5. DISCUSSION

When we see evolution in the plant kingdom from a simpler life forms to a more complex life forms, the position of the plant group bryophytes comes somewhere in between in the scheme. *Myo*-inositol and by extension the principal enzyme for its biosynthesis, L-*myo*-inositol-1-phosphate synthase is essential for various vital cellular functions in the life of plants including membrane formation, cell wall biogenesis, stress response and signal transduction (Lackey *et al*., 2003). Therefore, it's no surprise that the occurrence of the enzyme have been documented in different groups of plants viz., algae (Dasgupta *et al*., 1984); fungi (Donahue and Henry, 1981; Dasgupta *et al.*, 1984); ptridophytes (Benaroya *et al*., 2004; Chhetri *et al.*, 2006a, 2006b, 2006c), gymnosperms (Gumber *et al.*, 1980; Chhetri and Chiu, 2004) and angiosperms (Majumder and Biswas, 1973; Loewus and Loewus, 1980; Johnson and Wang, 1996; Ray Choudhuri *et al*., 1997; Majee *et al*., 2004; Majumder and Biswas, 2006 etc.). In the evolutionary scheme of things, the plants must have progressed through the groups like bryophytes to ptridophytes, gymnosperms and angiosperms (Fig-5.1). Naturally, the occurrence of *myo*-inositol in this group too is a foregone conclusion. This is the *raison d'etre* of the present work. Indeed, there have been sporadic reports regarding the occurrence of the enzyme L-myo inositol-1-phosphate synthase in bryophytes (Dasgupta *et al.*, 1984; Chhetri *et al.*, 2006, 2009). These studies were preliminary in nature and represented only a couple of thalloid bryophytes. However, complete study of the enzyme involving its functional biochemical characterization and the end product of the pathway, free *myo*-inositol is still lacking. This present work endeavours to fill that gap.

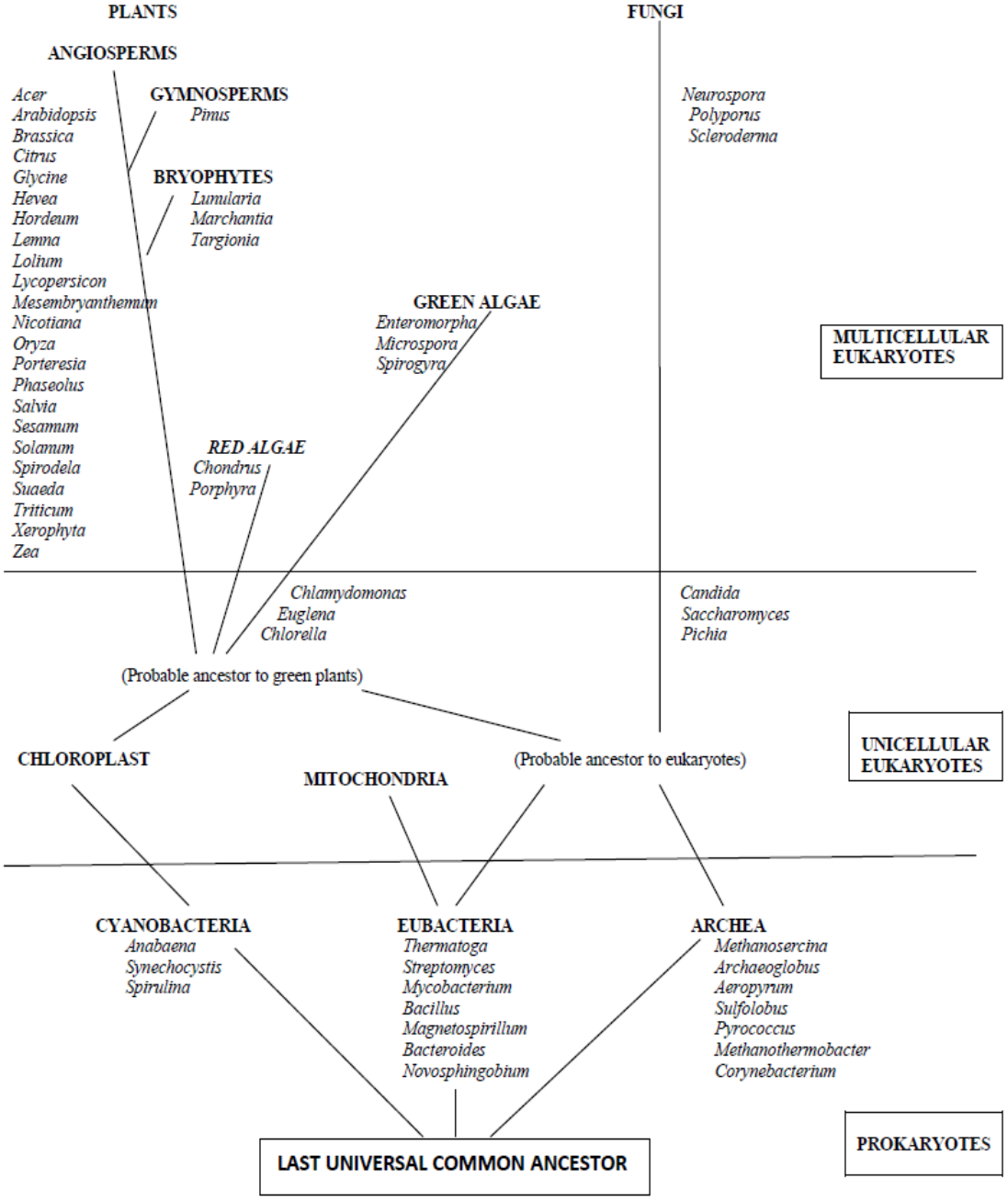

Fig-5.1: Distribution of MIPS across different life forms in plants

(Modified from Majumder *et al.*, 2003)

