DISCUSSION

Studies on high temperature stable enzymes from natural sources had so far directed investigators' attention to bacterial sources that grew in hot springs under extremes of temperature and pressure. The usability of high temperature stable enzymes from these bacterial sources in industrial processes found commercial use for these enzymes worldwide. However, difficulties in the procurement of patented thermostable enzymes on the one hand, and the procurement of natural bacterial sources from hot springs not available in India, combined with the need to culture them in larger amounts at considerable cost for obtaining the thermostable enzymes for industrial processes, directed our attention to seek and identify alternate natural sources of high temperature stable enzyme forms for their potential industrial and commercial applications in the country.

In seeking out alternate sources for high temperature enzymes within the ecology and environment of the Indian subcontinent, the existence of
several xerophytic species of plant varieties growing in very arid and semi-arid regions of this country attracted attention as a possible source of high temperature enzymes. Investigations were therefore initiated and directed to characterize the enzyme activities such as the amylase, xylose isomerase, xylanase and cellulases, enzymes that had an established industrial importance, and for which the assay, isolation, purification and characterization can be approached in a conventional manner. The Xerophytic plant species Opuntia vulgaris and Cereus pterogonus were therefore identified as the enzyme source for the purpose, as they were found growing abundantly in the coastal as well as in the arid neighborhoods of Pondicherry, India.

Using conventional protein isolation and characterization methods as reported here, studies carried out employing the two xerophytic plant species (Opuntia vulgaris and Cereus pterogonus) has yielded material and data that establishes the existence of high temperature stable amylase enzyme isoforms in these species, indicative of their significant role in starch processivity in these plant varieties.

By definition, high temperature stable enzymes, also called thermophilic enzymes, are those that exhibited their catalytic activities above 70 °C. The presence of the T₈₀ and T₉₀ amylase isoforms in the respective xerophytic species employed in this study can therefore be categorized as thermophilic isoforms. The T₅₀ variety from both the species by default is not
therefore thermophilic. The existence of the T$_{50}$ and the T$_{90}$ isoforms on the one hand in *Cereus pterogonus* versus the existence of T$_{50}$ and T$_{90}$ isoforms on the other hand in *Opuntia vulgaris* raised doubt if both the isoforms were constitutively expressed in these plant species, or the thermophilic T$_{80}$ and T$_{90}$ isoforms were inducible enzymes, since the habitat temperature for these plant species that grew in this geographical region is around 30 °C. The additional feature of alkali stability of these enzymes as exemplified by their pH optima at 8.5 in our studies, offers added advantage for the potential use of these enzymes in industrial applications wherein both, high temperature stability as well as alkaline stability was a matter of advantage for use of these enzymes. It is to be noted that alkaliphilic bacterial enzymes are not reported as of common occurrence.

Krystyna Zoltowska (2001) reported the existence of two isoforms of amylase from the intestine and the muscle of *Ascaris suum* (Nematoda). The intestinal α-amylase showed two optimum activities, at 40 °C and at 50 °C. The muscle α-amylase also exhibited two optimum activities, at 30 °C and at 50 °C. The identification of two peaks of enzyme activity in these species was therefore considered as due to the different thermal sensitivities of these amylase isoforms.

Gashaw Mamo (1999) reported the presence of two extracellular amylases designated as *Amy1* and *Amy11* obtained from the cell free culture supernatant of *Bacillus sp.* WN11. The enzyme showed an optimum activity at
80 °C. This amylase activity was considered as a proteolytic degradation product of a precursor high molecular weight amylase molecular form as reported by (Egelseer, 1996).

Gregory J. Zeikus (2001) reported the existence of a thermophilic α-amylase in microbes that was active in the temperature range 80 °C to 100 °C and was therefore a great potential source of thermophilic amylase for the breakdown of starch.

Lohf (2001) reported on alkaline amylase of different species of Bacillus that was not thermostable, and Arikan Burhan (2003) reported on the existence of a thermophilic and alkaliphilic amylase in alkaliphilic Bacillus sp. isolate ANT-6 having an optimum activity at 80 °C and an optimum pH at 10.5. This report served as an indirect support to findings of thermophilic and alkaliphilic amylases in the plant species Cereus pterogonus and Opuntia vulgaris. Observations made in this dissertation are further supported by the findings of Long-Liu Lin (1998) on the presence of thermophilic and alkaliphilic amylase in Bacillus. Sp. TS-23 having a temperature optimum at 70 °C and a pH optimum at 9.0.

