) and HCl (1N) before sterilization. The influence of age and size of the inoculum on the culture growth was examined by selecting four sets of inocula of varying ages (1,2,3 and 4 day old) and by providing a range of 1-5% (v/v) inocula respectively.

Culture medium optimization: The organism was cultivated in 500 ml conical flasks each holding 100 ml of four different media (M1,M2,M3 and M4; for composition see Table B.1). The flasks were incubated on rotary shaker with 180 rpm at 27 ± 1°C for 14 days. Samples (as whole flasks; in duplicate) were withdrawn at different time intervals and analysed for biomass, pH, residual glucose, total saccharides, amino nitrogen levels and for product detection.

Analytical Methods:

Chemicals and reagents used for the assays were procured from M/s. SD Fine Chemicals, India. Biomass was separated by centrifuging the culture at 8000 rpm for 15 minits and expressed as dry matter after drying at 80°C overnight in a hot air oven (Kemi Instruments, Madras, India). pH of the cultures was measured using a Systronics µ-361 model (Ahmedabad, India) digital pH meter. Reducing sugars were estimated according to the method of Miller (1959).

A known volume (500µl) of the culture broth after centrifuging (microcentrifuge 12C Remi instruments, India) was made up to 2.5 ml with distilled water and 3 ml of dinitro salicylic acid reagent was added to it and the mixer was boiled in a boiling water bath for 12 minutes followed by cooling at room temperature. It was then diluted by adding 16 ml of distilled water and mixed well before reading the absorbance at 546nm in UV-VIS spectrophotometer (Shimadzu 160-A, Japan) against glucose standard.

Total saccharides in the medium were estimated by the method of Dubois *et al.* (1956).

A known volume (100 µl) of the sample was made up to 1.0 ml with distilled water. To this 1.0 ml of 5% (v/v) solution of phenol in water was added followed by addition of 5.0 ml of concentrated H_2SO_4 after exposing the sample tubes to ice cold conditions. The tubes were then kept at room temperature for 10 minutes followed by incubation at 30°C for 20 minutes. The absorbance was read at 490 nm using UV-VIS spectrophotometer against glucose standard.

Amino nitrogen was estimated by Sorensen's formaldehyde titration (Levy 1957).

The sample initially was adjusted to pH 6.5 using HCl (1N) or NaOH (1N). Formol solution (commercial 37% HCHO after neutralization with alkali to get a final pH of 6.0) was added to it so that the final formal concentration was 6% of the mixture. Prior to the addition of alkali (standard NaOH) from the burette one drop of phenolpthalein solution was added to the sample mixture. At reaching a definite pink color (pH 8.8) the alkali addition was stopped and the volume recorded.

The product was detected using T L C (silica gel G; solvents hexane: chloroform: methanol 12.5 : 11 : 1.5) and HPLC (Shimadzu LC 6A, Japan) using a reverse phase C-8 bonded silica column and acetonitrile-water (70:30) as the solvent system at a flowrate of 1ml/minute. The column (C8, waters) was maintained at 60°C by means of a column oven (CTO, 6A, Shimadzu, Japan).

The product, CsA was identified and quantified based on the similarity of retention time with that of standard CsA used for calibrating the HPLC. For monitoring CsA thus the modified HPLC method of Kreuzig (1984) was chosen with the induction of 0.1% trifluoro acetic acid in to the solvent system.

Results and Discussion

Of the 889 colonies isolated only six were found to develope inhibition zones in either of the test organisms. They were then selected for secondary screening. Only the culture designated as B-58 exhibited consistantly and considerably good antifungal activity (Fig B.1) against *A. niger*. It did not exhibit any antibacterial activity.

The culture on identification was proved to be *Tolypocladium inflatum* (Weiser 1980; Bisselt 1983; Samson and Soare 1984) known to produce cyclosporin (Dreyfuss *et al.* 1976; Kobel and Traber 1982). Fig. B.2 exhibits the fungal filaments under scanning electron microscope.

The culture grew well on SD broth/agar supplemented with 0.4% yeast extract and produced large quantities of conidia. The colony was white to cream-cushiony (Fig. B.3), wooly flocky, and the size ranged from 20-30 mm. The back of the colony was creamy yellow. Conidiophores were short and cylindrical with terminal or lateral whorls of phialids. Phialids had an inflated basal part and long (6 µm) filiform neck. Elliptic-globose conidia formed in the heads.

Among various carbon sources, maltose and glucose supported maximum culture growth. While the addition of maltose yielded a biomass of 3.03 g/l glucose managed to produce 2.96 g/l of biomass. Lactose was least supportive among all the sugars resulting a biomass of only 0.99 g/l. Significantly, starch also supported the cellular growth giving 2.36 g/l of biomass in 10 days (Table B.2). During fermentation, concentration of carbon source (glucose) was also found to be influencing the mycelial biomass. To attain sufficient culture growth 1% glucose was found to be sufficient, (Table B.2). The biomass yield higher than 2.76 obtained with 1% initial glucose concentration was not contemplated with further rise in carbon source level in the medium. Studies on the influence of initial pH of the medium on the growth of *T.inflatum* revealed that though, a pH range of 5-6 was well

tolerated, maximum biomass was observed for pH 5. Growth media with lower (4 or less) or higher (7 or more) pH showed drastic reduction in fungal growth. Studies on the optimization of inoculum showed that a 24 hour old inoculum transferred at the rate of 2% (v/v) supported best mycelial growth. Further increase in the size of inoculum, i.e. 3 & 4% were not as effective as 2%, the biomass yield with 5% was comparable to that obtained with 2% (Table B.2)

The results of the growth of *T inflatum* in synthetic (M1&M2) and complex (M3&M4) media are depicted in Fig B.4. In synthetic media the growth was relatively lower with medium M1 extending the least support. Maximum biomass attainable with medium M1 was only 6.7 g/l and the same was observed on day 6. With medium M2 the maximum dry weight of 8.2 was detected on day 8. Of the two complex media used, medium M4 supported the growth effectively. In complex media as the fermentation progresses, the pH was found to be rising slightly towards the basic side of neutrality (Fig. B.5). In medium M2 also the pH was maintained around 6.0 for a maximum period. But in medium M1, the pH droped to 2.5 from 48 hours onwards and it did not raise thereafter.

Though a complete carbon source utilization was contemplated in media M1 & M2, in media M3 & M4 not more than 30% of sugars were consumed (Fig. B.6). By day 8, complete utilization of readily available sugar namely glucose was observed in complex media. Maltose was consumed largely during the late growth and stationary phases.

The consumption of amino acids was found to be relatively higher in medium M3 (Fig. B7). and the same was reflected in the product titre too. Medium M3 supported maximum production (Fig. B8). A CsA titre of 205 mg/l was attained on 10th day of fermentation in medium M3 which was slightly less on subsequent days. In medium M4, the maximum level of CsA detected was on day 14 and the same was only 44% of what was attained in medium M3 during the same period. In media M1 and M2 very low CsA levels were reported showing a minute period rise in the drug titre till the termination of fermentation.

Cyclosporin though exhibiting antifungal activity, was recognised more for the impact it could make in the field of immunopharmacology (McIntosh and Thomson 1980). The undesirable immunosuppression caused by this antibiotic when tested as antifungal agent led researchers to develop it as an immunosuppressive agent (Omura 1992). Because of its specificity of action within the immune system it is considered as the prototype of new generation of immunosuppressants and it has now became the drug of choice for all transplantation protocals around the globe (Borel 1981b; Balakrishnan and Pandey 1996a). This emphasises the necessity for the development of effective screening systems based on recent knowledge of pharmacology, basic medicine and receptor biochemistry. Such

Table B 1

Composition of media M1-M4 (conc. in g/l) used for the media optimization studies.

M1		M2		M3		M4	
Sucrose	30.0	Sucrose	30.0	Glucose	10.0	Glucose	10.0
Ammonium sulphate	10.0	Ammonium sulphate	10.0	Maltose	10.0	Maltose	10.0
Potassium dihydrogen phosphate	0.75	Potassium dihydrogen phosphate	0.75	Casein acid hydrolysate	10.0	Peptone	10.0
TMS[a]	1.0 ml	TMS[a]	1.0 ml	Sodium acetate	1.0	Yeast extract	5.0
						Sodium acetate	1.0
pH	5.4	L-Valine	0.05	L-Valine	0.05	L-Valine	0.05
		α-Aminobutyric acid	0.05	α-Aminobutyric acid	0.05	α-Aminobutyric acid	0.05
		pH	5.4	pH	5.4	pH	5.4

a Trace metal solution (in mg per 100 ml distilled water): $FeSO_4 . 7H_2O$, 500; $ZnSO_4 . 7H_2O$, 440; $MnCl_2 . 2H_2O$, 180; $CuSO_4.5H_2O$, 8; $(NH_4)_6 Mo_7 O_{24}.4H_2O$, 2 ; H_2SO_4, 0.2 ml.

Table B.2

Effect of some cultivation conditions on biomass production (dry matter, g/l) by *T. inflatum.*

Carbon Source, 2% each	g/l	Initial glucose, % (w/v)	g/l
Glucose	2.96	0.5	1.87
Fructose	2.34	1	2.67
Xylose	1.32	2	2.61
Lactose	0.99	3	2.60
Maltose	3.03	4	2.63
Sucrose	1.18	5	2.68
Starch	2.36		
Initial pH	**g/l**	**Age of inoculum, d**	**g/l**
2	0.062	1	2.97
3	0.380	2	2.31
4	0.391	3	1.57
5	2.681	4	1.37
6	2.214	**Size of inoculum, % (V/V)**	**g/l**
7	0.638	1	1.54
8	0.486	2	2.48
		3	1.83
		4	1.94
		5	2.57

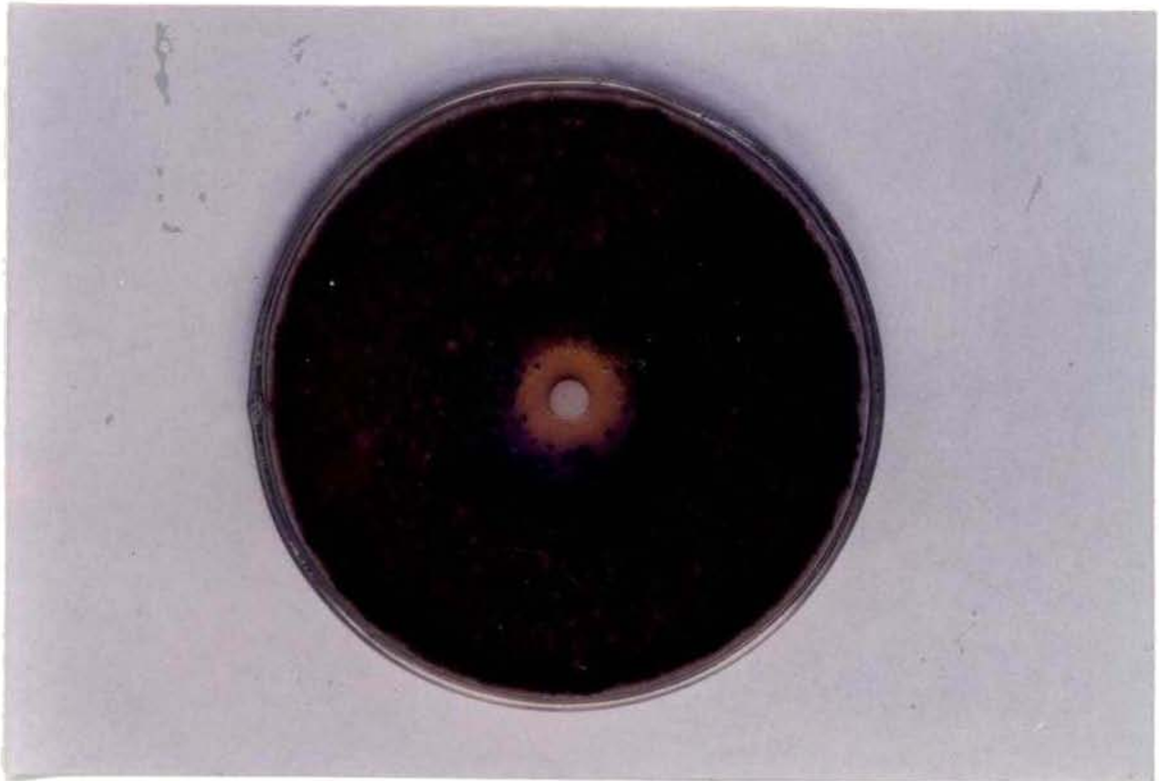


Fig B.1 : Antifungal activity of the extract of *T.inflatum* B-58 against *A. niger*

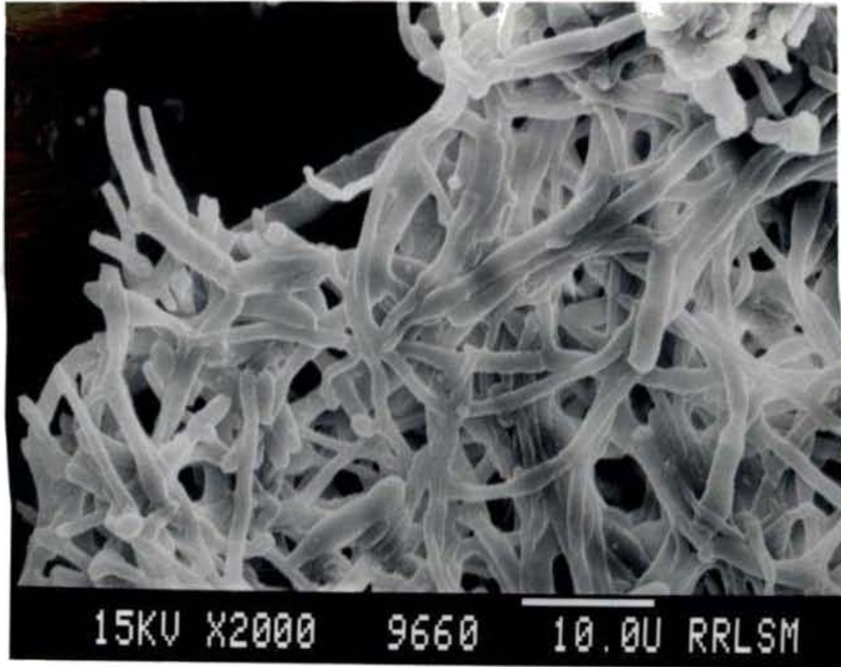


Fig B. 2: Scanning electron micrograph of *T.inflatum* grown in medium M3.

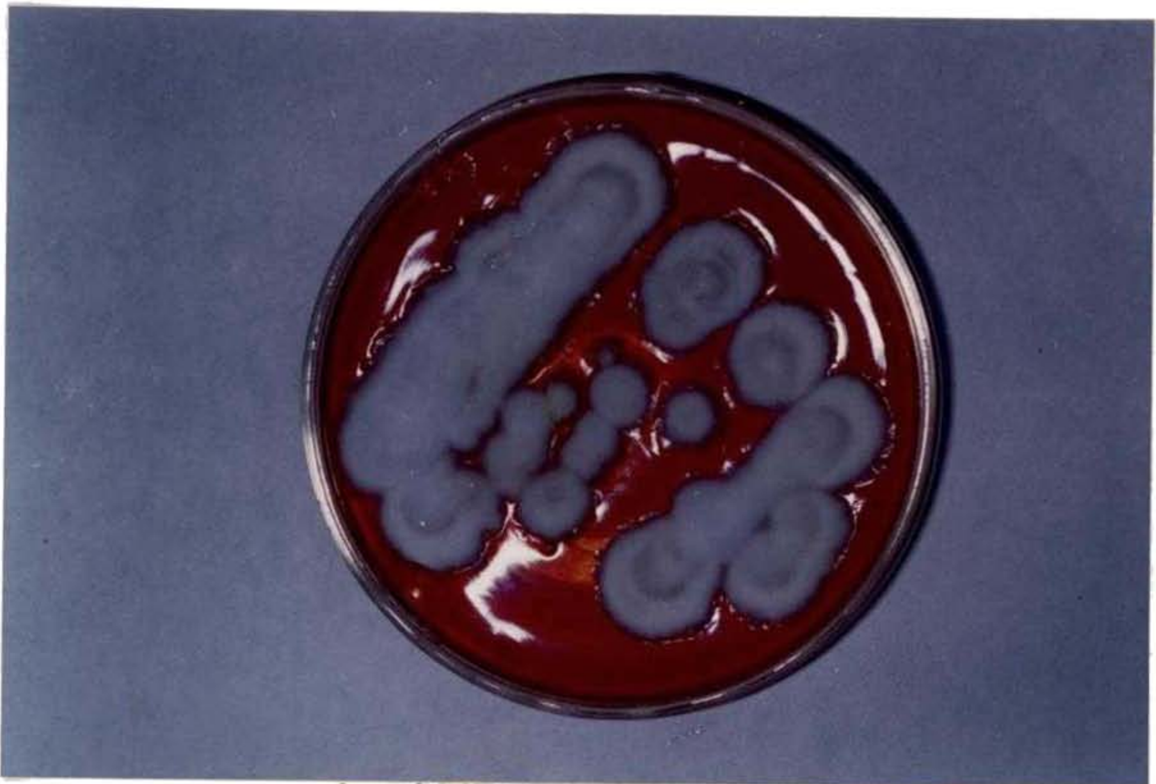


Fig B. 3: Colony of *T.inflatum* B-58 on Sabouraud Dextrose agar medium supplemented with yeast extract.

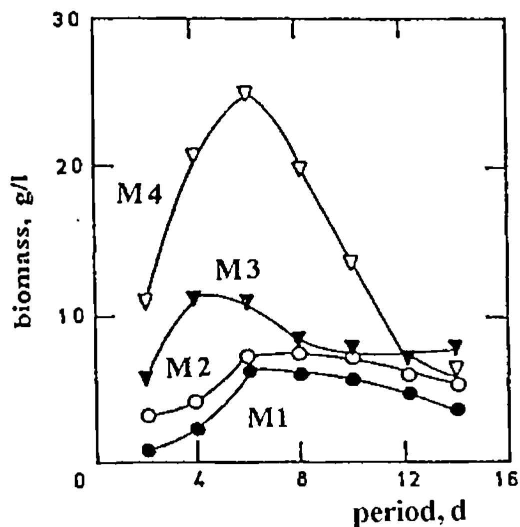


Fig B. 4: Growth of *T.inflatum* in media M1-M4

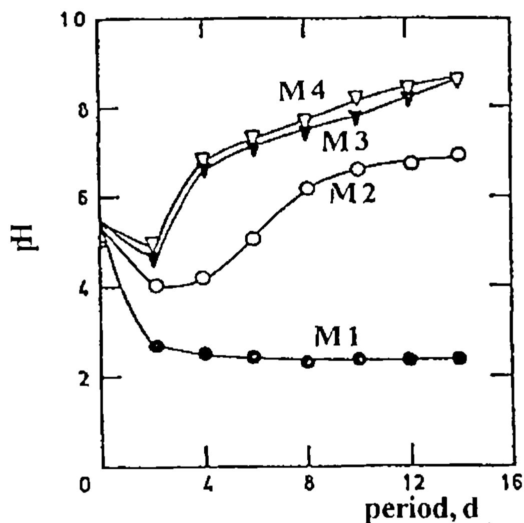


Fig B. 5: Change of the pH during growth of *T.inflatum* in media M1-M4

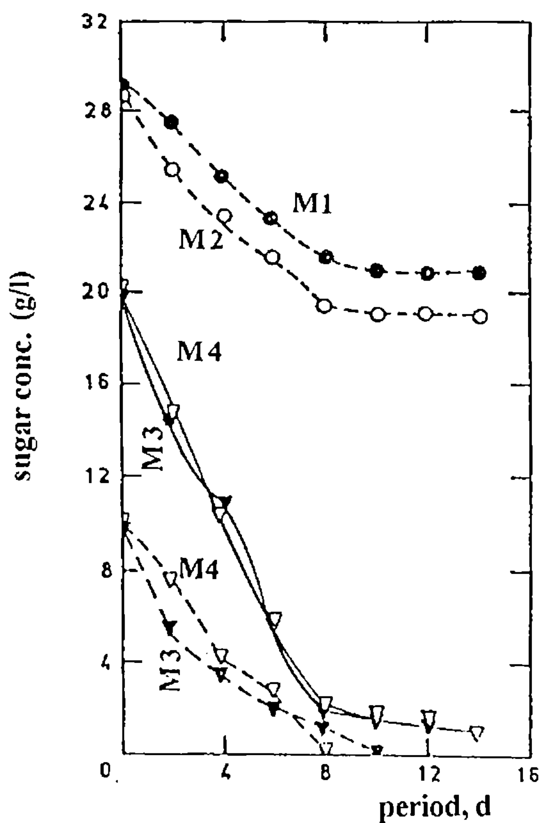


Fig. B. 6: Saccharide utilization during growth of *T.inflatum* in media M1-M4

--- consumption of reducing sugars

___ total saccharide consumption

compound based assay methods and gene induction assays (Kirsch *et al.* 1991) have all the more relevant now not only in terms of rapidity but they are powerful enough to unleash microbial products with highly specific activities from diverse localities.