This study enumerates the basic physico-chemical properties of the enzyme isolated from a two species of bryophytes available in Darjeeling hills, *A. khasiana* and *S. junghuhnianum* belonging to the group liverworts and mosses respectively. The fundamental question of occurrence of *myo*-inositol-1-phosphate synthase in different phylogenetically related families of bryophytes have been included in Table 4.1 which satisfied the preliminary answer about the occurrence of the enzyme MIPS in different groups of bryophytes. The results showed the highest titre of activity of the enzyme with consistent output in *A. khasiana* and *S. junghuhnianum* hence their utilization for further purification and characterization experiments. Activity of the enzyme presented in Table 4.1, clarifies that the enzyme may remain functional in the whole plant body, the vegetative part or the reproductive part among the tested organisms. Furthermore, it has been revealed that the titre of the enzyme-protein does not follow any cardinal rule regarding its activity in one or the other group of bryophytes. Hence, the activity is highest in *A. khasiana*, a liverwort and *S. junghuhnianum*, a moss. Additionally, it has been revealed that the enzyme exhibited significantly higher activity in the reproductive parts than in the vegetative portion of the same plant which is the general feature of other plants also (Majumder and Biswas, 1973; Donahue and Henry, 1981; Das Gupta *et al.*, 1984; Gumber *el al.*, 1984; Chhetri *et al.*, 2005, 2006a). Although these experiments have been made with relatively crude enzyme preparations, the unequivocal presence of the enzyme across different groups throws light on its universal prevalence, especially the distribution of MIPS among various bryophytic groups.

A. khasiana showed a four-fold higher enzyme activity and *S. junghuhnianum* showed a two-fold higher activity in its reproductive parts as compared to the vegetative parts

(Table-4.3 and Table-4.12). The product of this enzyme, L-*myo*-inositol-1-phosphate (the immediate precursor of free *myo*-inositol) fulfils the essential requirement for the formation of sex-linked structures and also the requirements during the development of life in many stages. This indicates a greater degree of inositol demand in reproductive plant parts than in the vegetative structures of bryophytes like other living organisms. Though greater specific activity of this enzyme has been recorded in the reproductive parts of *A. khasiana* and *S. junghuhnianum*, the enzyme MIPS may have some other metabolic significance in the vegetative parts. In ptridophytes, similar results of greater activity of the enzyme in reproductive parts as compared to the vegetative structures have already been reported (Chhetri *et al.*, 2005; Basak, 2013).

The vegetative and reproductive structures are characteristically associated with the production and utilization of *myo*-inositol. Therefore, this study has undertaken to estimate the free myo inositol also in addition to the activity of the enzyme MIPS. During the present studies, appreciable level of free *myo*-inositol has been detected in all bryophytes studied. However, the content was higher in *Bryum argenteum, Asterella khasiana* and *Anthoceros angustus* etc. studied (Table-4.2). The content of free myo-inositol was in general higher in the mosses as compared to the thalloid bryophytes. Whether this higher concentration of myo-inositol in mosses is due to more biosynthesis of the same or whether in the thalloid bryophytes the free myo-inositol is readily converted to other metabolites is still a lingering question.

As in the case of MIPS activity, the content of free *myo*-inositol, the end product of MIPs reaction was much higher in the reproductive parts when compared to the

vegetative parts. Thus the reproductive parts of *A. khasiana* showed 1.36 fold more *myo*-inositol than the vegetative parts (Table-4.4) and the reproductive parts of *S. junghuhnianum* showed 1.32 fold more *myo*-inositol than the vegetative parts (Table-4.13). Naturally, reproductive parts were chosen for the partial purification of the enzyme from the species concerned. The content of *myo*-inositol may point towards the status of *myo*-inositol biosynthesis of each species under the period of plant collection.

Biochemical characterization of the enzyme, MIPS is a prerequisite for deciphering the fundamental metabolic regulation with respect to the biosynthesis of inositol in the target organisms, *A. khasiana* and *S. junghuhnianum*. In turn isolation and purification of the L-*myo*-inositol-1-phosphate synthase from the above two bryophytic samples, *A. khasiana* and *S. junghuhnianum* was carried out to the extent possible which is presented in sections 4.4 and 4.7. Consequent upon the partial purification of the enzyme concerned from the target samples, study of the different parameters of the enzyme and it behaviour under the influence of altering internal and external environment was undertaken. The enzyme, MIPS was purified up to about 46.34 fold over the homogenate fraction in case of the liverwort, *A. khasiana* by employing low-speed centrifugation, streptomycin sulphate precipitation, ammonium sulphate fractionation, and successive chromatography on DEAE cellulose, hexylagarose and one final molecular sieve chromatography through BioGel A 0.5m (Table-4.5). The recovery of the enzyme based on total activity was about 36.35 % at this stage of purification. A comparable yield of 49 fold purification with 34% recovery has been reported from *Ulva lactuta* (Basak, 2013). Similarly, in the moss, *S. junghuhnianum*, the same techniques as described above including the different chromatography steps were

employed for partial purification of the enzyme. As a result the enzyme could be purified to about 58.67 fold over its homogenate fraction and the recovery in this case was about 32.86% based on total activity at this stage of purification (Table-4.14). Similarly, 61 fold purification and 54% recovery had been reported from pteridophyte, *Lycopodium clavatum* while 80.9 fold purification and 13.4% recovery had been reported from *Diplopterygium glaucum* (Chhetri *et al.*, 2006a; Basak *et al.*, 2012).