The purification strategy reported here for the isolation and purification of alpha amylase from the two xerophyte species employed conventional approaches such as ammonium sulphate precipitation of protein, ion exchange chromatography, and gel permeation chromatography,
to recognize and establish basic characteristics of the enzyme under these conditions. The cost factor involved in strategizing immuno affinity approaches as well as the procedural protocols required to process expensive import supplies offered significant hurdles and precluded working out improved methodologies for the isolation and purification needs. Hence compromises had to be made for optimizing possible methods for our investigative purpose without having to miss out on the novelties of our observations.

Though the purification fold (59 and 82 fold respectively) and the yield of protein (34%) obtained following the purification strategy employed here matched well with earlier reports on amylase purification, strategic improvements in the purification process and the yield of material was possible only if more modern approaches had been attempted. The lower recovery of the purified protein is probably due to the sensitivity of certain plant enzymes to extremely low abundance contaminants like heavy metals present in the chemicals employed for the isolation methods.

Results of the ICP-AES analysis carried out lately, of ammonium sulphate used for protein precipitation indicated the presence of chromium (21.43 ppm) in the chemical. Whether the level of chromium available to the enzyme during the precipitation process affected the enzyme activity was not specifically determined. Since, the ICP-AES facility was recently established, its' analytical sensitivity level of < 0.1 ppm for elemental analysis could not be
employed as a co-detection strategy for the determination of contaminant ions during the purification processes carried out in this study. ICP-AES analysis of the tissue homogenate 10,000 x g supernatant also indicated the presence of Mn$^{2+}$ (19-22 ppm) and Mg$^{2+}$ (527-568 ppm). The EPR results did not however establish the presence of manganese in the isoforms studied, probably due to the relatively lower level of sensitivity of this detection technique. While it may be contended that manganese did not co-purify with the amylase isoforms (based on EPR analysis alone), it cannot be emphasized similarly for diamagnetic elements that remained undetectable by the EPR method, even though Mg$^{2+}$ levels were indicated to be greater (by ICP-AES) than Mn$^{2+}$ levels in the homogenate supernatant. Lack of adequate purified protein samples also precluded wider studies on elemental analysis of the purified material employing the ICP-AES method. It was therefore believed that the plant enzyme was sensitive to contaminant factors and/or cold temperature during the purification process and that therefore resulted in a lower yield of the enzyme activity.

The SDS-PAGE analysis identified the existence of four distinct polypeptides in the crude homogenate 10,000 x g supernatant. These polypeptides were also seen enriched in the T$_{50}$, T$_{80}$ and T$_{90}$ ion exchange fractions following ion exchange column chromatography. Of these, two polypeptides, namely SDS-PAGE 66 kDa and 25 kDa, appeared following reduction of T$_{50}$ isoform of Cereus pterogonus as well as the T$_{90}$ isoform of Opuntia vulgaris with 2-mercaptoethanol. The Cereus pterogonus T$_{80}$ isoform
how ever exhibited only a single polypeptide of 66 kDa molecular mass under similar conditions. In all the cases however, the 66 kDa polypeptide band alone yielded the enzyme activity following the SDS-PAGE activity stain method although the proteins were treated with DTT, suggesting that the 66 kDa polypeptides singularly exhibited the enzyme activity and that each of the polypeptide bands existed as a holo enzyme unaffected by the sulfhydryl reduction process. It became possible to detect this enzyme activity due to the fact that in the activity PAGE gels, SDS was replaced with Triton-X-100 (Jei-Fu Shah, 1995) prior to detecting the enzyme activity. The retention of the amylase activity in the activity stained gels was not surprising since studies carried out here employing DTT as a reductant of the inter or intra chain disulphide bonds had established that the amylase protein of *Cereus pterogonus* and *Opuntia vulgaris* continued to exhibit enzyme activity following such a treatment.