Isaac *et al.* (1990) reported the isolation of *Tolypocladium* sp. from a host of habitats such as humified organic material, mite (*Mycobates* sp.) surface, humus of alpine soil, washed organic particles from alpine meadow, muskeg soil under *Pinus contorta* and water.

In the studies encompassing *Tolypocladium* sp carbon sources favouring biomass production were found to effect a different physiological state necessary for product biosynthesis (Zhao *et al.* 1991). Production and growth were not found to be identically influenced by the carbon sources in the fermentation of many antibiotics (Demain *et al.* 1983; Rêhacek 1972, 1986, 1990) and ergot alkaloids (Rêhacek and Sajdl 1990). Agathos *et al.* (1987) reported a similar scenario involving glucose and maltose exerting diverse influence in growth and cyclosporin biosynthesis. This necessitates the need to test each carbon source seperately as to its optimal concentration for cyclosporin production. There are also reports of subsequent addition of another carbon source irrespective of the initial saccharide for an enhanced CsA production (Agathos *et al.* 1987).

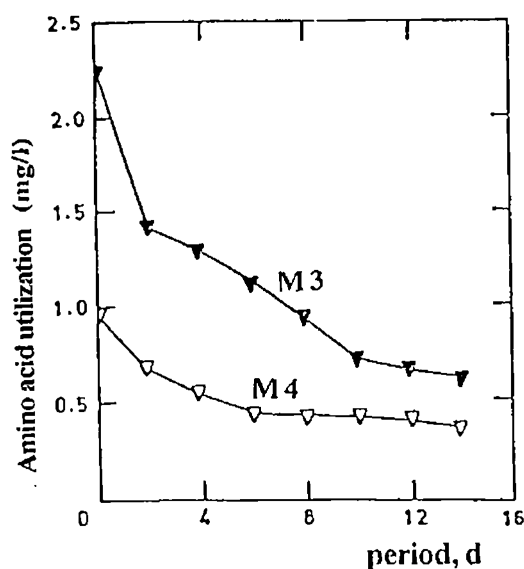


Fig. B.7: Amino acid utilization during growth of *T.inflatum* in media M3 & M4

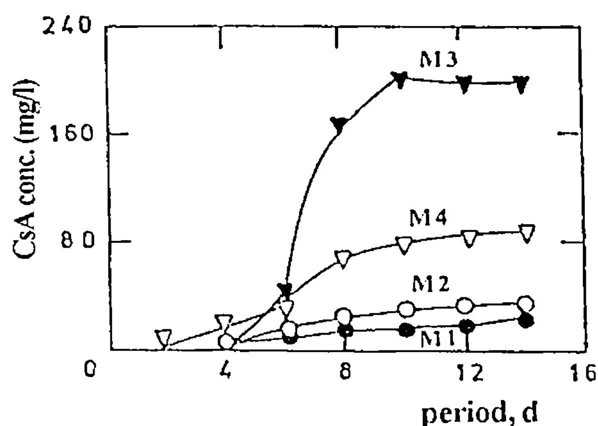


Fig. B.8: CsA level during fermentation of *T.inflatum* in media M1-M4

Among the four media tested only medium M4 could produce an enhanced biomass highlighting the role of peptone as a nitrogen source on the growth of the fungus. Aarino and Agathos (1990) reported that in *T. inflatum* fermentation pH and final cyclosporin titres were directly related to each other. Low final pH resulted in low product formation whereas higher final pH was associated with higher product titres. The complex media and the synthetic medium with the amino acid supplementation (M2) could take care of the medium pH in a desirable range indicating the role of amino acid in acting as a buffer. Similarly the complex media components also could ensure a good buffering capacity and thereby higher pH.

Amino acid addition could have some precursorial effect as advocated by Lee and Agathos (1989) in medium M2 as indicated by an enhanced product titre. Among media M3 and M4, the former could produce maximum productivity indicating the usefulness of aminoacid rich casein acid hydrolysate as a nitrogen source for cyclosporin production.

In all the four media product synthesis increased only after the attainment of maximum mycelial growth. The impact of specific incorporation of amino acids could also be visualized taking a cue from the finding by Kobel and Traber (1982) that specific production of a particular cyclosporin type could be influenced by the amino acid incorporation.

B.2 : Iron Requirements and Siderophore Search in the Producing Organism

Most of the recommended defined and semidefined growth media for *T. inflatum* contained escalating amounts of iron salts (Lee and Agathos 1989; Margritis and Chalal 1989) , yet it is not well established if iron was needed for the fungal growth. There are also reports on the little or no need for iron contrary to the usually recommended levels in many microbial strains (Archibald 1983; Bruyneel *et al* 1989; Pandey *et al.* 1994) and on the involvement of medium iron concentration in the regulation of production of siderophores, the iron-chelating compounds of microbial origin produced during conditions of iron starvation (Winkelmann 1991; Brait 1992). Therefore it was decided to investigate the iron status of the culture in a given environment.

Material and Methods

Microorganism : The locally isolated strain of *T. inflatum* B-58 was used. Refer section B.1 for details on storage, inoculation and cultivation conditions. Chemicals and reagents were supplied by M/s HiMedia, Bombay, India and Spectrochem Ltd. India.

Medium used : Modified synthetic medium of Lee and Agathos (1989) was used. It contained (in g/l).

Sucrose	30.0
$(NH_4)_2SO_4$	10.0
KH_2PO_4	0.75
Modified Trace Metal Solution (*ml/l*)	1.00
Deionized distilled water	1.0 *l*

Modified trace metal solution of Kobel and Traber (1982) had (in mg/l). $ZnSO_4 . 7H_2O$, 440.0, $MnCl_2 . 2H_2O$, 180; $CuSO_4 . 5H_2O$, 180.0; $(NH_4)_6 Mo_7O_{24} . 4H_2O$, 2.0; H_2SO_4 0.2 ml in 100 ml of distilled deionized water.

pH of the aforesaid medium was adjusted to 6.0 with 1N NaOH.

Fermentation : The influence of iron on the growth of the culture was examined by supplementing different predetermined quantities, ranging from 10 to 5000 μg/l, of iron to the above

mentioned synthetic medium by exogenously supplying different amounts (2 - 1000 μl) of $FeCl_3$ solution which was prepared by dissolving 0.5 g of $FeCl_3$ in 100 ml of deionized distilled water.

For studies involving synthetic chelating agents, liquid medium and solid medium, the composition of which was the same as that of the liquid medium supplemented with 2% (w/v) agar, were supplemented with ethylenediamine dihydroxyphenyl acetic acid (EDDHA) at different levels ranging from 20 to 1000 μg/l. For this mother solution (5% ; w/v) of the complexing agent EDDHA was prepared in deionized distilled water and sterilized by membrane filtration (Millipore filters of porosity 0.22 μ). A control culture, characterized by the iron sufficient conditions of growth, was prepared by supplementing the autoclaved media with sterile $FeCl_3$ (1M, prepared in concentrated HCl) to get a final concentration of 100μM of iron.

Agitated culturing was performed at 27 ± 1 °C in 100ml conical flasks each holding 10ml of medium and incubated in a rotory shaker (Certomat, B Brawn Biotech International, Germany). Static culturing was done in petri plates holding the solid medium and incubated in a laboratory incubator (M. B. instruments, Bombay, India) set to 27 ± 1°C.

To remove the contaminating iron from the medium, this (after reducing the pH to 4.0 with 1N HCl) was mixed with a 5% (w/v) solution of 8-hydroxyquinoline in chloroform in the ratio 5:1 and shaken well in a seperating funnel. The aqueous phase after seperation was again treated with chloroform . The pH of the aqueous phase was then increased to 6.0 prior to autoclaving. After cooling, flasks were inoculated and incubated in the rotory shaker set to the conditions described earlier. For studies on enrichment of iron this specially treated medium was supplemented with different concentration of iron (20-1000 μg/l) by supplementing with a range of 10 to 500μl of sterile 1M $FeCl_3$ before inoculating it.

Analytical Methods

Growth was determined by estimating the optical density (OD) at 610 nm in UV-VIS specrophotometer (160 A, Shimadzu, Japan) using the six day old cultures. Growth in the petri plates was measured by counting the number of colony forming units (cfu) after four days of incubation using a colony counter (Lapiz digital, Medica Instruments Manufacturing Co., Bombay).

The presence of siderophores in the supernatant obtained on centrifuging (5000 rpm for 3 minutes) the six day old culture was tested employing the Chrome Azurol S (CAS) Assay (Schwyn

and Neilands 1987). This was carried out by taking 0.5 ml of the supernatant in 8ml glass tube and to this was added 0.5 ml of CAS solution. The contents after mixing well were kept undisturbed for 1hour. The colour developed was compared with the colour produced with the standard samples of desferal (Ciba-Geigy, Switzerland).

Results and Discussion

Significant difference in the biomass concentration was observed with iron supplementation to the synthetic medium with maximum value recorded for 1mg/l of iron supplementation. i.e. 0.68 (Table B.3). In the control with no supplemented iron in it, the biomass was only 0.28 which followed a rise with the increase in the level of supplemented iron. This was not observed after 1 mg/l of iron supplementation.

Addition of EDDHA affected the growth of the fungus (Table B.4). No growth was recorded for the synthetic chelator concentration greater than 500 µg/l. The lowering of biomass with the rise in EDDHA was in line with a earlier experiment (Table B.3) which showed a clear iron dependant growth.

Biomass concentration was found attenuating in 8-hydroxyquinoline treated medium in the same pattern as that of the untreated synthetic medium on supplementation with different concentration of iron (Table B.5). But, for all similar iron concentrations, a relatively less growth was observed in treated medium as against the untreated medium as was evidenced by a maximum biomass of 0.186 reported for 1 mg/l of initial iron concentration as against 0.68 observed for the untreated culture (Table B.3). Treatment of the medium with 8-hydroxyquinoline apparently resulted removal of some other neutients from the medium, thus resulting in poor cellular growth of the fungal culture. The biomass yield with this medium was 28 times less than that obtained with original (untreated) medium. This confirmed from the fact that supplementation of iron could not revert the growth pattern to normal values (Table B.3 & B.5)

Supernatents from none of the samples on CAS assay could result positively indicating the non production of any siderophores even when the fungus was exposed to desferrated conditions. While the controls containing desferal gave an yellowish orange colour as was expected.

The results of these protocols show the significance of oligonutrients generally and iron in particular in providing conducive environment for the growth of this producing strain. This is noteworthy in studies encompassing media design strategies .

The inhibitory effect of EDDHA *T. inflatum* could be attributed to its toxicity on the culture, as there was no reversal of growth inhibition in the culture exposed to medium containing as much as 1mg/l of iron. The lowering of pH due to the addition of $FeCl_3$ solution could be another reason contributed towards this absence of growth.

Table B.3

Influence of iron on biomass concentration of *T. inflatum* in synthetic medium

Iron concentration in the medium (in µg/l)	OD (at 610 nm) after 6 days
control	0.28
10	0.39
100	0.58
200	0.60
1000	0.68
5000	0.60

Table B.4

Growth of *T. inflatum* in synthetic medium supplemented with EDDHA

EDDHA concentration in the medium (in µg/l)	OD(at 610nm) after 6 days	cfu*s after 4 days
control	0.24	31
20	0.08	17
100	0.06	11
500	0.01	nil
1000	no growth	nil
1000+1000 µg/l of Fe	no growth	nil

* *Colony forming units in agar plates*

Table B.5

Influence of iron on biomass conc. of *T. inflatum* in 8-hydroxyquinoline treated medium

Iron Conc. in the medium (in µg/l)	OD (at 610nm) after 6 days
control	0.01
20	0.012
100	0.092
200	0.162
1000	0.186

This study involving supplementation and depletion of iron assumes greater significance as it could be further extended to find the importance of oligonutrients in providing sufficiently suitable product biosynthesis environment. Salmon and Faris (1994) evidenced the negative influence of iron in the production of microcin-25 by *Escherchia coli*. In the absence of any studies on the oligonutrient requirements of *T. inflatum*, this findings from controlled iron starvation conditions is noteworthy. A concentration up to 1mg/l in the medium was found to be positively influencing the culture growth. This kind of search for siderophores must be of significant in view of they being used as drug delivery agents (Miller MJ and Malouin F 1993). Further Cheng *et al.* (1995) reported the stimulatory effect of Fe^{2+} on rapamycine production at concentrationshigher than that required for the culture growth.

B.3 : Submerged Fermentation of *T.inflatum* for the Production of Cyclosporin A

Synthesis of cyclosporins is reported to be influenced heavily by the external addition of the amino acid constituents of the molecules (Kobel and Traber 1982; Lee and Agathos 1989). Such experiments encompassing directed synthesis of CsA and its analogues in suspension cultures indicated that the composition and titre of each analogue produced were strongly determined by the externally supplemented amino acids. The precursor effect of the constituent amino acids supported the view that the multifunctional synthetases involved in the non ribosomal synthesis of peptide antibiotic and some other secondary metabolites exhibited a lack of specificity as was contemplated in some earlier studies. (Kleinkauf and von Dohran 1983; Billich and Zocher 1987; Lawen *et al.* 1989; Lawen and Zocher 1990a; Lawen *et al.* 1994).

Atttempts were made to produce CsA by fermentation in liquid medium (synthetic, SM and semisynthetic, SSM). Addition of amino acid precursors for maximum enhancement of product biosynthesis during fermentation were also studied.

Materials and Methods

Microorganism : The indigenously isolated fungal strain of *T. inflatum* B-58 was subcultured in glucose containing medium (SSM, described below). Storage, seed culturing and inoculum preparation were done the same way as is described in section B.1. Chemical and reagents used in this study were obtained from M/s HiMedia, Bombay, India.

Media composition (in g/l):

SM (Lee and Agathos 1989): pH 5.8 [adjusted with KOH(1N)]

Glucose	30.0
$(NH_4)_2SO_4$	10.0
KH_2PO_4	0.75
Trace metal solution (ml/l)	1.0*

SSM:pH 5.8[pH adjusted as in SM]

Glucose	20.0
Peptone	10.0
KH_2PO_4	5.00
Trace metal solution (ml/l)	1.0*

* *The composition of Trace Metal solution remained same as is described in section* B.1

Fermentation : Fermentations were performed in 250 ml conical flasks holding 50 ml medium and by incubating them at 27 ± 1°C at 180 rpm on a rotary shaker (Certomat MO, B Braun Biotech International, Germany). Inoculum was maintained as 5% ($^v/_v$) of the fermentation medium volume.

Fermentation media (SM & SSM) were supplemented with L-leucine, L-valine, α aminobutyric acid (a b u), D-valine, glycine, sarcosine and L-methionine (each at 4 g/l level). The effect of amino acid supplementation was investigated by providing each amino acid individually in both the media and each one in tandem with 4g/l of L-valine in SM. Using L-valine as a supplementary component, the optimal amount and time of addition recommendable for the best production were also worked out in SM. For this, fermentations were performed with initial L-valine concentration ranging from 0-12 g/l and by supplementing 4 g/l of L-valine at different time intervals ranging from 0 to 160 minutes after the commencement of fermentation respectively.

CsA analysis

Monitoring of CsA was done making use of a modified HPLC method of Kreuzing (1984). A 10 ml portion of the culture broth was extracted in each case by adding an equal volume of n-butyl acetate. The organic extracts thus obtained were flash evaporated, (Rotavapor R-124 with water bath B-480 and vaccum system B-169, Buchi, Switzerland). The resulting pasty residues were dissolved in equal volumes of acetonitrile and filtered through a microfilter (0.45 μm, Gelman) and were injected (20 μl) onto a reversed phase C8 bonded silica-gel column in a Shimadzu LC 6A high performance liquid chromatograph (Japan). The column was maintained at 60 °C by means of a column oven (CTO 6A, Shimadzu, Japan). Throughout the operation acetonitrile and water (80:20) containing 0.1% trifluoro acetic acid at a flow rate of 1 ml/min was used as the mobile phase. CsA was identified and quantified (detection at λ 210 nm) on the basis of the similarity of retention time to that of the standard CsA, used for the calibration of the system .

Results and Discussion

Of all the amino acids tested, L-valine produced the maximum enhancement on CsA production in both SM & SSM (Fig. B.9a, B.9b). The effect of this amino acid on the biosynthesis of CsA by *T.inflatum* was remarkable in SM with a 10 fold rise in productivity compared to the unsupplemented control culture (from 28 to 278 mg/l). In SSM, an increase of about 1.8 times was observed (from 180 to 324 mg/l).

L-Leucine was also found to be as effective as L-valine in supporting higher productivity mainly in SM where the CsA titre was found to be 255 mg/l as against 28 mg/l in the control culture. In SSM only a slight increase in drug titre was detected.

Glycine and abu produced comparable rise in productivity in SM and the CsA levels attained were 180 and 175 mg/l respectively. While abu maintained the positive effect it generated, in SSM also at a comparable level, glycine could not produce any positive effect on product yield in SSM.

D-valine failed to produce any rise in product synthesis either in SM or in SSM. Supplementing the medium with methylated amino acids viz. L-methionine and sarcosine only lead to a reduction in product titres both in SM & SSM. In SM, L-methionine resulted in as much as 46% inhibition in production compared to the unsupplemented control.

When added together L-valine and L-leucine lead to a dramatic increase in product titres from 26 mg/l to 480 mg/l (Fig. B.10). L-valine added at higher concentrations did not give any rise in product levels. Abu and glycine, in combination with L-valine gave slightly good productivity in comparison with their individual effects in SM. The rise in product level thus obtained were 22% and 48.6% respectively.

The stimulatory effects of L-valine was completely reversed when added along with sarcosine or methionine. Sarcosine brought down the productivity attained when 4 g/l of L-valine was added in SM, by 10 folds and methionine reduced the same by 8.75 folds.

Fig. B.11 indicates that the precursor role of L-valine was consistent after reaching a saturation level at 4 g/l of initial L-valine concentration. Further increase in precursor level did not produce any enhancement in product biosynthesis. Optimum time of addition of L-valine was found to be 20 hours after the commencement of fermentation to effect the best productivity (Table B.6). Till 46 hours after the commencement of ferementation addition of precursor aminoacid could produce a higher CsA titre than 297 mg/l, the level obtained with the addition of L-valine during the begining of fermentation.

Results of the aforesaid study clearly established the heavy influence of the external addition of constituent amino acids both positively and negatively. These results are in line with the earlier findings on CsA biosynthesis by Kobel and Traber (1982); Lee and Agathos (1989); Pandey *et. al.* (1995).