A question may naturally arise, why the enzyme was only partially purified? Though extremely desirable, purification of an enzyme at the level of homogeneity demands extensive standardization of several successive techniques. Such experiment in plant, a hitherto unknown system demands extremely sophisticated laboratories. Under the present laboratory conditions, several attempts were taken to purify this enzyme further by employing other chromatographic matrix like Superdex-200 and also by affinity chromatography using epoxy-activated glucose-6-phosphate, sepharose CL 6B etc. as the matrix. However, these techniques in the present bryophytic enzymes did not exhibit a very good result as was with the animal enzyme (Maeda and Eisenberg Jr., 1980). It may be mentioned here that the number of homogeneous preparation of this enzyme is still very little covering plant, animal and microbial systems and only a few homogeneous enzyme from the plants have been recovered either from natural populations or when it was overexpressed in microbial systems (Ogunyemi *et al.*, 1978; Pittner *et al*., 1979; Mauck *et al.*, 1980; Maeda and Eisenberg Jr., 1980; Donahue and Henry, 1981; Escamilla *et al*., 1982; Johnson and Sussex, 1995; RayChaudhuri *et al*., 1997; Smart and Flores, 1997; Majee *et al.*, 2005)

L-*myo*-inositol-1-phosphate synthase has been purified and characterized by conventional enzyme purification techniques such as ammonium sulphate precipitation, ion-exchange chromatography, gel filtration chromatography etc. Loewus and Loewus (1971) have purified this enzyme from *Acer pseudoplatanus* and the M_r of this preparation came to about 150 kDa. However, plant MIPS purified by employing the techniques of gel-filtration chromatography through Ultrogel AcA-34, anion exchange chromatography through DEAE-Sephacel, another gel filtration through BioGel A-0.5m found that the molecular weight of the native cytosolic MIPS from *Euglena gracilis* and *Oryza sativa* were 179KDa while that from *Spirulina platensis* was 200.32 kDa (RayChaudhuri *et al.*, 1997). In the pteridophyte, *Diplopterygium glaucum,* the apparent M_r of the enzyme was determined as 170.8 (Chhetri *et al*., 2006a). The apparent molecular weights of the cytosolic MIPS from the sample bryophytes during the present studies were found to be 183 kDa for *Asterella khasiana* and 174 KDa for *Sphagnum junghuhnianum.* This result was in consonance with the previous studies which describes the apparent molecular weight of the cytosolic enzyme from plant sources ranging from 135 kDa in *Lemna gibba* (Ogunyemi *et al*., 1978), 155 kDa in *Pinus ponderosa* (Gumber *et al*., 1984), 157 kDa in *Lilium longiflorum* (Loewus *et al*., 1984), 240 kDa in *Saccharomyces sp.* (Donahue and Henry, 1981) to 260 kDa in *Neurospora crassa* (Escamilla *et al.*, 1982). The native PAGE profiles of both the enzyme preparations exhibited 3-5 major bands (Fig-4.1 and Fig-4.10) which indicated that the preparations were not homogeneous. Therefore, no further attempts were made to determine its subunit molecular weight through SDS-PAGE.

MIPS obtained from the bryophytes did not exhibit any activity in absence of its substrate glucose-6-phosphate, both in case of *A. khasiana* and *S. junghuhnianum*. This is in consonance with the behaviour of the same enzyme obtained from pteridophytes (Chhetri *et al*., 2006a, 2006b), gymnosperms (Chhetri and Chiu, 2004), angiosperms (Chhetri *et al*., 2008) and human foetus (Chhetri *et al*., 2012) since G-6-P is the specific substrate of these enzymes. Presence of the co-enzyme NAD^+ was found to be essential in order for the enzyme to express its 100% activity. In the present instance, when the specific co-enzyme NAD^+ was deducted from the reaction mixture, the enzyme from *A. khasiana* exhibited approximately 59.17% loss of activity (Table-4.6) and that from *S. junghuhnianum* exhibited about 68.41% reduction of activity (Table-4.15). However, there was discernible MIPS activity to the tune of approximately 41% and 32% in case of *A. khasiana* and *S. junghuhnianum* respectively. This points towards the existence of some bound NAD^+ in the molecular architecture of this enzyme (Chhetri *et al*., 2012). Earlier studies on MIPS have shown that the enzyme from a number of other sources also exhibited variable degree of NAD^+ independent activity which is responsible for the residual enzyme activity even in absence of added NAD^+ in the reaction mixture, obviously due to the presence of endogenous NAD^+ in the enzyme system (Pittner and Hoffmann-Ostenhof, 1976; Barnet *et al*., 1970; DasGupta *et al*., 1984, Adhikari and Majumder, 1988). Deduction of NH_4Cl and ME was responsible for the loss of MIPS activity to the tune of 31.80% and 34.51% respectively in case of *A. khasiana* (Table-4.6) and the same caused the loss of 40.01% and 33.35% activity respectively in case of *S. junghuhnianum* (Table-4.15).