Mishra and Maheshwari (1996) reported on an α-amylase from thermophilic fungus *Thermomyces lanuginosus* as having a molecular weight of 24 kDa while Ratanakhanokchai (1992) reported a molecular weight of 210 kDa for α-amylase isolated from the thermophilic photosynthetic bacteria *Chloflexus aurantiacus*. Alian Ferrer (1999) reported that DTT had no effect on the membrane amylase isolated from *Toxoplasma gondii* but observed an increase in the cytoplasmic amylase activity of this parasite due to DTT.
The generation of polyclonal antibody serum against the fungal amylase aided to establish cross reactivity between the antibodies and the *Cereus pterogonus* and the *Opuntia vulgaris* amylase isoforms, suggestive of regions of commonality in the polypeptide sequences of these different isoforms. This was further emphasized by the observation that the rabbit antiserum brought about significant inhibition of these amylase activities during invitro assays.

It was interesting to note that the temperature related activity of the amylase isoforms was stabilizable by divalent Mg$^{2+}$ and Ca$^{2+}$, by employing critical concentration of these ions, whereas Mn$^{2+}$ invariably contributed to a reduction in the enzyme activity. This aspect can be appreciated based on the understanding that protein-metal interactions generally led to the formation of non covalent protein-metal complexes (Boel, 1990), yielding conformational alternatives exhibiting differences in activities. It is to be recognized that the formation of protein-metal aggregates at higher temperature acted as a protection mechanism exhibited by thermophilic enzymes in general, and contributed to the higher thermostability of these species. It may therefore be considered a mechanism for high temperature starch processivity in the xerophytes when growing at elevated higher ambient temperature in the environment. Experimental results obtained confirm the fact that the amylase isoforms were capable of functioning 10-12 minutes at 100 °C, making them a viable thermophilic alternative where required, to the thermophilic enzyme activity available from bacterial sources.
Jei-Fu-Shah (1995) reported the presence of α-amylase in *Thermus sp* and showed that Na\(^+\) stabilized the enzyme activity to a larger extent. Metal ions like Fe\(^{2+}\), and Cu\(^{2+}\) however inhibited the enzyme activity. Long-Liu Lin (1998) reported that Hg\(^{2+}\), Zn\(^{2+}\) and Cu\(^{2+}\) were strong inhibitors, while Ni\(^{2+}\) inhibited the enzyme activity moderately, suggestive of some amylases being metallo enzymes, and the inhibition by specific ions as being due to a competition between the exogenous cations and protein-associated cations, resulting in decreased metalloenzyme activity. The complete loss of enzyme activity by the chelating agent EDTA, demonstrated that metal ions were necessary for the enzyme activity of amylase and was supported by the findings of (Farez-vidal, 1995).

Even though Mn\(^{2+}\) reportedly is (Nguyen, 2002) an activator for most of the amylase enzyme activities, (Odile Mayzaud, 1985) observed that Mn\(^{2+}\) is an inhibitor for the amylase activity isolated from *Copepod Acartia Clausi*. This information is in direct support of our findings that Mn\(^{2+}\) inhibited the *Cereus pterogonus* and *Opuntia vulgaris* amylase activities.

Ca\(^{2+}\) is necessary for the stability and activity of amylases from most sources (Hsiu, 1964; Vallee, 1959). According to Wanderley (2004) the α-amylase from the yeast *Cryptococcus flavus*, and α-amylase from *Thermus sp* (Jei-Fu-Shah, 1995) did not require calcium for enzyme activity. This fact was observed again during the current studies.
Arikan Burhan (2003) reported that α-amylase from Bacillus sp isolate ANT-6 was inhibited by urea, and that SDS reduced the amylase enzyme activity by 63% (Jei-Fu-Shah 1995). Our findings are in good agreement with these reports.