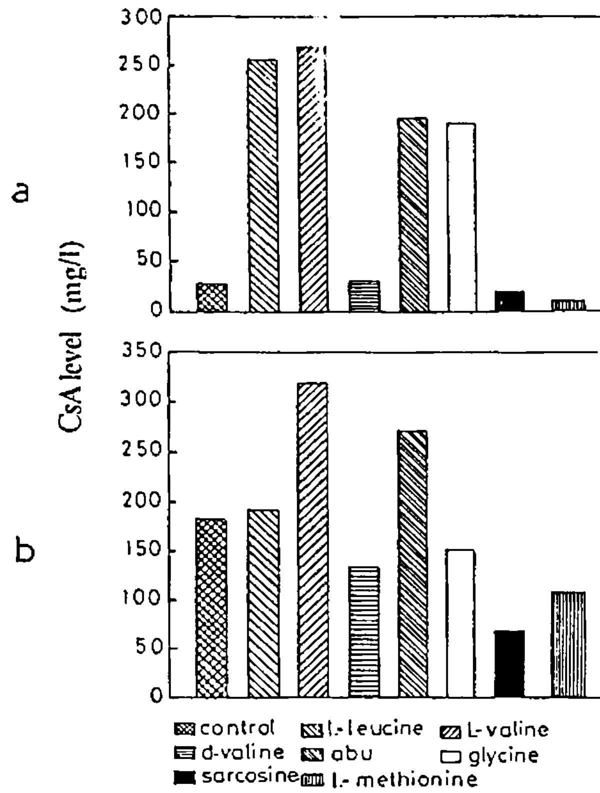


Fig B. 9: Effect of individual amino acids on CsA level in (a) SM and (b) SSM

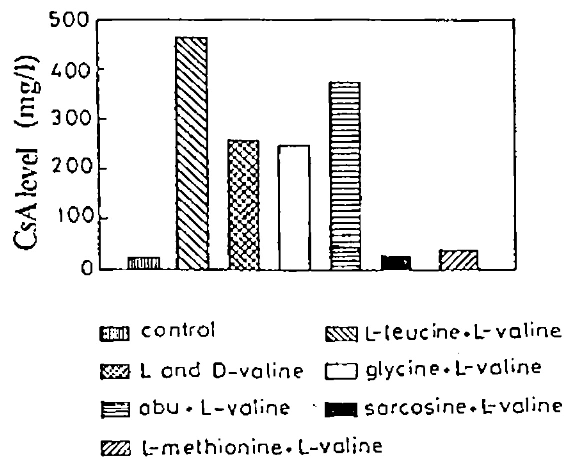


Fig B. 10: Effect of combinations of amino acids on CsA level in SM

Table B.6

Effect of time of L-Valine addition on CsA production in synthetic medium; 4g/l L-valine was added at each time. ND - not determined

Time of addition(h)	CsA (mg/l)	
	At the time of addition	At 240 h
---	ND	39
0	0	297
20	0	368
46	9	302
66	14	238
90	25	189
112	33	82
138	36	72
160	36	71

L-Valine, L-leucine, abu and glycine produced positive influence on production in SM. But the same was not prominantly pronounced in SSM with glycine producing even inhibiting effects (Fig.B.9b). This could be attributed to some means of modulation of the transport system in the cell wall by any medium component of SSM. While SM, lacks many unnecessary medium components SSM contain such complex medium components like peptone.

Stereo specific incorporation of amino acids is another aspect that becomes evident from the difference in the precursoring abilities of L and D-Valines in SM as well as in SSM. D-Valine was ineffective in producing any enhancement of biosynthesis. Haavik (1981) observed specific incorporation of L but not D-phenyl alanine in bacitracin biosynthesis by *Bacillus licheniformis*.

Methylated amino acids viz. sarcosine (N-methyl glycine) and L-methionine failed to influence biosynthesis positively, akin to the finding by Zocher *et al.*(1984) that cyclosporin biosynthesis *in vivo* was interfered by methylated amino acids. As suggested by Zocher *et al.* (1986), N-methylation could be the final step in CsA biosynthesis and that these methyl groups are derived from L-methionine via its activated form S-adenosyl-L-methionine. A feed back mechanism on this is expected to exist (Balakrishnan and Pandey 1996a). By dint of this it could be assumed that the N-methylated amino acid members of the CsA molecule could not be incorporated in to the molecule in contrast to their non methylated counterparts.

When given together, L-valine and L-leucine seem to act independently of each other indicating the possibility of different modes of action resulting in a synergistic effect on product biosynthesis. In bacitracin biosynthesis a similar scenario was observed when the two individually stimulatory amino acids L-phenyl alanine and L-histidine were used (Haavik, 1981).

The precursor role of L-valine was evident only up to 4g/l of initial L-valine conc (Fig. B.11) as was observed for L-methionine in cephalosporin C fermentation (Matsumura *et al.* 1978) and for L-leucine in bacitracin fermentation (Haavik and Vessia 1978). It was also observed that for the maximum incorporation of supplemented L-valine and hence best productivity, it is enough if the supplementation is performed during the exponential phase rather than towards the commencement of fermentation (see Table B.6) as the same would enhance secondary-metabolite production by directing the cell development towards the transcription of genes concerned (Grafe 1982).

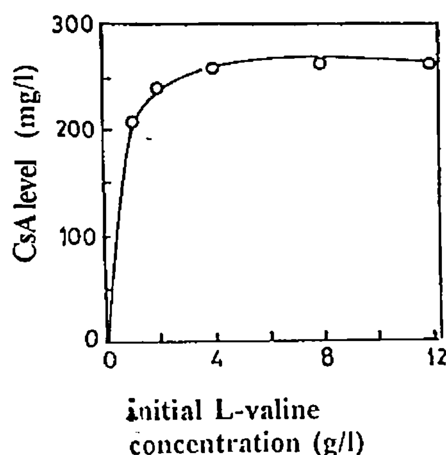


Fig. B.11 : Effect of Initial L-valine conc. on CsA yield in SM

Amino acid acting as precursor, inducer and/or developmental regulator were detected in many fermentations viz. L-methionine in cephalosporin C fermentation (Demain *et al.* 1963), tryptophan in ergot alkaloid fermentation (Krumpinski *et al.* 1976) and leucine in bacitracin fermentation (Haavik and Vessia 1978). The phenomenon of directed biosynthesis contemplated in many secondary metabolite biosynthesis especially in the case of peptide antibiotics is presumed to be due to the lack of specificity of multifunctional synthetases involved in the nonribosomal synthesis (Kleinkauf and von Dohran 1983, Lawen and Zocher 1990a). Cyclosporin synthetase, one of the most complex multifunctional polypeptides known so far, is reported to be holding a broad substrate specificity at most of its active sites (Lawen *et al.* 1989; Lawen and Zocher 1990b; Balakrishnan and Pandey 1996a) and hence a lack of specificity (Zocher *et al.* 1986; Billich and Zocher 1987 ; Lawen *et al.* 1989 & 1994).

B.4 : Solid State Fermentation of *T. inflatum* for the Production of Cyclosporin A

SSF systems, involving the growth of microbes in the absence of free water, stimulate the fermentation reactions occuring in nature (Lonsane *et al.* 1982, 1985; Pandey 1992). Though this technique, has been found to be successful over the years for the bioconversion of multitudes of agro-industrial residues in to liquid or gaseous fuels, scores of feed proteins, enzymes, organic acids (Smith and Berry 1975; Abdullah *et al.* 1985; Cuero *et al.*1985; Nigam *et al.* 1987) and other products such as oils (Jacob 1991), mycotoxins (Margaret and Damoglon 1986), aflotoxins (Massod and Rajan, 1990) etc, is limited for the production of biopharmaceuticals. A critical analysis of the literature reveals a resurgence of interest in SSF and oflate, a wide spectra of compounds, many of whose production through SSF was not even contemplated earlier were found emerging through this route (Lonsane 1994). The concentrated efforts in these direction with an aim to establish significant economic advantages over convensional submerged processes, offer a bright picture for the prospects of the production of small volume-high value products like CsA through SSF.

In this study *T. inflatum* was grown in afew solid substrates and the fermentation parameters were optimised for the production of CsA.

Microorganism : The locally isolated strain of *T. inflatum* B-58 was used for this study. Refer section B.1 for details. All the chemicals used in this study were of analytical grade. Corn steep liquor was procured from Sigma Chemical Co.., USA.

Inoculum preparation : The inocula were prepared by transferring 2% (v/v) spore suspension (spore count = 2×10^7 spores per ml) from the agar slants into SD broth held in 250 ml conical fask which was incubated in the rotary shaker for one day at 27 ± 1°C.

Fermentation Profile : Wheat bran medium made up of commercial quality wheat bran and distilled water in the ratio 40:60 was used in all experiments unless specified otherwise. Fermentation was carried out in 250 ml wide mouthed conical flasks each holding 25g wet weight, medium. Flasks were autoclaved at 121°C for 15 minutes. Initial pH of the medium was set to 6.0 uniformly in all flasks . Inoculum was added at 5% (v/w) level. Flasks were incubated for 14 days at 27 ± 1°C. Duplicate flasks were removed at every 48 hours interval for sampling.

Comparison of solid substrates to efficiently support the growth and activity of *T. inflatum* was carried out employing commercial quality rice bran and coconut cake as solid substrates besides

the wheat bran medium. Different samples of wheat bran media autoclaved for different time intervals ranging from 15 to 75 minutes were used for fermentation seperately to study the influence of time of autoclaving of the substrate on the fermentation process. To study the influence of initial moisture content (IMC) on the growth and activity of the fungus wheat bran medium with different IMC ranging from 55 to 75% was prepared by varying the wheat bran : water ratio of the media and the same were used for fermentaion. Moisture contents of the samples were determined using LP 16 infra red moisture analyser (Metter- Toledo AG, Switzerland) meant for the thermogravimetric determination of moisture.

In order to elecit the role of particle size of substrates, two different samples of wheat bran (A and B) with particle sizes $\geq$ 1mm and $\leq$ 850 μm respectively were used along with three different combinations (C, 50% each of A and B ; D, 75% of A and 25% of B; E, 25% of A and 75% of B) of these two wheat bran samples. Water activity (a_W) of the samples were measured using water activity meter (Thermoconstanter TH 2 RTD 33, Novasina, Switzerland). Different initial a_W values (0.942, 0.914, 0.843 and 0.77) were set using glycerol and water at different proportions and a_W changes along with growth and activity of the culture were followed in all cases at constant intervals.

In order to assess the role of nitrogen supplementation on CsA fermentation, different nitrogen sources viz. corn steep liquor (CSL), corn flour, yeast extract, peptone and inorganic nitrogen sources like $(NH_4)_2SO_4$ and $(NH_4)_3PO_4$ were introduced seperately each at 1% (w/w) level into the wheat bran medium. Also standerdised was a method to monitor the rate of CO_2 evolution from the flasks as a measure of biomass up to 200 hours from the commencement of fermentation using an online CO_2 monitoring device (Riken Keito, model Ri - 411A, Japan).

Analytical Methods

Mycelial growth was determined by estimating the concentration of glucosamine in the fermenting substrate (Sakurai *et al.* 1977).

Glucosamine assay : Fermented substrate was taken at 0.5g level and the same was mixed with 2.0ml of concentrated H_2SO_4. This mixture was kept at 30°C for 24 hours and the same was diluted with 36 ml of water and cooked at 120°C for 1hour. Each sample was then filtered through ordinary filter paper. The filtrate was then neutralized with NaOH (1N). A known volume (0.5ml) of the sample was mixed with equal amount of acetyl acetone reagent (1 ml acetyl acetone was mixed with 0.5 N sodium carbonate solution and made up to 50 ml). The mixture was kept in a boiling water bath for 20 minutes. After cooling it to room temperature, 0.5 ml of paradimethyl benzaldehyde

reagent (2.67 g of p-dimethyl amino benzaldehyde in a 1:1 mixture of AG ethanol and concentrated HCl and made up to 100ml) was added. 3 ml of alcohol was added to the above mixture and the tubes were kept in a water bath (Superfit, India) at 65°C for 10 minutes. The mixture was cooled to room temperature and the absorbance was read at 530 nm in UV-VIS spectrophotometer (UV 160 A Shimadzu, Japan) against a reagent blank.

pH measurements were made by a standard pH meter (Systronics µ361, Ahmedabad, India).

CsA levels in the samples were determined using a modified HPLC method of Kreuzig (1984). Fermented bran at 10 g level was processed each time for HPLC analysis as described in section-B.3. Qualitative determination of CsA in the extracts was done using thin layer chromatography (silica gel G; solvent mixture; hexane: chloroform : methanol-12.5 : 11 : 1.5).

Reducing sugars were estimated using the dinitrosalicylic acid reagent (Miller 1959) as described in section B.1 Total charbohydrate was determined by the method of Dubois *et al.* (1956) as stated in section B.1

Results and Discussion

Growth profile of *T. inflatum* on different solid substrates is potrayed in Fig.B.12 Wheat bran was found to be supporting maximum growth.. Highest biomass in wheat bran medium, 11.6 mg/gds was encountered on day six. Whereas growth was found to be prolonging up to 10th day in rice bran medium with the maximum value reaching only 5.85. Coconut cake failed to act as a good solid substrate for this culture. CsA production was also relatively higher in wheat bran medium as was indicated by the TLC spots. CsA production in wheat bran medium at different time intervals is depicted in Fig. B.13 Maximum production was contemplated on day 8. Thereafter the product profile exhibitted a slight reduction till the completion of fermentation on day 14. Fig. B.14 shows the PH profile observed during fermentation. As the reaction proceeds, the PH was found to be rising slightly towards the basic side of neutrality reaching a maximum of 7.9 on day 14. The reducing sugars were found to be consumed during the early days followed by complex sugars during the late growth phase (Fig. B.15 and B.16). On day 4 about 68% of reducing sugars were consumed but it took 10 days for consuming 67% of complex sugars.

Production of CsA was found to be slightly increasing with increasing the time of autoclaving of the substrate (Table B.7). CsA level went upto 449 mg/gds when the autoclaving of the substrate was raised upto 55 minutes. CsA production was highest at 60% of IMC (Table B.8). The product

biosynthesis was affected at higher and lower IMCs indicating the significance of the moisture level in the substrates. While 60 to 65% of IMC resulted a higher biomass of 11.34, an IMC of 55 to 60% favoured higher CsA level i.e 441 mg/gds.

Better growth and CsA biosynthesis were contemplated in a wheat bran medium prepared by equally mixing bigger and smaller brans (Table B.9). The variation in the consumption pattern of reducing sugars and complex sugars in different wheat bran media (A to E) is depicted Table B.10. The consumption level was found to be higher with smaller particle size. Both the biomass and CsA level were on the rise when wheat bran of mixed particles especially type C medium was used. Lower a_W values adversely affected the fungal growth as is indicated in Fig. B.17. With higher available free water at a_W 0.942, the biomass was 11.7 mg/gds on day 8 and same came down to 1.2 mg/gds for a_W=0.77.

Table B.7 :

Effect of autoclaving time at 121°C on the growth and CsA production by *T. inflatum*

Time in minutes	Biomass (glucosamine conc., mg/gds)	CsA titre (mg/kg wet fermented matter)
15	11.53	416
30	11.81	429
40	12.26	444
55	13.80	449
75	13.79	448

Table B.8

Effect of initial moisture level on the growth and CsA production by *T. inflatum*

Initial moisture level (%, w/w)	Biomass (glucosamine conc., mg/gds)	CsA titre (mg/kg wet fermented matter)
40-45	10.79	302
45-50	11.13	321
50-55	11.44	429
55-60	11.45	441
60-65	11.76	430
65-70	11.34	428
70-75	10.75	419

Inorganic nitrogen supplementation resulted in depleted levels of CsA production. (Fig. B.18). Corn flour, yeast extract and peptone could only enhance the growth and had no positive influence on CsA production (Table B.11). On the other hand CSL could enhance the glucosamine concentration up to 16.34 mg/gds on 8th day (Fig. B.17) and exert a positive impact on product biosynthesis by shifting the CsA titre from 428 in the control culture to 468 mg/gds (Table B.11).

Fig. B.19 show the biomass variations during fermentation as a measure of the rate of CO_2 evolution. A maximum of 136 ppm/gds was attained on 120 hours after the initiation of fermentation. The results were comparable with the conventional method of glucosamine estimation.

In the industrial exploitation of microbes, greater attention is expected to be given to culture design and standardization of the physicochemical parameters of the medium since microbes exhibit diverse patterns of nutritional and environmental requirements (Kumar and Lonsane 1990). Hence, various physical and nutritional factors were taken care of towards their optimization for obtaining good yields of CsA.

Wheat bran medium was found to be the medium of choice for CsA production through SSF. The relatively poor response of rice bran could be attributed to the fairely poor water retention capacity of starchy substrates (Oriol *et al.* 1988). Due to heavy oil content coconut cake could not act as an excellent solid support. In wheat bran medium the product titre was highest on day 8th and the same was 2.13 fold more than the productivity in liquid cultures.

As the fermentation progresses the pH of the medium was found to be on the rise and was maintained slightly towards the basic side of neutrality. Rise in pH was reported to be associated with higher product titre (Aarino and Agathos 1990). This was contemplated in suspension cultures also (Balakrishnan and Pandey 1996b).

Table B.9:
Effect of particle size of the substrate on the growth and CsA production by *T. inflatum*.

Nomenclature & type of wheat bran media used	**Biomass** (glucosamine conc., (mg/gds)	**Intensity of TLC spots**
A ($\geq$1 mm)	8.798	+
B ($\leq$850 um)	11.200	+ +
C (50% A ; 50% B)	11.316	+ + +
D (75% A ; 25% B)	9.568	+ +
E (25% A; 75% B)	11.258	+ +

Table B: 10

Percentage consumption of carbon source by *T. inflatum* in wheat bran media of different particle sizes

Type of wheat bran media used	% (w/w) of reducing sugars consumed	% (w/w) of total carbohydrate consumed
A	82.54	56.62
B	77.84	49.89
C	80.01	46.00
D	81.40	48.11
E	78.80	45.78

Table B. 11

Effect of nitrogen source supplementatioon (each at 1% (w/w) level) on CsA titre in wheat bran medium

Nitrogen sources	**CsA titre** (on 10th day) in mg/kg wet substrate
Control	428
$(NH_4)_2\ SO_4$ / $(NH_4)_3PO_4$	410
Corn flour	422
CSL	468
Yeast extract	412
Peptone	434

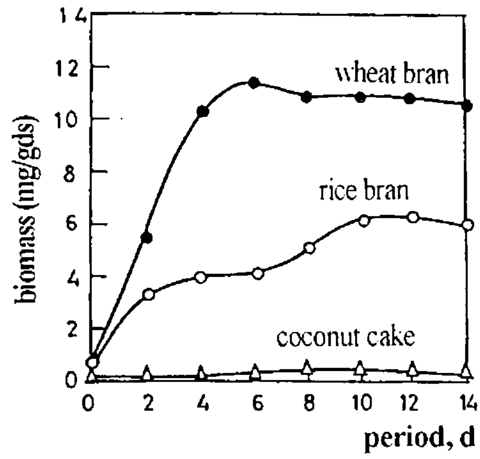


Fig B. 12: Growth profile of *T. inflatum* on different solid substrates used for fermentation.

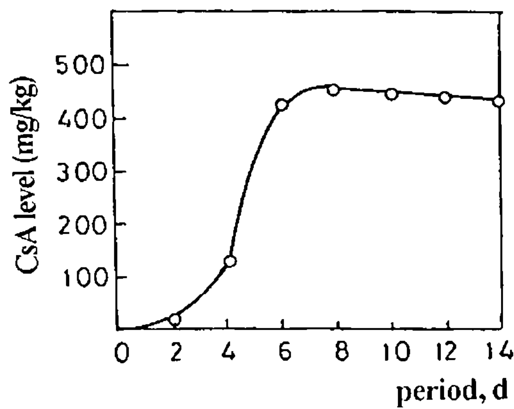


Fig B. 13: CsA production during fermentaion in wheat bran medium.