L-*myo*-inositol-1-phosphate synthase activity in both *A. khasiana* and *S. junghuhnianum* increased in a linear fashion with respect to time at least upto 90 minutes of incubation at 37°C (Fig-4.3 and Fig-4.12). Similarly, pteridophytic MIPS from *Diplopterygium glaucum* showed time linearity of 90 minutes (Chhetri *et al.*, 2006a) while the same from *Lycopodium clavatum* and *Selaginella monospora* exhibited time linearity between 75 and 90 minutes (Basak, 2013)

MIPS activity also increased linearly with respect to the concentration of enzyme protein in the assay mixture at least up to a concentration of 250 µg in case of *A. khasiana* enzyme and 300 µg of protein under optimal assay condition in case of *S. junghuhnianum* (Fig. 4.4 and Fig-4.13). The enzyme protein linearity *vis-a-vis* MIPS activity in *Diplopterygium glaucum* was found to be 280µg (Chhetri *et al*., 2006a) and the same effect in relation to the enzyme concentration in *Lunularia cruciata* was 300µg (Chhetri *et al*., 2009). These results were in consonance with the present results. However, the value for the same in the MIPS from algae was divergent in having its optimal activity at approximately 120pg (Basak, 2013).

The bryophytes MIPS from both *A. khasiana* and *S. junghuhnianum* were highly specific for its substrate, glucose-6-phosphate which is universal for the enzyme from different sources. However, *A. khasiana* MIPS exhibited 8.92 % activity when glucose-6-phosphate was replaced with galactose-6-phosphate and 3.38% activity when the same specific substrate was replaced with mannose-6-phosphate in comparison to the standard assay mixture containing glucose-6-phosphate as substrate (Table-4.7). Similarly, in case of *S. junghuhnianum*, replacement of glucose-6-phosphate with

galactose-6-phosphate and mannose-6-phosphate resulted in 11.23% and 3.81% enzyme activity respectively as compared to that when glucose-6-phosphate was used as substrate (Table-4.16). In the same scale, 9.38% activity of the enzyme MIPS was recorded with galactose-6-phosphate and 1.42% with mannose-6-phosphate as substrates in a leafy pteridophyte while the activity in *Selaginella monospora* was found to be 21.34% and 13.23% respectively, when galactose-6-phosphate and mannose-6-phosphate were used as substrates. However, the mammalian enzyme from human fetal liver was completely dependent on glucose-6-phosphate as its substrate and the above two hexose-6-phosphate could not act as substitutes of the enzyme (Chhetri *et al*., 2006a, Chhetri *et al*., 2012, Basak, 2013).

It has been found that a NAD^+-dependent oxido-reductase which is functionally identical with the L-*myo*-inositol -1-phosphate synthase is able to isomerize galactose-6-phosphate to *muco*-inositol-1-phosphate (Adhikari and Majumdar, 1988). In plants, the occurrence of different esters of *muco*-inositol is not uncommon. In addition, the utilization of mannose-6-phosphate as substrate, even to a limited extent also indicates the synthesis of the same or another isomer of inositol. Thus in the bryophytic systems studied, the probability of *muco*-inositol biosynthesis through a metabolic by-pass cannot be ruled out. It is pertinent to mention here that different isomers of *myo*-inositol are readily interconvertible by rotation of the C-C bonds (Ogawa, 1999) which may explain the existence of some activity of MIPS in presence of galactose-6-phosphate and mannose-6-phosphate even when glucose-6-phosphate was not there in the assay mixture. The identification of the isomeric *myo*-inositol product of galactose-6-

phosphate and mannose-6-phosphate through chemical studies may throw some light on the biosynthesis of inositol in bryophytes.

Kinetic studies on bryophytic L-*myo*-inositol-1-phosphate synthase was performed and the rate of reaction was found to increase with respect to its specific substrate (glucose-6-phosphate) concentration upto 6 mM in case of both the liverwort (*A. khasiana*) and the moss (*S. junghuhnianum*) (Fig-4.6 and Fig-4.15). The K_m value for glucose-6-phosphate of this enzyme from *A. khasiana* was calculated and was about 3.56mM while the same from *S. junghuhnianum* was found to be about 1.81 mM. Thus the K_m for glucose-6-phosphate in case of *A. khasiana* MIPS was comparable to that of rat testis enzyme which was about 3.89 (Maeda and Eisenberg Jr., 1980) and the K_m in case of *S. junghuhnianum* MIPS was somewhat similar to that of *Euglena gracilis* enzyme which was about 2.1 mM (DasGupta *et al*., 1984). In contrast, the pteridophytic MIPS from *Diplopterygium glaucum* showed a K_m for glucose-6-phosphate of about 0.83 mM (Chhetri *et al*., 2006a) and the K_m for the same for MIPS from the gymnosperm, *Taxus baccata* was about 1.05mM (Chhetri and Chiu, 2004). The V_{max} values for the substrate glucose-6-phosphate have also been worked out and it was found that the same for MIPS from *A. khasiana* was 0.71mM while the MIPS from *S. junghuhnianum* showed a value of 1.42 mM. Thus the V_{max} value for glucose-6-phosphate in case of MIPS obtained from *S. junghuhnianum* was exactly identical to that of the V_{max} value of pteridophytic enzyme from *Diplopterygium glaucum* calculated as 1.42 mM but less than those of yeast enzyme calculated at 1.6 mM (Donahue and Henry, 1981), *Euglena gracilis* at 4.0, *Oryza sativa* at 4.42 and *Spirulina platensis* at 5.05 (RayChaudhury *et al*., 1997). The V_{max} for glucose-6-phosphate in case of MIPS from *A. khasiana* at 0.71

was less than that from yeast (Donahue and Henry, 1981), human fetal brain and rat brain (Adhikari and Majumder, 1988) but more than those from the enzymes from other plant sources mentioned above.