Fluorescence spectroscopic analysis enabled to recognize that the intrinsic tryptophan fluorescence of these proteins correlated well with an environment in which the tryptophan residues remained totally exposed, followed by decremental changes in the intrinsic fluorescence of these proteins with increasing temperature, reaching a minimum in the emission $\lambda_{\text{max}}$ at 100 °C, suggestive of significant burial of tryptophan residues within the protein structure (or) within the protein aggregates that formed at higher temperature as evidenced employing Transmission Electron Microscopy of glutamine synthetases from thermophilic and mesophilic Bacillus species (Merkler, 1988). Fitter (2001) reported on a comparative analysis of the thermophilic and mesophilic amylases, and observed that changes in the fluorescence emission of α-amylase reflected changes occurring to the enzyme protein conformation such as unfolding, contributing to considerable differences in the tryptophan fluorescence emission. In the folded state, tryptophan residues generally remained buried in the hydrophobic interior of the protein structure, and during the unfolding process these residues became exposed more and more to the solvent, thereby changing the micro
environment. The shift into a more hydrophobic environment therefore reduced the fluorescent emission intensity at 340 nm due to the shifting and quenching effects (Schmidt, 1989; Pace, 1989) that accompanied the structural perturbations.

Laderman (1993) reported that the fluorescence emission of α-amylase from *P. furiosus* at 20 °C exhibited maximum intensity at 345 nm. When the fluorescent spectrum was monitored over a range of temperatures, there was no shift observed in the emission λ max, suggestive of that the tryptophan residues occupied a polar environment independent of temperature. A decrease in fluorescent intensity with increase in temperature is also reportedly due to increased quenching as a consequence of greater thermal motion (Galley and Edelman, 1964) in solution.

Denaturation kinetics of α-amylase from *Thermus* sp carried out by (Jei-Fu-Shah 1995) at various temperatures employing 25 mM phosphate buffer, at (pH 7.0), and for 0-45 minutes yielded an activity profile for the enzyme, that decreased significantly with increase in temperature and as a function of time. This enzyme was observed to retain about 80% of its’ original activity at 60 °C upto 15 minutes.

The denaturation kinetic studies reported here were undertaken to establish the thermophilic nature of the amylase isoforms. Denaturation assays conducted for various intervals of time at selected higher temperature
enabled to heat treat the enzyme species for a given length of time and
allowed to measure the residual enzyme activity to determine the enzyme
stability at higher temperatures. Denaturation assays when repeated using the
isoforms in the presence of specific divalent metal ions yielded denaturation
profiles that exhibited augmented thermal stability for the enzyme protein at
the higher temperatures. Generation of phase differences in the decay profile
of the denaturing enzymes at each selected temperature was also noticed.
Fast, slow and intermediate phases were identifiable in the decay profiles
enabling to determine the decay rate constants, from which the
thermodynamic indicator for free energy (ΔG) was calculated. Arrhenius plots
were then employed to determine the ΔH values. Comparative evaluation of
estimated ΔH values with experimental results obtained using the Differential
Scanning Calorimeter technique confirmed that the calculated enthalpies were
in good agreement with the experimentally determined enthalpy values,
suggestive of internal consistency in the experimental methods employed for
denaturation kinetics. The estimated entropy (ΔS) of decay yielded negative
values suggestive of structural rigidity or therefore conformational
inflexibility that developed in the decaying enzyme species, finally yielding
the residual enzyme activity. Negative entropy was indicative of lower degree
of disorderliness in the decaying molecular species, tending towards a final
state of disarray.

Attempts to amplify the amylase gene nucleotide sequence employing
*Cereus pterogonus* and *Opuntia vulgaris* genomic DNA templates and three
different pairs of sense-antisense primers synthesized as indicated in the text and using the polymerase chain reaction technique, resulted in difficulty to obtain a 1.3-1.5 kb ethidium bromide stained PCR band on agarose gels. The anticipation of 1.3-1.5 kb PCR band was based on the reported average size of eukaryotic amylase genes in selected mesophilic plant sources, available in the data bank. The use of Poly A+ RNA from these two species that was used to serve as an alternate template initially for the synthesis of full length cDNA and thereafter for the PCR based sequence amplification process involving each of the three different pairs of sense-antisense primers designed for the purpose, and employing the reverse transcriptase-PCR method also resulted in difficulty to obtain the 1.3-1.5 kb amylase gene PCR band on the agarose gels.