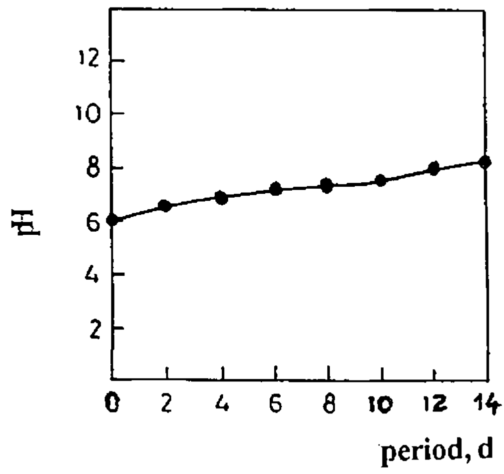


Fig B. 14: Change of the pH during growth of *T.inflatum* in wheat bran medium.

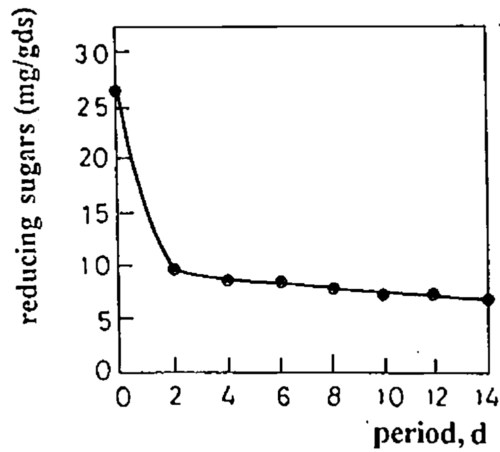


Fig. B. 15: Saccharide utilization during growth of *T.inflatum* in wheat bran medium.

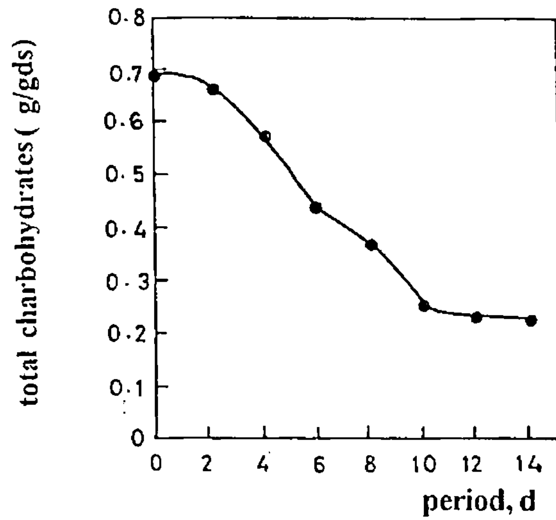


Fig B. 16: Utilization pattern of total charbohydrates during fermentation of *T.inflatum* in wheat bran medium.

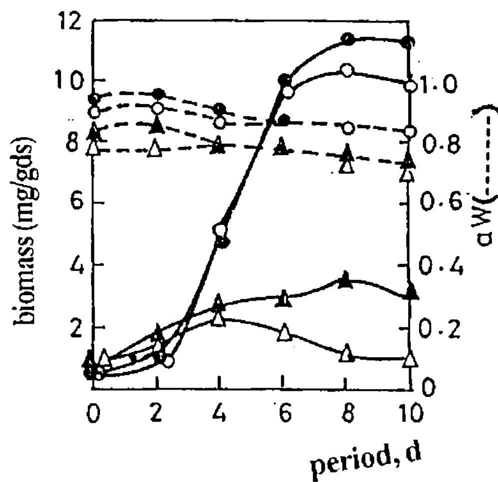


Fig B. 17: Growth profile of *T.inflatum* on wheat bran media, with different initialwater activity values, used for fermentation

● 0.942 o 0.914
▲ 0.842 △ 0.770

Growth and production were found to be positively influenced by the duration of autoclaving as the moist solid medium on prolonged autoclaving is likely to become more amenable to degradation during fermentation. This was reported earlier in gibberellic acid fermentation (Kumar and Lonsane 1990). Poor production of gibberellic acid was attributed to the insufficient modification of the substrate achieved in 15 minutes of autoclaving.

IMC was found to be another determining factor in CsA fermentation. Growth of the strain was limited for IMC less than 65%. Productivity was higher at 55 to 60% of IMC. The significance of moisture levels in SSF media on productivity of diverse products were discussed by Lonsane *et al.* (1985).

In solid cultures, the particle size of the substrate determines the void space and hence the supply of oxygen to the organism. For an enhanced mass transfer, rate of oxygen transfer into the void space is of prime significance which it is expected would be higher with bigger particles. Also, the degree of degradation is expected to be higher in particles with increased surface area (Molony *et al.* 1984). Degree of solubilization is higher in smaller particles (Pandey *et al.* 1988). Çoupling these two versions a uniform mixture of bigger and smaller brans was found to be suitable for CsA fermentation.

Another factor which has a say on the mass transfer occuring in SSF systems is water activity. Decreased a_w leads to low water availability and thereby reduced mass transfers. This contributes to the incomplete conversion of the substrate. Growth and metabolism were expected to be at stake during low a_w values in *T. inflatum* SSF also. Since water acts both as a reactant and as a vehicle for the substrate transport, it is expected that a_w might affect the enzyme transformation during fermentation and thus the drug biosynthesis.

The dwindling of production levels during inorganic nitrogen supplementation could be attributable to the poor maintenance of desired levels of pH during fermentation. Towards the end of fermentation this was detected to be 5.3 as against 8.2 in unsupplemented controls. The positive impact of CSL on CsA production could be possible by dint of the presence of various precursor amino acids in CSL. These results indicated that the low growth intensity resulted in a non-supplemented medium could not become a rate limiting step in CsA biosynthesis. But the existence of amino acids in the substrate lead to enhanced levels of product titres. These views also harmonize the findings by

Rêhacek (1972) that at the cessation of cell multiplication there is a positive relationship between CsA biosynthesis and culture viability.

The method of relating the rate of CO_2 evolution and biomass concentration assumes paramount significance in view of its ability to monitor the biomass concentration on line (Narahara *et al.* 1982 Desgranges 1991a) . More exact depiction of the physiological state of the organism during fermentation was attainable in this method rather than the glucosamine method. Though this method is comparable to other methods of biomass estimation, it appears to be bit more sensitive (Desgranges *et al.* 1991 b; Sugama and Okazaki 1979).

Higher product titres, lower waste water output, reduced energy requirement, absence of foam problem along with many other positive features that SSF enjoys over SmF in the production of bioactive primary and secondary metabolites coupled with the plenty of reports on the enhanced production of secondary metabolites in solid substrates (Ohno *et al.* 1993; Shahab 1994) indicate the feasiblity of this technique. Further our study supports the findings that certain strains could lead to better production of metabolites if they were adapted to surface growth on solid substrates.

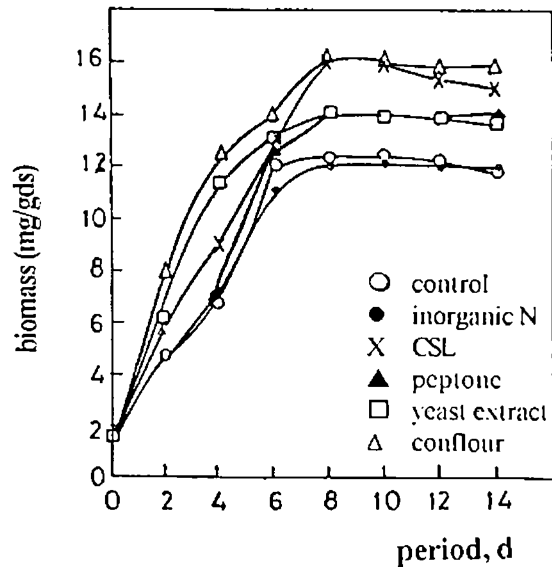


Fig. B.18 : Effect of N supplimentation on the growth of *T.inflatum* in wheat bran medium

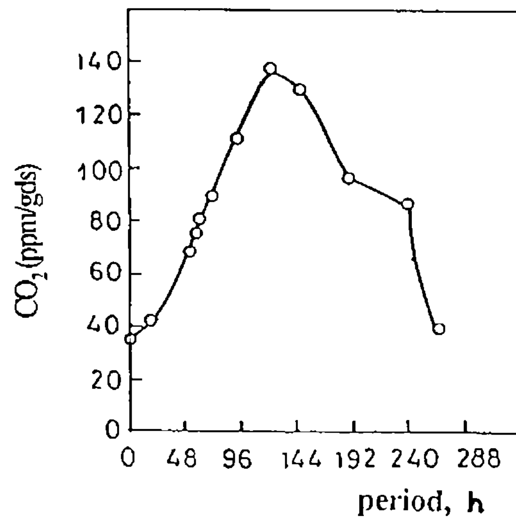


Fig. B.19 : Rate of evelution of CO_2 during fermentation of *T.inflatum*

B.5 : Cyclosporin A Production in Specially Treated Solid Substrates

Reports on the optimization of the medium constitutions for the maximization of the yield of CsA are rare amidst plenty of published data on clinical and immunological properties of this drug. Having identified SSF as an ideal route for the production of many high value - low volume secondary metabolites (Balakrishnan and Pandey 1996c) an investigation was planned to study the ability of wheat bran, the commonly used solid substrate, after some special treatments, to create a suitable production environment and hence the exploitation of such treated wheat bran media for an enhanced yield of CsA.

A defatted wheat bran medium (DWB) and a couple of wheat bran extract based media (WBE I and WBE II) were employed for this study.

Materials and Methods

Microorganism : The locally isolated strain of *T. inflatum* B-58 was used in this study. Storage and inoculation were done as described earlier in section B.1.

Media preparation for SSF : Locally purchased commercial quality wheat bran was used for this study . To obtain DWB, wheat bran was soaked in chloroform for 12-15 hours. This was reflexed for 3 hours at 50°C followed by filtration and air drying for 48 hours.

EWB I and II were prepared by soaking wheat bran in distilled water (the volume of water was 4 times the weight of wheat bran) and cooking the mix for 2 hours at 121°C under pressure. The liquid contents from the cooked material were squeezed out using muslin cloth. The filtrate (EWB-I) and the residue (EWB-II) were used for fermentation.

SSF : For every 10 g DWB 15 ml of distilled water was added and taken in 250 ml wide mouthed conical flasks for autoclaving. Inoculum (5% v/w) was added and all the flasks were incubated (MB instruments, Bombay, India) at 27± 1°C for 20 days under static condition.

EWB-I supplemented with 0.2% of agar was taken at 25 ml level in 250 ml conical flasks and at 10 ml level in petri plates. Autoclaved vessels were cooled, inoculated (5% v/v) and incubated at 27± 1°C. EWB - II was also used for SSF (25 g in 250 ml conical flasks) maintaining the foretold fermentation conditions.

Assay methods

Samples as whole flasks, in duplicate were withdrawn after every 48 hours interval. Glucosamine content of the cell wall was measured as an indication of biomass concentration (Sakurai *et al.* 1977) as described earlier in section B.4 On line measurement of rate of CO_2 evolution from the fermentation vessels were also monitored using CO_2 analyzer (Riken Keito Ri-411A, Japan) for comparison of the two methods of biomass estimation. CsA was estimated using HPLC analysis by the method of Kreuzig (1984) as described in section B.3

Results and Discussion

DWB system could support neither the growth adequately (Table B.12) nor the activity of the culture resulting in a poor CsA yield (Fig. B.20). Growth was initially very poor and it started picking up only from day 6. The exponential phase was found prolonging up to day 16 because of which fermentation was followed up to 20 days instead of the usual 14 days. On day 16, the biomass reached 10 mg/gds and it came down to 8 mg/gds on day 20. CsA production was detected in this system only from 8th day. Maximum production of 110 mg/kg wet defatted wheat bran was noticed on 15th day indicating the unsuitability of this system for the commercial production of CsA.

On the other hand, the EWB I greatly supported the growth (Table B.13) and activity of *T. inflatum* resulting in better product formation (Fig. B.21).

Though the biomass concentration initiated slowly in EWB - I system, after 4th day it raised tremendously reaching a maximum of 18.00 on 10 day (Table B.12). But in petri plates such a higher biomass was not contemplated, where highest glucosamine concentration encountered was only 6.8 on day 8. The residue of EWB I, the EWB II failed to exert a better support for the growth of *T. inflatum* (Table B.12). The growth prolonged upto day 10 reaching a maximum glucosamine concentration of 8.68. Fig. B.22 shows the comparative growth profiles of *T. inflatum* between EWB I & II as a measure of rate of CO_2 evolution. In both cases maximum physiological activity was found to be during 120 hours after the commencement of fermentation.

CsA production pattern in EWB I & II was documented in Fig. B.21. In EWB I, the product biosynthesis was found to be encouraging with the values reaching 600 mg/gram dry substrate (gds). In petry plates, the maximum value attained was only 385 mg/gds. In EWB II the production was only 18.3% of what was contemplated in EWB I.

Table B.12
Biomass concentration produced by *T. inflatum* in defatted wheat bran medium

Day	Concentration of glucosamine (mg/gds)
0	---
2	0.002
4	0.09
6	2.19
8	4.89
10	6.00
12	7.80
14	9.80
16	10.00
18	8.80
20	8.00

Table B. 13
Biomass concentration produced by *T. inflatum* in extract of wheat bran I & II

Day	Conc. of glucosamine (mg/gds)		
	EWB I		EWB II
	Flask	Petri plate	
0	00.01	0.01	0.12
2	01.49	0.69	1.39
4	03.88	2.00	4.34
6	10.18	6.80	6.07
8	14.82	6.70	8.28
10	18.01	6.00	8.68
12	11.60	5.90	6.19
14	11.11	5.90	6.00

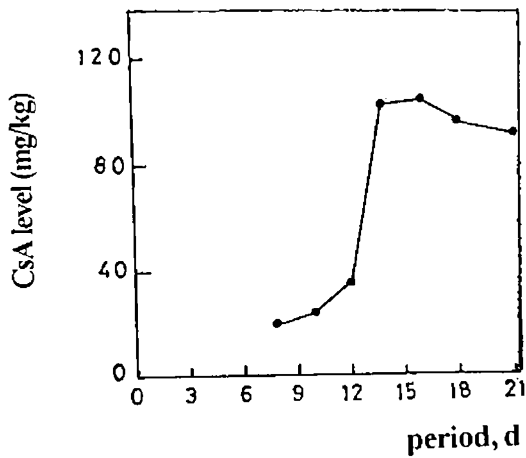


Fig B. 20: CsA production during fermentation in defatted wheat bran medium

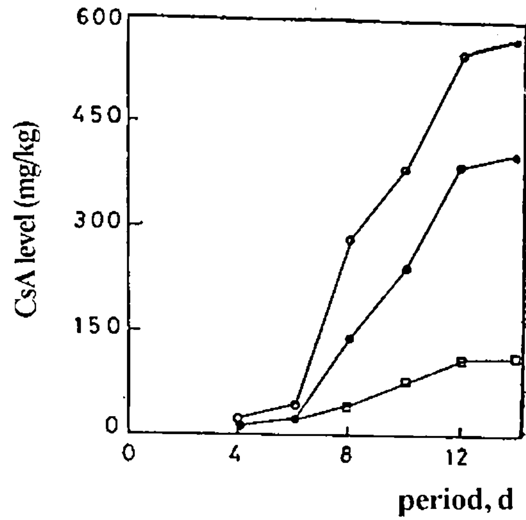


Fig B. 21: CsA production in extract of wheat bran media

○ EWBI (flasks) ●EWBI (petri plates) □EWBII

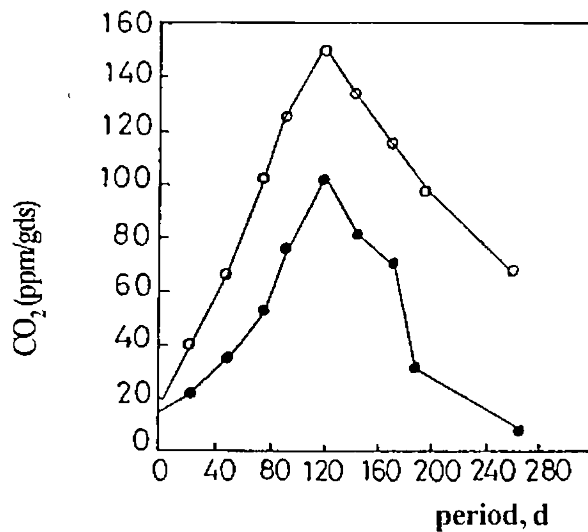


Fig B. 22: Rate of CO_2 evolution in extract of wheat bran media during growth of *T.inflatum*

○ EWBI ●EWBII

A comparison with the ability of untreated wheat bran medium to support the CsA production by *T. inflatum* (see section B.4) would underline the inability of DWB to act as a suitable substrate for CsA SSF. The idea of defatting of the solid substrate was taken up as the drug is known to be highly lipophilic and it is likely that considerable amount of the product was either not leached out during the extraction or lost due to cumbersome repetitive extraction procedures. However, during the process of defatting some of the essential nutrient ingredients of wheat bran could have been lost and hence a very unencouragable growth and activity of *T. inflatum* making the system unsuitable for CsA SSF.

Though EWB I was found to be very promising, the results in flasks were relatively good than that obtained with the same medium in petri plates. This could be due to the suitability of the flask bioreactor for the gaseous exchange and the availability of more non reaction space inside the vessel. The fabrication of suitable bioreactor taking into consideration the various features characteristic of SSF would further improve the yields considerably (Charyulu 1994).

Most of the chemical ingredients in wheat bran, it is assumed under went changes during the higher autoclaving period and they become amenable for degradation by the culture and thus offered better support than the untreated wheat bran. This is in line with the findings from Kumar and Lonsane (1990) on the influence of autoclaving time on the production of gibberellic acid.

The inability EWB II to support CsA fermentation efficiently could be due to its lower water activity (a_w) compared to that of the EWB I as most of the free and bound water were squeezed out during the extraction procedure .

Lowering of the physiological activity of the culture in EWB I & II after 180 hours is well documented in CO_2 measurement method. This method is thus sensitive than the glucosamine method where the biomass was continuously on the rise up to 10 days.

These results are highly significant in view of the current resurgence of interest in SSF and is reported at a time when there is higher inclination for the development of processes towards the enhanced production of high value microbial metabolites. Further, this appears to be a system which takes care of all the advantages that SSF employs over conventional submerged fermentation . viz higher product titres, lower waste water output, reduced energy requirements, absence of foam problem etc and the substrate treatment method is one of the least expensive way of treating a very commonly available and cheap source of substrate to arrive at higher product titres.

B.6 : Immobilization Studies

B.6.1 : Studies on the Properties of Gel Beads Used for the Fungal Immobilization

The process of immobilization of microbial cells leads to diverse advantages in any bioprocess intended to exploit such cells. They include (1) cells could be held in a fraction of the overall reactor volume, enabling materials to be produced at higher concentrations and less cumbersome downstream processing (2) changing of fluid in contact with the cells is easier (3) cells are exposed to less physically demanding environments (4) higher cell concentration could be availed for the reactions (5) possibility of prolonged periods of operation (6) lessening of the chances of contamination etc.

Gels have been most widely used for the entrapment of cells. They have higher porosities decreasing the diffusional problems. Further they do not subject embedded cell to surface forces making them sufficiently accomodated and suited for the process under exploitation (Rosevear and Lambe, 1982). The utility of entrapped cells for biotransformations and for the production of pharmaceutically active chemicals is highly promising . Hence, as a prelude to investigate the efficiency of the gel entrapped *T.inflatum* for CsA production, a study was planned to evaluate the diffusional and mechanical properties of a few gels viz. calcium alginate, agar and polyacrylamide.