The influence of the co-enzyme NAD^+ with respect to its concentration on this enzyme activity was determined and found that the rate of reaction increased with the increase in the concentration NAD^+ in the reaction mixture reaction upto its concentration of 0.8 mM in case of *A. khasiana* (Fig-4.7) and up to a concentration of 0.6mM in case of *S. junghuhnianum* (Fig-4.16). The K_m for the coenzyme, NAD^+ was found to be about 0.56 mM for MIPS from *A. khasiana* and the K_m for the MIPS from *S. junghuhnianum* was about 0.25 mM. These values were somewhat comparable to that of pteridophytic MIPS from *Diplopterygium glaucum* which showed K_m of about 0.44mM (Chhetri, 2004). In contrast, the K_m for NAD^+ for MIPS from many other plants were quite different e.g., 8mM for the yeast enzyme (Donahue and Henry, 1981), 0.11mM for the *Spirulina platensis*, 0.16mM for *Euglena gracilis*, 0.13mM for *Oryza sativa* (RayChaudhuri *et al*., 1997). Strangely enough, the K_m for NAD^+ for the same enzyme from human fetal brain showed a comparable value at 0.45mM (Adhikari and Majumder, 1988). The V_{max} for NAD^+ for the enzyme isolated from the bryophytes was also determined and found to be 0.68mM for the *A. khasiana* enzyme which was less than those from other plant sources like *Euglena gracilis* at 3.98 mM, *Spirulina platensis* at 4.24mM and *Oryza sativa* at 5.08 mM (RayChaudhuri *et al*., 1997) but more than those from some other sources (Majumder *et al*., 1997). The V_{max} for NAD^+ for the enzyme isolated from *S. junghuhnianum* was found to be about 1.12mM/hr which was comparable to that of 1.14 mM for the same obtained for the yeast enzyme (Donahue and Henry, 1981). Similarly,

the pteridophytic enzyme from *Diplopterygium glaucum* showed a V_{max} *of* 1.8 mM (Chhetri, 2004). Earlier studies have shown the V_{max} for NAD^+ for the same enzyme from *Marchantia nepalensis* to be about 1.11mM (Chhetri *et al*., 2006d) and *Lunularia cruciata* to about 1.21mM (Chhetri *et al*., 2009) which is in consonance with the present studies at least in case of *S. junghuhnianum.*

The bryophytic L-*myo*-inositol-1-phosphate synthase isolated from *A. khasiana* and *S. junghuhnianum* was specific for NAD^+. However, trials were made to obtain conclusive evidence for the same and from the results it was found that NAD^+ was not replaceable by $NADP^+$ in any of the two cases (Fig-4.8 and Fig-4.17). Interestingly, even in absence of the added NAD^+ some basal enzyme activity was observable, making the case for enzyme-bound endogenous NAD^+ more reasonable as already discussed. The residual enzyme activity, in presence of $NADP^+$ certifies the hypothesis.

The bryophytic MIPS from *A. khasiana* and *S. junghuhnianum* showed that it operates significantly through a narrow pH range i.e., from pH 7.0 to 7.5 with maximum at pH 7.0 in both the cases (Fig-4.9 and Fig-4.18). In earlier studies the pH optima for the same was found to be at pH 7.0 for the enzyme from yeast (Donahue and Henry, 1981), pH 7.2 from rat mammary gland (Naccarato *et al*., 1974), pH 7.5 from *Euglena gracilis* (RayChaudhuri *et al*., 1997), rat brain and human fetal brain (Adhikari and Majumder, 1988), pH 7.6 from *Entamoeba histolytica* (Lohia *et al*., 1999), pH 7.7 from *Lemna gibba* (Ogunyemi *et al*., 1978), rat testis (Maeda and Eisenberg, Jr., 1980) and *Neurospora* (Escamilla *et al*., 1982), pH 7.8 from *Spirulina platensis* (RayChaudhuri *et al*., 1997), pH 8.0 from *Acer pseudoplatanus* (Loewus and Loewus, 1971), pH 8.4 from

Oryza sativa (Funkhouser and Loewus, 1975). Similarly, the enzyme from *Diplopterygium glaucum* showed pH optima at pH 7.0 to 7.5 (Chhetri *et al*., 2006a) while those from *Pinus ponderosa* pollen and *Lilium longiflorum* pollen showed pH optima between pH 7.2-7.7 and pH 7.8-8.5 respectively (Gumber *et al*., 1984; Loewus *et al*., 1984).

