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 a few 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 secrete 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 devoid 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 synthetase (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. Mg²⁺ 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 conclusions 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.
Supplementation of nonmethylated constituent amino acids with the fermentation media favoured better product biosynthesis with L-valine resulting maximum enhancement. The precursor effect was neither visible 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 \( \text{CO}_2 \) 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 \( T.\text{inflatum} \) and CsA production. No much difference between immobilizing 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 varying 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 protocol 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.