Nonetheless, PCR amplification efforts resulted in the generation of a ethidium bromide stained specific PCR band, having a nucleotide length 450bp when the primer pair BMBF2–R2 was used for the purpose. Surprisingly, this specific PCR band was found to be generated irrespective of whether the genomic DNA template was used for the amplification process or the Poly A+ RNA were employed. The 450 bp PCR band resulted using either the Cereus pterogonus or the Optunia vulgaris polynucleotide sequences.

An estimation of the gene size for the 60-66 kDa amylase enzyme activity detected through the activity stained method on poly acrylamide gels would result in a 1.2-1.32 kb nucleotide length coding for a 400-440 amino
acid containing protein. The generation of a 450 bp PCR amplified polynucleotide sequence will however translate into a 150 amino acid polypeptide product. Even though SDS-PAGE under reducing conditions consistently yielded a 24-25 kDa polypeptide band for the T50 and T90 samples corresponding with a possibility that this polypeptide can be encoded by a 450 bp PCR amplified polynucleotide sequence, the fact remained that no 24-25 kDa polypeptide exhibiting enzyme activity upon activity staining process was observed in the T50, T80 or T90 fractions. It was therefore intriguing as to what did the 450 bp represent; and even more, as to whether either of the sense and antisense primers of the BMBFz -R2 pair went on to anneal with nucleotide sequences farther removed form the conserved sequence domains in the 5'- and 3' regions that probably served as an alternate template sequence both in the Poly A+ RNA as well as in the genomic DNA samples. In other words it is suspected that the 450 bp PCR band may remain an artefact.

Work is however in progress to determine if the 450 bp PCR band could be used as a probe to hybridize it with the genomic template through a southern blot analysis method. Time constrains requiring the submission of this dissertation prevents incorporation of further data to present a complete picture of the molecular biology study.

It was not surprising to observe the generation of PCR amplified bands representing the amylase gene that renders itself inconsistent in size to the expected nature and the size of its protein product. Database searches
identified the existence of a high degree of molecular divergence in the amylase gene sequences from the various species studied, suggesting that the molecular biological investigation of the amylase gene is a very complex process that will be fraught with extreme difficulties, and certainly beyond the scope of the present brief interrogative attempt initiated in this work.

The primers designed for the PCR amplification process targeted the conserved nucleotide sequence in the 5'- end and the 3'- end of eukaryotic amylase genes of selected plant varieties reportedly exhibiting a nucleotide length of 1.3-1.4 kb. The BMB primers exhibited an inter primer fidelity in the range 44-50% and a G+C content in the range 50-67%, considering all the three pairs of sense-antisense combinations synthesized. The data base primers for the amylase gene on the other hand exhibited an inter primer fidelity in the range 18-33 % and a G+C content in the range 41-68%. The potential for inter primer annealing, was therefore expected to greater for the BMB primers, with a propensity to generate PCR amplified primer control bands. The G+C content of the various primers were observed to be well with in the range of each other. Since the bioinformatics investigations (data mining) did not support the prediction of a low size template based PCR band, it was surprising to observe the generation of the 450 bp band reported in this work.

The existence of several intron-exon boundaries in the amylase gene probably caused severe limitations to the polymerase enzyme during the amplification process. Lack of knowledge on the complete nucleotide
sequence of the eukaryotic thermophilic amylase gene precluded a clear understanding of the intron-exon location as well as their boundary sequences that would have enabled the use of intra sequence oligonucleotide primers to compliment the needs of the polymerase during the chain extension processes as had been the practice for the use of nested primers. The extreme molecular divergence exhibited by the amylase family of genes became clearer when a comparison of the conserved nucleotide sequence in the conserved domains of the eukaryotic species studied and reported in the data base was made with the nucleotide sequence in the conserved domains of the eukaryotic species reported in this work. Corresponding overlapping nucleotide sequence were not observed between each domain. Sequence variations and segmental shift were both observed with in each conserved domain. Additionally, sequence homology between the 5' sense and the 3' antisense regions with in each conserved domains found to be much less than 50 % made it further difficult to use knowledge on the number of introns in the reported eukaryotic amylase genes for the benefit of predicting probable numbers and nature of the intron-exon junctions with in the thermophilic amylase genes. Extensive work therefore has to be carried out to achieve the goal.