The effects of varying concentrations (0-100mM) of different salts viz., $MgCl_2$, NH_4Cl and EDTA on bryophytic L-*myo*-inositol-1-phosphate synthase activity have been worked out (Table-4.8 and Table-4.17) and found that $MgCl_2$ acted as a mild stimulator, increasing the enzyme activity up to 1.6 fold and 1.2 fold in *Asterella khasiana* and *S. junghuhnianum* respectively. NH_4Cl was a strong stimulator of the enzyme and increased the rate of reaction by 7.5 and 9.3 fold in *A. khasiana* and *S. junghuhnianum* respectively. Earlier 1.3 fold stimulation of enzyme activity by the influence of 12mM NH_4Cl was recorded in case of pteridophytic MIPS (Chhetri *et al*., 2006a). In *Euglena gracilis*, the enzyme activity was stimulated 2.0 fold by the effect of NH_4Cl (Dasgupta *et al*., 1984) while in *Acer pseudoplatanus*, the enzyme activity was stimulated 2.3 fold by NH_4Cl (Loewus and Loewus, 1971). In both cytosolic and chloroplastic forms of MIPS from *Oryza sativa*, *Vigna radiata* and *Euglena gracilis*, NH_4Cl produced about 5.0 fold stimulation of activity (RayChaudhuri *et al*., 1997). On the other hand, EDTA acted as a mild inhibitor and at its highest concentration (100mM), it decreased the enzyme activity by 42.8% and 47.3% in *A. khasiana* and *S. junghuhnianum* respectively, though up to a concentration of 40mM the salt did effect any change in the enzyme activity in the bryophytes studied. In ptridophytes too, EDTA affected the enzyme activity in similar concentration dependent manner (Chhetri, 2004) which is an

established character of this enzyme in different plant species (Loewus and Loewus, 1980; Dasgupta *et al*., 1984). Ogunyemi *et al*., (1978) have found that MIPS from *Lemna gibba* could be inhibited by 30% by the influence of 1mM EDTA while 10mM EDTA completely inhibited the enzyme activity. EDTA at a concentration of 10mM did not cause any stimulation or inhibition of the enzyme activity; however, at 50mM it caused 1.5 fold stimulation of bovine MIPS (Maeda and Eisenberg Jr., 1980). The enzyme MIPS isolated from *Streptomyces griseus* was found to be completely inhibited by 60mM EDTA, however, this inhibition could be reversed by Mg^{2+} (Pittner *et al*., 1979). It appears that the bryophytic MIPS from both the sources were not affected by low concentration of EDTA, while at higher concentration it played an inhibitory role. Mg^{2+} is required for MIPS activity and addition of EDTA hinders the MIPS activity probably by inactivation of Mg^{2+} concentration (Sipos and Szabo, 1989). Mg^{2+} is an absolute necessity for conversion of *myo*-inositol-1-phosphate to *myo*-inositol in *Acer pseudoplatanus* (Loewus and Loewus, 1971). In *Neurospora crassa* 2.0 fold stimulation of the enzyme activity occur in presence of 2mM of Mg^{2+} (Sherman *et al*., 1981). Bryophytic MIPS was stimulated by 1.6 fold in *A. khasiana* and 1.3 fold in *S. junghuhnianum* (Table-4.8 and Table-4.17) by $MgCl_2$ in a concentration dependent manner. At very high concentrations, the role of Mg^{2+} has been found to be inhibitory in case of *Neurospora crassa* (Escamilla *et al*., 1982). Similar effects have also been observed in case of MIPS from rat testis (Maeda and Eisenberg, 1980). However, the bryophytic enzyme did not behave in the same way even at quite high a concentration.

Effects of other monovalent cations were also studied whereby the assay mixture contained varying concentration (0-10mM) of different chloride salts. It was found that the monovalent cation, K^+ showed stimulatory effect on bryophytic L-*myo*-inositol-1-

phosphate synthase enhancing the rate of reaction by 1.6 fold in *A. khasiana* and 1.8 fold in *S. junghuhnianum* in a concentration dependent manner. This is in consonance with 2.0 fold stimulation of the enzyme activity in presence of K^+ in MIPS from bovine testis (Mauck *et al*., 1980). In the present instance, Na^+ acted as a mild inhibitor of this enzyme from bryophytes, inhibiting the activity up to 47.6% in *A. khasiana* and 22.0% in *S. junghuhnianum* in a concentration dependent manner. High concentration of Na^+ inhibited the enzyme activity in case of *Neurospora crassa* (Escamilla *et al*., 1982). On the other hand, like in other cases, Li^+ acted as a strong inhibitor of the enzyme inhibiting the activity of the same by up to 81.9% and 76.1% in *A. khasiana* and *S. junghuhnianum* respectively. Li^+ exhibited this exclusive inhibition in a concentration dependent manner (Table-4.9 and Table-4.18). Rat testes enzyme had shown identical character (Maeda and Eisenberg, Jr., 1980; Loewus and Loewus, 1980). Li^+ was also inhibitory to *Oryza sativa, Vigna radiata* and *Euglena gracilis* enzyme in a concentration dependent manner and even its 5mM concentration proved to be detrimental to the enzyme activity (RayChaudhuri *et al*., 1997). In *Diplopterygium glaucum,* Li^+ inhibited the MIPS activity by 60% at 10 mM concentration (Chhetri, 2004).

Influence of some other divalent cations on the bryophytic MIPS was also studied. The studies revealed that Ca^{2+} was a mild stimulator of this enzyme causing 1.53 fold and 1.45 fold stimulation of the enzyme activity in *A. khasiana* and *S. junghuhnianum* respectively. At the same time, Mn^{2+}, Cu^{2+} and Cd^{2+} had some inhibitory effect which inhibited the enzyme activity by 9.5%, 6.6% and 5.7% respectively in the liverwort, *A. khasiana*. In case of MIPS isolated from the moss, *S. junghuhnianum,* Cu^{2+} showed a