Materials and Methods

The chemicals and reagents were procured from M/s SD Fine Chemicals, India

B.6.1.1 : ***Influence of alginate concentration on the properties of calcium alginate beads :*** Calcium alginate beads were prepared following the methods of Tanaka *et al.* (1984). Sodium alginate solutions of different concentrations ranging from 0.5 to 4% (w/v) each at 50 ml level was dropped into 500 ml of 1.0 M calcium chloride solution. Bovine serum albumin (BSA; MW 68 KDa) at 200 mg level was added to all concentrations of alginate solutions as molecular weight markers. The beads thus formed were cured in calcium chloride solution itself for 30 minutes followed by washing in distilled water. The beads were then transferred to conical flasks containing 50 ml of distilled water and kept on a rotary shaker (Certomat MO B Bran, Germany) (180 rpm) at 27± 1°C . Samples were withdrawn after 30 minutes of incubation and albumin level in the distilled water was worked out by the method of Lowry *et al.* (1951).

A known volume, 50 µl of the sample (water from the above experiment) was mixed with 4.5 ml of alkali mixture prepared with the aqueous solutions (in %, w/v) of $CuSO_4$, 1; sodium potassium

tartate, 2 and sodium carbonate, 2 mixed in the ratio 1:1:98 respectively. After 10 minutes of incubation, 0.5 ml of Folin's reagent was added to each tube and the colour developed after 30 minutes of incubation was read using UV-VIS Specrtrophotometer (160A Shimadzu, Japan) at 640 nm. using a standard curve prepared with different concentrations of BSA.

The diffusional property of beads was expressed in relation to the diffusion of BSA from beads prepared from the different concentrations of alginate.

B.6.1.2 : ***Influence of calcium chloride strength on the bead characteristics of alginate beads :*** Beads were prepared with 2% sodium alginate solution containing 200 mg BSA using different strengths of $CaCl_2$ solutions (100 to 1000 mM) as described earlier in section B.6.1.1. Diffusional abilities were measured after 30 minutes of incubation as mentioned in section B.6.1.1

B.6.1.3 : ***Influence of agar concentration on the bead characteristics of agar beads*** : Solutions of different concentrations of agar viz. 1 to 4% (w/v) containing 200 mg BSA were used for bead preparation following the method of Gogoi *et al.* (1994) by allowing them to fall drop by drop through a fine nostril into a hydrophobic phase. Diffusional characteristics was studied as described earlier in section B.6.1.1.

B.6.1.4 : ***Diffusional properties of various gel beads when molecules of different molecular weights were used :*** Glucose (0.18 KDa) and BSA (68 KDa) were employed for the comparison of the diffusional properties of alginate, agar and polyacrylamide beads.

Polyacrylamide beads were prepared according to the method of Woodward (1985) 1.98g acrylamide and 0.1g methylene bis acrylamide in 11ml of distilled water was supplemented with ammonium persulphate solution (15 mg in 3 ml distilled water) and 0.1 ml of tetramethyl-ethylene-diamines (TEMED). This was mixed with 6 ml distilled water containing 200mg of glucose or BSA. Cubes (3 x 2 mm) were cut from the gel after plating in petri plates and were washed in distilled water before use.

The glucose diffused out was detected using the dinitrosalicylic acid method of Miller (1959) as described in section B.1 and albumin by the Lowry's method (Lowry *et al.* 1951) as described in section B.6.1.1.

B.6.1.5 : ***Comparison of the rate of diffusion in alginate and agar beads :*** Cyanocobalamine (molecular weight 1350) was employed in 100 mg level to study the diffusion pattern from and into the beads. The beads were incubated by keeping them in a rotary shaker with 180 rpm at 27$\pm$1 °C.

The cyanocobalmine levels were measured by observing absorbance at different time intervals using UV-VIS Spectrophotometer (160A Shimadzu, Japan) at 540 nm. The rate of diffusion (D) was calculated from the following formula

$$D = \frac{\text{O.D at zero time - O.D at a particular time}}{\text{O.D at zero time}} \times 100$$

Results and Discussion

Table B.14 shows the impact of alginate concentration on the hardness and diffusional properties of bead. Size of the beads was in the range of 3 to 3.5 mm. Diffusion was quiet fast with beads produced with lower concentrations of alginate. On the contrary as the alginate concentration increased the beads tend to become hard lowering the porosity and hence a lowered diffusion of albumin (Table B.14). After 30 minutes of incubation the BSA diffused out was 89 mg/50 ml in the case of beads prepared with 0.5% alginate while the same for 4% alginate was only 38 mg/50 ml.

The effect of altering the concentration of the polyvalent cations used for the bead formation with sodium alginate on the bead characteristics are depicted in Table B.15. There is virtually no major change in the properties in terms of hardness and diffusional pattern when a range of 100 to 1000 mM of $CaCl_2$ solutions were used.

When the strength of agar solution was altered, hardness and diffusional characters got changed (Table B.16) the same way when alginate concentration was altered in calcium alginate beads ie. soft beads lead to higher diffusion. Size of the beads remind the same i.e 3 to 3.5 mm.

Table B.17 quantitatively describes the efficiency in terms of better diffusional property among 2% (w/v) alginate beads, 2%(w/v) agar beads and polyacrylamide granules. For the small molecule like glucose the diffusion was relatively faster in alginate and agar beads with polyacrylamide showing less glucose diffused out in the given period of time. In 2 hours almost all glucose was diffused out in alginate beads. In agar and polyacrylamide beads it was only 192 and 154 mg/50 ml respectively. But among alginate and agar the former showed good pattern of diffusion while in the latter, there is earlier levelling off of the values (Table B.17).

In the case of big molecules such as BSA, the diffusion is slow in all the three cases with polyacrylamide trailing far behind. The levelling off of the figures could be noted little earlier in agar beads as was the case with glucose diffusion also. But in alginate beads periodic response in diffusion was noticed (Table B.17).

Table B.14

Effect of alginate concentration on the nature of calcium alginate beads

concentration of alginate solution (%, w/v)	Bead Characteristics	
	Hardness	**Nature of diffusion** (mg BSA/50 ml)
0.5	soft	89
1	partly niggard	73
2	firmly mild	62
3	very firm	51
4	fully obdurate	38

Table B.15

Effect of strength $CaCl_2$ solution on the nature of calcium alginate beads

Strength of $CaCl_2$ solution (mM)	**Bead Characteristics**	
	Hardness	**Nature of diffusion** (mg BSA/50 ml)
100	firmly mild	63
200	firmly mild	62.6
500	firmly mild	62.4
1000	firmly mild	62.4

Table B.16

Effect of concentration of agar solution on the nature of agar beads

Strength of agar solution (%, w/v)	**Bead Characteristics**	
	Hardness	**Nature of diffusion** (mg BSA/50 ml)
1	soft	84
2	slightly firm	74.6
3	firmly mild	50
4	very firm	48

•

Table B.17

The diffusion pattern of glucose and BSA in beads of alginate, agar and polyacrylamide

Time of incubation (minutes)	Glucose (mg/50ml)			BSA(mg/50 ml)		
	alginate	agar	polyacry-lamide	alginate	agar	polyacry-lamide
0	---	---	52.0	---	---	19.5
30	186.0	185.7	150.0	61.9	79.3	60.2
60	193.5	191.0	153.6	87.5	120.0	76.3
90	196.5	191.7	153.9	111.2	128.5	92.6
120	198.2	191.9	154.1	130.6	129.0	109.0

Table B.18

Rate of diffusion of cyanocobalamine in alginate and agar beads

Time of incubation (minutes)	Alginate beads		Agar beads	
	from beads	into beads	from beads	into beads
0	---	---	---	---
5	68.0	48.0	63.9	36.0
10	76.8	65.0	71.4	44.0
15	88.0	71.0	74.0	53.0
20	89.6	77.0	78.0	60.8
25	90.0	78.0	78.6	62.0
30	90.0	78.4	78.9	62.8

The rate of diffusion of cyanocobalamine both inward and outward the beads show more periodic change with time in 2% alginate beads rather than in 2% agar beads (Table B.18) indicating the suitability of alginate beads for the proposed study. In alginate beads maximum diffusion from the beads (90.0) was noticed in 25 minutes itself but even after 30 minutes of incubation only 70% of what diffused out from the alginate beads only could move out from the agar beads. Diffusion into the beads was also good in alginate beads as was indicated by the difference in the cyanocobalamine diffusion into these beas which was 78.4 for the former and 62.8 for the later after a period of 30 minutes.

Mass transport of the substrates and products has been adjudged as the most important of the properties of the microbial cells getting altered during their immobilization to a natural or synthetic carrier (Norton and Amor 1994). Hence, it is an important prerequisite that pore size and hardness of the beads which in turn depend on the viscosity of the carrier were optimized initially to ensure the required diffusional and mechanical properties of the beads (White 1960; White and Dorion 1961; Gogoi *et al.* 1994).

The present study convinsingly established the superiority of alginate and agar beads over polyacrylamide beads. Diffusional limitations encountered in polyacrylamide immobilization by dint of the smaller pore size in the gel. This kind of limitation were not contemplated in alginate beads just because of the relatively larger pore size (Scot 1987; Smidsrod and Skjak 1990).

This study clearly established the significance of the concentration of alginate and agar solutions used for entrapment on the porosity and hardness of the gel beads. The inability of the concentration changes of the polyvalent cations of $CaCl_2$ solutions in influencing the bead characteristics were also observed. Considering the relatively better time dependant diffusion of molecules in alginate beads over agar beads the former was chosen for the further studies on fungal entrapment. For packed bed reactors (PBR) and continous stirred reactors, by dint of their better diffusional and mechanical properties, 2% alginate has largly been the universal choice.

This study also stressed the relatively free movements of low molecular weight substrates in comparison with the high molecular weight substrates which require more time and was not freely moving into Cyanocobalamine having almost the similar molecular weight as that of CsA, on rate of diffusion trials gave a vivid picture of the likely better results obtainable with 2% alginate beads.

B.6.2 : Cyclosporin A Production by Immobilized *T. inflatum*

Many advanced bioreactor systems employing immobilized cells have been used successfully over the years and the same have been unleashing impacts aplenty by dint of their far reaching therapeutic and analytical applications (Kolot 1983; Koshcheyenko *et al.* 1983; Vandamme 1983; Koshcheyenko and Sukhodolskaya 1985; Karube *et al.* 1984). PBR of immobilized cells have the advantage of simplicity of operation, high mass transfer rates and higher reaction rates. This seem to be promising and worth trying for CsA fermentation in view of the relatively lower yields contemplated through many other routes viz. submerged fermentation (Zhao *et al.* 1991; Agathos 1987; Balakrishnan and Pandey 1996b) solid state fermentation (Pandey *et al.* 1995) and immobilization (Foster *et al.* 1983). Thus this investigation was planned with an aim on the development of a PBR system filled with immobilized mycelia or spores (biocatalyst).

Materials and Methods

Microorganism : The locally isolated strain of *T. inflatum* B-58 was used in this study. Storage and maintenance of the culture and inoculum development were done the same way as described in section B.1. The chemical were from M/s Qualigens Ltd., & HiMedia, India.

Culture growth : The organism was cultivated in 250 ml conical flasks each holding 50 ml medium with the following composition (in g/l) and pH 5.6

Glucose	3.0
Maltose	2.0
Peptone	4.0
Glycerol	3.0
Malt extract	2.0
Casein acid hydrolysate	2.0

The flasks were incubated in rotary shaker at 150rpm and 27±1°C for 10 days. The mycelia were harvested by centrifugation (cooling centrifuge C-24, Remi Instruments, India) at 10 °C and 7000 rpm for 15 minutes followed by washing with normal saline.

Immobilization : Different entrapment materials viz. calcium alginate, agar and polycrylamide were used for immobilizing the mycelia. Mycelial entrapment in calcium alginate was done according to the method of Tanaka *et al.* (1984) Mycelia at 25% (wet weight) level were uniformly mixed with

sodium alginate solution (2%, w/v) and this mixture was dropped through fine nozzle into calcium chloride solution to obtain beads of 3 to 3.5 mm size (Fig. B.23).

The beads containing about 0.25g mycelium per ml of alginate solution were cured by allowing them to remain in calcium chloride solution for 30 minutes after which they were washed with sterile distilled water repeatedly.

Immobilization of mycelia in agar was done following the method of Gogoi *et al.* (1994). Mycelia at 25% (wet weight) level were mixed with the sterilized aqueous solution of agar (2% w/v) at 41°C and the mixture was placed drop wise at room temperature quickly onto a hydrophobic phase to get spherical beads of 3 to 3.5 mm size. Beads contained the same mycelial concentration as in alginate beads. Beads were repeatedly washed in normal saline before use.

Granules of polyacrylamide (2 x 3mm) containing fungal mycelia were prepared employing the method of Woodword (1985). To every 6ml of mycelial suspension, containing 1.5 g (wet weight) of mycelia, in order to maintain the same cell concentration as in the above two cases of entrapment, the monomers were added in the following concentrations

Acrylamide	1.9 g.
Methylene-bis-acrylamide (MBA)	0.10 g

both dissolved in 11 ml of distilled water followed by ammonium per sulphate (15 mg in 3 ml of distilled water) for inducing polymerization and tetramethyl-ethylene-diamine (TEMED) (0.1 ml) as a polymerization catalyst.

For spore immobilization, the fungus was cultivated for 7 days in a medium of the following composition (in g/1) and pH 5.6

Malt extract	30.0
Yeast extract	04.0

spores were harvested by centrifuging (cooling centrifuge, C24, Remi Instruments, India) at 10°C and 8000 rpm for 30 minutes. Spores after washing with normal saline were entrapped (0.2% wet weight) in 2% alginate as described above. Such immobilized spores were allowed to germinate by growing them in a medium of the following composition (in g/l) and pH 5.6.

Glucose	20.0
Casein acid hydrolysate	20.0
Peptone	10.0

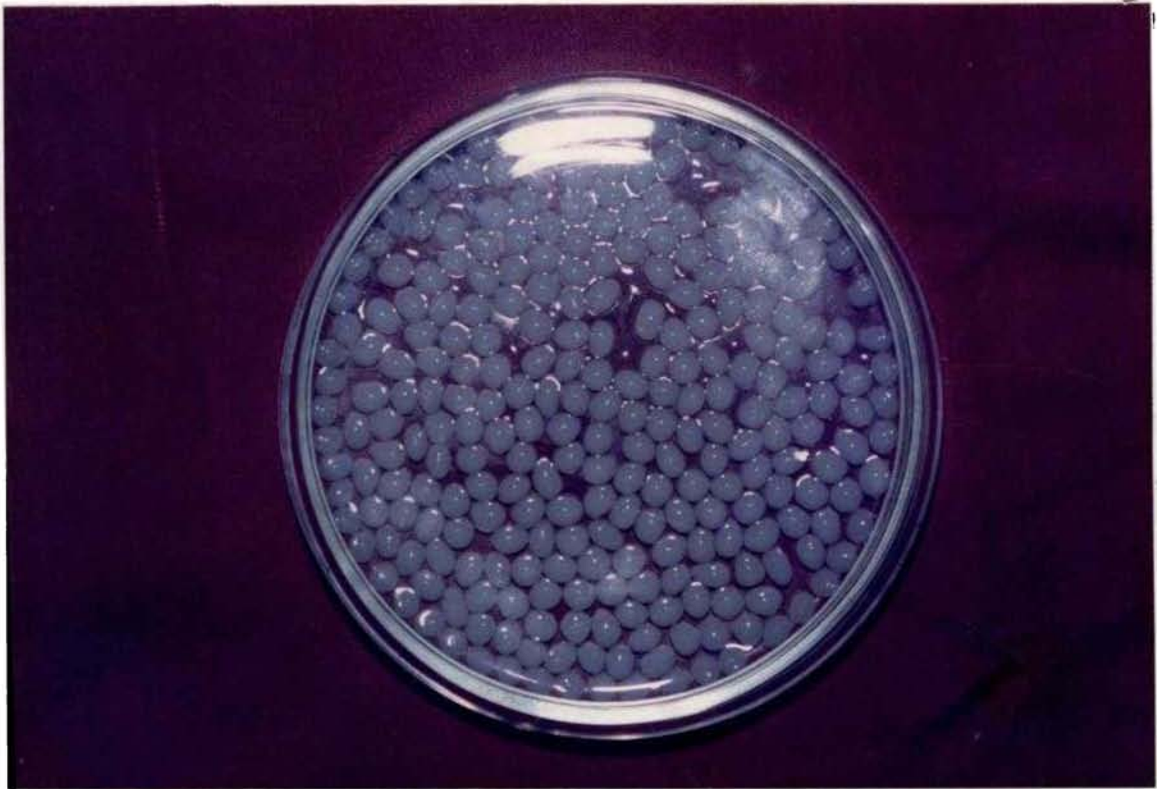


Fig. B.23 : Calcium Alginate Beads used for the Entrapment of *T. inflatum*

This medium (100 ml) was held in 500ml conical flasks. The flasks were incubated in a rotory shaker (100 rpm 27± 1°C). The beads containing immobilized cells or spores of *T.inflatum* (before packing the column) were filtered using filter paper and washed with normal saline.

Scanning electron micrograph of the growing mycelia inside the beads was obtained from a cross section of the 5 day old bead using the procedure discussed in section B.1.

Packing the bioreactor and feeding : The set up of PBR (Fig. B.24) consisted of an air pump (1), an air filter unit (2), a medium reservoir unit (3), a peristaltic pump (4), a glass column (5) (volume 123cm^3, length 25 cm, diameter 2.5 cm) and a mixing chamber (6). For packing the bioreactor approximately 550-575 beads were taken and poured into the column with water and later water was allowed to drain out. For batch mode of operation, medium was added to the column up to the level of beads and incubated under stationary condition at 27±1°C. PBR was operated under continuous mode also. This was attained by medium (substrate) recycling. Medium was pumped into the column in reverse flow mode (ie, from bottom to top flow) using the peristaltic pump (Eyela Microtube Pump MP-3, Tokyo Rikakikai Co. Ltd., Japan) at a flow rate of 0.5 ml/minute. The fermented medium collected from the outlet of the column (after quantification of CsA) was allowed to mix equally with the fresh medium from the reservoir and pumped into the reactor so as to make the feeding flow rate equal to the recycling flow rate, ie. 0.5/2 = 0.25 ml/minutes This was followed up to 24 hours with sampling at every 4 hours (ie. 24/4 = 6 cycles).

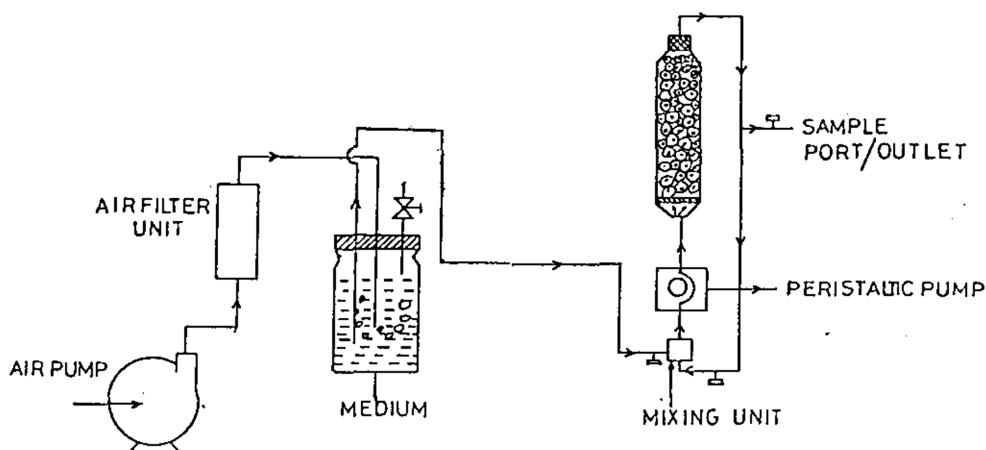


Fig. B.24. Schematic set-up of the packed bed bioreactor system comprising the immobilized *T.inflatum* for the production of CsA

To test the influence of constituent amino acids of CsA molecule, they were supplied to the medium at different concentrations and also in different combinations. Refer Table B.19 for the composition and nomenclature of each medium. For this 10 different media were fed in to the reactor separately. PBR was operated twice for each medium and results reported are the average values.

To monitor the effect of the concentration of biocatalyst, the number of beads in the PBR was altered to 420-440 and 710-730 besides the usual 550-575. This was tried in batch mode keeping the amount of the fermentation medium in the reactor constant.