Bioinformatics approach attempted to identify and establish the presence of conserved sequence regions in the 5' end- and the 3' end- of selected eukaryotic and prokaryotic amylase gene structure. Specific bioinformatic tools as cited under 'methods' were used for the purpose and
the existence of conserved domains were recognized in these species. Oligo nucleotide primer sequences were therefore designed to serve as the 5’ end sense and the 3’ end antisense oligos. These primer nucleotide sequences were then compared with the primer sequences generated by other investigators in the data bank that had been used for the amplification of amylase gene isolated from their various reported sources. Such a comparison offered a larger opportunity to confirm whether the BMB primers that were synthesized for the work reported here, would actually recognize and anneal to the conserved nucleotide sequences identified for the work in the eukaryotic and prokaryotic species, to yield the desired PCR amplified product size. Surprisingly, the reported primer nucleotide sequences were not found appropriate for the PCR amplification of the amylase gene since, significant differences in the localization as well as in the nucleotide sequences of the conserved regions of the different species considered were noted. A multiple sequence alignment approach was therefore used to determine the regions/segments of complimentarity between the primer sequences and the gene sequence of amylase from different sources. Inter primer fidelity, G+C content, primer melting temperature were all determined and compared between the synthesized BMB primers and the reported counter parts. No regions of complimentarity were observed between the various BMB primer sequences or between the primer and the amylase gene sequence excepting in the conserved sequence domains of the amylase gene for the primers. Yet a PCR product not greater than 450 bp
alone was obtained making this investigation deeply difficult. Whereas the minimum size of the eukaryotic gene can be expected in the range 1.2-1.5 kb (based on a protein MW 60 kDa), a maximum size of 3.2 kb can also be considered possible based on the largest size for the amylase protein product reported (210 kDa). Investigations are therefore continuing to establish the fact.

**Conclusion**

Based on the results obtained in the present study, it is concluded that:

Xerophytic species are a good alternate source of thermostable enzymes (Discovery)

- The amylase enzyme activity in *Cereus pterogonous* and *Opuntia vulgaris* existed as two isoforms in each species, one being normal, while the other being a thermophilic isoform.

- The thermophilic isoforms eluted at 0.30 M and 0.37 M NaCl concentration from the Dowex-1 ion exchange matrix employed.

- These isoforms may be the result of a proteolytic processing mechanism of a precursor form of amylase polypeptide, or the result of alternate splicing of a pre mRNA.

- The $T_{80}$ and $T_{90}$ isoforms are independently active for different time periods, in the temperature range 30°C to 100°C, and upto a maximum
of 15 minutes at 100 °C in presence of specific metal ions as stabilizing factor.

- The addition of Ca²⁺ and Mg²⁺ stabilized the half-life of each of the thermophilic isoforms.

- The molecular weights of these isoforms were determined to be 66 kDa for T₅₀ and T₈₀ (Cereus pterogonus) and 60 kDa for T₅₀ and T₉₀ (Opuntia vulgaris).

- Temperature dependent denaturation profiles yielded three independent phases for the denaturation process of the thermophilic isoforms.

- Differences in the rate constants and thermodynamic properties (Eₐ, ΔG, ΔH, ΔS) were noted for each isoform undergoing the denaturation process in the temperature range 30 °C to 100 °C.

- Fluorescence intensity changes detected during the denaturation process confirmed conformational alterations occurring to the enzyme species. The intrinsic tryptophan fluorescence decreased with increasing temperature suggestive of possible molecular aggregation leading to burial of tryptophan residues within the protein structure.

- Immunological cross reactivity and sequence interrelationship existed between the isoforms.

- Living systems are considered open systems that can exchange matter and energy with the environment. Plants therefore remain especially
sensitive to the environmental changes in water availability, soil and air composition and light intensity. Changes in the behaviour of catalytic activities observed in our studies due to variation in divalent cations used, their concentration, and the temperature changes effected are therefore indicative of the adaptability of these plant enzymes to different habitats of plant growth. Consequently, changes in the thermodynamic parameters influenced the adaptability processes within the plant cell and the metal ion effects observed will therefore function as a regulatory mechanism within plant cells, induced by the abiotic stress in their habitats.

- Temperature stable plant enzymes should therefore find significant application potential in industrial processes.