comparable inhibition of the enzyme activity, slowing down the reaction by 5.7% while $Cd^{2+}$and Mn^{2+}were slightly more toxic which inhibited the enzyme activity by 19.0% and 30.4% respectively. The action of divalent cations Ca^{2+}, Mg^{2+} and Mn^{2+} were found to be inhibitory to different degrees at 5mM in some other plants (RayChaudhuri *et al*., 1997). On the bryophytic MIPS, Zn^{2+} also exhibited a pronounced inhibitory activity slowing down the enzyme reaction by 18.0% and 43.8% in *A. khasiana* and *S. junghuhnianum* respectively. As expected, the heavy metal Hg^{2+} acted as a strong inhibitor of the bryophytic L-*myo*-inositol-1-phosphate synthase which caused the inhibition of *A. khasiana* enzyme by approximately 74.2% and that of *S. junghuhnianum* enzyme by about 81.9% (Table-4.10 and Table-4.19). MIPS isolated from *Archaeglobus* have been found to be active in presence of divalent cations such as Zn^{2+} and Mn^{2+} (Majumder *et al*., 2003). Similarly Zn^{2+}has been found as an integral part of MIPS in *Mycobacterium* conferring proper structural conformation to its presumed active site (Norman *et al*., 2002). In general, the eukaryotic MIPS showed a preference for NH_4^+ over divalent cations for its optimal activity. However, whether bryophytic MIPS is a Class-I aldolase where an intermediate Schiff base is formed between an amine group on the enzyme and a carbonyl group on the substrate; a Class-II aldolase that require divalent metals for its reaction or a Class-III aldolase requiring NH_4^+ for its optimal activity is an open question. Interestingly, the bryophytic MIPS isolated from *A. khasiana* as well as from *S. junghuhnianum* exhibited appreciable stimulation of activity both in the presence of NH_4^+ as well as that of Mg^{2+}. Maybe, it belongs to a completely different category showing dual characters of Class-II and Class-III aldolases.

The sugar alcohol, *myo*-inositol itself, up to the experimental concentration of 10 mM showed very little influence in either *A. khasiana* as well as in *S. junghuhnianum* enzyme which is in consonance with the studies on the *Diplopterygium glaucum* L-*myo*-inositol-1-phosphate synthase activity (Chhetri, 2004). Another sugar alcohol, galactitol showed enhancement of the enzyme activity up to a concentration of 4 mM in both *A. khasiana* and *S. junghuhnianum* beyond which the compound became toxic causing about 35% and 34% loss of activity respectively in the above two bryophytes at a concentration of 10mM. However, mannitol did not follow any pattern in its general stimulatory activity on *A. khasiana* MIPS, though in *S. junghuhnianum* it followed somewhat concentration dependent influence in its stimulatory role (Table-4.11 and Table-4.20). In contrast, sugar alcohols such as inositol, mannitol and sorbitol inhibited both chlorophastic and cytosolic forms of MIPS from other plant sources at 4mM. Moreover, *Euglena gracilis* cells under culture showed reduced MIPS activity in even lesser concentration of sugar alcohols (RayChaudhuri *et al*., 1997).

Preliminary studies on L-*myo*-inositol-1-phosphate synthase of bryophytic origin have been started by Chhhetri *et al*., (2006c, 2009) and the present investigator was a member of the group. The presented study conclusively proves the occurrence of L-*myo*-inositol-1-phosphate synthase in bryophytes and thus completes the phylogenetic tree regarding the occurrence of the same in different plant groups starting from algae to angiosperms. The screening of the enzyme and its end product, *myo*-inositol in different families of bryophytes indicates the universal occurrence and activity of the enzyme across different bryophytic groups. Since the detailed studies relating to L-*myo*-inositol-1-phosphate synthase from a hitherto neglected plant group, the bryophyte has been

undertaken, it is a prerequisite that its metabolic regulation is also studied with different parameters of biochemical characterization. Consequently, isolation of L-*myo*-inositol-1-phosphate synthase from the vegetative as well as reproductive parts of the bryophytes was undertaken. During these screening experiments, it was found out that the vegetative tissues showed a higher titre of MIPS activity in homogenate fraction over the low speed supernatant fraction, specifically in case of vegetative tissues in both the bryophytes studied (Table-4.3 and Table-4.12). This provides circumstantial evidences explaining the probability of existence of different forms of L-*myo*-inositol-1-phosphate synthase in bryophytic system, with some form being cytosolic and some other being particulate in nature. Such differential forms of MIPS have already been reported in pteridophytes (Chhetri *et al*., 2006d). Earlier, chloroplastic forms of MIPS in addition to the cytosolic forms have been reported by different groups (Imhoff and Bourdu, 1973; Adhikari *et al*., 1987; RayChaudhuri *et al*., 1997). Different types of particulate MIPS have been isolated from membrane bound organelles like chloroplasts, mitochondria, plasma membrane, plastids, endoplasmic reticulum, nuclei etc. in *Phaseolus vulgaris* (Lackey *et al*., 2003). Naturally, multiple forms of MIPS cannot be ruled out in case of bryophytes too. However, this was beyond the purview of present studies and as such the present study remained restricted to the cytosolic MIPS in bryophytes, the most predominant source of the enzyme from different other sources too.