Under recycle mode besides the usual flow rate (ie. 0.5 ml/minute), two additional flow rates 0.4 and 0.8 ml/minute were chosen to study the effect of contact time (time allowed for medium components to remain in contact with the immobilized cells) which for the recycle mode was tx-t0. Where t0 is the time of entering of the medium in the reactor, tx is the time of medium coming out from the reactor for one cycle. CsA was estimated using HPLC as described in section B.3.

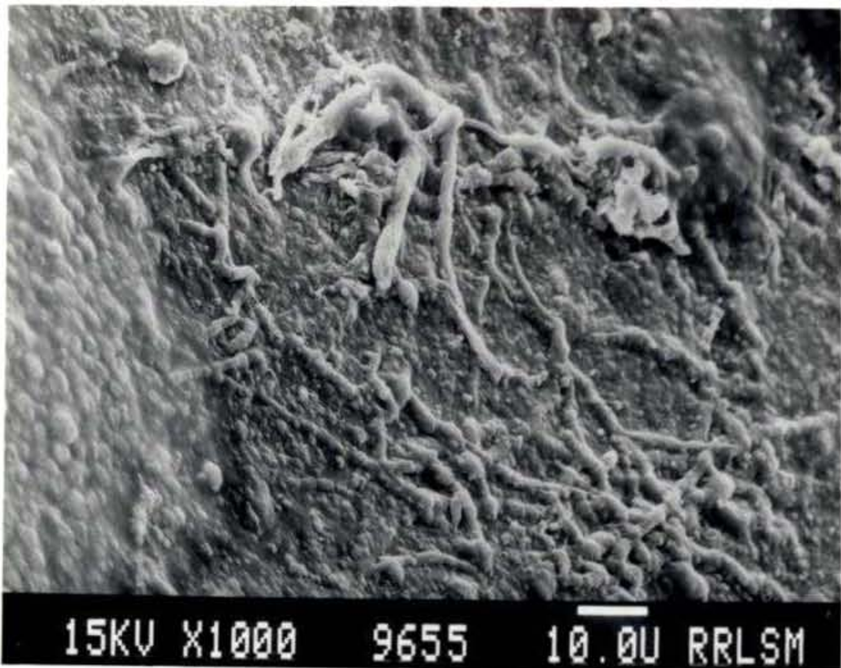
Results and Discussion

The yields of CsA after 24 and 48 hours of incubation in PBR under batch mode while packing with various entrapping materials is given in Table B.20. Alginate topped the list with 28.8 and 49.3 μg/ml of CsA.

Table B.19 shows the effect of different biotransformation media (I to X comprising different precursor amino acids in different concentrations and combinations) on the yields of CsA in PBR under batch mode. Positive influence of various amino acids on the yield are well documented both during 24 and 48 hours from the commencement of the reaction. Increasing the concentration of amino acids (with the exception of methylated amino acids) had a positive effect on the product biosynthesis especially with L-valine and L-leucine. L-valine resulted in 11 fold rise in productivity when an additional 1 g/l of it was supplied to the control culture and the rise was 13.5 folds when L-valine concentration was increased to 4 g/l in 24 hours. At 48 hours the same were 12 and 13 folds respectively. For L-leucine the rise in CsA titres were 11 and 12.5 folds at 24 hours and 11.7 and 12.8 folds at 48 hours for additional rise in L-leucine levels by 1 g/l and 3 g/l respectively. But increasing the concentration of these two amino acids together could not lead to a synergistic effect on product yields. Methylated amino acids brought down the product level as evidenced by the reduction to 23.0 from 28.0 and to 41.2 from 50.0 for 24 and 48 hours respectively when the medium was supplemented with 1 g/l of sarcosine and methionine. Increasing the concentration of the catalyst beyond a point served no better for CsA biosynthesis (Table B.21). The ideal biocatalyst concentration in constatant level of reaction medium in the PBR was found to be 0.06 which resulted 28 μg/ml of CsA in 24 hours.

Table B.22 shows the yields of CsA in PBR under continuous mode . At the end of each cycle both the mycelium and spore immobilized systems gave comparable levels of product yields. There was a rise in product titre with the increase in substrate recycle. Fig.B.25a&b show the scanning

Fig B. 25: Scanning electron micrograph of the cross section of cacium alginate bead with entrapped *T.inflatum* spores
(a) 0 hour bead



(b) 5 days old bead

electron micrograph of the cross section of spore immobilized beads immediately after immobilation and after 4 days of growth respectively. After four cycles the yield increased from 24.6 to 58.3 μg/ml for mycelial immobilized beads and from 20.3 to 56.6 μg/ml for spore immobilized biocatalysts. As is evident from Table B.21, higher flow rate (0.8 ml/minute) was not enhancing cyclosporin biosynthesis.

Microbial cells during immobilization in a natural or synthetic carrier are tend to be altered to a considerable extent with respect to many properties. The most important of the bioprocess parameter expected to get altered due to entrapment is the mass transport of the substrates and products (Norton and Amor1994). Of the three entrapment materials tried initially, calcium alginate supported better productivity indicating the suitable mechanical and diffusional properties of 2% alginate gels. It has been reported that alginate gels generally have relatively larger pore size and the diffusional limitations encountered with polyacrylamide gels due to small pore size are not encounted here (Scot 1987; Smidsrod and Skjak 1990).

Alginate beads are reported to be having no harmful effects on the biosynthetic machinery (Brodelius *et al.* . 1979). These kind of mycelial immobilized biocatalysts are preferred over enzyme immobilization as the latter can not easily be used for reactions involving cofactors (ATP, SAM etc) or multi-enzyme systems (Varlop and Klein 1982).

von Wartburg and Traber (1986) and Lee and Agathos (1989) reported the efficiency of exogenous feeding of amino acids precursors in positively influencing CsA fermentation. In an early study (Balakrishnan and Pandey 1996b) L-valine and α–amino butyric acid were found to be enhancing CsA levels in submerged cultures. Under entraped conditions this precursor role was more pronounced. While in liquid cultures, the rise in product titre was about 75% in SSM and 10 in SM (see section B.3), during immobilization the rise was of the order of 13 times with an initial concentration of 4 g/l of L-valine and for 4 g/l L-leucine, 12 times increase in entrapped condition was contemplated as against 8 times rise in SM and almost nil in SSM. This indicated the suitability of the immobilized reactors for the optimum conversion of externally supplied amino acids and enhanced CsA production over liquid fermentation. There are, however, reports of non-utilization of all constituent amino acids for CsA production and hence it could be assumed that higher levels of constituent amino acids especially L-valine and L-leucine might act as inducers or activators of the cyclosporin synthetase complex and might induce the specific incorporation and thus the production of particular cyclosporin type (Chun and Agathos 1989; von Wartburg and Traber 1986). While methylated amino acids failed to enhance CsA biosynthesis as was observed in suspension cultures (see section B.3) Zocher *et al.* (1984; 1986)

suggested N-methylation as a final step in CsA biosynthesis and N-methylated amino acid members of CsA molecule could not be incorporated in to the molecule *in vitro* . A feed back mechanism involving L-methionine is said to be involved in the methylation of constituent amino acids in CsA.

The CsA yield was more during substrate recycling. Better utilization of substrates was expected to occur on recycling the medium. During recycling a portion of the outflow was allowed to mix with the inlet stream of the reactor. Permitting relatively good fluid velocities was expected to provide the impetus to over come the bulk mass transfer resistance, if at all any, to the transport of substrate to the catalyst surface. On the other hand, if the reaction rates are expected to be slow, higher flow rates could be adversely affecting the productivity. Taking both into consideration it was decided to give the necessary bulk mass transfer coefficient values.

Table B. 19

Biotransformation media used and concentration of CsA obtained in PBR under batch mode of operation

Media	Composition	CsA titre (μg/ml)*	
		Period (hours)	
		24	48
M1	Glucose, 200 mg/L and amino acids viz. L-valine, L-leucine, α-aminobutyric acid, L-glycine and DL-alanine, 1 g/l each; pH 7.5	28.0	50.0
M2	M1-L valine and L leucine	16.8	32.0
M3	M2+ 1g/l each of all amino acids of M2	32.6	60.2
M4	M1 + 1g/l of L-valine	311.2	611.0
M5	M4+ 2g/l of L-valine	379.0	678.2
M6	M1+ 1g/l L-leucine	313.6	588.0
M7	M6+2g/l of L-leucine	350.8	642.2
M8	M1 + 1 g/l each of L-valine and L-leucine	326.0	685.0
M9	M8+ 2g/l each of L-valine and L-leucine	435.6	697.8
M10	M1+1g/l each of sarcocine and L-methionine	23.0	41.2

* *Standard deviations* $\pm$ *0.62*

Table B. 20

Comparison of the effectiveness of different encapsulation methods in CsA biosynthesis in a PBR under batch mode

Gelling agents	**CsA titres** (μg/ml)*	
	Period (hours)	
	24	48
Calcium alginate(2%; w/v)	28.8	49.3
Agar (2%; w/v)	20.8	43.6
Polyacrylamide	11.0	19.6

* *Standard deviations ± 0.52*

Table B. 21

Influence of the amount of the catalysts per unit volume of the reaction medium in CsA production in a PBR under batch mode of operation

Number of beads used for packing the column	**Conc. of the catalysts in reaction medium** (g/ml)	**CsA titres at 24 h** (μg/ml)*
420-440	0.045	16.0
550-575	0.060	28.0
710-730	0.075	20.6

* *Standard deviation ± 0.98.*

Table B.22

CsA yield in PBR under continuous mode of operation

Cycle No. (4 hours each)	CsA titre (μg/ml.)*			
	Yield at flow rate 0.5 ml/minute		Influence of flow rate in mycelium immobilized beeds	
	mycelium immobilized	spore immobilized	0.4 ml/minute	0.8 ml/minute
1	24.8	19.0	27.0	22.0
2	40.0	35.0	45.0	37.0
3	53.0	46.0	58.2	49.0
4	56.0	53.0	62.2	52.0
5	56.2	53.2	62.2	51.0
6	56.2	53.2	62.0	51.5

* *Standard deviation* $\pm$ *1.30*

Hence, it was decided to maintain a required flow rate which was not very high. In this PBR it was significant and advisable to maintain a relatively lower recycling rate so that ultimately it could maintain the required hydraulic retention time in order to achieve the desired rate of conversion. For better yield of CsA, the contact time of the media components with the biocatalysts was found to be increased. This was evident from the positive role of lower flow rate and not so high concentrations of the catalysts in product titres.

B.7 : Selection of Morphological Variants & Yield Improvement Trials

A number of techniques varying in cost and complexity are at the disposal of the investigators concerned with the construction of improved industrial producers. Use of mutagenic agents to modify the genome of industrial microorganisms inorder to obtain improved strains exhibiting better production has been practised largely for the genetic improvement of the wild strains. Classical mutagenesis involves two stages viz. treating of the culture with the mutagenic agent and screening of the mutagenized culture followed by the isolation of the mutated clones (Spencer and Spencer 1994).

In this study the wild culture of *T. inflatum* was exposed to UV and X ray irradiations separately with an aim of isolating strains which would be better producers of CsA. Survival curve for *T. inflatum* was worked out for varying exposure times.

Materials and Methods

Microorganism : The locally isolated strain of *T. inflatum* B - 58 was used for this study. Refer section B.1 for details. HEPES buffer was obtained from Aldrich Chemical Co., USA. The other chemicals were of analytical grade.

Mutation : Spore suspension, in sterile distilled water (10 ml) containing tween at 0.05% level, prepared by scrapping the agar slant with a loop was filtered through a column of glass wool to remove the mycelial fragments. The filtrate at 2% (v/v) level was added to 50 ml medium of the following composition (g/l) and pH 5.6 held in 250 ml conical fasks.

Glucose	20.0
Peptone	10.0
Casein acid hydrolysate	10.0

The flasks were incubated in a rotary shaker (Certomat MO B Braun, Germany) at 100 rpm for 24 hours at 27±1°C.UV irradiation was done following the method of Viji *et al.* (1993). Just germinated spores from 24 hour old culture were suspended in 10 ml of 0.1 M HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid) buffer of pH 7.4 in petri plates and subjected to UV irradiation from germicidal lamp at 2537 Å. The exposure time and proximity with the lamp were altered (from 30 to 10 cm and 1 to 8 minutes respectively) to give a suitable survival rate. The exposed culture (1.0 ml) was pipetted from the plate and serially diluted before plating. Plates (medium composition same as mentioned previously), after streaking with the lowest dilutions, were incubated at 27±1°C in darkness.

For X-ray irradiation spore suspension in test tubes were exposed to X rays (Rigaku Dmax/2c Japan with Ni filter and radiation $\lambda = 1.5406$ Å) emitted at 30 KV level for 30 seconds and 60 seconds.

Plating of the exposed cultures was done by the same way as was done for UV irradiated cultures. Colonies of interest, mutants were isolated by repeat streaking technique.

Fermentation : For shake flask cultivation, a loopful of each colony (isolated mutant) was transferred into 250 ml conical flasks containing 50 ml medium (composition was same as mentioned earlier). The flasks were incubated in rotory shaker at 27±1°C and 180 rpm for 14 days. Samples were withdrawn at the end of fermentation period (14 days). Colour of the final broth and pH were noticed.

Biomass was worked out on dry cell weight basis (see section B.1) and volumetric and specific production of CsA were worked out on the basis of HPLC analysis (see section B.3) and expressed as mg/l and mg/g wet biomass respectively.

Extracellular enzymes viz amylase, invertase and protease were assayed using samples from the growing culture. For this, samples of mutants were withdrawn on day 3 and centrifuged (cooling centrifuge C24 Remi Instruments, India) at 5000 rpm for 20 minutes. The clear supernatant was used for enzyme assays.

Amylase assay : The saccharogenic activity of the enzyme was measured by the method of Morita *et al.* (1996). 0.2 ml of the enzyme solution was incubated with 1.8 ml of substrate (soluble starch; 1%, w/v in distilled water) for 15 minutes at room temperature. The reaction was stopped by adding 2 ml of DNS reagent followed by boiling for 5 minutes in water bath. The amount of glucose released was measured by observing the absorbance at 575 nm in a UV-VIS Spectrophotometer (160 A Shimadzu, Japan). The blank was prepared in the same way with distilled water instead of the enzyme solution. One unit of amylase was expressed as the amount of glucose (in μ moles) by 1 ml of enzyme in 1 minute. For the sake of comparison the enzyme secreated out by untreated culture was also assayed at regular intervals upto 14 days of fermentation.

Invertase assay : The method of Malliwell (1961) was followed for estimating the invertase activity. For this 0.1 ml of the enzyme solution was mixed with 0.4 ml of distilled water. To this, 4 ml of 0.2 M acetate buffer pH 4.6 containing 1% (w/v) sucrose was added. After 5 minutes of incubation at 50°C and 5 minutes of boiling in water bath, 3 ml of the reaction mixture was withdrawn and to this 3 ml of DNS reagents was added followed by 5 minutes of boiling in water bath. The amount of fructose liberated was measured by reading the absorbance at 575 nm. One unit of enzyme activity was taken as equal to the amount of fructose released (in μ moles) by 1 ml of enzyme in 1 minute.

Protease assay : Protease assay was done by the method of Yoshida and Noda (1965). For this 1.0 ml of the enzyme solution was incubated with 4 ml of 1% (w/v) casein solution in 0.1 M tris HCl buffer (pH 7.8) at 37°C for 2 hours. The reaction was stopped by adding 10% trichloroacetic acid and filtered. The amount of acid soluble tyrosine released into the filtrate was measured by observing the absorbance at 273 nm. Blank contained water instead of the enzyme solution.

Results and discussion

Colonies which manifested coloured pigmented spots in the mycelial lawn, the pigmented variants and a non sporogenic variant were isolated along with one morphologically normal mutant on agar plates by repeat-streaking technique.

At the end of numerous sets of UV irradiation trials, the irradiation conditions were optimized. An exposure time of 8 minutes at a distance of 12 cm from the light source gave a survival rate of about 10% (Fig. B.26). Morphological variants, identified as the pigmented variants are shown in Fig. B.27 a&b.

All the isolated mutants on fermentation developed into different coloured broths as against the golden yellow broth developed by the parent culture (Table B.23). Final pH also showed marked difference from that of the wild culture, with the values reducing considerably reaching to a low of 2.4 for MV6. (Table B.23). The values were slightly basic only for mutants MV1 and MV2.

The levels of extracellular enzymes also displayed marked variation among the variants. MV1 released maximum amylase and invertase among the different mutants (Table B.23). Majority of the cultures did not result in protease production, nevertheless four of the mutants viz . MV2, MV5, MV7 and MV14 exhibited slight positivity in protease assay (Table B.23). The amylase enzyme profile of the parent culture is shown in Fig. 28. Maximum activity (0.59 units/ml) was noticed on day 3 for amylase while protease was not detectable. None of the mutants showed a higher amylase activity than the untreated culture except the marphologically normal mutant were the activity was retained (0.597 units/ml) but the same came down to as low as 0.248 units/ml in the case of MV6.

As is given in Table B.23 the biomass concentration contemplated at the end of fermentation varied considerably from 2.9 to 16.9 for various morphological variants.

None of the isolated cultures managed to produce higher volumetric production of CsA compared to that of the parent culture for which it was 210 mg/l. MV2, MV10 and MV13 could produce higher specific production of CsA. MV2 was the best among all in yielding 41 $\pm$ 2.6 mg/g of CsA (Table B.23). For the untreated culture under the best conidtions (in SSF) the specific production was observed to be only 26.25 mg/gds.

These results clearly indicate the different CsA producing capabilities of the selected mutants and their divergence from the parent culture in terms of many other features like biomass production, extracellular enzyme profiles, maintenance of medium pH, broth colour etc. It was obvious also from the findings that higher final pH was associated with higher specific production. The positive effect of pH on volumetric production was documented earlier (Aarino and Agathos 1990). Final cyclosporin titres were reported to be positively influenced by a higher final medium pH.

The difference in growth and pH indicate varying primary metabolism among the variants. As a mutagen UV rays were expected to introduce thiamine dimers into the DNA of the cell and alterations in the DNA might occur during excision of the dimers and enzymatic repair (Spencer and Spencer, 1994).

Though the effort to attain a higher producer of CsA could not be attained through this mutation trials. In view of the scanty information available in these kind of works involving *T. inflatum* the isolation and characterization of the morphological variants assumes importance

The variation in the extracellular enzyme profiles indicated the need to modulate the production medium divergently for different mutants. Further, this can well be used as a method to screen mutants in yield improvement trials.

This study assumes greater significance in view of the limited amount of information on the biology and genetics of the producing organism. Inspite of the numerous clinical studies of CsA and the availability of a whole host of techniques for the production of improved industrial strains, only little is done towards this goal in CsA producing strains. Aarino and Agathos (1990) reported the manifestation of a few pigmented variants. One such spontaneously developed red colony was 3 times higher producer of CsA in liquid cultures. Agathos *et al.* (1986) isolated a number of auxotrophs with less stability. Afew of them were higher producers than the stock isolate.

Stability of these morphological variants along with the characterization of the pigments produced by them need further examination. This study provided us with some ideas on the strain improvement possibilities of *T. inflatum*. Besides, it also offered significant potential for further studies on hybridization.