Experiments as described in Section-4.5.7 and Section-4.8.7 determine the thermal stability of the enzyme MIPS from *A. khasiana* and *S. junghuhnianum* respectively. Of the sample bryophytes, *A. khasiana* showed significant activity of L-*myo*-inositol-1-phosphate synthase between 20^{o}C and 50^{o}C with the temperature maxima at 30^{o}C

(Fig.4.5), similarly, the activity of L-*myo*-inositol-1-phosphate synthase isolated from *S. junghuhnianum* exhibited remarkable activity between temperatures of 10^{o}C and 40^{o}C with the temperature maxima at 30^{o}C (Fig-4.14). The temperature maxima of MIPS in both the bryophytes were 30^{o}C which is comparatively lower than the enzyme, isolated from a number of other sources as described in section-2 e.g., 35^{o}C for *Entamoeba histolytica* (Lohia *et al*., 1999) and some plants (RayChaudhuri *et al*., 1997), 40^{o}C for human fetal liver enzyme (Chhetri *et al*., 2012). Unlike the MIPS from other sources, the *A. khasiana* MIPS shows appreciable activity at the minimum temperature of 20ºC and the *S. junghuhnianum* MIPS exhibits its activation at even lower temperature of 10ºC. This may be called a unique feature of the bryophytic MIPS from these two sources though both exhibited temperature maxima at 30ºC. The activity of the enzyme in temperature as low as 10ºC is a significant information which has so far not been reported save from one source, *Xerophyta viscosa*, which is a resurrection plant and is extremely tolerant to osmotic and low temperature stress (Majee *et al*., 2005). This activity of bryophytic MIPS in comparatively lower temperature satisfies our hypothesis that bryophytic MIPS may have some cold temperature tolerance characteristics and it may be a potential future source of mining cold resistance specific MIPS genes. Studies have suggested that tolerance of MIPS to higher temperature is associated with the NAD^{+} bound to the enzyme (Adhikari and Majumder, 1983, Chhetri *et al*., 2006c). Whether bound NAD^{+} is also responsible for the activity of MIPS at lower temperature needs to be found out through further research.

Myo-inositol-1-phosphate synthase isolated from *Mycobacterium tuberculosis* H37Rv could functionally compensate for the yeast *INO1* mutation though the *INO1* of

Mycobacterium and other homologous prokaryotic genes revealed a distinct class of this enzyme (Bachhawat and Mande, 1999). *Myo*-inositol lipids are major components of mycobacterial plasma membrane and cell wall. Thus, *myo*-inositol is an essential metabolite that forms the building blocks for the synthesis of glycolipid and other metabolites in mycobacteria (Morita *et al*., 2011). Therefore, controlling these synthetic pathways is critical for controlling mycobacterial pathogenesis; as such this enzyme can be a potential drug target.

Disruption of inositol homeostasis has been associated with a number of illnesses, including metabolic syndrome, Alzheimer's disease, diabetes, epilepsy etc. (Gelber *et al*., 2001; Wang and Raleigh, 2014; Scioscia *et al*., 2007; Chang *et al*., 2014). Dysregulation of inositol levels has been reported in a wide variety of biomedical studies. Understanding the metabolism of myo-inositol will provide insight into the mechanism underlying these diseases (Frej *et al*., 2016). Altered inositol levels have also been demonstrated in patients with bipolar disorder (Shimon *et al*., 1997), major depressive disorder (Coupland *et al*., 2005) and schizophrenia (Shimon *et al*., 1998). For these reasons, modulating *myo*-inositol level was proposed as a therapy for these disorders (Chengappa *et al*., 2000; Palatnik *et al*., 2001).

MIPS expression during early stages of seed development is closely related to the essentiality of inositol biosynthesis for several biochemical pathways in plants (Downes *et al*., 2005). In *Passiflora edulis* the MIPS synthesizing gene was upregulated after a short exposure to cold stress (5ºC) (Abreu and Aragao, 2007), in *M. crystallinum*, salinity stress induced upregulation of MIPS mRNA expression by 5-fold and free inositol accumulation by approximately 10-fold (Ishitani *et al*., 1996). Indeed high and

low no-freezing temperatures and osmotic stresses appear to have several features in common (Abreu and Aragao, 2007). Thus MIPS plays role in basic metabolism as well as during the response of plants to environmental stress.

In plants, multiple physiological and biochemical characters are controlled by MIPS and in turn plants also possess multiple MIPS genes suggesting that different activities may be controlled by different MIPS genes (Valluru and Ende, 2011). Indeed two isoforms of MIPS gene were found in ice-plant (Ishitani *et al*., 1996), two in rice genome (Yoshida *et al*., 1999; Suzuki *et al*., 2007), seven in maize (Larson and Raboy, 1999), four in soybean (Hegeman *et al*., 2001), three in *Sesamum indicum* (Chun *et al*., 2003), two in chickpea (Kaur *et al*., 2008), three in *Arabidopsis* (Luo *et al*., 2011) and three in *Phaseolus vulgaris* (2012). The fate of the cell may be linked to differential gene regulation. MIPS may be involved in transcriptional regulation of chromatin metabolism in addition to being involved in myo-inositol biosynthesis. Thus in plants MIPS appear to have evolved as a protein that connects cellular metabolism, pathogen response and chromatin remodelling (Latrasse *et al*., 2013).

The thesis presented describes the fundamental evidence for the occurrence of L-*myo*-inositol-1-phosphate synthase in a different group of plants, the bryophytes thus completing the report on the occurrence of the enzyme in diverse groups from algae to angiosperms. Since a long time, the distribution, physiological role, biochemical characteristics and metabolic regulation of this enzyme have been documented from bacteria, algae, fungi, ptridophytes, gymnosperms, angiosperms and mammals. However, the information was meagre regarding the occurrence and function of *myo*-

inositol synthesizing potentiality from the amphibians of the plant kingdom, the bryophytes. Therefore, the presented study completes that gap of information regarding the biosynthesis and regulation of *myo*-inositol covering all plant groups through the evolutionary scale.