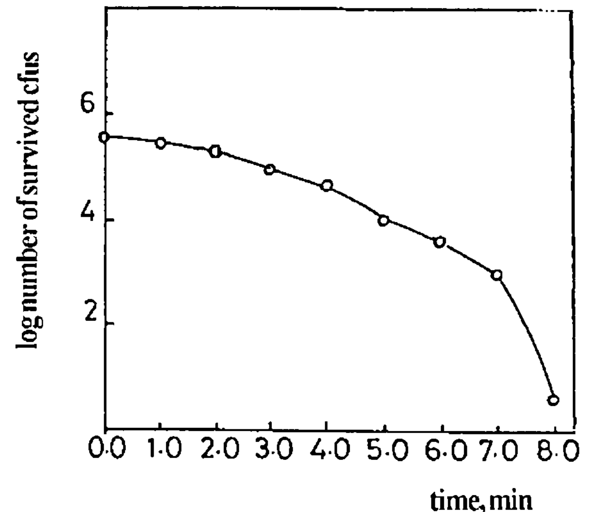


Fig. B.26 : Survival rate of *T.inflatum* after UV irradiation

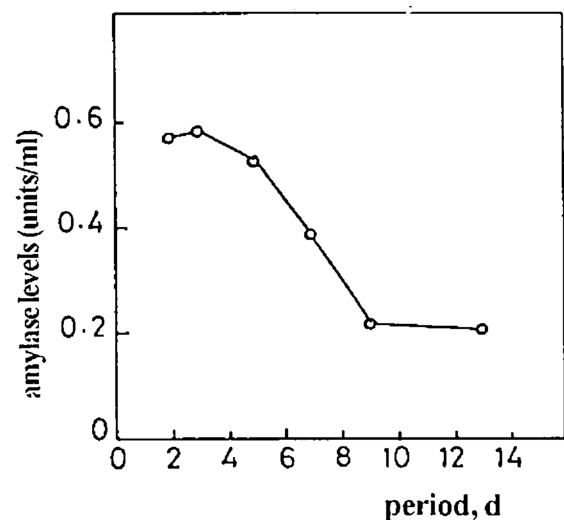


Fig. B.28 : Amylase production during the growth of *T.inflatum*

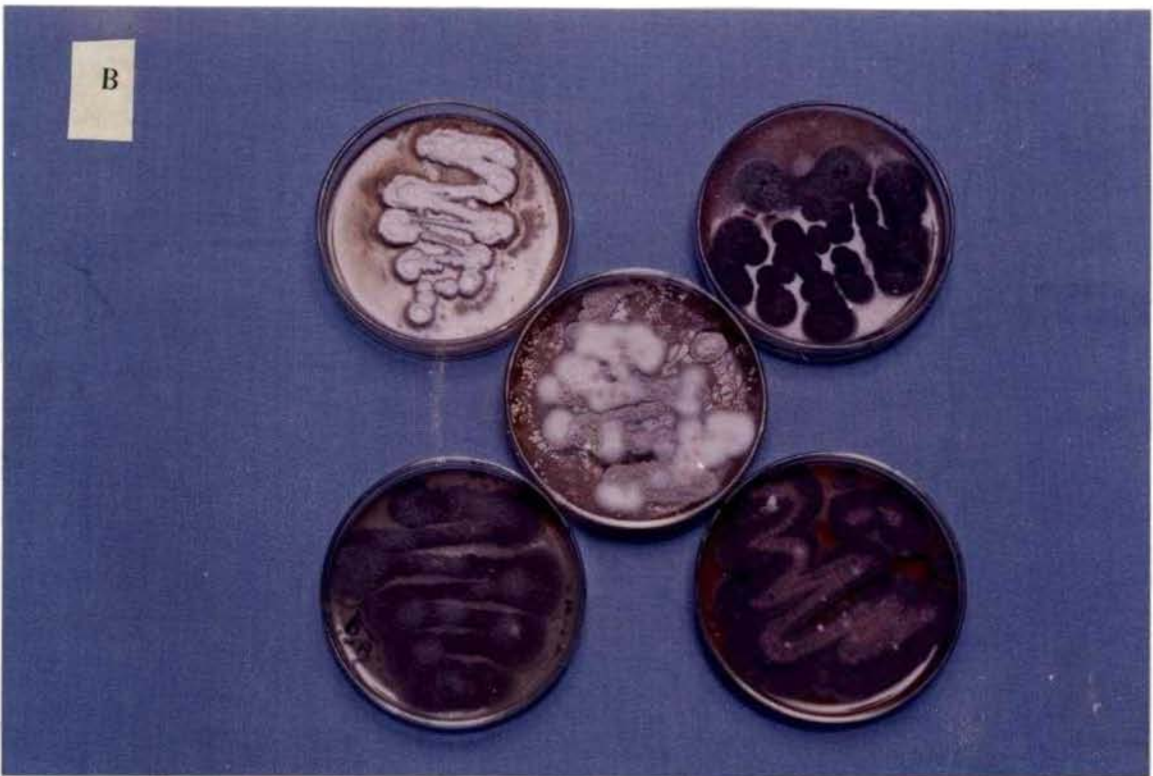


Fig. 27 A & B: Pigmented variants of *T.inflatum*

Table B.23

Characteristics of *T.inflatum* mutants

Entry	Colour/ type of colony	Colour of final broth	pH	Extracellular enzyme levels (units/l)			Biomass g/l	CsA level	
				Amylase	Invertase	Protease		Volumetric Production (mg/l ± SD)	Specific Production (mg/g ± SD)
MV1	normal white	yellow	8.1	597	795	---	8.0	195 ± 3	24 ± 0.02
MV2	pale pink	turbid yellow	7.4	447	285	92	3.3	138 ± 12	41 ± 2.6
MV3	green	pale yellow	3.6	581	40	---	9.0	42±2	4.6 ± 0.8
MV4	yellow	pale yellow	3.2	569	35	---	16.9	63 ± 8.2	37 ± 0.4
MV5	black	pale yellow	5.5	317	---	32	9.3	56 ± 2	6 ± 0.8
MV6	ash	pale yellow	2.4	248	10	---	8.6	---	---
MV7	brown	yellow	3.3	517	---	---	12.9	66 ± 9	5.6 ± 0.5
MV8	grey	pale yellow	3.0	424	---	46	3.9	22 ± 0.6	5.6 ± 0.9
MV9	pink	yellow	2.8	547	15	---	7.8	---	---
MV10	pale green	pale yellow	6.2	572	---	---	3.0	98 ± 0.6	33 ± 0.3
MV11	non sporous	yellow	4.2	584	300	---	3.2	60 ± 3	18 ± 0.5
MV12	pale yellow	yellow	4.0	497	230	---	3.4	48 ± 2	20 ± 0.6
MV13	white	brown	6.6	553	180	---	3.0	111 ± 2	37 ± 2
MV14	dark green	black	4.6	584	185	55	4.4	69 ± 1	15 ± 0.8
MV15	red	brownish yellow	3.0	303	40	---	2.9	16 ± 3	5.5 ± 0.2
MV16	pale green	yellow	4.9	420	20	---	2.9	30 ± 0.6	10 ± 0.1
MV17	pink	light yellow	3.6	290	30	---	5.4	26 ± 0.9	4.8 ± 0.1

B.8 : Purification Studies

B.8.1 : Partial Purification of Enzymes Involved in the Biosynthesis of Cyclosporin A

The occurance of unusual amino acids, α-amino butaric acid, D-alanine and the (2S, 3R, 4R, 6E)-2-amino-3-hydroxy-4-methyloct-6-enoic acid coupled with the presence of a number of N-methylated peptide bonds suggested the involvement of multienzymes in the biosysnthesis of CsA as was described in the biosysnthesis of many fungal depsipetides (Zocher *et al.* 1982; Peeters *et al.* 1988). A nonribosomal process of biosynthesis was envisaged by dint of the cyclic structure of the compound and the presence of unusual amino acids. The results of the feeding experiments conducted with labelled amino acids suggested the origination of N-methyl groups from L-methionine and amino acids from the natural precursors (Kobel *et al.* 1983; Zocher *et al.* 1984). Zocher *et al.* (1986) characterized a multienzyme from *T.inflatum*, involved in CsA biosysnthesis. In this work an attempt was made to isolate a fraction containing such a multienzyme. Also the S-adinosyl methionine systhetase (SAMS) involved in the methylation process was partially purified and characterized.

Organism and cultivation conditions : Culturing was enacted employing the indegenously isolated strain of *T. inflatum* B-58. The parameter settings were identical to the one described in previous chapters (refer section-B.1). Erlenmeyer flasks of 250 ml capacity holding 50 ml medium of the following composition (in %) and pH 5.6 was used. All the chemicals and reagents were of analytical grade. Chemicals used for the assay were procured from Sigma Chemical Co., USA.

Medium composition

Maltose	4.0
Glycerol	4.0
Peptone	4.0
Malt extract	2.0
L-valine	0.5
L-threonine	0.5
L-leucine	0.5

Samples, withdrawn at different time intervals, were analysed for biomass (by dry weight method as described in section B.1), CsA level, (HPLC method as explained in section B.3) and enzyme activity.

Crude enzyme preparation : The late growth phase (10 days old) mycelia harvested by centrifugation (cooling centrifuge C24 Remi instruments, India) at 6000 rpm for 15 minutes were

subjected to washing (3 times) in normal saline followed by sonication (Julabo USR-3, Germany) in a buffer of the following composition (in mM).

Sonication buffer composition

Tris-HCl (pH 8.0)	200
KCl	200
EDTA	300
Phenylmethane sulphonyl flouride	1000
$MgCl_2$	20
DTT	20
Glycerol (w/v)	30%

Removal of the cell debris by centrifugation at 10,000 rpm for 20 minutes yielded a clear supernatant which was taken as the crude enzyme.

Ammonium sulphate precipitation : Solid ammonium sulphate was added upto a final concentration of 60% (w/v) of the crude enzyme at 4°C. The preparation was then stirred in the cold for 12 hours and centrifuged at 10,000 rpm for 30 minutes.

Dialysis : The precipitate obtained from the aforesaid treatment was redissolved in minimum volume of the buffer and dialysed against distilled water in the cold for 12 hours. This dialysed enzyme was used for further purification protocals.

Chromatography : Gel filtration was carried out in a sephacryl S300 column (length 30 cm & diameter 1.5 cm) with end fittings. Equilibration of the column was effected employing 100mM tris-HCl buffer containing 0.1 M NaCl (pH 7.5).

A known volume (2 ml per injection) of the dialysed enzyme was loaded on to the column set at a flow rate of 2 ml/minute. The aforesaid buffer was used for the elution also. Fractions (5ml each) were collected at every 2.5 minutes Elution of the proteins were detected at 280 nm using a UV-VIS spectrophotometer (160 A, Shimadzu, Japan)

Fractions were subjected to enzyme assay (SAMS assay) and the positive fractions were further purified on a sephadex G200 columns enacting the earlier mentioned conditions and the fraction likely to contain the enzyme cyclosporin synthetase (CSS) was spared.

Enzyme assay : SAMS assay. This was dose making use of the method of Cantoni (1955). It is based on the amount of pyrophosphate liberated from ATP.

The reaction was initiated by the addition of the enzyme to the assay mixture described below at 37 °C followed by incubation for about 30 minutes. The reaction was ceased by the addition 1 ml

of 10% trichloro acitic acid. The orthophosphate was estimated in the protein free filtrates of test and blank.

Composition of SAMS enzyme assay mixture (in ml)

ATP [0.06M]	0.15
Tris (hydroxymethyl)amino methane buffer (0.5 M; pH:7.5) [0.5M]	0.2
$MgCl_2$ [1.0M]	0.25
Reduced glutathione [25mM]	0.1
L-methionine [0.1M]	0.1

For the blank water was used instead of L-methionine

One unit of enzyme activity was taken as that amount which in the presence of methionine caused an additional formation of 3 μg of orthophosphate from ATP in 30 minutes. Specific activity was calculated as units per mg of protein.

***Determination of molecular weight*:** Andrews method (Andrews 1965) was employed to determine the molecular weights (of SAMS and the fraction corresponding to the CSS). The sephacryl S-300 column (1.5 x 30 cm) was equilibrated with 100mM tris-HCl buffer containing 0.1 M NaCl (pH 7.5). The elution was carried out at a constant flow rate of 2ml/minute. The elution volumes, Ve for the sample, the marker proteins and blue dextran (used for void volumes, Vo determination) were calculated from the corresponding retension times. The molecular weight strandards used for the column calibration were

	Molecular Weights (KDa)
Carbonic anhydrase (bovine erythrocytes) (1)	29.0
Albumin (egg) (2)	45.0
Albumin (bovine) (3)	66.0
Phosphorylase b (rabbit muscle) (4)	97.4
β-galactosidase (*E.coli*) (5)	116.0
Myosin (rabbit muscle) (6)	205.0
Thyroglobulin (7)	668.0

Kav values were calculated as given below

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

Where Vt = total bed volume

The Kav values of the molecular weight markers arrived at from the aforementioned

relation were plotted against their log molecular weights and the molecular weight of enzymes were calculated from the graph thus obtained.

Biochemical characterization of SAMS

(1) *Determination of temperature optimum of SAMS* : The assay mixture for SAMS activity determination was incubated at various temperatures ranging from 20^0 C to 60 °C for 30 minutes each in a water bath maintained accordingly.

(2) *Determination of pH optimum of SAMS* : Buffers ranging in pH 6.0 to 9.0 were employed for the enzyme assay.

(3) *Influence of the inhibitors and metal ions on the activity of SAMS* : Inhibitors such as EDTA, IAA & Sodium fluoride (all at 10 mM level) were added to the assay mixture and the change in the residual enzyme activity were detected after incubation for 30 minutes Mg^{2+} and Mn^{2+} were also separately added to test their impact on the enzyme activity.

Results and Discussion

Synthesis profile of SAMS : SAMS activity measured in comparison with biomass production and product formation during diverse phases of culture growth is presented in Fig.B.29 The specific activity of SAMS shooted up during the active growth phase of the mycelium with a corresponding increase in the product titres. On day 8 when the growth was at its peak, SAMS activity increased to 7 units thereafter it rose sharply to 14 units with a corresponding increase in product level from 10 to 190 mg/l during the same period.

Purification of the enzymes : The purification details of SAMS are summerised in table B.24. The crude enzyme was having the specific activity only 46. Ammonium sulphate precipitation resulted in a 3 fold purified enzyme. Further purifications in sephacryl S300 and sephadex G200 gel filtration columns yielded, 17.5 and 50.2 fold purified enzymes respectively. After the first gel filtration, mainly 3 fractions were obtained of which the fraction 2 of intermediate molecular mass exhibited SAMS activity and that alone was further purified. The first fraction of higher molecular weight was expected to possess, CSS activity and hence the same was also used for molecular weight determination.

Molecular weight determination of enzymes : By gel filtration using sephacryl S-300 column, SAMS and the first fraction likely to possess CSS activity were found to have molecular weights 29,600 and 715,000 respectively (Fig. B.30).

Table B.24

Details on the purification of SAMS from *T.inflatum* B-58

Method used	Initial volume (ml)	Total enzyme activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification achieved (in folds)
Dialysis	100	255000	5600	46	100	1
$(NH_4)_2SO_4$ precipitation	25	165000	1200	138	64	3
Sephacryl S-300	25	160500	200	805	63	17.5
Saphadex G-200	15	120000	52	2308	53	50.2

Table B.25

Effect of inhibiters (10μM) and metal ions (100 μM) on SAMS activity

inhibitor/metal ion	%inhibition* / % enhancement**
Na F	100*
EDTA	100*
IAA	100*
Mg^{2+}	215**
Mn^{2+}	75**

Temperature optimum of SAMS enzyme : The enzyme was found to be not stable at higher temperatures. It showed maximum activity at 35° C. (Fig.B.31a). The activity came down sharply with further rise in temperature reaching a low of 35 units at 60° C.

pH optmum of SAMS enzyme : The enzyme activity was more pronounced (96 units) at pH 7.8 with sharp decrease in activity after pH 8.0 (Fig.B.31b)

Inhibition of SAMS activity : At 10 mM level NaF, EDTA and IAA resulted in complete inhibition of the enzyme activity. (Table B.25)

Requirement of metal ions for SAMS activity : Mg^{2+} was found enhancing the optimum activity. Mn^{2+} could also restore the activity to some extent in the absence of Mg^{2+} ions. (Table B.25)

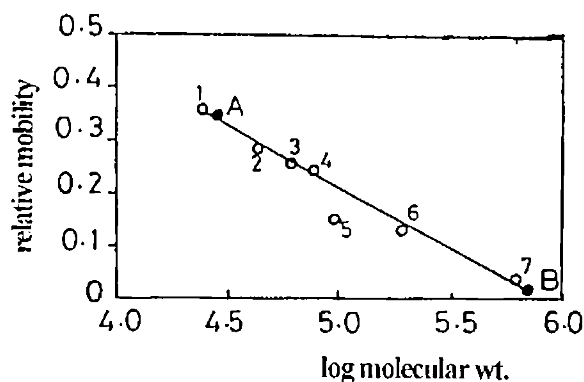


Fig B.30: **Determination of molecular weight of SAMS and CSS containing fraction by gel filtration on sephacryl S-300**

A-SAMS B-CSS

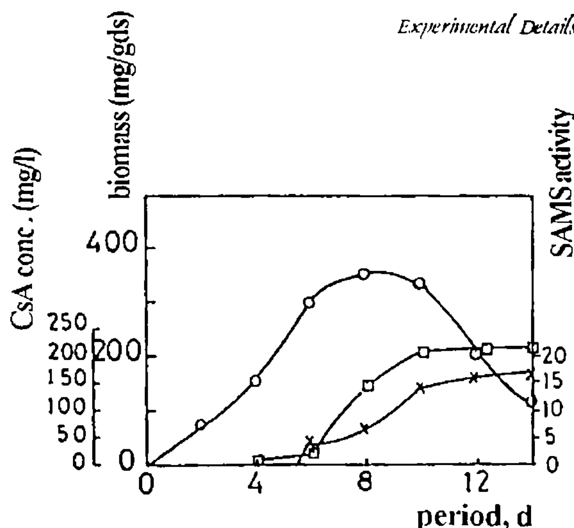


Fig B.29: **Profile of SAMS activity is comparisom with the dynamics of CsA synthesis during the growth of *T.inflatum*-B-58**

O biomass □ CsA level X SAMS activity

The pattern of rise in the product titres with a corresponding increase in SAMS activity during the growth of the culture illustrated the involvement of SAMS in the product synthesis. Methylation of aminoacids, an important step in the biosynthesis of CsA, is reported to be governed by S-adenosyl methionine (SAM), the methyl donor for the methylation process (Kobel 1983; Zocher *et al.* 1992). SAMS enzyme is taking part in the synthesis of SAM and hence the supply of SAM for the methylation of CsA is said to be influenced by the action of SAMS.

Researchers worked towards the realization of total synthesis of CsA reported the isolation of an enzyme fraction powerful in catalysing the activation of constituent aminoacids

of CsA from the crude exracts of *T. inflatum* (Zocher *et al.*1986). A similar N-methylation of aminoacids of the peptide chain was demonstrated in enmiatin also (Zocher *et al.*1982). Billich and Zocher (1987) have demonstrated an enzyme fraction from *T. inflatum* with a molecular weight 70,000 which was able to synthesise complete CsA molecule *in vitro* in the presence of the constituent aminoacids, SAM and ATP.

In our study SAMS was purified to 50.2 fold than the crude enzyme from the fungal mycelium. Inhibition of SAMS by NaF & EDTA indicated the necessity of metal ions for the enzyme activity. Inhibition of the enzyme by IAA, the thiol directed agent, exhibited the presence of -SH groups in the enzyme which are a prerequisite for the activity. Also it is found to be a Mg^{2+} requiring enzyme very much like other ATP requiring enzymes. These properties were analogous to the finding of Cantori (1955) on SAMS from rat liver.

The fraction having a molecular weight of >700 KDa isolated from the crude extracts of *T.inflatum* could be similar to the 700 KDa polypeptide chain by Billich & Zocher (1987) and hence considered as the CSS containing fraction. Since the enzyme activity for the total synthesis is expected to be residing in this single polypeptide, this is in sharp contrast with other antibotic synthetases such as gramicidin S or tyrocidine synthetase.

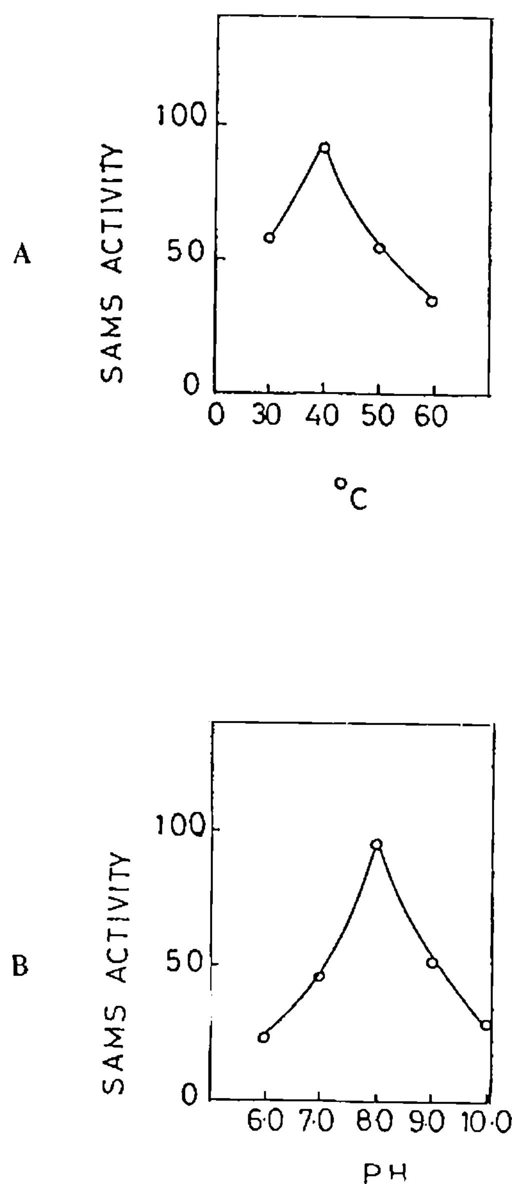


Fig B.31: A and B temperature and pH profiles of SAMS.

B.8.2 : Purification of Cyclosporin A from the Fermentation Broth

CsA being a neutral cyclic polypeptide lacking any active functional groups, designing a purification protocal would be challenging. However, a method comprising gel filtration of the fungal extract through sephadex LH 20 with an initial run through a silica gel column yielded apparently pure product exhibiting comparable IR spectra and amino acid sequences besides a good biological activity as showed by the standard sample of CsA.

Materials and Methods

Microorganism : The local isolate of *T. inflatum* B-58 was used as the production strain. All the chemicals and reagents were from M/s HiMedia, India.

Seed preparation : A slope culture of the organism was transferred to 50 ml medium of the following composition (in g/l) and pH 5.6 held in a 250 ml conical flask.

Maltose	30.0
Casein acid hydrolysate	20.0
Peptone	10.0

The flask was incubated in the rotory shaker (Certomat MO B Brawn Biotech International, Germany) at 27±1°C and 180 rpm for 3 days.

Second stage seed was developed by transferring the primary seed at 30%(v/v) level to the medium of the following composition (in g/l) and pH 5.6.

Maltose	20.0
Casein acid hydrolysate	20.0
Peptone	05.0

Cultivation conditions were the same as mention above.

Production conditions: Secondary seed was transferred at the rate of 15%(v/v) to the production medium of the following composition (in g/l) and pH 5.6 held in 500 ml conical flasks.

Maltose	20.0
Casein acid hydrolysate	10.0
Precursor amino acids	4.0

(L-valine, L-leucine, α-amino butyric acid) (each)

Mineral solution (ml) 1.0

(see section B. 1)

Cultivation condition were same as described for the seed preparation. Fermentation was continued for 6 more days in this medium.

Down stream processing : Biomass from the culture flasks was harvested by filtering through a muslin cloth. Culture filtrate was discarded and the wet weight of the fungal biomass was determined.

The biomass obtained after the macrofiltration was subjected to grinding using pestle and mortar. The resulting mix was extracted using methanol twice at the ratio of 1:2 (w/v). This was achieved by shaking the mixture in a water bath shaker (Metrex, New Delhi, India) at 150 rpm and room temperature for 60 minutes. The extracts were seperated by filtration through a filter paper. The extracts were then pooled up.

The extract thus obtained was evoperated using a rotary evoperator (R 124 Buchi, Switzerland) with a vaccum pump (B169 Buchi, Switzerland) and a water bath (B480 Buchi, Switzerland). The pasty residue was collected by suspending it in sterile distilled water.

The residue in water was then extracted with ethyl acetate in the ratio 1:2. The ethyl acetate fraction was washed with aqueous solution of 0.1% (w/v) sodium bicarbonate followed by flash evapouration using the rotary evoperator. The resulted residue was dissolved in hexane : chloroform and methenol in the ratio 12.5 : 11 : 1.5.

Purification

Chromatography : (a) *Silica gel column* : Silica gel (S.D. fine chemicals, India) of mesh size 60-120 was washed with distilled water followed by methanol and was used to pack the column (length, 30cm and diameter, 1.5 cm). Methanol was then replaced with the mobile phase, hexane:chloroform:methanol; 12.5:11:15. The sample (residue dissolved in the mobile phase) was loaded at 10% level of the bed volume. By withdrawing mobile phase, the sample was allowed to enter into the stationary phase. Ten fractions, each one equalent to 1/5 of the bed volume, were collected. The fractions were tested in TLC (see section B.1) for the presence of CsA. All the active fractions were pooled up.

(b) *Sephadex LH 20 column* : Sephadex LH 20 (Sigma Chemical Co., USA) suspended in methenol was used for packing the column (same as in the earlier run). The sample from the previous step of

purification was loaded at 5% level of the bed volume. Samples were withdrawn and 5 fractions, each one equalent to 1/5 of the bed volume, were collected and tested for the presence of CsA using TLC. Active fractions were pooled up and the process was repeated twice using the same column following washing with methanol.

Comparison of the chemical identity of CsA with the standard sample of CsA.

(1) *IR spectral analysis* : IR spectral analysis of the compound was done using a Perkin Elmer (USA) Model 882 infrared spectrometer.

(2) *Comparison of the CsA hydrolysate* : Amino acid profiles of the hydrolyzed compound was compared by running a thin layer chromatogram. About 2 mg each of standard and test preparations of CsA were hydrolyzed in HCl (6N) by boiling for 40 h followed by neutralization with NaOH and made up to 10ml with distilled water. The mix was then spotted on TLC plates (silica gel G, butanol:water:acetic acid; 5:3:2). Spots were visualized with a ninhydrin reagent.

Test for biological activity : For this, antifungal activity was tested against *A.nigar* as described in section B.1.

Results and discussion

The medium components for the seed development and production medium were chosen based on the knowledge of the response of *T. inflatum* B-58 to various carbon and nitrogen sources form the earlier experiments. Maltose was found to be suitable for better growth of the culture among the various carbon sources used to prepare defined media (Balakrishnan and Pandey 1996b). The ratio of the two nitrogen sources were altered as the fermentation progresses, in favour of casein acid hydrolysate because of its ability to support higher CsA biosynthesis (Balakrishnan and Pandey 1996b)

Considering the impact of the precursor amino acids esp. L-valine, L-leucine and α-amino butyric acid (see section B1, B3 and B.6.2) they were included at the optimum level.

After macrofiltration, the biomass was found to be 160 mg/litre. The cellular extract after initial purification through silica gel column was further purified in sephadex LH 20 considering the lipophilicity of the compound. Purified CsA yield was found to be 125 mg/l. Dreyfuss *et al.* (1976) reported a purification procedure to obtain CsA and CsC from the fermentation growth where the yield were reported to be 150 to 200 mg/l and 50 to 100 mg/l for CsA and CsC respectively.

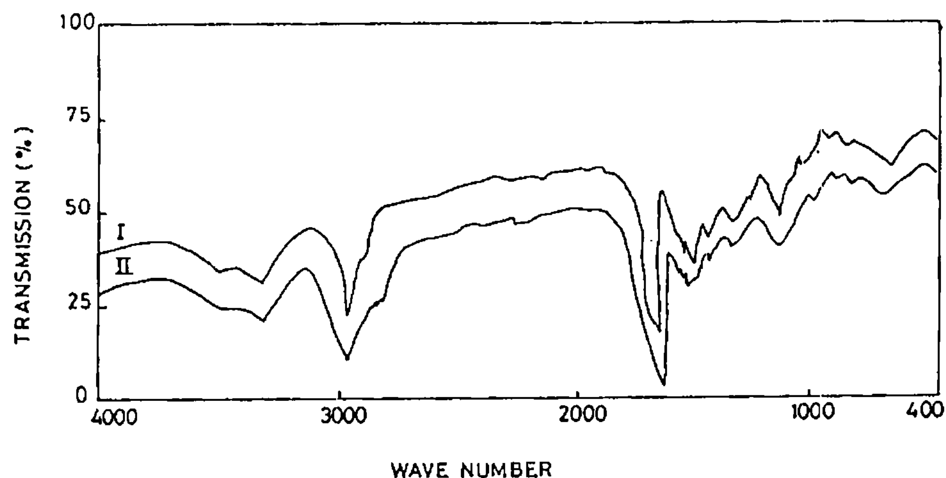


Fig. B.32: IR spectra of standard (I) and purified (II) samples of CsA

The final purified extract on IR spectral analysis gave a superimposible spectra with that of the standard CsA (Fig. B.32). Further the TLC profiles of the hydrolysates of the purified and standard CsA preparations were found to be identical.

The inhibition zone around the filter paper disc indicated the antifungal property of the purified preparation (Fig. B.33). This is an indication of retention of biological activity even after undergoing cumbersome purification proceedures.

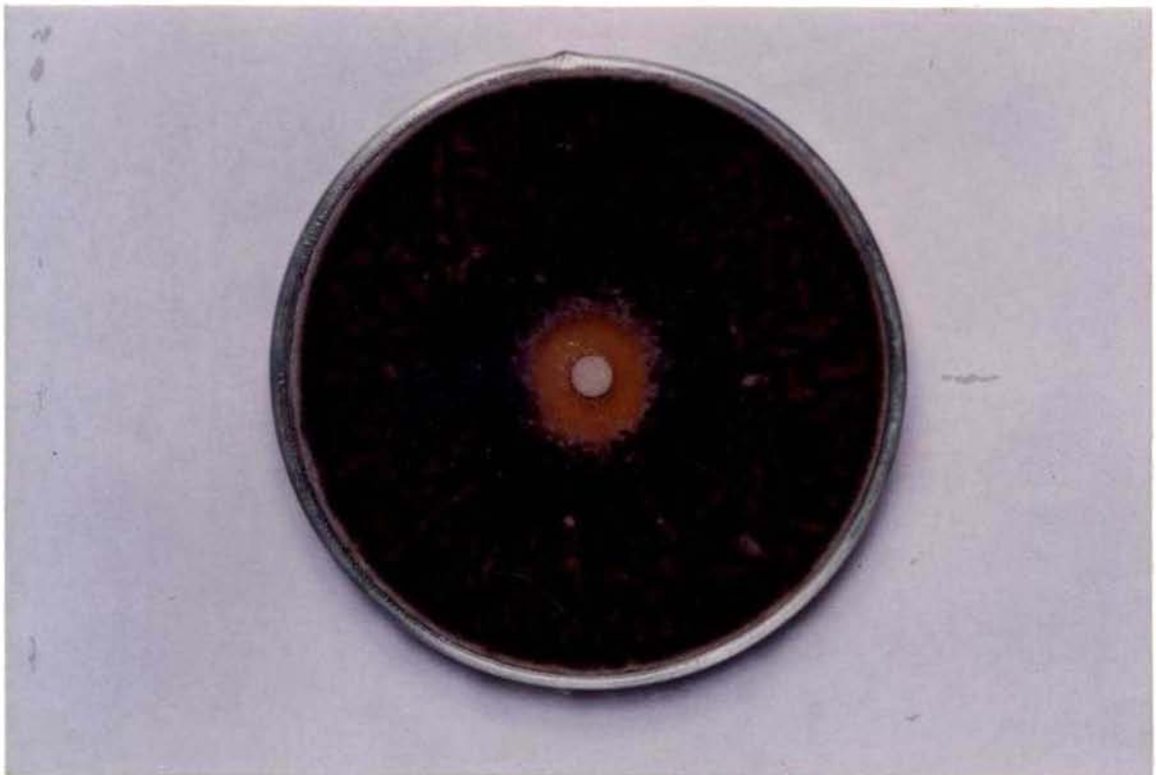


Fig B.33: Antifungal activity of the purified CsA from the extract of *T.inflatum* B-58 against *A. niger*

C. SUMMARY AND CONCLUSIONS

Soil samples were collected and processed as part of a screening programme initiated to isolate microbial strains producing bioactive microbial metabolites. Based on the inhibition zones produced by the extracts of isolated organisms in test cultures, a few colonies were isolated. One of the colonies designated as B-58 produced consistent and considerably good antifungal activity against *A.niger*. On subsequent identification, it turned out to be *T.inflatum*, the potential producer of the immunosuppressive agent, CsA.

Use of maltose or glucose as carbon source, maintenance of pH in the range of 5 to 6, use of 24 hours old inoculum at 2% level were afew situations which resulted in an optimal culture growth and at 1% level of carbon source sufficient mycelial biomass was produced. Growth and activity of the culture were less pronounced in defined media. In complex media as the fermentation progressed, the pH was found to be shifting towards the basic side of neutrality. Nature of nitrogen source was of significance in altering the product biosynthesis towards the positive side efficiently as was indicated by the higher CsA titres when casein acid hydrolysate was used in one of the complex media instead of peptone in another medium where only an enhanced growth was encountered. Supplementation of the medium with constituent amino acids of CsA was proved to be an effective methodology towards attaining an enhanced product biosynthesis. Under submerged condition the product titre was maximum on day 6 and this was attained on completion of the log phase of growth.

As part of media design efforts the significance of oligonutrients in creating a suitable environment during fermentation was appreciated by evaluating the iron requirements of the producing strain. The experiments carried out in synthetic, semisynthetic and chemically treated media pointed out the requirement of iron for an optimal culture growth. A concentration of 1 g/l of Fe^{2+} was well tolerated by the fungus. Under iron depleted conditions, however, the culture did not secreate out any siderophores.

The precursor effect of the constituent amino acids of CsA was confirmed in suspension cultures using a synthetic and a semi synthetic media deviod of any unnecessary complex media components. L-valine produced maximum enhancement in CsA titre. All the nonmethylated constituent amino acids managed to shift the biosynthesis rate positively. The effects were more visible in synthetic medium with many amino acids failed to make a difference in semi synthetic medium. Methylated amino acids, sarcosine and methionine and D-valine produced a negative

impact on productivity. Combined supplementation of L-valine and L-Leucine was effective in raising CsA titre from 26 to 480 mg/l. But in combination with methylated amino acids a reversal of the positive effects that L-valine produced when used alone was observed. Optimum level of addition of L-valine was found to be 4 g/l of initial amino acid concentration and the optimum time of addition was 20 hours after the initiation of fermentation. The influence of external addition of amino acid constituents on CsA biosynthesis in *T.inflatum* B-58 fermentation was thus confirmed.

Among the various solid substrates tried for SSF, wheat bran was found to be favouring the production and the productivity was found to be 2.14 times higher than what was contemplated in submerged culturing. Increasing the duration of autoclaving, maintenance of an initial moisture content of 55-60% (v/w), employment of wheat bran of mixed particle size as substrate, keeping the a_w values higher than 0.9 and supplementation of corn steep liquor at 10% (w/w) level positively influenced the productivity. On-line monitoring of biomass as a measure of the rate of CO_2 evolution was compared with the conventional glucosamine content estimation and the former was found to be a more sensitive and reliable method for biomass estimation.

Though wheat bran turned out to be the medium of choice for CsA production, it was treated further to condition the solid media for a still enhanced CsA titre. Defatting of the wheat bran however, resulted in poor productivity. On the other hand, extract of the over cooked wheat bran was found to be an ideal support for better biosynthesis.

Studies were performed to identify the suitable immobilizing agent for CsA production by whole cell immobilized bioreactor assembly. 2% Calcium alginate showed a good time bound diffusion, better mechanical properties and higher CsA productivity. The biotransformation of diverse constituent amino acids into CsA in PBR indicated once again the precursor role of L-valine, L-leucine and α-amino butyric acid. But no synergistic effect of combinations of amino acids were detected. Under continuous mode of operation part of the out flow was mixed with the in flow of fresh medium resulting in a rise in productivity upto four cycles. Mycelial and spore immobilized biocatalysts produced comparable levels of CsA. Increasing the flow rate above 0.5 ml/minute and rising the concentration of the catalyst above 0.06 g/ml of the entrapping agent were found to be detrimental.

Strain improvement trials were undertaken making use of the classical mutation techniques effected through the use of UV and X-rays. Survival curve for UV irradiation was

worked out and the irradiation conditions were optimised. An exposure time of 8 minutes at a distance of 12 cm from the source of light resulted a survival rate of about 10%. Morphological variants differing in the nature of pigment production were isolated and they were subjected to fermentation. They were different from the parent culture and were also exhibiting variability among themselves in terms of colour of the final broth, final pH, extra cellular enzyme levels, biomass and CsA productivity. However, none of them managed to produce a higher volumetric productivity. Three of them yielded higher specific productivity than what was attained with the wild culture.

In an effort to partially purify and characterize the enzyme(s) in the biosynthetic machinery of CsA, a fraction of the mycelial extract on purification in sephacryl S-300 was found to posses SAM sythetase (SAMS) activity and yet another fraction was found to be the likely cyclosporin synthetase(CSS) as indicated by its molecular weight which for the former was 29,600 and for the latter was 7,15,000. SAMS on further purification yielded a 50.2 fold purified enzyme. Temperature and pH optima of SAMS were found to be 35°C and 8.0 respectively. Mg^{2+} was found to be enhancing its optimum activity.

A product purification strategy was also worked out employing two stages of seed development followed by production under submerged conditions. The organic extracts on silica gel column followed by sephadex LH 20 gel filtration column yielded a relatively pure sample of CsA exhibiting superimpossible IR spectrum and identical amino acid sequence with the standard sample of CsA.

The major conclussions drawn from the above studies include :

- Altering of the carbon and nitrogen sources in the medium provided diverse physiological conditions for CsA production. With the progression of fermentation maintenance of pH on the basic side of neutrality was found to be suitable for a more sustainable product biosynthesis. The extend of buffering exerted by diverse medium components resulted in the maintenance of desired pH also was proved to be of significance while designing a production medium.

- Though there is a positive dependance of iron suplimentation as one of the oligonutrients on the growth of the culture, no sidephores were secreted out by this strain under desferrated conditions.

- Supplimentation of nonmethylated constituent amino acids with the fermentation media favoured better product biosynthesis with L-valine resulting maximum enhancement. The precursor effect was neither vissible with the alterations in the stereospecificity of the amino acids nor profound with higher initial amino acid concentration. Combined supplementation and supplementation at the initiation of idiophase were found to be favouring the productivity.

- With sufficiently moistent and autoclaved wheat bran higher CsA levels were attainable if a_w was maintained relatively high and with the supplementation of 1% CSL. Rate of evolution CO_2 could be successfully used as a measure to monitor the biomass. SSF showed an edge over SmF in terms of suitability of this strain to get induced for a better productivity.

- Extract of wheat bran was the best system for higher productivity .

- Alginate beads were highly effective for immobilizing *T.inflatum* and CsA production. No much difference between immobolizing spores and mycelia on productivity. Media recycling at medium flow rate highly favoured better production.

- Morphological variants isolated as mutants exhibited diverse properties on fermentation suspected to be due to varrying primary metabolism. Such a screening could be employed to pick up variants with different product biosynthetic efficiency.

- SAMS, the enzyme that controls the methylation process in product biosynthesis and the other fraction of higher molecular weight, the CSS fraction were separated and molecular weights determined. Further purification yielded a 50 fold purified SAMS which on characterisation was found to be identical to other such enzymes contemplated elsewhere.

- Purification protocal of CsA isolation from the fermentation broth after submerged cultivation was performed to get an yield of little more than 50% of what was attainable from the crude extract.

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